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The effects of postnatal and maternal diet-induced obesity on physiology and vascular function

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Abstract

In recent years there has been an explosion in the rates of obesity, defined as a body mass index greater than 30kg/ m^2, and associated cardiovascular disease. Alterations in peripheral glucocorticoid metabolism have been suggested to play a role in the development of obesity. Obesity occurs in both sexes, but the risk of associated metabolic disturbance and vascular dysfunction is greater in men. Although there is no accepted definition of obesity in rodents, the term is used to describe animals with a significant increase in fat pad mass often achieved by feeding a high fat diet. Although animal models of obesity have been useful in delineating potential mechanisms linking obesity with its metabolic and vascular sequelae, most studies have been in male animals and, thus, have not addressed sex differences. Additionally, emerging evidence shows that obesity during pregnancy is associated with increased cardio-metabolic and vascular disease in offspring, although the processes underlying such 'programming' effects are unclear.

This thesis addresses the hypothesis that exposure to postnatal, or maternal obesity will alter both metabolism and vascular function in mice.

Male and female mice maintained on a high fat and sugar diet from 5 weeks of age had increased adipose tissue deposition in adulthood. However there were striking sex differences in glucose homeostasis, mRNA levels and glucocorticoid metabolism, with males being more severely affected. Treatment of male mice with 17β-estradiol ameliorated a number of the effects of the high fat diet, including weight gain and altered glucose homeostasis; additionally estradiol altered glucocorticoid metabolism in the adipose so that it resembled that of females. Suprisingly, given the changes in metabolism, obesity in adult mice produced only small changes in vascular function and did not alter vascular remodelling following injury.

The effects of maternal obesity were studied using male offspring aged 3 and 6 months. The offspring of obese mothers had similar body weight, adiposity, plasma lipid and plasma hormone concentrations to controls. In contrast, exposure to obesity in utero was associated with receptor specific changes in agonist-mediated contraction and decreased endothelium-dependent relaxation in male offspring. Despite these changes in vascular function, no alterations in blood pressure or vascular remodelling following injury were present.

These results demonstrate that the more profound changes in glucose-insulin homeostasis associated with obesity in male humans can be recapitulated in rodent models and imply that

estradiol plays a role in protecting the metabolism of female mice, potentially by alteration of glucocorticoid metabolism. Despite altered metabolism in postnatal obesity vascular function remained normal suggesting metabolic and vascular dysfunction are not intrinsically linked. Conversely, maternal obesity did not cause any overt changes in offspring metabolism but caused vascular dysfunction implying these parameters can be programmed independently.

Declaration

I declare that this thesis and the data presented within are a result of my own work carried out at the University of Edinburgh, with the following exceptions:

Dr Patrick Hadoke and Dr Nicholas Kirkby performed the luminal wire injury surgery, and Dr Patrick Hadoke and Dr Karolina Duthie assisted in mounting femoral arteries on wire myographs for functional analysis (Chapter 6). All data in Table 5.1 was provided by Dr V King who generated the model of maternal obesity.

I declare that this work has not been previously submitted for any other degree or qualification.

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A person who never made a mistake never tried anything new.

Albert Einstein

Abstracts and presentations

Maternal diet-induced obesity in C57BL/6 mice alters vascular function in their offspring but not metabolism or vascular remodelling after intravascular injury. R.S. Dakin, P.W.F. Hadoke, B.R. Walker, J.R. Seckl, A.J. Drake. *J. DOHaD (2011)*; **2** (s1): PI-019

Diet-induced obesity is associated with sex-specific differences in glucocorticoid metabolism.. R.S. Dakin, J.R. Seckl, P.W.F. Hadoke, B.R. Walker, A.J. Drake. *Endocr Rev (2011)*; **32** (3): P2-442

Diet-induced obesity with metabolic dysfunction does not alter vascular function or remodelling in young C57BL/6 mice. R.S. Dakin, A.J. Drake, B.R. Walker, J.R. Seckl, P.W.F. Hadoke. *Endocrine Abstracts (2011)*;**25** P145

Maternal obesity is associated with sex-specific effects on offspring metabolism. R. Dakin, L. Liu, V. King, P. Hadoke, B. Walker, J. Seckl, A. Drake. Oral presentation. *Power of programming meeting, Munich, May 2010*.

Diet-induced obesity in C57BL/6 mice is associated with sex-specific changes in glucocorticoid metabolism. R.S. Dakin, P.W.F. Hadoke, J.R. Seckl, B.R. Walker, A.J. Drake. *Endocrine Abstracts (2010)*; **21** P185

Diet-induced obesity is associated with sex-specific alterations in metabolic, but not vascular, function in mice. R.S. Dakin, B.R. Walker, J.R. Seckl, P.W.F. Hadoke, A.J. Drake. Oral presentation. *Scottish Society for Experimental Medicine meeting, Aberdeen, May 2009.*

Contents

Chapter 6

The effects of maternal and postnatal obesity on vascular function and remodelling 168

List of figures

List of tables

Abbreviations

3α-HSD	3α-hydroxysteroid dehydrogenase
5αR	5α-reductase
5βR	5β-reductase
5HT	5-hydroxytryptamine
11β-HSD	11β-hydroxysteroid dehydrogenase
ACh	Acetycholine
ACTH	Adrenocorticotrophic hormone
ANOVA	Analysis of variance
ApoE	Apolipoprotein E
AR	Androgen receptor
B	Corticosterone
BABB	benzyl alcohol, benzyl benzoate
bp	Base pairs
BSA	Bovine serum albumin
CBG	Corticosterone binding globulin
cDNA	Complementary DNA
CON	Control
CONoff	Offspring of control dams
Cp	Crossing point
CRH	Corticotrophin releasing hormone
DIO	Diet-induced obesity
DIOoff	Offspring of obese dams

DHT	Dihydrotestosterone
DNA	Deoxyribonucleic acid
EDHF	Endothelium-derived hyperpolarising factor
EDTA	Ethylene diamine tetraacetic acid
EEL	External elastic lamina
ELISA	Enzyme-linked immunosorbent assay
Epi	Epididymal
ER	Estrogen receptor
FAS	Fatty acid synthase
GH	Growth hormone
GnRH	Gonadotrophin releasing hormone
GR	Glucocorticoid receptor
GTT	Glucose tolerance test
H	Hours
HPA	Hypothalamic-pituitary-adrenal
HPG	Hypothalamic-pituitary-gonadal
HPLC	High performance liquid chromatography
HRP	Horseradish peroxidase
HSL	Hormone sensitive lipase
IEL	Internal elastic lamina
IL	Interleukin
i.p.	Intraperitoneal
KPSS	Potassium physiological saline solution

KO	Knock-out
LDL	Low density lipoprotein
LPL	Lipoprotein lipase
Mes	Mesenteric
MR	Mineralocorticoid receptor
mRNA	Messenger ribonucleic acid
MW	Molecular weight
NAD	Nicotinamide adenine dinucleotide
NADP	Nicotinamide adenine dinucleotide phosphate
NEFA	Non-esterified fatty acid
NO	Nitric oxide
NOS	Nitric oxide synthase
OPT	Optical projection tomography
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PE	Phenylephrine
PEPCK	Phosphoenolpyruvate carboxykinase
POMC	Pro-opiomelanocortin
PPAR	Peroxisome proliferator-activated receptor
PSS	Physiological saline solution
qPCR	Quantitative polymerase chain reaction
Ret	Retroperitoneal
RNA	Ribonucleic acid

RT-PCR	Reverse transcriptase polymerase chain reaction
SC	Subcutaneous
SHBG	Sex hormone binding globulin
SNP	Sodium nitroprusside
SPA	Scintillation proximity assay
STZ	Streptozotocin
UST	United States trichrome
VSMC	Vascular smooth muscle cell
WT	Wild type

Chapter 1

Introduction

Background

The prevalence of obesity has escalated dramatically in recent years and is believed to be the leading cause of type-2 diabetes and cardiovascular disease (WHO, 2011). Scotland has one of the highest rates of obesity in the world, and in 2010 27% of adults in Scotland were classed as obese (Keenan *et al.*, 2011). It has been estimated that 47% of type2 diabetes can be attributed to obesity, as well as 36% of hypertension and 18% of myocardial infarction (Grant *et al.*, 2007). Interestingly in most countries there is a greater proportion of obese women than men, including women of child-bearing age (Brooks and Maklakov, 2010). Despite the higher incidence of obesity, pre-menopausal women have less cardiovascular disease than men (British HeartFoundation, 2010) and are, therefore, somewhat protected from the effects of excess adipose deposition. Evidence suggests that visceral obesity, primarily seen in men, is more likely to be associated with insulin resistance than the peripheral distribution of adipose tissue which is more commonly found in pre-menopausal women (Fox *et al.*, 2007), although the mechanisms for this remain unclear. One potential mechanism which may be important in the metabolic sequelae associated with obesity is altered glucocorticoid metabolism (Seckl *et al.*, 2004; Walker and Andrew, 2006); and glucocorticoid metabolism differs between the sexes (Endres *et al.*, 1979; Handa *et al.*, 1994; Mitev *et al.*, 2003; Mattsson *et al.*, 2007; Paulsen *et al.*, 2007; Weiser and Handa, 2009) until menopause (Andersson *et al.*, 2009). The first half of this thesis explores sex differences in a murine model of obesity and the potential role of glucocorticoid metabolism in mediating these.

While changes in diet and lifestyle are believed to be the primary factors in the increasing prevalance of obesity, evidence suggests that the developmental environment can programme future disease risk. Many epidemiological studies have demonstrated a link between low birth weight and the subsequent development of hypertension, type-2 diabetes, and cardiovascular disease (Barker *et al.*, 1993a). With rising levels of obesity in women of child-bearing age attention has turned to the potential for programming as a result of exposure to maternal obesity during development. Early evidence suggests babies born to obese mothers have increased adiposity (Catalano *et al.*, 2009b) and are heavier during childhood (Gale *et al.*, 2007). This thesis also uses a new murine model to investigate the effects of maternal obesity on offspring metabolism.

There is a link between obesity and vascular dysfunction (Stapleton *et al.*, 2008) which may underlie the associated increase in cardiovascular disease. However, in rodent models of obesity, vascular dysfunction is not consistently reported. Recent studies in animal models have suggested that programming of vascular function may result from exposure to maternal obesity (Koukkou *et al.*, 1998; Samuelsson *et al.*, 2008). The final part of this thesis uses the models of postnatal and maternal obesity, established for metabolic studies, to investigate the link between obesity and altered vascular function and arterial lesion formation.

This chapter will introduce the sex differences in obesity and associated cardiovascular disease as well as the key processes in lipid and glucose metabolism. A detailed review of glucocorticoid metabolism and the evidence implicating its dysregulation in the pathogenesis of obesity is also included. Finally, changes in vascular physiology induced in obesity and type-2 diabetes are presented along with evidence for programming by maternal obesity.

1.1 Obesity

Obesity is now one of the biggest health problems in the world. The World Health Organisation (WHO) predicts that by 2015 there will be approximately 2.3 billion overweight people aged 15 years and above, and of these over 700 million will be obese (defined as a body mass index (BMI) greater than 30kg/m^2) (WHO, 2011). Worryingly, the number of obese children has also increased at an alarming rate, with the prevalence predicted to worsen with advancing age (Deckelbaum and Williams, 2001).

A very small proportion of people suffer from inherited diseases which are associated with marked obesity, with most of these resulting from genetic disruption of the leptin-melanocortin pathway (Farooqi and O'Rahilly, 2005). However, most obesity is believed to be due to the relatively rapid increase in food availability and sedentary lifestyles in Westernised countries in the late 20th century, resulting in energy intake exceeding usage. Additionally, with the decreasing cost of food and increasing portion sizes the opportunity to over-eat has become the norm. A shift from careers requiring physical exertion to office based jobs and increasing sedentary leisure time has also added to the increase in obesity. Changes in lifestyle and eating have been reasonably widespread; however, the susceptibility to obesity varies among people in the same environment. This suggests that the propensity to become obese is polygenic in origin, and involves many signalling pathways and molecules

both in the central nervous system and periphery, in addition to the imbalance of energy intake and usage.

1.1.1 Sex differences in obesity

The rates of obesity differ enormously among countries, although in most the incidence of obesity is higher in women than in men. The Scottish Health Survey 2003 found 22% of males and 24% of females aged between 16-64 years were obese, (Government, 2005). To date, few studies have explained the reason for the disparity in obesity between the sexes although socioeconomic state (Wardle *et al.*, 2002) and reproductive capacity (Brooks and Maklakov, 2010) have both been correlated with obesity in women. Lean women naturally have a higher percentage of body fat than men and the difference in rates of obesity has been attributed to the expansion of this larger depot thought to be necessary for reproduction (Power and Schulkin, 2008). Despite the higher prevalence of obesity in women rates of associated cardiovascular diseases are usually lower; part of the explanation for this may lie in the sex differences in adipose deposition.

1.1.1.1 Adipose distribution

The anatomical distribution of adipose tissue is the most noticeable sex difference in obesity. In men and post-menopausal women an ‘apple’ shape is common which is characterised by a greater abdominal or visceral adipose deposition. In pre-menopausal females a ‘pear’ shape predominates which is characteristed by a peripheral deposition around the hips and gluteals. In some cases excess lipid is also deposited in organs other than the adipose, the most common being the liver leading to non-alcoholic fatty liver disease (NAFLD) (Mehta *et al.*, 2002). This is particularly dangerous as the liver, unlike adipose tissue, is not designed to accommodate excess lipid. Sex steroids have been proposed as key regulators of sex specific patterns of adipose distribution, exemplified by the changes in fat distribution observed following puberty and after the menopause (further details in section 1.3.3). The precise mechanisms through which sex steroids regulate deposition of adipse tissue are not clear, but altered lipid metabolism, through sex steroid effects on the transcription of lipoprotein lipase (LPL) and leptin and the activation of hormone sensitive lipase (HSL), has been proposed as mediators of these effects (reviewed in (Mayes and Watson, 2004)).

Amount of visceral adipose tissue increases with age in both sexes and for all body weights, though the increase is greater in males (Enzi *et al.*, 1986). Visceral adipose is associated with increased incidence of the metabolic syndrome and insulin resistance (Despres *et al.*, 2008). This may be due to the proximity of visceral adipose to the portal system into which it can

release hormones and, thus, gain direct access to the liver (Bergman *et al.*, 2006). Subcutaneous adipose tissue area is greater than visceral adipose area in women, regardless of overall weight, until the menopause when the ratio shifts to favour an android deposition (Enzi *et al.*, 1986). In contrast to visceral adipose tissue, hormones synthesised in subcutaneous adipose are released into the systemic circulation. In addition the different adipose depots have unique patterns of gene expression and hormone synthesis. For example the expression and secretion of interleukin-6 (IL-6) is relatively high in the visceral adipose compared with the subcutaneous, where the expression and secretion of leptin is greater (Fain *et al.*, 2004). Receptor expression is also depot specific with greater androgen and glucocorticoid receptor expression in the visceral adipose (Kershaw and Flier, 2004).

1.1.1.2 Sex differences in cardiovascular disease

The pathogenesis of cardio-metabolic disease is introduced later in this chapter (section 1.1.6); however, the incidence of these diseases is normally higher in men and correlated with the abdominal deposition of adipose. Insulin resistance and type-2 diabetes are more prevalent in males than females. The amount of intra-abdominal adipose tissue has been shown to be a strong predictor of insulin resistance (Ross *et al.*, 2002a; Ross *et al.*, 2002b) and, as this distribution pattern is more common in men, it may be one reason for the higher incidence of the disease. In addition to insulin resistance, an epidemiological study of dyslipidemia in hypertensive individuals also found the prevalence to be higher in men than women (O'Meara *et al.*, 2004). Currently, no studies have addressed this disparity which suggests a lack of lipid storage, or greater propensity to release free fatty acids (FFAs) from adipose, in males. As well as higher prevalence of primarily metabolic conditions in men; obesity also affects the vasculature and blood pressure in a sex specific manner. Hypertension is more common in men than pre-menopausal women, although after the menopause blood pressure levels are comparable between men and women and may increase with age (Reckelhoff, 2001). Interestingly, sex differences in hypertension have also been reported in animal models such as the spontaneously hypertensive rat and Dahl salt-sensitive rat (Reckelhoff, 2001). The exact mechanisms controlling these sex differences are unknown but are likely to involve many molecular pathways. Measurements of intima-media thickness, an early predictor of atherosclerosis and cardiovascular events (Lorenz *et al.*, 2007), are lower in younger females than in males but become comparable in older women, in association with increased atherosclerotic plaque formation (Sinning *et al.*, 2011). Sex steroids have been suggested to mediate the differences in atherosclerosis by altering FA

metabolism and circulating lipid concentrations in addition to exerting direct effects on the vasculature such as estrogen-mediated nitric oxide production (Majmudar *et al.*, 2000).

1.1.2 Fatty acid metabolism

Lipids are a primary energy source in the mammalian diet and their storage and metabolism is tightly regulated. Obesity is associated with excess calorie intake which can alter the balance of lipid storage and metabolism such that storage predominates and adipose tissue expands.

1.1.2.1 Lipogenesis, fatty acid re-esterification and lipolysis

The synthesis of fatty acids (FAs) is called lipogenesis and involves *de novo* synthesis of FAs from glucose precursors, while the production of triglycerides (TGs) from their FA and glycerol substrates is called re-esterifcation (Figure 1.1). Lipogenesis is stimulated by insulin in the fed state to generate FAs and TGs for storage, and inhibited during fasting when insulin levels fall. Insulin promotes lipogenesis by increasing glucose uptake in adipocytes through the recruitment of glucose transporters (GLUT-4) and activating lipogenic enzymes through covalent modification (Kersten, 2001). The adipokine leptin can inhibit lipogenesis, opposing the effects of insulin and repressing the expression of genes involved in FA and TG synthesis (Soukas *et al.*, 2001) and additionally stimulating FA oxidation (Bai *et al.*, 1996). Leptin deficiency in humans is extremely rare but is associated with severe early onset obesity which resolves when recombinant leptin is administered (Licinio *et al.*, 2004). An important transcription factor in adipose tissue is peroxisome proliferator-activated receptor γ (PPARγ), which triggers adipocyte differentiation. Evidence suggests that PPARγ can be stimulated by insulin, and regulates many genes in the adipose inducing those involved in lipogenesis and adipogenesis (Lehrke and Lazar, 2005). PPARγ agonists are used therapeutically as insulin sensitising agents, although patients using them frequently gain fat mass (Fuchtenbusch *et al.*, 2000).

As well as synthesising lipids, adipose tissue can take up and store free fatty acids (FFAs) in the form of TGs, known as FA re-esterification. Uptake is believed to occur either by diffusion of FAs to the inner membrane of the cell and/or active transport of FAs by transmembrane proteins. FFA uptake can also occur following hydrolysis of TGs by LPL. Due to the insolubility of lipids they travel through the bloodstream in lipoproteins, along with cholesterol and apolipoproteins. LPL is found on the luminal surface of capillaries and arteries where it hydrolyses circulating TGs from lipoproteins, producing FFAs which can be taken up by adipocytes (Wang and Eckel, 2009).

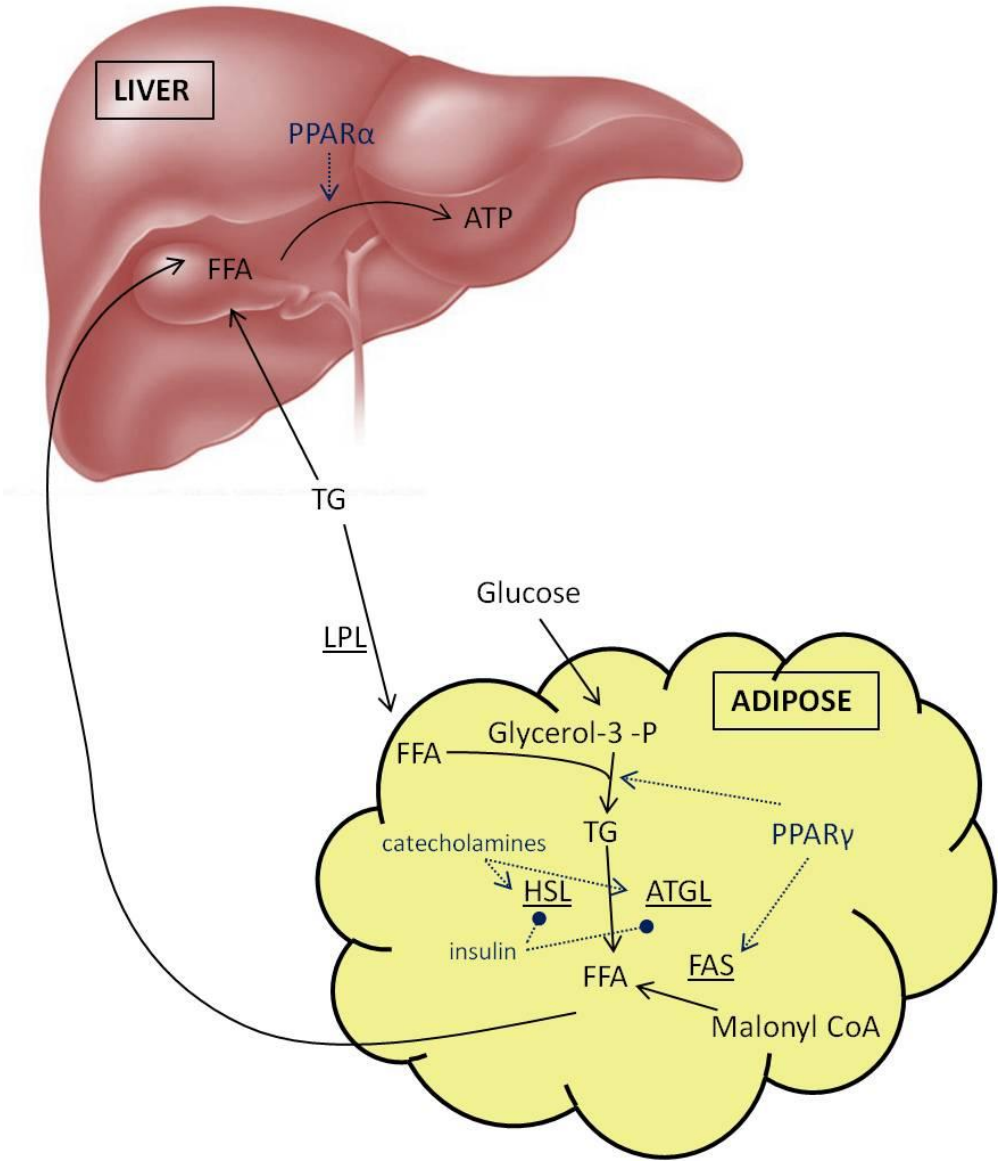


Figure 1.1 Lipogenesis, fatty acid re-esterification and lipolysis

Dietary lipids circulate in the blood stream as triglycerides (TGs), these can be metabolised by lipoprotein lipase (LPL) to release free fatty acids (FFAs) that enter the adipose tissue. TGs also undergo lipolysis inside the adipocytes in reactions catalysed by hormone sensitive lipase (HSL) and adipose triglyceride lipase (ATGL). The enzymes are activated and move to their site of action in response to catecholamines, whereas insulin deactivates them. FAs are oxidised in the liver to produce energy, the processes involved are up-regulated by the transcription factor peroxisome proliferator receptor (PPAR) α
Opposing lipolysis FFAs, which can be produced by fatty acid synthase (FAS), and glycerol-3-phosphate (P) are combined to produce TGs for storage. Lipogenesis and FA re-esterification are enhanced by PPARγ.

Lipolysis is the process of hydrolysing TGs to produce FAs and glycerol which can be released in to the bloodstream and used for energy. This occurs in times of fasting or exercise when energy is required. Lipolysis, like lipogenesis, is under hormonal regulation and can be stimulated by catecholamines during fasting or exercise. Catecholamines bind to β-adrenoreceptors which cause a signalling cascade ending in the activation and translocation of HSL and adipose triglyceride lipase (ATGL) (Greenberg *et al.*, 2011). HSL and ATGL are believed to be the rate determining enzymes in stimulated lipolysis due to their high expression in adipose tissue and high hydrolytic capacity (Schweiger *et al.*, 2006). Insulin inhibits lipolysis in the fed state when energy sources are abundant and storage necessary, by inhibiting the activation and translocation of HSL (Holm *et al.*, 2000; Su *et al.*, 2003). Obesity is associated with altered lipolysis, including increased basal rates of lipolysis and FA release which contribute to the development of insulin resistance (Arner, 1999).

1.1.2.2 Fatty acid oxidation

FAs can be oxidised in all tissues except the brain to produce energy. The enzymes involved in FA oxidation are situated in the mitochondria although very long chain FAs are preferentially metabolised in peroxisomes (Bronfman *et al.*, 1979). Before entering into the mitochondria for oxidation, FAs must be activated in the cytoplasm to produce Acyl-CoA. The subsequent process inside the mitochondrion, β-oxidation, occurs through sequential removal of 2-carbon units by oxidation at the β-carbon position of the fatty acyl-CoA molecule. These reactions generate NADH and $FADH_2$ which enter the electron transport chain and produce ATP. Each round of β-oxidation also produces a molecule of acetyl-CoA which enters the tricarboxylic acid cycle generating further cofactors to produce ATP (reviewed in (Rinaldo *et al.*, 2002). Acetyl-CoA also has a regulatory role; it inhibits its own production from glucose-derived pyruvate precursors, promoting gluconeogenesis (section 1.1.3.2). The oxidation of FAs produces much more energy per gram of substrate than that of carbohydrates, denoting their importance as an energy source.

Changes in FA oxidation have been suggested to play a role in the pathogenesis of obesity. Obese humans oxidise less fat after a meal than lean controls (Astrup *et al.*, 1997), and rats with inherited susceptibility to obesity have reduced capacity for fatty acid oxidation when lean or obese (Ji and Friedman, 2007). Lipid-lowering drugs, such as fibrates, have been proposed to elicit some of their effects by enhancing FA oxidation. This is due to their capacity to activate the perixosome-proliferator receptor α (PPARα) which induces expression of the enzymes that control β-oxidation (Minnich *et al.*, 2001). The role of

PAPRα in FA metabolism has been confirmed by the generation of KO mice which have both reduced FA oxidation and gluconeogenesis in the fasted state (Le May *et al.*, 2000).

1.1.3 Glucose metabolism

Glucose is the other primary source of energy in the body and can be used for metabolism or stored for later release. The product of glucose metabolism, 3-glycerophosphate (glycerol) is vital in lipid synthesis as it combines with FAs to generate TGs. Due to the importance of glucose and glycerol derived energy they can both be synthesised from non-carbohydrate sources in the liver and adipose tissue when their natural precursors are unavailable. This section discusses glucose synthesis and metabolism which primarily occurs in the liver, while most FA re-estrification and breakdown occurs in the adipose tissue.

1.1.3.1 Glucose storage

Although FA metabolism yields a greater amount of energy than carbohydrate, the brain and red blood cells can only metabolise glucose and thus a constant supply is required. Following feeding and increases in insulin, the liver removes excess glucose from the circulation and stores it as glycogen (which can be depolymerised when blood glucose begins to fall, in order to maintain glucose supply to the body). The first step in hepatic glycogenesis is the retention of glucose. This is achieved by the enzyme hexokinase which phosphorylates glucose, preventing it from leaving the cell. Enzymes involved in glycogen synthesis are activated by secondary messengers in response to insulin receptor activation (Miller and Larner, 1973). When insulin concentrations fall enzymes capable of glycogen degradation are activated.

1.1.3.2 Gluconeogenesis

When glycogen stores are exhausted following extended periods of fasting or starvation the liver begins *de novo* production of glucose from non-carbohydrate sources (Figure 1.2). This process, termed gluconeogenesis, drives the conversion of pyruvate to glucose and requires energy input. Due to their bi-directional nature most of the enzymes required for gluconeogenesis are also used in glycolysis, the process of generating energy from glucose. The main exception is phosphoenolpyruvate carboxykinase (PEPCK) which catalyses the conversion of oxaloacetate to phosphoenolpyruvate and is believed to be the rate limiting enzyme in gluconeogenesis (Rosella *et al.*, 1993). There are two forms of PEPCK, one cytosolic and one mitochondrial, although little is known about the function and regulation of the mitochondrial form (Hanson, 2009). The following information and references in this

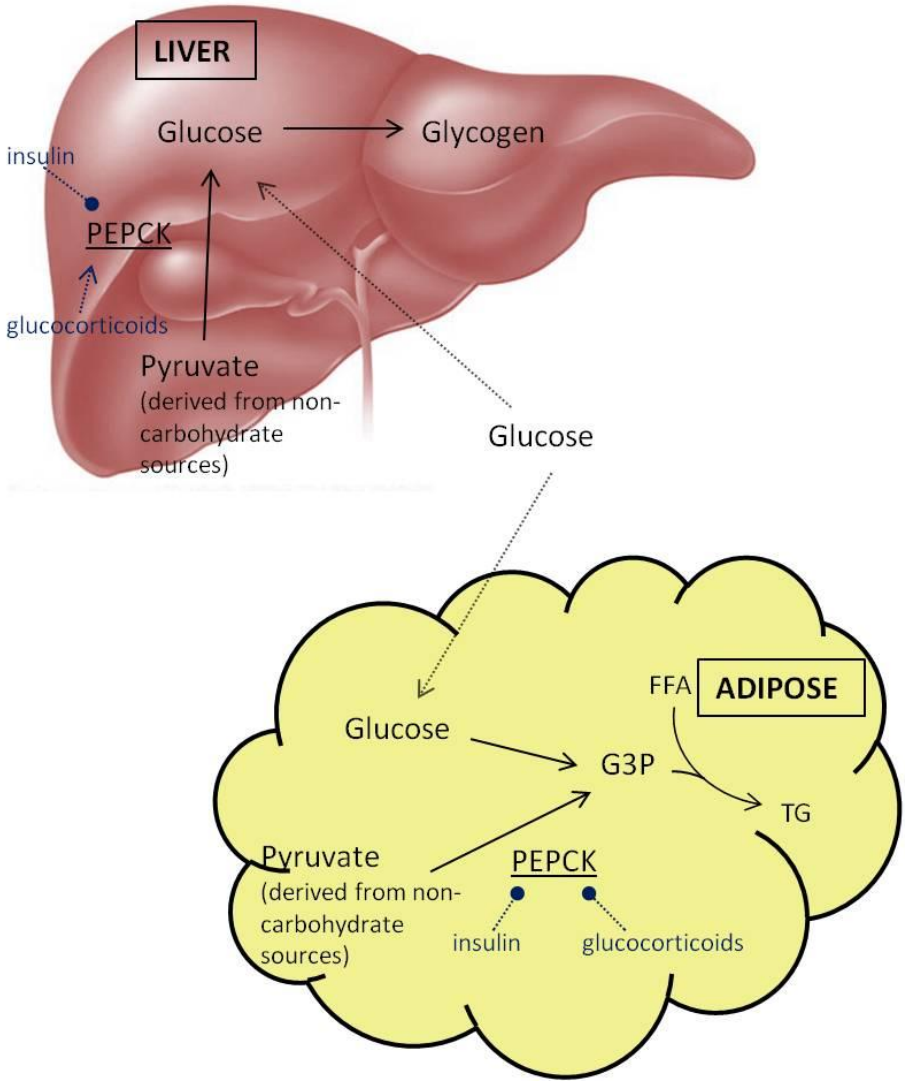


Figure 1.2 Gluco- and glyceroneogenesis

When glucose is not available in the liver it can be produced by gluconeogenesis from pyruvate. The rate limiting step in the process is the production of phosphoenolpyruvate from oxaloacetate which is catalysed by phosphoenolpyruvate carboxykinase (PEPCK). In the adipose tissue the same enzyme is responsible for glyceroneogenesis; generating glycerol 3-phosphate (G3P) from pyruvate. This can be used to store fatty acids in the form of triglycerides (TG). PEPCK is regulated by hormones; in the liver and adipose tissue insulin inhibits transcription of the enzyme whereas glucocorticoids activate transcription in the liver but inhibit it in the adipose.

thesis refer to the cytoplasmic enzyme. Transcription of PEPCK is rapidly inhibited by insulin, mediated by the insulin receptor-induced reduction of cAMP, which alters the transcription of many factors that normally initiate and activate PEPCK transcription (such as PPARγ coactivator-1α and CREB binding protein) (O'Brien *et al.*, 1990). Insulin also activates protein kinases which alter the phosphorylation state of proteins required for PEPCK transcription such as forkhead transcription factor, rendering it inactive (Nakae *et al.*, 2001). In the fasting state when gluconeogenesis is vital, PEPCK is stimulated by glucagon and glucocorticoids. Glucagon increases cAMP concentrations, through the activation of adenylyl cyclase, which can phosphorylate proteins that bind to sites in the PEPCK promoter initiating transcription (reviewed in (Chakravarty *et al.*, 2005)). Cortisol binds to glucocorticoid receptors (GR) which bind to glucocorticoid response elements in the PEPCK gene promoter and up-regulate transcription (Imai *et al.*, 1990). Insulin is believed to have the greatest transcriptional control over PEPCK due to the number of pathways and transcription factors it affects, and also due to its ability to overcome glucocorticoid-mediated activation (Sasaki *et al.*, 1984).

1.1.3.3 Glyceroneogenesis

PEPCK is also present in adipose tissue where it modulates glyceroneogenesis, the synthesis of 3-glycerophosphate in an abbreviated version of gluconeogenesis. Glyceroneogenesis can also occur in the liver, when pyruvate is converted to glycerol rather than glucose as in gluconeogenesis (Beale *et al.*, 2002). Mice over-expressing PEPCK in adipose tissue become obese due to the increased production of glycerol backbones required for FA re-esterification (Franckhauser *et al.*, 2002). Interestingly, despite obesity these mice do not become diabetic, further implicating circulating FFAs in the pathogenesis of insulin resistance. PEPCK is under hormonal control in the adipose tissue, as in the liver, although the same hormones do not elicit the same responses. In the fed state, glyceroneogenesis is decreased as glucose can be used to generate glycerol-3-phosphate and subsequently TGs. In contrast, on fasting or a low carbohydrate diet the synthesis of glycerol-3-phosphate is initiated in the adipose tissue (reviewed in (Reshef *et al.*, 2003). This pathway must be used to generate glycerol-3-phosphate as adipose does not express the enzymes required to produce it from glycerol released as a result of TG lipolysis (Schlender and Reimann, 1977). Insulin suppresses the transcription of PEPCK as in the liver; however, glucocorticoids also repress transcription of PEPCK in adipose, highlighting the tissue specific regulation of the enzyme (Nechushtan *et al.*, 1987). A separate promoter region to that important in liver, which lies further upstream from the transcription start site, controls PEPCK expression in the adipose tissue (Short *et*

al., 1992). Glucocorticoids diminish the binding of adipocyte-derived nuclear proteins that normally activate the PEPCK promoter, inhibiting their effects and therefore transcription of the gene (Olswang *et al.*, 2003). The transcription factor PPARγ, implicated in adipogenesis, is required to initiate transcription of PEPCK, by binding to elements in the gene promoter (Olswang *et al.*, 2002).

Obesity is associated with insulin resistance and type 2 diabetes with increased endogenous glucose production. This is due to dysregulation of gluco- and glyceroneogenesis in the liver such that PEPCK expression is enhanced and glucose and glycerol are still produced in the fed state (Gastaldelli *et al.*, 2000). Increases in plasma FFAs also promote insulin resistance and reduced expression of PEPCK in adipose tissue (Forest *et al.*, 1997) which stops their re-esterification into TGs resulting in further release of FFAs in to the plasma. PPARγ agonists have been promoted as insulin-sensitising agents and are believed to elicit their effects partly by enhancing expression of PEPCK in adipose and promoting FA re-esterification (Glorian *et al.*, 2001).

1.1.4 Adipose tissue

Whether genetic or dietary in origin, obesity is a disease of weight gain and, more specifically, fat accumulation. When the energy input is greater than demand the body stores the excess in adipose tissue for release in periods of fasting. Obesity does not occur instantly, but is a gradual process of adipose expansion occurring as the energy balance is consistently tipped in favour of input. For this reason, until recently, obesity was mainly seen in middle aged adults; however increasing rates of obesity are now seen in children. Adipose tissue is made up of adipocytes which contain lipid droplets. They are dynamic cells in normal physiology responsible for lipid uptake, synthesis, storage, and release.

1.1.4.1 Adipocyte hypertrophy and hyperplasia

Adipocytes are formed by differentiation of stromal preadipocytes. This requires growth arrest and clonal expansion of the precursors in response to several transcription factors including PPARγ (Rosen *et al.*, 1999). During the terminal phases of differentiation insulin sensitivity is conferred on the cell and the expression of many lipid metabolising enzymes increases (Gregoire *et al.*, 1998; Wu *et al.*, 1999). Evidence suggests adipogenesis can occur throughout life (Deslex *et al.*, 1987; Gregoire *et al.*, 1995) and in response to high fat and carbohydrate diets (Miller *et al.*, 1984) leading to the expansion of the tissue by increasing cell number.

In addition to hyperplasia, adipocytes have the capacity to increase in size and, therefore, in capacity for lipid storage. This hypertophy can occur by increased lipogenesis and/or decreased lipolysis in response to physiological changes. Adipose hypertrophy is important in protecting the liver and muscle from lipid accumulation. Indeed, a lack of adipose tissue, as in lipodystrophy, is associated with insulin resistance in the muscle and liver steatosis (Carr *et al.*, 1998; DeFronzo, 2004). However, increases in adipocyte size have also been correlated with insulin resistance, and obese individuals with fewer but larger adipocytes are more glucose intolerant and hyperinsulinemic than comparable individuals with a greater number of smaller adipocytes (Arner *et al.*, 2009).

1.1.4.2 The endocrine capacity of the adipose tissue

Over the last two decades much research has resulted in the recognition that adipose tissue is a metabolically active endocrine organ, rather than a simple lipid storage site (reviewed in (Ahima and Flier, 2000)). Adipocytes produce a wide range of factors involved in glucose-insulin homeostasis, inflammation, appetite regulation and vascular function. These include adiponectin, leptin, resistin, tumour necrosis factor α, IL-6, LPL, 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1), aromatase and unidentified vaso-dilatory factors. Of particular interest to this thesis is the capacity of adipose to metabolise steroid hormones, which will be introduced later (sections 1.2.3 & 1.3.1).

1.1.5 Rodent models of obesity

To allow further studies of the mechanisms involved in the progression of obesity and the associated metabolic disturbance, many rodent models have been developed. Unlike in humans where BMI is widely used to classify obesity there is no accepted definition in rodents. In addition to using BMI the WHO state that obesity is an abnormal or excessive fat accumulation that may impair health (WHO, 2011), therefore for the purpose of this thesis the term obesity is used to describe rodents with a significant increase in fat mass who have been consuming a high fat and sugar diet. The earliest models were found in large colonies of rodents and resulted from spontaneous genetic mutations. Subsequently, polygenic models of obesity were generated by breeding of selected obese individuals. Most recently attention has turned to diet-induced models of obesity that involve feeding rodents high fat and/or sugar diets somewhat reflective of the nutritional habits of obese humans.

1.1.5.1 Monogenic models

Monogenic models of obesity, often in combination with diabetes, exist in rats and mice and have proved useful in understanding energy expenditure. Ob/ob and db/db mice and the Zucker fatty rat were all discovered following spontaneous mutations generating obesity. Subsequent characterisation of the mutations revealed a defect in leptin production in the ob/ob mice (Zhang *et al.*, 1994b) and the leptin receptor in both the db/db mouse (Chen *et al.*, 1996a) and Zucker fatty rat (Phillips *et al.*, 1996). The loss of either the signalling molecule or receptor in ob/ob and db/db mice and Zucker fatty rats causes hyperphagia, obesity and insulin resistance. Both the ob/ob mouse (Herberg and Coleman, 1977) and Zucker fatty rat can maintain normal circulating glucose concentrations (Bray, 1977). However, the db/db mouse develops hyperglycaemia by 8 weeks of age (Herberg and Coleman, 1977), as does a sub-strain of Zucker fatty rats termed Zucker diabetic fatty rats (Friedman *et al.*, 1991), establishing these strains as models of obesity and diabetes. Models of monogenic obesity have been useful in understanding the function of leptin signalling and the regulation of energy intake. Some models also display similar microvascular complications to humans, such as diabetic retinopathy and nephropathy (Oltman *et al.*, 2005; Drel *et al.*, 2006). However, mutations in leptin are only responsible for a handful of obesity/diabetes cases in humans and have a different etiology to the worldwide epidemic of obesity and type 2 diabetes (Loos and Bouchard, 2003).

A third monogenic mouse model of obesity results from inheritance of the dominant *agouti* alleles A^{Y} or A^{vy}. The agouti gene normally functions to control production of melanin that gives rise to coat colour (Lu *et al.*, 1994). A range of dominant and recessive alleles of *agouti* exist, with those encoding yellow (^{Y}) being dominant. Expression of these ^{Y} alleles leads to maturity onset obesity, increased hepatic lipogenesis and hyperinsulinemia (Bultman *et al.*, 1992). In contrast to models in which leptin signalling is disrupted, these phenotypes do not arise as a consequence of marked hyperphagia.

1.1.5.2 Genetic modifications

In 2005 the murine obesity gene map identified 248 genes which affect body weight and adiposity when mutated or over-expressed in mice (Rankinen *et al.*, 2006). This number has increased since that report, particularly with the advances in technology which allow for the generation of tissue-specific modification of gene expression. Introducing transgenic models is, therefore, beyond the spectrum of this introduction. However, models pertinent to the

understanding of the results are introduced and discussed as necessary in subsequent sections and chapters.

1.1.5.3 Polygenic models

Most human obesity is likely to be mediated by multiple genes rather than single polymorphisms, suggesting polygenic rodent models may be more relevant in laboratory studies. Male New Zealand Obese mice are hyperphagic and have increased adiposity and type-2 diabetes (Herberg and Coleman, 1977) and their phenotype is believed to be due to a combination of hyperphagia, reduced energy expenditure and insufficient physical activity. A fat (F) line of mice was generated by Bunger and colleagues in Edinburgh following long-term selection for fatness over 60 generations; with the phenotype shown to be independent of leptin and the leptin receptor (Bunger *et al.*, 2002). These mice have increased weight gain and adiposity compared to controls, as well as reduced physical activity. Polygenic models of obesity are often fed high fat and sugar diets to exacerbate their phenotypes.

1.1.5.4 Diet-induced models

Although particular genes are likely to play a role in human obesity, the imbalance of energy input and usage is the primary causative factor. To reflect this, many rodent models of diet-induced obesity have been generated. Initial studies fed rats a 'cafeteria' type diet, this involved refined human foods high in fat and sugar which promote hyperphagia (Rolls *et al.*, 1980). The main problem with cafeteria diets is the unknown micro and macronutrient composition which makes it difficult to ascertain which additional dietary components may be important in the generation of the phenotype. Despite this problem, cafeteria diets are very good at promoting obesity and the associated metabolic consequences and, as such, are still used in both rat and mouse models. To try and control for micro and macronutrient content, highly palatable homogenous commercial diets are more frequently used. These can be high in fat and cholesterol, known as the 'Western' diet, or high in fat and sugar - the 'obesogenic' diet. Body fat accumulates in correlation with the amount of dietary fat in rats and mice; this increase is gradual and only plateaus when extreme fat deposition occurs (Boozer *et al.*, 1995; West *et al.*, 1995). The effects of obesogenic and cafeteria diets are not uniform across strains, with some displaying apparent resistant to weight gain. A study investigating 9 strains of mice found 6 to be sensitive to the effects of consuming a high fat diet, with the other 3 apparently resistant to the development of obesity (West *et al.*, 1992). Outbred Sprague-Dawley rats have a spectrum of responses within the strain when fed a high fat diet; some animals show excessive weight gain while others are resistant (Levin and

Keesey, 1998). These studies support the proposed polygenic nature of obesity, and the use of dietary obesity in the selection of obesity sensitive animals.

One of the most frequently used murine models of diet-induced obesity (DIO) is the C57BL/6 strain. This was originally identified as being obesity sensitive by Surwit and colleagues in 1988. It remains lean when fed a chow diet but becomes obese when fed an obesogenic diet (Surwit *et al.*, 1988). The mice develop many of the physiological changes associated with human obesity including insulin resistance and dyslipidemia. Because of their propensity to develop obesity and type 2 diabetes they have been extensively used to investigate the molecular events which may underlie changes in feeding, and fuel storage and usage in obesity

1.1.6 Obesity increases risk of cardiovascular disease

With the rise in obesity comes an increase in the incidence of many other conditions and diseases, particularly those which increase risk of cardiovascular disease. This is a huge economic burden and a review of the published literature from Europe suggested an obesity-related healthcare cost of €10.4 billion in 2009 (Muller-Riemenschneider *et al.*, 2008).

1.1.6.1 Insulin resistance and type-2 diabetes

Insulin resistance is a pre-cursor for type 2 diabetes and is characterised by excess insulin production due to lack of a physiological effect. Insulin enhances glycogenesis and glucose uptake whilst inhibiting gluconeogenesis, thus, promoting the usage and storage of ingested carbohydrate (see section 1.1.3). In insulin resistance this shift of metabolism does not occur and the body responds by increasing insulin synthesis and release. It is possible to maintain blood glucose within the normal range by increasing insulin production; however, type-2 diabetes develops when glucose can no longer be kept in the normal physiological range, due to increased insulin resistance at a tissue level and inadequate insulin secretion. In Scotland, over 80% of people diagnosed with type-2 diabetes are overweight or obese (SDSM Group, 2011), and the correlation between the prevalence of the two conditions has led to the term ‘diabesity’ being used. The exact aetiology of insulin resistance and type 2 diabetes is not clear, although improvement in insulin sensitivity following weight loss highlights the detrimental effects of adipose deposition. It has been proposed that elevated FFAs and the expansion of adipose tissue mediate the development of insulin resistance. Data suggests that FFAs may: (1) compete with glucose as the energy substrate, decreasing glucose oxidation, (2) inhibit insulin signalling which reduces glucose uptake, (3) antagonise the effects of insulin promoting gluconeogenesis and (4) potentially affect insulin secretion (reviewed in

(Boden, 1999)). In addition to the changes in lipid metabolism, obesity is accompanied by chronic low-grade inflammation in the adipose tissue which may also play a role in the development of insulin resistance (Dandona *et al.*, 2004). Diabetes can lead to other health problems including nephropathy and retinopathy; both due to the effects of elevated blood glucose concentrations on the vasculature.

1.1.6.2 Dyslipidemia

An elevated lipid concentration in the blood may be mediated by an increase in dietary cholesterol and triglycerides, increased lipid storage and release by lipolysis, and changes in FA oxidation. Whilst lipids are vital in many physiological functions, in excess they can disrupt metabolism causing conditions such as insulin resistance (section 1.1.6.1) or accumulate in atherosclerositic plaques in the vasculature (section 1.1.6.5).

1.1.6.3 Vascular dysfunction

Obesity is associated with hypertension and atherosclerosis (discussed in subsequent sections 1.1.6.4 & 1.1.6.5), both of which may involve changes in vascular function. In addition, poorly controlled type 2 diabetes can cause microvascular diseases, such as retinopathy, by decreased tissue perfusion. The association of these diseases suggest that vascular physiology can be modulated by excess adiposity and glucose. In obese subjects (Arcaro *et al.*, 1999; Van Guilder *et al.*, 2006) and those who are insulin resistant (Steinberg *et al.*, 1996; Steinberg and Baron, 2002) endothelium-dependent, such as response to acetylcholine, but not -independent vasodilation is impaired. Insulin can activate endothelial nitric oxide synthase (eNOS) resulting in local production of nitric oxide and the relaxation of smooth muscle cells (Steinberg *et al.*, 1994). In obesity it is proposed that insulin resistance affects endothelium-dependent relaxation as a consequence of inadequate insulin receptor activation and inhibition of eNOS-dependent nitric oxide production. A blunted response to endothelium-dependent vasodilators is found in some animal models of obesity and type 2 diabetes (Bourgoin *et al.*, 2008; Belin de Chantemele *et al.*, 2009), in association with decreased Akt and eNOS phosphorylation as well as reduced basal transcription of eNOS and dimer formation (Molnar *et al.*, 2005; Kim *et al.*, 2008; Fulton, 2009; Symons *et al.*, 2009). One study in mice found evidence for insulin resistance in the vasculature before liver and adipose tissue (Kim *et al.*, 2008) suggesting it may be an early complication of obesity. In addition to altered nitric oxide production in obesity and insulin resistance, studies suggest increased removal may also be responsible for the changes in vasodilation. This is due to higher concentrations of reactive oxygen species, which act as scavengers to clear nitric

oxide, reported in obesity (Cai and Harrison, 2000; Williams *et al.*, 2002; Bakker *et al.*, 2008). Increased concentrations of NEFAs, due to increased endogenous lipolysis seen in obesity, may also affect endothelium-dependent vasodilation. Infusion of NEFAs blunts the response to metacholine, a muscarinic receptor agonist activating the same pathways as acetylcholine. The mechanisms involved are not clear but reduced nitric oxide production and release, and increased release of vasoconstrictor substances have been proposed (Steinberg and Baron, 2002).

1.1.6.4 Hypertension

Hypertension is a chronic medical condition in which the arterial blood pressure is elevated. Around 90% of hypertension does not have an identified cause but obesity is considered to be a major risk factor. Activation of the renin-angiotensin-aldosterone system, increased sympathetic nervous system activity, insulin resistance, leptin resistance, increased pro-coagulatory activity and endothelial dysfunction have all been proposed as mechanisms linking obesity to hypertension (reviewed in (Davy and Hall, 2004). Normal weight hypertensive individuals are more likely to become obese than normotensive individuals, suggesting that the link may be bidirectional (Julius *et al.*, 2000).

1.1.6.5 Atherosclerosis

Obesity is an independent risk factor for atherosclerosis which is characterised by lesions in medium and large sized arteries due to lipid accumulation in vessel walls. The development of atherosclerosis involves an interaction between plasma lipids, the vascular endothelium, inflammatory cells, platelets, and vascular smooth muscle cells (VSMCs); initiated by chronic vascular injury (reviewed in (Ross, 1999; Libby, 2002). The lesions are largely asymptomatic but can limit blood flow leading to tissue hypoxia such as in angina. The most damaging consequence of atherosclerosis occurs when a plaque erodes or ruptures often leading to myocardial infarction and stroke. Endothelial cell dysfunction is proposed as an early event in the pathogenesis of atherosclerosis (Bonetti *et al.*, 2003), and its occurrence is increased in obesity. In addition to changes in vascular physiology, the increased circulating lipid concentrations in obesity are likely to increase risk of the disease.

1.1.6.6 Lesion formation

Treatment for atherosclerosis includes changes to diet and lifestyle as well as pharmacotherapy. However, mechanical procedures such as percutaneous coronary intervention (PCI) can revascularise the affected vessel and are the most common treatment

in advanced stages of the disease. Although this treatment is successful, it inevitably causes a degree of damage to the vessel wall particularly when a stent is implanted. The acute injury results in formation of intimal lesions (Kibos *et al.*, 2007) which are rich in VSMCs and extracellular matrix, unlike atherosclerotic lesions. Greater intimal-media thickness is found in obese subjects, which is correlated with increased risk of lesion formation and cardiovascular events (O'Leary *et al.*, 1999; Kotsis *et al.*, 2006). In addition restenosis following bare metal stent implantation is more common in obese patients (Asakura *et al.*, 1998; Nikolsky *et al.*, 2005). Increases in insulin concentrations, common in obesity, can contribute to increased arterial wall thickness by direct trophic effects as well as by changes in second messengers which stimulate growth and proliferation of VSMCs and may alter lesion formation in response to vascular injury (Stapleton *et al.*, 2008).

1.2 Glucocorticoids

In addition to the effects of dietary composition already introduced, steroid hormones play a role in controlling metabolism and have been implicated in the pathogenesis of obesity. Glucocorticoids are synthesised from cholesterol in the adrenal cortex. They are involved in many physiological processes including development, metabolism, response to stress and immunity. In recent decades evidence has emerged proposing a link between altered glucocorticoid metabolism and the development of obesity and associated cardiovascular diseases. The link will be further explored in this thesis and therefore glucocorticoids are introduced in the following section.

1.2.1 Synthesis and secretion of glucocorticoids

Both the synthesis and the secretion of glucocorticoids are controlled by the hypothalamic-pituitary-adrenal (HPA) axis (Figure 1.3). The hypothalamus secretes corticotrophin-releasing hormone (CRH) and arginine vasopressin (AVP) which cleave pro-opiomelanocortin (POMC) in the anterior pituitary to produce adrenocorticotrophic hormone (ACTH). This hormone is released in to the circulation to travel to the adrenal glands and stimulate glucocorticoid synthesis. ACTH can also induce the synthesis and secretion of mineralocorticoids and androgen precursors from the adrenal.

Glucocorticoids are synthesised from cholesterol in the zona fasciculata of the adrenal cortex. Their synthetic pathway is catalysed by many enzymes including members of the cytochrome P450 family and 3β-dehydrogenase (Figure 1.4). ACTH mediates this synthesis

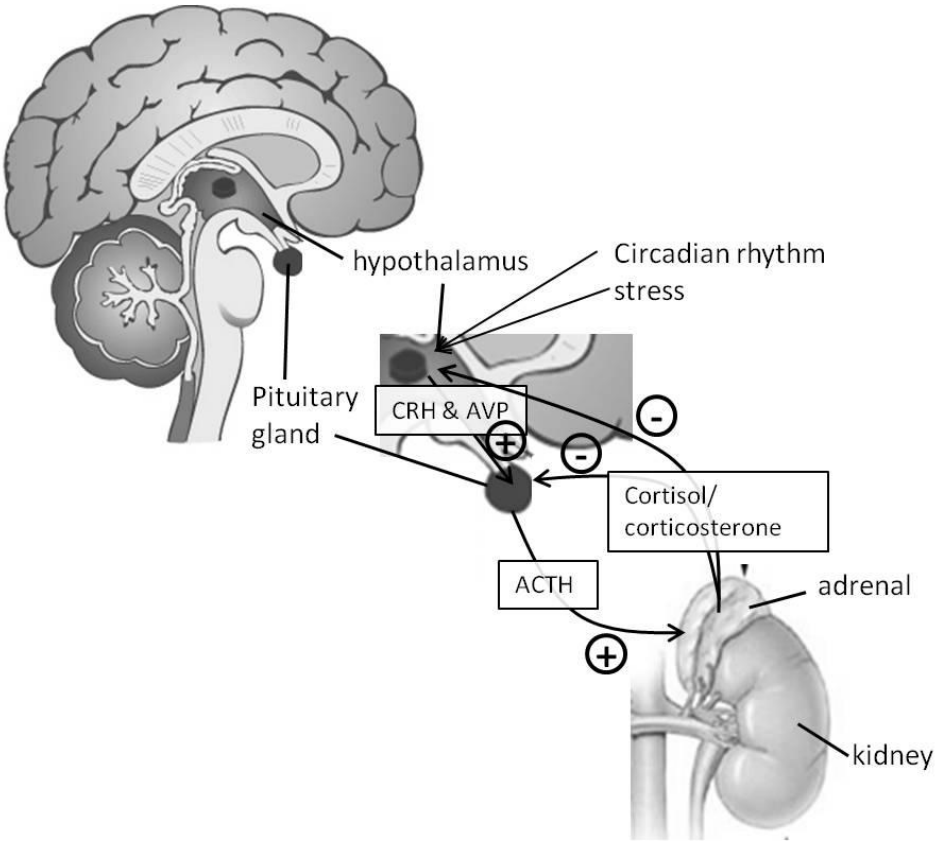


Figure 1.3 Hypothalamic-pituitary-adrenal axis

The hypothalamic-pituitary-adrenal (HPA) axis is responsible for the production of glucocorticoids from their cholesterol precursors in the adrenal glands. The HPA axis is regulated by circadian rhythms and negative feedback. Stimulation of the hypothalamus induces secretion of corticotrophin-releasing hormones (CRH) and arginine vasopressin (AVP). This causes release of adrenocorticotrophin (ACTH) from the pituitary gland which travels through the circulation to the adrenal gland where it instigates production of cortisol in humans and corticosterone in rodents. Glucocorticoids inhibit production of CRH and ACTH controlling their own release.

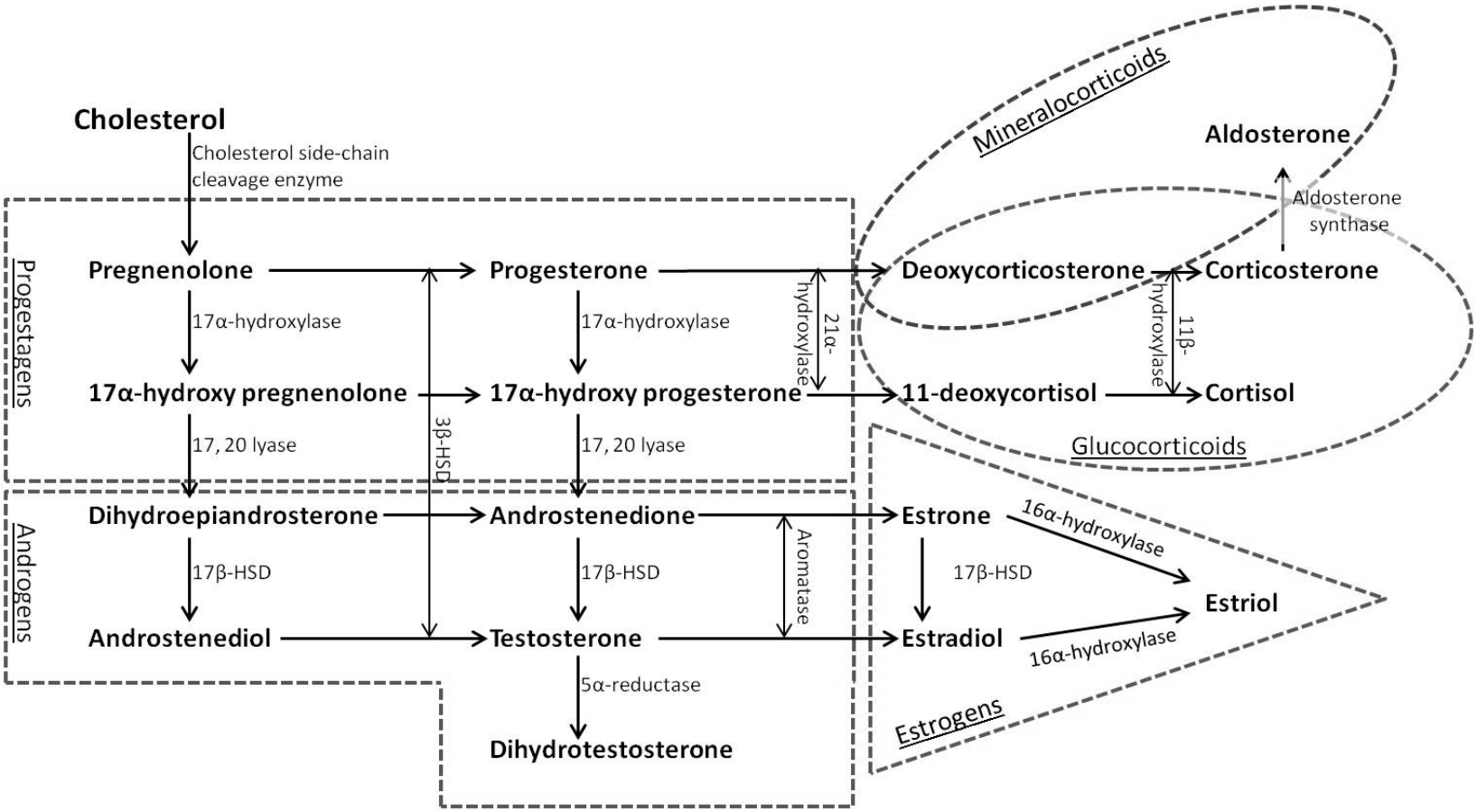


Figure 1.4 Steroid synthesis

Pathways involved in the biosynthesis of steroids from cholesterol. HSD=hydroxysteroid dehydrogenase.

by increasing cholesterol uptake in cortical cells, stimulating the transport of cholesterol to the mitochondria and increasing cholesterol binding to an enzyme which cleaves cholesterol to pregnenolone (Simpson and Waterman, 1988). In man, cortisol is the primary glucocorticoid, whereas in rodents corticosterone is dominant due to the lack of 17α hydroxylase (van Weerden *et al.*, 1992).

The HPA axis is tightly controlled by many different mechanisms. CRH is released in response to physical and psychological stressors such as heat, infection or emotional stress. Glucocorticoids regulate their own concentrations in plasma by negative feedback, with elevated levels inhibiting the transcription and release of CRH, and the transcription and cleavage of POMC (Gagner and Drouin, 1985; Ma *et al.*, 2001). Additionally there is a 24hr circadian rhythm of circulating glucocorticoid levels. In humans, glucocorticoid concentrations are highest in the morning with the nadir in the evening (Nussey and Whitehead, 2001). In nocturnal animals, levels are lowest in the morning, rising over the day during the rest phase (Windle *et al.*, 1998). Glucocorticoid availability is also controlled by its interaction with carrier proteins; 80-90% is bound to corticosteroid binding-globulin (CBG) and 10-15% is bound to albumin (Lewis *et al.*, 2005). The remaining ~5% is free to diffuse into cells and bind to receptors. Hormones bound to carrier proteins are believed to be inactive and provide a 'back-up' of circulating hormone if required.

1.2.2 Glucocorticoid signalling

The effects of glucocorticoids are mediated by two nuclear receptors; glucocorticoid receptors (GR) and mineralocorticoid receptors (MR). GRs are widely expressed and are responsible for the majority of the physiological effects of glucocorticoids. MRs have a higher affinity for glucocorticoids than GRs but are only expressed in certain tissues (Rupprecht *et al.*, 1993). Illicit activation of MR by glucocorticoids in these tissues is controlled by co-localisation of MRs to the enzyme 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2) (introduced later in section 1.2.3.2) which can metabolise glucocorticoids to inert metabolites.

When glucocorticoids are not present, GR is held in the cytoplasm as part of a large protein complex which maintains an inactive conformational state which is favourable to high-affinity ligand binding. Upon binding of glucocorticoids the protein complex dissociates and the active receptor dimerises and translocates to the nucleus. Once in the nucleus the activated GR binds to DNA at glucocorticoid response elements (GRE), upstream of the promoter region in glucocorticoid sensitive genes (Smoak and Cidlowski, 2004). The

resulting effect is to either induce or repress transcription of the target gene. The number of target genes of GR differs between cell types with at least 100 in some (Barnes, 1998; Reddy *et al.*, 2009). GR can also indirectly regulate gene expression in its monomeric form through interaction with other transcription factors (Beck *et al.*, 2009). Recent studies have shown that glucocorticoids can also exert effects through many non-genomic pathways. This includes incorporation into lipid membranes to alter the activity of membrane bound proteins, association with membrane-bound receptors and changing the phosphorylation state of caveolin resulting in activation of the protein kinase B/Akt pathway (Stahn and Buttgereit, 2008).

1.2.3 Glucocorticoid metabolism

Glucocorticoid concentrations are additionally regulated by metabolism within target tissues (Figure 1.5). The steroids can be converted between active and inactive forms by two isozymes of the 11β-hydroxysteroid dehydrogenase (11β-HSD) enzyme, thus controlling the activation of GR and MR. Permanent clearance and metabolism of glucocorticoids predominantly occurs in the liver controlled by a series of enzymes which catalyse A-ring reduction. The enzymes involved in glucocorticoid metabolism will be introduced below.

1.2.3.1 11β-HSD1

11β-HSD1 is a bi-directional enzyme *in vitro* but reductase activity predominates *in vivo*, converting inactive 11-keto metabolites into their active form. Transgenic mice lacking functional 11β-HSD1 cannot convert 11-dehydrocorticosterone to corticosterone (Kotelevtsev *et al.*, 1997). The direction of the reaction *in vivo* is controlled by a second enzyme hexose-6-phosphate dehydrogenase (H6PDH). Both 11β-HSD1 and H6PDH are found in the lumen of the ER, where H6PDH generates the reduced cofactor nicotinamide adenine dinucleotide phosphate (NADPH) which is required for the reductase activity of 11β-HSD1 (Bujalska *et al.*, 2005). The reliance of 11β-HSD1 on the provision of this cofactor is demonstrated in H6PDH knock-out mice which cannot perform the reductase reaction (Lavery *et al.*, 2006). 11β-HSD1 is widely expressed in the body, mainly in tissues with a high proportion of GR such as liver, adipose tissue, skeletal muscle, lung, brain, gonads and VSMCs and its expression and activity is controlled by many factors including cytokines, insulin, growth factors, sex steroids and glucocorticoids. Studies suggest that the expression and activity of 11β-HSD1 is lower in adipose tissue of lean women than men (Paulsen *et al.*, 2007) which may result in sex specific profiles of glucocorticoids. It is widely believed that 11β-HSD1 is important in amplifying local glucocorticoid

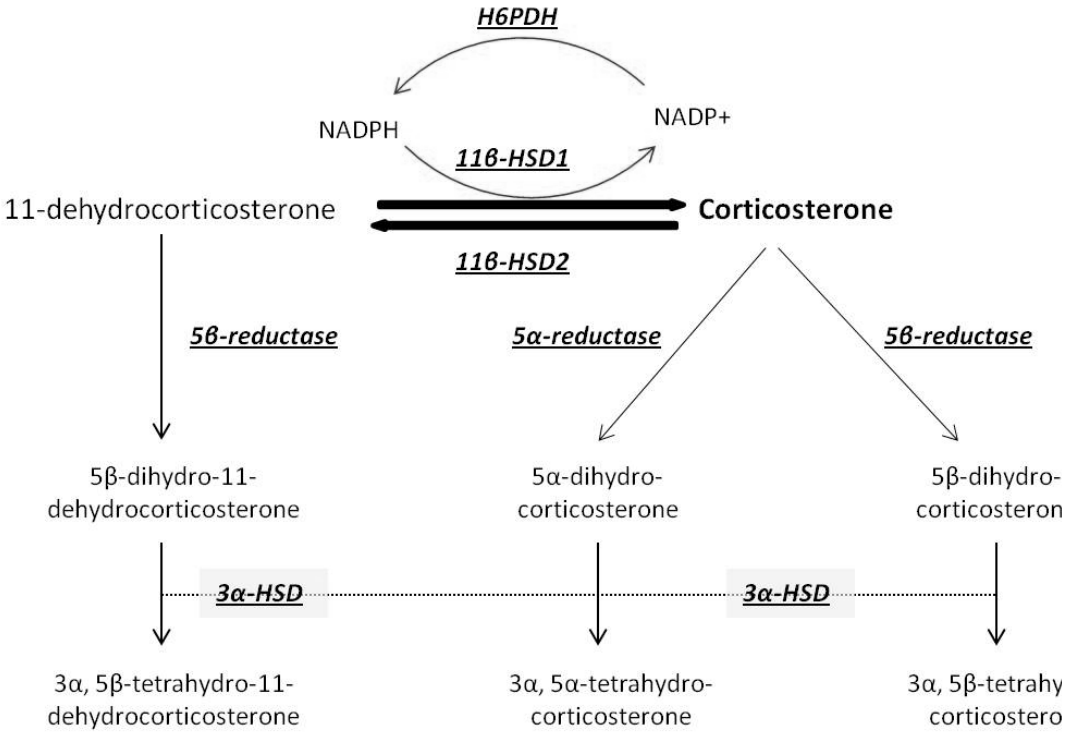


Figure 1.5 Glucocorticoid metabolism

Pathways and enzymes involved in the metabolism of corticosterone, the active glucocorticoid in rodents. HSD= hydroxysteroid dehydrogenase, H6PDH = hexose-6-phosphate dehydrogenase.

concentrations in target tissues as well as contributing to circulating concentrations (Seckl and Walker, 2001).

1.2.3.2 11β-HSD2

The second isozyme of 11β-HSD is dependent on the cofactor NAD+ for dehydrogenation of glucocorticoids *in vivo* (Brown *et al.*, 1993). 11β-HSD2 catalyses the conversion of active glucocorticoids, cortisol and corticosterone, to their inactive 11-keto metabolites. The enzyme is predominantly expressed in mineralocorticoid target tissues such as the kidney, salivary glands and sweat glands (Walker and Stewart, 2003). It is believed that the primary function of 11β-HSD2 is to inactivate glucocorticoids and thus protect MRs from inappropriate activation (Funder *et al.*, 1988). 11β-HSD2 is also highly expressed in the placenta, where it is thought to protect the developing fetus from unnecessary glucocorticoid receptor activation which could affect development (see section 1.4.1).

1.2.3.3 5α-Reductase

5α-Reductase (5αR) catalyzes the reduction of a double bond in active glucocorticoids yielding 5α-dihydro metabolites. Two isozymes of 5αR exist, which are products of 2 different genes (Russell and Wilson, 1994). While both are involved in the reduction of steroids, their location and substrate preferences differ. 5αR (type 1) is found in the liver, adipose tissue and brain; this isozyme is predominantly discussed in this thesis due to its role in glucocorticoid metabolism (thus any references to the enzyme should be considered to be type 1 unless otherwise stated). 5αR2 (type 2) is expressed in male reproductive tissues such as prostate, epididymis and seminal vesicles (Russell and Wilson, 1994). The type 2 isozyme reduces testosterone to 5α-dihydrotestosterone, which is a more potent ligand at the androgen receptor than testosterone (Siiteri and Wilson, 1974). In rodents 5αR1 has a greater affinity for androgens, progesterones and glucocorticoids than 5αR2 (Normington and Russell, 1992). In the liver and adipose tissue, 5αR1 is involved in the reduction of active glucocorticoids, though the products of the reaction are still able to activate the glucocorticoid receptor (McInnes *et al.*, 2004).

1.2.3.4 5β-Reductase

5β-Reductase (5βR) is also involved in the metabolism of steroids, reducing the double bond within the A ring. Unlike the products of 5αR, the resulting 5β-dihydro metabolites have a cis formation. The expression of this reductase is also more limited, mostly found in the liver with a small amount expressed in kidney (Charbonneau and The, 2001). Mutations in 5βR

lead to defects in bile acid metabolism suggesting this is one of its key functions (Palermo *et al.*, 2008). In terms of glucocorticoid metabolism, 5βR can reduce both active and inactive metabolites, with the resulting products unable to elicit a response at the glucocorticoid receptor (McInnes *et al.*, 2004).

1.2.3.5 3α-Hydroxysteroid dehydrogenase

5α- and 5β-dihydro metabolites are rapidly reduced *in vivo* by 3α-hydroxysteroid dehydrogenase (3α-HSD) to produce 5α- or 5β-tetrahydro metabolites. It is believed that the role of 3α-HSD is to catalyze permanent metabolism of steroids to inactive forms. However *in vivo*, 3α-HSD can function in both directions depending on the availability of cofactors $NADP^+$ and NADPH. In prostate 3α-HSD regenerates 5α-dihydrotestosterone (Rizner *et al.*, 2003) which, as mentioned, is more potent than testosterone itself, suggesting that 3α-HSDs are important in controlling the concentrations of biologically active 5α-dihydro metabolites.

1.2.4 Physiological effects of glucocorticoids

Glucocorticoids have a broad range of effects throughout the body; these depend upon the target tissue, the receptor that is activated and the length of exposure to the steroid. The importance of glucocorticoids is highlighted in two diseases: Addison's disease which is due to a deficiency in glucocorticoid production, and Cushing's syndrome associated with glucocorticoid excess. Patients with Addison's disease suffer muscle weakness, hypotension, weight loss, hypoglycaemia and depression. Cushing's syndrome is characterised by abdominal obesity, hypertension, insulin resistance, dyslipidemia and increased cardiovascular disease risk. Glucocorticoids are involved in many physiological processes and have a key role in mediating inflammation; those discussed below are of particular relevance to this thesis.

1.2.4.1 Developmental effects

Glucocorticoids are vital for normal fetal development. Evidence for this comes from both mouse and man; mice with genetic disruption of GR die within a few hours of birth due to respiratory failure (Cole *et al.*, 1995a) and synthetic glucocorticoids are administered clinically to fetuses at risk of pre-term delivery in order to accelerate lung development (Crowley *et al.*, 1990). GR is expressed from mid-gestation, corresponding with a rise in maternal cortisol levels which correlates with increased organ maturity of the fetus (Cole *et al.*, 1995b). Glucocorticoids are important for the growth and development of the brain, heart, lungs and many other organs. While the evidence clearly demonstrates glucocorticoid

signalling is vital during development, excess exposure of the fetus is associated with an increased risk of diabetes, hypertension and obesity in adulthood (Doyle *et al.*, 2000; Dalziel *et al.*, 2005), discussed in more detail in section 1.4.1.

1.2.4.2 Metabolism

Glucocorticoids mobilise energy sources for usage in the form of glucose and NEFAs, opposing the effects of insulin which induces fuel storage. The effects are mediated by many tissues; in the brain glucocorticoids increase appetite, to boost intake of fat and sugar (Dallman *et al.*, 2004). In the liver, glucocorticoids activate gluconeogenic pathways through increased transcription of the enzymes PEPCK (Imai *et al.*, 1990) and glucose-6- phosphate (G6P) (van Schaftingen and Gerin, 2002). Additionally, glucocorticoids initiate the release of hepatic fatty acids and decrease liver mitochondrial β-oxidation (Macfarlane *et al.*, 2008). In skeletal muscle, glucocorticoids induce proteolysis to generate substrates for gluconeogenesis in the liver (Jackman and Kandarian, 2004), and reduce glucose uptake to maintain a circulating energy source. The effects of glucocorticoids in the adipose tissue are dependent on other hormones, namely insulin and catecholamines. When insulin is low and catecholamines high, glucocorticoids induce lipolysis to provide an energy source in the form of NEFAs to the surrounding tissues. When insulin is high and catecholamines low, and therefore lipolysis is suppressed, glucocorticoids have the opposite effect and promote lipid storage (Macfarlane *et al.*, 2008).

1.2.4.3 Blood pressure

Glucocorticoids act through multiple mechanisms in the kidney, heart and vasculature to maintain normal blood pressure. The importance of glucocorticoids in the maintenance of blood pressure is evident in the clinical conditions Cushing's disease, in which excess glucocorticoids cause hypertension, and Addison's disease, in which a lack of glucocorticoids is associated with hypotension. In the kidney, glucocorticoids increase salt retention leading to water retention and an increase in plasma volume. This is due to the effects of GR and MR on sodium retention in the nephron, although subtle effects in other organs may also be involved (Grunfeld, 1990). Glucocorticoids have direct effects on the vasculature through GR, and are proposed to increase contractility and therefore peripheral resistance. The exact mechanisms of glucocorticoid-dependent arterial contraction are imperfectly understood. Although glucocorticoids characteristically contract vessels *in vivo* they do not directly induce contraction in isolated arteries and veins (Hadoke *et al.*, 2006b). The changes in peripheral resistance may also reflect a reduction in vasodilation as

glucocorticoids have been shown to reduce-acetylcholine mediated vasorelaxation and also reduce the synthesis of nitric oxide and prostaglandins (Mangos *et al.*, 2000). However, this has not been reproduced in the literature or our laboratory without concomitant inhibition of 11β-HSD1 which may also impair endothelial cell function (Personal communication Dr. P Hadoke, University of Edinburgh). Glucocorticoids can cause hypertension by activation of MR, which is highly expressed in the kidney and has a higher affinity for the steroid than GR. Renal MR is normally protected by 11β-HSD2 which metabolises cortisol; however, deficiency of this enzyme in humans (Ferrari, 2010) and rodents (Kotelevtsev *et al.*, 1999) results in hypertension.

1.2.5 Glucocorticoids and obesity

There is undoubtedly a correlation between circulating levels of cortisol and the development of obesity and metabolic complications seen in Cushing's disease. Diet-induced obesity is also associated with many cardiovascular risk factors such as insulin resistance, dyslipidemia and hypertension; collectively termed the metabolic syndrome (see section 1.1.6). The similarities between Cushing's disease and the metabolic syndrome led to a number of studies investigating the hypothesis that alterations in glucocorticoid production or secretion may contribute to the development of obesity and the metabolic syndrome. Secretion of cortisol from the adrenals is increased in obese subjects and plasma concentrations correlate with features of the metabolic syndrome (reviewed in (Seckl *et al.*, 2004). Many studies in humans and animal models have demonstrated the importance of local, or pre-receptor, glucocorticoid metabolism in the liver and adipose tissue in the development of obesity and the associated cardiovascular risk factors.

1.2.5.1 Human studies

The development of stable isotope tracers enabled the activity of 11β-HSD1 to be measured in humans and demonstrated substantial peripheral regeneration of cortisol (Sandeep *et al.*, 2005). In obese subjects, hepatic glucocorticoid metabolism by 11β-HSD1 is reduced whereas expression and activity of the enzyme is increased in subcutaneous adipose tissue biopsies (Rask *et al.*, 2001), with the latter confirmed by *in vivo* microdialysis (Sandeep *et al.*, 2005). However, this is not the case in all studies; one study reported no difference in 11β-HSD1 transcription in subcutaneous adipose tissue from lean and obese subjects, and a negative correlation between BMI and 11β-HSD1 activity in omental preadipocytes (Tomlinson *et al.*, 2002). More heterogeneity in the human literature exists with respect to glucocorticoid regeneration in visceral adipose tissue, with some studies reporting that this is

increased in obesity (Desbriere *et al.*, 2006; Michailidou *et al.*, 2007; Veilleux *et al.*, 2009), whereas others report no change (Tomlinson *et al.*, 2002; Simonyte *et al.*, 2009). It is likely that conflicting results can be attributed to the type of adipose material (preadipocyte, adipocytes or whole tissues), the health of the patients, and the level of analysis (mRNA expression or enzymatic activity). Interestingly, most studies investigating the activity of 11β-HSD1 in humans report higher levels in visceral adipose than subcutaneous (Bujalska *et al.*, 1999; Simonyte *et al.*, 2009; Veilleux *et al.*, 2009) which may suggest greater glucocorticoid regeneration in males due to their propensity to store adipose in this depot. In addition the enzyme activity is correlated with adipocyte size (Veilleux *et al.*, 2009).

1.2.5.2 Rodent models

Obese Zucker rats, which are leptin resistant due to a mutation in the leptin receptor gene, have tissue specific alterations in glucocorticoid metabolism. In the liver, 11β-HSD1 expression and activity are decreased whilst 5αR activity is increased (Livingstone *et al.*, 2000a), suggesting reduced local concentrations of active glucocorticoids. In contrast, in visceral adipose tissue, 11β-HSD1 activity is increased, predicting enhanced regeneration of glucocorticoids and potentially promoting obesity (Livingstone *et al.*, 2000a). Another group demonstrated changes in 11β-HSD1, and GR and MR signalling in the brains of obese Zucker rats (Mattsson *et al.*, 2003) which may influence the HPA axis. Studies in *ob/ob* mice report similar tissue-specific changes in glucocorticoid metabolism to those seen in Obese Zucker rats. In *ob/ob* mouse liver, 11β-HSD1 activity, but not mRNA, is decreased and 5βR expression and activity increased, whereas no changes in 5αR expression were seen (Livingstone *et al.*, 2009). In adipose tissue, 11β-HSD1 expression and activity were increased in visceral depots but decreased in subcutaneous depots (Livingstone *et al.*, 2009). This suggests that the anatomical location of adipose may be important in controlling glucocorticoid metabolism and is in agreement with the findings that the metabolic complications associated with visceral adipose accumulation are more profound than those seen with subcutaneous deposition. Whilst the obesity in Zucker rats and *ob/ob* mice is primarily due to changes in leptin signalling, treatment with glucocorticoid receptor antagonists reverses the obese phenotype (Yukimura *et al.*, 1978; Shimomura *et al.*, 1987). This highlights alterations in glucocorticoid metabolism as a secondary, or additional, mechanism leading to obesity and the metabolic syndrome in these models.

Changes in glucocorticoid metabolism are also apparent in rodent models of diet-induced obesity. Wistar rats fed a high fat, normal carbohydrate, diet had reduced 11β-HSD1 activity in liver, subcutaneous and omental adipose tissue after just 3 weeks. However, after 20

weeks of the feeding regimen these changes were no longer present (Drake *et al.*, 2005a). Interestingly, mice fed an obesogenic diet for 18 weeks showed decreased 11β-HSD1 activity in adipose tissue (Morton *et al.*, 2004), similar to the short term changes seen in rats. Given that increased local glucocorticoid regeneration causes adipose accumulation, this may reflect a protective mechanism to stop lipid deposition and maintain insulin sensitivity. In support of this, mice with adipose-specific over-expression of 11β-HSD1 show features of the metabolic syndrome (Masuzaki *et al.*, 2001), and mice with targeted disruption of the enzyme have improved glucose tolerance and insulin sensitivity when fed a high-fat diet (Morton *et al.*, 2001). Transgenic over-expression of 11β-HSD1in the liver leads to insulin resistance and dyslipidemia (Paterson *et al.*, 2004) suggesting that tissue specific patterns of glucocorticoid regeneration may be important in determining the deleterious effects of an obesogenic diet.

As well as changes in 11β-HSD1, increased glucocorticoid metabolism by the A-ring reductases was reported in the obese Zucker rat and *ob/ob* mouse as mentioned above. Increased activity of hepatic 5βR was found in high fat fed rats (Drake *et al.*, 2005a) and *ob/ob* and *db/db* mice (Livingstone *et al.*, 2009). Furthermore, 5αR knock-out mice develop fatty liver and glucose intolerance (Livingstone, 2009). These studies support the suggestion that increased concentrations of glucocorticoid have a role in the pathogenesis of the metabolic syndrome in rodents.

The exact mechanisms controlling peripheral glucocorticoid metabolism, and the changes in obesity, are not clear. However, the consistent finding of alterations in obese humans and rodents, as well as the results of deletion or over-expression of key enzymes in the metabolic pathway, have led to the consensus that reduction of active glucocorticoids is beneficial to metabolism.

1.2.5.3 Pharmacological inhibition of 11β-HSD1

Glucocorticoids are administered as replacement therapy in glucocorticoid deficiency, and in higher doses as anti-inflammatory agents or as immunosuppressants. Following the discovery that changes in peripheral glucocorticoid metabolism are evident in obese humans and animals, many laboratories have focused on developing inhibitors of 11β-HSD1 in recent years. Successful and selective pharmacological inhibition of 11β-HSD1 has been reported by many companies. Treatment with these inhibitors has been consistently shown to improve insulin sensitivity in both dietary and genetic rodent models of obesity (Alberts *et al.*, 2002; Alberts *et al.*, 2003; Hermanowski-Vosatka *et al.*, 2005). Translation of these

inhibitor studies to the clinical environment has recently been undertaken with trials in early stages; one randomised placebo controlled study of patients with type 2 diabetes has reported that treatment with an 11β-HSD1 inhibitor for 12 weeks improved hyperglycaemia (Rosenstock *et al.*). However, studies using a second inhibitor did not report alterations in fasting plasma glucose but did find modest improvements in body weight and blood pressure (Feig *et al.*, 2011). The results of ongoing trials by several drug companies are therefore eagerly awaited.

1.3 Sex steroids

A potential role for sex hormones in the development of obesity and associated cardiovascular disease is alluded to by the sex specific incidence of these conditions (introduced in section 1.1.1). There are three main classes of sex steroid, androgens, estrogens and progestogens which all act on nuclear receptors in a variety of tissues. The relative concentrations of androgens, estrogens and progestogens are sex dependent with androgens predominant in males and estrogens and progestogens predominating in females pre-menopause. In recent years, sex steroids have been shown to play a role in determining the anatomical deposition of adipose tissue (section 1.1.1.1) and development of cardiovascular diseases (section 1.1.1.2). Estrogen is of particular interest due to the apparent protection of women from cardiovascular disease despite high incidence of obesity. Therefore, this thesis and introduction will concentrate on the role of estrogen in the development of obesity and cardiometabolic disease, however, a role for androgens has also been proposed which has been extensively reviewed elsewhere (Pasquali, 2006; Renato, 2006; Mammi *et al.*, 2012).

1.3.1 Synthesis and secretion

Sex hormones are primarily synthesised and secreted from the gonads and their production is regulated by the hypothalamic-pituitary-gonadal (HPG) axis (Figure 1.6). The hypothalamus secretes gonadotrophin releasing hormone (GnRH) which binds to receptors in the pituitary inducing the release of the gonadotrophins, luteinising hormone (LH) and follicular stimulating hormone (FSH). In the ovaries the gonadotrophins activate production of estrogens and progesterone which control the ovarian and menstrual cycle. In males, LH stimulates testosterone production in the Leydig cells of the testes while FSH induces spermatogenesis.

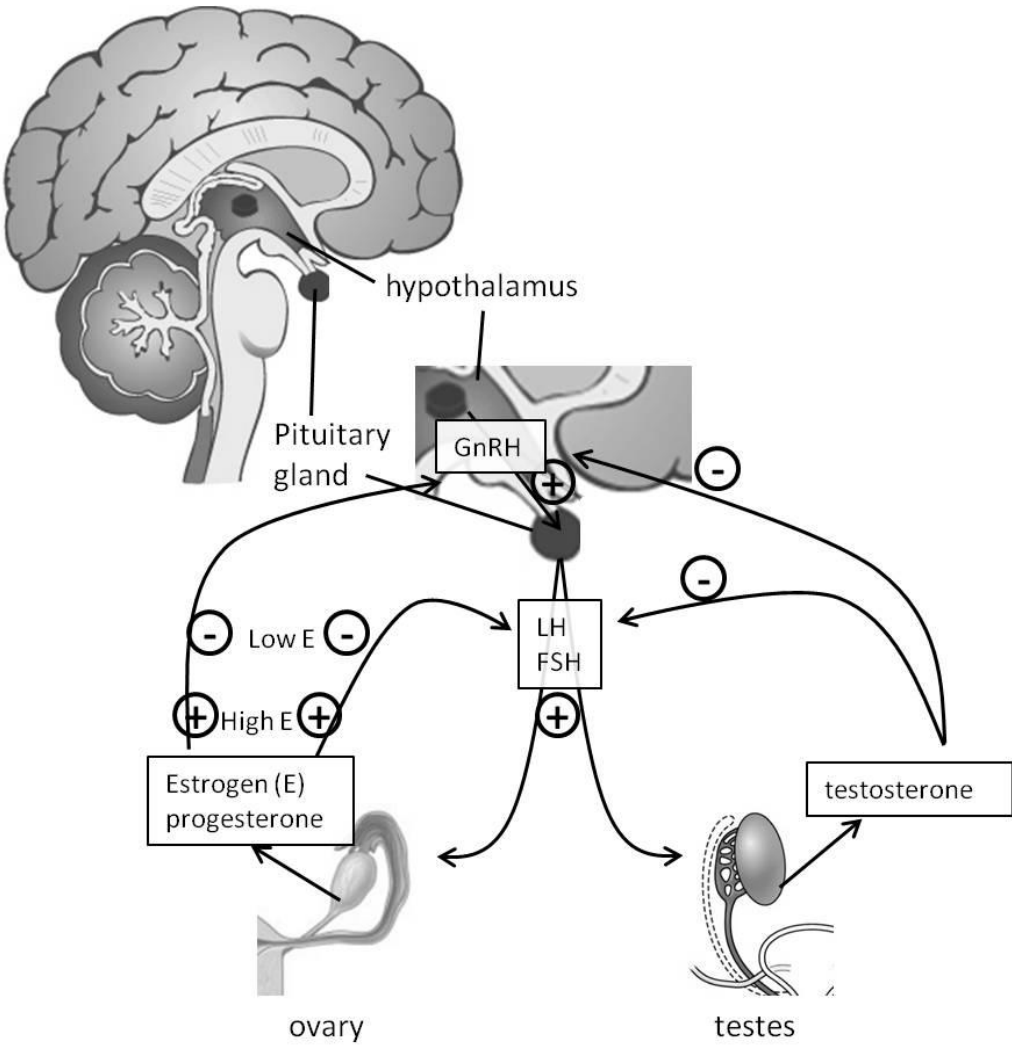


Figure 1.6 Hypothalamic-pituitary-gonadal axis

The hypothalamic-pituitary-gonadal (HPG) axis controls the production of sex steroids in the gonads. Stimulation of the hypothalamus induces secretion of gonadotrophin-releasing hormone (GnRH). This causes release of luteinising hormone (LH) and follicular stimulating hormone (FSH) from the pituitary gland which travel through the circulation to the gonads instigating production of estrogen (E) and progesterone in the ovary and testosterone in the testes. Sex steroids regulate their own production; testosterone inhibits production of GnRH, LH and FSH; low concentrations of estrogen similarly regulate negative feedback but high concentrations of estrogen stimulate positive feedback, leading to a surge of LH, FSH, and estrogen.

Like all steroids, sex hormones are synthesised from pregnenolone, a derivative of cholesterol. Oxidation reactions produce progestogens which can, in turn, be oxidised to androgens and, finally, estrogens (Figure 1.4). While the gonads are the primary sites of sex steroid synthesis, the placenta has an important role in their production during pregnancy and the adrenal cortex produces some progestogens and androgens in humans which can then be converted to more active metabolites in the gonads. Other tissues contain the necessary enzymes to produce sex steroids, including bone and adipose. In women this is particularly noticeable following the menopause when ovarian function reduces. Estrogens can then be formed by aromatisation of androgens, catalysed by the enzyme aromatase (CYP19A1) (Figure 1.4). In women, aromatase expression increases after the menopause (Cleland *et al.*, 1985), this has also been found in rats following ovariectomy (Zhao *et al.*, 2005).

The HPG axis is regulated by negative feedback. Both estrogen and testosterone can inhibit the production of GnRH in the hypothalamus, and gonadotrophins in the pituitary. In women the feedback is dependent on the concentrations of estrogen; low levels are associated with negative feedback but high levels can have a positive effect, increasing GnRH and LH release to induce ovulation. The two mechanisms of feedback activated by estrogens control the maturation and release of the ovum for fertilisation, and explain the cyclic nature of its production. In humans, the bioavailability of androgens and estrogen is also controlled by sex hormone binding globulin (SHBG). SHBG is synthesised and secreted from the liver into the bloodstream where it binds androgens and estrogens, leaving only low concentrations free to activate receptors. SHBG synthesis is increased by high concentrations of estrogens, decreased by androgens and glucocorticoids, and reduced in obesity in children and adults. While SHBG is an important determinant of the bioavailability of sex steroids in humans, rodents do not have circulating binding proteins for androgens and estrogens postnatally (Corvol and Bardin, 1973; Renoir *et al.*, 1980).

1.3.2 Androgen and estrogen signalling

Estrogens mediate their effects via α and β estrogen receptors (ER) and both isoforms of the ER are expressed in a wide range of tissues. ERα and ERβ are expressed in classic estrogen target tissues, such as the female reproductive system, as well as in blood vessels and adipose. ERα alone is found in the hippocampus, hepatocytes, and interstitial cells of the kidney while ERβ is expressed in the thyroid and non hippocampal areas of the brain (Taylor and Al-Azzawi, 2000). Androgens bind the androgen receptor (AR) which is found in testis, prostate, adipose tissue (Pedersen *et al.*, 1996) and many other cells. The intracellular 5α-

reduced form of testosterone, dihydrotestosterone, has a much higher affinity for the receptor (Siiteri and Wilson, 1974).

ERs and ARs are both nuclear receptors which act as ligand regulated transcription factors to induce or repress gene expression. Upon ligand activation the receptors dimerise and bind to hormone response elements in the promoter regions of target genes in concert with other proteins and transcription factors. ERs and ARs can also have rapid effects on proteins which do not have hormone response elements in their genes, demonstrating non-genomic actions of the steroids. These are suggested to be mediated by membrane or cytosolic receptors which activate protein kinase cascades resulting in alterations in the function of the target protein (Heinlein and Chang, 2002; Bjornstrom and Sjoberg, 2005).

1.3.3 Estrogens and obesity

Although estrogen has been proposed to play a role in obesity and the associated metabolic complications the exact mechanisms through which it acts are incompletely understood. It is likely to involve many factors including; changes in lipid metabolism, glucose metabolism, and the anatomical distribution of adipose tissue.

1.3.3.1 Humans

The effect of estrogen on obesity and cardiovascular disease is demonstrated by the increased prevalence of both following the menopause. The discontinuation of estrogen synthesis in the ovaries is associated with increased adipose accumulation in central or visceral depots (Tchernof and Poehlman, 1998). Treatment of post-menopausal women with hormone replacement therapy (HRT), which includes estrogens, reduces visceral adipose accumulation and alters the plasma lipid profile (Sorensen *et al.*, 2001; Sumino *et al.*, 2003). Discrepancies have been found between observational and randomised clinical trials of HRT, with an increased incidence of cardiovascular disease reported in some clinical trials (Manson *et al.*, 2003; WHISC, 2004). This often occurred in the early years of taking HRT and diminished with time, and has been suggested to be due to an increase in thrombogenesis which is not sustained in the longer term (Stevenson, 2004). Further analysis of studies has suggested the benefits of HRT may be limited in certain populations, such as those with diabetes (Salpeter *et al.*, 2006) and may be dependent on the dose of steroids, type of preparation used and route of administration (Stevenson, 2004). Another population of patients with abnormal concentrations of endogenous estrogen are those suffering from polycystic ovary syndrome (PCOS), in whom estrogen concentrations are within the normal range for half of the cycle but fail to increase pre-ovulation (Franks, 1995). Aside from

chronic anovulation and fertility issues, PCOS is associated with central obesity and insulin resistance (Gambineri *et al.*, 2002). However, in addition to changes in estrogen PCOS is characterised by hyperandrogenism which is also likely to play a role in the associated metabolic complications.

1.3.3.2 Animal Models

In animals (rats and mice) ovariectomy is used as a model of menopause and results in increased adiposity which can be ameliorated by exogenous estradiol treatment (McElroy and Wade, 1987; Hong *et al.*, 2009). The role of estrogen in controlling adiposity is further demonstrated in transgenic mice with altered estrogen signalling and synthesis. Following deletion of ERα, both male and female mice become obese and develop insulin resistance (Heine *et al.*, 2000). Aromatase KO mice, which are unable to synthesise estrogen from testosterone, also accumulate intra-abdominal adipose tissue and have fasting hyperinsulinemia (Jones *et al.*, 2001). Interestingly, ERβ KO mice are not obese, suggesting that the effects of estrogen on adiposity are mediated by the α-receptor. Recently, an in depth study of neuron specific ERα deletion found that lack of estrogen signalling increases adiposity and causes changes in metabolism (Xu *et al.*, 2011). This suggests the effects of estrogen on obesity may be modulated by both central and peripheral mechanisms in a variety of tissues.

1.3.4 Estrogens and the vasculature

In addition to its beneficial effects on metabolic status (section 1.3.3), it has been proposed that estrogen has direct effects on the vasculature (White, 2002). This may play a role in the decreased incidence of cardiovascular disease seen in women despite greater prevalence of obesity.

1.3.4.1 Estrogen and vascular function

Both ERα and ERβ are expressed in endothelial cells and VSMCs, though their expression is heterogeneous and depends on vascular bed and gender (Mendelsohn and Karas, 2005). Sex differences have been reported in the expression and function of endothelium-derived vasodilators (Kauser and Rubanyi, 1994; McCulloch and Randall, 1998; Villar *et al.*, 2008). Estrogen treatment can induce endothelium-dependent and independent vasodilation in the forearm of postmenopausal women, as well as increasing nitric oxide (Tagawa *et al.*, 1997). In addition it has been reported that endothelium-dependent hyperpolarizing factors can mediate vasodilation in the absence of nitric oxide to a greater extent in females than males

(McCulloch and Randall, 1998). Although sex differences in nitric oxide and vasodilation are consistently found, changes in vasoconstriction are more controversial. Some studies report a decreased response to vasoconstrictors in females, while others suggest no sex differences or even an increased reactivity (Sader and Celermajer, 2002; Thompson and Khalil, 2003). Many studies have proposed a role for estrogen in calcium flux, either in attenuating influx or stimulating efflux, which would reduce vascular contraction and enhance dilation (Ho and Liao, 2002).

1.3.4.2 Estrogen and vascular lesion formation

Estrogen has been proposed to slow the progression of atherosclerosis in humans and animals (Bourassa *et al.*, 1996). Although changes in plasma lipids and a reduction in other metabolic risk factors contribute to this effect, a further protection independent of these parameters suggests specific effects on the vessel wall (Bourassa *et al.*, 1996). Estrogen treatment of rabbits undergoing balloon injury surgery to the aorta and iliac arteries reduces neointimal proliferation (Foegh *et al.*, 1994). This may be due to reduced expression of adhesion molecules such as vascular cell adhesion molecule-1, intercellular adhesion molecule-1, and E selectin (Frazier-Jessen and Kovacs, 1995; Caulin-Glaser *et al.*, 1996) which recruit inflammatory cells to sites of vascular injury, though this has not been consistently reported. Estrogen has been found to inhibit the expression of other pro-inflammatory cytokines which enhance lesion formation and reduce mitogenic cytokine expression and activity, reducing VSMC proliferation (Foegh *et al.*, 1994; Chen *et al.*, 1996b). Interestingly, exogenous estrogen was shown to reduce neointimal proliferation to a similar extent in ERα KO and WT mice (Iafrati *et al.*, 1997), suggesting a role for ERβ or another mechanism independent from the classic receptor activation.

1.4 Early-life programming

The environment experienced *in utero* and during early-life has been hypothesised to program disease risk in adulthood (Barker, 1998). In many populations around the world, insults during pregnancy have been shown to increase the prevalence of later disease, independent of other known risk factors (such as smoking and social class). Programming effects were first described in association with low birth weight, suggesting exposure to an adverse environment *in utero* as a stimulus (Barker *et al.*, 1989). Two major hypotheses have been advanced to explain this link between low birth weight and later disease: over-exposure to glucocorticoids during development (e.g. as a consequence of exposure to maternal stress)

and inadequate fetal nutrition (e.g. as a consequence of a poor or restricted maternal diet during pregnancy). It has been proposed that the fetus adapts in order to maximise survival in a poor extra-uterine environment; however this results in a predisposition to disease when the pre- and postnatal environments are not matched (Hales and Barker, 1992).

1.4.1 Glucocorticoid exposure

Steroids have long-term effects on tissue structure and function. Neonatal exposure to androgens programs hepatic metabolism of steroids, alters sexually dimorphic structures in the brain and affects sexual behaviour (Gustafsson *et al.*, 1975; Gustafsson *et al.*, 1983). Over-exposure of the fetus to glucocorticoids has been proposed as a potential mechanism of early-life programming. GRs are expressed in the fetus (Cole *et al.*, 1995b) and placenta (Sun *et al.*, 1997) from early in development. MRs, which can also be activated by glucocorticoids, are expressed in fetal tissues but with a more distinct pattern and at a later stage in gestation (Brown *et al.*, 1996). As discussed (section 1.2.4.1) glucocorticoids are important in the growth and maturation of organs, such that they are administered to women at risk of pre-term delivery.

In humans, antenatal glucocorticoid treatment results in reduced birth weight and long-term increases in blood pressure and changes in myocardium structure (Doyle *et al.*, 2000; Bloom *et al.*, 2001). As well as affecting the cardiovascular system, prenatal exposure to glucocorticoids alters brain structure associated with changes in memory and behaviour (Welberg *et al.*, 2001). Intrauterine growth retardation itself is associated with increased circulating concentrations of cortisol in the fetus (Goland *et al.*, 1993). Additionally, cortisol can affect placenta size, by inhibiting the production of growth factors, with subsequent effects on the fetus such as reduced nutrient supply and growth retardation. In sheep and rat models of *in utero* glucocorticoid exposure birth weight is reduced. When offspring of rats treated with the synthetic glucocorticoid dexamethasone are followed to adulthood they have higher blood pressure, changes in vascular function and structure, hyperglycaemia and hyperinsulinemia (Nyirenda *et al.*, 1998; O'Regan *et al.*, 2004; Hadoke *et al.*, 2006a). Exposure to glucocorticoids during development can also permanently program HPA axis activity, which, given the many functions of glucocorticoids (section 1.2.4), is likely to influence physiology and disease risk. The timing of exposure to glucocorticoids is critical in programming these long term outcomes, in rats, glucocorticoid administration in the last week of pregnancy confers the changes in adult physiology, whereas exposure in the first week has no effects on glucose-insulin homeostasis and blood pressure (Levitt *et al.*, 1996). As well as exogenous treatment, fetal glucocorticoid over-exposure can also occur as a

consequence of inhibition or transgenic deletion of 11β-HSD2 which deactivates glucocorticoids in the placenta. The resulting outcome is similar to that seen in the dexamethasone-treated rat, with reduced birth weight and altered behaviour (Holmes *et al.*, 2006). Maternal glucocorticoids are likely to be increased by stress as a consequence of many stimuli including unbalanced nutrition. It is therefore possible that early life programming occurs as a consequence of both changes in nutrition (introduced below) and glucocorticoids.

1.4.2 Maternal under-nutrition

The 'Dutch Hunger Winter', a period of famine in 1944-1945 which affected the western Netherlands, provided a data set to study the effects of maternal under-nutrition. Babies exposed to the famine *in utero* have higher incidence of glucose intolerance (Ravelli *et al.*, 1998) and hypertension (Roseboom *et al.*, 1999) in adulthood. Interestingly, the same increase in later diseases was not seen in a population from Leningrad that were conceived and born during a severe famine (Stanner *et al.*, 1997). However, the children exposed to malnutrition *in utero* also had little food post-natally and this consistency in diet is hypothesised to have mitigated any effects of programming. In many other populations food restrictions or specific diets during pregnancy have led to low birth weight and increases in cardiovascular disease have been reported (Prentice *et al.*, 1987; Godfrey *et al.*, 1994; Reynolds *et al.*, 2001; Reynolds *et al.*, 2010). Dietary composition, as well as calorific or nutritional value, has been shown to influence fetal development with studies suggesting that deficiencies in specific micronutrients may also program the fetus (Boucher, 1998). Although trials providing expectant mothers with micronutrient supplementation have not shown effects on offspring birth weight (Fall *et al.*, 2003; Merialdi *et al.*, 2003), effects on adult health are still possible so that long-term follow up studies are required.

Animal studies have demonstrated similar effects of maternal under-nutrition during pregnancy. Rats fed a low protein diet during pregnancy have low birth weight offspring, which display insulin resistance and hyperglycaemia in later life (Langley *et al.*, 1994; Langley-Evans *et al.*, 1996). In mice maternal dietary restriction also causes decreased birth weight and increased weight gain and features of type 2 diabetes in adult offspring (Bol *et al.*, 2009). Maternal caloric or protein restriction increases blood pressure in offspring, and alters vascular function, with increased vasoconstriction response (Ozaki *et al.*, 2001) and decreased endothelium-dependent vasodilation (Brawley *et al.*, 2003) in adult offspring. While results differ slightly between models used and parameters tested data is consistent in suggesting an increased risk of cardiovascular disease is associated with low birth weight. As

well as alteration of maternal diet, fetal nutrition can be reduced by surgical ligation of uterine vessels (McMillen and Robinson, 2005). This also leads to reduced birth weight and long term changes in cardiovascular health.

1.4.3 Maternal obesity

As maternal undernutrition has been shown to program the long-term health of the offspring, it has been hypothesised that maternal/fetal overnutrition may also exert programming effects.

1.4.3.1 Human studies

Obese mothers have more complications during pregnancy and spend longer in hospital following delivery of their children. Neonates born to mothers with a pregravid BMI over 25 had increased adiposity when compared with controls (Catalano *et al.*, 2009a) and preliminary follow up studies of children born to obese mothers show that they are heavier and have a higher BMI (Whitaker, 2004; Ogden *et al.*, 2006; Mesman *et al.*, 2009), which is still evident when they are young adults (Stuebe *et al.*, 2009; Tequeanes *et al.*, 2009). In addition to overall weight gain, there is also increased adipose mass in the children of obese mothers (Gale *et al.*, 2007) which is maintained into adulthood independent of current lifestyle factors (Mingrone *et al.*, 2008; Reynolds *et al.*, 2010). As has been introduced previously obesity is associated with hyperglycaemia and hyperinsulinaemia (section 1.1.6.1). One small study found neonates of obese mothers were more insulin resistant than those of lean mothers (Catalano *et al.*, 2009a). Recent evidence from the large hyperglycaemia and pregnancy outcome study found an association between maternal BMI and fetal hyperinsulinaemia even after adjustment for maternal glycaemia (Study Cooperative Research Group, 2008). A higher prevalence of insulin resistance is starting to be reported in offspring followed to early adulthood whose mothers were obese (Mingrone *et al.*, 2008) or had diabetes (Boerschmann *et al.*, 2010). Gestational diabetes, which is often associated with obesity, causes macrosomia in the offspring as well as hyperglycaemia, both of which are believed to predict obesity in adulthood (Gillman *et al.*, 2003). Although incidence of obesity and gestational diabetes correlate they are not characterised by the same parameters and could induce different programming stimuli and therefore data should be interpreted accordingly.

Studies investigating maternal obesity and cardiovascular risk in their offspring are currently very limited. Some isolated studies have shown an association between blood pressure and gestational weight gain (Lawlor *et al.*, 2004; Mamun *et al.*, 2009; West *et al.*, 2011), though

this may be due to increased BMI in the offspring. Attention is turning to potential interventions to improve maternal health, which may impact on offspring health in the short and long term. One study found exercise during pregnancy reduced offspring birth weight but did not alter maternal insulin sensitivity (Hopkins *et al.*, 2010). Longitudinal studies of such interventions are obviously lacking, and this may mean offspring birth weight is being over-analysed as an indicator of future health. Birth weight is a crude measure of the *in utero* environment and is not always elevated in offspring of obese mothers (Artal *et al.*, 2007; Hopkins *et al.*, 2010).

Despite variation most studies demonstrate extra or excess growth *in utero* may lead to increases in cardiovascular disease risk as has been well documented in those with growth retardation in early life. One major consideration of human studies is the 'obesogenic environment' that the children are being born in to. Obese mothers are likely to pass on their eating habits and behaviours to their children, and thus they are likely to show the effects of diet and lifestyle induced obesity in addition to any programmed effects.

1.4.3.2 Rodent models

Whilst studies in humans are on-going, animal models have proved invaluable in predicting the future impact of maternal obesity, as well as in attempting to uncover the mechanisms by which early-life programming occurs. In animals, the post-weaning environment can be tightly regulated, reducing the environmental effects which confound human studies. Models have been developed in a variety of species and have used females that are already obese or females placed on an obesogenic diet during pregnancy and lactation. The effects of maternal obesity can already be seen in the unborn fetus: maternal obesity in sheep increases pancreatic weight and β-cell number in the fetus (Ford *et al.*, 2009) while in Japanese Macaques it increases fetal hepatic triglyceride content (Aagaard-Tillery *et al.*, 2008).

1.4.3.2.1 Timing of insult

Unlike glucocorticoid-induced programming, in which the timing of the insult is critical in determining the programming effects, most models of maternal over-nutrition (including pre-conception and during gestation), lead to the same metabolically-compromised phenotype in the offspring, as does offspring exposure to excess nutrition during the suckling period (Armitage *et al.*, 2005b; Khan *et al.*, 2005; Bayol *et al.*, 2007; Samuelsson *et al.*, 2008; Bruce *et al.*, 2009). Some debate remains, however, with a study demonstrating the offspring of rats rendered obese before mating but maintained on standard diet during pregnancy became obese in adulthood (Shankar *et al.*, 2008) and another suggesting pre-conception

obesity has no additional effect over the exposure to a high fat diet during pregnancy in terms of programming effects on offspring adiposity (Howie *et al.*, 2009). While White *et al.* found maternal obesity was necessary for the programming effects of a high fat diet on offspring adiposity in a rat model (White *et al.*, 2009). Studies of the physiology of obese dams are beginning to be reported and may shed some light on the mechanisms leading to the differential effects on the offspring.

1.4.3.2.2 Programming of offspring obesity and metabolism

Another major difference between programming by maternal obesity and under-nutrition or glucocorticoid exposure is the lack of influence of maternal obesity on offspring birth weight (Boullu-Ciocca *et al.*, 2005; Buckley *et al.*, 2005; Bruce *et al.*, 2009; White *et al.*, 2009). However, in adulthood the offspring of obese rats and mice are generally heavier, with more adipose tissue than controls, and this effect is amplified by feeding an obesogenic diet post-weaning (Buckley *et al.*, 2005; Khan *et al.*, 2005; Taylor *et al.*, 2005; Srinivasan *et al.*, 2006; Bayol *et al.*, 2007; Samuelsson *et al.*, 2008; Elahi *et al.*, 2009). Rats exposed to a junk food diet *in utero* have increased intramuscular lipid content (Bayol *et al.*, 2005) and increased liver triglyceride content has been reported in offspring of obese rats (Buckley *et al.*, 2005) and mice (Elahi *et al.*, 2009). Increases in body weight and adiposity may be due to hyperphagic activities of offspring (Kirk *et al.*, 2009; Nivoit *et al.*, 2009) and decreased energy expenditure (Samuelsson *et al.*, 2008) and alterations in brain regions controlling appetite are also apparent in the offspring of obese dams (Chang *et al.*, 2008)

In addition to increased weight gain and adiposity, changes in metabolism have also been described in offspring of obese dams. Hyperglycaemia and hyperinsulinemia have been reported following either a period of fasting or a glucose tolerance test, and lipid handling is also affected with higher plasma concentrations of triglycerides and cholesterol (Guo and Jen, 1995; Buckley *et al.*, 2005; Khan *et al.*, 2005; Samuelsson *et al.*, 2008; Elahi *et al.*, 2009). These changes are likely to be associated with the increase in weight and adiposity discussed though reduced β-cell number, volume (Cerf *et al.*, 2005) and insulin secretory capacity (Taylor *et al.*, 2005) have been described. On a molecular level changes in the expression of glucose transporter 2 (Cerf *et al.*, 2005) and components of the insulin signalling pathway (Buckley *et al.*, 2005; Shelley *et al.*, 2009; Martin-Gronert *et al.*, 2010) are evident.

Despite the known association between altered local glucocorticoid metabolism and obesity (section 1.2.5) few studies have investigated glucocorticoid metabolism in models of

maternal obesity. Neonatal over-feeding studies in rats (achieved by reducing litter size) showed that the mRNA abundance and/or activity of 11β-HSD1 was increased in visceral (Boullu-Ciocca *et al.*, 2005) and retroperitoneal (Hou *et al.*, 2011) adipose tissue. An increase in GR expression in visceral adipose was also reported in one study (Boullu-Ciocca *et al.*, 2005). These results suggest that maternal obesity might be associated with altered glucocorticoid regeneration and signalling in the offspring, changes which may be associated with adiposity and insulin resistance (section 1.2.5).

1.4.3.2.3 Programming of vascular function

In addition to metabolic changes, exposure to maternal obesity increases cardiovascular disease in offspring, as is seen in other models of early-life programming. There is evidence for hypertension (Khan *et al.*, 2003; Khan *et al.*, 2004; Samuelsson *et al.*, 2008; Elahi *et al.*, 2009) in offspring of obese dams and this may deteriorate with advancing age (Samuelsson *et al.*, 2008; Liang *et al.*, 2009). In rats blood pressure can be measured from as early as 30days using remote telemetry. Using this technology it has been reported that blood pressure is raised in offspring of obese dams before changes in adiposity are evident, although the mechanisms are not clear a sympathetic origin was proposed (Samuelsson *et al.*, 2010). Other changes in the vasculature may also play a role in the development of hypertension, particularly differences in structure and function which may increase peripheral resistance. Rodent offspring of obese dams demonstrate endothelial dysfunction seen as reduced endothelium-dependent vasodilation (Samuelsson *et al.*, 2008; Koukkou *et al.*, 1998; Ghosh *et al.*, 2001; Taylor *et al.*, 2004; Chechi *et al.*, 2009). In addition reports suggest altered vascular fatty acid content (Ghosh *et al.*, 2001), increased aortic stiffness and reduced smooth muscle cell number (Armitage *et al.*, 2005a) which may all contribute to hypertension.

To date, lesion formation following vascular injury has not been studied in offspring of obese dams. However, a study of ischemia/reperfusion injuries of the myocardium found increased susceptibility in male offspring of genetically obese mothers (Calvert *et al.*, 2009) though the mechanism was not investigated. Atherosclerosis is hypothesised to form in response to chronic vascular injury (introduced in section 1.1.6.5), though lesions are seen in young children and animals. Offspring of hypercholesterolemic rabbits have increased lesion size at birth, however swine exposed to a high cholesterol diet *in utero* were not different from those of control sows at birth or at 5months (Norman and LeVeen, 2001). Interestingly the study in swine included an amplification protocol which found those fed the atherosclerotic diet during early and postnatal life had less plaques than those reared by

control sows (Norman and LeVeen, 2001). It was hypothesised that programming of cholesterol metabolism inferred a protection from atherosclerosis in this animals (Norman and LeVeen, 2001), whether this is true in other species is unknown and warrants further investigation.

1.4.4 Potential programming mechanisms

The exact mechanisms by which the environment experienced in early life can cause long-term changes in tissue structure and function are still unclear. Many changes in gene expression and enzyme activity have been reported in animal models but how these are elicited or maintained is unclear. Programming of the HPA-axis and epigenetic regulation of gene expression have both been proposed though further research is required to confirm their involvement. It is possible that similar mechanisms are involved in programming regardless of stimulus, though this is currently not known. To allow for further investigation, and intervention studies, robust reproducible models of programming are required. This is particularly important when considering the future health of children being born to obese mothers during the current epidemic of obesity.

1.5 Hypotheses and aims

The work described in this thesis addressed the hypotheses that:

(1) As a consequence of the protective effects of estrogens, the metabolic and cardiovascular changes caused by obesity are more severe in male than in female mice.
(2) Exposure to maternal obesity *in utero* programmes metabolic and cardiovascular dysfunction in adult offspring.

In order to address these hypotheses, the aim of the specific studies performed was to answer the following questions:

- Are obesity-induced changes in metabolic function less severe in female, than in male, mice?
- Does maternal obesity predispose offspring to metabolic dysfunction in adulthood?
- Does obesity in adulthood, or exposure to obesity *in utero*, induce vascular dysfunction and promote neointimal proliferation in response to arterial injury?

Chapter 2

Materials and methods

2.1 Materials

All chemicals and reagents were from Sigma-Aldrich (Dorset, UK) unless otherwise stated.

2.2 Buffers and Solutions

2.2.1 1kb DNA ladder

20% v/v 20µg 1Kb ladder (Invitrogen, Strathclyde, UK) in distilled water with 20% v/v loading buffer.

2.2.2 10x TBE buffer

0.9M Tris base, 0.9M boric acid, 20mM EDTA in distilled water. Autoclaved, diluted to 0.5x before use.

2.2.3 Acidified Potassium Permanganate

0.3%v/v concentrated sulphuric acid (Fisher Scientific, Leicestershire, UK) and 0.3% v/v potassium permanganate were mixed with distilled H_2O immediately prior to use and kept for no more than 48 hours.

2.2.4 BABB

34% v/v benzyl alcohol (Acros Organics, Fisher Scientific, UK), 66% v/v benzyl benzoate (Acros Organics)

2.2.5 Borate buffer

8.25g boric acid, 2.7g NaOH, 3.5ml conc. HCl (33%), 5g BSA made up to 1L with distilled water, pH 7.4. Stored at -20°C, thawed at room temperature before use.

2.2.6 Gomori's Aldehyde fuschin

Pararosaniline was dissolved 1% w/v in 60% ethanol, followed by addition of 1% v/v HCl (VWR, Leicestershire, UK) and 2% v/v paraldehyde. The solution was left in sunlight for 2 days to develop then vacuum filtered using Whatman #1 paper (GE Healthcare, Buckinghamshire, UK).

2.2.7 Gomori's Trichrome Reagent

The following compounds were dissolved in distilled H_2O; 0.6% w/v phosphotungstic acid, 0.6% w/v chromotrope 2R and 0.3% w/v fast green FCF, mixed vigorously and finally 1% v/v acetic acid (Fisher Scientific) was added.

2.2.8 Homogenisation Buffer (for microsome preparations)

250mM sucrose, 10mM HEPES in distilled water, pH 7.5. Stored at 4°c for no more than one week

2.2.9 Krebs Buffer

2.54mM $CaCl_2$, 1.19mM $MgSO_4$, 3.80mM KCl, 1.19mM KH_2PO_4, 25mM $NaHCO_3$, 118mM NaCl in distilled water, pH 7.4. Stored at 4°C for no more than one week.

2.2.10 Mobile Phase for HPLC (WAM)

WAM 60: 15: 25 (v/v/v) HPLC grade water (Fisher Scientific): acetonitrile (VWR): methanol (Fisher Scientific), was mixed and filtered through 0.45µm filter paper (Millipore, MA, USA).

2.2.11 Phosphate buffered saline (PBS)

1 tablet was dissolved in 200ml deionised water to give 0.01 M phosphate buffer, 0.0027 M potassium chloride and 0.137 M sodium chloride, pH 7.4.

2.2.12 Potassium Physiological Saline Solution (KPSS)

124.6mM KCl, 2.5mM $CaCl_2$, 1.2mM $MgSO_4$, 25mM $NaHCO_3$, 1.2mM KH_2PO_4, 27µM EDTA, 5.5mM glucose. Stored at 4°C until use, bubbled with 95% O_2/ 5% CO_2 during myography.

2.2.13 Physiological Saline Solution (PSS)

119mM NaCl, 3.7mM KCl, 2.5mM $CaCl_2$, 1.2mM $MgSO_4$, 25mM $NaHCO_3$, 1.2mM KH_2PO_4, 27µM EDTA, 5.5mM glucose. Stored at 4°C until use, bubbled with 95% O_2/ 5% CO_2 during myography

2.2.14 Weigert's Haematoxylin

Weigert's Haematoxylin solution A and solution B (Sigma Aldrich) were mixed 1:1 immediately prior to use and kept for no more than 48 hours.

2.3 *In vivo* procedures

2.3.1 Animal maintenance

All animal procedures were carried out under the terms of the Animals Scientific Procedures Act 1986 (project licence numbers 60/3962 and 60/5867, personal licence for Rachel Dakin 60/11838). Animals were under the care of the animal research technicians at the University of Edinburgh Biomedical Research Facility at Little France throughout the experimental period. C57BL/6 mice were bred in house for studies. Mice were maintained in a temperature (19-23°C) and humidity controlled environment on a 12h light/dark cycle (lights on 0700 daily). Mice were weaned from dams at ~21 days and ears notched to allow identification throughout studies. Mice were kept 4-6 per cage, except where stated, and cages were cleaned weekly. Mice had free access to standard laboratory chow (Special Diet Services, Essex, UK) and drinking water unless stated.

2.3.2 Generation of obese mice

C57BL/6 mice were bred in house; pups were weaned at 3 weeks of age and housed in single sex experimental groups. Groups were selected randomly from as many litters as possible. From 5 weeks of age mice were fed ad libitum with either control (CON; 11% kcal from fat, 73.1% Kcal from carbohydrate as corn starch, D12328) or obesogenic (DIO; 58% kcal from fat, 25.5% Kcal from carbohydrate as sucrose, D12331) diet (details in Table 2.1), both from Research Diets Inc. (NJ, USA). Animals were maintained on experimental diet for the remainder of the study as outlined in each chapter.

2.3.3 Generation of offspring exposed to maternal obesity

Obese and control female C57BL/6 mice were generated as in section 2.3.2 in collaboration with Dr V King. The mice, which were first time breeders, were mated with C57BL/6 males (maintained on standard diet) at 15 weeks of age and remained on experimental diet during gestation and suckling. Maternal characteristics are listed in Table 5.1, chapter 5. Litters were reduced to 5 pups on postnatal day one and offspring were weaned at postnatal day 23,

	D12331 High fat and sucrose			D12328 Control fat and corn starch	
	gm %	kcal %		gm %	kcal %
Protein	23.0	16.4		16.8	16.4
Carbohydrate (sucrose)	35.5	25.5		74.3	73.1
Fat	35.8	58		4.8	10.5
Total		100			100
	5.56 kcal/gm			4.07 kcal/gm	

	D12331			D12328	
	gm	kcal		gm	kcal
Casein	228	912		228	912
DL-Methionine	2	0		2	0
Maltodextrin 10	170	680		170	680
Corn Starch	0	0		835	3340
Sucrose	175	700		0	0
Soybean Oil	25	225		25	225
Coconut Oil (hydrogenated)	333.5	3001.5		40	360
Mineral Mix	40	0		40	0
Sodium Bicarb`onate	10.5	0		10.5	0
Potassium citrate	4	0		4	0
Vitamin Mix	10	40		10	40
Choline Bitartrate	2	0		2	0
Coloured dye	0.1	0		0.1	0
Total	1000.1	5558.5		1366.6	5557

Table 2.1 Diet composition

High fat and control fat diets were purchased from Research Diets Inc. (NJ, USA). Diets were matched for micro and macronutrients with the exception of carbohydrate source and quantity of fat. Table shows the % of grams and kcalories (kcal) provided by protein, carbohydrate and fat and the full composition of the diets as provided by the manufacturers.

housed in single sex cages and fed standard laboratory chow diet. Only one mouse from each litter was used in subsequent experiments; this is because litter mates cannot be considered independent as they shared the same *in utero* environment.

2.3.4 Terminal procedures and harvesting of tissues

Animals were killed by CO_2 asphyxiation or perfusion fixation at the times reported in individual chapters. Adult female mice were killed in estrus assessed by vaginal smear (section 2.3.10).

2.3.4.1 CO_2 asphyxiation

Mice were placed in a tank filled with increasing concentrations of CO_2 until breathing stopped. Upon removal from the tank death was confirmed by checking for pedal reflex reactions.

2.3.4.2 Perfusion fixation

Mice which underwent femoral artery injury surgery were killed by perfusion fixation. Formalin, an aldehyde fixative, helps maintain tissue structure by forming methylene crosslinks between tissue amino groups. By perfusing formalin through the vasculature the rigidity and therefore lumen structure of the vessels was maintained.

Firstly animals were deeply anaesthetised by intraperitoneal injection with 80mg/kg sodium pentobarbital (Ceva Animal Health Ltd., Buckinghamshire, UK). Once anaesthesia was induced, determined by lack of reflex reactions, the chest cavity was opened and the heart exposed. The circulation was flushed with PBS containing heparin 10U/ml (Leo Laboratories, Buckinghamshire, UK) by placing a 23 gauge needle into the left ventricle and making a small cut in the right atrium for blood and perfusate to escape. The PBS flow was maintained at 6ml/min by a peristaltic pump (Gilson, Bedfordshire, UK) for ~3 min. Once the blood was cleared, 10% neutral buffered formalin was perfused through the system in the same way for approximately 7 min. Fixation was determined by bleaching of the liver and muscle rigidity.

2.3.4.3 Tissue collection

Blood was collected from the Inferior Vena Cava with 1%(v/v) 0.5M EDTA; samples were centrifuged at 5,000rpm for 10min at 4°C and the supernatant plasma aliquotted and stored at -80°C. Liver, kidney, adrenal gland, spleen and adipose depots (subcutaneous, mesenteric, retroperitoneal and epididymal) were dissected, weighed where appropriate, snap frozen on

dry ice and stored at -80°C. For studies of vascular function femoral arteries were collected and immediately stored in PSS.

Mice that underwent luminal wire injury surgery had their femoral arteries dissected; from the lowest suture left in place after surgery past the femoropopliteal branch to the branch with the iliac artery. Any residual adipose and connective tissue was removed and vessels stored in formalin for a further 24 hours then transferred into 70% ethanol until processed for optical projection tomography (OPT).

2.3.5 Surgical techniques

2.3.5.1 Preparation for surgery

Mice were anesthetised in a chamber with increasing concentrations of 4-5% isoflurane (Merial Animal Health Ltd, Essex, UK). Once unconscious, determined by lack of reflexes, mice were transferred to a heat pad where administration of anaesthetic (2% isoflurane in oxygen) was maintained via a nose cone. For post-operative analgesia, $0.1mg.kg^{-1}$ buprenorphine (Alstoe Animal Health, Yorkshire, UK) was administered subcutaneously before the surgical procedure. Surgical instruments were sterilised using a bead steriliser prior to surgery and between each animal.

2.3.5.2 Recovery from surgery

Immediately following surgical procedures animals were placed in a cage alone, to recover from the anaesthesia. Once fully alert and active, normally within 1 hour, the animals were returned to the home cage. Animals and wounds were checked for the subsequent 4 days; firstly for general health and well being, and secondly for signs of infection. No major problems occurred as a result of the surgical procedures undertaken. A small number of mice required repeated suturing one or two days post femoral artery injury, due to the stitches having been chewed and removed by the animals.

2.3.5.3 Estradiol pellet implantation

Ninety-day release, 0.25mg 17β-estradiol pellets (Innovative Research of America, FL, USA) were implanted in male mice to administer the hormone (~2.8µg/day) over the duration of the study. The lipophilic properties of the pellet allow the drug to be slowly and consistently released into the interstitial fluid of the animals and thus into the circulation. This method of estradiol administration has previously been reported in the literature in studies of obesity in both rats and mice (Geisler *et al.*, 2002; D'Eon *et al.*, 2005; Andersson

et al., 2010). Sham surgery was performed on control animals; mice underwent the same surgical procedure with the exclusion of the pellet being inserted.

Animals were prepared for surgery as described (section 2.3.5.1). The surface of the surgery site, the left mid-dorsal region, was shaved and a small incision the size of the pellet made. A superficial pocket was created by blunt dissection of subcutaneous tissue, distal to the site of incision. The pellet was inserted, the skin incision closed with 6-0 silk sutures and the animals left to recover (section 2.3.5.2).

2.3.5.4 Femoral artery injury surgery

Intra-luminal injury was performed on murine femoral arteries to induce neointima hyperplasia. The model used was previously described by Sata *et al.* (Sata *et al.*, 2000).

Mice were prepared for surgery as described (section 2.3.5.1). The inner surface of the left hind limb was shaved and an incision made at the top of the leg. The femoral neurovascular bundle was exposed by blunt dissection of the surrounding muscles. The femoral artery and vein were isolated from the nerve around the femoropopliteal bifurcation. Temporary suture loops (6-0 silk sutures, Mersilk, Ethicon, West Lothain, UK) were placed around the vessels both proximal and distal to the popliteal branch point to allow a temporary halt to blood flow in the artery and vein.

The popliteal artery was also ligated and tension placed upon the ligature in order to bring the artery into the plane of the femoral artery. The area was irrigated with 1% w/v lignocaine (1% lidocaine hydrochloride, Hamlen Pharmaceuticals, Gloucestershire, UK) to dilate vessels as necessary. An angioplasty guidewire (0.014" diameter, Cook Inc., IN, USA) was inserted through an arteriotomy in the isolated popliteal artery, 2mm from the femoropopliteal bifurcation. The temporary ligature was loosened to allow the wire to be advanced approximately 5mm along the femoral artery. The wire was left in place for 1 minute then tension re-applied to the temporary ligature as it was removed. The artery was permanently ligated proximal to the arteriotomy and blood flow to femoral artery and surrounding tissues restored by removal of temporary sutures. The skin incision was closed with 6-0 silk sutures and animals left to recover (section 2.3.5.2).

2.3.6 *In vivo* blood sampling

Blood samples were taken from mice (2.3.7 & 2.3.8) allowed free movement on top of their cage. The tip of the tail ~1mm was cut off with a pair of fine scissors and the blood gently massaged through the tail. Drops of blood were collected from the distal end of the tail by

capillary action into EDTA coated tubes, centrifuged at 2.3g for 10min at 4°C and the supernatant plasma stored at -20°C or -80°C. Following blood collection mice were returned to the cage immediately. This procedure can be used repeatedly without harm to the mice.

2.3.7 Intraperitoneal glucose tolerance test

Mice were fasted for 6 hours prior to the test; free access to water was permitted. A basal blood sample was taken by tail nick within 1 minute of cage disturbance (to reduce glucose response to stress). Glucose (40% w/v) was injected intraperitoneally to give a dose of 2g glucose per kg body weight. Blood samples were taken at 15, 30, 60 and 90 min post injection as in section 2.3.6. Animals were returned to clean cages with access to experimental diet and water immediately following the test.

2.3.8 Diurnal blood sampling

Tail tip blood samples were taken at 7am and 7pm, when the lights were changing from dark and light and light to dark. Samples were taken within 1 minute of cage disturbance before stress response would be detected in the blood. Samples were collected and stored as in section 2.3.6.

2.3.9 Tail cuff plethysmography

Systolic blood pressure was measured using tail cuff plethysmography as previously described (Krege *et al.*, 1995). This technique uses light transmittance to monitor blood flow through the rodent tail, which is inversely proportional to tail volume. The transmittance is traced during inflation and deflation cycles of a pressurised cuff surrounding the mouse tail. The pressure at which the cyclical changes in tail volume, and therefore blood flow, stop indicates systolic blood pressure.

Mice were placed in a 40°C chamber for approximately 8 min prior to measurement, to ensure good circulation particularly through the tail. The mouse was then placed in a small restraint tube above a heated pad to limit movement during the blood pressure measurement. The tail cuff containing a photo sensor sleeve (Harvard Appartus, Kent, UK) was placed around the base of the tail and equipment adjusted until a cyclic change in tail volume, representative of pulse, was seen on the connected data acquisition computer (IBM, Hampshire, UK). Pulse traces were recorded during 4 inflation and deflation cycles to just above and below the point where pulse was no longer apparent, or reappeared. The average

of the last 3 deflate measurements was recorded as systolic blood pressure. This was repeated on 3 days and the average was calculated for each mouse.

2.3.10 Vaginal Smear

Vaginal smears were used to determine the stage of estrus cycle in adult female mice. Day of estrus can be determined by microscopically inspecting the type and relative numbers of cells present in the vagina.

The mice were allowed to move freely on top of their home cage. The tip of a plastic pipette containing 0.5ml 0.9% w/v saline solution was gently inserted approximately 3mm into the vagina. The vagina was lavaged with saline, samples smeared onto histology slides and left to air dry.

2.4 *Ex vivo* functional analysis

Wire myography was used to determine the functional response of femoral arteries.

2.4.1 Tissue preparation

Mice were killed (section 2.3.4.1) and femoral arteries dissected under 10x magnification, from the abdominal wall to the bifurcation of the common femoral artery. Vessels were stored in PSS (2.2.13) and further cleaned of peri-adventitial adipose and connective tissue before two rings approximately 2mm long were cut from each vessel. Exact vessel lengths were measured with a calibrated graticule eyepiece. The arterial rings were mounted in the organ baths of a Mulvany-Halpern myograph (Multi-myograph 610M, Danish Myotech, Aarhus, Denmark) containing PSS, to record isometric tension. The vessel was mounted using two wires thread through the lumen and secured on two opposing supports, one connected to a force transducer. The output from the transducer was passed to a data acquisition computer (Apple Inc, CA, USA) via a converter (Maclab/8, AD Instruments, Oxfordshire UK) and recorded using Chart 4.3.4 software (AD Instruments). Two rings from each animal were mounted, one left intact and the other denuded of endothelium by lightly rubbing the lumen with a hair. Vessel diameter was not measured in arteries used for functional anaylsis. Measurements were made in histology slides from separate cohorts of animals generated using the same protocols (Table 6.5 & 6.6). No differences between groups were found; diameters ranged from 210-336µm.

Myography baths were kept at 37°C and bubbled with 95% O_2 / 5% CO_2. Resting tension of approximately 7.5mN was gradually applied to vessels over 30min by adjustment of the micrometer. This optimal resting force has been previously determined in our laboratory (Dr N Kirkby, personal communication) and is in accordance with published literature investigating function in femoral arteries (Widmer *et al.*, 2006; Bhattacharya *et al.*, 2008).

2.4.2 Functional response

To determine contractility of vessels and 'rejuvenate' responses following dissection and mounting PSS was replaced with KPSS (2.2.12), ~6ml. The contraction was left to stabilise then baths emptied and tissues washed with PSS until they returned to resting tension. This was repeated twice more, the tension reached with the third KPSS wash was recorded as the maximum contractility for that tissue.

For all arterial rings cumulative concentration-response curves to the α_1-adrenoreceptor agonist, phenylephrine (PE) were recorded over the range $1x10^{-9}$ to $3x10^{-5}$ using half log unit steps. Rings were then washed with PSS until baseline tension was returned. The same protocol was then used with the G-protein coupled receptor agonist 5-hydroxytryptamine (5HT). To measure relaxation responses vessels were pre-contracted to 80% of maximal contraction (EC_{80}) with 5HT. Once contraction was stabilised the endothelium-dependent vasodilation was measured with acetylcholine (Ach) again in half log units from $1x10^{-9}$ to $3x10^{-5}$. Tissues were again washed with PSS before pre-contraction (EC_{80}) with 5-HT and the response to the NO donor sodium nitroprusside was measured from $1*10^{-10}$ to $3*10^{-6}$.

2.4.3 Data analysis

Data was recorded as tension developed following administration of drug to the bath. Functional responses were expressed as force per length of muscle contracted normalised to the third response to KPSS (maximum contraction).

$$Response\ to\ drug\ (mN/mm) = \frac{Tension\ (mN)}{2x\ vessel\ length\ (mm)}$$

$$Response\ to\ drug\ (\%\ of\ KPSS\ maximum\ contraction) = \frac{Response\ to\ drug\ (mN/mm)}{Response\ to\ KPSS\ (mN/mm)}\ x\ 100$$

Dose-response curves were created using non-linear regression and compared by 2-way ANOVA followed by Bonferroni's post-hoc analysis. Responses to dilators were expressed

as percentage of the pre-existing tension prior to drug addition. Summary data for dose-response curves (Emax, maximum response; pD2 or –logEC50, concentration eliciting 50% Emax) were calculated using iterative least squares fit equation. All graphs and statistics were generated using GraphPad Prism 5.0 software (Graphpad Software, CA, USA)

2.5 Molecular Biology

2.5.1 Quantitative real time PCR (qPCR)

The transcript (mRNA) abundance of specific genes of interest was quantified using quantitative real time PCR (qPCR). Firstly RNA was extracted from tissues, and reverse transcribed to generate cDNA. The cDNA underwent qPCR using specific primers and *Taq* DNA polymerase to amplify the gene of interest. The 5' to 3' exonuclease activity of *Taq* DNA polymerase allows the use of florescent probes to quantify the amplification. The probes have a fluorescent reporter and quencher attached; the proximity stops any emission of fluorescence. As the probes are broken up by the *Taq* DNA polymerase the fluorescent reporter is released and the rate of change of fluorescence followed to determine mRNA abundance. The mRNA levels of genes are assessed relative to the mRNA levels of reference genes, to control for reverse transcription efficiency (Zhu and Altmann, 2005).

2.5.1.1 RNA extraction from liver

RNA extraction was carried out using RNeasy mini kits from Qiagen, West Sussex, UK.

Liver tissue (~30mg) was homogenised in RLT buffer (600µl), samples were then centrifuged at 12,500g for 3 min. The supernatant was removed and mixed with an equal volume of 70% v/v ethanol; this solution was placed on the RNeasy spin column and spun 8,000g 15 sec and the eluate discarded. The column was washed with RW1 buffer (700µl) followed by RPE buffer (700µl), spun at 8,000g for 15 sec, the eluate being discarded after each spin. A final wash with buffer RPE (500µl) was carried out followed by centrifugation at 8,000g for 2 min, the spin column was then transferred to a clean collection tube and spun at 12,500g for 1 min to fully dry the membrane and eliminate any carryover of wash buffers. The spin column was then placed in a clean eppendorf tube and RNA eluted with 50µl RNAse free water and centrifuged at 8,000g for 1 min. RNA was immediately placed on ice and stored at -80°C.

2.5.1.2 RNA extraction from adipose tissue

RNA extraction from adipose used the same method as liver (2.5.1.1) except for tissue homogenisation. Tissue (~60mg) was homogenised in 1ml Qiazol Lysis reagent (Qiagen). 300µl chloroform was added, mixed thoroughly and incubated at RT for 5min. Samples were centrifuged 4°C, 13,000g, 15min, to separate phases. The supernatant containing RNA was transferred to a new tube and an equal volume of 70% ethanol added as with the liver samples. The remainder of the procedure was the same as with liver (2.5.1.1), except RNA was eluted with 30µl RNase free water.

2.5.1.3 RNA quality assessment

Quality of RNA was assessed by separating ribosomal RNA (rRNA) using electrophoresis. 8µl loading dye was added to 2µl RNA, centrifuged and loaded on an agarose (Lonza, Berkshire, UK) gel (1.5%w/v in 0.5x TBE buffer) containing SYBR safe DNA dye (Invitrogen), 1:10,000. Gels were electrophoresed at 100v for ~1hr. RNA integrity was determined by the existence of two clear rRNA bands, 28s and 18s, with no smearing, and no genomic DNA band. If degradation was found RNA was extracted again from a new piece of tissue. If DNA contamination was found samples were treated with DNAse (section 2.5.1.5).

A 2100 Bioanalyzer system (Agilent Technologies, Cheshire, UK) was also used to investigate RNA quality in one experiment, although desirable due to cost it could not be used in all experiments. The Bioanalyzer uses micro chips containing a sieving polymer and fluorscent dye. Samples and ladder are loaded in to the wells and the chip becomes an electrical circuit. As in normal gel electrophoresis the RNA is electrophoretically driven by the voltage gradient and molecules separated by size. The dye molecules intercalate in to the RNA so that the complexes can be detected and speed of movement compared to a reference ladder. Data is translated in to gel like images and electropherograms (Figure 2.1b) by the provided software and an RNA integrity number (RIN) calculated. A RIN value of 10 indicates intact RNA and a value of 1 fully degraded, values over 7.15 are believed to be acceptable for microarray anaylsis (Kiewe *et al.*, 2009) the RIN of the sample shown was 8.3 (Figure 2.1a). This is a user-independent, automated system for RNA quality control and therefore robust in predicting integrity (Schroeder *et al.*, 2006). Although the gel images produced by the Agilent Bioanalzyer are of a very high resolution compared to standard gel electrophoresis the results were found to be comparable such that one sample containing degraded RNA and not suitable for downstream application was detected by both methods.

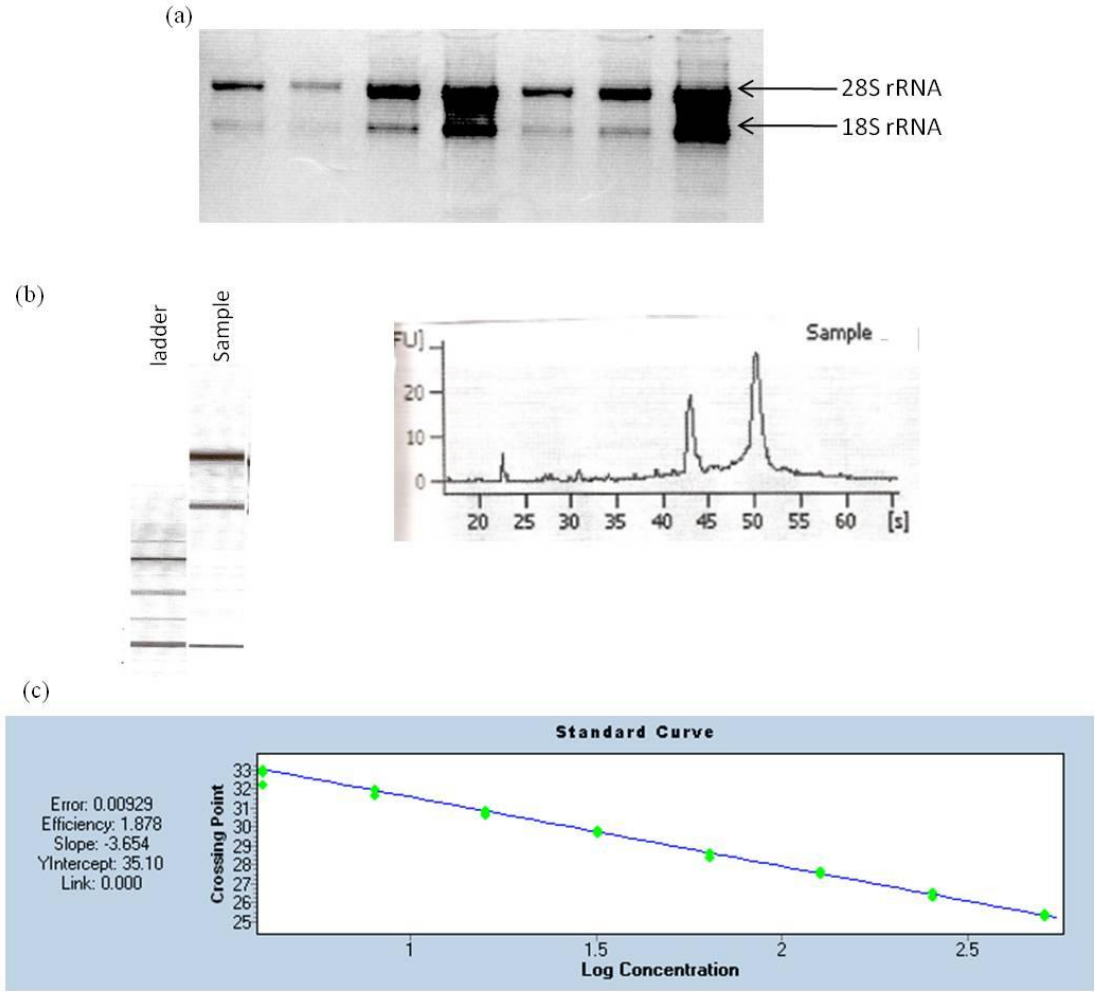


Figure 2.1 Representative gel electrophoresised RNA, Bioanalyzer gel and electropherogram and qPCR standard curve

Quality of RNA samples was analysed visually using gel electrophoresis. Samples were separated on a 1.5%w/v agarose gel at 100v for 1hr. RNA integrity was deemed acceptable if clear 18s and 28s ribosomal RNA bands were visible without smearing as is seen in these samples (a). The intensity of the band was reflective of RNA quantity, accurately assessed using spectrophotmetry (section 0). The quality of one set of RNA was also assessed using a Bioanalzyer (Agilent Technologies). This also separated RNA by electrophoresis but software provides user-independent gel images and electropherograms by measuring fluorescence (FU) against time (s). These images are used to generate a RNA integrity number (RIN) which was 8.3 for the sample shown.
qPCR was used to analyse the transcript abundance of mRNA. The crossing point (Cp), a measurement of fluorescence emission, decreases as log concentration of mRNA increases. Cp of standards was plotted against log 'concentration' (c) by Lightcycler Software (Roche). The mRNA abundance of unknown samples was determined from their Cp by interpolation of the standard curve and presented relative to the abundance of reference genes.

This sample had a RIN value of 5.2, while all others tested were above 7.15.

2.5.1.4 RNA quantification

RNA was quantified using a NanoDrop (ND) Spectrophotometer (Thermo Fisher, Leicestershire, UK) measuring absorbance at 260nm. The ND-1000 software provided concentrations in ng/µl using Beer Lamberts law. Purity of RNA with respect to contaminating protein and salts was also monitored by the software. The ratio of absorbance at 260nm was compared to 230nm (260/230), for phenol, and 280nm (260/280), for protein, with values between 1.8 and 2.1 deemed acceptable.

2.5.1.5 DNAse treatment

Genomic DNA contamination was removed using a DNA-*free* kit from Ambion (Applied Biosystems, Cheshire, UK) according to the manufacturers' protocol. DNase 1 buffer (10x, 4µl) and rDNase 1 (1µl) was added to 40µl RNA (diluted 1:4 in RNase free water) and incubated at 37°C for 30 min. DNase inactivation reagent (4µl) was added; samples agitated before centrifugation 10,000g 1.5min. Supernatant containing RNA was removed and underwent RNA quality (2.5.1.3) and quantity (0) measurements.

2.5.1.6 Reverse transcriptase polymerase chain reaction (RT-PCR)

Total RNA (liver -0.25µg, adipose – 0.5µg) was reverse transcribed using the Access RT-PCR system (Promega, Hampshire, UK) according to manufacturers' protocol, samples were incubated in a PCR machine (G-Storm1, Labtech International, East Sussex, UK) Briefly, each RNA sample was diluted to the appropriate concentration (liver – 30ng/µl, adipose - 60ng/µl) with RNase free water, 8.7µl of which was incubated at 75°C for 10 min to denature samples. Following denaturation 11.3µl of 'mastermix' was added:

RT-PCR mastermix composition, reagents from Access RT-PCR system (Promega):

5x AMV-RT Buffer - 4µl
$MgCl_2$ (5mM) - 4µl
dNTP mixture (1mM) - 2µl
Oligo dTs – 0.2µl
Recombinant RNasin® Ribonuclease Inhibitor (1u/µl) – 0.5µl
AMV (Avian Myeloblastosis Virus) RT polymerase (15u/µg) – 0.6µl

Samples were incubated at room temp for 10min for annealing and extension, followed by 42°C, 15min for AMV-RT to transcribe cDNA and 95°C, 5min to inactivate enzymes. Two

negative controls were included, one with no RNA and the second with no AMV-RT enzyme to ensure reagents were not contaminated and did not contain genomic DNA. Samples of cDNA were stored at -20°C.

2.5.1.7 Quantitative real time polymerase chain reaction (qPCR)

Abundance of mRNA was quantified using a LightCycler 480 (Roche Applied Science, West Sussex, UK). Primers (details in Table 2.2) were designed to match intron-spanning boundaries wherever possible using the Roche Universal Probe Library (UPL). Where a satisfactory assay was not available using the UPL system a commercially designed assay was used (*Taq*Man® Gene Expression Assays; Applied Biosystems). Approximately 2µl of each cDNA sample to be analysed was pooled, a standard curve was created (1:4 – 1: 512) from the pool. Samples (diluted in LightCycler H_2O; liver 1:40, adipose 1:20), standards and controls, negative reverse-transcriptase control (diluted as samples) and H_2O, were added to the plate, 2µl of each in triplicate followed by 8µl of Master Mix.

qPCR mastermix composition:

	UPL system	ABI system
LightCycler H_2O	2.7µl	2.5µl
Roche Probes Master	5µl	5µl
Primers (12pmol/µl)	0.1µl	N/A
Probe (Roche) (4pmol/µl)	0.1µl	N/A
Primer Probe mix	N/A	0.5µl

qPCR plates were centrifuged at 8,000g for 2min, to ensure all samples and mastermix were at the bottom of the well. Samples were denatured (95°C, 5min), then underwent 50 PCR amplification cycles; denaturation (95°C, 10sec), annealing (60°C, 30sec) and elongation (72°C, 0.1sec). The LC480 Software (Roche) monitored fluorescence during the PCR and plotted amplification curves, fluorescence against PCR cycle number. The crossing point (Cp) was calculated as the maximum point of the second derivative of the amplification

Gene accession number	Primer sequence	UPL probe
5α reductase (5αR*)* *Srd5a1* (NM_175283.3)	For – gggaaactggatacaaaataccc Rev - ccacgagctccccaaaata	41
5β-reductase (5βR*)* *Akrd1* (NM_145364.2)	For – gaaaagatagcagaagggaaggt Rev - gggacatgctctgtattccataa	103
11β hydroxysteroid dehydrogenase (11β-HSD1*)* *hsd11b1* (NM_008288.2)	For – tctacaaatgaagagttcagaccag Rev - gccccagtgacaatcactt	1
Estrogen receptor (ER*)* *Esr1 (*NM_007956.4)	For – gctcctaacttgctcctggac Rev - cagcaacatgtcaaagatctcc	97
Fatty acid synthase (FAS*)* *fasn* (NM_007988.3)	For – ccaaatccaacatgggaca Rev - tgctccagggataacagca	34
Glucocorticoid receptor (GR*)* *Nr3c1* (NM_008173.3)	For – tgacgtgtggaagctgtaaagt Rev - catttcttccagcacaaaggt	56
Hormone sensitive lipase (HSL*)* *lip* (NM_010719.5)	For – gcgctggaggagtgttttt Rev - ccgctctccagttgaacc	3
Lipoprotein lipase *(*LPL*)* *lpl* (NM_008509.1)	For – ctcgctctcagatgccctac Rev - ggttgtgttgcttgccatt	95
Peroxisome proliferator activated receptor-α (PPARα*)* *ppara* (NM_011144.2)	For – ccttccctgtgaactgacg Rev - ccacagagcgctaagctgt	5
Peroxisome proliferator activated receptor-γ (PPARγ*)* *pparg* (NM_011146.1)	For – tgctgttatgggtgaaactctg Rev - ctgtgtcaaccatggtaatttctt	2
phosphoenolpyruvate carboxykinase (PEPCK*)* *pck1* (NM_011044.2)	For – gatgacattgcctggatgaa Rev - cgttttctgggttgatagcc	105
Tata box binding protein (TBP*)* *tbp* (NM_013684.2)	For – gggagaatcatggaccagaa Rev - gatgggaattccaggagtca	97
Taqman Gene Expression Assays purchased from ABI		
Cyclophilin (CYC*)* *ppia (*NM_008907.1)	Mm02342430_gl	
β-actin (β-actin*)* *actb (*NM_007393.3)	Mm01215647_gl	
Androgen receptor (AR*)* *ar* (NM_013476.3)	Mm00442688_m1	
Glyceraldehyde-3phosphate dehydrogenase (GAPDH*)* *Gapdh* (NM_008084.2)	Mm99999915_g1	

Table 2.2 Details of primers for qPCR

Primers were designed using the Roche Universal Probe Library (UPL) or a commercially designed assay was used (*Taq*Man® Gene Expression Assays; Applied Biosystems). Abbreviations in brackets are used in this thesis (for, forward; rev, reverse).

curve. Triplicates were deemed acceptable if the standard deviation of crossing point <0.3 cycles. Negative controls were deemed acceptable if Cp >10 cycles were higher than that of the lowest standard. A standard curve of Cp against log 'concentration'(serial dilution) was generated (Figure 2.1c) for each gene and deemed acceptable if reaction efficiency was between 1.8 and 2.1. The mRNA 'concentration' of unknown samples were interpolated from the standard curve and expressed relative to the abundance of a reference gene. Reference genes were chosen for each study and tissue independently and are listed in the relevant chapters. Ideally reference genes are not affected by any treatment in the study; however this was not always possible particularly when dealing with sex differences. Where there were group differences in mRNA abundance for reference genes, the pattern of abundance was comparable for all reference genes tried (CYC, β-actin, TBP,GAPDH) and therefore the variation could be due to actual amount of cDNA in the well rather than effect of treatment. mRNA abundance of 18S rRNA is often used as a reference gene for q PCR. This was not possible in these studies because cDNA was synthesised using dTT primers, as 18S does not have a polyA tail (Zhu and Altmann, 2005) efficient synthesis could not be ensured. The mRNA abundance was normalised to cyclophilin, β-actin or an average of both in all chapters. These were chosen as the mRNA was abundant in the tissues used, the Cp of the highest standard being between 23-25 cycles. The standard deviation between samples was also very low, <0.1 cycles further indicating high levels of mRNA. Finally the reference genes chosen have been used by other studies investigating murine metabolism (D'Eon *et al.*, 2005; Bruce *et al.*, 2009; Livingstone *et al.*, 2009).

2.6 Biochemical assays

All spectrophotometric measurements were carried out with an OPTImax Tunable Microplate Reader (Molecular Devices, CA, USA). Standard curves were plotted SoftMax Pro Software (Molecular Devices) using linear regression, with the exception of the corticosterone assay (2.6.8).

2.6.1 Quantification of protein

Protein concentrations were determined using the DC Protein Assay (Biorad, Hertfordshire, UK) based on the Lowry method (Lowry *et al.*, 1951)). Standards of BSA Protein concentrations were determined using the DC Protein Assay (Biorad) as directed by manufacturers; the assay is based on the Lowry method. Standards of BSA were made ranging from 2 to 0.1mg/ml. Standards and samples were added to a 96 well plate, 5µl in

duplicate, followed by 25µl of reagent A then 200µl reagent B. Plates were incubated at room temperature for between 20-50 minutes and absorbance measured at 750nm. Absorbance of protein standards was used to generate a standard curve (absorbance against concentration) which was deemed acceptable if r^2>0.95 and duplicates differed by less than <10%. Sample protein concentrations were calculated from the standard curve, where samples absorbance was not in the standard curve further dilutions were carried out and the assay repeated.

2.6.2 Quantification of plasma insulin using Enzyme-Linked Immunosorbent Assay (ELISA)

Insulin was quantified in plasma samples taken during glucose tolerance tests (section 2.3.7) using the Ultra Sensitive Mouse Insulin ELISA Kit (Crystal Chem Inc., IL, USA) as directed by the manufacturer. Briefly, a series of insulin dilutions were prepared (6.4-0.1ng/ml) in sample diluent provided with the kit. Samples (5µl, in singlicate due to the limited availability of plasma) and standards (5µl, in duplicate) and 95µl sample diluent were added to wells which had been precoated with primary guinea pig anti-insulin antibody, and incubated for 2hrs 4°C. Plates were washed 5 times with wash buffer to remove unbound insulin. 100µl horseradish peroxidise (HRP) conjugated anti insulin was added to each well and incubated for 30min. Plates were washed 7 times with wash buffer before the addition of 100µl of enzyme substrate solution containing tetramethylbenzidine (TMB). The plate was incubated in the dark for 40min and 100µl sulphuric acid (0.5M) added to stop the reaction. Absorbance was measured spectrophotomically at 630 and 450nm, and values at 630nm subtracted (deemed to be due to artefacts on the plate). A standard curve was fitted, absorbance against concentration and deemed acceptable if r^2>0.95 (Figure 2.2), duplicates of standards were accepted if they varied by <10%. Unknown sample concentrations were interpolated from the curve.

2.6.3 Quantification of testosterone in plasma by ELISA

Testosterone was quantified in plasma samples taken at post mortem (section 2.3.4.3) using the Demeditec Testosterone rat/mouse ELISA (Demeditec Diagnostics GmbH, Kiel, Germany) following manufacturer's instructions. The ELISA is based on the principle of competitive binding; an unknown amount of testosterone in the sample competes with a known amount of testosterone (provided) conjugated to HRP for the binding sites in the

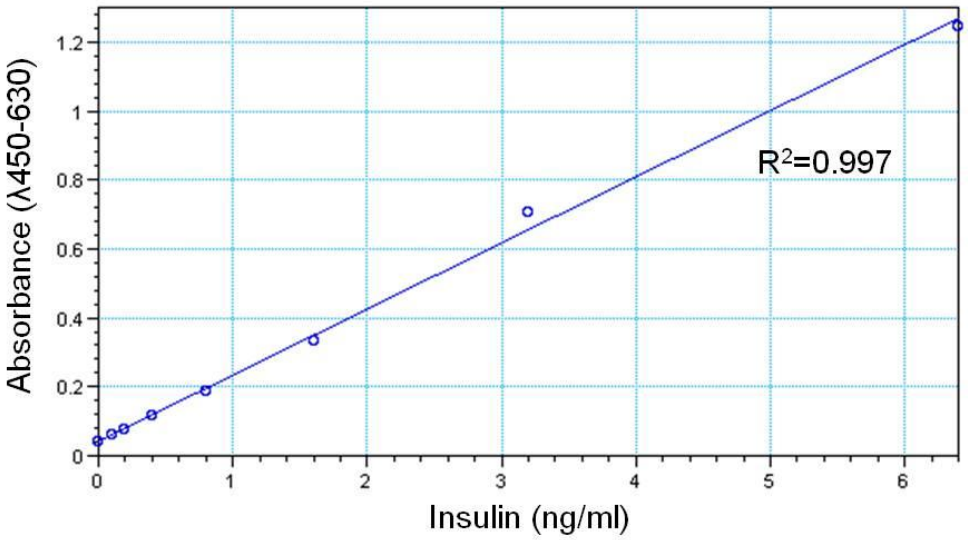


Figure 2.2 Example of insulin ELISA standard curve

Concentration of insulin bound to the plate increased with increasing concentrations of standards, as measured by absorbance (λ450-630). Graphs were plotted with SoftMax Pro Software (Molecular Devices) using linear regression. Standard curves were deemed acceptable if r^2 >0.95 and variation between standards was <10%. The insulin concentration of samples was determined from their absorbance (λ450-630) by interpolation against the standard curve.

wells of the plate. Following washing of unbound steroid and addition of substrate for HRP the concentration of testosterone is inversely proportional to the optical density measured.

Samples (10µl, in singlicate) and standards (10µl, duplicate, provided with kit) were added to the plate, followed by 100µl incubation buffer and 50µl enzyme conjugate. The plate was incubated at RT with shaking for 60mins and unbound protein removed by washing (4 x 300µl), this was followed by the addition of 200µl substrate solution and further incubation for 30mins without shaking. The reaction was stopped with 50µl stop solution and absorbance read at 450nm. A standard curve (absorbance against concentration) was plotted on a semi-logarithmic scale and deemed acceptable if r^2>0.9 and duplicates of standards varied by <10%. Sample concentrations were then interpolated from the curve.

2.6.4 Quantification of plasma glucose

Glucose was quantified in plasma samples taken during glucose tolerance tests (section 2.3.7) using the Infinity Glucose Hexokinase Liquid Stable Reagent (Thermo Electron, PA, USA). The reagent contained substrates, cofactors and enzymes which phosphorylate and oxidise glucose by reducing NAD^+ to NADH, this cofactor can be measured spectrophotomically.

Serial glucose standards were made (22-2.5mmol/L) from standard provided diluted in distilled water. Samples (2µl, in singlicate due to scarcity of plasma) and standards (2µl, duplicate) were added to a 96-well plate followed by 200µl reagent and incubated in darkness (10min, RT). Absorbance was measured at 340nm and a standard curve generated (absorbance against concentration), deemed acceptable if r^2>0.95 and duplicates of standards varied by <10%. Sample concentrations were then interpolated from the curve.

2.6.5 Quantification of cholesterol in plasma

Cholesterol was quantified using the Infinity Cholesterol Liquid Stable Reagent (Thermo Electron). The reagent contained substrates, cofactors and enzymes which catalysed hydrolysis and oxidation of cholesterol to form H_2O_2; this was turned in to a chromophore, by peroxiadase, which was measured spectrophotometrically.

Serial dilutions of cholesterol standard (provided) were made (20-0.5mmol/L) in distilled water. Samples and standards (2µl, duplicate) were added to a 96-well plate followed by 200µl reagent and incubated 37°C, 5min. Absorbance was read at 600nm (plate background) and 500nm (proportional to cholesterol concentration). Difference in absorbance readings

($A_{500} - A_{600}$) was plotted against concentration to generate a standard curve, this was deemed acceptable if r^2>0.95 and duplicates of standards were accepted if they varied by <10%. Sample concentrations were then interpolated from the curve.

2.6.6 Quantification of triglycerides

Triglyceride was quantified in plasma and saponified liver samples using the Infinity Triglyceride Liquid Stable Reagent (Thermo Electron). The reagent contained substrates, cofactors and enzymes which break down triglycerides into glycerol and free fatty acids, these are then phosphorylated and reacted with oxygen to form H_2O_2; this was turned in to a chromophore, by peroxidase, which was measured spectrophotometrically.

Serial dilutions of triglyceride standard (provided) were made (5.65-0.28mmol/L) in distilled water. Samples (2µl, in singlicate due to scarcity of plasma, duplicate for liver samples) and standards (2µl, duplicate) were added to a 96-well plate followed by 200µl reagent and incubated 37°C, 5min. Absorbance was read at 600nm (plate background) and 500nm (proportional to triglyceride concentration). Difference in absorbance readings ($A_{500} - A_{600}$) was plotted against concentration to generate a standard curve, this was deemed acceptable if r^2>0.95 and duplicates of standards were accepted if they varied by <10%. Sample concentrations were then interpolated from the curve.

2.6.7 Quantification of Non-Esterified Fatty Acids (NEFAs) in plasma

NEFAs were quantified using the HR Series NEFA-HR kit (Wako Diagnostics, VA, USA). The reagents contained substrates, cofactors and enzymes which converted NEFAs into fatty acyl-CoA esters, these were then reacted with oxygen forming H_2O_2; this was turned in to a chromophore, by peroxidase, which was measured spectrophotometrically.

Serial dilutions of NEFA standard (provided) were made (2-0.1mmol/L) in distilled water. Samples (5µl, in singlicate due to scarcity of plasma) and standards (5µl, duplicate) were added to a 96-well plate followed by 150µl Reagent R1 and incubated 37°C, 10min. Reagent R2 was then added, 75µl incubated for a further minute and the absorbance read at 550nm (proportional to NEFA concentration) and 660nm (plate background). The difference in absorbance ($A_{550} - A_{660}$) was calculated and plotted against concentration to generate a standard curve. This was deemed acceptable if r^2>0.95 and duplicates of standards varied by <10%.

2.6.8 Quantification of corticosterone in plasma by radioimmunoassay (RIA)

Plasma corticosterone was quantified using an in house assay samples collected at the peak and nadir of the diurnal variation (section 2.3.8). Plasma was diluted 10% (v/v) with borate buffer and incubated (80°C, 45min) to denature corticosteroid-binding globulin (CBG) and release corticosterone. Serial dilutions of corticosterone were prepared from a standard (320-0.06nM) using borate buffer. Standards and samples (20µl) were added to a 96-well plate in duplicate. A mix of primary antibody (against corticosterone) and $[^3H]_4$-corticosterone was prepared; 3µl of $[^3H]_4$-corticosterone (62.2 Ci/mmol, GE Healthcare) was added to 6ml borate buffer and adjusted to 8,000cpm, followed by 60µl sheep anti-mouse primary antibody (pre-diluted 1:100, to give a final dilution of 1:10,000), a gift from Dr Christopher Kenyon, University of Edinburgh. The primary antibody mix (50µl) was added to each well followed by 50µl Scintillation proximity assay (SPA) beads (5mg/ml in borate buffer, GE Healthcare), coated with anti-sheep secondary antibody, the plate sealed, inverted to mix and incubated in the dark for 16 hours.

Scintillation occurred when the SPA beads bound to primary antibody which was associated with $[^3H]_4$-corticosterone. The scintillation decreased as the concentration of unlabelled corticosterone increases, due to the increase in competition to bind the primary antibody. The scintillation was quantified using a liquid scintillation counter (Microbeta Plus, Wallac 1450, Turku, Finland), and data used to construct a semi-log standard curve (bound/bound at zero concentration against corticosterone concentration) on AssayZap software (Biosoft, Cambridgeshire, UK) (Figure 2.3). Plasma corticosterone in each sample was interpolated from the graph and the original dilution taken in to account. Duplicates of standards were deemed acceptable if they varied by <20%.

2.7 Extraction of hepatic triglyceride

To analyse hepatic triglyceride concentrations, lipids were extracted from tissues using a saponifcation method. This hydrolyses triglycerides under basic conditions to form glycerol and fatty acid salts, unwanted proteins are removed by salting out with $MgCl_2$. Triglyceride content can be analysed by measuring the amount of glycerol produced in the hydrolysis.

Liver tissue (~100mg) was digested at 55°C overnight in 10% (w/v) potassium hydroxide in ethanol; undigested tissue was removed by centrifugation at 10,000g 5min. Supernatant was

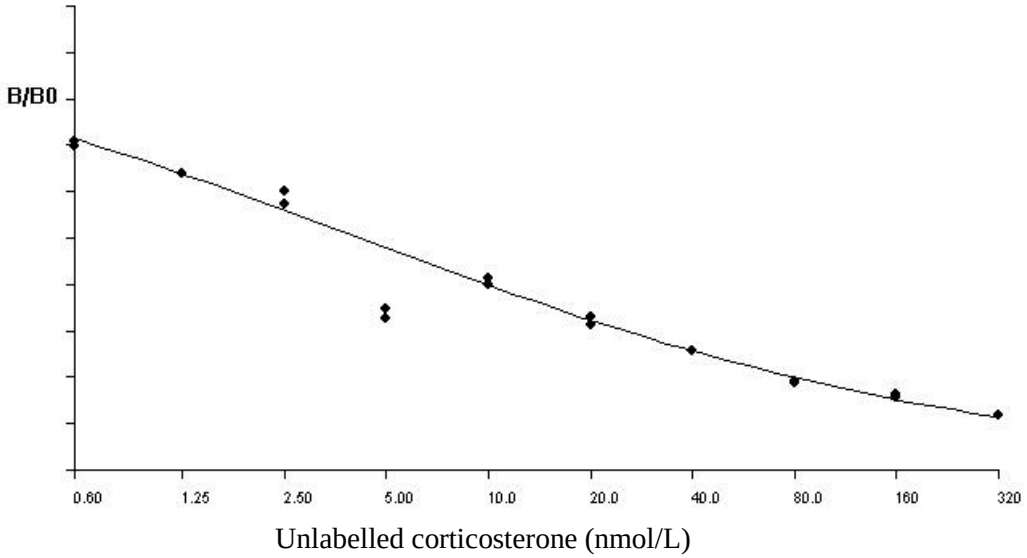


Figure 2.3 Example corticosterone radioimmunoassay standard curve

The proportion of bound [H] corticosterone ($B/B_{0,}$ y axis) decreases as it is displaced by increasing concentrations of unlablelled corticosterone (x-axis). Graph points were fitted with a sigmoidal concentration-response curve using AssayZap software. The corticosterone concentration of samples was determined from their B/B_0 by interpolation from the standard curve.

removed and mixed with 1M magnesium chloride (1:1), samples were then vortexed, incubated on ice for 10min and centrifuged at 10,000g, 5min. The supernatant was used to measure triglyceride as in section 2.6.6. While the saponification generates glycerol the first step in the assay hydrolyses triglycerides into glycerol therefore the same assay can be used.

2.8 Protein

2.8.1 Tissue preparation for enzymatic activity assays

To allow for enzyme activity analysis tissue homogenates of liver and adipose were made. In liver the homogenates were further processed to separate microsomes, the location of 11β-HSD1, and cytosol as previously described (Livingstone *et al.*, 2000; Livingstone *et al.*, 2005).

2.8.1.1 Microsome and cystosol preparation

Tissue (~100mg) was homogenised in 1.8ml homogenisation buffer (2.2.8), followed by centrifugation at 1000g ,4°C, 15 min to pellet heavy membranes. Supernatant was removed and subjected to ultra centrifugation using an Optima TLX, with a TLA100.3 rotor (Beckman Coulter, High Wycombe, UK) at 48,600rcf, 4°C, 30min to pellet membrane fraction. Supernatant was again removed and samples spun at 163,350rcf, 4°C, 1hr. Supernatant containing cytosol was aliquotted and stored at -80°C. The pellet containing microsomes was resuspended in 500µl Krebs buffer (2.2.9), aliquotted and stored at -80°C.

2.8.1.2 Adipose tissue homogenates

Tissue (~100mg) was homogenised in 1.8ml Krebs buffer (2.2.9) and centrifuged 1,000rcf, 4°C, 5 min. The infranatant, a clear layer above the pellet and below the glycerol top containing the protein was removed, aliquotted and stored at -80°C.

2.8.2 Enzymology

2.8.2.1 11β-HSD1 activity

11βHSD1 acts as a reductase *in vivo*, converting inactive 11-dehydrocorticosterone to corticosterone. However, *in vitro*, dehydrogenase activity predominates; therefore 11β-HSD1 activity was quantified through conversion of corticosterone to 11-dehydrocorticosterone.

Enzyme kinetics were determined from hepatic microsomal fractions (section 2.8.1.1) and whole tissue homogenates from adipose (section 2.8.1.1). Tissue was incubated at 37°C in Krebs buffer containing glucose (1% w/v), NADP (2mM), 1,2,6,7-[$^{3}H]_4$-corticosterone (GE Healthcare) and unlabelled corticosterone. Protein concentration and time points were optimised for each tissue in each study to ensure first order enzyme kinetics; details are listed in relevant chapters. First order kinetics occurs when the rate of reaction is dependent on the concentration of only one reactant, such that if you double the amount of reactant the reaction velocity will also double. This linear relationship is only present up to a certain concentration, or time, after which the line starts to curve and flatten off. An example of a graph used during optimisation can be seen in Figure 2.4a, this shows the linear relationship between time and product formed is present. Other studies in our laboratory have shown the activity of 11β-HSD1 is in first order kinetics until 30% of substrate has been converted to product (Personal communication Dr R Andrews, University of Edinburgh).

2.8.2.2 Steroid extraction and separation

After incubations steroids were extracted into fresh tubes with 10x ethyl acetate, the organic phase was then evaporated under nitrogen at 60°C. The dried extracts were dissolved in 500µl mobile phase (60% H_2O, 15% Acetonitrile, 25% Methanol). Steroids were injected using a 717plus Autosampler (Waters Ltd., Hertfordshire, UK) and separated by high performance liquid chromatography (HPLC). Steroids were eluted (600 Controller pump, Waters Ltd.) with mobile phase (1.5ml/min, 45ºC) from a C_{18} Sunfire column (length 15cm; internal diameter, 4.6mm, pore size 5µm; Waters Ltd.) and quantified by on-line scintillation counting after mixing with Goldstar scintillation fluid (2ml/min; Meridian, Surrey, UK) using a scintillation pump (Berthold, Hertfordshire, UK). The proportion of corticosterone converted to metabolites was calculated by integrating the peak areas of the analytes in the chromatograms (

Figure 2.4) using Chromeleon software (Dionex, CA, USA). Specific activity was determined using the concentration of protein and substrate and time of incubation. Assays were performed in duplicate and deemed acceptable if they varied by <10%. Minimum peak height was set at 3x background. First order kinetics were determined by ensuring conversion <40%.

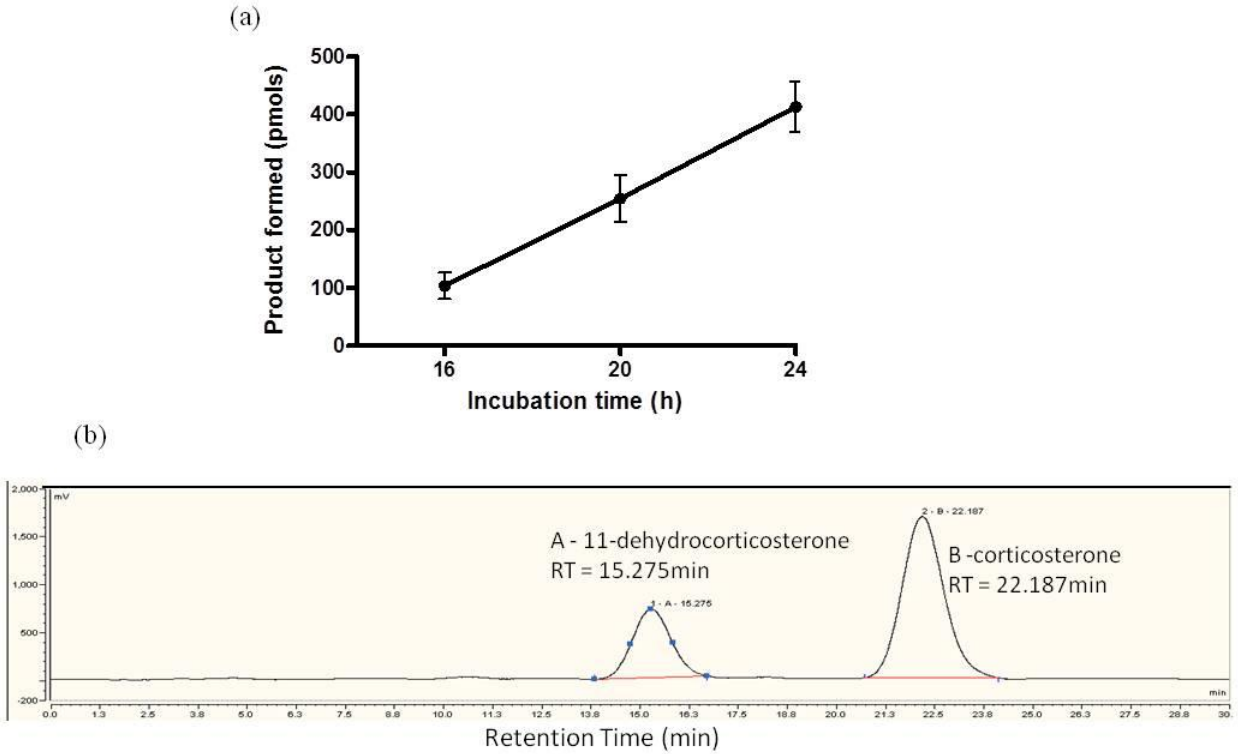


Figure 2.4 Optimisation of first order kinetics for 11β-HSD1 assay and chromatogram showing separation of 11-dehydrocorticosterone (A) and corticosterone (B) by HPLC

To ensure first order kinetics when determining activity of 11β-HSD1 product formed (pmols) from 2μM corticosterone was determined at 3 time points. 3 samples from each group were used, in this case pre-pubertal animals investigated in chapter 4, and it shows the linear relationship was present over the range of incubation times used (a). Products of 11β-HSD1 assays were separated by HPLC and quantified using on-line scintillation counting. Standards were used to determine retention times (RT) of expected metabolites as is shown in (b). Enzyme activity was quantified by comparing the relative area of the substrate and product peaks following integration.

2.1 Imaging techniques

2.1.1 Optical projection tomography (OPT)

OPT is a relatively new 3D imaging technique which can be used to image murine neointimal lesions generated using the luminal wire injury model (section 2.3.5.4) (Kirkby *et al.*, 2011). OPT provides images of a greater resolution to other methods available at a lower cost (Sharpe *et al.*, 2002). While analysis by histology offers superior resolution than OPT, generating volumetric data by 3D reconstructions of 2D sections is lengthy, labour intensive and destructive. OPT can quickly provide 3D images of blood vessels, as well as leaving tissue intact allowing for further analysis with histology (Kirkby *et al.*, 2011).

The technique works by suspending the sample in a refractive index-matching solution and illuminating it with an ultraviolet source shone through a filter selective for a specific excitation wavelength. Photons emitted by endogenous fluorophores in the specimen are focussed through an emission filter to the detector. Multiple projections covering a 360° rotation of the sample are reconstructed using a modified computed tomography back-projection algorithm to produce a 3D image.

2.1.1.1 Tissue preparation

Femoral arteries to be analysed by OPT were excised as described (2.3.4.2 & 2.3.4.3). Each femoral artery was suspended in 1.5% (w/v, 40ml) low melting point agarose which was cooled to 40-50°C and filtered through 113v Whatman paper (GE Healthcare). Arteries were suspended as straight as possible and agarose rapidly cooled. Gelled agarose blocks were fixed to magnetic mounts using cyanoacrylate adhesive (Henkel, Hertfordshire, UK) with the artery in the axis of the mount. The mounted blocks were trimmed into a conical shape to minimise back-reflection of light. Mounted samples were dehydrated in methanol (Fisher Scientific) for 24 hours then transferred to the refractive index matching solution BABB (2.2.4) and allowed to clear for >16 hours.

2.1.1.2 Scanning and reconstruction

Before scanning the optical projection tomograph (Bioptonics 3001, Bioptonics, Edinburgh, UK) was calibrated to the axis of rotation using the pin provided and applying an appropriate correction factor. Mounts with tissue attached were placed in the scanning chamber using the magnetic attachment. The sample position was adjusted until revolving around its own centre

of axis in the centre of the field of view. Magnification was set to 1pixel = 6µm, this was the maximum magnification possible to view the vessel. For scanning, samples were illuminated by a UV source with a 425/20nM band-pass excitation filter and 475nM low-pass emission filter (GFP filter) which strongly detects auto fluorescence. The exposure time was adjusted to maximise the resolution of the resulting image and the scanner focused by eye. Raw data was acquired by automated capture of images (1024 x 1024 pixels) at 0.9° rotation increments to give 400 images covering 360° rotation. After scanning the mounted femoral arteries were cleared of BABB in methanol >24hrs, detached from mounts and trimmed of excess agarose before processing for histology (section 2.2).

Images were visually assessed for focus and rotation misalignment using appropriate software (Data Viewer, Sky Scan, Kontich, Belgium), before computed tomography reconstructions were calculated. A misalignment correction was applied to each dataset using NRecon Software (Skyscan) to compensate for defects in the axis of rotation. The compensation was calculated by manually viewing reconstructions of 2D planes at various points in the vessel.

2.1.1.3 Quantification

Reconstructed OPT data was quantified using CT data analysis software (CTan, SkyScan). In each vessel the region of interest, containing the intimal lesion, was defined. The border between the media and neointima (the internal elastic lamina) was estimated and traced ~every 40 scans. This was possible due to the greater fluorescence emitted by the media. The intima/media border for the interleaved scans was interpolated by the software; this was checked and corrected where necessary. The defined volume, inside the internal elastic lamina, was subjected to a manually defined intensity threshold which produced a binary image with white pixels representing neointima and black pixels lumen. Volumes (μm^3) of neointima and lumen were calculated from the stack of cross-section areas by the software. The volume of lesion was expressed according to the following equation

$$Volume\ of\ neointima\ inside\ IEL\ (\%) = \frac{neointima\ volume\ (\mu m^3)}{volume\ inside\ IEL\ (\mu m^3)}\ x\ 100$$

As 3D volumetric data is calculated from a stack of 2D cross-section images, the maximum cross-sectional area of lesion can also be found in each vessel. The image with the largest neointima was selected as representative for each vessel. Stenotic ratio was calculated according to the following equation

$$Stenotic\ ratio = \frac{Neointima\ area\ (\mu m^3)}{Area\ inside\ IEL\ (\mu m^3)}$$

2.2 Histological techniques

2.2.1 Tissue preparation

2.2.1.1 Preparation of vaginal smears

Samples from vaginal smears (section 2.3.10) were air dried on to slides, to further fix the cells slides were immersed in 100% methanol (Fisher Scientific) for 2 minutes before staining.

2.2.1.2 Preparation of femoral arteries

To allow thin sections of tissues to be cut and analysed for morphology and composition samples were first processed. This removes water from the tissue and replaces it with paraffin wax which is solid at room temperature. The wax blocks containing tissue can then be cut and the wax is later removed when the tissue is stained.

Femoral artery samples were processed in agarose due to prior OPT analysis (2.1.1). Samples were loaded into cassettes and processed to wax using a Tissue-Tek vacuum infiltration processor (Sakura Finetek Europe, Alphen aan den Rijn, The Netherlands) by the University of Edinburgh Histology Lab. Briefly, cassettes were dehydrated in increasing alcohol concentrations (50-100%), cleared in xylene and immersed in molten wax (Thermo-Shandon, Cheshire, UK) at 60°C. This was performed under vacuum with agitation to ensure infiltration of solvent/wax. Samples were correctly positioned in molten paraffin embedding moulds and cooled on a Tissue-Tek Cryo-console (Sakura Finetek Europe) to solidify wax.

Sequential 4µm sections were cut using a Leitz 1512 microtome (Leica Microsystems, Buckinghamshire, UK), then floated on a waterbath ~35°C to smooth the sections before being mounted 2 per slide (Superfrost Plus electrostatically-coated microscope slides, VWR). Slides were baked at 37°C overnight to fully adhere sections to slides. Femoral artery sections were mounted using the following pattern, 20 sections mounted then 20 sections (80µm) discarded, this was repeated until vessel morphology was normal, determined by microscopic analysis. Where possible OPT scans were used to determine the largest area of lesion and therefore when to stop sectioning.

2.2.2 Staining

2.2.2.1 Grunewald-Giemsa staining

Vaginal smears were stained with Grunewald-Giemsa, this visualises different cell types, specifically platelets and leukocytes which can be used to determine stage of estrus as previously described (Meziane *et al.*, 2007).

Fixed vaginal smears were immersed in 5%v/v Giemsa stain (stock 0.4% v/v, Sigma Aldrich) for 4 min, and washed in dH_2O for 1 min. Smears were mounted in DPX mountant (BDH, Dorset, UK) and left to dry before microscopic analysis.

2.2.2.2 United States Trichrome (UST) staining

For morphological analysis the first femoral artery slide of each set was stained using a UST method (Hadoke *et al.*, 1995). This stain uses a Gomori's aldehyde Fucshin to stain Elastin, as well as Gomori's trichrome which outlines collagen (blue-green), elastin (deep purple), and cells (cytoplasm – red, nuclei – black).

Staining was carried out using an automated cytology stainer (Varistain Gemini, Thermo-Shandon) as previously described (Hadoke *et al.*, 1995). Briefly slides were deparaffinised in xylene (Fisher Scientific) and rehydrated through decreasing concentrations of alcohol (100%, 95% and 70%) to water. Sections were then bleached with potassium permanganate (2.2.3) followed by washing in 2% v/v oxalic acid. Sections were then stained with Gomori's aldehyde fucshin (2.2.6), differentiated in 70% v/v alcohol and washed in running tap water. Nuclei were stained with Weigert's iron haematoxylin (2.2.14), slides washed in distilled water then placed in 5% w/v phosphotungstic acid to de-stain collagen fibres. Slides were washed again then finally placed in Gomori's trichrome reagent (2.2.7) to stain collagen and cytoplasm and rinsed in 0.2% v/v acetic acid. Sections were dehydrated through graded alcohols and cleared in xylene. Coverslips were mounted using Consulmount (Thermo-Shandon) with an automated coverslipping device (Consul, Thermo-Shandon). The full staining protocol is detailed in Table 2.3.

2.2.2.3 Photomicrograph acquisition

Colour photomicrographs of stained tissue were taken to allow further analysis. Images were captured with a CoolSNAP colour camera (Photometrics, AZ, USA) using 10x objective magnification (Axioskop stage microscope, Carl Zeiss Inc, Hertfordshire, UK) with a

Step	Reagent	Duration
1	Xylene	2 x 5min
2	100% v/v ethanol	2 x 5min
3	95% v/v ethanol	1 x 5min
4	Running tap water	1 x 5min
5	0.3% w/v potassium permanganate in 0.3% v/v sulphuric acid	1 x 1min
6	Running tap water	1 x 1min
7	2% oxalic acid	1 x 5s
8	70% v/v ethanol	1x5min
9	Gomori's aldehyde fuchsin	1 x 5min
10	70% v/v ethanol	1 x 2min
11	Running tap water	1 x 5min
12	Weigert's iron haemotoxylin	1 x 5min
13	5% w/v phosphotungstic acid	1 x 5min
14	Running tap water	1 x 2min
15	Gomori's trichrome	1 x 20min
16	0.2% v'v acetic acid	1 x 1min
17	95% v/v ethanol	1 x 1min
18	100% v/v ethanol	2 x 2min
19	Xylene	2 x 2min

Table 2.3 United States trichrome staining protocol

MicroColour liquid crystal RGB filter (Cambridge Research and Instrumentation Inc., MA, USA) using MCID basic 7.0 software (Interfocus Imaging Ltd., Cambridgeshire, UK).

2.2.2.4 Analysis of vaginal smears

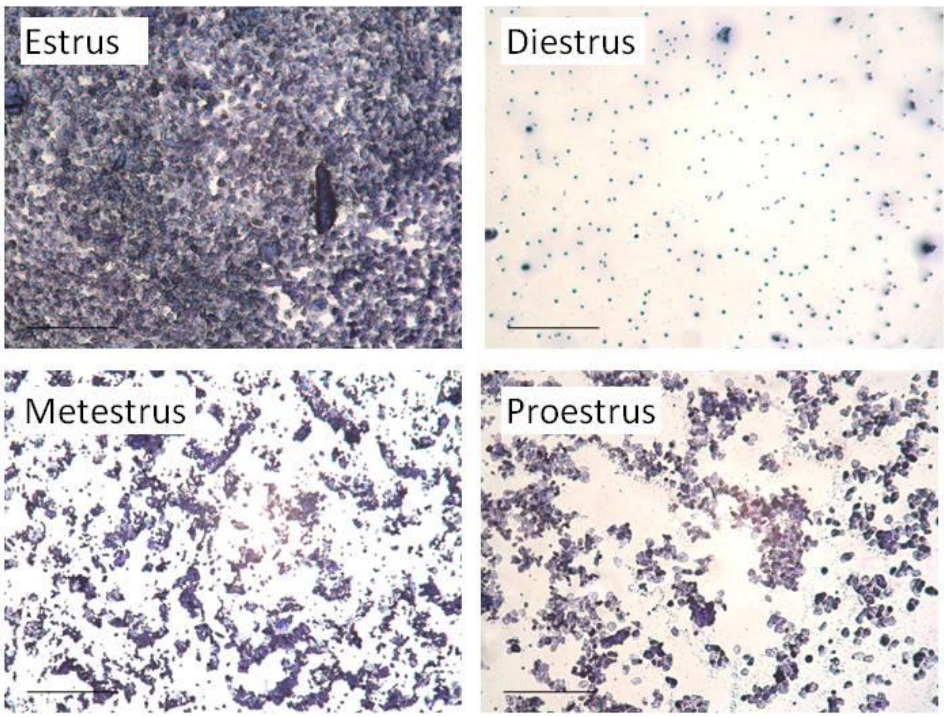
Cell type/s present and abundance were determined on photomicrographs of smears. The stage of estrus was determined according to the details in Figure 2.5.

2.2.2.5 Morphometric analysis of femoral artery

UST staining allowed morphometric analysis of the femoral arteries. Both the external (EEL) and internal (IEL) elastic laminae could be seen in the sections and allowed measurements of medial area (between EEL and IEL), intimal lesion area (any cellular or thrombotic material between the IEL and lumen) and lumen (any empty space within IEL) to be made. Measurements were made using Photoshop CS3 software (Adobe systems, CA, USA) by tracing the lamina and areas calculated according to the scale 1 pixel = 0.5126μm^2, determined by graticule slide. The section with the largest intimal area was chosen to represent each artery, and the stenotic ratio calculated as in section 2.1.1.3.

2.3 Statistics

Data are presented as mean ± SEM; statistical tests used are listed in individual chapters. Data were processed using Microsoft Office Excel 2007 (Microsoft, Berkshire, UK), graphs plotted using Prism 5.0 (GraphPad software, CA, USA) and statistical analysis carried out in Prism 5.0 or Statistica (Statsoft, Buckinghamshire, UK). In all studies n refers to the number of individual animals from which data were acquired. The numbers used in the studies were based on others in the published literature, where possible, which showed statistical differences in the parameters investigated. Our department has carried out and published many studies of metabolic function and glucocorticoid metabolism, similar to those in chapter 3, and found 8 animals per group provides adequate statistical power to find differences (Morton *et al.*, 2005; Livingstone *et al.*, 2009). Numbers used in studies of offspring metabolic and vascular physiology in chapters 5 and 6 were based on studies by Samuelsson *et al.* (Samuelsson *et al.*, 2008) and Calvert *et al.* (Calvert *et al.*, 2009). Studies investigating vascular function and remodelling numbers were based on the study by Molnar *et al.* (Molnar *et al.*, 2005). Statistical significance was taken at the 5% level.



Stage of estrus cycle	Details of cell histology
Estrus	Exclusively cornified epithelial cells,
Diestrus	Only leukocytes often few in number
Metestrus	Leukocytes in clumps, tendency to cluster around nucleated epithelial cells
Proestrus	Mostly nucleated epithelial cells with dispersed leukocytes

Figure 2.5 Analysis of vaginal smears using Giemsa staining

Smear samples were obtained by vaginal lavage and stained using Giemsa. Stage of estrus cycle was determined by cell type, morphology and number as described in photomicrographs and text. Scale bar = 200µm.

Chapter 3

Sex differences in the response to diet-induced obesity in C57BL/6 mice

3.1 Introduction

There has been an increase in the prevalence of obesity over recent years, associated with an increase in cardiometabolic disorders such as type 2 diabetes, dyslipidemia and hypertension (Guh *et al.*, 2009; WHO, 2011). Increasing evidence suggests the manifestation of obesity and associated metabolic disease is influenced by sex, with males at greater risk, and as a consequence the World Health Organisation is promoting gender specific research (Kautzky-Willer A., 2009).

Anatomical variations in the sites of deposition of adipose tissue have been suggested to play an important role in susceptibility to the metabolic sequelae of obesity. Men are prone to accumulate visceral adipose tissue, which is associated with metabolic abnormalities; whereas women tend to store fat in peripheral sites, such as subcutaneous and gluteal depots (Geer and Shen, 2009). Patients matched for the amount of subcutaneous adipose tissue, but with contrasting high or low visceral adipose accumulation, have very different insulin and glucose profiles (Ross *et al.*, 2002a; Ross *et al.*, 2002b). The sex-specific pattern of adipose distribution may be influenced by circulating androgens and estrogens. In men, post menopausal women, and ovariectomised animals, lower estrogen levels are associated with increased visceral adiposity and insulin resistance (Kotani *et al.*, 1994; D'Eon *et al.*, 2005). Exogenous estrogen treatment has been shown to be beneficial in reducing cardiovascular disease in observational studies of post-menopausal women (Stampfer *et al.*, 1991) while randomised controlled trials found no benefit or, in fact, greater risk of disease (Herrington, 1999). In males historical studies show estrogen increases coronary heart disease (Marmorston *et al.*, 1962; CDPR Group, 1973). Changes in the concentrations of one sex steroid are unlikely to occur in isolation, as estrogen can be synthesised from testosterone (section 1.3.1) and an association between lower testosterone concentrations and increased cardiovascular disease risk in men has been found in some human and animal studies (reviewed in (Alexandersen *et al.*, 1996), while women suffering from polycystic ovary syndrome with elevated testosterone concentrations are at increased risk (Talbott *et al.*, 1995). Additionally the balance of free androgens and estrogens can be controlled by sex hormone binding globulin (SHBG) in humans, which has itself been proposed to play a role in cardiovascular disease risk (Pugeat *et al.*, 1995).

The reasons for increased susceptibility to obesity and the associated metabolic dysfunction in certain individuals are unclear. However, similarities between some of the clinical features associated with glucocorticoid excess as a consequence of Cushing's syndrome and those found in the metabolic syndrome suggest a potential role for glucocorticoids. Increased peripheral glucocorticoid regeneration is found in the adipose tissue of obese humans (Rask *et al.*, 2001; Rask *et al.*, 2002; Wake *et al.*, 2003) and rats (Livingstone *et al.*, 2000a; Livingstone *et al.*, 2000b), and transgenic over-expression of 11β-HSD1 in adipose tissue of mice causes obesity and metabolic dysfunction (Masuzaki *et al.*, 2001). Interestingly male mouse models of dietary obesity have decreased 11β-HSD1 expression and activity in adipose tissue (Morton *et al.*, 2004). This has been proposed to be a protective mechanism as mice with a targeted disruption of the gene show improved insulin sensitivity when fed a high fat diet (Kotelevtsev *et al.*, 1997; Morton *et al.*, 2001). Glucocorticoid metabolism in obese females has not been explored in mouse models.

To allow further studies of the mechanisms involved in the progression of obesity and metabolic dysfunction, animal models have been extensively used. Although models of monogenic obesity are useful in studying the control of energy expenditure, only a very small percentage of the human population have similar loss of function mutations (Farooqi and O'Rahilly, 2005). More relevant to the modern obesity epidemic, which is believed to be polygenic in origin, are models of diet-induced obesity (DIO). Studies in rats (Pagliassotti *et al.*, 1994; Pagliassotti *et al.*, 1996; Levin *et al.*, 1997) and mice (West *et al.*, 1992; Surwit *et al.*, 1995) have shown that induction of obesity is possible using high fat and/or sugar feeding, although the time taken for obesity and metabolic consequences to become manifest varies between strains. The C57BL/6 mouse strain is susceptible to the development of obesity and its metabolic consequences on a high fat and sugar diet (Surwit *et al.*, 1988; West *et al.*, 1992; Surwit *et al.*, 1995) and was therefore chosen for the studies in this thesis. Generating the model in mice also gives the possibility for genetic manipulation in future studies to dissect potential mechanisms. However, few studies have investigated the effects of gender in models of rodent obesity. Studies in two genetic models of obesity and diabetes in rats suggest that there are sex-specific differences in the response to high fat diet (Maher *et al.*, 1996; Corsetti *et al.*, 2000) and studies in mouse models of dietary obesity suggest male animals are more prone to weight gain (Noonan and Banks, 2000; Nishikawa *et al.*, 2007) and metabolic dysfunction (Hwang *et al.*, 2010) than females. Although the mechanisms controlling the sex-specific responses to diet-induced obesity in rodent models are unclear, studies showing that ovariectomised females gain more weight than males (Hong *et al.*, 2009) suggest an important role for estrogen.

3.1.1 Hypothesis

This chapter explores the hypothesis that the metabolic effects of consuming a high fat diet are more profound in male than female mice.

3.1.2 Aims

To investigate this hypothesis a model of dietary obesity in C57BL/6 mice was used to answer the following questions;

- Does consumption of a high fat diet cause comparable increases in body weight and adipose deposition in male and female mice?
- Presuming comparable increases in adipose tissue; do plasma lipid, glucose, insulin and corticosterone concentrations increase to a greater extent in male mice?
- Does consumption of a high fat diet cause hepatic lipid accumulation in both sexes?
- Does a high fat diet alter the mRNA abundance of genes involved in glucose and lipid metabolism in a sex specific manner?
- Does a high fat diet alter the mRNA abundance and/or activity of glucocorticoid metabolising enzymes in the liver and adipose in a sex specific manner?

3.2 Methods

3.2.1 Experimental Outline

The experimental outline is presented in Figure 3.1. Blood pressure measurements are reported in chapter 6.

3.2.2 Animal Maintenance and terminal procedures

Male and female C57BL/6 mice (n=8/group from 8 litters) were fed control (CON, research diets D12328) or obesogenic (DIO, research diets D12331) diets *ad libitum* from 5 weeks of age and maintained as described (2.3.2). Five days prior to glucose tolerance testing animals were individually housed to allow for acclimatisation.

Mice were killed by CO_2 asphyxiation between 2-5pm, following a 6 hour fast, and tissues collected as described (0). Female mice were killed during estrus, as determined by vaginal smear (2.3.10) and Giemsa staining (2.2.2.1). The purpose of fasting and controlling for estrus cycle stage was to minimise variability in mRNA abundance and enzyme activity measured in tissues *post mortem*.

3.2.3 Metabolic tests

Male and female mice underwent a glucose tolerance test (2.3.7) at least 5 days after being individually housed and one week later diurnal blood samples were taken in line with the changing of the lights (2.3.8).

3.2.4 Quantification of nutrients and hormones in plasma and hepatic triglycerides

Levels of glucose (2.6.4) and insulin (2.6.2) were measured in plasma samples taken during the glucose tolerance test. Non-esterified fatty acids (NEFAs) were measured (2.6.7) in the first two glucose tolerance test samples. Plasma triglycerides were measured (2.6.6) in the fasting plasma sample. Corticosterone concentrations were measured (2.6.8) in diurnal blood samples, and cholesterol (2.6.5) in the nadir sample. Liver triglyceride was extracted using a saponfication method (2.7) and quantified using a colourimetric assay as in plasma.

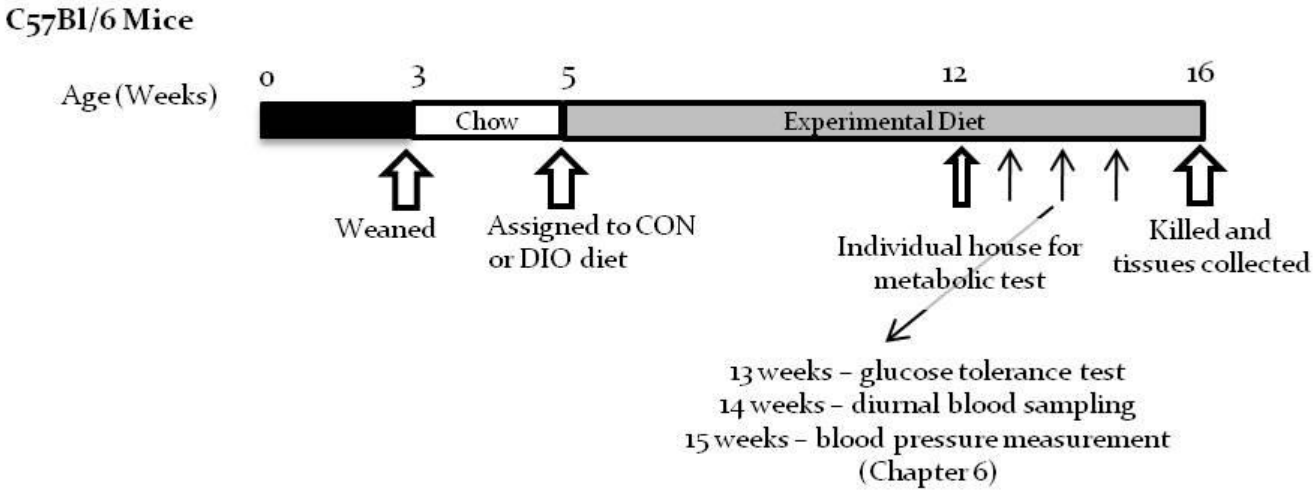


Figure 3.1 Schematic of experimental outline

Male and female C57BL/6 mice were fed control (CON, Research Diets D12328) or obesogenic (DIO, Research Diets D12331) diets from 5 weeks of age (n=8/group). At 12 weeks of age mice were individually housed and underwent metabolic tests. Animals were then killed and tissues weighed and collected.

3.2.5 Quantification of mRNA abundance

RNA was isolated from liver (2.5.1.1) and subcutaneous adipose tissue (2.5.1.2), quantified (0) and used for cDNA synthesis (2.5.1.6). RNA was also extracted from the mesenteric adipose tissue, however the quality was not deemed high enough for use in quantitative PCR due to presence of degradation products despite several attempts. The abundance of mRNA encoding 5α-reductase type1 (5αR), 5β-reductase (5βR), 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1), estrogen receptor-α (ERα), fatty acid synthase (FAS), glucocorticoid receptor (GR), hormone-sensitive lipase (HSL), lipoprotein lipase (LPL), phosphenolpyruvate carboxykinase (PEPCK), peroxisome proliferator receptor α (PPARα) and peroxisome proliferator receptor γ (PPARγ) were determined as appropriate using qPCR (2.5.1.7). Due to the large sex differences in the abundance of mRNA encoding ER, separate standard curves of cDNA were made for males and females and the mRNA abundance analysed in the two sexes separately. In liver, mRNA transcript levels were normalised to the abundance of cyclophilin mRNA and in adipose tissue mRNA was normalised to β-actin mRNA abundance.

3.2.6 Enzymology

11β-HSD1 reductase activity was measured as described (2.8.2). Conditions were optimised for each tissue individually to ensure kinetics were in the first order (Table 3.1). While 5αR is active in both the liver and adipose, its activity is unstable *ex*-vivo and therefore 5αR activity could not determined. The activity of 5βR has been measured in previous studies (Livingstone *et al.*, 2000b; Drake *et al.*, 2005a; Barat *et al.*, 2007; Livingstone *et al.*, 2009) but conditions to produce reproducible results, with first order kinetics, could not be ascertained in this study.

3.2.7 Statistics

Data are presented as mean ± SEM and were analysed using two-way analysis of variance (ANOVA) or repeated measures ANOVA as appropriate to assess the overall effects of the obesogenic diet and sex. Fisher's least squares difference post-hoc testing was used when appropriate to determine differences between individual groups. When applicable (i.e. in epididymal adipose tissue and for ER mRNA abundance), data were analysed in each sex separately using Student's unpaired t test.

Tissue	Substrate concentration (µM)	Protein concentration (mg/ml)	Incubation time (hrs)
Hepatic microsomes	2	0.025	2 – ♂ 4 - ♀
Subcutaneous adipose homogenate	2	0.025 - CON ♂ 0.05 - CON ♀ 0.1 – DIO ♂ & ♀	16 - CON ♂ 20 - CON ♀ 24 - DIO ♂ & ♀
Mesenteric adipose homogenate	9	0.1 - CON 0.3 - DIO	24

Table 3.1 Conditions for quantification of 11β-HSD1 activity

Activity of 11β-HSD1 was quantified using radiolabelled corticosterone. The substrate and protein concentration as well as incubation time were optimised to give first order kinetics in each tissue.

3.3 Results

3.3.1 The effects of diet-induced obesity and sex on physiology

3.3.1.1 Body and Tissue Weights

DIO caused a significant increase in weight gain between 5 and 13 weeks age in both sexes (Figure 3.2). At culling male DIO mice were 19.7% and females 14.4% heavier than their respective CON groups (Table 3.2).

DIO increased the wet weights of subcutaneous, mesenteric, retroperitoneal and, for males only, epididymal adipose tissue (Table 3.2), this was also seen when tissue weights were expressed as a percentage of body weight (Figure 3.3). Female mice had smaller adipose depots than male mice (Table 3.2), though relative to body weight this was only true for mesenteric adipose (Figure 3.3). No interaction between the effects of diet and sex were found in wet (Table 3.2) or relative (Figure 3.3) adipose tissue weights.

Liver and kidneys were smaller in females than males, DIO increased liver and kidney weight in both sexes (Table 3.2). When organ weights were expressed relative to body weight, only reduced liver size in females remained significant (Table 3.3). DIO and sex did not affect the wet weights of the adrenal glands or spleen (Table 3.2), however both were found to be larger in females when expressed relative to body weight (Table 3.3).

3.3.1.2 Glucose Tolerance Test

DIO caused an increase in plasma glucose and insulin concentrations during a glucose tolerance test (Figure 3.4A & B). A striking sex difference was found in that females had lower concentrations of both glucose and insulin, and only a small upwards shift of the response curve following DIO (Figure 3.4A & B). In contrast male DIO mice had elevated fasting plasma insulin concentrations and neither the plasma glucose nor insulin returned to baseline during the 90 minutes of the test.

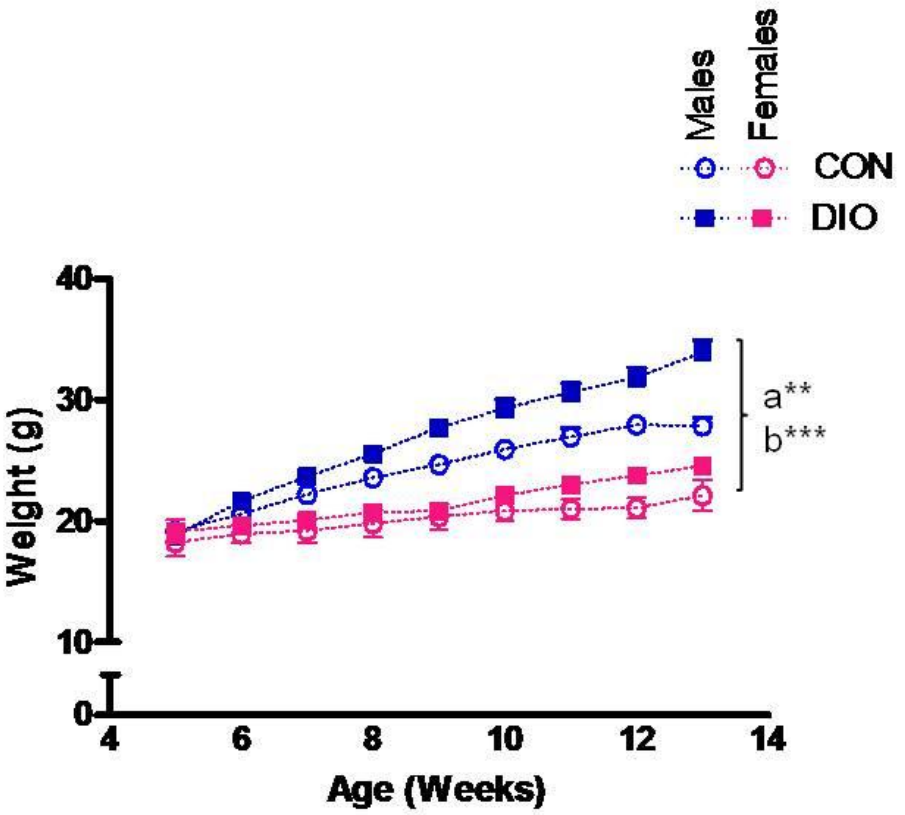


Figure 3.2 Consumption of an obesogenic diet increases weight gain

Male (blue) and female (pink) mice fed control (CON, open circles) or obesogenic (DIO, closed squares) diet from 5 weeks of age were weighed at regular intervals. **a**: effect of DIO to cause increased weight gain. **b:** effect of sex, such that females weighed less than males. Data are mean ± SEM, analysed by repeated measures 2-way ANOVA, n=8/group **p<0.01, ***p<0.001.

	Body, adipose and organ weights						
	Males		**Females**		**Effect of diet**	**Effect of sex**	**Interaction**
	CON	**DIO**	**CON**	**DIO**	**P value**	**P value**	**P value**
Cull weights (g)	27.4 ± 0.6	32.8 ± 1.0	22.2 ± 0.6	25.4 ±0.7	**<0.001**	**<0.001**	0.19
SC. adipose (mg)	231 ± 17	404 ± 17	141 ± 14	267 ± 29	**<0.001**	**<0.001**	0.25
Mes. adipose (mg)	198 ± 11	256 ± 19	106 ± 10	171 ± 23	**<0.001**	**<0.001**	0.86
Ret. adipose (mg)	68 ± 9	129 ± 13	48 ± 5	80 ± 14	**<0.001**	**<0.01**	0.18
Epi. adipose (mg)	186 ± 25	414 ± 42			**<0.001**		
Liver (g)	1.21 ± 0.07	1.30 ± 0.05	0.90 ± 0.02	1.13 ± 0.02	**<0.01**	**<0.001**	0.16
Kidney (mg)	148 ± 20	195 ± 10	128 ± 04	142 ± 04	**<0.05**	**<0.01**	0.16
Adrenal (mg)	3.6 ± 0.7	2.5 ± 0.3	4.1 ± 0.8	4.2 ± 0.7	0.43	0.11	0.38
Spleen (mg)	96 ± 6	89 ± 6	96 ± 10	101 ± 2	0.41	0.41	0.83

Table 3.2 Body, adipose and organ weights from obese (DIO) and control (CON) mice

Male and female mice were fed control (CON) or obesogenic (DIO) diets from 5 weeks of age and killed at 16 weeks of age. Wet tissue weights were recorded at culling. DIO increased the weight of subcutaneous (SC.), mesenteric (Mes.), retroperitoneal (Ret.) and epididymal (Epi) adipose as well as liver and kidney. The wet weights of the same adipose depots and organs were smaller in females. There was no significant interaction between the effects of diet and sex. Adrenal gland and spleen weight were not altered as a result of DIO or sex. Data are mean ± SEM, analysed by 2 way ANOVA. Epididymal (Epi.) adipose weight was analysed by Student's unpaired t-test. n=8/group.

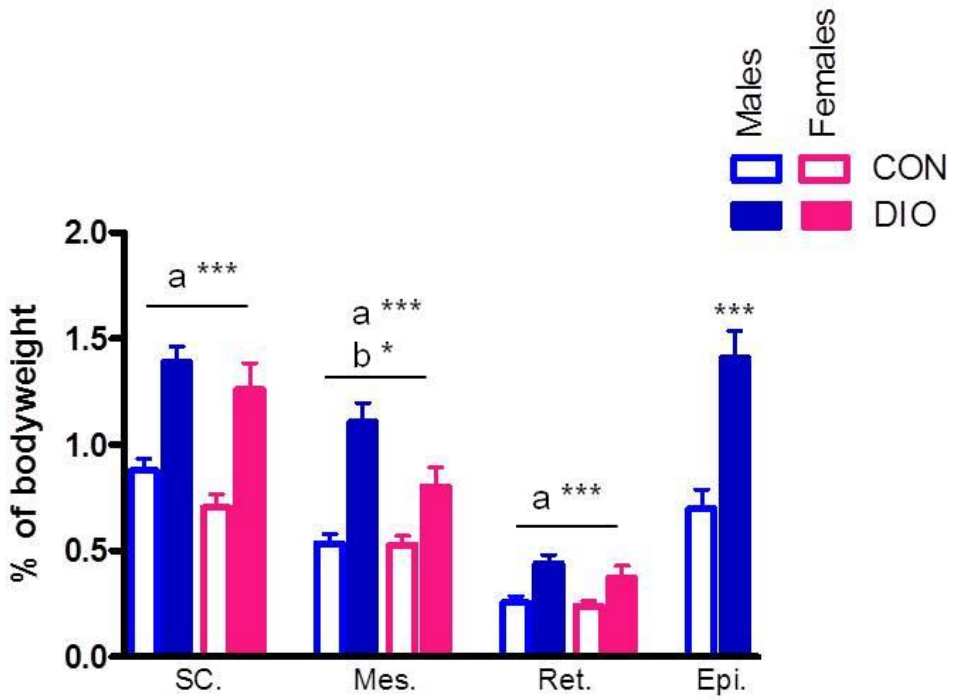


Figure 3.3 Consumption of an obesogenic diet increased adiposity

Male (blue) and female (pink) mice were fed control (CON, open bars) or obesogenic (DIO, closed bars) diet from 5 weeks age and killed at 16 weeks of age. Wet weights of; subcutaneous (SC.), mesenteric (Mes.), retroperitoneal (Ret.) and epididymal (Epi) adipose were recorded, and are expressed as a percentage of bodyweight. **a**: effect of DIO to increase percent of body weight contained in adipose tissue. **b**: effect of sex on the size of mesenteric adipose depots. Data are mean ± SEM, analysed by 2 way-ANOVA. Epididymal (Epi.) adipose weight was analysed by Student's unpaired t-test. n=8/group, *$p<0.05$, ***$p<0.001$.

	Relative organ weights						
	Males		**Females**		**Effect of diet**	**Effect of sex**	**Interaction**
	CON	**DIO**	**CON**	**DIO**	**P value**	**P value**	**P value**
Liver (%bw)	4.5 ± 0.1	4.4 ± 0.3	3.8 ± 0.1	3.9 ± 0.1	0.88	**<0.001**	0.62
Kidney (%bw)	0.57 ± 0.07	0.70 ± 0.04	0.64 ± 0.01	0.68 ± 0.03	0.12	0.36	0.54
Adrenal (%bw)	0.014 ± 0.003	0.009 ± 0.001	0.021 ± 0.004	0.020 ± 0.003	0.33	**<0.01**	0.47
Spleen (% bw)	0.34 ± 0.03	0.33 ± 0.02	0.48 ± 0.04	0.48 ± 0.01	0.90	**<0.001**	0.80

Table 3.3 Relative organ weights from obese (DIO) and control (CON) mice

Male and female mice were fed control (CON) or obesogenic (DIO) diets from 5 weeks of age and killed at 16 weeks of age. Wet tissue weights were recorded at culling and are expressed as a percentage of body weight. DIO did not affect the relative weights of the organs measured. Data are mean ± SEM, analysed by 2 way ANOVA n=8/group.

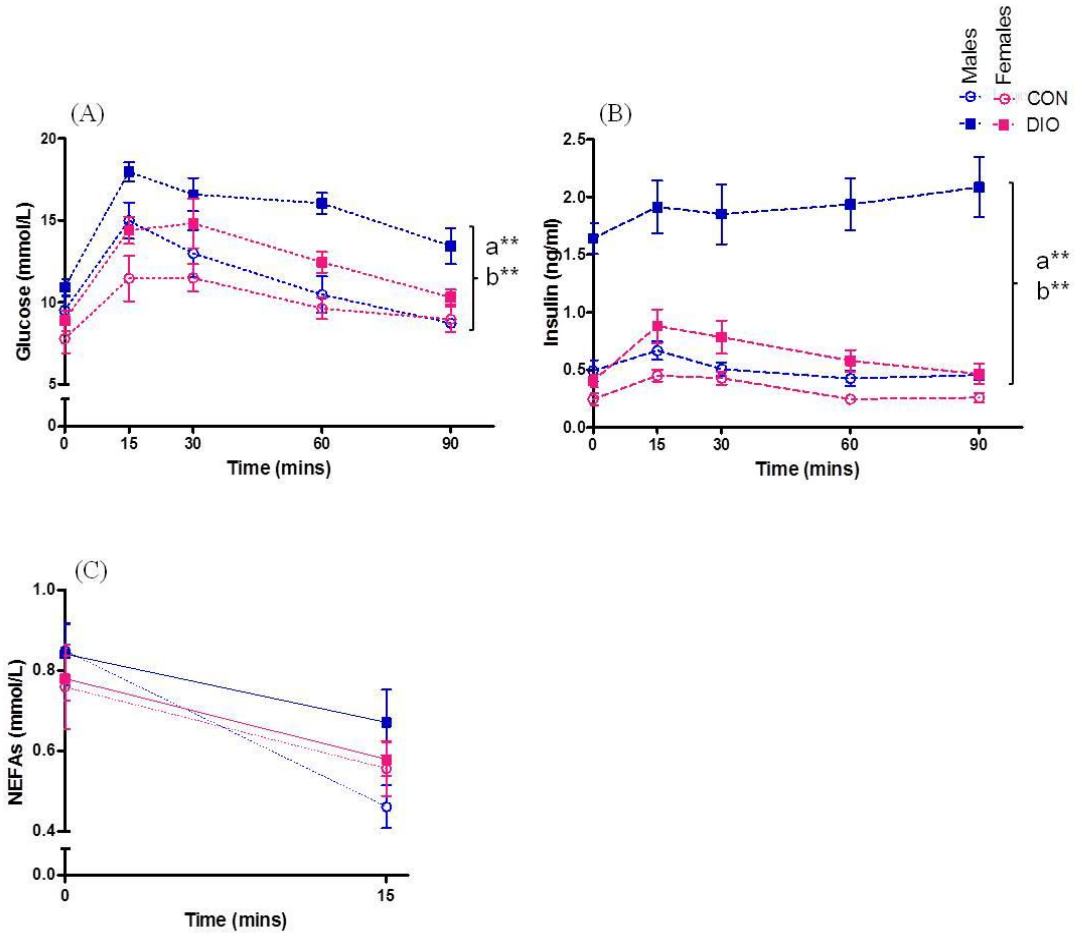


Figure 3.4 Obesogenic diet has profound effects on plasma glucose and insulin in males

Male (blue) and female (pink) mice were fed control (CON, open circles) or obesogenic (DIO, closed squares) diet from 5 weeks of age and underwent a glucose tolerance test at 13 weeks of age. Glucose (A), insulin (B) and non-esterified fatty acids (NEFAs) (C) were measured in plasma samples collected during a glucose tolerance test. **a**: the effect of DIO to increase plasma glucose and insulin concentrations. **b:** effect of sex to decrease parameters in females. Data are mean ± SEM, analysed by repeated measures 2-way ANOVA; n=8/group **p<0.01.

Lipid and hormone concentrations							
	Males		**Females**		**Effect of diet**	**Effect of sex**	**Interaction of effects**
	CON	**DIO**	**CON**	**DIO**	**P value**	**P value**	**P value**
Fasting plasma triglyceride (mmol/L)	0.57 ± 0.03	0.78 ± 0.05	0.41 ± 0.03	0.54 ± 0.02	**<0.001**	**<0.001**	0.21
Total plasma cholesterol (mmol/L)	1.71 ± 0.13	2.67 ± 0.23	1.53 ± 0.17	2.86 ± 0.16	**<0.001**	0.55	0.56
Hepatic triglyceride (nmol/mg)	27.5 ± 3.7	60.6 ± 8.6	26.5 ± 3.0	58.5 ± 3.8	**<0.001**	0.77	0.93
Nadir plasma corticosterone (nM)	64.8 ± 8.0	70.8 ± 7.6	66.6 ± 14.3	62.1 ± 10.0	0.94	0.74	0.60
Peak plasma corticosterone (nM)	282.0 ± 43.0	177.0 ± 26.2	457.3 ± 62.0	375.0 ± 33.1	**0.03**	**<0.001**	0.79

Table 3.4 Concentrations of lipids and corticosterone in obese (DIO) and control (CON) mice

Male and female mice were fed control (CON) or obesogenic (DIO) diets from 5 weeks of age. Plasma lipid and corticosterone levels were measured in samples taken during metabolic tests. Hepatic triglyceride was analysed in post-mortem tissue. DIO increased lipid levels and decreased peak corticosterone concentrations. There were sex differences in plasma triglyceride and corticosterone concentrations. Data are mean ± SEM, analysed by 2 way ANOVA n=8/group.

3.3.1.3 Lipid Concentrations

Neither sex nor diet had an effect on NEFA concentrations following a glucose bolus (Figure 3.4C). DIO was associated with an increase in the plasma concentrations of triglycerides and total cholesterol as well as hepatic triglycerides in both sexes (Table 3.4). Plasma triglyceride concentrations were lower in females than males (Table 3.4).

3.3.1.4 Corticosterone Concentrations

The expected rise in corticosterone concentration over the dark phase, when mice are active, was seen in all groups. Neither obesity nor sex had an effect on nadir concentrations of corticosterone. However, at the peak of the circadian rhythm DIO was associated with a decrease in circulating corticosterone concentrations (Table 3.4). Females had higher peak plasma corticosterone concentrations than males (Table 3.4).

3.3.1.5 Plasma concentrations of sex steroids

An ELISA for measuring murine plasma estradiol, proposed to be the most accurate of those commercially available (Haisenleder *et al.* 2011) was used. However, in all samples, estradiol levels from both sexes were below the limit of detection and therefore the data are not presented in this thesis. Due to the lack of plasma and assay cost this was not repeated. Testosterone was measured in plasma from female mice, but the values obtained were below the limit of detection of the assay and therefore these data are not presented. Testosterone concentrations were measured in a group of comparable male animals and the data are presented and discussed in chapter 4.

3.3.2 The effects of diet-induced obesity and sex on mRNA transcript abundance

3.3.2.1 Glucose and lipid metabolising genes

DIO did not affect the hepatic abundance of mRNAs encoding PEPCK, LPL, HSL or PPARα (Figure 3.5A). The abundance of mRNA encoding PPARα was higher in females than males (Figure 3.5B) and there was an interaction between the effects of DIO and sex on PEPCK, LPL and PPARα mRNA levels such that DIO was associated with an increase in males but either reduced or unaltered transcript abundance in females (Figure 3.5A).

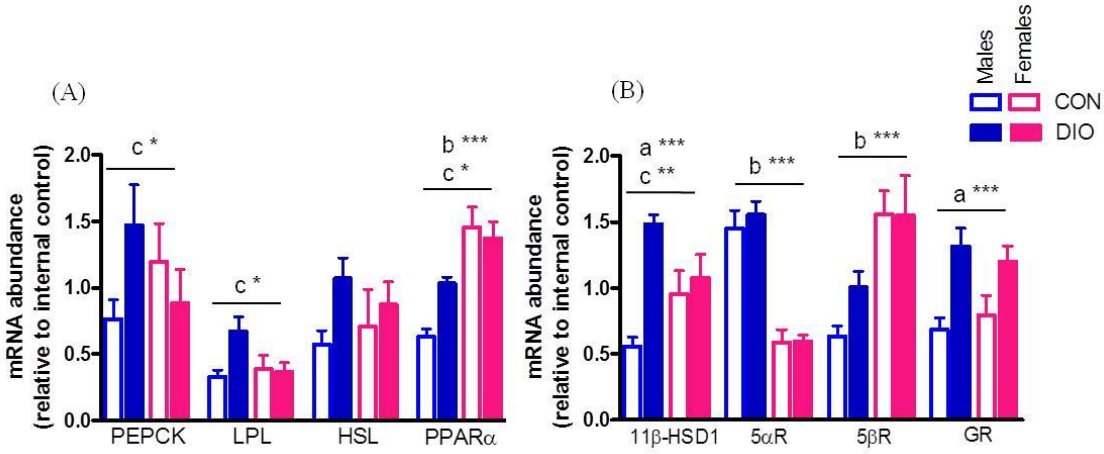


Figure 3.5 Obesity and sex influence hepatic mRNA abundance

Male (blue) and female (pink) mice were fed control (CON, open bars) or obesogenic (DIO, closed bars) diet from 5 weeks of age and killed at 16 weeks of age. Abundance of mRNA encoding proteins involved in (A) glucose and lipid metabolism and (B) glucocorticoid metabolism was quantified in liver and normalised to the abundance of mRNA encoding cyclophilin using qPCR. **a**: effect of DIO to increase mRNA abundance of 11β-HSD1 and GR. **b**:effect of sex on mRNA abundance of PPARα, 5αR and 5βR. **c**: interaction between the effects of diet and sex to affect the transcript levels of PEPCK, LPL, PPARα and 11β-HSD1. Data are mean ± SEM, analysed by 2 way ANOVA; n=8/group, *p<0.05, **p<0.01, ***p<0.001.

DIO was associated with a decrease in subcutaneous adipose abundance of mRNAs encoding PEPCK, LPL and PPARγ but did not affect HSL (Figure 3.6A). mRNA levels of both PEPCK and HSL were lower in females than males (Figure 3.6A) and additionally, there was an interaction between DIO

and sex so that DIO caused a large decrease in PEPCK mRNA levels in males but had a very small effect on females (Figure 3.6A).

3.3.2.2 Glucocorticoid metabolising genes

In the liver DIO was associated with increased levels of mRNA encoding 11β-HSD1 and GR (Figure 3.5B) but did not affect the abundance of 5αR or 5βr mRNA. Females had lower transcript levels of 5αR mRNA and higher transcript levels of 5βR mRNA than males (Figure 3.5B). There was an interaction between the effects of DIO and sex on the abundance of 11β-HSD1 mRNA; whilst DIO increased abundance in both sexes, the effect was much greater in males than females. As glucocorticoid metabolism was a focus of this chapter further post-hoc analysis of 11β-HSD1 and GR mRNA abundance was carried out using Fisher's least squares difference. This revealed higher levels of mRNA encoding 11β-HSD1 in lean females than lean males ($p<0.05$) but no difference in GR.

In the subcutaneous adipose DIO caused a significant reduction in the mRNA abundance of 11β-HSD1 in both sexes but did not affect levels of mRNA encoding GR (Figure 3.6B) and there were no differences between sexes. Additional post-hoc analysis did not find any difference between male and female control animals. 5βR is not expressed in adipose and additionally, the abundance of 5αR mRNA could not be reliably determined in subcutaneous adipose tissue as transcript levels were very low.

3.3.2.3 Estrogen receptor α

mRNA encoding ERα was not detectable in male liver, but in female liver DIO did not alter its abundance (Figure 3.7A). There was no effect of DIO on mRNA abundacne of ERα in subcutaneous adipose tissue in either sex (Figure 3.7B & C), mRNA encoding ERβ was not detectable in subcutaneous adipose in either sex.

3.3.3 The effects of diet-induced obesity and sex on 11β-HSD1 activity

There was an interaction between DIO and sex such that DIO was associated with a trend to decreased hepatic 11β-HSD1 activity in males and increased activity in females (Figure 3.8A).

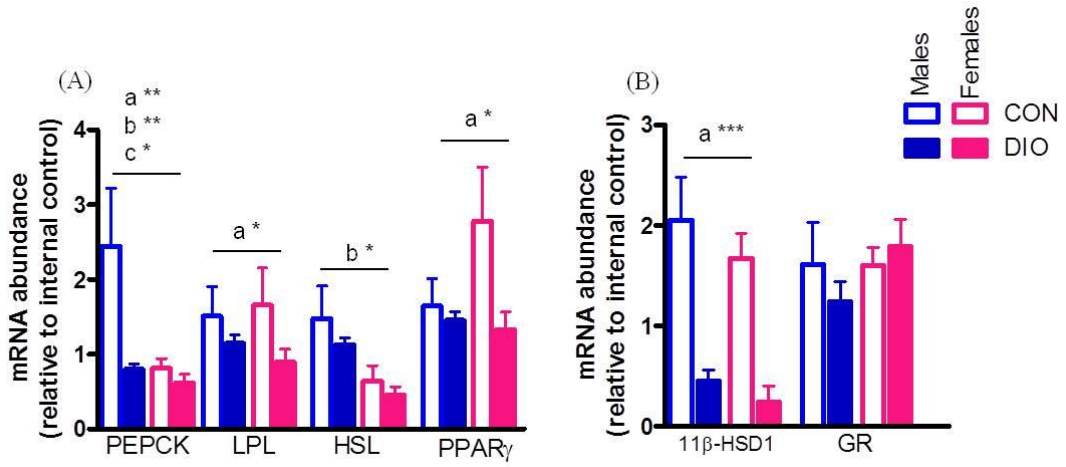


Figure 3.6 Obesity and sex influence mRNA abundance in subcutaneous adipose tissue

Male (blue) and female (pink) mice were fed control (CON, open bars) or obesogenic (DIO, closed bars) diet from 5 weeks of age and killed at 16 weeks of age. Abundance of mRNA encoding proteins involved in (A) glucose and lipid metabolism and (B) glucocorticoid metabolism was quantified in subcutaneous adipose tissue and normalised to the abundance of β-actin using qPCR. **a**: effect of DIO to decrease abundance of mRNA encoding PEPCK, LPL, PPARγ and 11β-HSD1. **b**:effect of sex on abundance of mRNA encoding PEPCK and HSL. **c**: interaction between the effects of diet and sex to affect the transcript levels of PEPCK. Data are mean ± SEM, analysed by 2 way ANOVA; a: effect of diet, b: effect of sex, c: interaction between diet and sex; n=5/CON group, 7/DIO group, *p<0.05, **p<0.01, ***p<0.001.

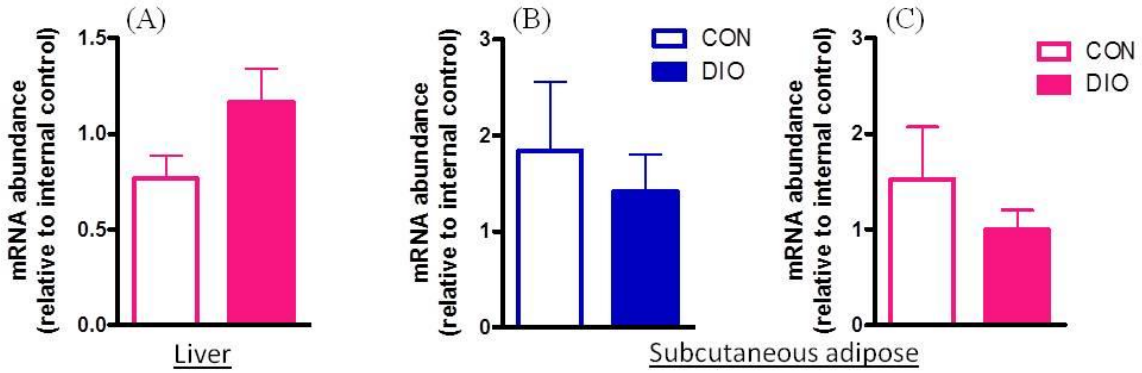


Figure 3.7 Obesity does not affect mRNA abundance of estrogen receptor α

Male (blue) and female (pink) mice were fed control (CON, open bars) or obesogenic (DIO, closed bars) diet from 5 weeks age and killed at 16 weeks of age. Abundance of estrogen-receptor α (ERα) mRNA was quantified in female liver, normalised to cyclophilin mRNA (A), and male (B) and female (C) subcutaneous adipose tissue, normalised to β-actin mRNA, using qPCR. DIO did not cause any significant differences in abundance of mRNA encoding ERα. Data are mean ± SEM analysed using Student's unpaired t-test; n=8/group in liver (A), 5/CON group, 7/DIO group in subcutaneous adipose (B & C).

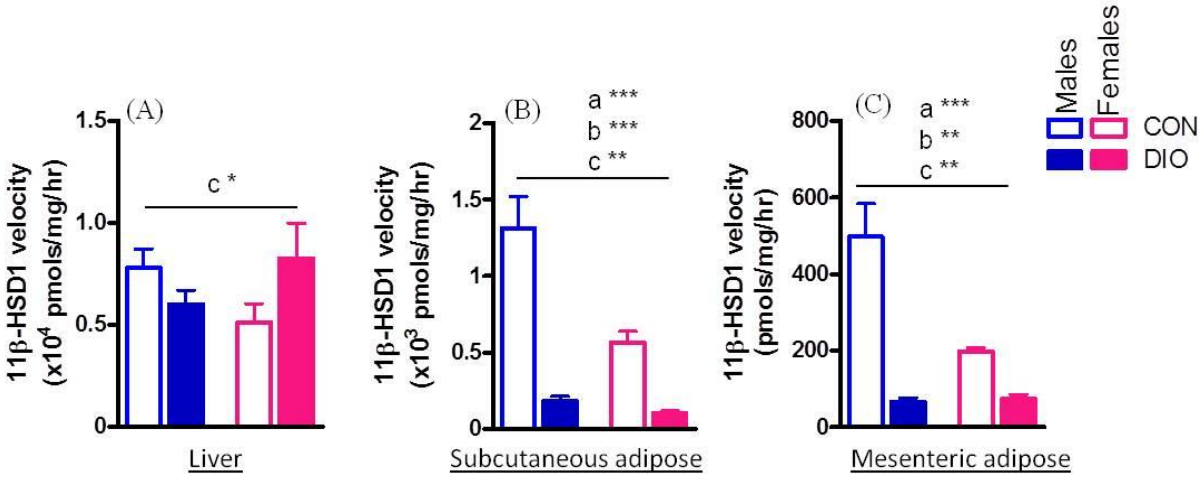


Figure 3.8 Obesity and sex influence 11β-HSD1 activity in the liver and adipose tissue

Male (blue) and female (pink) mice were fed control (CON, open bars) or obesogenic (DIO, closed bars) diet from 5 weeks of age and killed at 16 weeks of age. The activity of 11β-HSD1 was quantified using a radio-labelled tracer in hepatic microsomes (A), subcutaneous (B) and mesenteric (C) adipose tissue. **a**: effect of DIO to decrease 11β-HSD1 activity. **b**: effect of sex to lower 11β-HSD1 activity in females. **c**: interaction between the effects of diet and sex to affect 11β-HSD1 activity. Data are mean ± SEM, analysed by 2 way ANOVA; n=8/group, **p<0.01, ***p<0.001.

DIO caused a decrease in activity of 11β-HSD1 in subcutaneous (Figure 3.8B) and mesenteric (Figure 3.8C) adipose tissue. 11β-HSD1 activity was lower in adipose tissue in females (Figure 3.8B & C). The interaction between the effects of DIO and sex showed the relative reduction in activity was much larger in males (Figure 3.8B & C).

3.4 Discussion

The results presented in this chapter demonstrate consumption of a high fat diet causes comparable increases in adipose deposition in both sexes as well as raised plasma and hepatic concentrations of lipids. However, the effects on glucose-insulin homeostasis were more profound in males such that they demonstrated fasting hyperinsulinaemia, glucose intolerance and insulin resistance during a glucose tolerance test. Further sex specific responses to a high fat diet were found in the hepatic and adipose mRNA abundance of genes involved in glucose, lipid and glucocorticoid metabolism. The activity of 11β-HSD1 in adipose tissue was found to be sexually dimorphic, with lower activity in lean females than males and reduced activity in response to DIO in both sexes. These results suggest that the sex differences reported in human obesity and rates of related cardiovascular disorders may also be apparent in mice. They also suggest a role for reduced adipose glucocorticoid regeneration in the apparent protection of glucose-insulin homeostasis in females.

3.4.1 Body weight and adiposity

Female mice are lighter than males and the difference in body weight following consumption of an obesogenic diet was less obvious in females. Female mice consuming an obesogenic diet were significantly heavier than controls although the increase was less than that seen in males. While few studies use female mice, slower weight gain when exposed to obesogenic diets is consistently reported (Noonan and Banks, 2000; Hong *et al.*, 2009; Hwang *et al.*, 2010) despite comparable food intake between sexes (Noonan and Banks, 2000; Nishikawa *et al.*, 2007; Hwang *et al.*, 2010). It would have been beneficial to measure food intake and energy balance in this study to explore this sex difference. Whilst body weight is a useful measure of obesity, adipose depot weights may give a better indication of metabolic health as adiposity is associated with insulin resistance (Kaye *et al.*, 1991) and coronary heart disease (Casassus *et al.*, 1992) independently of body weight in humans. There was a similar increase in the percentage of body weight stored in adipose in both males and females on the obesogenic diet. In humans, females have a greater total body fat mass, with more subcutaneous adipose than males, although hepatic and intra-abdominal adipose are comparable between the sexes (Westerbacka *et al.*, 2004). These patterns of deposition were not detected in the mice, though DIO females did have less mesenteric adipose tissue than males. Interestingly mesenteric adipose is the part of the visceral depot which is most correlated with metabolic disease both in humans (Fox *et al.*, 2007) and mice (Rebuffe-

Scrive *et al.*, 1993). The differences in metabolism between males and females could reflect both the less severe weight gain and/or reduced amount of mesenteric adipose with DIO in females.

3.4.2 Lipid concentrations

DIO caused an increase in plasma and hepatic lipid concentrations consistent with previous studies (Gallou-Kabani *et al.*, 2007; Oosterveer *et al.*, 2009; de Meijer *et al.*, 2010; Fraulob *et al.*, 2010). DIO increased plasma triglyceride concentrations in both sexes but these were lower in control females than in males and the response to diet was also smaller in females. Interestingly this pattern was not seen with hepatic triglyceride or plasma cholesterol concentrations suggesting sex-specific lipid metabolism may be molecule and location specific. In normal physiology, NEFA concentrations fall in the fed state due to increased lipogenesis and reduced lipolysis (Kahn and Flier, 2000). Analysis of plasma NEFA concentrations did not reveal a clear effect of DIO or sex. Another study has reported lack of NEFA suppression in obese male mice (Burcelin *et al.*, 2002). The reason this was not apparent in this chapter is probably due to the variation in results between mice. The mean values for male DIO mice were above all other groups, different statistical analysis, such as separating the sexes may demonstrate a lack of NEFA suppression. Insulin induces the switch from lipolysis to lipogenesis in response to feeding (Kahn and Flier, 2000) and lack of NEFA suppression is suggestive of insulin resistance.

3.4.3 Glucose and insulin homeostasis

Evidence for insulin resistance was noted in male DIO animals in the form of fasting hyperinsulinemia, as well as raised glucose and insulin concentrations throughout a 90 minute glucose tolerance test, consistent with previous studies (Surwit *et al.*, 1988; Surwit *et al.*, 1991; Rebuffe-Scrive *et al.*, 1993; Surwit *et al.*, 1995; Collins *et al.*, 2004). A striking effect of sex was noted in response to an endogenous glucose bolus: whilst obesity caused increased glucose and insulin concentrations in females the difference was much smaller than in males, and both parameters returned to baseline during the test. This is consistent with the few other studies investigating obesity in both sexes of mice (Noonan and Banks, 2000; Gallou-Kabani *et al.*, 2007; Hwang *et al.*, 2010). The results in this chapter suggest females are more insulin sensitive than males when fed a control diet, and maintain sensitivity when fed an obesogenic diet, as has been demonstrated in human studies with pre-menopausal women (Nuutila *et al.*, 1995; Donahue *et al.*, 1996; Ross *et al.*, 2002b; Cnop *et al.*, 2003; Moran *et al.*, 2008).

The mechanism behind the greater insulin sensitivity in females, regardless of body weight, is unclear. As mentioned, human females have a greater percentage of body fat than males (Westerbacka *et al.*, 2004) though this was not found in either the CON or DIO mice investigated in this chapter. In fact all mice had comparable sized adipose depots when expressed relative to body weight, with the exception of a smaller mesenteric deposition in DIO females. The differences in mesenteric adipose may a play a role in the glucose-insulin homeostasis as visceral adipose has been associated with insulin resistance (Pouliot *et al.*, 1992; Fox *et al.*, 2007; Despres *et al.*, 2008). It has been suggested that the pattern of adipose deposition which occurs during and following puberty is a factor in insulin sensitivity, with females primarily accumulating peripheral adipose and males developing a more central pattern of adipose deposition (Kissebah and Krakower, 1994). The differences in the relative concentrations of sex steroids following puberty may account for these sex differences in adipose distribution and the associated insulin sensitivity seen in human and animal models. Estrogen, acting through the ERα receptor, has been shown to increase insulin sensitivity through inhibition of hepatic glucose production and increased glucose transport to muscle in mice (Bryzgalova *et al.*, 2006), which would predict a healthier metabolic phenotype in obese females if estrogen concentrations are maintained. The sex differences found in this study resemble the maintenance of insulin sensitivity despite increase in adiposity in human females and support the use of female as well as male mice in studies of obesity and insulin resistance.

3.4.4 mRNA abundance of glucose and lipid metabolising genes

In male mice DIO caused an increase in hepatic mRNA abundance of glucose and lipid metabolising genes, but no changes were observed in females. This may reflect the more marked changes in glucose homeostasis in males. In contrast DIO affected mRNA transcript levels in the adipose tissue of both sexes, consistent with increased lipid levels and expansion of adipose.

Levels of hepatic PEPCK are decreased in the fed state as a response to insulin release, and induced in the fasting state by glucagon to increase gluconeogenesis in liver (O'Brien *et al.*, 1990). The increase in hepatic PEPCK mRNA levels despite fasting hyperglycaemia suggests the males have hepatic insulin resistance resulting in a failure to suppress gluconeogenesis. Sex differences in PEPCK activity have been reported in rat liver (Wimmer, 1989a), as activity was not quantified in this study it is currently unclear if this is also found in mice. The same author reports anatomical differences in PEPCK activity throughout the liver with highest activity in the periportal zone declining towards the

perivenous zone (Wimmer, 1989b). While a sample of the left lobe was always used to extract RNA for the studies in this thesis the specific area within the lobe was not kept consistent and may explain why the mRNA abundance was quite variable.

In adipose tissue PEPCK is regulated by the same hormones as in the liver, though glucocorticoids have the opposite effect. PEPCK catalyses glyceroneogenesis in adipose tissue, generating glycerol 3-phosphate and promoting fatty acid re-esterification (Pilkis and Granner, 1992). DIO males had fasting hyperinsulinaemia which would be predicted to decrease PEPCK mRNA as was found in the subcuaneous adipose. Transgenic over-expression of PEPCK in adipose tissue results in increased lipid storage with the maintenance of insulin sensitivity (Franckhauser *et al.*, 2002). However, when the same transgenic mice are fed an obesogenic diet they become more obese and are metabolically impaired compared with controls (Franckhauser *et al.*, 2006), suggesting that lower adipose PEPCK expression may be beneficial when animals are exposed to an obesogenic diet. The female mice in this chapter had much lower subcutaneous adipose abundance of mRNA encoding PEPCK, regardless of diet. As the females have a preferable metabolic state and response to diet, this further suggests the change seen in obese males may be an attempt to compensate for, or reduce the consequences of, an obesogenic diet.

In the liver the role of HSL is believed to be small (Reid *et al.*, 2008), though the enzyme can hydrolyse cholesterol as well as intracellular triglyceride, as in adipose tissue (its primary site of action) (reviewed in (Holm *et al.*, 2000)). The induction of hepatic HSL mRNA with fasting in DIO males may increase rates of lipolysis and direct free fatty acids to β-oxidation or back in to the circulation to be stored in a 'metabolically safe' manner in adipose, as is seen in HSL hepatic over-expressing mice (Reid *et al.*, 2008). Interestingly, no increase of HSL mRNA abundance was found in DIO female mice, even though they have comparable hepatic triglyceride accumulation to males. Sex differences in HSL have been reported in human adipose tissue (Kolehmainen *et al.*, 2002), consistent with the sex-specific expression in adipose tissue reported here. The activity of HSL is controlled by phosphorylation which is induced by catecholamines and insulin (Holm *et al.*, 2000) so that hormone-induced phosphorylation may have a more important role in controlling tissue activity than mRNA abundance.

LPL also hydrolyses triglycerides to release free fatty acids, though unlike HSL it acts on the lipid rich core of lipoproteins rather than intracellular triglycerides (Wang and Eckel, 2009). The enzyme is most abundant in muscle and adipose tissue, and on the epithelial cells of the blood vessels where it controls the uptake of fatty acids into tissues (Wang and Eckel, 2009).

Hepatic synthesis of LPL is low in adult compared to neonatal rat (Burgaya *et al.*, 1989) and mouse (Yacoub *et al.*, 1990) livers and its presence is believed to be mainly due to uptake from the circulation (Vilaro *et al.*, 1988; Camps *et al.*, 1991), which may explain why low levels of hepatic mRNA encoding LPL were detected in this study. Given that LPL is normally synthesised at low levels in the liver it is intriguing that DIO in male mice caused an increase in mRNA levels; predicting an increase in tissue fatty acid levels, which could then be incorporated into triglycerides for storage. Hepatic over-expression of LPL causes lipid accumulation and insulin resistance (Kim *et al.*, 2001) which suggests the increase in mRNA in male DIO mice could be contributing to the metabolic phenotype seen.

LPL synthesised in adipose tissue is transported to the capillary endothelial cells where it hydrolyses circulating lipoproteins to allow for uptake by the adipocytes. Studies have shown that LPL expression increases in adipose in line with triglyceride accumulation (Amri *et al.*, 1996) and is induced in response to insulin (Picard *et al.*, 2002). The decrease in LPL mRNA observed here would seem counterintuitive as it predicts a decrease in lipid uptake in adipocytes which would potentially contribute to lipid accumulation in other tissues such as liver. The transcription of LPL is controlled by many regulatory elements binding to its 5' region (Preiss-Landl *et al.*, 2002) and, in addition, LPL is under post-transcriptional, translational and post-translational regulation (reviewed in (Wang and Eckel, 2009), so that the differences found in mRNA may not be indicative of activity.

Activation of PPARα induces transcription of genes involved in lipid oxidation (Gulick *et al.*, 1994), and in the clinic agonists of this receptor are used as lipid lowering agents (reviewed in (Fruchart, 2009)). Increased mRNA abundance of PPARα in DIO males, along with increased concentrations of its endogenous ligand, free fatty acids, may represent a compensatory mechanism to increase fatty acid oxidation and reduce hepatic accumulation of triglycerides. The higher abundance of mRNA encoding PPARα in females, regardless of diet, is consistent with the literature, suggesting its control is sexually dimorphic (Yoon, 2009) and indeed, receptor agonists are only effective in treating dyslipidemia in male mice (Yoon *et al.*, 2002) or female mice following ovariectomy (Jeong *et al.*, 2004). This implies an interaction between PPARα and estrogen; so that higher PPARα expression in females may be protective from some effects of obesity, resulting in higher basal oxidation of fatty acids. It therefore follows that the increase in mRNA abundance in response to DIO may be beneficial in males but the already higher levels in females cannot, or need not, be further modulated.

Adipose tissue PPARγ activation causes an increase in the transcription of molecules involved in lipid uptake and storage and modulates glucose metabolism (reviewed in (Lehrke and Lazar, 2005). Agonists of PPARγ are used clinically as insulin sensitising agents (Kintscher and Law, 2005) and were originally shown to have similar effects on insulin sensitivity in obese rodents (Chang *et al.*, 1983a; Chang *et al.*, 1983b; Chang *et al.*, 1983c). Although there is an effect of an obesogenic diet to reduce PPARγ transcript levels, the difference is obviously greater in female mice. This reduction in PPARγγ in response to DIO would predict a reduction in adipose lipid uptake and increased insulin resistance. However, contradictory results have been reported from two PPARγ knock-out models when fed a high fat diet; one study reported reduced adipose deposition and maintenance of insulin sensitivity (Jones *et al.*, 2005), whilst the other reported insulin resistance in fat and liver (He *et al.*, 2003). It is, therefore, unclear whether the reduction in mRNA abundance seen in female mice following DIO, and the lower levels in males would have an adverse or beneficial effect in obesity.

3.4.5 Hepatic glucocorticoid metabolism

Studies in humans and mice have shown decreased or comparable hepatic 11β-HSD1 expression and activity in obesity (Rask *et al.*, 2001; Rask *et al.*, 2002; Morton *et al.*, 2004; Livingstone *et al.*, 2009). However the results of this chapter show increased mRNA abundance in response to DIO, particularly in male mice. Whilst this may reflect a compensatory response to the lower circulating corticosterone concentrations found in the plasma, increased expression may be deleterious, as hepatic over-expression of 11β-HSD1 results in fatty liver and insulin resistance (Paterson *et al.*, 2004). Changes in enzyme activity differed from those in mRNA abundance, suggesting either post-transcriptional mechanisms of regulation or enzyme inhibition. Biochemical studies of 11β-HSD1 show it to be folded and glycosylated in the ER (Ozols, 1995) and it is possible that alterations in this ER processing could underlie the differences between mRNA abundance and activity. Levels of circulating androgens, estrogens and/or growth hormone may underlie the sex differences in 11β-HSD1 in CON animals. An inhibitory effect of pharmacological levels of estrogen on transcription of 11β-HSD1 has been found in rats (Low *et al.*, 1994; Jamieson *et al.*, 1999; Andersson *et al.*, 2010) although mRNA abundance was higher in female than male mice. Conversely, one study showed that testosterone treatment in gonadectomised rats caused up-regulation of 11β-HSD1 mRNA and activity (Liu *et al.*, 1998); although another study found no effect of androgen treatment in intact animals (Nwe *et al.*, 2000).

Whilst the diet-induced changes seen in 11β-HSD1 were sex specific, GR mRNA abundance increased in DIO in all mice. This may reflect a compensatory response to decreased circulating corticosterone concentrations. However, the consistency in both sexes, despite potential differences in glucocorticoid regeneration by 11β-HSD1, suggests it may be a response to increasing adiposity or other metabolic parameters occurring in both male and female mice.

Increased metabolism of glucocorticoids by 5αR has also been reported in human (Andrew *et al.*, 1998) and rodent obesity (Livingstone *et al.*, 2005; Livingstone *et al.*, 2009). In this study obesity did not affect the abundance mRNA encoding 5αR but was associated with increased levels of 5βR mRNA in males. This may be important as 5α-reduced metabolites can still activate the glucocorticoid receptor (McInnes *et al.*, 2004). Therefore increased metabolism specifically through the 5β-pathway would be predicted to reduce local glucocorticoid concentrations and signalling. Thus, the higher baseline abundance of 5βR mRNA in females may be protective and the increase seen in DIO males may be a compensatory mechanism. Changes in metabolism may also reflect the dual function of the liver to maintain circulating corticosterone concentrations as a consequence of increased regeneration by 11β-HSD1, whilst reducing local concentrations by increased degradation by 5βR (Mayes and Watson, 2004). Sex differences have been reported in rat 5αR expression (Normington and Russell, 1992) with much higher expression in female livers. However the opposite was found in the results of this chapter. Whether the species differences are due to the nutritional state in which the animals were killed, as is seen with PEPCK expression (Wimmer, 1989b), is unclear. Interestingly, in lean humans, disproportionally low concentrations of 5α-reduced metabolites are found in urine of females compared with males (Finken *et al.*, 1999), which suggests the metabolism in the female mice shares some similarities to that seen in humans.

3.4.6 Adipose glucocorticoid metabolism

The results of this chapter support previous studies demonstrating that male mice fed an obesogenic diet have reduced subcutaneous adipose expression and activity of 11β-HSD1 (Morton *et al.*, 2004) and this may be a protective mechanism to reduce local glucocorticoid signalling. Additionally, the results here demonstrate that subcutaneous adipose 11β-HSD1 mRNA abundance and mesenteric and subcutaneous adipose activity are lower in females than in males, consistent with data in humans (Paulsen *et al.*, 2007). These results suggest a new hypothesis; that a lower level of adipose glucocorticoid regeneration in females may be advantageous, reducing the metabolic complications associated with obesity. Evidence

suggests that both estrogen and testosterone can modulate the transcription and activity of 11β-HSD1 in the adipose of humans (Zhu *et al.*, 2010) and rodents (Paulsen *et al.*, 2008; Tagawa *et al.*, 2009). These observations suggesting interactions between sex hormones, obesity and 11β-HSD1 will be explored further in the next chapter.

The activity of 11β-HSD1 was much lower in mesenteric than subcutaneous adipose but the sex-specific and diet-induced changes were maintained across both depots. Changes in 11β-HSD1 following obesity have been shown to be specific to the particular adipose depot studied in some rodent models (Livingstone *et al.*, 2000a; Livingstone *et al.*, 2009). However a study comparing subcutaneous and visceral adipose tissue from humans found no differences in mRNA abundance between depots (Paulsen *et al.*, 2007). Depot-specific glucocorticoid metabolism may be important in the development of the obesity-related metabolic syndrome. If increased local glucocorticoid regeneration causes fat accumulation and insulin resistance, the effects on metabolism may be less marked if this occurs in subcutaneous rather than visceral adipose. Given the sex differences in adipose distribution, depot specific differences in glucocorticoid metabolism could be important in the sexually dimorphic effects seen in obesity.

3.4.7 Study limitations

Although there was a significant increase in adipose tissue weight in the DIO mice of both sexes the increase in body weight was less profound in females. A greater difference in bodyweight was found in another cohort of female mice used to study the effects of maternal obesity (Chapter 5). The animals were a similar age to those studied in this chapter but had not undergone any metabolic studies at this point. This may suggest the female DIO mice were highly affected by the inevitable stress of the procedures, studies of food intake would help to address this issue. In addition the levels of obesity achieved in these studies were modest in comparison to others investigating metabolism (Black *et al.*, 1998; Surwit *et al.*, 1995) and glucocorticoid metabolism (Morton *et al.*, 2004), primarily due to longer feeding periods. Extending the feeding time in future experiments may allow for easier comparison to the published data. This is of particular importance in female mice; if the increase in body weight could be comparable to that found in males greater interpretation of sex differences could be made.

While many interesting changes in response to DIO and due to sex were found in the abundance of mRNAs whether this translates to protein levels is unknown. Many mechanisms can alter the amount of mRNA which is translated to protein. Additionally post-

translational modifications can affect protein structure and function and enzyme activity. In order to determine if the differences found in mRNA are playing a role in the metabolic response to obesity more investigations are required. Overall protein concentrations could be determined using western blotting and more specific cellular location by immunohistochemistry. Where enzymes are concerned activity assays should also be completed. In addition experiments investigating the abundance of mRNAs in subcutaneous adipose tissue may have been statistically under powered. Whilst 8 animals were used in each group good quality RNA could not be extracted from all of them, therefore reducing the n numbers from what was planned. This may lead to type 2 errors; failing to reject a false null hypothesis. Using a larger cohort of animals would ensure the investigations would be adequately powered even if some samples were not useable.

3.4.8 Conclusions

In summary, exposure to an obesogenic diet is associated with weight gain and increased adipose deposition in both sexes. The accompanying changes in metabolism are sex specific, with males becoming insulin resistant whilst females remain insulin sensitive. The differences in physiology may be influenced by sexually dimorphic changes in genes involved in glucose and lipid metabolism. Furthermore, the sex specific profiles of glucocorticoid metabolising enzymes, specifically lower activity of 11β-HSD1 in females, may play a role in determining the metabolic response to a high fat and sugar diet.

Chapter 4

Effects of estradiol on the response to diet-induced obesity in C57BL/6 mice

4.1 Introduction

The work described in chapter 3 shows that the physiological response to obesity in mice differs between the sexes, with females maintaining insulin sensitivity despite diet-induced increases in adiposity. Many studies have proposed that estrogen may play an important role in protecting females from the deleterious effects of obesity (reviewed in (Mayes and Watson, 2004; Meyer *et al.*, 2011)). The risk of developing obesity and the metabolic syndrome increases after the menopause (Poehlman *et al.*, 1995) or ovariectomy (Hong *et al.*, 2009) and this effect is reversed after treatment with exogenous estrogen (McElroy and Wade, 1987). In addition, estrogen regulates body fat distribution, energy balance, insulin synthesis, hepatic glucose production and lipid deposition (reviewed in (Meyer *et al.*, 2011)). However, many of the pathways through which estrogen elicits these effects remain to be discovered. The findings in transgenic mouse models lacking estrogen or its receptor suggest that estrogen may also have a role in male adipose tissue deposition. Male mice lacking aromatase (Fisher *et al.*, 1998) or estrogen receptor (ER) (Heine *et al.*, 2000) have increased adipose depots and develop insulin resistance, although both models are also associated with elevations in testosterone concentrations. Whilst these studies have described the deleterious effects of removing estrogen signalling on adiposity and metabolism, the effects of increased estrogen levels in male animals have been less well characterised.

The changes in circulating sex steroids as a consequence of sexual maturation are associated with the development of obesity in male ERα KO mice (Ohlsson *et al.*, 2000). Since these mice lack estrogen action through ERα receptor, this suggests that estrogen action may be associated with protection from the development of obesity following the maturational changes which occur at puberty. In addition to the changes in circulating steroids occurring during puberty, in rats the sexually dimorphic expression of some liver enzymes is programmed by sex steroid exposure during early life, with reinforcement of these differences occurring at puberty (Gustafsson *et al.*, 1977). The sex differences in mRNA abundance and enzyme activity reported in chapter 3 may, therefore, occur as a result of exposure to sex steroids during prenatal or early postnatal development or as a consequence of the changes in sex steroids following puberty.

As discussed in chapter 3, the lower activity of 11β-HSD1 in adipose tissue of lean females may be one mechanism protecting females from the deleterious effects of exposure to an obesogenic diet. Although the pathways underlying the sexually dimorphic transcript levels

and activity of 11β-HSD1 in the mouse are not known, in rats estradiol inhibits the transcription of hepatic 11β-HSD1 (Low *et al.*, 1994; Jamieson *et al.*, 2000) and acts as a non-competitive inhibitor in adipose tissue (Tagawa *et al.*, 2009). Additionally, 11β-HSD1 activity in subcutaneous adipose increases following estrogen depletion at the menopause in humans (Andersson *et al.*, 2009). This evidence suggests a role for estrogen in mediating the sex differences in local glucocorticoid regeneration.

4.1.1 Hypothesis

Estrogen exerts a protective effect against the metabolic consequences associated with diet-induced obesity in C57BL/6 adult mice. This is mediated in part through effects on peripheral glucocorticoid metabolism.

4.1.2 Aims

To investigate this hypothesis two groups of mice were used. The first cohort was killed pre-puberty to answer the following questions:

- Are sex differences in glucose, lipid and glucocorticoid metabolising genes already present before puberty?

The second group used obese and lean male mice generated as described in chapter 3, with the addition of continuous estradiol treatment from a slow-release pellet to answer the following questions:

Does administration of estradiol:

- Alter body weight and adiposity caused by DIO in male mice?
- Modulate the development of obesity-associated insulin resistance?
- Modify the increases in plasma and hepatic lipids seen in DIO?
- Alter the mRNA abundance of genes involved in glucose and lipid metabolism?
- Alter the mRNA abundance and/or activity of glucocorticoid metabolising enzymes in the liver and adipose, and if so are the changes maintained in obesity?

4.2 Methods

4.2.1 Experimental outline

C57BL/6 mice (n=8/group from 8 litters) of both sexes used in the pre-pubertal experiments were culled between 3 and 4 weeks of age, following a physical examination for signs of puberty. Female mice were assessed for vaginal opening and male mice assessed for balano-preputial separation. If either characteristic was detected the animal was deemed to be in puberty and not used in this cohort. Onset of puberty varies between strain and animal facility: in our animal facility puberty in C57BL/6 mice has been found to begin at 33.1 ± 0.8 days in both sexes (Personal communication Dr Caitlin Wyrwoll, University of Edinburgh and University of Western Australia).

The experimental outline for adult mice used in this chapter can be seen in Figure 4.1.

4.2.2 Animal Maintenance and terminal procedures

Male C57BL/6 mice were fed control (CON, research diets D12328) or obesogenic (DIO, research diets D12331) diets *ad libitum* from 5 weeks and maintained as described (2.3.1). At 6 weeks mice underwent estradiol pellet implantation surgery (see below) providing four groups (Sham CON n=11, Sham DIO n=11, Estradiol CON n=12, Estradiol DIO n=12). 5 days prior to glucose tolerance testing animals were individually housed to allow for acclimatisation.

Mice were killed by CO_2 asphyxiation and tissues collected as described (0). Pre-pubertal mice were killed between 2-4pm following a 2 hour fast and adult mice between 2-5pm, following a 6 hour fast. The purpose of fasting was to minimise variability in gene expression and enzyme activity measured in tissues post mortem.

4.2.3 Estradiol pellet implantation surgery

Ninety-day release, 0.25mg 17β-estradiol pellets were implanted in male mice at 6 weeks age (2.3.5.3), to consistently release the hormone for the remainder of the study (Estradiol CON n=12, Estradiol DIO n=12). A control group underwent sham surgery (Sham CON n=11, Sham DIO n=11), which involved all surgical procedures with the exception of the pellet being implanted. Animals were left to recover as described (2.3.5.2) before being

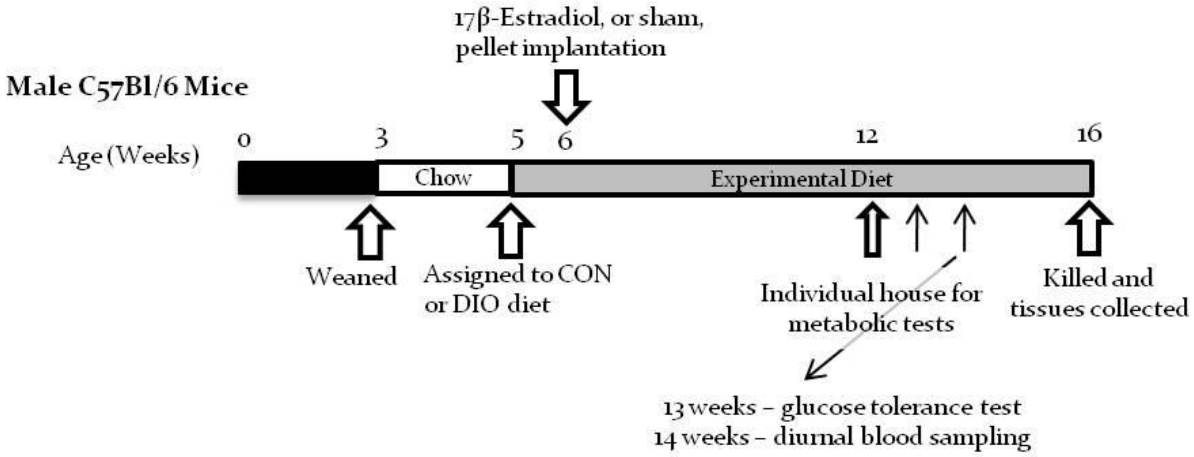


Figure 4.1 Schematic of experimental outline

Male C57BL/6 mice were fed control (CON, Research diets D12328) or obesogenic (DIO, Research diets D12331 diets from 5 weeks of age. One week later mice underwent surgical implantation of an estradiol pellet, or sham surgery giving 4 groups; Sham CON n=11, Sham DIO n=11, Estradiol CON n=12, Estradiol DIO n=12. At 12 weeks of age mice were individually housed and underwent metabolic tests. Animals were then killed and tissues weighed and collected.

returned to the home cage with the relevant experimental diet. Cages housed a mixture of estradiol and sham treated animals on the same experimental diet.

4.2.4 Metabolic tests

Mice underwent a glucose tolerance test (2.3.7) at least 5 days following individual housing; a week later diurnal blood samples were taken in line with the light cycle (2.3.8).

4.2.5 Quantification of nutrients and hormones in plasma and hepatic triglycerides

Levels of glucose (2.6.4) and insulin (2.6.2) were measured in plasma samples taken during the glucose tolerance test. Non-esterified fatty acids (NEFAs) were measured (2.6.7) in the first two glucose tolerance test samples. Plasma triglycerides were measured (2.6.6) in the fasting plasma sample. Corticosterone concentrations were measured (2.6.8) in diurnal blood samples, and cholesterol (2.6.5) in the nadir sample. Liver triglyceride was extracted using a saponification method (2.7) and quantified using a colourimetric assay as in plasma. Testosterone was measured (2.6.3) in blood samples collected at post-mortem; estradiol could not be quantified. For further discussion see sections 3.3.1.3 & 4.4.6.

4.2.6 Quantification of mRNA abundance

RNA was isolated from liver (2.5.1.1) and subcutaneous adipose tissue (2.5.1.2), quantified (0) and cDNA synthesised using RT-PCR (2.5.1.6). The abundance of mRNA encoding 5α-reductase type 1 (5αR), 5β-reductase (5βR), 11β-hydroxysteroid dehydrogenase (11β-HSD1), androgen receptor (AR), estrogen receptor-α (ERα), fatty acid synthase (FAS), glucocorticoid receptor (GR), hormone-sensitive lipase (HSL), lipoprotein lipase (LPL), phosphoenolpyruvate carboxykinase (PEPCK), peroxisome proliferator receptor α (PPARα) and peroxisome proliferator receptor γ (PPARγ) were determined using qPCR (2.5.1.7). In adult mice hepatic and adipose mRNA abundance were normalised to the average abundance of mRNAs encoding cyclophilin and β-actin. In pre-pubertal mice hepatic and adipose mRNA transcript levels were normalised to the abundance of cyclophilin mRNA.

4.2.7 Enzymology

11βHSD1 reductase activity was measured as described (2.8.2). Conditions are shown in Table 4.1, these were optimised for each tissue individually to ensure kinetics were in the first order.

Tissue	Substrate concentration (μM)	Protein concentration (mg/ml)	Incubation time (hrs)
Hepatic microsomes	2	0.025	3
Subcutaneous adipose homogenate	2	0.025 – Estradiol CON & DIO, Sham DIO 0.05 – Sham CON 0.05 - Pre-pubertal	20 – Estradiol & Sham 24 – Pre-pubertal

Table 4.1 Conditions for quantification of 11β-HSD1 activity

Activity of 11β-HSD1 was quantified using a radiolabelled substrate. The substrate and protein concentration as well as incubation time were optimised to give first order kinetics in each tissue.

4.2.8 Statistics

Data are presented as mean ± SEM. Differences between males and females pre-puberty were assessed using unpaired Student's t-test. For the adult study, 2-way analysis of variance (ANOVA) or repeated measures 2-way ANOVA was used to analyse the effects of the obesogenic diet or estradiol and the interaction between the two.

4.3 Results

4.3.1 Sex differences in mRNA abundance and enzyme activity pre-puberty

4.3.1.1 Glucose and lipid metabolism

There were no differences between males and females in the hepatic mRNA levels of PEPCK, LPL or HSL, while PPARα transcript levels were higher in females (Figure 4.2A). In subcutaneous adipose tissue, there were no differences between the sexes in the abundance of mRNA encoding PEPCK, LPL, FAS or PPARγ, however females had higher transcript levels of HSL (Figure 4.3A).

4.3.1.2 Glucocorticoid metabolism

There were no differences between males and females in the hepatic abundance of mRNA encoding 11β-HSD1, 5αR or 5βR, but females had greater abundance of GR (Figure 4.2B). In subcutaneous adipose tissue, sex did not affect the transcript levels of GR or 11β-HSD1 (Figure 4.3B). In addition there were no sex differences in the activity of 11β-HSD1 in the liver (Figure 4.4A) or subcutaneous adipose (Figure 4.4B).

4.3.2 The effects of estradiol treatment and diet-induced obesity on physiology in adulthood

4.3.2.1 Body and tissue weights

DIO caused a significant increase in weight gain over the duration of the study (Figure 4.5). Estradiol administration reduced weight gain regardless of diet (Figure 4.5) and at culling sham DIO mice were 27% heavier than CON while estradiol-treated DIO mice were only 8.8% heavier than their controls (Table 4.2).

Estradiol treatment had a striking effect on adipose weight such that it blocked the majority of the DIO induced increase in adipose tissue seen in sham animals when expressed either as wet tissue weight (Table 4.2) or relative to body weight (Figure 4.6). DIO increased wet liver weight (Table 4.2), but caused a reduction relative to body weight (Table 4.3). In addition

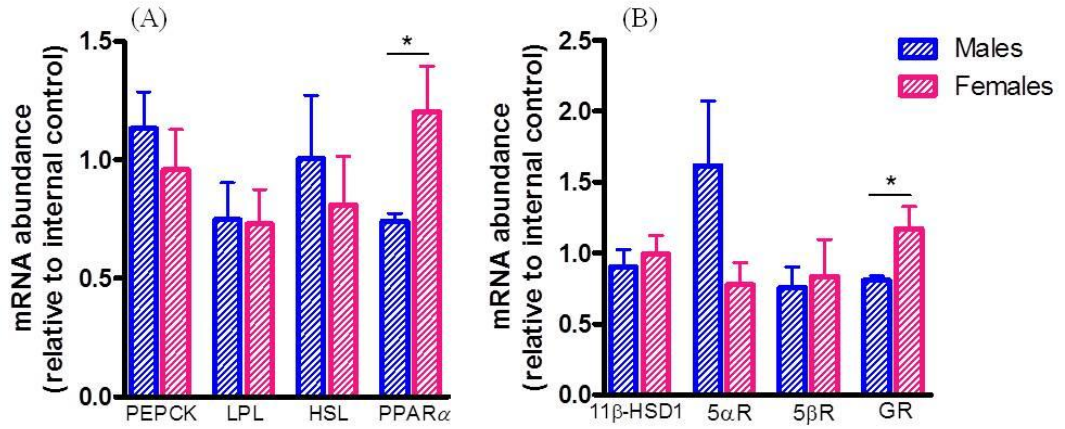


Figure 4.2 Sex determines hepatic abundance of mRNA encoding PPARα and GR in mice pre-puberty

Male (blue bars) and female (pink bars) mice were killed before the onset of puberty, between 21-28 days. Abundance of mRNAs encoding proteins involved in (A) glucose and lipid metabolism and (B) glucocorticoid metabolism were quantified in liver and normalised to mRNA levels of cyclophilin using qPCR. Females had greater abundance of PPARα and GR mRNA. Data are mean ± SEM, analysed by Student's t-test; n=8/group, *p<0.05.

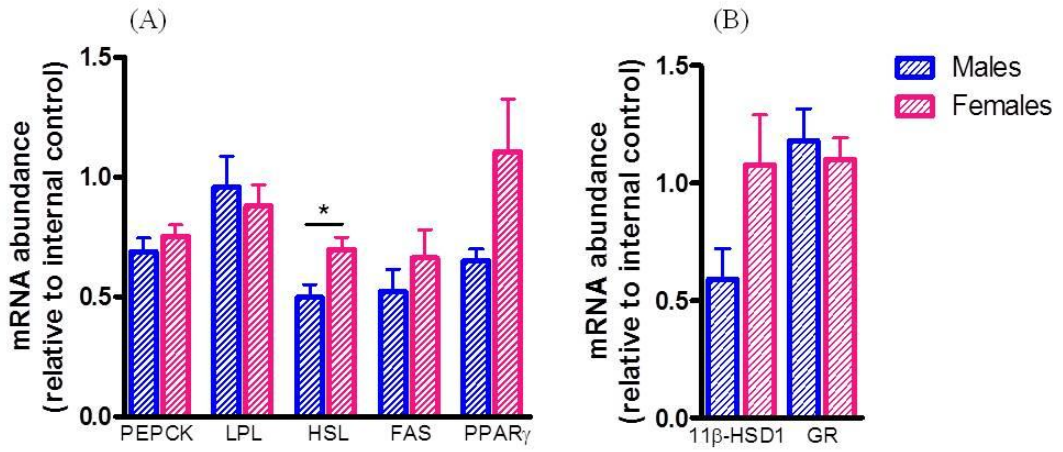


Figure 4.3 Sex determines subcutaneous adipose mRNA abundance of HSL in mice pre-puberty

Male (blue bars) and female (pink bars) mice were killed before the onset of puberty. Abundance of mRNAs encoding proteins involved in (A) glucose and lipid metabolism and (B) glucocorticoid metabolism were quantified in subcutaneous adipose and normalised to mRNA levels of cyclophilin using qPCR. Females had a greater abundance of mRNA encoding HSL. Data are mean ± SEM, analysed by Student's t-test; n=8/group, *$p<0.05$.

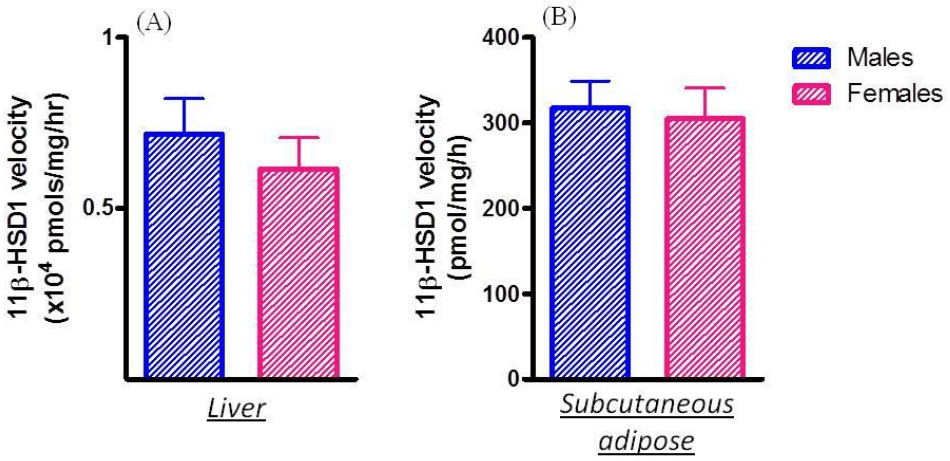


Figure 4.4 Sex does not affect 11β-HSD1 activity in mice pre-puberty

Male (blue bars) and female (pink bars) mice were killed before the onset of puberty. The activity of 11β-HSD1 was quantified in liver (A) and subcutaneous adipose (B) tissue collected at *post mortem* using a radio-labelled tracer. Data are mean ± SEM; n=8/group.

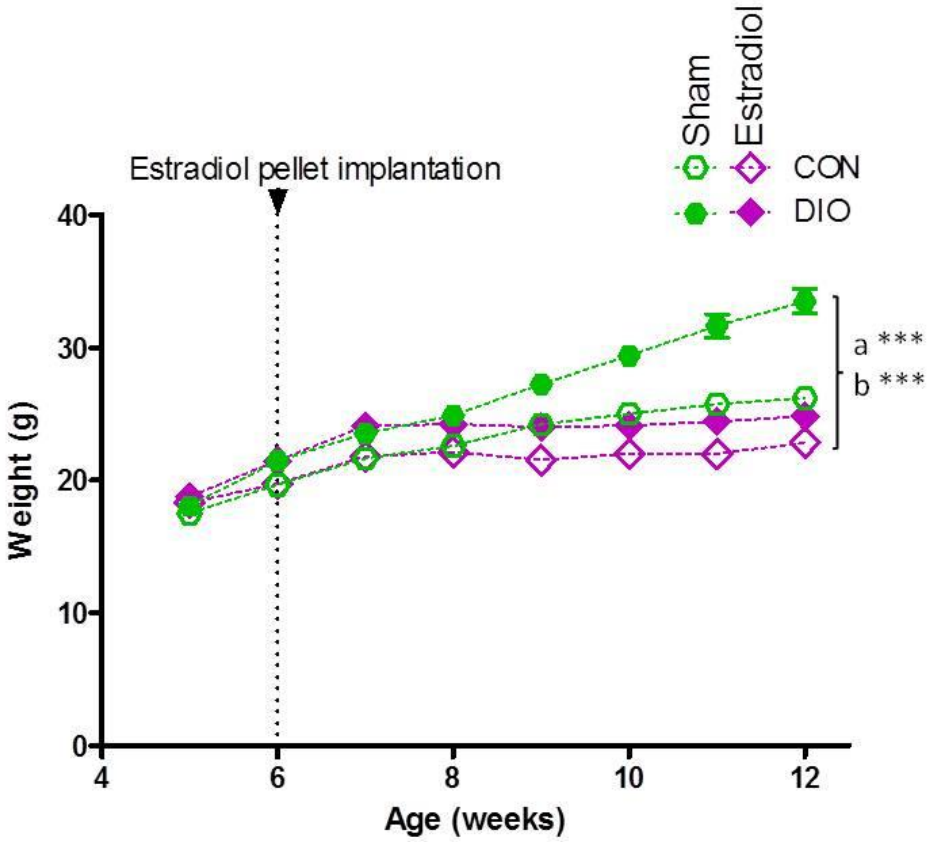


Figure 4.5 Continuous estradiol treatment ameliorates DIO-induced weight gain in male mice

Male mice were fed control (CON, open symbols) or obesogenic (DIO, closed symbols) diets from 5 weeks of age and at 6 weeks of age underwent implantation of a continuous release 17β-estradiol pellet (Estradiol, purple symbols) or sham surgery (sham, green symbols). Mice were weighed weekly. **a:** effect of DIO to increase weight gain, **b:** effect of estradiol to reduce body weight. Data are mean ± SEM, analysed by repeated measures 2-way ANOVA; n=11/Sham group, 12/Estradiol group ***p<0.001.

	Body, adipose and organ weights						
	Sham		**Estradiol**		**Effect of diet**	**Effect of estradiol**	**Interaction**
	CON	**DIO**	**CON**	**DIO**	**P value**	**P value**	**P value**
Cull weights (g)	26.3 ± 0.4	33.4 ± 1.0	23.8 ± 0.3	25.9 ±2.1	**<0.001**	**<0.001**	**<0.001**
SC. Adipose (mg)	205 ± 15	579 ± 58	169 ± 15	225 ± 22	**<0.001**	**<0.001**	**<0.001**
Mes. Adipose (mg)	151 ± 19	389 ± 33	130 ± 58	191 ± 19	**<0.001**	**<0.001**	**<0.01**
Ret. Adipose (mg)	52 ± 6	218 ± 21	33 ± 3	64 ± 7	**<0.001**	**<0.001**	**<0.001**
Epi Adipose (mg)	189 ± 12	750 ± 50	120 ± 12	224 ± 32	**<0.001**	**<0.001**	**<0.001**
Liver (g)	1.00 ± 0.03	1.17 ± 0.05	0.97 ± 0.03	1.02 ± 0.04	**<0.01**	**<0.05**	0.11
Kidney (mg)	153 ± 4	171 ± 10	139 ± 3	159 ± 6	**<0.01**	**<0.05**	0.80
Adrenal (mg)	3.3 ± 0.05	4.6 ± 0.08	3.5 ± 0.03	4.7 ± 0.08	0.06	0.84	0.93
Testis (mg)	101 ± 4	112 ± 3	66 ± 5	71 ± 8	0.13	**<0.001**	0.57

Table 4.2 Body, adipose and organ weights from male obese (DIO) and control (CON) mice treated with estradiol

Male mice were fed control (CON) or obesogenic (DIO) diets from 5 weeks of age and at 6 weeks of age underwent implantation of a continuous release 17β-estradiol pellet (Estradiol) or sham surgery (sham). Mice were killed at 16 weeks of age and wet tissue weights recorded. DIO mice were heavier than controls, and had larger adipose depots, livers and kidneys. Additionally estradiol affected body weight, and wet weights of adipose tissue, liver, kidney and testis. There was an interaction of the effects of DIO and estradiol on body and adipose tissue weight. Data are mean ± SEM, analysed by 2 way ANOVA n=11/Sham group, 12/Estradiol group.

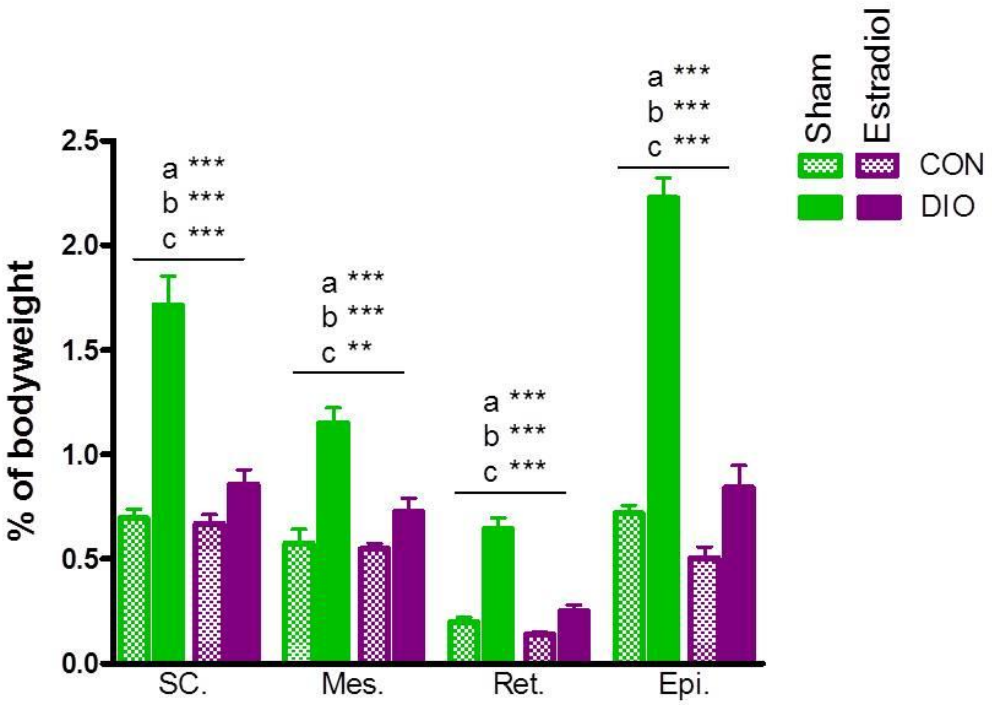


Figure 4.6 Continuous estradiol treatment attenuates obesity-associated adipose deposition

Male mice were fed control (CON, hatched bars) or obesogenic (DIO, closed bars) diets from 5 weeks of age and at 6 weeks of age underwent implantation of a continuous release 17β-estradiol pellet (Estradiol, purple bars) or sham surgery (sham, green bars). Mice were killed at 16 weeks of age and wet weights of; subcutaneous (SC.), mesenteric (Mes.), retroperitoneal (Ret.) and epididymal (Epi) adipose recorded. **a**: effect of DIO to increase percent of body weight contained in adipose tissue, **b**: effect of estradiol to reduce adipose deposition, **c:** interaction between the effects of diet and estradiol. Data are expressed as a percentage of bodyweight. Graph shows mean ± SEM, analysed by 2 way-ANOVA; n=11/Sham group, 12/Estradiol group ***p<0.001.

	Body and Organ weights						
	Sham		Estradiol		Effect of diet	Effect of estradiol	Interaction of effects
	CON	DIO	CON	DIO	P value	P value	P value
Liver (%bw)	3.8 ± 0.1	3.5 ± 0.1	4.1 ± 0.1	3.9 ± 0.1	**<0.05**	**<0.01**	0.69
Kidney (%bw)	0.59 ± 0.01	0.51 ± 0.03	0.58 ± 0.01	0.62 ± 0.03	0.44	**<0.05**	**<0.05**
Adrenal (%bw)	0.013 ± 0.002	0.014 ± 0.003	0.015 ± 0.002	0.018 ± 0.003	0.33	0.18	0.63
Testis (% bw)	0.38 ± 0.02	0.34 ± 0.01	0.28 ± 0.02	0.27 ± 0.03	0.20	**<0.001**	0.34

Table 4.3 Relative organ weights from male obese (DIO) and control (CON) mice treated with estradiol

Male mice were fed control (CON) or obesogenic (DIO) diets from 5 weeks of age and at 6 weeks of age underwent implantation of a continuous release 17β-estradiol pellet (Estradiol) or sham surgery (sham). Mice were killed at 16 weeks of age and wet tissue weights were recorded; these are expressed as a percentage of body weight. DIO mice had relatively larger livers and estradiol affected liver, kidney and testis size. Data are mean ± SEM, analysed by 2 way ANOVA n=11/Sham group, 12/Estradiol group.

estradiol reduced liver weight but increased it relative to body weight. Estradiol caused a reduction in actual and relative testes size (Table 4.2 & Table 4.3).

4.3.2.2 Glucose tolerance test

DIO was associated with an increase in plasma glucose and insulin during glucose tolerance testing in all mice (Figure 4.7A & B). Estradiol-treated mice had lower glucose and insulin concentrations when compared with sham animals. Estradiol treatment reduced the increase in plasma insulin and glucose associated with DIO (Figure 4.7A & B).

4.3.2.3 Lipid concentrations

Neither diet nor estradiol-treatment affected the suppression of NEFAs in the plasma following a glucose bolus (Figure 4.7C). DIO caused an increase in the concentrations of plasma triglycerides and total cholesterol as well as hepatic triglycerides (Table 4.4). Estradiol did not affect any of these parameters in CON mice, but reduced the DIO associated increase in hepatic triglycerides (Table 4.4).

4.3.2.4 Corticosterone concentrations

DIO did not affect the concentrations of corticosterone at the peak or nadir of the circadian rhythm (Table 4.4). Estradiol treatment increased nadir corticosterone concentrations in CON and DIO mice but did not alter peak levels (Table 4.4).

4.3.2.5 Testosterone concentrations

Estradiol caused a reduction in plasma testosterone concentrations in CON and DIO mice. DIO was not associated with any effect on plasma testosterone concentrations (Table 4.4).

4.3.3 The effects of estradiol and diet-induced obesity on mRNA abundance

4.3.3.1 Glucose and lipid metabolising genes

DIO and estradiol both increased hepatic abundance of mRNA encoding PPARα such that estradiol DIO mice had the greatest abundance (Figure 4.8A). Estradiol decreased levels of mRNA encoding PEPCK in the liver of CON and DIO mice but had no effect on LPL (Figure 4.8A).

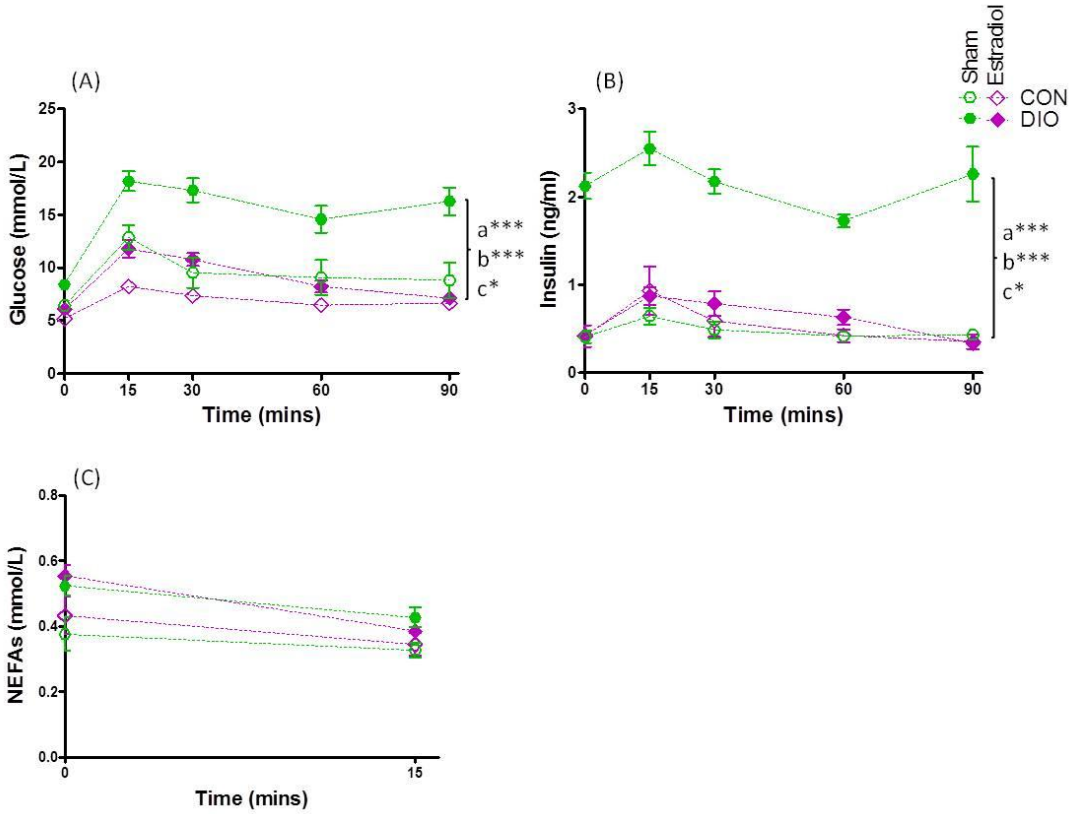


Figure 4.7 Continuous estradiol treatment ameliorates the obesity-associated increases in plasma glucose and insulin

Male mice were fed control (CON, open/hatched symbols) or obesogenic (DIO, closed symbols) diets from 5 weeks of age and at 6 weeks of age underwent implantation of a continuous release 17β-estradiol pellet (Estradiol, purple symbols) or sham surgery (sham, green symbols). Glucose (A), insulin (B) and non-esterified fatty acids (NEFAs) (C) were measured in plasma samples collected during a glucose tolerance test. **a**: the effect of DIO to increase plasma glucose and insulin, **b:** effect of estradiol to decrease glucose and insulin, **c:** interaction between the effects of diet and estradiol. Data are mean ± SEM, analysed by repeated measures 2-way ANOVA; n=11/Sham group, 12/Estradiol group *p<0.05, ***p<0.001.

Lipid and hormone concentrations							
	Sham		**Estradiol**		**Effect of diet**	**Effect of estradiol**	**Interaction**
	CON	**DIO**	**CON**	**DIO**	**P value**	**P value**	**P value**
Fasting plasma triglyceride (mmol/L)	0.55 ± 0.05	0.94 ± 0.09	0.59 ± 0.06	0.81 ± 0.06	<**0.001**	0.49	0.20
Total plasma cholesterol (mmol/L)	2.19 ± 0.31	3.59 ± 0.44	2.05 ± 0.22	3.16 ± 0.21	<**0.001**	0.34	0.62
Hepatic triglyceride (nmol/mg)	19.0 ± 2.7	48.8 ± 6.9	25.9 ± 2.8	27.9 ± 3.3	<**0.001**	0.10	<**0.01**
Nadir plasma corticosterone (nM)	15.3 ± 3.5	17.0 ± 2.9	50.0 ± 11.7	39.6 ± 6.0	0.55	<**0.001**	0.41
Peak plasma corticosterone (nM)	148.7 ± 18.1	178.9 ± 24.5	240.2 ± 35.5	173.1 ± 30.6	0.52	0.14	0.09
Plasma testosterone (ng/ml)	2.5 ± 1.3	2.2 ± 1.4	0.4 ± 0.1	0.5 ± 0.2	0.70	<**0.05**	0.95

Table 4.4 Concentrations of lipids, corticosterone and testosterone in male obese (DIO) and control (CON) mice treated with estradiol

Male mice were fed control (CON) or obesogenic (DIO) diets from 5 weeks of age and at 6 weeks of age underwent implantation of a continuous release 17β-estradiol pellet (Estradiol) or sham surgery (sham). Plasma lipid and corticosterone levels were measured in samples taken during metabolic tests. Hepatic triglyceride was analysed in post-mortem tissue. DIO increased lipid levels in mice, whereas estradiol caused an increase in nadir corticosterone concentration and decrease in testosterone concentration. Data are mean ± SEM, analysed by 2 way ANOVA, plasma testosterone concentrations were log transformed before analysis as the variance within groups was not comparable. n=11/Sham group, 12/Estradiol group.

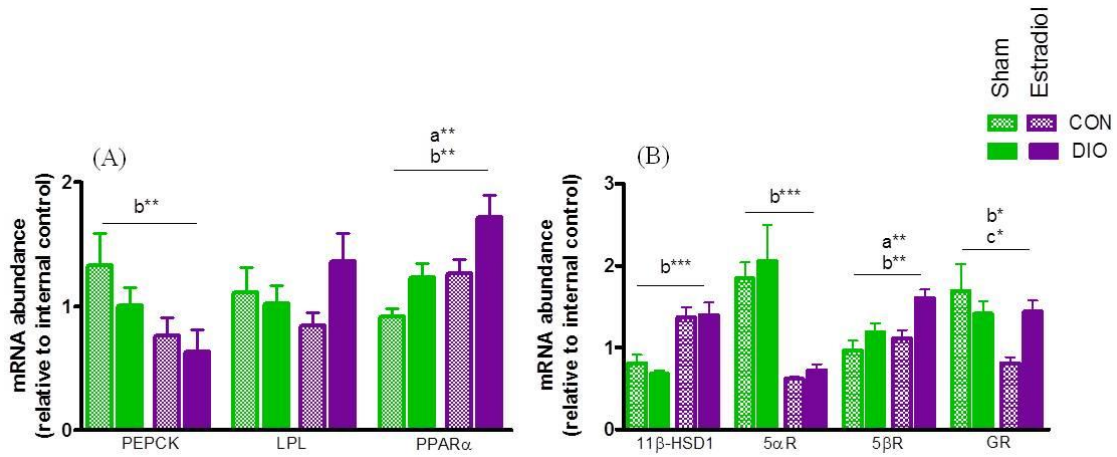


Figure 4.8 Continuous estradiol treatment affects hepatic abundance of mRNAs encoding proteins involved in glucose, lipid and glucocorticoid metabolism

Male mice were fed control (CON, hatched bars) or obesogenic (DIO, closed bars) diets from 5 weeks of age and at 6 weeks of age underwent implantation of a continuous release 17β-estradiol pellet (Estradiol, purple bars) or sham surgery (sham, green bars). Mice were killed at 16 weeks of age and abundance of mRNAs encoding proteins involved in (A) glucose and lipid metabolism and (B) glucocorticoid metabolism was quantified in liver using qPCR. Abundance was normalised to the average abundance of cyclophilin and β-actin mRNA. **a**: effect of DIO to increase abudance of PPARα and 5βR mRNA. **b**:effect of estradiol on mRNA abundance of PEPCK, PPARα, 11β-HSD1 5αR, 5βR and GR. **c**: interaction between the effects of diet and estradiol to affect the transcript levels of GR. Data are mean ± SEM, analysed by 2 way ANOVA; n=11/Sham group, 12/Estradiol group, *p<0.05, **p<0.01, ***p<0.001.

In subcutaneous adipose tissue DIO increased mRNA abundance of LPL and PPARγ, and decreased that of FAS (Figure 4.9A). Estradiol administration reduced the abundance of mRNA encoding PEPCK, LPL and HSL (Figure 4.9A) in CON and DIO mice. Estradiol increased FAS transcript levels in the adipose of CON mice, but DIO reduced it to levels comparable to those seen in sham DIO animals (Figure 4.9A).

4.3.3.2 Glucocorticoid metabolising genes

DIO increased mRNA abundance of hepatic 5βR regardless of estradiol treatment but did not affect transcript levels of the other glucocorticoid metabolising genes (Figure 4.8B). Estradiol caused an increase in hepatic 11β-HSD1 and 5βR mRNAs and a decrease in 5αR and GR mRNAs (Figure 4.8B). There was an interaction between the effects of diet and estradiol on GR mRNA such that DIO did not affect the transcript level in sham animals, but increased it in the estradiol group.

In subcutaneous adipose both high fat diet and estradiol caused a reduction in mRNA abundance of 11β-HSD1, there was also an interaction between the effects, as the reduction induced by DIO was much smaller in estradiol treated mice than sham animals (Figure 4.9B). Neither diet nor estradiol altered abundance of mRNA encoding GR in adipose (Figure 4.9B).

4.3.3.3 Androgen and estrogen receptors

mRNAs encoding AR and ERα could not be detected in the liver using tissues from this study. In subcutaneous adipose tissue DIO caused an increase in mRNA abundance of AR, while estradiol had no effect (Figure 4.10A). Neither diet nor estradiol-treatment altered transcript levels of ERα (Figure 4.10B).

4.3.4 The effects of diet and estradiol on 11β-HSD1 activity

No significant effect of diet or estradiol on hepatic 11β-HSD1 was found (Figure 4.11A). In subcutaneous adipose tissue, both DIO and estradiol reduced the activity of 11β-HSD1. There was also an interaction between DIO and estradiol such that the DIO induced-reduction in 11β-HSD1 activity was large in sham animals and much smaller in the estradiol group (Figure 4.11B).

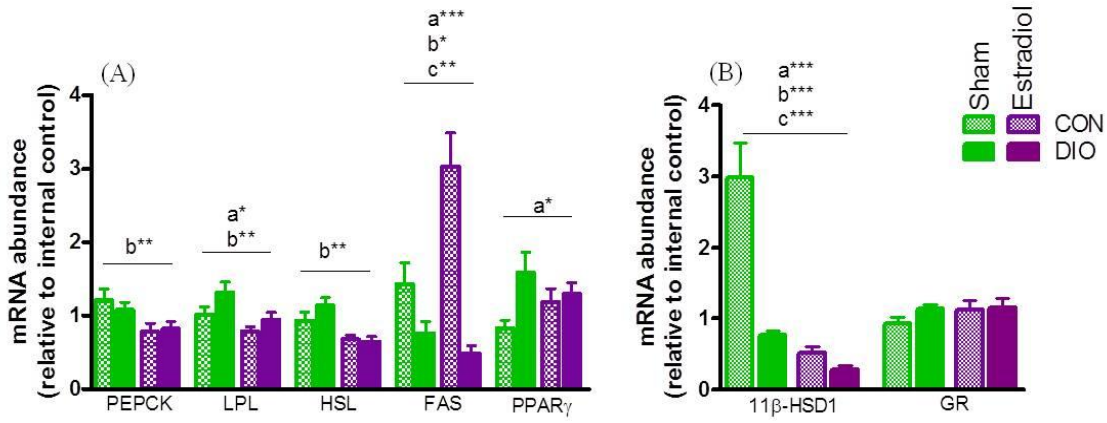


Figure 4.9 Continuous estradiol treatment affects subcutaneous adipose abundance of mRNAs encoding proteins involved in glucose, lipid and glucocorticoid metabolism

Male mice were fed control (CON, hatched bars) or obesogenic (DIO, closed bars) diets from 5 weeks of age and at 6 weeks of age underwent implantation of a continuous release 17β-estradiol pellet (Estradiol, purple bars) or sham surgery (sham, green bars). Mice were killed at 16 weeks of age and abundance of mRNAs encoding proteins involved in (A) glucose and lipid metabolism and (B) glucocorticoid metabolism was quantified in subcutaneous adipose using qPCR. Abundance was normalised to the average abundance of cyclophilin and β-actin mRNA. **a**: effect of DIO on levels of mRNAs encoding LPL, FAS, PPARγ and 11β-HSD1. **b**:effect of estradiol on mRNA abundance of PEPCK, LPL, HSL, FAS and 11β-HSD1, **c**: interaction between the effects of diet and estradiol to affect the transcript levels of FAS and 11β-HSD1. Data are mean ± SEM, analysed by 2 way ANOVA; n=11/Sham group, 12/Estradiol group, *p<0.05, **p<0.01, ***p<0.001.

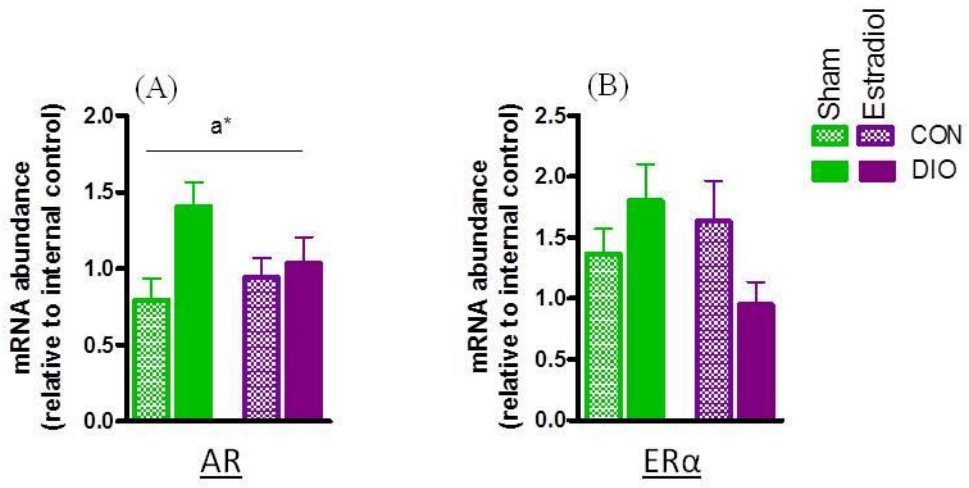


Figure 4.10 Continuous estradiol treatment does not alter levels of mRNAs encoding androgen or estrogen receptor-α in subcutaneous adipose tissue

Male mice were fed control (CON, hatched bars) or obesogenic (DIO, closed bars) diets from 5 weeks of age and at 6 weeks of age underwent implantation of a continuous release 17β-estradiol pellet (Estradiol, purple bars) or sham surgery (sham, green bars). Mice were killed at 16 weeks of age and abundance of mRNAs encoding (A) androgen receptor (AR) and (B) estrogen receptor-α (ERα) quantified in subcutaneous adipose using qPCR. Abundance was normalised to the average abundance of cyclophilin and β-actin mRNA. **a**: effect of DIO to increase abundance of mRNA encoding AR. Data are mean ± SEM, analysed by 2 way ANOVA; n=11/Sham group, 12/Estradiol group, *p<0.05.

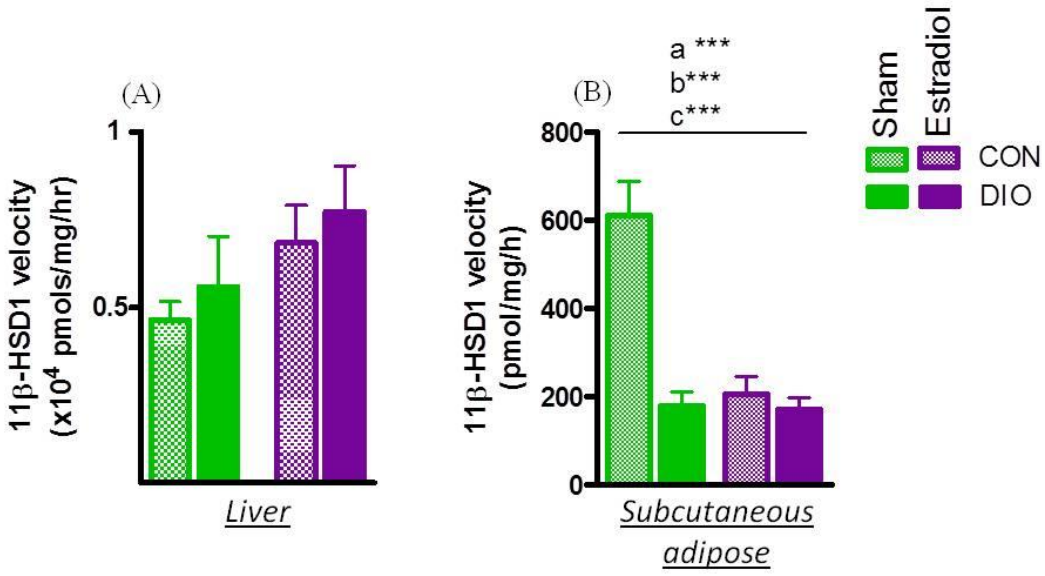


Figure 4.11 Continuous estradiol treatment and DIO reduces 11β-HSD1 activity in adipose tissue but not liver

Male mice were fed control (CON, hatched bars) or obesogenic (DIO, closed bars) diets from 5 weeks of age and at 6 weeks of age underwent implantation of a continuous release 17β-estradiol pellet (Estradiol, purple bars) or sham surgery (sham, green bars). Mice were killed at 16 weeks of age and the activity of 11β-HSD1 was quantified in liver (A) and subcutaneous adipose (B) using a radio-labelled tracer. **a**: effect of DIO to decrease 11β-HSD1 activity in adipose. **b**: effect of estradiol to lower 11β-HSD1 activity, **c**: interaction between the effects of diet and estradiol. Data are mean ± SEM, analysed by 2 way ANOVA; n=11/group, ***p<0.001.

4.4 Discussion

The results of this chapter support the hypothesis that estradiol treatment in male mice alters the response to an obesogenic diet and affects glucocorticoid metabolism. These data will be discussed with reference to chapter 3, which demonstrated sex differences in the response to obesity and glucocorticoid metabolism, providing the hypothesis for these experiments. Sex differences in the transcript levels of some genes are evident before puberty, and are likely to be 'programmed' during early-life. Continuous administration of estradiol to male mice reduced weight gain, adipose deposition and changes in glucose-insulin homeostasis associated with obesity. Additionally, estradiol exposure altered glucocorticoid metabolism in the liver and adipose tissue, resulting in mRNA levels and enzyme activity profiles similar to those found in females (chapter 3). These data support the hypothesis that sexually dimorphic patterns of glucocorticoid metabolism play a role in the sex specific response to an obesogenic diet.

4.4.1 Body weight, adiposity and organ size

Estradiol-treated mice did not gain weight at the same rate as sham mice, regardless of diet. This difference in body weight may be due to the reduction in adipose tissue induced by alterations in appetite or metabolism and energy expenditure. The differences in body weight could also reflect a lack of linear growth, though no visible phenotype was recorded at post-mortem; measurements of body length and muscle size would provide useful information in future studies to eliminate this possibility. To date, there have been no reports of the effect of estradiol on diet-induced obesity in male animals although daily injections of estradiol for one week decreased body weight and adipocyte size in male rats, presumably fed chow though not reported (Pedersen *et al.*, 1991). Conversely, deletion of ERα (Heine *et al.*, 2000) or aromatase (Jones *et al.*, 2001), and therefore ablation of estrogen signalling or synthesis, increases weight gain and adipose deposition in male mice fed a normal diet. In ERα KO mice this increase in weight was due to reduced energy expenditure rather than increased food consumption (Heine *et al.*, 2000), as well as adipocyte hypertrophy and hyperplasia, parameters which were not examined in this thesis. Estrogen concentrations also have an impact on energy balance in females, as ovariectomy in mice leads to increased weight gain and adiposity without increased food intake (Witte *et al.*, 2010). The alterations in energy balance induced by estrogen are likely to be, at least in part, controlled by the brain. Direct injections of estradiol into the paraventricular nucleus result in a reduction in food intake,

body weight and increased wheel running (Butera and Czaja, 1984), whereas brain specific deletion of ERα leads to hyperphagia and abdominal obesity (Xu *et al.*, 2011). The receptor-interacting protein 140 (RIP140) is a negative transcriptional regulator of nuclear receptors required for maintainence of energy homeostasis, including ER (Cavailles *et al.*, 1995). RIP140 is involved in fat accumulation and mice deficient in the protein are lean and resistant to diet-induced obesity (Leonardsson *et al.*, 2004). Interestingly estrogen has been shown to increase the expression of RIP140 (Augereau *et al.*, 2006) therefore investigating its expression of would be of interest in future studies.

Effects of estradiol were also seen on liver and testis weights. The reason for a decrease in liver weight relative to body weight following DIO is unclear. This was not seen in the previous chapter using the same model (chapter 3), but is consistent with another study from our group (Personal communication Dr. Vicky King, University of Edinburgh). The reason for this is unclear since liver weight would be expected to increase in obesity as a consequence of lipid accumulation. The reduction in testicular size is as expected since estrogen will suppress the HPG axis by negative feedback mechanisms; this will reduce the synthesis and release of gonadotrophins so both Leydig cell and spermatogenesis function is reduced and the testes shrink.

4.4.2 Glucose and insulin homeostasis

Maintenance of normal glucose tolerance and insulin sensitivity in estradiol-treated males may be a consequence of the reduction in adipose tissue, since adipose tissue releases FFAs and adipokines into the circulation which can lead to insulin resistance (Boden, 1999; Rabe *et al.*, 2008). Male ERα (Heine *et al.*, 2000) and aromatase (Jones *et al.*, 2001) KO mice have moderate insulin resistance which may be due to the increase in adiposity associated with models lacking estrogen signalling. Additionally, females subjected to ovariectomy (Riant *et al.*, 2009) also develop moderate insulin resistance and glucose intolerance which can be reversed with estrogen treatment (Riant *et al.*, 2009), and this is also seen with hormone replacement therapy in post-menopausal women (Tchernof *et al.*, 1998). Estrogen can alter glucose metabolism and insulin sensitivity in many tissues including liver, muscle and adipose tissue. Estrogen treatment in rats up-regulates ERα mRNA abundance and protein levels in the liver (Koritnik *et al.*, 1995), and in mice ER binding domains are found in hepatic genes which control metabolism and maintain insulin sensitivity (Gao *et al.*, 2008). Estrogen treatment increases hepatic insulin sensitivity by decreasing gluconeogenesis and glycogenolysis in female rats (Matute and Kalkhoff, 1973) and ovariectomised mice (Ahmed-Sorour and Bailey, 1981). In this study, the decreased levels of

mRNA encoding PEPCK in the estradiol treated males suggests reduced gluconeogenesis. Exogenous estrogen treatment in post-menopausal women suppresses lipolysis and free-fatty acid release in adipose, which also helps maintain insulin sensitivity (O'Sullivan and Ho, 1995). In models of reduced estrogen signalling, reduced glucose uptake into skeletal muscle (Puah and Bailey, 1985), increased hepatic expression of genes involved in lipid synthesis (Bryzgalova *et al.*, 2006), hepatic lipid accumulation (Takeda *et al.*, 2003) and loss of insulin-mediated suppression of glucose production (Bryzgalova *et al.*, 2006) have all been proposed to play a role in the development of insulin resistance and may warrant further investigation in the model used in this chapter.

Estradiol can also affect insulin synthesis and secretion in the pancreas (Wong *et al.*, 2010). Diabetes can be induced in mice by injection of streptozotocin (STZ) which enters beta-cells and causes oxidative stress, DNA damage and cell death. STZ treatment is much more effective in male mice and exogenous estrogen treatment reduces its effectiveness (Paik *et al.*, 1982). Other models of diabetes are more severe in males than females (Corsetti *et al.*, 2000; Geisler *et al.*, 2002), suggesting a role for estradiol in protecting the pancreas. *In vitro,* estradiol can protect isolated pancreatic islets from apoptosis, and this effect is partially reversed by ER antagonists (Contreras *et al.*, 2002) suggesting a direct role of estradiol in preserving islet function.

4.4.3 Lipid concentrations

Consistent with the data presented in chapter 3 (Table 3.4), DIO increased plasma and hepatic lipid concentrations. Although estradiol-treated mice had less adipose tissue and a reduction in hepatic triglyceride levels, estradiol treatment had no effect on plasma lipid concentrations. While no changes in total cholesterol were found, estradiol may be athero-protective through its effects on LDL and HDL (Kushwaha and Hazzard, 1981; Tang *et al.*, 1991), which were not measured independently in this study.

Estradiol-treated male mice, like female mice, maintained insulin sensitivity despite increased plasma lipid concentrations following consumption of an obesogenic diet. The results of this chapter suggest the lower plasma triglyceride concentrations in females, despite consumption of an obesogenic diet (chapter 3); cannot be induced in males by estradiol treatment used in this chapter, and may be due to a non er-dependent mechanism. However, in male humans estrogen treatment can correct the dyslipidemia resulting from mutations in ER or aromatase (Grumbach and Auchus, 1999) suggesting some ER pathways play a role in controlling plasma lipids in humans.

4.4.4 mRNA abundance of glucose and lipid metabolising genes

The abundance of mRNAs encoding PPARα and PPARγ was sexually dimorphic before puberty, in the same pattern as found in adults (chapter 3), suggesting that this difference is programmed during early development as has been reported for other genes in the rat (Gustafsson *et al.*, 1977). A number of studies have suggested that there is cross talk between ER and PPARs (Keller *et al.*, 1995; Ma *et al.*, 1998; Wang and Kilgore, 2002) and the results of this chapter suggest that estradiol can increase abundance of mRNAs encoding the PPARs in adult mice. Thus, the surge in sex steroids at puberty could exaggerate the differences seen earlier in life or, alternatively, the mechanisms which induced changes in early life can still be activated by high concentrations of estradiol in adulthood.

The increase in hepatic PPARα mRNA levels induced by DIO is consistent with the results previously reported in males (chapter 3). As this response was not found in females, it may be mediated by other pathways specific to male mice that are not affected by estradiol treatment. An increase in adipose PPARγ mRNA following DIO was not found in the previous study (chapter 3) and the reasons for this are unclear as the same protocol was used. Increasing PPARγ enhances lipid storage and promotes insulin sensitivity, suggesting the increase in DIO sham males may be an attempt to compensate for the effects of obesogenic diet.

The sex difference in adipose HSL mRNA levels before puberty is opposite to that seen in adults (chapter 3). Estradiol treatment of males resulted in mRNA levels comparable to adult females suggesting that this sex difference is induced following increases in estrogen at puberty. The lack of an effect of DIO is consistent with the previous chapter and suggests HSL is not a primary target in modulating metabolism following consumption of an obesogenic diet.

Although estradiol treatment reduced abundance of hepatic PEPCK mRNA in males, there were no sex differences identified in chapter 3. This suggests that pepck mRNA might be modulated by high concentrations of estrogen and/or that other hormones such as glucocorticoids, glucagon and insulin may have a greater effect on PEPCK mRNA in normal physiology. In the adipose tissue the lower PEPCK transcript levels in estradiol-treated males is consistent with the results found in females (chapter 3). This supports the hypothesis that reduced PEPCK mRNA abundance and the consequent reduction in glyceroneogenesis may be one mechanism protecting females from the deleterious metabolic consequences of DIO. Sex differences in transcript levels of PEPCK mRNA were not present before puberty,

suggesting that the changes in hormone concentrations in females following puberty are responsible for the differences in adulthood. Interestingly the diet-induced change in adipose PEPCK mRNA levels found in chapter 3 was not found in this experiment. Given the similarity of the metabolic results in the male animals in chapter 3 and sham male animals in this chapter, and the use of the same protocols, the reasons for this difference are unclear.

Due to technical issues mRNA encoding FAS could not be measured in subcutaneous adipose tissue chapter 3, which would have been of interest given the large increase in levels seen following estradiol treatment. The increased mRNA abundance in the estradiol-treated CON mice may reflect the relative lack of adipose tissue and the need to synthesise fatty acids to be released into circulation and oxidized in the liver to generate energy. Despite the high transcript levels of FAS in estradiol CON mice, DIO reduced FAS transcript abundance to lower levels than those found in sham CON mice with similar amounts of adipose tissue. The resultant reduction in fatty acid synthesis may be a key pathway targeted by estradiol, in response to increased dietary lipids, to reduce lipogenesis and utilise stored and circulating FFAs.

4.4.5 Glucocorticoid metabolism

4.4.5.1 Estradiol and hepatic glucocorticoid metabolism in lean animals

The sexually dimorphic patterns of hepatic 11β-HSD1 mRNA abundance found in chapter 3 were not present before puberty. Alterations in transcript levels of 11β-HSD1 were induced by estradiol treatment, suggesting the differences seen in adults are induced by changes in sex steroids following puberty. Sex differences in mRNA and activity of 11β-HSD1 have been reported in rats (Low *et al.*, 1994), though the differences are thought to be partly induced by sex differences in growth hormone (GH) secretion during early life (Low *et al.*, 1994). The results of this chapter propose a role for this to occur after puberty coinciding with a dramatic increase in estrogen concentrations.

5αR plays a key role in sexual differentiation by converting testosterone into dihydrotestosterone (DHT) and, while this is mainly catalysed by the type 2 isozyme, the type 1 enzyme measured in this study can also bind this substrate (Normington and Russell, 1992). No significant difference in mRNA levels of hepatic 5αR was found in pre-pubertal animals, although a trend for lower levels in females was present as was found in adulthood (Chapter 3). It is likely this difference did not reach significance because of the large variability between animals and because the experiment was under powered. No studies have

investigated sex differences prepuberty, and therefore n numbers were based on those used in adult studies. In future experiments with young animals a larger cohort should be used to ensure adequate power. Additionally, estrogen-treated males had lower 5αR mRNA levels. The difference may be programmed *in utero* due to the local milieu of hormones involved in sexual development with the difference becoming more evident in adulthood, following the increase in estrogen concentrations in females. The decreased transcript levels of mRNA encoding 5αR type 1 in females may result in the preferential metabolism of glucocorticoids by 5βR. This hypothesis is supported by the sex differences in 5βR induced following increases in estrogen post puberty and by estrogen treatment in males. Tipping the balance in favour of 5βR metabolism may reduce GR activation by metabolising 11-dehydrocorticosterone, the substrate for 11β-HSD1 and also reducing the local levels of 5α-metabolites which are still active at the receptor (McInnes *et al.*, 2004).

Hepatic abundance of mRNA encoding GR was higher in females than males before puberty, this difference was no longer apparent in adults and estradiol exposure reduced abundance in males. This suggests a role for estradiol in the regulation of GR, reducing mRNA levels at puberty in females. This is consistent with data showing treatment of human breast cancer cells with estrogen reduces GR expression (Krishnan *et al.*, 2001).

4.4.5.2 Estradiol and adipose tissue glucocorticoid metabolism in lean animals

Activity of 11β-HSD1 was not affected by sex prepuberty as was found in adulthood (chapter 3). Additionally, adipose 11β-HSD1 mRNA and activity was reduced in males by estradiol treatment. This suggests that the sex differences in 11β-HSD1 mRNA abundance are due to the changes in circulating concentrations of estrogen which occur following puberty. The abundance of mRNA encoding GR was not sexually dimorphic before puberty or in adulthood (chapter 3) and consistent with these results estradiol treatment did not affect GR mRNA. This suggests that the estradiol-induced reduction in glucocorticoid regeneration by 11β-HSD1 does not result in a compensatory increase in gr mRNA abundance.

4.4.5.3 Obesity, estradiol and hepatic glucocorticoid metabolism

As in females (chapter 3), the mRNA abundance of 11β-HSD1 and 5αR in liver was not affected by DIO in estradiol-treated males however DIO did increase the abundance of 5βR, in addition to the induction by estradiol, consistent with the data from male animals in the previous chapter. This may reflect a physiological response to dietary composition, such that increased glucose and/or lipid intake increases 5βR mRNA levels in males regardless of the circulating concentrations of sex steroids, as is the case for adipose ApoE expression in

response to cholesterol (Laffitte *et al.*, 2001). Changes in 5βR may also be a compensatory mechanism to increase local glucocorticoid metabolism or decrease circulating corticosterone concentrations, though the circulating concentrations were not different from CON mice.

4.4.5.4 Obesity, estradiol and adipose tissue glucocorticoid metabolism

DIO and estradiol reduced mRNA abundance of 11β-HSD1 in adipose tissue. The activity of 11β-HSD1 was also reduced by estradiol treatment, but there was little additional effect of combining these interventions. These results suggest a similar level of glucocorticoid regeneration in sham DIO and all estradiol-treated males, in addition to comparable GR mRNA abundance. Due to the differences in adiposity and glucose-insulin homeostasis between groups these results support the published hypothesis that the reduction of adipose 11β-HSD1 in male mice is a compensatory mechanism in response to obesity (Morton *et al.*, 2004). The results also support the hypothesis for the chapter that reduced glucocorticoid regeneration, mediated by estradiol, is associated with protection from some of the effects of exposure to an obesogenic diet.

4.4.5.5 Potential mechanisms for estradiol regulation of 11β-HSD1

Interestingly, the mRNA abundance of hepatic 11β-HSD1 did not correlate with activity of the enzyme in adult mice, in this or the previous chapter. This disparity becomes apparent post-puberty suggesting that sex steroids may play a role in controlling activity as well as gene expression in mice. Although the sexually dimorphic expression of 11β-HSD1is affected by GH secretion in rats (Low *et al.*, 1994), estradiol can also repress transcription (Low *et al.*, 1993; Andersson *et al.*, 2010). Estrogen can still down regulate 11β-HSD1 in adrenalectomised rats (Jamieson *et al.*, 1999) suggesting the effect of estrogen is not mediated by changes in glucocorticoid synthesis in the adrenals. Furthermore, whilst GH secretion patterns affect 11β-HSD1 expression and activity, in hypophysectomised rats estrogen still has a suppressive effect (Low *et al.*, 1994), suggesting it is not mediated by the pituitary. Other hormones and inflammatory factors have been shown to regulate 11β-HSD1, and estrogen may be acting with or upon such molecules. In the mouse, leptin increases hepatic 11β-HSD1 activity (Liu *et al.*, 2003), and estradiol may be activating leptin expression with a consequent increase in 11β-HSD1. Increasing leptin would also be expected to protect against the metabolic complications of obesity due to its effects on adipose mass and appetite regulation through receptors in the brain (Campfield *et al.*, 1996). In primary cultures from rat mesenteric adipose, estradiol acts as a non-competitive inhibitor

of 11β-HSD1 (Tagawa *et al.*, 2009). Removal of estradiol production *in vivo* by ovariectomy increases 11β-HSD1 in rat visceral adipose tissue and the enzyme can then be repressed by estrogen treatment (Andersson *et al.*, 2010). Adiposity and adipocyte hyperplasia has been proposed as the most likely explanation for estradiol regulation of 11β-HSD1 in rats. In pair-fed female rats, changes in 11β-HSD1 following ovariectomy and estrogen treatment were due to the lack of adipose rather than increases in estradiol (Paulsen *et al.*, 2008). Complementary to this study, Andersson *et al.* reported that estradiol induced a decrease in visceral, but not subcutaneous, adipose deposition in female rats in addition to a decrease in mesenteric, but not subcutaneous adipose tissue, 11β-HSD1 expression and activity (Andersson *et al.*, 2010). In contrast to the findings in DIO females in which a significant increase in adipose deposition was associated with a reduction in 11β-HSD1 (chapter 3), estradiol-treated male mice had very small increases in adipose when fed the obesogenic diet and no change in 11β-HSD1 activity compared to their respective controls. Consistent with the rat data this implies that diet or adipose expansion can alter 11β-HSD1 despite differences caused by estradiol in lean animals. It is possible the differences are induced by estradiol if its local production by aromatase is amplified in obesity as has been suggested in humans due to adipocyte hyperplasia (Cleland *et al.*, 1985).

4.4.6 The androgen-estrogen balance

Due to the technical issues described in chapter 3 (3.3.1.3) the plasma concentrations of estradiol in the sham group could not be determined. The concentrations obtained with plasma from the estradiol-treated group were not adequate for presentation or analysis as some samples fell below the limits of detection and those that did not were hugely variable. In female mice plasma estradiol concentrations vary throughout the estrus cycle reaching their maximum during proestrus (Saito *et al.*, 2009). Other groups have reported inconsistent plasma estradiol concentrations following implantation of pellets from the same source as used in this experiment. One group using 0.1mg, 21-day (~ 4.7µg/day) pellets in females, reported plasma estradiol concentrations in the range of 115-148pg/ml stating it as a dose that restored physiological levels (Karas *et al.*, 2001), presumably those in proestrus. While another group reported plasma estradiol concentrations of 323pg/ml from pellets administering ~2µg/day in male mice and 13.8pg/ml in the vehicle treated group (Geisler *et al.*, 2002), comparable to other reports in WT males (Couse and Korach, 1999; Saito *et al.*, 2009). One potential reason for the disparity in results is the time the plasma used to measure estradiol was collected from the mice. The study in female mice collected plasma at the end of the 21-day release period of the pellet (Karas *et al.*, 2001); whereas that of Geisler *et al.*

analysed estradiol concentrations in plasma collected 28 days into a 90-day release period (Geisler *et al.*, 2002). The pellets do not actively pump the hormone in to the circulation and therefore the concentrations are likely to reduce towards the end of the release period. In addition the groups used different commercially available assays to measure plasma estradiol which have been shown to be highly variable (Haisenleder *et al.*, 2011). The lack of plasma estradiol measurements in this chapter is a current weakness in this study. The results of other studies discussed above suggest the plasma concentrations are likely to exceed those found physiologically in female mice, though at present this cannot be confirmed.

Whilst plasma testosterone concentrations were variable, as has been reported in rodents (Bartke *et al.*, 1973), a decrease occurred in response to estradiol treatment as has been previously demonstrated (Akingbemi *et al.*, 2003). This is likely to be due to increased negative feedback on the HPG axis, which decreases GnRH and gonadotrophin secretion and therefore reduces testosterone production. ERs are found in the hypothalamus where they can regulate the transcription of gonadotrophin genes (Gharib *et al.*, 1990; Shughrue *et al.*, 1997) such that reduced negative feedback in er KO mice increases testosterone concentrations (Akingbemi *et al.*, 2003). DIO did not alter plasma testosterone concentrations in either estradiol-treated or sham mice, in contrast to studies of morbidly obese human males (reviewed in (Cohen, 1999)). In fact, morbidly obese male humans suffer from low testosterone and high estradiol (Kley *et al.*, 1979; Cohen, 1999), similar to the hormonal environment in the model used in this chapter, though in the mouse this was associated with resistance, rather than propensity, to obesity.

The changes in testosterone found in this model should not be neglected when discussing the phenotype induced by estradiol treatment. Treatment of men with testosterone reduces waist/hip ratio (Marin *et al.*, 1992) and visceral fat mass (Marin *et al.*, 1993) and male AR KO mice have smaller adipose depots, with fewer and smaller adipocytes when young (Yeh *et al.*, 2002) but develop obesity with age (Sato *et al.*, 2003). Global AR KO mice maintain insulin sensitivity despite obesity (Fan *et al.*, 2005), while an adipose specific AR KO model has hyperinsulinemia without obesity (McInnes, 2010). The relative estrogen concentrations in these models were not reported, but the phenotypes of AR KOs propose a role for androgens in adiposity and insulin sensitivity. Additionally, in ER and aromatase KO mice obesity and the associated metabolic complications are confounded by increased testosterone concentrations as well as the lack of estrogen signalling. Data from all of these transgenic mouse models suggest that the relative concentrations of estrogen and testosterone may be important in normal physiology, such that the changes in adiposity and insulin sensitivity

presented in this chapter may have been a consequence of the alterations in either sex hormone or a change in the androgen-estrogen balance. The effects of altering the balance have rarely been addressed but one study found treatment of ovariectomised mice with dihydro-testosterone caused greater weight gain and adipose deposition than is seen with physiological testosterone concentrations (McInnes *et al.*, 2006). Further studies are required to investigate the importance of the androgen-estrogen balance in males.

The alteration in the androgen-estrogen balance of the estradiol-exposed males may have also affected glucocorticoid metabolism and, therefore, the changes in physiology. Testosterone increases the expression and activity of 11β-HSD1 in omental adipose tissue from pre-pubescent boys (Zhu *et al.*, 2010). Therefore the decrease in testosterone following estradiol treatment could account for the reduced activity. However, the decrease in adipose 11β-HSD1 following DIO in sham mice was not accompanied by a change in testosterone suggesting this is unlikely. Additionally, adipose tissue aromatase can modulate levels of testosterone and estrogen, and, therefore, the balance of the two may control the sexual dimorphism of 11β-HSD1 and the changes seen in enzyme activity in obesity and following the menopause.

4.4.7 Sex steroid receptors

Whilst transcript levels of mRNA encoding AR in subcutaneous adipose were increased by DIO, no changes were found in response to estradiol or the reduction in testosterone. This suggests the receptor may be responsive to the metabolic environment rather than changes in sex steroids. This may be tissue specific as a study in the reproductive tract suggests increasing estrogen reduces AR protein levels (Rivas *et al.*, 2002). As discussed above data from transgenic AR KO mice suggest a role for androgens in metabolism. The increase in AR mRNA abundance with DIO in this study may be a compensatory mechanism to maintain insulin sensitivity, or to decrease adipose deposition as is seen in humans given endogenous testosterone (Marin *et al.*, 1992). The lack of change in abundance of mRNA in estradiol treated males which do not have impaired metabolism or increased adipose deposition, supports the idea of a compensatory mechanism. Interestingly global AR deletion leads to obesity in adulthood in male but not female mice (Sato *et al.*, 2003) which further indicates the importance of the balance of sex steroids. Local adipose levels of estrogen are increased in obesity due to adipocyte hyperplasia increasing the abundance of aromatase (Cleland *et al.*, 1985). Increased tissue concentrations of estrogen may affect receptor expression, with one study showing the steroid upregulates AR mRNA in human preadipocytes (Dieudonne *et al.*, 2006).

The abundance of mRNA encoding ERα was altered in response to diet but the direction of change was dependent on estradiol treatment. mRNA encoding ERβ could not be quantified in this study; this is likely due to the lower expression than α isoforms, and additionally the reduced mRNA in males compared to female animals. Tissue specific concentrations of estrogen and testosterone in adipose tissue may modulate ER expression (Rodriguez-Cuenca *et al.*, 2005) and the response to sex steroids in different adipose depots may play a role in the metabolic consequences of obesity (Rodriguez-Cuenca *et al.*, 2005). It would be of interest to determine mRNA levels of ER and AR in other adipose depots in this study given greater time. The results of this chapter support studies showing there is sufficient ER to be quantified in adipose tissue of male mice, and to play a role in normal physiology (Heine *et al.*, 2000; Cooke *et al.*, 2001).

4.4.8 Study limitations

The estradiol treatment obviously had a profound effect on weight gain and adiposity such that the DIO estradiol mice were comparable in weight to sham CON mice. It is therefore unclear if the relative lack of changes in metabolism are due to low food intake and reduced adipose tissue in estradiol mice or changes in basal physiology. Studies of food intake and pair feeding sham mice would provide a more strigent control phenotype and allow for greater interpretation of the data. As discussed in section 4.4.6 the concentrations of plasma estradiol in the mice has not been measured to date. This information is vital for full interpretation of the results and comparison to the concentrations found in females. Reducing the concentration of estradiol used may lead to a greater degree of obesity being achieved in the mice. This would also help address the issue of whether the differences found are based on adiposity, estradiol or a combination of both.

The differences in mRNA abundance should also be investigated on a protein level (see section 3.4.7). The effects of estradiol on 11β-HSD1 have been proposed to be depot specific (Andersson *et al.*, 2010), therefore studying the enzyme in visceral adipose would allow for further comparisons to published literature.

4.4.9 Conclusions

In summary, estradiol-treatment of male mice consuming an obesogenic diet blocked the adipose tissue deposition and insulin resistance found in controls. Indeed, the physiology of male mice treated with estradiol resembled that of female mice presented in the previous chapter with the exception that the obese females accumulated a comparable amount of adipose to males, whereas estradiol-treated males did not. Treatment with estradiol induced

changes in many genes involved in glucose, lipid and glucocorticoid metabolism and, consistent with the hypotheses for this study; estradiol-treated mice had reduced glucocorticoid regeneration in adipose tissue, suggesting that this may be part of the mechanism determining the metabolic response to an obesogenic diet. These results support the use of pharmacological inhibition of 11β-HSD1 as a potential insulin sensitising strategy in men, and suggest further investigations into their efficacy in females. The results presented in this chapter also highlight the need to increase the understanding of the importance of the balance of androgens and estrogens in obesity.

Chapter 5

The effects of maternal obesity on offspring metabolism

5.1 Introduction

Many epidemiological studies have demonstrated a link between the environment in early-life and long term risk of cardiovascular disease (Barker *et al.*, 1989; Barker *et al.*, 1993b; Ravelli *et al.*, 1998; Doyle *et al.*, 2000; Shiell *et al.*, 2000; Gillman *et al.*, 2003; Martin-Gronert and Ozanne). In recent times there has been an explosion in the rates of obesity, including in women of child bearing age (Kanagalingam *et al.*, 2005; Guelinckx *et al.*, 2008). This has led to a plethora of studies suggesting links between exposure to obesity *in utero* and during suckling (referred to as early-life) and long term health (Laitinen *et al.*, 2001; Ogden *et al.*, 2006; Gale *et al.*, 2007; Koupil and Toivanen, 2008; Catalano *et al.*, 2009a). Due to the limited opportunities for experimental manipulations and time required to study this link in humans, animal models have proved useful experimental tools. In rodent models, exposure to maternal obesity induces a variety of adverse metabolic changes in the offspring, including increased rates of obesity, insulin resistance and hyperlipidemia (Guo and Jen, 1995; Bayol *et al.*, 2008; Samuelsson *et al.*, 2008; Howie *et al.*, 2009; McCurdy *et al.*, 2009; Nivoit *et al.*, 2009). Furthermore some groups have demonstrated maternal obesity can cause long-term alterations in tissue structure or function (Cerf *et al.*, 2005; Ford *et al.*, 2009, Armitage *et al.*, 2005a; Calvert *et al.*, 2009) and signalling pathways (Rodrigues *et al.*, 2009, Bruce *et al.*, 2009; Martin-Gronert *et al.*, 2010) of the offspring. These data suggest that exposure to obesity during development and suckling can program the offspring, though the mechanisms controlling this are still unclear.

To investigate potential mechanisms of programming a model of early life programming by obesity was required. Studies have suggested that over feeding dams post conception is enough to program changes in offspring adiposity (Khan *et al.*, 2005; Bayol *et al.*, 2008) (Tamashiro *et al.*, 2009) whilst others show obesity pre-conception is also required (White *et al.*, 2009). The model chosen for this study used pre-existing obesity before conception, to replicate the problem of increasing levels of obesity in women of child bearing age which occurs before and not just during pregnancy. C57BL/6 mice are known to be predisposed to the effects of high fat and sugar diets (Surwit *et al.*, 1988; West *et al.*, 1992; Surwit *et al.*, 1995), and therefore provide a good representation of the human condition (see section 1.1.5.4). Generating the model in mice gives the potential for genetic manipulation in future studies to dissect mechanisms.

In chapter 3 the effects of an obesogenic diet on C57BL/6 mice were described. To allow for comparison of the effects of exposure to maternal obesity or post natal obesity the same diet

and time points were used in this study. The results of chapter 3 showed female C57BL/6 mice given an obesogenic diet have increased adiposity and mild elevations of glucose and insulin during a glucose tolerance test, but no fasting hyperglycaemia or hyperinsulinemia. While females become more insulin resistant during pregnancy (Catalano *et al.*, 1991), the results of chapter 3 suggest the dams would start with comparable insulin and therefore provide a model of the effects of maternal obesity and not maternal diabetes. The second reason for using this model and experimental design was to allow for the comparison of the vascular effects of post-natal obesity, with those induced by exposure to maternal obesity (described in chapter 6).

5.1.1 Hypothesis

This chapter explores the hypothesis that exposure to maternal obesity during development and suckling adversely programmes offspring metabolism in later life.

5.1.2 Aims

To investigate this hypothesis we generated a murine model of maternal obesity and studied male offspring to answer the following questions:

- Does exposure to maternal obesity increase weight gain and adiposity?
- Does maternal obesity increase plasma concentrations of glucose, insulin, lipids and corticosterone in the offspring?
- Does exposure to maternal obesity alter the mRNA abundance of genes involved in metabolism in the liver and adipose tissue?

5.2 Methods

5.2.1 Experimental protocol

The experimental outline can be seen in Figure 5.1. Only male offspring were used, one from each litter.

5.2.2 Animal maintenance and terminal procedures

Male offspring of obese (DIOoff) or control (CONoff) dams, first time mothers, were generated as described in chapter 2 (2.3.3). All offspring were fed a standard diet (RMI 801002; Special Diet Services, Witham, UK) *ad libitum* following weaning and maintained as described (2.3.1). For subsequent experiments one offspring per litter was used. Offspring were individually housed at 3 months (n=8/group) and 6 months (n=10 CONoff, 6 DIOoff) and left to acclimatise for at least 5 days before metabolic experiments commenced.

Animals were killed ~ 10 days after diurnal blood sampling; their blood pressure was measured during this time (see chapter 6 for results). All mice were killed using CO_2 asphyxiation and tissues were collected and stored as described (0). Offspring aged 3 months were killed between 8-10am, whilst those aged 6 months were killed between 2-5pm following a 6 hour fast. Although ideally all mice would have been killed in the fasted state and at the same time of day to minimise variability in mRNA levels, the 3 month offspring were killed in the morning to allow for the analysis of vascular function in newly dissected vessels (see chapter 6).

5.2.3 Metabolic tests

Offspring aged 3 and 6 months underwent a glucose tolerance test (2.3.7) at least 5 days after individual housing. One week later diurnal blood samples were taken in line with the light cycle (2.3.8).

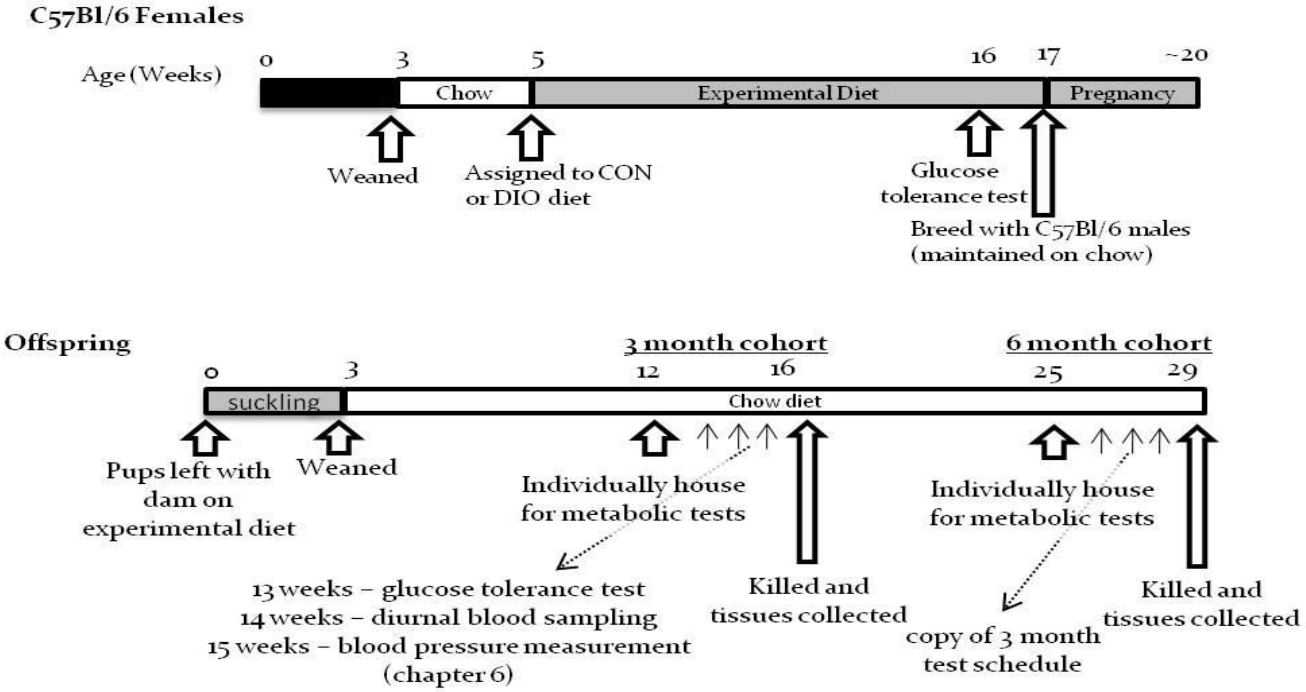


Figure 5.1 Schematic of Experimental Protocol

C57BL/6 females fed control (CON, Research diets D12328) or obese (DIO, Research diets D12331) diets were bred with C57BL/6 males (maintained on chow diet) at ~17 weeks and dams remained on experimental diet during gestation and suckling. All litters were reduced to 6 pups on postnatal day 1 and at 3 weeks age offspring of control (CONoff) and obese (DIOoff) dams were weaned and housed in single sex groups 3-5/cage,. Two cohorts of offspring were studied, one at 3 months and one at 6 months. At the ages specified mice were individually housed and underwent metabolic tests. Animals were then killed and tissues weighed and collected.

5.2.4 Quantification of plasma nutrients and hormones and hepatic triglycerides

Concentrations of glucose (2.6.4) and insulin (2.6.2) were measured in plasma samples taken during the glucose tolerance test. Non-esterified fatty acids (NEFAs) were measured (2.6.7) in the first two glucose tolerance test samples. Plasma triglycerides were measured (2.6.6) in the fasting plasma sample. Corticosterone concentrations were measured (2.6.8) in diurnal blood samples, and cholesterol (2.6.5) in the nadir sample. Liver triglyceride was extracted using a saponfication method (2.7) and quantified using a colourimetric assay as in plasma

5.2.5 Quantification of mRNA levels

RNA was isolated from liver (2.5.1.1) and subcutaneous adipose tissue (2.5.1.2), quantified (0) and cDNA synthesised using RT-PCR (2.5.1.6). The abundance of mRNAs encoding; 5α-reductase type1 (5αR), 5β-reductase (5βR), 11β-hydroxysteroid dehydrogenase (11β-HSD1), fatty acid synthase (FAS), glucocorticoid receptor (GR), hormone-sensitive lipase (HSL), lipoprotein lipase (LPL), phosphoenolpyruvate carboxykinase (PEPCK), peroxisome proliferator receptor α (PPARα) and peroxisome proliferator receptor γ (PPARγ) were determined using qPCR (2.5.1.7) and normalised to the abundance of cyclophilin.

5.2.6 Statistics

Data are presented as mean ± SEM, where n numbers represent the number of offspring used. Data was analysed by unpaired Student's t-test or repeated-measures analysis of variance (ANOVA) as appropriate.

5.3 Results

5.3.1 The effects of maternal obesity on offspring physiology

5.3.1.1 Maternal Charateristics

DIO female mice were heavier than controls and had hyperglycaemia during a glucose tolerance test (IPGTT) pre-mating, this weight difference was maintained at e18 (Table 5.1). DIO females had less pups per litter but the birth weights were comparable (Table 5.1). Over all the studies set-up by Dr V. King the plug rate for both CON and DIO females was found to be 90% and the rate of successful pregnancies was 70% (Personal communication Dr V. King).

5.3.1.2 Body and tissue weights

At weaning, 3 weeks of age, DIOoff were heavier than controls (13.1 ± 0.64 vs 8.9 ± 0.56 g, $p<0.001$ n=27CONoff, 19 DIOoff each from different litters). Repeated measures ANOVA showed maternal diet did not affect body weight over time (Figure 5.2); weights were not taken once animals were individually housed.

At 3 and 6 months liver, kidney, adrenal, subcutaneous, mesenteric, retroperitoneal and epididymal adipose weights were all comparable to controls (Table 5.22).

5.3.1.3 Glucose tolerance test

Concentrations of plasma glucose and insulin during a glucose tolerance test were comparable in DIOoff and CONoff at both 3 months (Figure 5.3a & c) and 6 months (Figure 5.3 b & d). Both parameters increased with age in line with previous literature (Bailey *et al*, 1982).

5.3.1.4 Lipid concentrations

There were no differences in plasma NEFAs on fasting or in response to a glucose bolus in either age group (Figure 5.3 e & f). At 3 months of age fasting plasma triglyceride concentrations were lower in DIOoff (3), whereas hepatic triglycerides were comparable to controls (3). There was no difference in total plasma cholesterol (3) between groups at 3 months. At 6 months of age DIOoff and CONoff had

	CON	DIO	P value
Weight at mating (g)	22.2 ± 0.4 (6)	30.2 ± 0.9 (6)	<0.001
Plasma glucose during IPGTT (area under curve -mmol/l/min)	45.8 ± 3.1 (6)	64.0 ± 4.0 (6)	0.004
Weight at e18 (g)	33.9 ± 0.4 (7)	38.3 ± 1.1 (7)	<0.001
Number of pups per litter	6.5 ± 0.5 (21 litters)	5.0 ± 0.4 (22 litters)	0.033
Offspring birth weights	1.32 ± 0.02 (7 litters)	1.28 ± 0.03 (6 litters)	0.25

Table 5.1 Maternal characteristics and offspring statistics

Female C57BL/6 mice were fed con (CON) or obesogenic diets (DIO) from 5 weeks age. Mice underwent an intraperitoneal glucose tolerance test (IPGTT) at 15 weeks pre-mating. Data are mean ± SEM analysed by Student's unpaired t-test, n numbers shown in brackets. All data in table provided by Dr V. King, University of Edinburgh.

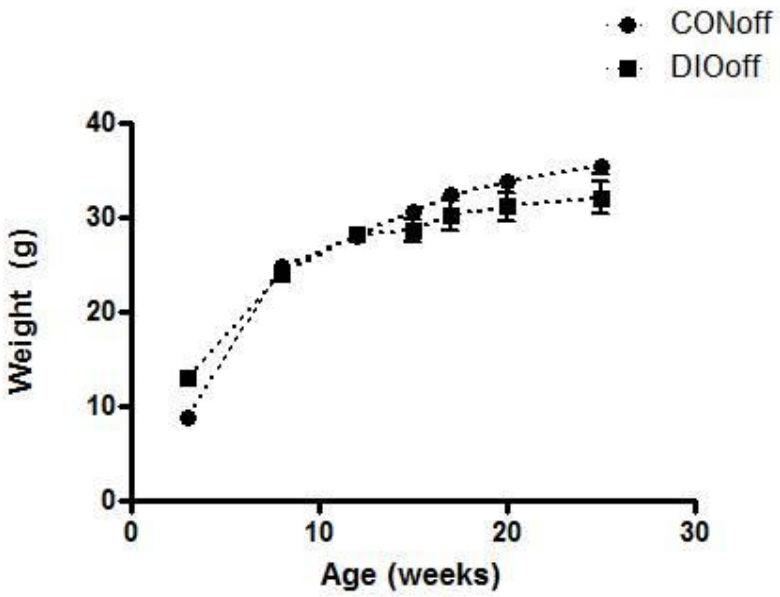


Figure 5.2 Exposure to maternal obesity causes increased weight at 3 weeks of age, but does not affect body weight after weaning

Offspring of obese (DIOoff, squares) and control (CONoff, circles) dams were weighed at regular intervals. Exposure to maternal obesity did not alter offspring body weights over the duration of the study (3-25 weeks age). Data are mean ± SEM, analysed by repeated measures ANOVA. n=27-CONoff, 19-DIOoff until 12 weeks, n=10-CONoff, 6-DIOoff until 25 weeks.

	Body, organ and adipose weights					
Tissue	**3 months**			**6 months**		
	CONoff	**DIOoff**	**p Value**	**CONoff**	**DIOoff**	**p Value**
Final body weight (g)	28.0 ± 0.5	28.4 ± 0.5	0.57	33.7 ± 0.8	31.1 ± 1.9	0.17
Liver (g)	1.43 ± 0.09	1.49 ±0.08	0.63	1.36 ±0.05	1.25 ± 0.07	0.21
Kidney (mg)	183 ± 15	203 ± 6	0.21	182 ± 8	168 ± 9	0.28
Adrenal (mg)	2.8 ± 0.5	2.6 ± 0.04	0.75	3.9 ± 0.9	3.3 ± 0.2	0.60
Subcutaneous adipose (mg)	195 ± 22	154 ± 16	0.39	298 ± 50	269 ± 60	0.72
Mesenteric adipose (mg)	163 ± 27	221 ± 19	0.08	323 ± 29	284 ± 60	0.52
Retroperitoneal adipose (mg)	51 ± 7	48 ± 6	0.74	180 ± 23	122 ± 20	0.09
Epididymal adipose (mg)	191 ± 20	159 ± 17	0.20	438 ± 39	326 ± 35	0.07

Table 5.2 Body and tissue weights from offspring of control (CONoff) and obese (DIOoff) dams

Offspring were killed aged 3 or 6 months and wet tissue and organ weights recorded. No differences in organ or adipose weights were observed. Data are mean ± SEM, n= 6-10/group. Comparisons between groups analysed using Student's unpaired t-test.

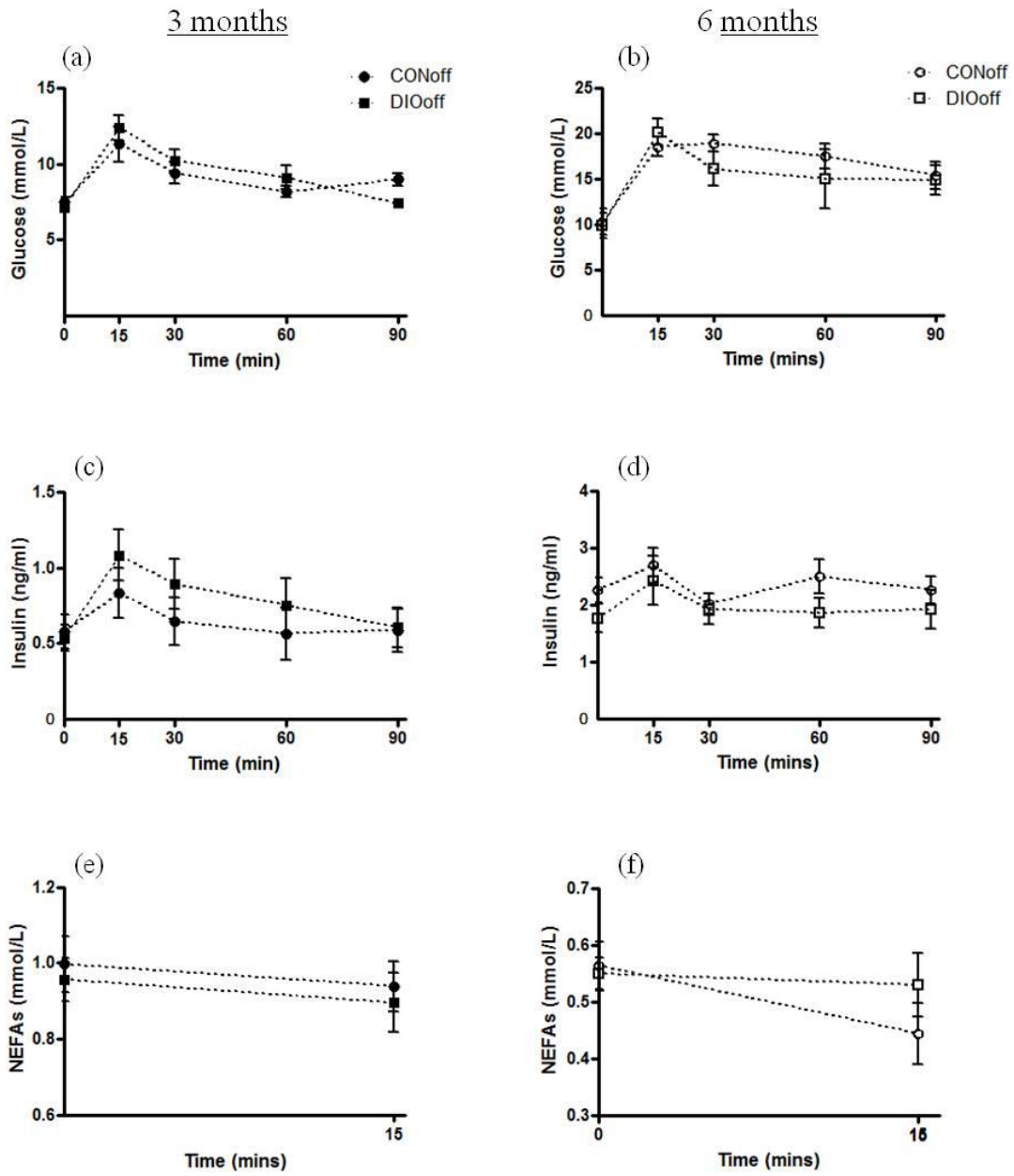


Figure 5.3 Exposure to maternal obesity does not affect glucose, insulin or free fatty acids during a glucose tolerance test

Offspring of obese (DIOoff, squares) or control (CONoff, circles) dams underwent a glucose tolerance test at 3 (filled symbols) or 6 months (open symbols) of age. Glucose (a & b), insulin (c & d) and non-esterified fatty acids (NEFAs) (d & f) were measured in plasma samples collected at times indicated. No differences were found in any of the parameters. Data are mean ± SEM, n=6-10/group, data were analysed using repeated-measures ANOVA.

	Lipid and Corticosterone levels					
	3 months			6 months		
	CONoff	**DIOoff**	**p Value**	**CONoff**	**DIOoff**	**p Value**
Fasting plasma triglyceride (mmol/L)	0.62± 0.06	0.46 ± 0.02	**0.04**	1.10 ±0.02	1.03±0.07	0.23
Total plasma cholesterol (mmol/L)	1.25 ± 0.13	1.04 ±0.07	0.19	2.22 ± 0.14	2.14 ± 0.24	0.78
Hepatic triglyceride (nmol/mg)	15.0 ± 0.20	21.0 ± 2.7	0.09	29.5 ± 1.8	32.1 ± 5.8	0.61
Nadir plasma corticosterone (nM)	26.7 ± 2.2	44.7 ± 7.0	**0.04**	35.3 ± 5.7	27.3 ± 5.0	0.35
Peak plasma corticosterone (nM)	217.4 ± 21.5	259.7 ± 14.6	0.12	222.1 ± 16.5	196.4 ± 17.0	0.31

Table 5.3 Lipid and corticosterone concentrations in offspring from obese (DIOoff) and control (CONoff) dams

Fasting plasma and hepatic triglycerides, total cholesterol and basal and peak corticosterone concentrations were analysed at 3 and 6 months. Data are mean ± SEM, n=6-10/group, values compared using Student's unpaired t test.

comparable concentrations of plasma triglycerides, cholesterol and hepatic triglycerides (3), though an increase due to age was noted.

5.3.1.5 Plasma corticosterone

The expected difference in corticosterone during the active and inactive phase was seen in all offspring. At 3 months of age DIOoff had higher concentrations of plasma corticosterone than CONoff at the diurnal nadir; however, there was no difference at the peak time point (3). At 6 months of age, the concentrations of plasma corticosterone were comparable at both the peak and nadir of the circadian rhythm (3).

5.3.2 The effects of exposure to maternal obesity on mRNA abundance

5.3.2.1 Hepatic mRNA levels

At 3 months of age DIOoff had higher transcript levels of mRNAs encoding LPL, HSL and PPARα than CONoff and comparable FAS mRNA (Figure 5.4a). These differences were not maintained at 6 months (Figure 5.4c). The mRNA levels of PEPCK was not affected by exposure to maternal obesity at either time point (Figure 5.4a & c). Note that the assays to measure abundance of HSL and FAS mRNAs were not deemed acceptable for use at the 6 month time point due to high variability between replicates both of samples and standards.

Analysis of glucocorticoid metabolising genes showed an increase in transcript levels of 5αR and 5βR in DIOoff at 3 months (Figure 5.4b). However, at 6 months there was no difference in 5αR or 5βR mRNA levels (Figure 5.4d). The abundance of 11β-HSD1 and GR mRNA was not changed by early-life exposure to obesity in either age group studied (Figure 5.4b & d).

5.3.2.2 Subcutaneous adipose mRNA levels

Maternal obesity caused a decrease in transcript levels of mRNA encoding PPARγ in subcutaneous adipose but did not alter the transcript levels of PEPCK, LPL or HSL at 3 months (Figure 5.5a). However, at 6 months DIOoff had increased mRNA levels of LPL and HSL, but mRNA abundance of PEPCK, FAS and PPARγ were comparable to controls (Figure 5.5c). The abundance of FAS could not be quantified at 3 months due to problems with the assay, specifically large variability between replicates.

The mRNA levels of 11β-HSD1 and GR were comparable in DIOoff and CONoff at 3 months age (Figure 5.5b). This remained the case for both genes at 6 months (Figure 5.5d).

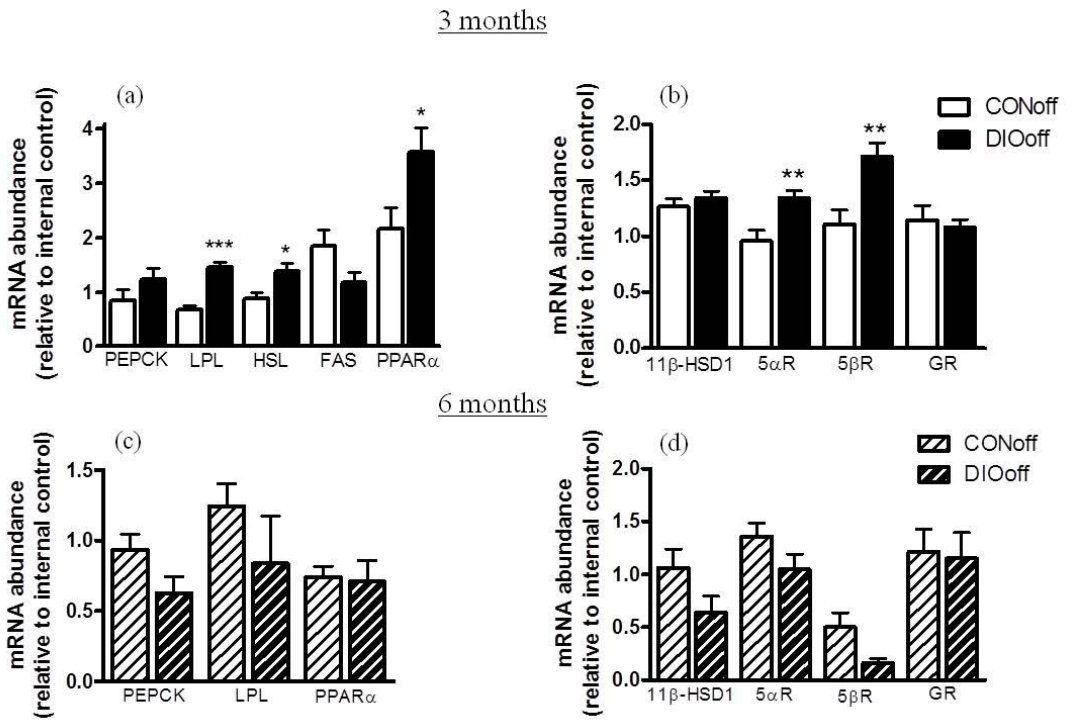


Figure 5.4 Exposure to maternal obesity causes changes in hepatic mRNA abundance at 3 months

Hepatic mRNA levels were analysed by qPCR in offspring of obese (DIOoff) and control (CONoff) dams at 3 (solid bars) and 6 (hatched bars) months. Abundance was normalised to the abundance of cyclophilin mRNA. At 3 months DIOoff had a significant increase in abundance of mRNAs encoding LPL, HSL, PPARα, 5αR and 5βR. At 6 months mRNA abundance was comparable for all genes quantified. Data are mean ± SEM, n=6-10/group, groups compared using Student's unpaired t test *p<0.05, **p<0.01, ***p<0.001.

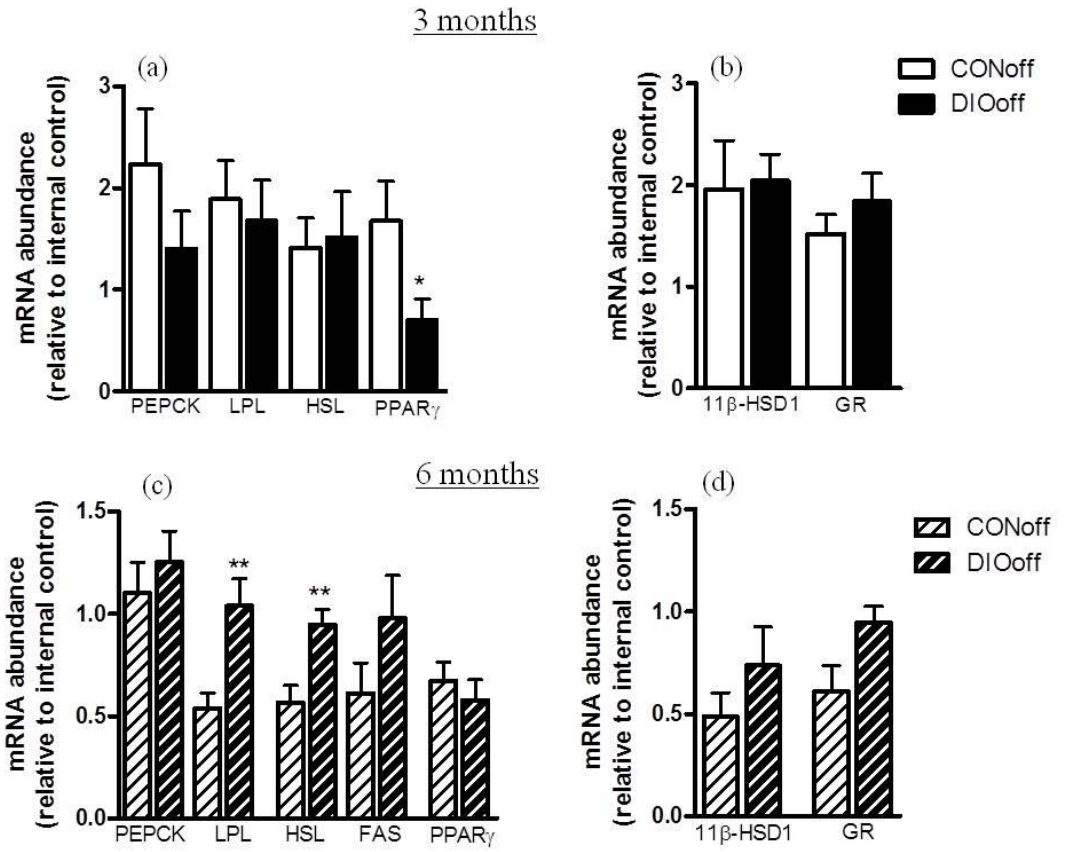


Figure 5.5 Exposure to maternal obesity causes alterations in subcutaneous adipose mRNA abundance

Subcutaneous adipose mRNA levels were analysed by qPCR in offspring of obese (DIOoff) and control (CONoff) dams at 3 (solid bars) and 6 (hatched bars) months. Abundance was normalised to the abundance of cyclophilin mRNA. At 3months DIOoff had a significant decrease in abundance of mRNA encoding PPARγ. At 6months maternal obesity caused increased transcript levels of LPL and HSL. Data are mean ± SEM, n=6-10/group, groups compared using Student's unpaired t test, *p<0.05, **p<0.01.

5.4 Discussion

The experiments in this chapter addressed the hypothesis that exposure to maternal obesity during development and suckling adversely programmes offspring metabolism in later life. The results show that this model of maternal obesity does not programme alterations in offspring body weight, adiposity or plasma concentrations of glucose, insulin or lipids but does induce changes in mRNA levels at both 3 and 6 months of age. This suggests in some circumstances an obesogenic developmental environment can be tolerated by mice, without causing long term changes in metabolism.

5.4.1 Offspring body and tissue weights

The difference in body weight developed during the first 3 weeks of life has been shown in other models of maternal obesity (Samuelsson *et al.*, 2008; Guo and Jen, 1995; Rodrigues *et al.*, 2009; Tamashiro *et al.*, 2009) though not all (Srinivasan *et al.*, 2006; Calvert *et al.*, 2009). This is likely to be due to suckling milk from an obese mother still consuming an obesogenic diet, since maternal diet during the suckling period has a large effect on milk composition, so that it contains less protein and more fat, altering the overall energy density (Rolls *et al.*, 1986). As the birth weights of both groups were comparable this suggests an increased growth rate in DIOoff in the first 3 weeks of life. Although accelerated growth has been suggested to have negative effects on insulin sensitivity in neonates that are light for gestational age (Rotteveel *et al.*, 2008; Hermann *et al.*, 2009), the same findings have not been described in 'normal' weight neonates, and the lack of difference in weight, adiposity or glucose-insulin homeostasis of the offspring later in life suggests this initial period of rapid growth may not be of great significance.

The results show maternal diet did not affect offspring body weight between 3-25 weeks, despite the difference seen at weaning. Some studies of maternal obesity suggest offspring of obese dams are heavier, divergences occurring at 12 weeks in males (Samuelsson *et al.*, 2008) and transiently from 5 weeks (Samuelsson *et al.*, 2008) or permenantly from 10 weeks in females (Srinivasan *et al.*, 2006), others report no differences (Khan *et al.*, 2005; Khan *et al.*, 2003; Han *et al.*, 2005; Bruce *et al.*, 2009; Calvert *et al.*, 2009). Increased adiposity has also been reported in offspring of obese mothers (Buckley *et al.*, 2005; Khan *et al.*, 2005; Bayol *et al.*, 2008) though body weight was not reported. Larger adipose depots suggest greater lipid storage and a propensity for obesity and its related metabolic consequences in later life, however in this study no significant differences in adipose size were found.

5.4.2 Offspring plasma hormone and lipid concentrations

Maternal obesity did not cause a difference in fasting plasma glucose or insulin concentrations, or response to an intraperitoneal glucose tolerance test in offspring, in agreement with a number of other studies (Han *et al.*, 2005; Bayol *et al.*, 2008). Those that do report increases in glucose and insulin (Tamashiro *et al.*, 2009) or insulin alone (Buckley *et al.*, 2005; Khan *et al.*, 2005; Srinivasan *et al.*, 2006), as a result of exposure to maternal obesity, also report increases in body weight and/or adiposity. The results of previous chapters (3 & 4) demonstrate changes in glucose-insulin homeostasis normally occur alongside increases in weight gain and adiposity, with smaller increases in weight correlating with less severe metabolic disturbance. This suggests that alterations in glucose and insulin concentrations in offspring of obese dams may occur as a consequence of weight gain, rather than specific programming of individual pathways.

Exposure to maternal obesity reduced plasma triglyceride concentrations but did not alter hepatic triglycerides in offspring aged 3 months. These results may suggest an altered lipid flux and deposition pattern in the offspring. Diet consumption and physical activity of offspring were not monitored in this study. It is possible that the DIOoff did not eat as much as CONoff, and/or they were more metabolically active. However, in fasting, plasma triglyceride is mainly determined by liver VLDL secretion and is not very sensitive to food intake. Short term adaptations may be the consequence of adjustment from the obese environment in early life to the standard diet post weaning. Increased hepatic lipid content in older offspring of obese mothers has been reported (Bruce *et al.*, 2009) in association with changes in the inflammatory marker CD44, hepatic mitochondria and genes involved in oxidative stress (Bruce *et al.*, 2009).

Plasma concentrations of glucose, insulin and lipids increased with age in our study, age related increases in these parameters are well established (Bailey and Flatt, 1982). Early-life exposure to obesity may worsen metabolic health by programming accelerated aging in the offspring as has been hypothesised in other models of early-life programming (Sayer *et al.*, 1997; Entringer *et al.*,2011).

5.4.3 Offspring mRNA levels

The increases in abundance of LPL, HSL and PPARα mRNAs at 3 months, if translated to protein levels, could increase fatty acid hydrolysis and increase β-oxidation of stored triglyceride. This may result in reducing hepatic lipid storage and plasma lipid concentrations which correlates with maintaining insulin sensitivity (Hansen *et al.*, 1997;

Griffin *et al.*, 1999). These changes in mRNA abundance are transient and may cause the decreased plasma triglyceride concentrations found at 3 months. In the study suggesting priming of steatohepatitis by maternal obesity (Bruce *et al.*, 2009) a microarray was used to evaluate hepatic expression of many genes. In comparison to the results shown here Bruce *et al.* report offspring of obese dams have a 2.6 fold increase in mRNA levels of FAS (Bruce *et al.*, 2009), the only gene investigated in common to this study. There was no evidence for hepatic lipid accumulation at this time point in the Bruce study. However, at 30 weeks when steatosis was present they report a 1.6 fold increase in FAS expression a reduction from the earlier time point (Bruce *et al.*, 2009). Again this supports the observation that mRNA levels are highly dynamic, can react to changes in physiology and are, therefore, unlikely to be the sole mechanism behind programming.

More temporal changes were found in mRNA levels in the subcutaneous adipose tissue. At 3 months there was an increase in PPARγ mRNA. This transcription factor increases adipogenesis and, therefore, provides storage for lipid molecules (Medina-Gomez *et al.*, 2007). It is an important drug target in humans with agonists to the receptor prescribed to help maintain insulin sensitivity (Yki-Jarvinen, 2004). While adipose weights in the DIOoff were not increased, the increase in abundance of mRNA encoding PPARγ at 3months may be an attempt to provide more metabolically 'safe' storage if the excess hepatic lipids were to be hydrolysed. This change in transcript level is again lost with age and by 6 months DIOoff have increased transcript levels of mRNA encoding LPL and HSL in subcutaneous adipose. As in the liver these lipases are responsible for hydrolysing lipids and generating free fatty acids from stored sources which may be beneficial in times of starvation. The mice were killed in a fasted state which may have caused an increase in the mRNA abundance of these genes, though why it would be greater in the DIOoff compared with CONoff is unclear. If this is maintained long term it may have adverse affects on the animals' metabolism by raising circulating free fatty acids.

It is possible other changes in mRNA levels found at 3 months age and not 6 months are due to nutritional state and timing of killing. Offspring were killed un-fasted in the morning when 3 months of age to allow for vascular function analysis (Chapter 6), but killed fasted in the afternoon when 6 months of age. Although fasting would be expected to have the same effect on both CON and DIO offspring, it is possible the response may have been programmed *in utero.*

As in the other chapters to further investigate the functional relevance of changes in mRNA protein levels and enzyme activities should be determined.

5.4.4 Plasma corticosterone and glucocorticoid metabolism in offspring

The 3 month DIOoff offspring had higher concentrations of corticosterone at the nadir but not at the peak of the circadian cycle, in agreement with other studies (Boullu-Ciocca *et al.*, 2005). The reason for this increase is unclear but could include differing stress responses, an alteration in the circadian rhythm potentially due to the transcription factor 'clock' (Kino and Chrousos, 2011a; Kino and Chrousos, 2011b), or changes in corticosteroid binding globulin (CBG) (Breuner and Orchinik, 2002). As with most of the differences seen in the 3 month offspring the difference in corticosterone concentrations was lost by 6 months, suggesting the adaptations in early life are enough to return physiology to normal by 6 months. The DIO offspring had increased levels of mRNA encoding the metabolising enzymes 5αR and 5βR which would lower hepatic glucocorticoid concentrations. This may be a compensatory mechanism to reduce the increased circulating corticosterone though it should be noted that 5α-metabolites can activate the glucocorticoid receptor (McInnes *et al.*, 2004). It may also be a mechanism to protect from the development of obesity as several animal models have shown that reducing glucocorticoid concentrations helps protect against the effects of diet-induced obesity (Morton *et al.*, 2001; Morton *et al.*, 2004). An alternative explanation for changes in these enzymes is the potential for these offspring to have been exposed to elevated glucocorticoids *in utero* as this is known to affect the HPA axis in other models of programming (Levitt *et al.*, 1996; Nyirenda *et al.*, 1998). Concentrations of glucocorticoids greatly increase in maternal blood towards the end of pregnancy (Nolten *et al.*, 1980; Montano *et al.*, 1991), but no differences were found between control and obese dams used in this study (personal communication Dr. V King). Additionally, maternal diet is known to affect the activity of the enzyme 11β-HSD type 2 (11β-HSD2) in the placenta (Bertram *et al.*, 2001, Langley-Evans *et al.*, 1996); this enzyme inactivates maternal corticosterone and alterations in its activity can affect fetal glucocorticoid exposure (Lindsay *et al.*, 1996; Holmes *et al.*, 2006). It would be possible to test the integrity of this barrier using radio-labelled tracers in future studies.

At 6 months there was no difference in GR mRNA levels in adipose tissue, although increased expression has been seen in a study of perinatal overfeeding in rats (Boullu-Ciocca *et al.*, 2005). This would be predicted to increase glucocorticoid signalling in the adipose which has been shown to have a negative impact on metabolism, specifically insulin sensitivity and hyperlipidemia (Masuzaki *et al.*, 2001). One proposed mechanism to explain long term alterations in gene expression, and increased risk of cardiovascular disease, is epigenetic modification of the genome (Handy *et al.*, 2011). Epigenetic marks are laid down

in development and persist throughout the life of an animal and include methylation of DNA, histone modification and non-coding RNAs (Handy *et al.*, 2011). The GR has been proposed as a target of epigenetic modification in models of early-life under-nutrition (Lillycrop *et al.*, 2005; Lillycrop *et al.*, 2007), altered maternal behaviour (Weaver *et al.*, 2004) and maternal stress (Szyf *et al.*, 2005). Investigating possible epigenetic modifications of the glucocorticoid receptor gene in the adipose tissue of DIOoff would be interesting. Nevertheless, this study found no major effects of maternal obesity on whole body physiology; suggesting any epigenetic changes may not be having an influence or may only cause effects with ageing.

5.4.5 Models of maternal obesity

Overall, this study shows that maternal obesity in C57BL/6 mice does not have major effects on offspring physiology at 3 or 6 months age, contrary to other published work (Samuelsson *et al.*, 2008; Bruce *et al.*, 2009; Elahi *et al.*, 2009). Many of the changes reported in other studies are reported in older offspring and do not affect all the parameters studied. Sex specific affects of maternal obesity have been reported with suggestions of more metabolic dysfunction in females (Han *et al.*, 2005; Bayol *et al.*, 2008; Samuelsson *et al.*, 2008; Elahi *et al.*, 2009), suggesting programming of alterations in sex steroids as well as, or instead of, metabolic parameters. Studying female offspring may provide a physiological phenotype as a consequence of maternal obesity; published data would also suggest aging the offspring further would bring about changes in metabolism.

Other major differences between this study and others, which may explain the variation in outcomes, is the model of maternal obesity used. Some studies have utilised genetic models of obesity (Han *et al.*, 2005; Calvert *et al.*, 2009), an obesogenic diet very briefly before pregnancy (Guo and Jen, 1995; Khan *et al.*, 2005) or just during pregnancy and suckling (Bayol *et al.*, 2007; Tamashiro *et al.*, 2009) and also postnatal over-nutrition by reducing litter size (Rodrigues *et al.*, 2009). These models will not generate the same extent of obesity in females, and some may be more likely to promote alterations in insulin signalling giving a phenotype of gestational diabetes, not maternal obesity. Not only does the type and 'length' of obesity vary but also the age of female rodents used to become mothers. Some studies begin high fat feeding at weaning (Howie *et al.*, 2009), another at 100 days age (Samuelsson *et al.*, 2008). However the weights of the dams in this study are comparable to those reported in two others (Samuelsson *et al.*, 2008; Bruce *et al.*, 2009) that effected offspring physiology. Finally the composition of obesogenic diets used in other studies is varied; some having just increased fat content as lard (Howie *et al.*, 2009) or palm oil (Ferezou-Viala *et*

al., 2007) with no change in sucrose content, whilst other studies use diets supplemented with human foods (Bayol *et al.*, 2007; Samuelsson *et al.*, 2008). The biggest problem with these diets is the choice of control, it is extremely important, particularly when looking at programming effects, to consider the content of all other micro and macronutrients in the control diet. If increased fat intake occurs at the expense of protein, the widely accepted programming effects of prenatal exposure to a low protein diet (reviewed in (Barker, 1998)) may occur. Secondly, in comparison to refined diets, cereal based chow diets (often used as controls) contain plant-derived ingredients, which may vary throughout the year (Barnard *et al.*, 2009). They also contain markedly different micronutrients and phytoestrogens not found in refined diets which affect physiology and behaviour (as reviewed in (Warden and Fisler, 2008)). These variables were minimised in this study by using two matched refined diets from the same supplier. This suggests that some of the programming effects reported in other studies may be due to differences in other micro- or macronutrient content between obesogenic and control diets.

Another important difference between the study in this thesis and others is the use of first time mothers. The study by Samuelsson *et al.* (Samuelsson *et al.*, 2008) used proven breeders which improved the success rate of pregnancy (Personal communication Professor L. Poston, King's College, London). Multiparity itself is an independent risk factor for obesity in humans (Gunderson *et al.*, 2009) and was recently shown to increase adiposity in female mice and their male offspring (Rebholz *et al.*, 2011). Therefore using proven breeders may amplify the effects of maternal obesity alone.

5.4.6 Conclusions

In summary we did not find any marked changes in offspring physiology as a result of maternal obesity. We did see some changes in mRNA abundance, though these were not consistent at 3 and 6 months. Plasticity in programmed changes may allow for a transitional period early in life to minimise the effects of the maternal physiology which are not required with advancing age and/or consistency in environment

Chapter 6

The effects of maternal and postnatal obesity on vascular function and remodelling

6.1 Introduction

Obesity is associated with hypertension (Berchtold *et al.*, 1981), atherosclerosis (McGill *et al.*, 2002) and coronary artery disease (Jousilahti *et al.*, 1999). Recent data suggest the risk of cardiovascular disease is also increased in those whose mother was obese (reviewed in (Drake and Reynolds, 2010)). The exact link between obesity and CVD is not fully understood, but evidence suggests a role for vascular dysfunction (Drexler and Hornig, 1999; Cai and Harrison, 2000; Bonetti *et al.*, 2003; Caballero, 2003). This includes alterations in vascular tone, which can increase peripheral resistance, and modified vascular remodelling following injury such as is seen in atherosclerosis. The Barker hypothesis, supported by many clinical studies, proposes a link between adverse fetal environment and risk of adult hypertension and cardiac events (Barker *et al.*, 1989; Barker *et al.*, 1990; Barker *et al.*, 1993a), however, this work is centred around maternal under-nutrition and intrauterine growth restriction rather than maternal obesity.

Many animal models have been used to explore the relationship between obesity and cardiovascular disease but the results generated are not consistent. Increased blood pressure has been shown in some rat (Kurtz *et al.*, 1989; Dobrian *et al.*, 2001) and mouse (Noronha *et al.*, 2005; Rahmouni *et al.*, 2005; Symons *et al.*, 2009) models of dietary and genetic obesity, but not in others (Mark *et al.*, 1999; Monassier *et al.*, 2006). Vascular dysfunction as a result of obesity has been shown in many relevant animal models (Bourgoin *et al.*, 2008) some suggesting it even precedes the metabolic manifestation (Kim *et al.*, 2008). However, in rodent models discrepancies exist with some suggesting only certain arteries are affected (Oltman *et al.*, 2006; Bhattacharya *et al.*, 2008), or receptor specific effects (Molnar *et al.*, 2005; Mundy *et al.*, 2007). Vascular remodelling and lesion formation have also been studied in rodent models of obesity; in Zucker fatty rats neointima formation following arterial injury was greater than in controls (Desouza *et al.*, 2006), however, in genetic mouse models of obesity lesion formation was decreased (Schafer *et al.*, 2004). Furthermore murine models of dietary obesity have reported both increased (Schafer *et al.*, 2004) and comparable (Molnar *et al.*, 2005) lesion formation following arterial injury.

Recently, with the explosion in rates of obesity, models investigating the long term effects of exposure to maternal obesity have been generated, discussed in chapter 5. Some models produce increased blood pressure in rodent offspring exposed to maternal obesity during development and suckling (Khan *et al.*, 2005; Samuelsson *et al.*, 2008; Elahi *et al.*, 2009). However, the offspring in these studies were also shown to have increased adiposity or

plasma lipid concentrations, making it difficult to distinguish between the effects of exposure to maternal obesity, versus the consequences of postnatal obesity. Rabbit (Napoli *et al.*, 2000) and mouse (Napoli *et al.*, 2002) models of maternal hypercholesterolaemia produce offspring with an increased risk of atherogenesis, suggesting programming of vascular remodelling can occur *in utero*, though no models investigating response to arterial injury have been published to date.

Obesity is associated with insulin resistance both in humans and in animals (Kahn and Flier, 2000). Insulin has a role in controlling vascular tone, and therefore blood pressure, via the synthesis and release of nitric oxide in the endothelial cells, which causes vasodilation (Muniyappa *et al.*, 2007). Endothelial dysfunction is a common occurrence in diabetic patients (Steinberg *et al.*, 1996), but whether the same mechanisms are affected in obesity is unclear. It has also been demonstrated that plasma lipid concentrations can affect vascular function (Jayakody *et al.*, 1987; Liao, 1994). Overall these studies indicate obesity may affect the vasculature directly through unknown mechanisms, as well as through changes in metabolic or endocrine state.

The experimental models of postnatal and maternal obesity presented in this thesis (chapter 3 & 5), provide an opportunity to further investigate the link between obesity and vascular function.

6.1.1 Hypothesis

This chapter explores the following hypothesis: exposure to high fat diet either (a) *in utero* and throughout suckling, or (b) in postnatal life, causes arterial dysfunction and an increase in lesion formation following arterial injury.

6.1.2 Aims

To investigate the proposal that either postnatal obesity or exposure to maternal obesity during development and suckling adversely affects the vascular system, the following questions were addressed;

- Does exposure to obesity alter systolic blood pressure?
- Can exposure to obesity cause changes in vasoconstriction and relaxation?
- Does exposure to obesity cause changes in arterial smooth muscle or endothelial cell function?
- Does exposure to obesity alter the extent of vascular remodelling following intra-luminal injury?

Mice do not spontaneously develop atherosclerosis on a high fat or high cholesterol diet without transgenic alteration such as deletion of the gene coding for apolipoprotein E (ApoE) (Zhang *et al.*, 1992; Zhang *et al.*, 1994a) or the LDL-receptor (Ishibashi *et al.*, 1993). Atherosclerosis is a physiological response to vascular injury, as is re-stenosis that can occur in humans following stent implantation, an event more common in obese subjects (Piatti *et al.*, 2003; Nikolsky *et al.*, 2005). Therefore a model of femoral artery intra-luminal injury (Sata *et al.*, 2000; Dover *et al.*, 2007) was used to assess vascular remodelling in the mice. The response to intra-luminal injury is a cellular proliferation resulting in a smooth muscle rich neointima, similar to that seen in re-stenosis.

6.2 Methods

6.2.1 Experimental Outline

Mice were exposed to either:

1. Postnatal obesity, induced by a high fat and sugar diet administered (to male and female mice) from 5 weeks of age.

2. Maternal obesity, in the form of obese mothers maintained on a high fat and sugar diet prior to pregnancy, during gestation and suckling. The effects on male offspring were studied

One cohort of animals underwent blood pressure measurements when aged 15 weeks (following the metabolic tests presented in chapters 3 and 5) and were killed the following week. Femoral arteries were removed for *ex vivo* functional analysis which was performed on the same day. For studies of post-natal obesity n= 8/group, and studies of exposure to maternal obesity n= 10 CONoff and 8DIOoff all from different litters also used for studies of metabolism reported in chapter 5. However, due to technical difficulties in successfully mounting and denuding arteries on myographs the number of animals used in each experiment is stated in the figure legend. Additional cohorts of animals (not used for metabolic studies and therefore group housed) underwent intra-luminal wire injury surgery at 15 weeks of age, were left to recover for 4 weeks and were then killed to allow collection of femoral arteries for analysis. For studies of post-natal obesity n=7/group were fed experimental diets, however 2 animals did not survive the surgical procedure meaning the number of animals available for analysis was as follows; n=6 CON males and DIO females, n=7 DIO males and CON females. All offspring of dams fed control or high fat diet survived the surgical procedure, n=8 CONoff and 6 DIOoff.

6.2.2 Animal maintenance and terminal procedures

In the study of post-natal obesity, mice were fed either control (CON, Research Diets D12328) or obesogenic (DIO, Research Diets D12331) diets from 5 weeks of age (2.3.2). The vascular impact of maternal obesity was addressed using male offspring of obese (DIOoff) or control (CONoff) dams which were first time mothers, generated as described (2.3.3), and fed a standard diet (RMI 801002; Special Diet Services, Witham, UK) from

weaning *ad libitum*. One animal from each litter was used in experiments. All animals were maintained as described (2.3.1).

For functional analysis of the femoral artery, mice were killed using CO_2 asphyxiation (2.3.4.1) between 8-10am, femoral arteries were removed and placed into PSS. To analyse response to intra-luminal wire injury mice were killed by perfusion fixation (2.3.4.2) with neutral buffered formalin 28 days post surgery. Perfusion fixation was used as it preserved a patent lumen, allowing for easier and more informative image analysis. After fixation, femoral arteries were removed and stored in formalin for a further 24 hours before transfer to 70% ethanol.

6.2.3 Blood pressure measurements

Systolic blood pressure was measured by tail cuff plethysmography on 3 consecutive days and an average of the three readings was taken (2.3.9). As mice must be restrained for this procedure it is known to be stressful and therefore the results were interpreted as such. Wherever possible, serial measurements were taken at the same time of day, to reduce extraneous sources of variability.

6.2.4 Intra-luminal wire injury surgery

To induce intra-luminal injury the femoral artery was isolated and an angioplasty guidewire (0.015" diameter) advanced into the lumen through an arteriotomy in the popliteal branch, left for 1 minute, removed, and the artery ligated just above the arteriotomy (2.3.5.4). Animals were killed 28 days post-injury by perfusion fixation (see section 6.2.2) and femoral arteries collected. This is the optimal time point for the maximal stable lesion size following intra-luminal wire injury (Sata *et al.*, 2000; Dover *et al.*, 2007) and in a similar model of mouse carotid artery ligation (Kumar and Lindner, 1997, Godin *et al.*, 2000).

6.2.5 *Ex vivo* functional analysis of femoral arteries

Vascular function was investigated by wire myography (section 2.4) using ~2mm long arterial rings taken from a point immediately proximal to the femoropopliteal bifurcation. Two rings cleaned of peri-adventitial material (which is known to alter vascular responsiveness (Lohn *et al.*, 2002; Verlohren *et al.*, 2004), were prepared from each artery; one ring was left intact, and the second denuded of endothelium by rubbing the lumen with a human hair. This allowed for the effects of obesity on endothelial cell function to be explored.

All vessels were stimulated with three successive exposures to high (125mM) potassium PSS (KPSS) to check viability and reproducibility of contraction. This was followed by generation of cumulative concentration-response curves to phenylephrine (PE; $1x10^{-9}$ to $3x10^{-5}$) and 5-hydroxytryptamine (5-HT; $1x10^{-9}$ to $3x10^{-5}$) (2.4.2). Two vasoconstrictors were chosen as they agonise different receptors; PE working primarily through α1-adrenergic receptors and 5-HT acting on 5-HT_1 and 5-HT_2-receptors, but both activate G-proteins and similar downstream signalling pathways. The sensitivity to the vasodilators acetylcholine (ACh) and sodium nitroprusside (SNP) (following pre-constriction with $3x10^{-7}$M 5-HT, to give 80% of the maximal response,) was also analysed (2.4.2). ACh is an endothelium-dependent vasodilator acting on muscarinic receptors to induce release of vasodilators including nitric oxide (Furchgott and Zawadzki, 1980) whereas SNP is an endothelium-independent vasodilator causing activation of guanylate cyclase in smooth muscle cells (Schultz and Schultz, 1977).

6.2.6 Quantification of lesion formation using optical projection tomography

Injured femoral arteries were imaged using optical projection tomography (OPT). This recently published technique (Kirkby *et al.*, 2011) scans the femoral artery in 0.9° increments and individual scans are then reconstructed to form a 3-dimensional representation of the vessel. The position of the intima/media border, the internal elastic lamina (IEL), was traced on scans at regular intervals from the remaining ligature and the position interpolated for the regions in between. The neointima and lumen were distinguished using image segmentation and the absolute size of the neointima was expressed as total volume or 2D area for each scan line. The largest area of neointima at a single scan line was used as a measure of 2D neointimal area in the section and this allowed for comparison with histological analysis (2.1.1).

6.2.7 Quantification of lesion formation using histology

Following OPT, femoral arteries were processed and embedded in paraffin wax for histology. Sequential 4µm sections were cut in a repeating pattern where 20 sections were kept (2 per slide) then 20 discarded (2.2.1.2). The first slide from each set was stained with United States Trichrome (UST) (2.2.2.2) to distinguish between the different layers of the vessel, particularly the elastic laminae which border the media and intima. The slide with the

largest neointima was identified to represent the arterial sample, and the area of the vessel layers was measured to allow for calculation of the stenotic ratio, as described (2.2.2.5).

6.2.8 Statistics

Concentration-response curves were created using non-linear regression and compared by 2-way ANOVA followed by Bonferroni's post-hoc analysis. Myography summary data were generated using a least squares fit model (2.4.3). Other comparisons in this chapter were performed using unpaired Student's t-test, with adult males and females and offspring analysed separately. Data shown are mean ± SEM.

6.3 Results

6.3.1 Systolic blood pressure

Systolic blood pressure was not altered following induction of diet-induced obesity in male or female mice, nor in male offspring following exposure to maternal obesity (Table 6.1).

6.3.2 Femoral artery function

Vessel viability was confirmed using KPSS in all groups; there were no differences in basal responses of intact or denuded vessels in mice subjected to postnatal or maternal DIO (Table 6.2, Table 6.3, Table 6.4). Lumen diameter was not measured in arteries used for myography. However, no differences were found when measurements were made in comparable cohorts of animals which underwent the intraluminal femoral artery injury surgery (Table 6.5 & 6.6). Functional data are expressed as a percentage of KPSS response to minimise variability due to unavoidable damage caused by removal of the endothelium and mounting on myographs. Arteries from all groups elicited concentration-dependent contractions to PE and 5-HT, and relaxation with SNP. Denuding the vessels of endothelium caused the expected reduction in relaxation to ACh in all groups (Furchgott and Zawadzki, 1980).

6.3.2.1 Postnatal diet-induced obesity in male mice

There was no difference in contractile response to PE in intact male femoral arteries (Figure 6.1a). When denuded of endothelium there was a small leftward shift of the concentration-response curve of DIO males (Figure 6.1b), though no difference in pD_2 was found when compared by Student's t-test (Table 6.2). DIO caused a leftward shift and higher maximum of the response curve to 5-HT in both intact and (Figure 6.1c) and denuded (Figure 6.1d) vessels. Consequently, the maximum contraction (Emax) was significantly higher in intact vessels from DIO mice compared with controls (Table 6.2).

A rightward shift of the concentration-response curve to ACh was seen in DIO mice (Figure 6.2a) when compared using analysis of variance. However no difference in $-\log IC_{50}$ was found when compared using Student's t-test (Table 6.2). DIO vessels denuded of endothelium lost less tone than CON when treated with increasing concentrations of ACh (Figure 6.2b). Despite the reduced response, no differences were found when $-\log EC_{50}$ or maximum relaxation were compared (Table 6.2). DIO caused a small rightward shift in the

		Systolic Blood pressure (mmHg)		P value
		CON	DIO	
Postnatal obesity	Males	121 ± 3	118 ± 3	0.57
	Females	119 ± 3	118 ± 3	0.85
Maternal obesity	Males	117 ± 4	121 ± 2	0.53

Table 6.1 Exposure to an obesogenic diet does not alter systolic blood pressure

Male and female mice were fed obesogenic (DIO) or control (CON) diets from 5 weeks of age. Male offspring from control (CON) and obese (DIO) mothers were fed a standard diet from weaning. Systolic blood pressure was measured at 14 weeks of age in all mice. Neither postnatal nor maternal obesity altered blood pressure. Data are mean ± SEM, postnatal obesity; n=8/group, maternal obesity; CON n=10, DIO n=6, analysed by Student's unpaired t test.

		Intact			Denuded		
		CON	DIO	P value	CON	DIO	P value
KPSS	Emax (mN/mm)	2.37 ± 0.26	2.26 ± 0.11	0.70	1.31 ± 0.26	1.58 ± 0.42	0.60
Phenylephrine	pD_2	5.90 ± 0.15	5.68 ± 0.11	0.28	6.00 ± 0.12	6.24 ± 0.16	0.26
	Emax (mN/mm)	2.00 ± 0.28	2.04 ± 0.15	0.90	1.37 ± 0.35	1.69 ± 0.49	0.61
	Emax (%KPSS)	88.4 ± 3.7	87.6 ± 7.0	0.92	96.5 ±10.3	101.6 ± 4.1	0.66
5-hydroxytryptamine	pD_2	7.04 ± 0.14	6.68 ± 0.46	0.46	6.99 ± 0.14	6.59 ± 0.49	0.45
	Emax (mN/mm)	2.14 ± 0.22	2.34 ± 0.10	0.43	1.30 ± 0.28	1.59 ± 0.38	0.56
	Emax (%KPSS)	**91.2 ± 3.3**	**104.0 ± 4.4**	**0.04**	94.0 ± 8.2	101.9 ± 4.7	0.42
Acetylcholine	-(log)IC_{50}	7.88 ± 0.17	7.59 ± 0.19	0.28	7.77 ± 0.35	8.05 ± 0.41	0.61
	Max relaxn (%)	94.7 ± 3.3	95.6 ± 2.3	0.82	60.9 ± 3.0	43.0 ± 9.4	0.10
Sodium Nitroprusside	-(log)IC_{50}	8.60 ± 0.12	8.38 ± 0.20	0.36	8.55 ± 0.19	8.30 ± 0.29	0.48
	Max relaxn (%)	99.0 ± 0.4	99.3 ± 0.3	0.54	102.5 ± 1.6	103.3 ± 1.0	0.66

Table 6.2 Myography summary data from male mice fed obese (DIO) or control (CON) diets

Male mice were fed control (CON) or obesogenic (DIO) diets from 5 weeks of age. Femoral arteries were removed at ~15 weeks of age and function assessed using wire myography. Summary data for the four drugs used are provided. Emax is expressed in force (mN/ mm) or as a percentage of response to 125mM KPSS; Maximum relaxation is expressed as a percentage of pre-constriction to $3x10^{-7}$M 5-hydroxytryptamine. Data are mean ± SEM, analysed using Student's unpaired t-test; n=6/group except for phenylephrine n=5/group.

		Intact			Denuded		
		CON	DIO	P value	CON	DIO	P value
KPSS	Emax (mN/mm)	2.50 ± 0.17	2.70 ± 0.08	0.42	1.00 ± 0.13	1.30 ± 0.41	0.51
Phenylephrine	pD_2	5.99 ± 0.14	6.10 ± 0.06	0.50	6.41 ± 0.07	6.58 ± 0.07	0.11
	Emax (mN/mm)	2.16 ± 0.30	2.20 ± 0.18	0.91	0.94 ± 0.23	1.53 ± 0.47	0.30
	Emax (%KPSS)	84.6 ± 7.3	83.0 ± 6.7	0.88	97.2 ± 0.4	106.3 ± 6.5	0.29
5-hydroxytryptamine	pD_2	6.92 ± 0.11	7.13 ± 0.05	0.12	6.99 ± 0.08	7.21 ± 0.10	0.12
	Emax (mN/mm)	2.56 ± 0.25	2.84 ± 0.12	0.35	1.10 ± 0.18	1.48 ± 0.52	0.50
	Emax (%KPSS)	101.5 ± 2.8	112.4 ± 6.6	0.16	103.7 ± 3.2	111.5 ± 6.9	0.33
Acetylcholine	$-(\log)IC_{50}$	7.75 ± 0.10	7.90 ± 0.14	0.44	7.44 ± 0.30	7.52 ± 0.26	0.84
	Max relaxn (%)	95.5 ± 2.8	93.5 ± 4.4	0.71	45.6 ± 4.3	28.2 ± 5.9	**0.04**
Sodium Nitroprusside	$-(\log)IC_{50}$	8.34 ± 0.29	8.43 ± 0.27	0.82	8.02 ± 0.02	7.87 ± 0.12	0.25
	Max relaxn (%)	98.2 ± 0.5	98.8 ± 0.6	0.49	102.2 ± 1.1	100.5 ± 1.1	0.29

Table 6.3 Myography summary data from female mice fed an obesogenic (DIO) or control (CON) diet

Female mice were fed control (CON) or obesogenic (DIO) diets from 5 weeks of age. Femoral arteries were removed at ~15 weeks of age and function assessed using wire myography. Summary data for the four drugs used are provided. Emax is expressed in force (mN/ mm) or as a percentage of response to 125mM KPSS, maximum relaxation is expressed as a percentage of preconstriction to $3x10^{-7}$M 5-hydroxytryptamine. Data are mean ± SEM, analysed using Student's unpaired t-test; n=6/group except for phenylephrine n=5/group.

		Intact			Denuded		
		CONoff	DIOoff	P value	CONoff	DIOoff	P value
KPSS	Emax (mN/mm)	1.61 ± 0.19	1.18 ± 0.2	0.21	1.12 ± 0.28	0.60 ± 0.24	0.23
Phenylephrine	pD_2	6.14 ± 0.20	5.91 ± 0.11	0.39	**6.52 ± 0.12**	**5.70 ± 0.34**	**0.02**
	Emax (mN/mm)	1.35 ± 0.20	0.82 ± 0.24	0.10	0.83 ± 0.22	0.42 ± 0.17	0.19
	Emax (%KPSS)	93.5 ± 3.6	86.6 ± 6.4	0.35	97.1 ± 4.8	103.8 ± 9.3	0.50
5-hydroxytryptamine	pD_2	7.10 ± 0.04	7.14 ± 0.09	0.95	7.14 ± 0.06	7.06 ± 0.07	0.41
	Emax (mN/mm)	1.45 ± 0.22	1.37 ± 0.24	0.82	0.83 ± 0.22	0.46 ± 0.18	0.24
	Emax (%KPSS)	**99.2 ± 3.1**	**112.5 ± 5.3**	**0.04**	97.9 ± 5.6	122.8 ± 18.0	0.16
Acetylcholine	-(log)IC_{50}	**7.56 ± 0.11**	**6.68 ± 0.19**	**<0.001**	7.59 ± 0.18	7.31 ± 0.21	0.35
	Max relaxn (%)	94.5 ± 2.2	84.3 ± 8.2	0.23	62.0 ± 4.6	54.0 ± 8.3	0.38
Sodium Nitroprusside	-(log)IC_{50}	8.52 ± 0.14	8.34 ± 0.12	0.33	8.29 ± 0.16	8.36 ± 0.17	0.79
	Max relaxn (%)	100.5 ±0.8	99.5 ± 0.4	0.33	112.3 ± 7.0	109.6 ± 8.3	0.81

Table 6.4 Myography summary data from male mice born to obese (DIOoff) or control (CONoff) dams

Femoral arteries were removed at ~15 weeks of age and function assessed using wire myography. Summary data for the four drugs used are provided. Emax is expressed in force (mN/ mm) or as a percentage of response to 125mM KPSS and maximum relaxation is expressed as a percentage of preconstriction to $3x10^{-7}$M 5-hydroxytryptamine. Data are mean ± SEM; analysed using Student's unpaired t-test, n=9/ CONoff group, n=8 DIOoff intact, n=7 DIOoff denuded.

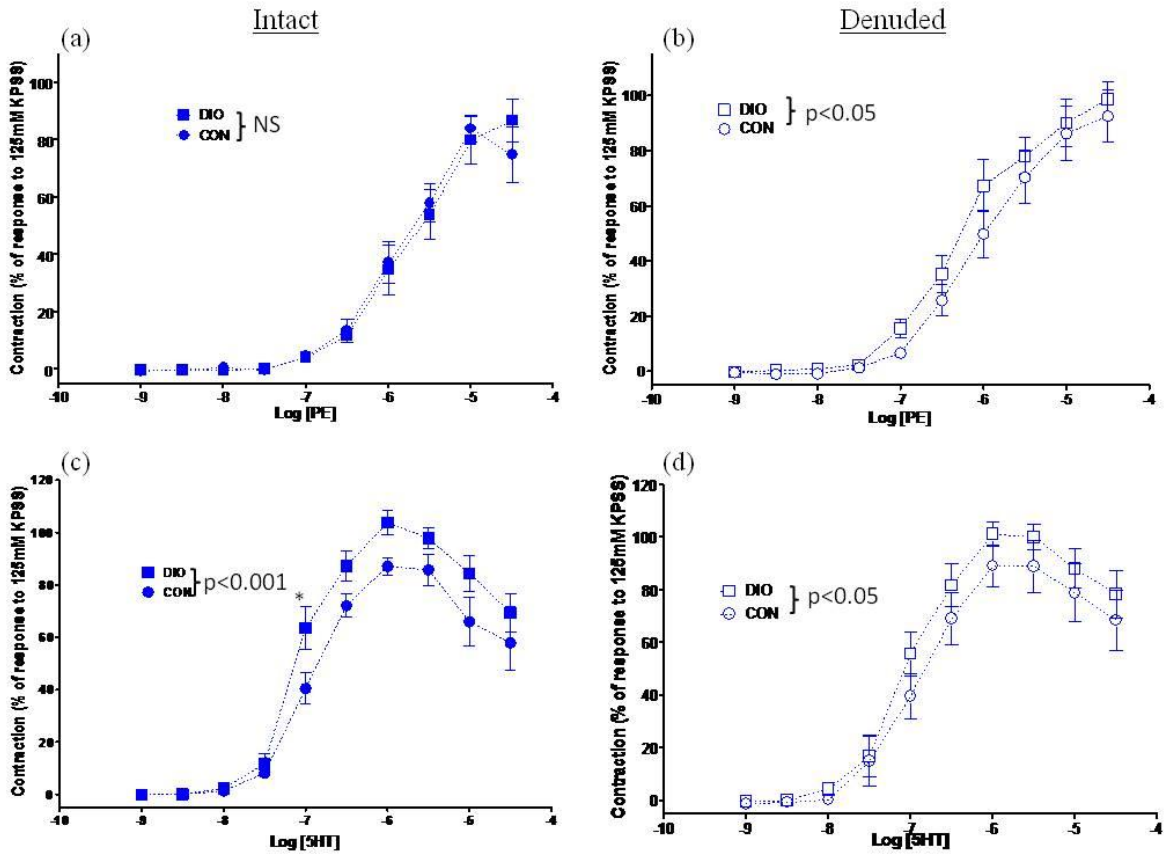


Figure 6.1 DIO caused agonist-dependent increases in femoral artery contraction in male mice.

Male mice were fed obesogenic (DIO, squares) or control (CON, circles) diet from 5 weeks of age. Femoral arteries were removed at ~15 weeks of age and responses to phenylephrine (PE) (a & b) and 5-hydroxytryptamine (5-HT) (c & d) assessed using wire myography. Two rings were created from each vessel, one left intact (closed symbols) and one denuded of endothelium (open symbols). Denuded, but not intact, DIO vessels had increased sensitivity to PE. Increased sensitivity and greater maximum contraction to 5-HT was seen in DIO vessels. Data are mean ± SEM; analysed by 2 way ANOVA, n=5/group phenylephrine, n=6/group 5-hydroxytryptamine. NS- not significant.

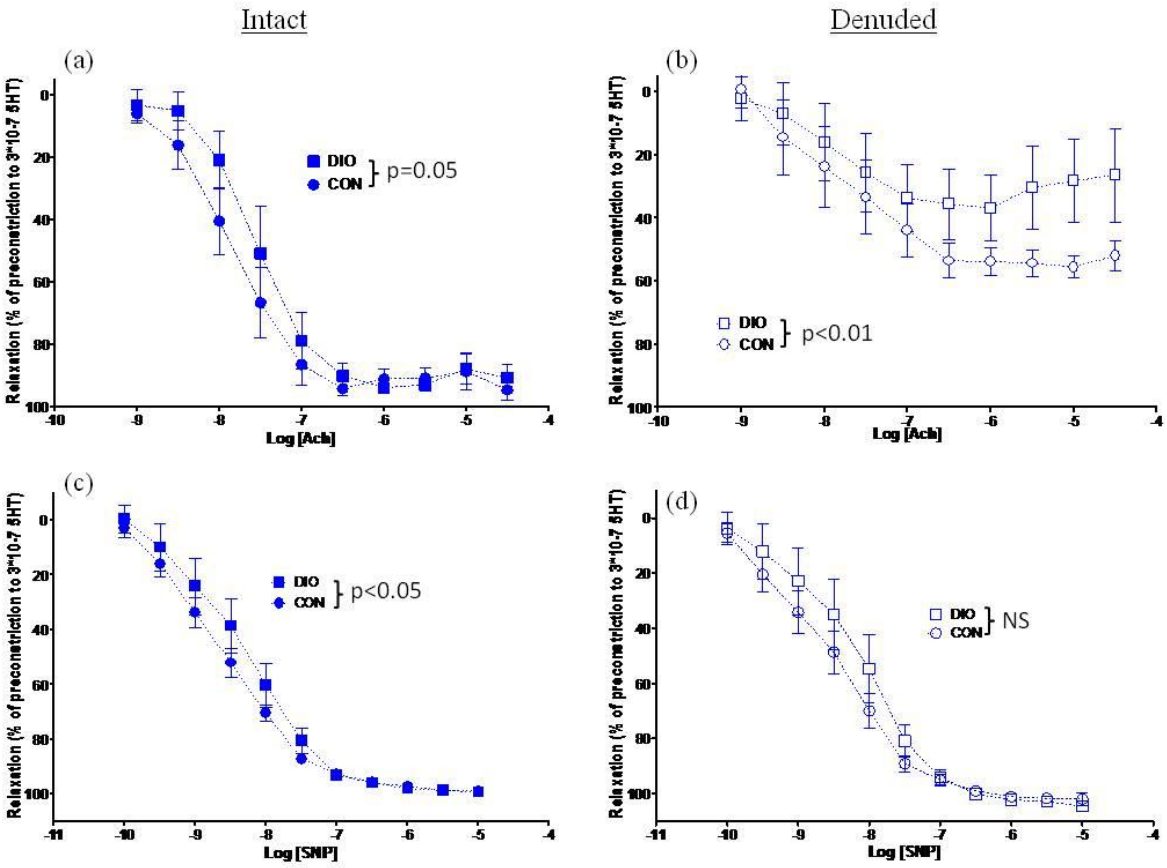


Figure 6.2 DIO reduced endothelium-dependent and -independent relaxation in femoral arteries from male mice

Male mice were fed obesogenic (DIO, squares) or control (CON, circles) diet from 5 weeks of age. Femoral arteries were removed at ~15 weeks of age and responses to acetylcholine (ACh) (a & b) and sodium nitroprusside (SNP) (c & d) assessed following a preconstriction with 5-hydroxytryptamine using wire myography. Two rings were created from each vessel, one left intact (closed symbols) and one denuded of endothelium (open symbols). DIO concentration-response curves were shifted to the right in intact vessels. Denuded CON vessels lost more tone during ACh treatment than DIO. Data are mean ± SEM; analysed by 2 way ANOVA, n=6/group. NS- not significant.

SNP concentration-response curve in intact vessels when compared with analysis of variance (Figure 6.2c). This small shift did not result in differences in the summary statistics (Table 6.2). SNP-induced relaxation was comparable in femoral arteries from male DIO and CON mice (Figure 6.2, Table 6.2).

6.3.2.2 Postnatal diet-induced obesity in female mice

In female mice the response to PE in intact vessels was not affected by DIO (Figure 6.3a & Table 6.3). When denuded of endothelium a small rightward shift of the concentration response-curve was observed (Figure 6.3b) though comparison of pD_2 values with Student's t-test did not show any significant differences (Table 6.3). DIO caused a leftward shift and higher maximum in the concentration response curves for 5-HT both in intact (Figure 6.3c) and in denuded vessels (Figure 6.3d). However, changes in pD_2 and maximum contraction did not reach statistical significance when compared using Student's t-test (Table 6.3).

DIO did not alter the vascular response to ACh or SNP in intact femoral arteries (Figure 6.4a & c, Table 6.3). However, as seen in the males, female DIO arteries did not lose as much tone with increasing concentrations of ACh when denuded of endothelium (Figure 6.4b); this was also reflected in the reduced maximum relaxation (Table 6.3). Removal of endothelium resulted in a small rightward shift in the concentration-response curve for SNP when compared by analysis of variance (Figure 6.4d). However comparison of $-\log IC_{50}$ using Student's t-test did not show any differences (Table 6.3).

6.3.2.3 Mice exposed to maternal obesity

Denuded femoral arteries from DIOoff had reduced sensitivity to PE seen both as a decrease in pD_2 (Table 6.4), and a large rightward shift of the concentration-response curve (Figure 6.5b). Post-hoc analysis revealed differences specifically at $1x10^{-6}$ M and $3x10^{-6}$ M. Intact vessels from DIOoff also had a rightward shift of the concentration-response curve to PE (Figure 6.5a) but no difference was found in pD_2 when compared using Student's t-test (Table 6.4). Maximum vasoconstriction to PE was comparable between groups in both intact and denuded vessels (Table 6.4). In response to 5-HT, concentration response-curves from DIOoff and CONoff were significantly different, when compared using analysis of variance, post-hoc analysis revealed this to be due to a greater maximum in DIOoff which was maintained at the highest concentrations of 5-HT (Figure 6.6c & d). The maximum contraction to 5-HT in was greater in DIOoff intact vessels when summary statistics were compared using Student's t-test (Table 6.4), this did not reach statistical significance in vessels denuded of endothelium (Table 6.4).

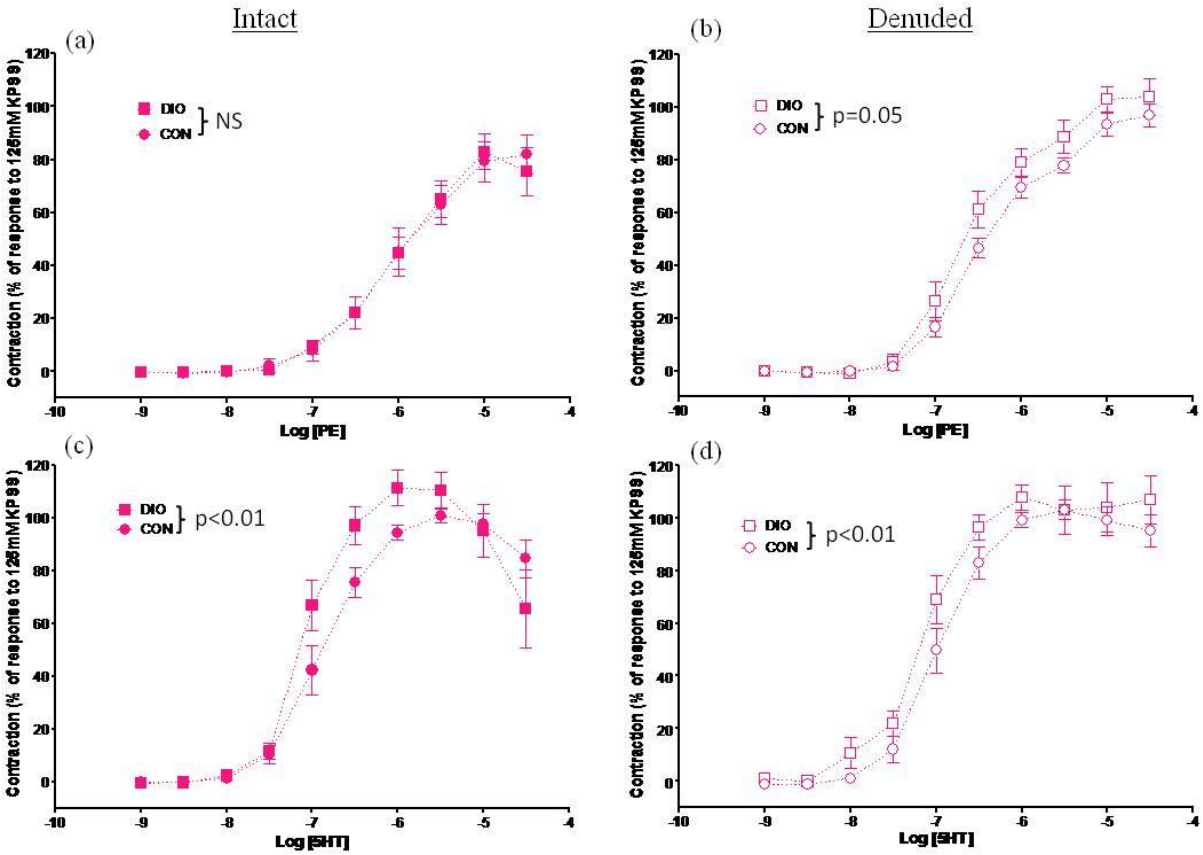


Figure 6.3 DIO caused agonist-dependent increases in femoral artery constriction in female mice

Female mice were fed obesogenic (DIO, squares) or control (CON, circles) diet from 5 weeks of age. Femoral arteries were removed at ~15 weeks of age and responses to phenylephrine (PE) (a & b) and 5-hydroxytryptamine (5-HT) (c & d) assessed using wire myography. Two rings were created from each vessel, one left intact (closed symbols) and one denuded of endothelium (open symbols). DIO concentration-response curves to PE were shifted to the left in denuded, but not intact vessels. Increased maximum contraction to 5-HT and a left shift of the concentration-response curve was seen in all DIO vessels. Data are mean ± SEM; analysed by 2 way ANOVA, n=5/group phenylephrine, n=6/group 5-hydroxytryptamine. NS- not significant.

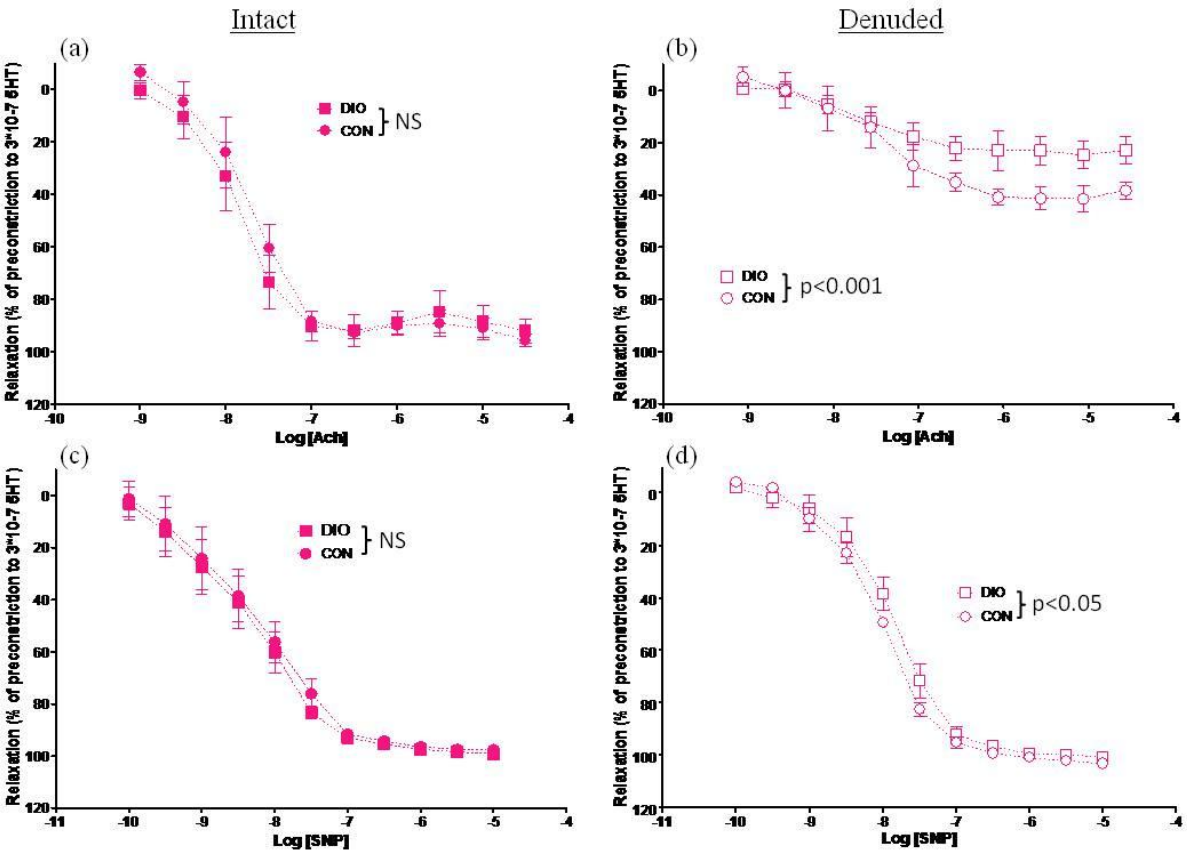


Figure 6.4 DIO causes a reduction in endothelium -independent relaxation in femoral arteries from female mice with DIO.

Female mice were fed obesogenic (DIO, squares) or control (CON, circles) diet from 5 weeks of age. Femoral arteries were removed at ~15 weeks of age and responses to acetylcholine (ACh) (a & b) and sodium nitroprusside (SNP) (c & d) assessed, following a preconstriction with 5-hydroxytryptamine, using wire myography. Two rings were created from each vessel, one left intact (closed symbols) and one denuded of endothelium (open symbols). Intact vessels had comparable sensitivity to both ACh and SNP, denuded DIO vessels lost less tone than CON during ACh treatment and had a small rightward shift of the concentration-response curve to SNP. Data are mean ± SEM; analysed by 2 way ANOVA, n=5/group. NS- not significant.

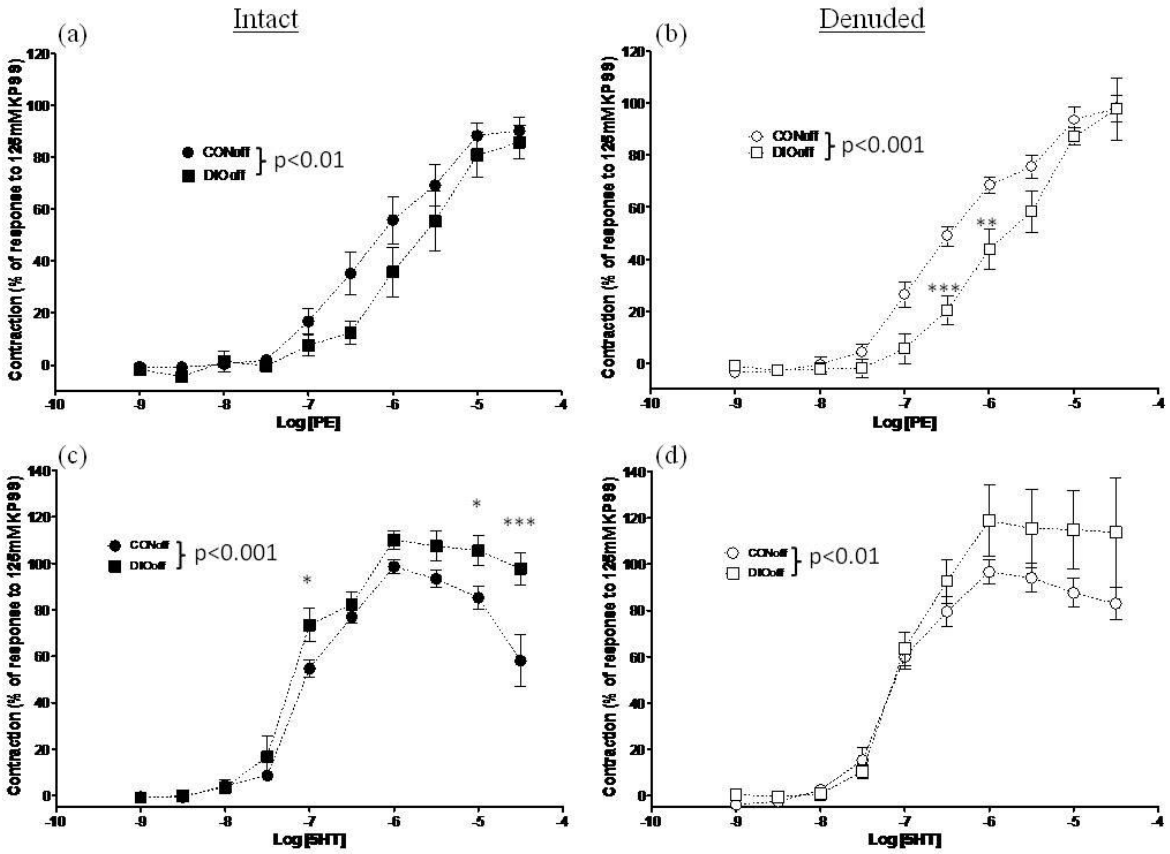


Figure 6.5 Maternal obesity causes a decrease in phenylephrine sensitivity and an increase in contraction to 5-hydroxytryptamine in offspring femoral arteries

Vascular contraction was analysed in male mice born to obese (DIOoff, squares) or control (CONoff, circles) dams. Femoral arteries were removed at ~15 weeks of age and responses to phenylephrine (PE) (a & b) and 5-hydroxytryptamine (5-HT) (c & d) assessed using wire myography. Two rings were created from each vessel, one left intact (closed symbols) and one denuded of endothelium (open symbols). DIOoff had reduced sensitivity to PE and a greater contraction at high concentrations of 5-HT. Data are mean ± SEM; analysed by 2 way ANOVA, Bonferroni's post-hoc test, n=9/ CONoff group, n=8 DIOoff intact, n=7 DIOoff denuded, *p<0.05, **p<0.01, ***p<0.001.

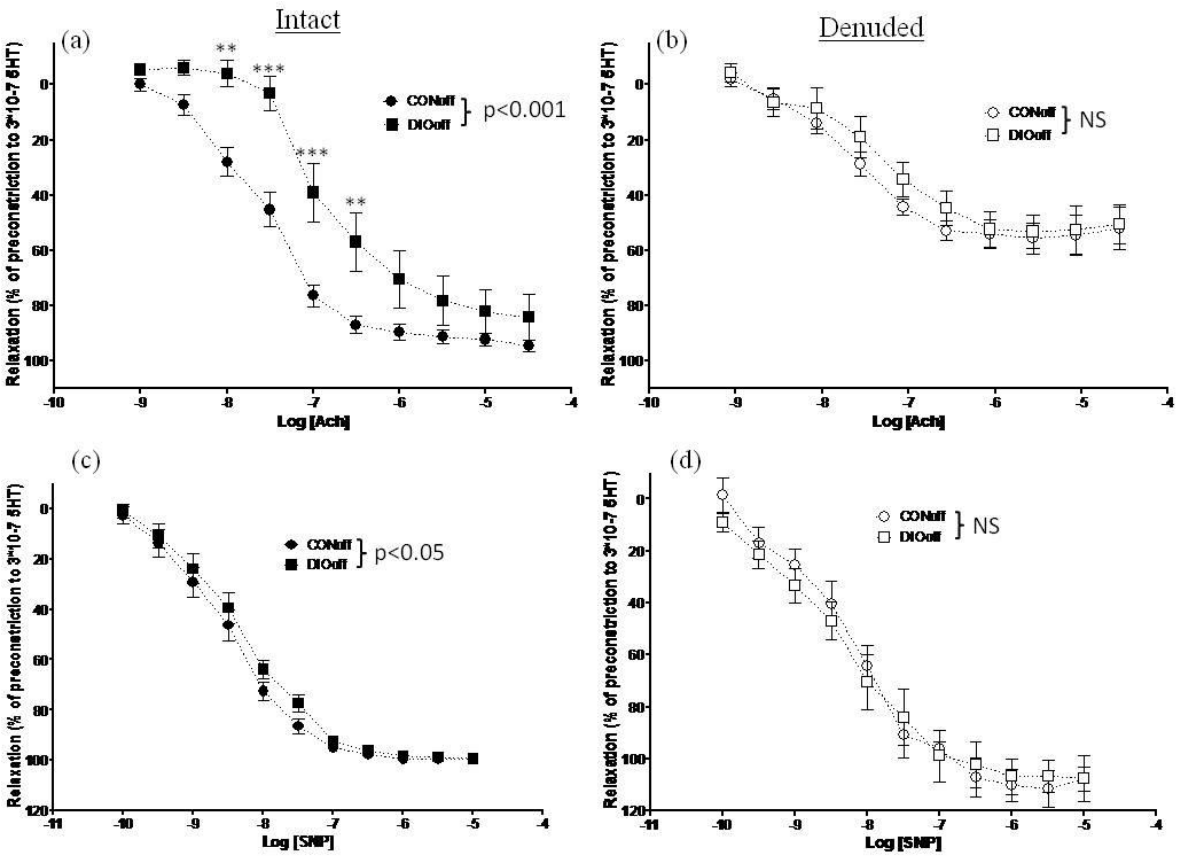


Figure 6.6 Maternal obesity causes an endothelium-dependent reduction in relaxation in femoral arteries of offspring

Vascular relaxation was analysed in male mice born to obese (DIOoff, squares) or control (CONoff, circles) dams. Femoral arteries were removed at ~15 weeks of age and relaxation response to acetylcholine (ACh) (a & b) and sodium nitroprusside (SNP) (c & d) assessed following a preconstriction with 5-hydroxytryptamine using wire myography. Two rings were created from each vessel, one left intact (closed symbols) and one denuded of endothelium (open symbols). Intact DIOoff vessels had reduced sensitivity to ACh and SNP; these differences were lost when the endothelium was removed. Data are mean ± SEM; analysed by 2 way ANOVA, Bonferroni's post-hoc test, n=9/ CONoff group, n=8 DIOoff intact, n=7 DIOoff denuded, NS- not significant, **p<0.01, ***p<0.001.

Exposure to maternal obesity caused a rightward shift of the concentration-response curve to ACh in intact femoral arteries, specifically at concentrations between $1x10^{-8}$-$3x10^{-6}$M (Figure 6.6a). This was also demonstrated by a reduction in $-\log IC_{50}$ (Table 6.4). When the endothelium was removed from vessels no difference in the response to ACh was seen (Figure 6.6b). A small right shift of the response-curve to SNP was seen in intact DIOoff femoral arteries when compared using analysis of variance (Figure 6.6c). However no difference in $-\log IC_{50}$ was found when compared using Student's t-test (Table 6.4). No change in the response to SNP was found when vessels were denuded (Figure 6.6d). Maximum relaxation to either ACh or SNP was not affected by exposure to maternal obesity (Table 6.4).

6.3.3 Vascular remodelling following intra-luminal wire injury

Analysis of femoral arteries revealed consistent lesion development, in the form of neointima, as a result of intra-luminal wire injury analysed by both OPT and histology (Figure 6.7). Due to variability of vessel size, volumetric data are presented as a percentage of volume inside the IEL and 2-dimensional data are presented as a stenotic ratio.

6.3.3.1 Mice subjected to post-natal obesity

Postnatal obesity did not affect the volume of neointimal lesion formed in response to luminal wire injury (Figure 6.8a & d, Table 6.5). The same result was found when the largest area of lesion from each artery was analysed by OPT (Figure 6.8b & e, Table 6.5) and histology (Figure 6.8c & f, Table 6.5).

6.3.3.2 Offspring of obese dams

Exposure to maternal obesity did not affect lesion volume (Figure 6.9a, Table 6.6) or area (Figure 6.9b & c, Table 6.6) in male offspring. Analysis of lesion by OPT found both the total area inside the IEL and neointimal area were not affected by exposure to maternal obesity (Table 6.6). Lumen size (Table 6.6) and stenotic ratio (Figure 6.9) were also comparable when analysed by histology.

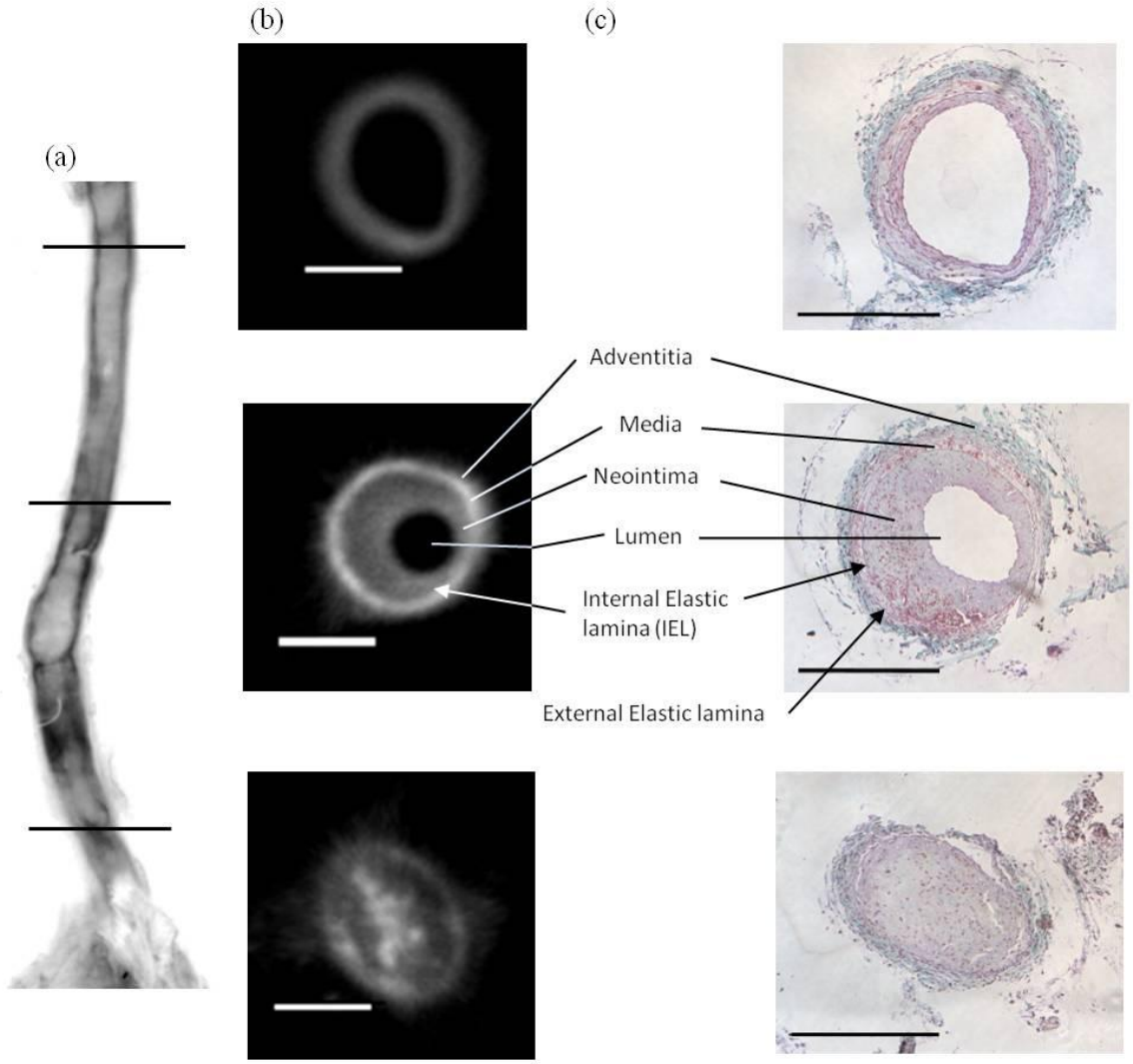


Figure 6.7 Representative optical projection tomography (OPT) scan, 2-D reconstructed cross sections and histology sections showing neointima formation following intra-luminal wire injury

Following intra-luminal wire injury femoral arteries were perfusion fixed and embedded in agarose. Neointima was visualised by OPT scanning which generated 3D images of vessels (a) by reconstructing a stack of 2D images (b). Arteries were then processed for histology and stained with United States Trichrome (c) for further analysis. Cross-sectional images (b & c) are taken from the 3 points indicated on the full artery scan (a). Scale bar 200µm.

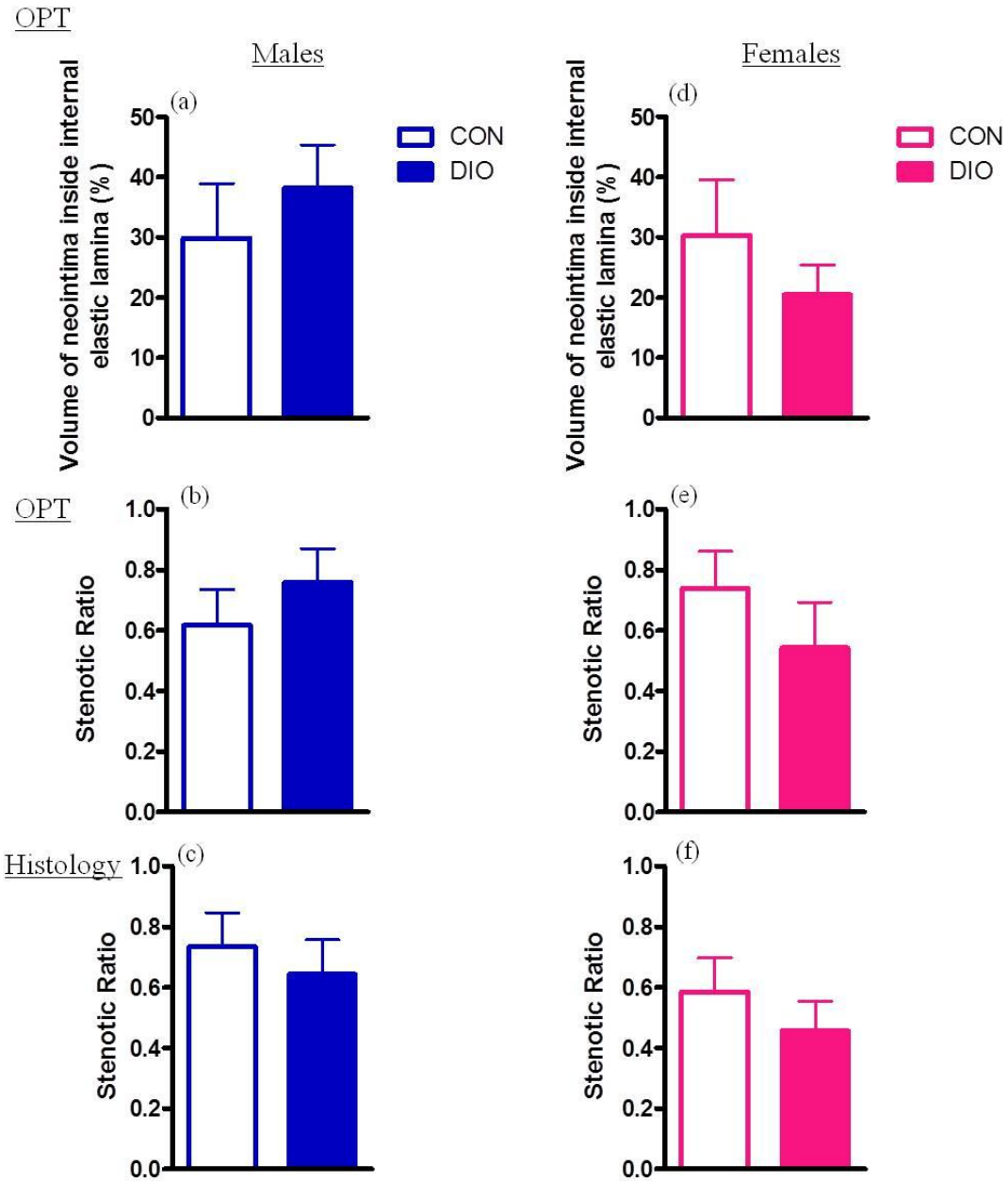


Figure 6.8 Postnatal obesity does not alter neointimal lesion formation following luminal injury

Male (blue) and female (pink) mice were fed obesogenic (DIO, closed bars) or control (CON, open bars) diet from 5 weeks of age and at 15 weeks underwent femoral artery intra-luminal wire injury. Neointimal volume was analysed using optical projection tomography and presented as a percentage of the total volume inside the internal elastic lamina (a & d) to account for variability in overall vessel size. The maximum stenotic ratio (neointima:lumen) from each artery was calculated from the 2D stack of images generated for optical projection tomography (OPT) reconstruction (b & e) and sections stained with United States Trichrome (c & f). Postnatal obesity did not alter the volume or area of neointimal lesion formed post injury in either sex. Data are mean ± SEM, analysed using students unpaired t-test; n= 6 CON males and DIO females, n=7 DIO males and CON females.

		Males			Females		
Imaging technique		CON	DIO	p value	CON	DIO	p value
OPT	Volume inside IEL ($x10^7$ μm^3)	20.4 ± 3.5	33.8 ± 7.7	0.20	20.3 ± 2.1	18.7 ± 3.6	0.70
	Neointimal volume ($x10^7$ μm^3)	5.7 ± 1.2	13.8 ± 5.1	0.18	6.2 ± 2.1	3.9 ± 1.5	0.42
	Lumen Volume ($x10^7$ μm^3)	11.3 ± 4.9	20.1 ± 3.7	0.17	14.1 ± 2.5	14.7 ± 2.6	0.87
	Area inside IEL ($x10^3$ μm^2)	59.5 ± 9.9	74.2 ± 18	0.51	46.5 ± 6.0	45.6 ± 6.9	0.92
	Neointima area ($x10^3$ μm^2)	41.7 ± 11.2	45.8 ± 4.6	0.72	31.7 ± 4.9	28.0 ± 11.2	0.75
	Lumen area ($x10^3$ μm^2)	17.8 ± 4.1	28.3 ± 15.1	0.54	14.9 ± 8.0	17.6 ± 6.2	0.80
Histology	Media area ($x10^3$ μm^2)	13.6 ± 1.6	24.8 ± 8.5	0.22	12.9 ± 3.9	13.3 ± 1.8	0.94
	Area inside IEL ($x10^3$ μm^2)	63 ± 8.9	130 ± 58	0.30	54 ± 4.5	54 ± 5.3	0.90
	Neointimal area ($x10^3$ μm^2)	44.4 ± 8.3	86.1 ± 52.0	0.48	31.9 ± 6.8	22.6 ± 3.7	0.27
	Lumen area ($x10^3$ μm^2)	18.6 ± 8.2	30.3 ± 8.3	0.42	22.5 ± 5.7	30.9 ± 6.8	0.36
	Lumen diameter (μm)	248.3 ± 12.4	267.5 ± 18.0	0.41	268.0 ±14.4	264.1 ± 10.7	0.84

Table 6.5 The effects of post-natal obesity on vascular morphology and composition following intra-luminal injury

Male and female mice were fed obesogenic (DIO) or control (CON) diet from 5 weeks of age and at 15 weeks underwent intra-luminal wire injury of the femoral artery. Volumes inside the internal elastic lamina (IEL) and of neointima formed in a defined region were determined using optical projection tomography (OPT). The area inside the IEL and neointima were determined from the 2D stack of OPT images and using histology at the point where the neointima was greatest. Lumen diameter was measured distal from the point of wire insertion where the vessel morphology had returned to normal. Data are mean ± SEM, analysed using Student's unpaired t-test; n= 6 CON males and DIO females, n=7 DIO males and CON females.

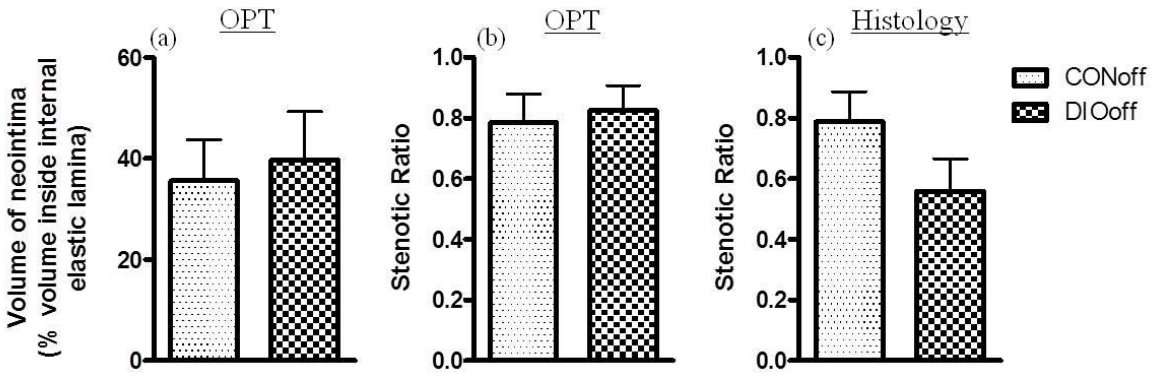


Figure 6.9 Exposure to maternal obesity does not alter neointimal lesion formation in the offspring

Male mice born to obese (DIOoff, checked bars) or control (CONoff, dotted bars) dams underwent femoral artery injury at 15 weeks of age and neointimal hyperplasia was analysed. Neointimal volume was analysed using optical projection tomography and presented as a percentage of the total volume inside the internal elastic lamina (a) to account for variability in overall vessel size. The maximum stenotic ratio (neointima:lumen) from each artery was calculated from the 2D stack of images generated for optical projection tomography (OPT) reconstruction (b) and from sections stained with United States Trichrome (c). Exposure to maternal obesity did not alter the volume or maximum area of neointima formed following vascular injury. Data are mean ± SEM, analysed using Student's unpaired t-test; n= 8 CONoff, n=6 DIOoff.

Imaging technique		CONoff	DIOoff	p value
OPT	Volume inside IEL ($x10^7$ μm^3)	14.2 ± 2.0	10.1 ± 1.7	0.19
	Neointimal volume ($x10^7$ μm^3)	5.2 ± 1.4	3.3 ± 0.6	0.30
	Lumen Volume ($x10^7$ μm^3)	9.5 ± 2.4	6.8 ± 2.0	0.46
	Area inside IEL ($x10^3$ μm^2)	74.3 ±10.4	47.6 ± 4.6	0.07
	Neointima area ($x10^3$ μm^2)	42.7 ± 8.0	34.4 ± 3.6	0.44
	Lumen area ($x10^3$ μm^2)	16.7 ± 7.7	13.2 ± 3.6	0.74
Histology	Media area ($x10^3$ μm^2)	10.6 ± 2.3	11.5 ± 2.4	0.80
	Area inside IEL ($x10^3$ μm^2)	54.6 ± 8.9	59.3 ± 6.4	0.69
	Neointimal area ($x10^3$ μm^2)	38.0 ± 11.9	27.1 ± 5.9	0.47
	Lumen area ($x10^3$ μm^2)	9.1 ± 4.7	22.9 ± 6.2	0.09
	Lumen diameter (μm)	245.3 ±13.3	281.8 ± 24.4	0.18

Table 6.6 The effects of obesity in early life on vascular morphology and composition following luminal injury

Vascular remodelling following luminal wire injury was analysed in male mice born to obese (DIOoff) or control (CONoff) dams. Volumes inside the internal elastic lamina (IEL) and of neointima formed in a defined region were determined using optical projection tomography (OPT). The area inside the IEL and neointima were also determined from the 2D stack of OPT images and using histology at the point where the neointima was greatest. Lumen diameter was measured distal from the point of wire insertion where the vessel morphology had returned to normal. Data are mean ± SEM, analysed using Student's unpaired t-test; n= 8 CONoff, n=6 DIOoff.

6.4 Discussion

The experiments described in this chapter addressed the hypothesis that mice exposed to obesity develop hypertension, alterations in vascular function and increased remodelling following intra-luminal wire injury. Two experimental paradigms of obesity were used: exposure to maternal obesity during development and suckling; or, diet-induced obesity during postnatal life. The results of this chapter must be interpreted with reference to the metabolic changes seen in the two models as presented in chapters 3 and 5. Briefly, postnatal DIO caused adiposity in both male and female mice, though the associated insulin resistance and dyslipidemia were more severe in males. In contrast maternal obesity did not have any overt effect on adiposity or metabolism. The results in this chapter show neither exposure to maternal, or post natal obesity, are associated with alterations in systolic blood pressure or lesion formation following intra-luminal wire injury. However, vascular function is affected by exposure to obesity, with exposure during development and suckling having a much more profound effect. These results imply exposure to postnatal or maternal obesity affect different vascular mechanisms. They also suggest maternal obesity can programme altered vascular function in the offspring, in the absence of overt alterations in metabolism described in chapter 5.

6.4.1 Postnatal obesity and hypertension

Rodent models of obesity have not consistently reported increased blood pressure and our study showed results comparable to Monassier *et al.* who found no difference in systolic blood pressure in diet-induced obese male and female mice (Monassier *et al.*, 2006). One potential reason for the discrepancy in studies is the degree of obesity induced by different dietary manipulations, since fatter animals may have higher blood pressures. Indeed, Williams *et al.* fed C57BL/6 male mice a high fat diet for 15 weeks, 5 weeks longer than this study, and reported a moderate (8.6mmHg) increase in blood pressure (Williams *et al.*, 2003). However studies by Symons *et al.* (Symons *et al.*, 2009) and Rahmouni *et al.* (Rahmouni *et al.*, 2005) reporting increased blood pressure in association with dietary-induced obesity showed equivalent levels of obesity, developed over the same time period, to those reported in our study. An alternative explanation for the difference in effects on blood pressure could be differences in the diets used. Notably Symons (Symons *et al.*, 2009) and Rahmouni (Rahmouni *et al.*, 2005) reported using ‘chow’ diet as a control which was not fully matched to the high fat diet for other nutrients such as salt. Differing salt concentrations

could affect blood pressure, particularly in salt sensitive individuals (Muntzel and Drueke, 1992) although C57BL/6 mice have not been shown to be salt sensitive unless mutant.

Other studies reporting hypertension in association with dietary induced obesity have used male mice (Rahmouni *et al.*, 2005; Symons *et al.*, 2009), which, given findings reported in chapter 3, are likely to have larger metabolic disturbances than females. Another important sex difference is the beneficial effects of estrogen on the vascular system (reviewed in (White, 2002)), this implies female mice may need an even greater degree of obesity/metabolic dysfunction, than males, before changes in blood pressure would be observed.

It has been proposed that leptin is a key mediator in the development of hypertension in obese mice. In leptin deficient *ob/ob* mice blood pressure is lower than in lean controls, despite significant increases in adiposity (Mark *et al.*, 1999). Leptin acts in the hypothalamus to increase renal sympathetic activity and blood pressure (Marsh *et al.*, 2003). In situations of leptin resistance, as is proposed to occur in obese subjects, some of the functions of this adipokine are lost (Van Heek *et al.*, 1997; Rahmouni *et al.*, 2005) but the renal response to leptin is preserved (Rahmouni *et al.*, 2005). Therefore it is unlikely that leptin resistance plays a role in the development of hypertension in dietary obesity. Recently a study has suggested inflammatory mediators are the neural link between obesity and high blood pressure (Purkayastha *et al.*, 2011). Purkayastha *et al.* propose NFκB and TNFα, which are elevated in obesity (Hotamisligil *et al.*, 1993; Zhang *et al.*, 2008), to be regulators of neural hypertension in mice (Purkayastha *et al.*, 2011). If this mechanism has not been activated it may explain the lack of changes in blood pressure.

6.4.2 Maternal obesity and hypertension

While no effect of maternal obesity on offspring blood pressure was reported here, a number of other studies have reported hypertension in offspring exposed to maternal obesity, in association with increased body weight or adiposity (Khan *et al.*, 2003; Samuelsson *et al.*, 2008; Elahi *et al.*, 2009). In contrast, no changes in glucose or insulin metabolism were found in the model used in this thesis (chapter 5). This, combined with the lack of increased blood pressure, may suggest that hypertension in offspring of obese dams is secondary to metabolic changes and results from an altered endocrine environment, rather than direct programming of hypertension as has been proposed to occur following maternal protein restriction (Langley-Evans *et al.*, 1994; Langley-Evans *et al.*, 1996). Sex differences in blood pressure have been found both in obese rodents (Monassier *et al.*, 2006) and in offspring

exposed to maternal obesity (Khan *et al.*, 2003). Khan *et al.* reported that hypertension occurred in female offspring of obese dams but not in males (Khan *et al.*, 2003), females were not studied in this chapter but the results are consistent in male offspring.

Blood pressure measurements in this study were determined using tail cuff plethysmography, a method demonstrated to give reproducible results comparable to those measured intra-arterially (Krege *et al.*, 1995). However, the technique can only measure systolic blood pressure and also is stressful as it involves restraint. These limitations of the technique may mask differences in diastolic, and resting or un-stressed, blood pressure. The use of radiotelemetery to measure arterial blood pressure has improved the accuracy of continuous readings in conscious free moving animals. Studies of rodent blood pressure using radiotelemetery have reported group differences in response to changing environmental temperature (Williams *et al.*, 2003) and stress (O'Regan *et al.*, 2008), which may be missed using a plethysmography method. Using radiotelemetery in the models used in this chapter would determine if the results found with the tail cuff procedure are also applicable in other situations (e.g. non-stressed). This may be particularly important in mice exposed to maternal obesity as there is potential that blood pressure could fluctuate between stressed and unstressed situations, as reported in offspring exposed to glucocorticoids *in utero* (O'Regan *et al.*, 2008). Comparing blood pressure results with those previously reported in the literature is difficult as the origins of the strain and environment in the housing facility may impact on rodent blood pressure. The consistency of our results suggests a systolic pressure of 120mmHg is normal for the colony of C57BL/6 mice used for the studies in this chapter.

Heart rate, cardiac output, and renal function all have roles in the regulation of blood pressure. These parameters were not measured in this study; however it has been shown that heart rate is unchanged in obese mice (Williams *et al.*, 2003). Peripheral resistance and vascular tone can also alter arterial blood pressure. Although peripheral resistance was not calculated, measures of vascular function can indicate if differences in vascular resistance are likely.

6.4.3 Femoral artery function following postnatal obesity

Mice subjected to post-natal obesity had small changes in their vascular responses to PE, 5-HT, ACh and SNP. The significant differences found when analysed using analysis of variance resulted from small but consistent shifts of concentration-response curves to the left or right of controls. However, when Student's t-test was used to compare single data points

such as pD_2 or maximum contraction, no changes were found. However, the differences found in the concentration-response curves are small and possibly unlikely to be physiologically relevant. Periadvential adipose tissue has been shown to release relaxation factors (Lohn *et al.*, 2002), promote endothelial dysfunction (Ketonen *et al.*, 2010) and regulate arterial tone of mesenteric arteries (Verlohren *et al.*, 2004). The adipose was removed from vessels before function was analysed, to determine changes in the vessels alone. If the function of the same arteries was analysed *in vivo* greater differences may be found due to the effects of the periadventital adipose. In addition, the small alterations in function described may become more problematic if the system was further stressed with increasing levels of obesity, by infection or by vascular damage.

Other groups have demonstrated anatomical differences in vascular dysfunction following obesity or diabetes (Crauwels *et al.*, 2000; Bhattacharya *et al.*, 2008; Oltman *et al.*, 2006); this may account for some of the inconsistencies seen when comparing studies. The results of this chapter are specific to the femoral artery and should not be presumed to be the same in all vessels of the animal. The femoral artery was studied in this chapter in order to determine any functional changes which may impact on lesion formation following intra-luminal injury which was subsequently investigated.

A study by Molnar *et al.* using a similar experimental protocol to that in this chapter, reported differences in femoral artery vasoconstriction response to phenylephrine and in relaxation following treatment with acetylcholine and sodium nitroprusside (Molnar *et al.*, 2005). The differences in results to those reported in this chapter may reflect small differences in study design, for example, while the degree of obesity was similar, the dietary composition and constituents and the timing at which the diet was introduced (and therefore age of animals) differed. Ageing is a risk factor in the development of atherosclerosis and vascular dysfunction (Spagnoli *et al.*, 1991; Lundberg and Crow, 1999). By using older animals Molnar *et al.* may have potentiated the small changes in vascular responses reported here.

The most consistent change seen in post-natal DIO in both sexes was increased contraction to 5-HT, both in intact and endothelium denuded vessels. This suggests an alteration in receptor sensitivity or numbers on the smooth muscle cells in the vessel media. The loss of relaxation at high concentrations of 5-HT in DIO mice may reflect a reduction of the endothelium-dependent vasodilatory response to this compound (Cocks and Angus, 1983; Toda and Okamura, 1990). Physiologically greater sensitivity to endogenous vasoconstrictors may lead to increased peripheral resistance with time and, consequently, to

raised blood pressure. No major differences in response to the other vasoconstrictor, PE, were found, which suggests the change is specific to the 5-HT receptor and less likely to be a difference in downstream signalling which is common to both agonists.

Most reports implicate insulin resistance as a key mediator in endothelial dysfunction in obese humans (Caballero *et al.*, 1999; Steinberg *et al.*, 1996; Rueda-Clausen *et al.*, 2011) and in some animal models (Molnar *et al.*, 2005; Erdei *et al.*, 2006; Traupe *et al.*, 2002). The results presented in chapter 3 demonstrate female DIO mice were not insulin resistant, which might explain the lack of change in ACh-mediated relaxation. However, DIO male animals had fasting hyperinsulinemia (chapter 3), but still a comparable ACh-mediated relaxation to controls. This result in males is consistent with two other groups who found no effect of obesity on thoracic (Noronha *et al.*, 2005) or femoral (Bhattacharya *et al.*, 2008) artery ACh-mediated relaxation. Bhattacharya *et al.* have reported increased sensitivity of femoral arteries to ACh only when males are given a very high fat diet (Bhattacharya *et al.*, 2008) which suggests specific diets may account for some of the differences between results. It is possible other vascular factors released in response to ACh, such as hydrogen peroxide (Matoba *et al.*, 2000), prostacyclin (Holzmann *et al.*, 1980), potassium (Savage *et al.*, 2003), or other unknown endothelium-derived hyperpolarising factors (EDHF) (Brandes *et al.*, 2000), can compensate for a lack of vasodilation by nitric oxide. Brandes *et al.* demonstrated a vasorelaxation response to acetylcholine is still elicited in mice with eNOS genetically deleted (Brandes *et al.*, 2000). They went on to find inhibition of prostacyclin did not reduce the vasodilation (Brandes *et al.*, 2000), demonstrating other EDHFs have a role to play in murine resistance artery dilation. Increased EDHFs were reported as an explanation for the lack of difference in ACh mediated vasodilation of thoracic arteries of obese, compared with control, mice (Crauwels *et al.*, 2000; Noronha *et al.*, 2005). Consistent with this explanation, some groups have proposed that ACh-mediated relaxation of femoral arteries is mediated more by endothelium-derived hyperpolarisation, which is unaffected by obesity, than nitric oxide in the mouse (Brandes *et al.*, 2000) and rat (Savage *et al.*, 2003). However, other groups investigating femoral artery relaxation in rat suggest nitric oxide is the primary or only mediator (Zygmunt *et al.*, 1995; Wigg *et al.*, 2001).

Endothelium denudation of vessels reduced response to ACh; however, vessels from obese animals maintained vascular tone to a greater degree than controls. This could be due to a greater loss of endothelial cells in the DIO group; if vessels were narrower the denuding method could conceivably have been more effective in this group. Inhibiting nitric oxide synthase before addition of ACh would determine if the difference in tone was due to

residual endothelial cells, following the denudation process, which could generate nitric oxide and cause relaxation in the CON group. Alternatively the difference may be due to a vasoconstriction response induced by ACh which has been reported in porcine (Kawamura *et al.*, 1989) and bovine (Brunner *et al.*, 1991) coronary arteries at high concentrations of agonist. By removing the endothelial cells, ACh will act on muscarinic receptors in the smooth muscle cells, if a greater response or number of receptors was present in the DIO mice it could account for the maintenance of vascular tone. Interestingly, the vasoconstriction response to ACh is augmented in intact vessels from humans with atherosclerosis and may form part of the pathogenesis of the disease (Ludmer *et al.*, 1986).

6.4.4 Maternal obesity and femoral artery function in the offspring

Despite lack of overt metabolic changes in offspring of obese dams (data presented in chapter 5), profound changes in femoral artery function were found. The decreased sensitivity to PE was modulated in the presence of endothelial cells suggesting altered signalling predominantly in the smooth muscle cells. As maximum contraction was similar to controls it is unlikely to be due to decreased receptor numbers, but a decreased affinity or alterations in downstream signalling. If maintained this reduced sensitivity to PE could act as a compensatory mechanism to stop increases in peripheral tone. Increased sensitivity to noradrenaline, also an adrenergic agonist, has been reported in other models of programming by maternal exposure to glucocorticoid (O'Regan *et al.*, 2008), or diabetes and obesity (Koukkou *et al.*, 1998). Two other studies have looked at the vascular effects of maternal obesity in mice, one found no difference in aortic response to PE (Chechi *et al.*, 2009), the other, reported increased sensitivity of mesenteric arteries to noradrenaline (Samuelsson *et al.*, 2008). As discussed, the type of artery used to assess function could explain the different results. While the study by Samuelsson *et al.* also showed increased arterial contraction, the offspring of obese dams were reported to have increased adiposity and plasma concentrations of triglycerides, insulin and leptin (Samuelsson *et al.*, 2008) making it difficult to determine to what extent the altered vascular contraction reflects programming effects and/ or the effect of altered metabolism.

Exposure to maternal obesity also altered the response to 5-HT in offspring femoral arteries, giving a greater maximum contraction rather than altered EC_{50}. The maximum contraction is greater than that elicited by KPSS, which is mediated by an influx of Ca^{2+}; this suggests an increase in sensitivity to Ca^{2+} in response to 5-HT rather than a greater contractile ability in DIOoff. The maximum contraction was only statistically greater in intact vessels suggesting an effect of the endothelial cells. The difference in contraction was in fact emphasised by a

loss of tone in CONoff at high concentrations of 5-HT. It has been shown that 5-HT can release vasodilator substances from endothelial cells (Cocks and Angus, 1983), this mechanism may be impaired in DIOoff accounting for the difference in maximum contraction. No other published studies have looked at the vascular response to 5-HT following maternal obesity, though the results reported here are suggestive of endothelial dysfunction which was supported by decreased response to ACh.

As well as decreased sensitivity to ACh, a reduction in maximum relaxation was also seen, though this was variable in DIOoff and did not reach statistical significance. Maternal obesity did not affect the offspring response to sodium nitroprusside, which causes relaxation by activation of guanylate cyclase. This indicates one downstream signalling pathway of nitric oxide in the smooth muscle cells is not affected by maternal obesity, and further suggests a dysfunction of the endothelial cells. The alteration in response to ACh could be due to reduced numbers of muscarinic receptors or reduced affinity for the ligand. Reduced sensitivity to ACh could also be mediated by nitric oxide, for example as a result of decreased production or increased scavenging in endothelial cells. As has already been discussed in this chapter, ACh causes release of other EDHFs (Brandes *et al.*, 2000) and the lack of release or action of these could also cause the reduction in relaxation. On the other hand increased released of endothelial derived contracting factors (EDCFs) (Luscher *et al.*, 1992) could cause the reduced response to acetylcholine.

Other studies of offspring of obese dams have found reduced relaxation response to ACh in rats (Ghosh *et al.*, 2001; Khan *et al.*, 2004) and mice (Samuelsson *et al.*, 2008). Interestingly the rat studies, somewhat comparable to the results of this chapter, reported only small changes in plasma triglycerides (Ghosh *et al.*, 2001) or no overt changes in metabolism (Khan *et al.*, 2004) accompanying the endothelial dysfunction. However, as discussed above, the study in mice resulted in many changes in metabolism (Samuelsson *et al.*, 2008) making the mechanism of endothelial dysfunction harder to pinpoint. In support of a programming effect distinct from altered metabolism; Koukkou *et al.* found reduced response to ACh in mesenteric arteries of rat offspring of obese dams aged only 15 days (Koukkou *et al.*, 1998).

The mechanism behind the endothelial dysfunction is unclear as some studies report reduced maximum relaxation (Ghosh *et al.*, 2001) and others, like this, show reduced sensitivity (Samuelsson *et al.*, 2008) to ACh. A study using specific inhibitors of nitric oxide and EDHF in offspring of obese dams suggests altered EDHF response in mesenteric but not femoral artery when treated with ACh (Taylor *et al.*, 2004), though this was carried out in 180day old rat offspring of maternal obesity dams fed only a high fat, not high sugar, diet. The study

also suggested up-regulation of nitric oxide and prostaglandins in response to reduced EDHF release or sensitivity (Taylor *et al.*, 2004). Whether similar mechanisms are involved in the reduction of response reported here are unclear but response to ACh following blockade of nitric oxide production and/or in the presence of potassium to depolarise cells and inhibit EDHFs would indicate the relative contribution of each factor.

Endothelial dysfunction is associated with cardiovascular disorders (Drexler and Hornig, 1999) and atherosclerosis (Bonetti *et al.*, 2003) in humans. It is also observed in human and animal models of diabetes (Steinberg *et al.*, 1996; Molnar *et al.*, 2005). Interestingly the vascular function of offspring from obese dams presented here shares some similarities to that reported by Molnar *et al.* in a study of postnatal obesity (Molnar *et al.*, 2005); specifically, decreased endothelium-dependent vasodilation. Despite hyperinsulinaemia, insulin stimulated phosphorylation of akt and eNOS were normal in the study by Molnar *et al.*, but reduced eNOS protein dimers were present, potentially as a result of peroxynitrite disruption (Molnar *et al.*, 2005). It could be assumed that insulin sensitivity is maintained in the DIOoff as no evidence for altered plasma insulin was found but, as shown by Molnar *et al.*, it is still possible for the downstream signalling pathway of Ach (production of nitric oxide) to be altered.

Whilst studies suggest a strong link between plasma lipids, hormones and vascular function, the results of this study suggest alternative mechanisms must also be present. In humans, obese pregnant women have been shown to have altered concentrations of lipids, hormones and inflammatory cytokines, similar to non-pregnant subjects (Ramsay *et al.*, 2002). During development these factors will enter fetal circulation and it is possible they can cause changes in gene expression which impact on femoral artery function but not on whole body physiology. Thus the effect is still due to altered concentrations of plasma lipids and hormones but during development rather than in adult life. DIOoff themselves did not have altered plasma insulin concentrations following a fast or glucose tolerance test, and in fact had lower plasma triglyceride levels than controls. Likewise, female mice subjected to postnatal obesity did not show signs of vascular dysfunction, and therefore it would be unlikely to be present in the dams of DIOoff pre-pregnancy.

Vascular dysfunction is often linked with hypertension as it can lead to changes in peripheral resistance. Increased contractility and/or decreased vasodilatory responses may increase peripheral resistance and therefore drive an increase in blood pressure unless other mechanisms can compensate. It is notable that the DIOoff have vascular dysfunction, but no change in blood pressure. It is possible that blood flow, controlled by heart rate which was

not measured in this study, could change to accommodate increases in resistance. The concentrations of agonist which were required to find differences in offspring vascular function are often greater than those that are found physiologically. The changes found in this study may represent differences seen with pharmacological doses and might not become physiologically relevant unless the system is stressed or another insult such as obesity is present. This study only investigated the function of femoral arteries, changes in vascular function have anatomical variation, as discussed above (section 6.4.3), and therefore the differences may not be present in other vessels. Resistance arteries are more important in regulating blood pressure than conduit arteries, such as the femoral, thus it would be of interest to look at the function of resistance arteries in this model.

The novel results reported in this chapter show maternal obesity is associated with endothelial dysfunction in the offspring independently from alterations in plasma concentrations of glucose, insulin, triglycerides and cholesterol.

6.4.5 Postnatal obesity and vascular remodelling

The lack of profound functional changes in the femoral artery following postnatal obesity was mirrored by a normal response to intra-luminal wire injury surgery. The use of OPT allowed for volumetric analysis enabling investigations into neointima length not seen in standard two-dimensional analysis.

There are many factors involved in vascular remodelling following injury, one being inflammation (Ross, 1999; Virdis and Schiffrin, 2003). As no difference in lesion size was found, further immunohistochemistry for specific cell types in lesions was not undertaken. However, descriptive notes of UST stained slides were made, and these revealed no increase in appearance of thrombosis, or suggestion of differential neointima composition. As was discussed with respect to vascular function, if obesity and metabolic dysfunction were the primary cause of increased lesion formation the results presented in chapter 3 would predict the effect to be greater in male mice than females. The OPT analysis did reveal a small pattern towards increase volume of neointima formed in male DIO, though the same pattern was seen in lumen volume, potentially suggesting outward remodelling of the vessel.

Obesity and insulin resistance has been shown to increase neointimal proliferation following balloon arterial injury in rats (Park *et al.*, 2001; J. Shelton, 2003; Desouza *et al.*, 2006). In mice, ferric chloride-induced arterial injury was shown to increase neointimal proliferation in the C57BL/6 strain but not in *ob/ob* mice (Schafer *et al.*, 2004) which lack functional leptin. When *ob/ob* mice were given recombinant leptin, lesion formation increased. In a

second genetic model of diabetes and obesity; *db/db* mice, which lack the leptin receptor, recombinant leptin administration did not alter lesion formation implicating a role for the leptin receptor (Schafer *et al.*, 2004) in the injury response.

Consistent with the results in this chapter, Molnar *et al.* used C57BL/6 mice fed high fat diet and did not find any significant difference in neointimal hyperplasia following femoral artery wire denudation injury (Molnar *et al.*, 2005). A degree of leptin resistance may account for the lack of changes in lesion size found both in this study and in that of Molnar et al. (Molnar *et al.*, 2005). However, if leptin were the only mediator of neointimal lesion formation it would seem logical that the transgenic models of obesity, which lack leptin signalling, would have smaller lesions than C57BL/6 mice which has not been described (Schafer *et al.*, 2004). Species-specific responses to vascular injury can be seen in the literature which may account for the variability in the effects of obesity. A dramatic reduction in lesion size was reported by Stephenson *et al.* following intra-luminal injury in *db/db* mice, believed to be due to reduced smooth muscle migration (Stephenson *et al.*, 2003). However, studies using type 2-diabetic rats report increased lesion size following vascular injury (Park *et al.*, 2001; J. Shelton, 2003; Desouza *et al.*, 2006).

Vascular injury has been proposed to initiate the build up of atherosclerotic plaques (Ross, 1999), however, it is not possible to produce atherosclerotic lesions in wild type mice. In the study reported in this chapter we chose to use a model of arterial denudation, which generates a cellular intimal lesion, to study vascular remodelling. As discussed above various models have been used to study vascular remodelling, such as balloon arterial injury, ferric chloride cuff and even radiation and whether the same results would have been found using a different model is unclear. If it is possible to generate an injury which incorporates lipids in to the remodelling response, this is more likely to be directly modulated by obesity and increased plasma lipid concentrations. It would also be interesting to use a model in which the endothelial cells are not damaged as they display many chemoattractant and adhesion molecules which can initiate vascular remodelling responses and atherogenesis, and are increased in obesity (Ferri *et al.*, 1999; Vachharajani *et al.*, 2005).

6.4.6 Maternal obesity and vascular remodelling in the offspring

Differences in femoral artery function were attributed to changes both in smooth muscle and endothelial cells. The model of injury used denuded the vessel of endothelium; removing the cells would mean their contribution to lesion formation may be minimal. This suggests the changes in endothelial cell function induced by maternal obesity are unlikely to affect

vascular remodelling following this injury. However, cells proximal to the injury site would also have a role in the remodelling response and their dysfunction could be important in lesion formation. Non-denuded models of arterial injury, as discussed above in relation to post-natal obesity (section 6.4.5), may seem appealing to investigate. The lesions formed in these injuries are more focal and therefore may lead to difficulties in analysis. It is also not clear how some models initiate injury or endothelial damage and if physical, vessel restrictions, as well as chemical parameters are involved.

No other studies investigating the response to intra-vascular injury in offspring of obese dams have been reported. The lack of overt metabolic dysfunction in the offspring in this study (chapter 5) may suggest no differences would be found, although the profound alterations in vascular function have already provided evidence for programming of the vasculature independent of changes in metabolism. Given that postnatal obesity does not consistently affect lesion formation following injury (as discussed above) maternal obesity might not be an influential factor on the same processes in offspring.

Maternal obesity has been associated with increased inflammation and reactive oxygen species in the offspring (Bruce *et al.*, 2009; McCurdy *et al.*, 2009; Bilbo and Tsang, 2010; Yan *et al.*, 2010) Alterations in inflammatory response may also provide a link between the changes found in vascular function, and the many varying metabolic and vascular phenotypes found as a result of maternal obesity in other studies. Atherosclerosis has a significant inflammatory component (Libby, 2002) and greater incidences of fatty streaks, an atherosclerotic precursor, are found in children of hypercholesterolaemic mothers (Napoli *et al.*, 1999) and offspring of atherosclerotic rabbits (Palinski *et al.*, 2001) and mice (Alkemade *et al.*, 2007) independent of metabolic status. If inflammatory changes are involved, vascular remodelling may differ from controls in older offspring or those further challenged with an obesogenic diet, or in alternative arterial injury models.

6.4.7 Study limitations

The blood pressures reported in this thesis were consistent with one study (Elahi *et al.*, 2009) but are higher than others (Rahmouni *et al.*, 2005; Samuelsson *et al.*, 2008; Symons *et al.*, 2009). This could be due to the technique used (discussed in section 6.4.2) or other intrinsic differences between housing environments and origin of the strain. It is possible that the control values are high and are therefore masking increases induced by exposure to obesity.

The resting tension applied to femoral arteries to investigate function was kept consistent for all groups. For accurate results this relies on vessel diameter being unaffected by exposure to

obesity. The vessel diameter was not measured in femoral arteries used to investigate vascular function and therefore this cannot be ascertained for certain. Measures were made in separate cohorts of animals generated using the same protocols but subjected to intraluminal wire injury surgery. The measurements were made in UST stained slides at the furthest point away from injury possible. At this point the vessel morphology had returned to normal and therefore was unlikely to have been exposed to the wire. Although these results showed no effect of exposure to obesity on vessel diameter measurements in the actual vessels used would be highly preferential and should be made in future studies.

6.4.8 Conclusions

In summary, postnatal obesity does not cause changes in blood pressure, femoral artery function or remodelling following intra-luminal wire injury, suggesting obesity and/or the associated metabolic dysfunction must be well established before its effects become apparent. Maternal obesity does not alter blood pressure or neointima formation following intra-luminal injury in the offspring, but it does cause potentially detrimental changes to femoral artery function. This suggests that maternal obesity can program signalling pathways in the vasculature, distinct from those controlling metabolism.

Chapter 7

General Discussion

In recent decades the prevalence of obesity has rapidly increased, such that it is now one of the biggest global health problems (WHO, 2011). Obesity is a huge economic drain because of its role in the development of cardiometabolic disease including type 2 diabetes, hypertension, and atherosclerosis. In humans the prevalence of cardiovascular disease differs between sexes, potentially in part due to the distribution of adipose tissue; however, the exact mechanisms controlling adipose deposition and its effects on metabolic health are unknown. Most research attempting to delineate the links between obesity and cardiovascular disease has focussed on male animals and, therefore, sex differences are relatively unexplored. The effect of obesity in women of childbearing age is of particular interest due to the potential for over-nutrition to result in programming the metabolic and vascular physiology of the unborn fetus. Therefore, improving our understanding of sex differences in obesity, and elucidating the effects of maternal obesity on the offspring are important in informing attempts to reduce the incidence of cardiovascular disease in current and future generations.

The overall objective of the work described in this thesis was to study the sex differences in murine postnatal obesity and also the effects of maternal obesity on offspring physiology. The results generated in the first chapter expanded this study to include an investigation into the effects of estradiol on male obesity and glucocorticoid metabolism. An additional aim of the project was to explore the link between obesity and vascular physiology in the models of postnatal and maternal obesity.

7.1 Obesity in females

In the 1990s the National Institutes for Health mandated that trials supported by them should be adequately powered to support the analysis of gender and racial subgroups, though interestingly many studies still fail to report their results by sex (Geller *et al.*, 2011). The same equality is not seen in pre-clinical research with the search for drug targets and molecular pathologies of disease primarily focused on effects in male animals. Evidence suggests females are somewhat protected from the effects of obesity since, despite a greater percentage of adipose tissue, the rates of associated cardiovascular diseases are lower. The lack of research in females may mean that some of the molecular pathways that confer protection from obesity are being neglected. The most likely reason for the lack of use of females is the cyclic changes in physiology due to the estrus cycle. This makes study design more difficult since investigations should ideally be performed at a similar time point in the estrus cycle to control for the effects of fluctuating levels of sex steroids.

The anatomical distribution, as well as quantity, of adipose tissue has been postulated as the driving force behind sex differences in obesity-induced cardio-metabolic disease. The study reported in chapter 3 found a comparable increase in wet subcutaneous and retroperitoneal adipose tissue weight in males and females. However, the interaction between the effects of sex and obesity on mesenteric adipose indicated less visceral deposition in the female mice. Whether this small difference in distribution is part of the mechanism behind the sex-specific metabolic consequences of murine obesity is unclear and studies with estradiol did not enable a conclusion to be reached. In fact, treatment of males with estradiol (chapter 4) almost completely attenuated the diet-associated increase in adiposity. It is possible that glucose tolerance and insulin sensitivity were maintained in estradiol-treated mice due to the lack of adipose tissue accumulation, rather than the effects of estradiol on glucocorticoid metabolism or other target systems. It should be noted that plasma lipid concentrations were increased in estradiol-treated DIO mice, demonstrating that diet did have some physiological effects regardless of the steroid treatment. Although not a surrogate for females, estradiol-treated males could be useful in understanding the mechanisms linking adiposity and metabolic dysfunction. The lack of adipose tissue deposition could be addressed by repeating the experiment with a lower dose of estradiol, or by pair feeding the obesogenic diet to estradiol and sham treated animals.

7.1.1 Obesity and glucocorticoid metabolism

The results of this thesis support a wealth of published data describing an association between the activity of 11β-HSD1 and the development of obesity and metabolic complications. In male obese mice, 11β-HSD1 activity is reduced in correlation with adipose accumulation and this may be a protective mechanism (Morton *et al.*, 2004). While also apparent in females, 11β-HSD1 activity in adipose tissue was lower in lean females than males. This sex difference in glucocorticoid metabolism could be one mechanism accounting for the sex-specific metabolic responses to obesity in mice. Although studies have shown that 11β-HSD1 KO mice (Morton *et al.*, 2001), or those treated with inhibitors (Alberts *et al.*, 2002; Alberts *et al.*, 2003; Hermanowski-Vosatka *et al.*, 2005) are protected from the effects of a obesogenic diets, these studies were all carried out in male mice. Future studies treating diet-induced obese female mice with inhibitors of 11β-HSD1 or using female 11β-HSD1 KO mice would help determine whether the sex differences seen in the activity of the enzyme contribute to the metabolic dysfunction associated with obesity. It would also be of interest to extend these studies to include tissue specific KO models as it is plausible that the

effects of 11β-HSD1 inhibition could be mediated through the brain, liver, and adipose tissue, independently or in combination, and this may also differ between sexes.

The interaction between 11β-HSD1 and estrogen is of particular interest as this may provide a new selective target to modulate enzyme activity. The exact mechanisms by which estradiol affects 11β-HSD1 are still to be elucidated; and while estrogen has been shown to act as a non-competitive inhibitor of 11β-HSD1 in primary rat adipocyte cultures (Tagawa *et al.*, 2009) the results presented in chapters 3 and 4 also suggest an effect on mRNA abundance. Increased glucocorticoid regeneration in post-menopausal, compared with pre-menopausal, women has been described (Andersson *et al.*, 2009); and it would be interesting to extend this study to include post-menopausal women receiving hormone replacement therapy (HRT). Both preliminary studies of 11β-HSD1 inhibition in humans (Rosenstock *et al.*, 2010; Feig *et al.*, 2011) used male and female patients, although the results were not analysed for the effect of sex. Additionally the patients studied were aged between 40-60 years in one trial, and 29-66 in the other, and therefore hormonal status is likely to be heterogeneous. Reporting of sex specific results as well as combined data in future trials would be of interest in the light of the results of this thesis which suggests the effect of enzyme inhibition will be larger in males.

Local glucocorticoid concentrations can be affected by A-ring reductase enzymes as well as 11β-HSD1. Higher abundance of mRNA encoding 5βR was found in females, consistent with higher levels induced by estradiol treatment. To date no work has been published on direct inhibition or deletion of 5βR, though this would provide a new model in which to further investigate sex differences in glucocorticoid metabolism. The results of this thesis suggest greater metabolism of glucocorticoids by 5βR in females and, therefore, it could be hypothesised that deletion of 5βR would result in higher tissue specific concentrations of glucocorticoids in female mice. 5βR KO mice would also provide a model for further investigations into the differential effects of 5α- and 5β-reduced glucocorticoid metabolites on obesity and metabolic dysfunction.

7.2 Sex steroid balance

While the results presented in chapter 4 propose a role for estrogen in protecting males from the effects of an obesogenic diet, one large limitation of this study was the concurrent reduction in testosterone concentrations. As discussed in the chapter (section 4.4.6) the relative concentrations of estrogen and testosterone may be important. This highlights the

importance of studying both hormones together rather than in isolation, as it seems likely that the most metabolically beneficial combination of hormone concentrations varies depending on sex. Aromatase, which converts testosterone to estrogen, is found in adipose tissue and adipocyte hyperplasia in obesity may increase sex steroid conversion and alter the hormone balance (Cleland *et al.*, 1985; Cohen, 1999). Androgen receptors are proposed as the primary route of sex steroid feedback in male mice, although estrogen administration to gonadectomised mice can also normalise luteinising hormone concentrations (Wersinger *et al.*, 1999). As estrogen can bind to androgen receptors (Yeh *et al.*, 1998), increased local concentrations in obesity may elicit an inappropriate negative feedback response and further alter hormonal balance. In humans, sex hormone binding globulin (SHBG) is a key regulator of the bioavailability of sex steroids so that measurements of SHBG as well as sex steroids are required to understand the changes in hormone milieu occurring in obesity. Androgens inhibit SHBG production, while estrogens stimulate it *in vivo.* Interestingly, glucocorticoids have also been proposed to stimulate SHBG production (Cunningham *et al.*, 1985) suggesting a possible interaction between the changes in 11β-HSD1 reported in obesity and the bioavailability of sex steroids. As animal models provide a unique opportunity to control sex steroid concentrations without the interference of SHBG it would be interesting to repeat the studies of chapter 4 in mice with a) low concentrations of testosterone and estrogen b) low estrogen and high testosterone and c) high concentrations of both hormones. This would allow for further investigations into the hormonal control, and thus sex differences, in 11β-HSD1 and the effects on diet-induced obesity. Complementary to this would be to measure the tissue concentrations of steroids and the activity of aromatase, to determine if conversion of testosterone to estrogen is enhanced with adiposity in mice.

7.3 Programming by maternal obesity

The well established links between low birth weight and future health outcomes in humans (Barker *et al.*, 1990; Barker *et al.*, 1993a) and animal models (reviewed in (Bertram *et al.*, 2001)) demonstrate the importance of the developmental environment as well as the plasticity of molecular physiology during early life. Over-nutrition has been proposed as an important environmental stimulus for early-life programming, so that the recent epidemic of obesity has great public health implications. Human studies require long-term follow up and are complicated by the impact of postnatal environmental factors such as diet, education and lifestyle, so that animal models provide a useful tool for the investigation of the mechanisms linking maternal obesity with offspring health.

7.3.1 Dietary composition

The results presented in chapter 5 demonstrate little evidence for the programming of glucose, lipid or glucocorticoid metabolism in the offspring of obese mothers in this model. Although there is a wealth of literature to suggest maternal obesity causes increased adiposity and related metabolic disorders in rodent models (reviewed in (Drake and Reynolds, 2010)), offspring phenotypes differ between reports. Whilst it is possible to control the nutritional composition of diets fed to laboratory rodents this is often not accurately reported in experimental methods or results. Diets high in fat and/or sugar may be limited in protein content and variable with respect to macro- and micro-nutrients. Protein restriction in pregnant rodents leads to offspring with low birth weight and insulin resistance in later life (Ozanne *et al.*, 1996a; Ozanne *et al.*, 1996b), such that failure to control for protein content may result in programming effects in the offspring of obese dams. Less well studied are the effects of other dietary components including micronutrients on offspring physiology.

Many programmed phenotypes become more apparent when the offspring are challenged with an additional stressor in postnatal life such as an obesogenic diet or infection. To extend the model used in this thesis, feeding offspring with the same diets consumed by the dams would be of interest. Although this may reveal an effect of exposure to maternal obesity it would need to result in a large exacerbation of the known detrimental effects of postnatal obesity reported in chapter 3 to be clinically relevant. In a population where obesity is rapidly becoming the predominant classification of body weight, a small variation due to maternal nutritional effects is unlikely to generate new health concerns, though it may extend the need for treatments for type 2 diabetes, hypertension and other cardiovascular diseases. The results of chapter 5 also raise the possibility of overcoming the effects of programming by maternal obesity through promotion of a healthy diet and lifestyle postnatally. Currently, the effects of maternal obesity are difficult to ascertain with any certainty but encouraging weight loss and a healthy lifestyle before pregnancy may reduce the chance of programmed effects, and potentially more importantly prevent the domino effect of passing on undesirable eating behaviours.

7.3.2 Inter-generational programming

An interesting proposal is the potential for inter-generational programming as has been described in other models of early-life programming (Drake *et al.*, 2005b). This suggests that the gametes forming in a fetus of an obese dam may be altered such that a phenotype is seen

when the F1 offspring themselves have children. In human terms, the effects of an obese mother would be seen in her grandchildren. This is an interesting hypothesis as it would bring together subtle changes in nutritional status programmed in the F1 generation with epigenetic changes laid down during gamete formation potentially resulting in a stronger phenotype in the F2 generation. Due to the potential role of dietary components in programming, any inter-generational experiments must be designed with appropriate caution. The effects of paternal nutrition on offspring physiology must also be considered; new evidence demonstrates paternal obesity causes adiposity and β-cell dysfunction in female offspring (Ng *et al.*, 2010).

7.3.3 Sex differences in programming

Although the results of this thesis have highlighted the sex differences in murine postnatal obesity, only male offspring of obese dams were studied, due to time and practical limitations. The studies of postnatal obesity would imply worse metabolic phenotypes in males; however, most studies investigating exposure to maternal obesity in both sexes find worse or more pronounced metabolic phenotypes in females (Han *et al.*, 2005; Bayol *et al.*, 2008; Samuelsson *et al.*, 2008; Elahi *et al.*, 2009). Although the reasons for this are unclear, female fetuses may be more sensitive to environmental changes in early life and/or programmed changes may be more detrimental to females in later life. Maternal nutritional status could also result in the programming of reproductive function including sex steroid concentrations in the fetus or adult offspring which may have detrimental effects, for example a programmed reduction in estrogen may increase the risk of obesity. Interestingly, rodent studies of intrauterine growth restriction demonstrate a more severe phenotype in male, compared with female, offspring (reviewed in (Grigore *et al.*, 2008)) suggesting that different mechanisms may be involved in programming by maternal obesity. Ideally, studies of programming effects should include both sexes and allow for investigation at several time points; however, time, space, technical and monetary practicalities rarely allow for such thorough investigations.

7.3.4 Potential mechanisms

Due to the comparable metabolic physiology of the offspring of control and obese dams, and practical constraints of a PhD, follow-up studies to investigate potential programming mechanisms were not undertaken. However, this should be a future direction of studies investigating programming by exposure to maternal obesity. One potential mediator of future offspring health is epigenetic modification which is important in establishing different

patterns of gene expression, without changing DNA sequence. Epigenetic modifications include DNA methylation, histone modifications, and non coding RNAs and are established early in development and maintained throughout life, which makes them prime candidates to explain programming. Maternal nutritional status can alter the epigenetic regulation of gene expression. For example, in rats, maternal protein restriction decreases methylation of the GR and PPARα promoters in offspring liver (Lillycrop *et al.*, 2005) while maternal total calorie restriction increases methylation of the same genes (Gluckman *et al.*, 2007). Additionally, feeding pregnant mice a diet high in methyl donors increases methylation of the agouti gene in many offspring tissues (Waterland and Jirtle, 2004). Changes in mRNA levels and gene expression have been repeatedly reported as a consequence of maternal obesity or fetal over-nutrition. However few studies have investigated the role of epigenetic regulation. Raising rats in small litters which increases postnatal nutrition has been shown to cause hypermethylation of the pro-opiomelanocortin *(*POMC*)* promoter, which inhibits the essential upregulation of POMC by hormones suppressing its anorexic effects (Plagemann *et al.*, 2009). In addition, offspring of obese Japanese macaques have altered hepatic chromatin structure due to covalent modifications of histones (Aagaard-Tillery *et al.*, 2008) further supporting the potential role of epigenetic modifications in programming by maternal obesity.

7.4 Vascular function

The majority of evidence suggests that changes in vascular function correlate with insulin resistance in addition to obesity. Studies have reported changes in vascular function in obese normoglycemic patients, although fasting plasma insulin concentrations and homeostatic model assessment (HOMA) insulin resistance index were higher in the obese subjects compared with controls (Van Guilder *et al.*, 2006). In mice fed a high fat diet, insulin resistance occurs in the vasculature before peripheral tissues (Kim *et al.*, 2008). If also true in humans, this suggests that the endothelial dysfunction consistently reported in obesity is a response to raised insulin concentrations, even when normal metabolic physiology is maintained. It is almost impossible to separate obesity and raised insulin concentrations in humans; however, the models presented in this thesis provided a unique opportunity to delineate the two by using obese male mice with fasting hyperinsulinemia and insulin resistance and obese female mice with normal plasma insulin concentrations. Interestingly, neither sex had any physiologically relevant changes in vascular contraction or relaxation. Mice may be able to protect the vasculature from the effects of an obesogenic diet and raised

insulin concentrations. The results presented in chapter 6 add to the inconsistency found in the literature with regard to vascular function in murine postnatal obesity. It would be interesting to see if the changes reported by other groups, particularly endothelial dysfunction, would occur in the model used in this thesis with more time/increasing obesity. Many other reports have suggested large species variability in vascular function which may be another reason for the inconsistency of results reported.

7.4.1 *In vivo* imaging

To understand the mechanistic links between obesity, changes in vascular function and lesion formation, *in vivo* monitoring would be a huge benefit. This would allow for lesion formation and progression to be tracked over time following the surgical injury. It would also allow for functional analysis of the vessels *in situ*, where additional vasoactive agents can be released by peri-adventital adipose tissue, or anatomical changes in diameter restricted. In humans, the vasculature of the forearm has been extensively used to study the response to dietary constituents, hormones and pharmacological agents in conscious patients. However, in animals this can only be carried out under anaesthetic which, depending on the specific drug used, may alter vascular resistance, cardiac output and blood pressure (Skolleborg *et al.*, 1990) potentially limiting the relevance of the results.

While the use of OPT has provided an excellent addition to vascular lesion quantification, in particular in allowing 3D measurements, *in vivo* quantification would be preferable. Non-invasive imagining techniques are available to quantify lesions, which due to their relatively solid structure are not altered by anaesthetics. Ultrasound biomicroscopy has been used to assess plaque morphologies in the carotid arteries of mice (Gan *et al.*, 2007), as has magnetic resonance imaging (Trogan *et al.*, 2004). Both of these techniques would be useful in the early stages of lesion formation when the response to injury is at its greatest, and could be combined with specific cell markers to understand the patterns of cellular infiltration, migration and differentiation.

7.4.2 Maternal obesity and vascular function

Whilst postnatal obesity only had subtle effects on vascular function, the effects of exposure to maternal obesity were much greater. This is particularly interesting in light of the lack of overt changes in the metabolism of the offspring and suggests the potential to program altered vascular contraction and endothelial dysfunction without insulin resistance. A study investigating the effects of maternal obesity in rat offspring found altered vascular function from as early as 15 days of age (Koukkou *et al.*, 1998). Although plasma glucose and

triglycerides were comparable to control offspring, one possible influence at this early time point is the composition of the mothers' milk, and differences may be due to current nutritional intake rather than programmed changes. However, differences were maintained at 60 days of age in the study by Koukkou *et al.* consistent with the results found in the mouse model presented in this thesis. The mechanisms behind the changes in vascular function are currently unknown but warrant investigation as maternal obesity may increase the incidence of cardiovascular disease through direct effects on the vasculature instead of, or as well as, being a consequence of offspring obesity. Investigations of the phenotype of the endothelial cells would be the immediate direction of future studies. This could be carried out by inhibition of endothelial nitric oxide synthase (eNOS) in functional studies. In addition, post mortem analysis of eNOS expression, location, phosphorylation, and dimer formation could be used to determine whether the potential to synthesise nitric oxide has been altered by programming.

7.4.3 Alternative models

In the experiments described in this thesis, lesion formation was investigated 28 days after luminal wire injury, when the neointima is believed to be stable (Sata *et al.*, 2000; Dover *et al.*, 2007). Even with the addition of *in vivo* imaging or increased length of study this model may not be the most appropriate to explore the relationship between obesity and vascular response to injury in mice. There are other models of vascular injury which do not damage the endothelium such as direct ligation or placement of a perivascular cuff. As endothelial dysfunction is the most consistently reported vascular response to obesity these injuries may demonstrate differences in lesion formation as a result of changes to the endothelium. This would be of particular relevance in the model of exposure to maternal obesity where evidence of endothelial dysfunction was found. The advantage of the wire injury model used in this thesis is the similarity to the vascular damage seen in angioplasty and the maintenance of blood flow within the region of injury. This method reproducibly produces large neointimal lesions and is well tolerated in the animals, thus it is the preferred model of neointimal hyperplasia in the department.

Endothelial dysfunction and altered lesion formation may indicate a propensity for atherosclerosis, though this does not spontaneously occur in mice. To study atherosclerosis in mice, transgenic models, often in combination with high cholesterol diet, are required. Whilst these models have been useful in understanding the molecular pathology of the disease, the high plasma concentrations of cholesterol and changes in lipid metabolism due to deletion of genes generates a model quite different to simple obesity. In fact, studies in our

department found feeding ApoE KO mice an obesogenic diet as opposed to a western diet high in cholesterol did not result in atherosclerotic lesion formation (personal communication Dr. P. Hadoke). Thus larger mammals may be more clinically relevant for studying the relationships between atherosclerosis or lesion formation and consumption of an obesogenic diet.

7.5 Conclusions

In conclusion, these studies have provided valuable insights into the complex, sex-specific nature of obesity. They demonstrate that the relative protection of women from the metabolic effects of obesity is also found in mice and can be induced in males by treatment with estrogen. In addition they suggest a role for glucocorticoid metabolism in the sex differences associated with obesity. The results also demonstrate that maternal obesity can program vascular dysfunction in the offspring, independent of metabolism, which could have major public health implications if true in humans. The animal models developed and utilised in this thesis will allow for further investigations into the mechanisms of the sex differences associated with obesity and potential long-term consequences of maternal obesity on subsequent generations.

References

Aagaard-Tillery, K. M., Grove, K., Bishop, J., Ke, X., Fu, Q., McKnight, R. and Lane, R. H. (2008). Developmental origins of disease and determinants of chromatin structure: Maternal diet modifies the primate fetal epigenome. *J Mol Endocrinol* **41**(2): 91-102.

Ahima, R. S. and Flier, J. S. (2000). Adipose tissue as an endocrine organ. *Trends in Endocrinology & Metabolism* **11**(8): 327-332.

Ahmed-Sorour, H. and Bailey, C. J. (1981). Role of ovarian hormones in the long-term control of glucose homeostasis glycogen formation and gluconeogenesis. *Annals of Nutrition and Metabolism* **25**(4): 208-212.

Akingbemi, B. T., Ge, R., Rosenfeld, C. S., Newton, L. G., Hardy, D. O., Catterall, J. F., Lubahn, D. B., Korach, K. S. and Hardy, M. P. (2003). Estrogen receptor-α gene deficiency enhances androgen biosynthesis in the mouse leydig cell. *Endocrinology* **144**(1): 84-93.

Alberts, P., Engblom, L., Edling, N., Forsgren, M., Klingstrom, G., Larsson, C., Ronquist-Nii, Y., Ohman, B. and Abrahmsen, L. (2002). Selective inhibition of 11beta-hydroxysteroid dehydrogenase type 1 decreases blood glucose concentrations in hyperglycaemic mice. *Diabetologia* **45**(11): 1528-32.

Alberts, P., Nilsson, C., Selen, G., Engblom, L. O., Edling, N. H., Norling, S., Klingstrom, G., Larsson, C., Forsgren, M., Ashkzari, M., Nilsson, C. E., Fiedler, M., Bergqvist, E., Ohman, B., Bjorkstrand, E. and Abrahmsen, L. B. (2003). Selective inhibition of 11 beta-hydroxysteroid dehydrogenase type 1 improves hepatic insulin sensitivity in hyperglycemic mice strains. *Endocrinology* **144**(11): 4755-62.

Alexandersen, P., Haarbo, J. and Christiansen, C. (1996). The relationship of natural androgens to coronary heart disease in males: A review. *Atherosclerosis* **125**(1): 1-13.

Alkemade, F. E., Gittenberger-de Groot, A. C., Schiel, A. E., VanMunsteren, J. C., Hogers, B., van Vliet, L. S. J., Poelmann, R. E., Havekes, L. M., Willems van Dijk, K. and DeRuiter, M. C. (2007). Intrauterine exposure to maternal atherosclerotic risk factors increases the susceptibility to atherosclerosis in adult life. *Arterioscler Thromb Vasc Biol* **27**(10): 2228-2235.

Amri, E. Z., Teboul, L., Vannier, C., Grimaldi, P. A. and Ailhaud, G. (1996). Fatty acids regulate the expression of lipoprotein lipase gene and activity in preadipose and adipose cells. *Biochem J* **314 (Pt 2)**: 541-6.

Andersson, T., Soderstrom, I., Simonyte, K. and Olsson, T. (2010). Estrogen reduces 11beta-hydroxysteroid dehydrogenase type 1 in liver and visceral, but not subcutaneous, adipose tissue in rats. *Obesity (Silver Spring)* **18**(3): 470-5.

Andersson, T. S., Simonyte, K., Andrew, R., Strand, M., Burén, J., Walker, B. R., Mattsson, C. and Olsson, T. (2009). Tissue-specific increases in 11β-hydroxysteroid dehydrogenase type 1 in normal weight postmenopausal women. *PLoS One* **4**(12): e8475.

Andrew, R., Phillips, D. I. and Walker, B. R. (1998). Obesity and gender influence cortisol secretion and metabolism in man. *J Clin Endocrinol Metab* **83**(5): 1806-9.

Arcaro, G., Zamboni, M., Rossi, L., Turcato, E., Covi, G., Armellini, F., Bosello, O. and Lechi, A. (1999). Body fat distribution predicts the degree of endothelial dysfunction in uncomplicated obesity. *Int J Obes Relat Metab Disord* **23**(9): 936-42.

Armitage, J. A., Lakasing, L., Taylor, P. D., Balachandran, A. A., Jensen, R. I., Dekou, V., Ashton, N., Nyengaard, J. R. and Poston, L. (2005a). Developmental programming of aortic and renal structure in offspring of rats fed fat-rich diets in pregnancy. *J Physiol* **565**(Pt 1): 171-84.

Armitage, J. A., Taylor, P. D. and Poston, L. (2005b). Experimental models of developmental programming: Consequences of exposure to an energy rich diet during development. *J Physiol* **565**(Pt 1): 3-8.

Arner, E., Westermark, P. O., Spalding, K. L., Britton, T., Ryden, M., Frisen, J., Bernard, S. and Arner, P. (2009). Adipocyte turnover: Relevance to human adipose tissue morphology. *Diabetes* **59**(1): 105-9.

Arner, P. (1999). Catecholamine-induced lipolysis in obesity. *Int J Obes Relat Metab Disord* **23 Suppl 1**: 10-3.

Artal, R., Catanzaro, R. B., Gavard, J. A., Mostello, D. J. and Friganza, J. C. (2007). A lifestyle intervention of weight-gain restriction: Diet and exercise in obese women with gestational diabetes mellitus. *Applied Physiology, Nutrition, and Metabolism* **32**(3): 596-601.

Asakura, Y., Suzuki, M., Nonogi, H., Haze, K., Sato, A., Inada, H., Okuda, Y., Yamashita, K. and Harano, Y. (1998). Restenosis after percutaneous transluminal coronary angioplasty in patients with non-insulin-dependent diabetes mellitus (niddm). *J Cardiovasc Risk* **5**(5-6): 331-4.

Astrup, A., Raben, A., Buemann, B. and Toubro, S. (1997). Fat metabolism in the predisposition to obesity. *Ann N Y Acad Sci* **827**: 417-30.

Augereau, P., Badia, E., Fuentes, M., Rabenoelina, F., Corniou, M., Derocq, D. l., Balaguer, P. and Cavailles, V. (2006). Transcriptional regulation of the human nrip1/rip140 gene by estrogen is modulated by dioxin signalling. *Molecular Pharmacology* **69**(4): 1338-1346.

Bai, Y., Zhang, S., Kim, K. S., Lee, J. K. and Kim, K. H. (1996). Obese gene expression alters the ability of 30a5 preadipocytes to respond to lipogenic hormones. *J Biol Chem* **271**(24): 13939-42.

Bailey, C. J. and Flatt, P. R. (1982). Hormonal control of glucose homeostasis during development and ageing in mice. *Metabolism* **31**(3): 238-246.

Bakker, W., Eringa, E. C., Sipkema, P. and van Hinsbergh, V. W. (2008). Endothelial dysfunction and diabetes: Roles of hyperglycemia, impaired insulin signaling and obesity. *Cell Tissue Res*.

Barat, P., Livingstone, D. E. W., Elferink, C. M. C., McDonnell, C. R., Walker, B. R. and Andrew, R. (2007). Effects of gonadectomy on glucocorticoid metabolism in obese zucker rats. *Endocrinology* **148**(10): 4836-4843.

Barker, D. J. (1998). In utero programming of chronic disease. *Clin Sci (Lond)* **95**(2): 115-28.

Barker, D. J., Bull, A. R., Osmond, C. and Simmonds, S. J. (1990). Fetal and placental size and risk of hypertension in adult life. *BMJ* **301**(6746): 259-62.

Barker, D. J., Gluckman, P. D., Godfrey, K. M., Harding, J. E., Owens, J. A. and Robinson, J. S. (1993a). Fetal nutrition and cardiovascular disease in adult life. *Lancet* **341**(8850): 938-41.

Barker, D. J., Hales, C. N., Fall, C. H., Osmond, C., Phipps, K. and Clark, P. M. (1993b). Type 2 (non-insulin-dependent) diabetes mellitus, hypertension and hyperlipidaemia (syndrome x): Relation to reduced fetal growth. *Diabetologia* **36**(1): 62-7.

Barker, D. J., Winter, P. D., Osmond, C., Margetts, B. and Simmonds, S. J. (1989). Weight in infancy and death from ischaemic heart disease. *Lancet* **2**(8663): 577-80.

Barnard, D. E., Lewis, S. M., Teter, B. B. and Thigpen, J. E. (2009). Open- and closed-formula laboratory animal diets and their importance to research. *J Am Assoc Lab Anim Sci* **48**(6): 709-13.

Barnes, P. J. (1998). Anti-inflammatory actions of glucocorticoids: Molecular mechanisms. *Clin. Sci.* **94**(6): 557-572.

Bartke, A., Steele, R. E., Musto, N. and Caldwell, B. V. (1973). Fluctuations in plasma testosterone levels in adult male rats and mice. *Endocrinology* **92**(4): 1223-1228.

Bayol, S. A., Farrington, S. J. and Stickland, N. C. (2007). A maternal 'junk food' diet in pregnancy and lactation promotes an exacerbated taste for 'junk food' and a greater propensity for obesity in rat offspring. *Br J Nutr* **98**(4): 843-51.

Bayol, S. A., Simbi, B. H., Bertrand, J. A. and Stickland, N. C. (2008). Offspring from mothers fed a 'junk food' diet in pregnancy and lactation exhibit exacerbated adiposity that is more pronounced in females. *J Physiol* **586**(13): 3219-3230.

Bayol, S. A., Simbi, B. H. and Stickland, N. C. (2005). A maternal cafeteria diet during gestation and lactation promotes adiposity and impairs skeletal muscle development and metabolism in rat offspring at weaning. *J Physiol* **567**(Pt 3): 951-61.

Beale, E. G., Hammer, R. E., Antoine, B. and Forest, C. (2002). Glyceroneogenesis comes of age. *FASEB J* **16**(13): 1695-6.

Beck, I. M., Vanden Berghe, W., Vermeulen, L., Yamamoto, K. R., Haegeman, G. and De Bosscher, K. (2009). Crosstalk in inflammation: The interplay of glucocorticoid receptor-based mechanisms and kinases and phosphatases. *Endocr Rev* **30**(7): 830-82.

Belin de Chantemele, E. J., Irfan Ali, M., Mintz, J. and Stepp, D. W. (2009). Obesity induced-insulin resistance causes endothelial dysfunction without reducing the vascular response to hindlimb ischemia. *Basic Res Cardiol*.

Berchtold, P., Jorgens, V., Finke, C. and Berger, M. (1981). Epidemiology of obesity and hypertension. *Int J Obes* **5 suppl 1**: 1-7.

Bergman, R. N., Kim, S. P., Catalano, K. J., Hsu, I. R., Chiu, J. D., Kabir, M., Hucking, K. and Ader, M. (2006). Why visceral fat is bad: Mechanisms of the metabolic syndrome. *Obesity* **14**(2S): 16S-19S.

Bertram, C., Trowern, A. R., Copin, N., Jackson, A. A. and Whorwood, C. B. (2001). The maternal diet during pregnancy programs altered expression of the glucocorticoid receptor and type 2 11beta-hydroxysteroid dehydrogenase: Potential molecular mechanisms underlying the programming of hypertension in utero. *Endocrinology* **142**(7): 2841-53.

Bhattacharya, I., Mundy, A. L., Widmer, C. C., Kretz, M. and Barton, M. (2008). Regional heterogeneity of functional changes in conduit arteries after high-fat diet. *Obesity (Silver Spring)* **16**(4): 743-8.

Bilbo, S. D. and Tsang, V. (2010). Enduring consequences of maternal obesity for brain inflammation and behavior of offspring. *The FASEB Journal* **24**(6): 2104-2115.

Bjornstrom, L. and Sjoberg, M. (2005). Mechanisms of estrogen receptor signaling: Convergence of genomic and nongenomic actions on target genes. *Molecular Endocrinology* **19**(4): 833-842.

Black, B. L., Croom, J., Eisen, E. J., Petro, A. E., Edwards, C. L. and Surwit, R. S. (1998). Differential effects of fat and sucrose on body composition in a/j and c57bl/6 mice. *Metabolism* **47**(11): 1354-9.

Bloom, S. L., Sheffield, J. S., McIntire, D. D. and Leveno, K. J. (2001). Antenatal dexamethasone and decreased birth weight. *Obstet Gynecol* **97**(4): 485-90.

Boden, G. (1999). Free fatty acids, insulin resistance, and type 2 diabetes mellitus. *Proceedings of the Association of American Physicians* **111**(3): 241-248.

Boerschmann, H., Pfluger, M., Henneberger, L., Ziegler, A. G. and Hummel, S. (2010). Prevalence and predictors of overweight and insulin resistance in offspring of mothers with gestational diabetes mellitus. *Diabetes Care* **33**(8): 1845-9.

Bol, V. V., Delattre, A.-I., Reusens, B., Raes, M. and Remacle, C. (2009). Forced catch-up growth after fetal protein restriction alters the adipose tissue gene expression program leading to obesity in adult mice. *Am J Physiol Regul Integr Comp Physiol*: 90497.2008.

Bonetti, P. O., Lerman, L. O. and Lerman, A. (2003). Endothelial dysfunction: A marker of atherosclerotic risk. *Arterioscler Thromb Vasc Biol* **23**(2): 168-75.

Boozer, C. N., Schoenbach, G. and Atkinson, R. L. (1995). Dietary fat and adiposity: A dose-response relationship in adult male rats fed isocalorically. *Am J Physiol* **268**(4 Pt 1): E546-50.

Boucher, B. J. (1998). Inadequate vitamin d status: Does it contribute to the disorders comprising syndrome 'x'? *Br J Nutr* **79**(4): 315-27.

Boullu-Ciocca, S., Dutour, A., Guillaume, V., Achard, V., Oliver, C. and Grino, M. (2005). Postnatal diet-induced obesity in rats upregulates systemic and adipose tissue glucocorticoid metabolism during development and in adulthood: Its relationship with the metabolic syndrome. *Diabetes* **54**(1): 197-203.

Bourassa, P. A., Milos, P. M., Gaynor, B. J., Breslow, J. L. and Aiello, R. J. (1996). Estrogen reduces atherosclerotic lesion development in apolipoprotein e-deficient mice. *Proc Natl Acad Sci U S A* **93**(19): 10022-7.

Bourgoin, F., Bachelard, H., Badeau, M., Melancon, S., Pitre, M., Lariviere, R. and Nadeau, A. (2008). Endothelial and vascular dysfunctions and insulin resistance in rats fed a high-fat, high-sucrose diet. *Am J Physiol Heart Circ Physiol* **295**(3): H1044-1055.

Brandes, R. P., Schmitz-Winnenthal, F. H., Feletou, M., Godecke, A., Huang, P. L., Vanhoutte, P. M., Fleming, I. and Busse, R. (2000). An endothelium-derived hyperpolarizing factor distinct from no and prostacyclin is a major endothelium-dependent vasodilator in resistance vessels of wild-type and endothelial no synthase knockout mice. *Proc Natl Acad Sci U S A* **97**(17): 9747-52.

Brawley, L., Itoh, S., Torrens, C., Barker, A., Bertram, C., Poston, L. and Hanson, M. (2003). Dietary protein restriction in pregnancy induces hypertension and vascular defects in rat male offspring. *Pediatric Research* **54**(1): 83-90 10.1203/01.PDR.0000065731.00639.02.

Bray, G. A. (1977). The zucker-fatty rat: A review. *Fed Proc* **36**(2): 148-53.

Breuner, C. and Orchinik, M. (2002). Plasma binding proteins as mediators of corticosteroid action in vertebrates. *Journal of Endocrinology* **175**(1): 99-112.

Bronfman, M., Inestrosa, N. C. and Leighton, F. (1979). Fatty acid oxidation by human liver peroxisomes. *Biochem Biophys Res Commun* **88**(3): 1030-6.

Brooks, R. and Maklakov, A. (2010). Sex differences in obesity associated with total fertility rate. *PLoS One* **5**(5): e10587.

Brown, R. W., Chapman, K. E., Edwards, C. R. and Seckl, J. R. (1993). Human placental 11 beta-hydroxysteroid dehydrogenase: Evidence for and partial purification of a distinct nad-dependent isoform. *Endocrinology* **132**(6): 2614-21.

Brown, R. W., Diaz, R., Robson, A. C., Kotelevtsev, Y. V., Mullins, J. J., Kaufman, M. H. and Seckl, J. R. (1996). The ontogeny of 11 beta-hydroxysteroid dehydrogenase type 2 and mineralocorticoid receptor gene expression reveal intricate control of glucocorticoid action in development. *Endocrinology* **137**(2): 794-7.

Bruce, K. D., Cagampang, F. R., Argenton, M., Zhang, J., Ethirajan, P. L., Burdge, G. C., Bateman, A. C., Clough, G. F., Poston, L., Hanson, M. A., McConnell, J. M. and Byrne, C. D. (2009). Maternal high-fat feeding primes steatohepatitis in adult mice offspring, involving mitochondrial dysfunction and altered lipogenesis gene expression. *Hepatology* **50**(6): 1796-808.

Brunner, F., Kuhberger, E., Groschner, K., Poch, G. and Kukovetz, W. R. (1991). Characterization of muscarinic receptors mediating endothelium-dependent relaxation of bovine coronary artery. *Eur J Pharmacol* **200**(1): 25-33.

Bryzgalova, G., Gao, H., Ahren, B., Zierath, J. R., Galuska, D., Steiler, T. L., Dahlman-Wright, K., Nilsson, S., Gustafsson, J. Å., Efendic, S. and Khan, A. (2006). Evidence that oestrogen receptor-α plays an important role in the regulation of glucose homeostasis in mice: Insulin sensitivity in the liver. *Diabetologia* **49**(3): 588-597.

Buckley, A. J., Keseru, B., Briody, J., Thompson, M., Ozanne, S. E. and Thompson, C. H. (2005). Altered body composition and metabolism in the male offspring of high fat-fed rats. *Metabolism* **54**(4): 500-7.

Bujalska, I. J., Draper, N., Michailidou, Z., Tomlinson, J. W., White, P. C., Chapman, K. E., Walker, E. A. and Stewart, P. M. (2005). Hexose-6-phosphate dehydrogenase confers oxo-reductase activity upon 11î²-hydroxysteroid dehydrogenase type 1. *Journal of Molecular Endocrinology* **34**(3): 675-684.

Bujalska, I. J., Kumar, S., Hewison, M. and Stewart, P. M. (1999). Differentiation of adipose stromal cells: The roles of glucocorticoids and 11beta-hydroxysteroid dehydrogenase. *Endocrinology* **140**(7): 3188-96.

Bultman, S. J., Michaud, E. J. and Woychik, R. P. (1992). Molecular characterization of the mouse agouti locus. *Cell* **71**(7): 1195-204.

Bunger, L., Forsting, J., McDonald, K. L., Horvat, S., Duncan, J., Hochscheid, S., Baile, C. A., Hill, W. G. and Speakman, J. R. (2002). Long-term divergent selection on body fatness in mice indicates a regulation system that is independent of leptin production and reception. *The FASEB Journal*.

Burcelin, R., Crivelli, V., Dacosta, A., Roy-Tirelli, A. and Thorens, B. (2002). Heterogeneous metabolic adaptation of c57bl/6j mice to high-fat diet. *Am J Physiol Endocrinol Metab* **282**(4): E834-842.

Burgaya, F., Peinado, J., Vilaro, S., Llobera, M. and Ramirez, I. (1989). Lipoprotein lipase activity in neonatal-rat liver cell types. *Biochem J* **259**(1): 159-66.

Butera, P. C. and Czaja, J. A. (1984). Intracranial estradiol in ovariectomized guinea pigs: Effects on ingestive behaviors and body weight. *Brain Research* **322**(1): 41-48.

Caballero, A. E. (2003). Endothelial dysfunction in obesity and insulin resistance: A road to diabetes and heart disease. *Obesity* **11**(11): 1278-1289.

Caballero, A. E., Arora, S., Saouaf, R., Lim, S. C., Smakowski, P., Park, J. Y., King, G. L., LoGerfo, F. W., Horton, E. S. and Veves, A. (1999). Microvascular and macrovascular reactivity is reduced in subjects at risk for type 2 diabetes. *Diabetes* **48**(9): 1856-62.

Cai, H. and Harrison, D. G. (2000). Endothelial dysfunction in cardiovascular diseases: The role of oxidant stress. *Circ Res* **87**(10): 840-844.

Calvert, J. W., Lefer, D. J., Gundewar, S., Poston, L. and Coetzee, W. A. (2009). Developmental programming resulting from maternal obesity: Effects on myocardial ischemia/reperfusion injury. *Experimental Physiology*: -.

Campfield, L. A., Smith, F. J. and Burn, P. (1996). The ob protein (leptin) pathway--a link between adipose tissue mass and central neural networks. *Horm Metab Res* **28**(12): 619-32.

Camps, L., Reina, M., Llobera, M., Bengtsson-Olivecrona, G., Olivecrona, T. and Vilaró, S. (1991). Lipoprotein lipase in lungs, spleen, and liver: Synthesis and distribution. *Journal of Lipid Research* **32**(12): 1877-88.

Carr, A., Samaras, K., Burton, S., Law, M., Freund, J., Chisholm, D. J. and Cooper, D. A. (1998). A syndrome of peripheral lipodystrophy, hyperlipidaemia and insulin resistance in patients receiving hiv protease inhibitors. *AIDS* **12**(7): F51-8.

Casassus, P., Fontbonne, A., Thibult, N., Ducimetiere, P., Richard, J. L., Claude, J. R., Warnet, J. M., Rosselin, G. and Eschwege, E. (1992). Upper-body fat distribution: A hyperinsulinemia-independent predictor of coronary heart disease mortality. The paris prospective study. *Arterioscler Thromb* **12**(12): 1387-92.

Catalano, P., Presley, L., Minium, J. and Mouzon, S. (2009a). Fetuses of obese mothers develop insulin resistance in utero. *Diabetes Care* **32**(6): 1076 - 1080.

Catalano, P. M., Presley, L., Minium, J. and Hauguel-de Mouzon, S. (2009b). Fetuses of obese mothers develop insulin resistance in utero. *Diabetes Care* **32**(6): 1076-1080.

Catalano, P. M., Tyzbir, E. D., Roman, N. M., Amini, S. B. and Sims, E. A. (1991). Longitudinal changes in insulin release and insulin resistance in nonobese pregnant women. *Am J Obstet Gynecol* **165**(6 Pt 1): 1667-72.

Caulin-Glaser, T., Watson, C. A., Pardi, R. and Bender, J. R. (1996). Effects of 17beta-estradiol on cytokine-induced endothelial cell adhesion molecule expression. *The Journal of Clinical Investigation* **98**(1): 36-42.

Cavailles, V., Dauvois, S., L'Horset, F., Lopez, G., Hoare, S., Kushner, P. J. and Parker, M. G. (1995). Nuclear factor rip140 modulates transcriptional activation by the estrogen receptor. *EMBO J* **14**(15): 3741-51.

CDPR (Coronary drug project research) Group. (1973). The coronary drug project. *JAMA: The Journal of the American Medical Association* **226**(6): 652-657.

Cerf, M. E., Williams, K., Nkomo, X. I., Muller, C. J., Du Toit, D. F., Louw, J. and Wolfe-Coote, S. A. (2005). Islet cell response in the neonatal rat after exposure to a high-fat diet during pregnancy. *Am J Physiol Regul Integr Comp Physiol* **288**(5): R1122-8.

Chakravarty, K., Cassuto, H., Reshef, L. and Hanson, R. W. (2005). Factors that control the tissue-specific transcription of the gene for phosphoenolpyruvate carboxykinase-c. *Critical Reviews in Biochemistry and Molecular Biology* **40**(3): 129-154.

Chang, A. Y., Gilchrist, B. J. and Wyse, B. M. (1983a). Ciglitazone, a new hypoglycaemic agent. 3. Effect on glucose disposal and gluconeogenesis in vivo in c57bl/6j-ob/ob and -+/? Mice. *Diabetologia* **25**(6): 514-20.

Chang, A. Y., Wyse, B. M. and Gilchrist, B. J. (1983b). Ciglitazone, a new hypoglycemic agent. Ii. Effect on glucose and lipid metabolisms and insulin binding in the adipose tissue of c57bl/6j-ob/ob and -+/? Mice. *Diabetes* **32**(9): 839-45.

Chang, A. Y., Wyse, B. M., Gilchrist, B. J., Peterson, T. and Diani, A. R. (1983c). Ciglitazone, a new hypoglycemic agent. I. Studies in ob/ob and db/db mice, diabetic chinese hamsters, and normal and streptozotocin-diabetic rats. *Diabetes* **32**(9): 830-8.

Chang, G.-Q., Gaysinskaya, V., Karatayev, O. and Leibowitz, S. F. (2008). Maternal high-fat diet and fetal programming: Increased proliferation of hypothalamic peptide-producing neurons that increase risk for overeating and obesity. *J. Neurosci.* **28**(46): 12107-12119.

Charbonneau, A. and The, V. L. (2001). Genomic organization of a human 5beta-reductase and its pseudogene and substrate selectivity of the expressed enzyme. *Biochim Biophys Acta* **1517**(2): 228-35.

Chechi, K., McGuire, J. J. and Cheema, S. K. (2009). Developmental programming of lipid metabolism and aortic vascular function in c57bl/6 mice: A novel study suggesting an involvement of ldl-receptor. *Am J Physiol Regul Integr Comp Physiol* **296**(4): R1029-1040.

Chen, H., Charlat, O., Tartaglia, L. A., Woolf, E. A., Weng, X., Ellis, S. J., Lakey, N. D., Culpepper, J., Moore, K. J., Breitbart, R. E., Duyk, G. M., Tepper, R. I. and Morgenstern, J. P. (1996a). Evidence that the diabetes gene encodes the leptin receptor: Identification of a mutation in the leptin receptor gene in db/db mice. *Cell* **84**(3): 491-5.

Chen, S.-J., Li, H., Durand, J., Oparil, S. and Chen, Y.-F. (1996b). Estrogen reduces myointimal proliferation after balloon injury of rat carotid artery. *Circulation* **93**(3): 577-584.

Cleland, W. H., Mendelson, C. R. and Simpson, E. R. (1985). Effects of aging and obesity on aromatase activity of human adipose cells. *Journal of Clinical Endocrinology & Metabolism* **60**(1): 174-177.

Cnop, M., Havel, P. J., Utzschneider, K. M., Carr, D. B., Sinha, M. K., Boyko, E. J., Retzlaff, B. M., Knopp, R. H., Brunzell, J. D. and Kahn, S. E. (2003). Relationship of adiponectin to body fat distribution, insulin sensitivity and plasma lipoproteins: Evidence for independent roles of age and sex. *Diabetologia* **46**(4): 459-69.

Cocks, T. M. and Angus, J. A. (1983). Endothelium-dependent relaxation of coronary arteries by noradrenaline and serotonin. *Nature* **305**(5935): 627-30.

Cohen, P. G. (1999). The hypogonadal-obesity cycle: Role of aromatase in modulating the testosterone-estradiol shunt - a major factor in the genesis of morbid obesity. *Medical Hypotheses* **52**(1): 49-51.

Cole, T. J., Blendy, J. A., Monaghan, A. P., Krieglstein, K., Schmid, W., Aguzzi, A., Fantuzzi, G., Hummler, E., Unsicker, K. and Schutz, G. (1995a). Targeted disruption of the glucocorticoid receptor gene blocks adrenergic chromaffin cell development and severely retards lung maturation. *Genes Dev* **9**(13): 1608-21.

Cole, T. J., Blendy, J. A., Monaghan, A. P., Schmid, W., Aguzzi, A. and Schutz, G. (1995b). Molecular genetic analysis of glucocorticoid signaling during mouse development. *Steroids* **60**(1): 93-6.

Collins, S., Martin, T. L., Surwit, R. S. and Robidoux, J. (2004). Genetic vulnerability to diet-induced obesity in the c57bl/6j mouse: Physiological and molecular characteristics. *Physiology & Behavior* **81**(2): 243-248.

Contreras, J. L., Smyth, C. A., Bilbao, G., Young, C. J., Thompson, J. A. and Eckhoff, D. E. (2002). 17beta-estradiol protects isolated human pancreatic islets against proinflammatory cytokine-induced cell death: Molecular mechanisms and islet functionality. *Transplantation* **74**(9): 1252-9.

Cooke, P. S., Heine, P. A., Taylor, J. A. and Lubahn, D. B. (2001). The role of estrogen and estrogen receptor-[alpha] in male adipose tissue. *Molecular and Cellular Endocrinology* **178**(1-2): 147-154.

Corsetti, J. P., Sparks, J. D., Peterson, R. G., Smith, R. L. and Sparks, C. E. (2000). Effect of dietary fat on the development of non-insulin dependent diabetes mellitus in obese zucker diabetic fatty male and female rats. *Atherosclerosis* **148**(2): 231-41.

Corvol, P. and Bardin, C. W. (1973). Species distribution of testosterone-binding globulin. *Biol Reprod* **8**(3): 277-82.

Couse, J. F. and Korach, K. S. (1999). Estrogen receptor null mice: What have we learned and where will they lead us? *Endocrine Reviews* **20**(3): 358-417.

Crauwels, H. M., Van Hove, C. E., Herman, A. G. and Bult, H. (2000). Heterogeneity in relaxation mechanisms in the carotid and the femoral artery of the mouse. *European Journal of Pharmacology* **404**(3): 341-351.

Crowley, P., Chalmers, I. and Keirse, M. J. (1990). The effects of corticosteroid administration before preterm delivery: An overview of the evidence from controlled trials. *Br J Obstet Gynaecol* **97**(1): 11-25.

Cunningham, S. K., Loughlin, T., Culliton, M. and McKenna, T. J. (1985). The relationship between sex steroids and sex-hormone-binding globulin in plasma in physiological and pathological conditions. *Ann Clin Biochem* **22 (Pt 5)**: 489-97.

D'Eon, T. M., Souza, S. C., Aronovitz, M., Obin, M. S., Fried, S. K. and Greenberg, A. S. (2005). Estrogen regulation of adiposity and fuel partitioning. Evidence of genomic and non-genomic regulation of lipogenic and oxidative pathways. *J Biol Chem* **280**(43): 35983-91.

Dallman, M. F., la Fleur, S. E., Pecoraro, N. C., Gomez, F., Houshyar, H. and Akana, S. F. (2004). Minireview: Glucocorticoids--food intake, abdominal obesity, and wealthy nations in 2004. *Endocrinology* **145**(6): 2633-2638.

Dandona, P., Aljada, A. and Bandyopadhyay, A. (2004). Inflammation: The link between insulin resistance, obesity and diabetes. *Trends Immunol* **25**(1): 4-7.

Davy, K. P. and Hall, J. E. (2004). Obesity and hypertension: Two epidemics or one? *Am J Physiol Regul Integr Comp Physiol* **286**(5): R803-813.

de Meijer, V. E., Le, H. D., Meisel, J. A., Sharif, M. R. A., Pan, A., Nosé, V. and Puder, M. (2010). Dietary fat intake promotes the development of hepatic steatosis independently from excess caloric consumption in a murine model. *Metabolism* **59**(8): 1092-1105.

Deckelbaum, R. J. and Williams, C. L. (2001). Childhood obesity: The health issue. *Obes Res* **9 Suppl 4**: 239S-243S.

DeFronzo, R. A. (2004). Dysfunctional fat cells, lipotoxicity and type 2 diabetes. *Int J Clin Pract Suppl*(143): 9-21.

Desbriere, R., Vuaroqueaux, V., Achard, V., Boullu-Ciocca, S., Labuhn, M., Dutour, A. and Grino, M. (2006). 11beta-hydroxysteroid dehydrogenase type 1 mrna is increased in both

visceral and subcutaneous adipose tissue of obese patients. *Obesity (Silver Spring)* **14**(5): 794-8.

Deslex, S., Negrel, R., Vannier, C., Etienne, J. and Ailhaud, G. (1987). Differentiation of human adipocyte precursors in a chemically defined serum-free medium. *Int J Obes* **11**(1): 19-27.

Desouza, C. V., Gerety, M. and Hamel, F. G. (2006). Neointimal hyperplasia and vascular endothelial growth factor expression are increased in normoglycemic, insulin resistant, obese fatty rats. *Atherosclerosis* **184**(2): 283-9.

Despres, J.-P., Lemieux, I., Bergeron, J., Pibarot, P., Mathieu, P., Larose, E., Rodes-Cabau, J., Bertrand, O. F. and Poirier, P. (2008). Abdominal obesity and the metabolic syndrome: Contribution to global cardiometabolic risk. *Arterioscler Thromb Vasc Biol* **28**(6): 1039-1049.

Dieudonne, M. N., Sammari, A., Dos Santos, E., Leneveu, M. C., Giudicelli, Y. and Pecquery, R. (2006). Sex steroids and leptin regulate 11beta-hydroxysteroid dehydrogenase i and p450 aromatase expressions in human preadipocytes: Sex specificities. *J Steroid Biochem Mol Biol* **99**(4-5): 189-96.

Dobrian, A. D., Davies, M. J., Schriver, S. D., Lauterio, T. J. and Prewitt, R. L. (2001). Oxidative stress in a rat model of obesity-induced hypertension. *Hypertension* **37**(2): 554-560.

Donahue, R. P., Prineas, R. J., DeCarlo Donahue, R., Bean, J. A. and Skyler, J. S. (1996). The female 'insulin advantage' in a biracial cohort: Results from the miami community health study. *Int J Obes Relat Metab Disord* **20**(1): 76-82.

Dover, A. R., Hadoke, P. W., Macdonald, L. J., Miller, E., Newby, D. E. and Walker, B. R. (2007). Intravascular glucocorticoid metabolism during inflammation and injury in mice. *Endocrinology* **148**(1): 166-72.

Doyle, L. W., Ford, G. W., Davis, N. M. and Callanan, C. (2000). Antenatal corticosteroid therapy and blood pressure at 14 years of age in preterm children. *Clin Sci (Lond)* **98**(2): 137-42.

Drake, A. J., Livingstone, D. E., Andrew, R., Seckl, J. R., Morton, N. M. and Walker, B. R. (2005a). Reduced adipose glucocorticoid reactivation and increased hepatic glucocorticoid clearance as an early adaptation to high-fat feeding in wistar rats. *Endocrinology* **146**(2): 913-9.

Drake, A. J. and Reynolds, R. M. (2010). Impact of maternal obesity on offspring obesity and cardiometabolic disease risk. *Reproduction*: REP-10-0077.

Drake, A. J., Walker, B. R. and Seckl, J. R. (2005b). Intergenerational consequences of fetal programming by in utero exposure to glucocorticoids in rats. *Am J Physiol Regul Integr Comp Physiol* **288**(1): R34-8.

Drel, V. R., Mashtalir, N., Ilnytska, O., Shin, J., Li, F., Lyzogubov, V. V. and Obrosova, I. G. (2006). The leptin-deficient (ob/ob) mouse. *Diabetes* **55**(12): 3335-3343.

Drexler, H. and Hornig, B. (1999). Endothelial dysfunction in human disease. *J Mol Cell Cardiol* **31**(1): 51-60.

Elahi, M., Cagampang, F., Mukhtar, D., Anthony, F., Ohri, S. and Hanson, M. (2009). Long-term maternal high-fat feeding from weaning through pregnancy and lactation predisposes offspring to hypertension, raised plasma lipids and fatty liver in mice. *Br J Nutr* **102**(4): 514 - 519.

Endres, D. B., Milholland, R. J. and Rosen, F. (1979). Sex differences in the concentrations of glucocorticoid receptors in rat liver and thymus. *Journal of Endocrinology* **80**(1): 21-26.

Entringer, S., Epel, E. S., Kumsta, R., Lin, J., Hellhammer, D. H., Blackburn, E. H., Wüst, S. and Wadhwa, P. D. (2011). Stress exposure in intrauterine life is associated with shorter telomere length in young adulthood. *Proceedings of the National Academy of Sciences* **108**(33): E513-E518.

Enzi, G., Gasparo, M., Biondetti, P. R., Fiore, D., Semisa, M. and Zurlo, F. (1986). Subcutaneous and visceral fat distribution according to sex, age, and overweight, evaluated by computed tomography. *The American Journal of Clinical Nutrition* **44**(6): 739-46.

Erdei, N., Toth, A., Pasztor, E. T., Papp, Z., Edes, I., Koller, A. and Bagi, Z. (2006). High-fat diet-induced reduction in nitric oxide-dependent arteriolar dilation in rats: Role of xanthine oxidase-derived superoxide anion. *Am J Physiol Heart Circ Physiol* **291**(5): H2107-15.

Fain, J. N., Madan, A. K., Hiler, M. L., Cheema, P. and Bahouth, S. W. (2004). Comparison of the release of adipokines by adipose tissue, adipose tissue matrix, and adipocytes from visceral and subcutaneous abdominal adipose tissues of obese humans. *Endocrinology* **145**(5): 2273-2282.

Fall, C. H., Yajnik, C. S., Rao, S., Davies, A. A., Brown, N. and Farrant, H. J. (2003). Micronutrients and fetal growth. *J Nutr* **133**(5 Suppl 2): 1747S-1756S.

Fan, W., Yanase, T., Nomura, M., Okabe, T., Goto, K., Sato, T., Kawano, H., Kato, S. and Nawata, H. (2005). Androgen receptor null male mice develop late-onset obesity caused by decreased energy expenditure and lipolytic activity but show normal insulin sensitivity with high adiponectin secretion. *Diabetes* **54**(4): 1000-1008.

Farooqi, I. S. and O'Rahilly, S. (2005). Monogenic obesity in humans. *Annu Rev Med* **56**: 443-58.

Feig, P. U., Shah, S., Hermanowski-Vosatka, A., Plotkin, D., Springer, M. S., Donahue, S., Thach, C., Klein, E. J., Lai, E. and Kaufman, K. D. (2011). Effects of an 11β-hydroxysteroid dehydrogenase type 1 inhibitor, mk-0916, in patients with type 2 diabetes mellitus and metabolic syndrome. *Diabetes, Obesity and Metabolism* **13**(6): 498-504.

Ferezou-Viala, J., Roy, A. F., Serougne, C., Gripois, D., Parquet, M., Bailleux, V., Gertler, A., Delplanque, B., Djiane, J., Riottot, M. and Taouis, M. (2007). Long-term consequences of maternal high-fat feeding on hypothalamic leptin sensitivity and diet-induced obesity in the offspring. *Am J Physiol Regul Integr Comp Physiol* **293**(3): R1056-62.

Ferrari, P. (2010). The role of 11beta-hydroxysteroid dehydrogenase type 2 in human hypertension. *Biochim Biophys Acta* **1802**(12): 1178-87.

Ferri, C., Desideri, G., Valenti, M., Bellini, C., Pasin, M., Santucci, A. and De Mattia, G. (1999). Early upregulation of endothelial adhesion molecules in obese hypertensive men. *Hypertension* **34**(4 Pt 1): 568-73.

Finken, M. J., Andrews, R. C., Andrew, R. and Walker, B. R. (1999). Cortisol metabolism in healthy young adults: Sexual dimorphism in activities of a-ring reductases, but not 11beta-hydroxysteroid dehydrogenases. *J Clin Endocrinol Metab* **84**(9): 3316-21.

Fisher, C. R., Graves, K. H., Parlow, A. F. and Simpson, E. R. (1998). Characterization of mice deficient in aromatase (arko) because of targeted disruption of the cyp19 gene. *Proc Natl Acad Sci U S A* **95**(12): 6965-70.

Foegh, M. L., Asotra, S., Howell, M. H. and Ramwell, P. W. (1994). Estradiol inhibition of arterial neointimal hyperplasia after balloon injury. *J Vasc Surg* **19**(4): 722-6.

Ford, S. P., Zhang, L., Zhu, M., Miller, M. M., Smith, D. T., Hess, B. W., Moss, G. E., Nathanielsz, P. W. and Nijland, M. J. (2009). Maternal obesity accelerates fetal pancreatic {beta}-cell but not {alpha}-cell development in sheep: Prenatal consequences. *Am J Physiol Regul Integr Comp Physiol* **297**(3): R835-843.

Forest, C., Franckhauser, S., Glorian, M., Antras-Ferry, J., Robin, D. and Robin, P. (1997). Regulation of gene transcription by fatty acids, fibrates and prostaglandins: The phosphoenolpyruvate carboxykinase gene as a model. *Prostaglandins Leukot Essent Fatty Acids* **57**(1): 47-56.

Foundation, B. H. (2010). Coronary heart disease statistics.

Fox, C. S., Massaro, J. M., Hoffmann, U., Pou, K. M., Maurovich-Horvat, P., Liu, C.-Y., Vasan, R. S., Murabito, J. M., Meigs, J. B., Cupples, L. A., D'Agostino, R. B., Sr and O'Donnell, C. J. (2007). Abdominal visceral and subcutaneous adipose tissue compartments: Association with metabolic risk factors in the framingham heart study. *Circulation* **116**(1): 39-48.

Franckhauser, S., Munoz, S., Elias, I., Ferre, T. and Bosch, F. (2006). Adipose overexpression of phosphoenolpyruvate carboxykinase leads to high susceptibility to diet-induced insulin resistance and obesity. *Diabetes* **55**(2): 273-80.

Franckhauser, S., Munoz, S., Pujol, A., Casellas, A., Riu, E., Otaegui, P., Su, B. and Bosch, F. (2002). Increased fatty acid re-esterification by pepck overexpression in adipose tissue leads to obesity without insulin resistance. *Diabetes* **51**(3): 624-30.

Franks, S. (1995). Polycystic ovary syndrome. *New England Journal of Medicine* **333**(13): 853-861.

Fraulob, J. C., Ogg-Diamantino, R., Fernandes-Santos, C., Aguila, M. B. and Mandarim-de-Lacerda, C. A. (2010). A mouse model of metabolic syndrome: Insulin resistance, fatty liver and non-alcoholic fatty pancreas disease (nafpd) in c57bl/6 mice fed a high fat diet. *J Clin Biochem Nutr* **46**(3): 212-23.

Frazier-Jessen, M. and Kovacs, E. (1995). Estrogen modulation of je/monocyte chemoattractant protein-1 mrna expression in murine macrophages. *The Journal of Immunology* **154**(4): 1838-1845.

Friedman, J. E., de Vente, J. E., Peterson, R. G. and Dohm, G. L. (1991). Altered expression of muscle glucose transporter glut-4 in diabetic fatty zucker rats (zdf/drt-fa). *Am J Physiol* **261**(6 Pt 1): E782-8.

Fruchart, J.-C. (2009). Peroxisome proliferator-activated receptor-alpha (ppar[alpha]): At the crossroads of obesity, diabetes and cardiovascular disease. *Atherosclerosis* **205**(1): 1-8.

Fuchtenbusch, M., Standl, E. and Schatz, H. (2000). Clinical efficacy of new thiazolidinediones and glinides in the treatment of type 2 diabetes mellitus. *Exp Clin Endocrinol Diabetes* **108**(3): 151-63.

Fulton, D. J. (2009). Mechanisms of vascular insulin resistance: A substitute akt? *Circ Res* **104**(9): 1035-7.

Funder, J. W., Pearce, P. T., Smith, R. and Smith, A. I. (1988). Mineralocorticoid action: Target tissue specificity is enzyme, not receptor, mediated. *Science* **242**(4878): 583-5.

Furchgott, R. F. and Zawadzki, J. V. (1980). The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature* **288**(5789): 373-6.

Gagner, J. P. and Drouin, J. (1985). Opposite regulation of pro-opiomelanocortin gene transcription by glucocorticoids and crh. *Molecular and Cellular Endocrinology* **40**(1): 25-32.

Gale, C. R., Javaid, M. K., Robinson, S. M., Law, C. M., Godfrey, K. M. and Cooper, C. (2007). Maternal size in pregnancy and body composition in children. *J Clin Endocrinol Metab* **92**(10): 3904-11.

Gallou-Kabani, C., Vige, A., Gross, M.-S., Rabes, J.-P., Boileau, C., Larue-Achagiotis, C., Tome, D., Jais, J.-P. and Junien, C. (2007). C57bl/6j and a/j mice fed a high-fat diet delineate components of metabolic syndrome[ast]. *Obesity* **15**(8): 1996-2005.

Gambineri, A., Pelusi, C., Vicennati, V., Pagotto, U. and Pasquali, R. (2002). Obesity and the polycystic ovary syndrome. *International journal of obesity and related metabolic disorders : journal of the International Association for the Study of Obesity* **26**(7): 883-96.

Gan, L.-m., Grönros, J., Hägg, U., Wikström, J., Theodoropoulos, C., Friberg, P. and Fritsche-Danielson, R. (2007). Non-invasive real-time imaging of atherosclerosis in mice using ultrasound biomicroscopy. *Atherosclerosis* **190**(2): 313-320.

Gao, H., Falt, S., Sandelin, A., Gustafsson, J.-A. and Dahlman-Wright, K. (2008). Genome-wide identification of estrogen receptor {alpha}-binding sites in mouse liver. *Mol Endocrinol* **22**(1): 10-22.

Gastaldelli, A., Baldi, S., Pettiti, M., Toschi, E., Camastra, S., Natali, A., Landau, B. R. and Ferrannini, E. (2000). Influence of obesity and type 2 diabetes on gluconeogenesis and glucose output in humans: A quantitative study. *Diabetes* **49**(8): 1367-1373.

Geer, E. B. and Shen, W. (2009). Gender differences in insulin resistance, body composition, and energy balance. *Gender Medicine* **6**(Part 1): 60-75.

Geisler, J. G., Zawalich, W., Zawalich, K., Lakey, J. R., Stukenbrok, H., Milici, A. J. and Soeller, W. C. (2002). Estrogen can prevent or reverse obesity and diabetes in mice expressing human islet amyloid polypeptide. *Diabetes* **51**(7): 2158-69.

Geller, S. E., Koch, A., Pellettieri, B. and Carnes, M. (2011). Inclusion, analysis, and reporting of sex and race/ethnicity in clinical trials: Have we made progress? *J Womens Health (Larchmt)* **20**(3): 315-20.

Gharib, S. D., Wierman, M. E., Shupnik, M. A. and Chin, W. W. (1990). Molecular biology of the pituitary gonadotropins. *Endocrine Reviews* **11**(1): 177-199.

Ghosh, P., Bitsanis, D., Ghebremeskel, K., Crawford, M. A. and Poston, L. (2001). Abnormal aortic fatty acid composition and small artery function in offspring of rats fed a high fat diet in pregnancy. *J Physiol* **533**(Pt 3): 815-22.

Gillman, M. W., Rifas-Shiman, S., Berkey, C. S., Field, A. E. and Colditz, G. A. (2003). Maternal gestational diabetes, birth weight, and adolescent obesity. *Pediatrics* **111**(3): e221-6.

Glorian, M., Duplus, E., Beale, E. G., Scott, D. K., Granner, D. K. and Forest, C. (2001). A single element in the phosphoenolpyruvate carboxykinase gene mediates thiazolidinedione action specifically in adipocytes. *Biochimie* **83**(10): 933-943.

Gluckman, P. D., Lillycrop, K. A., Vickers, M. H., Pleasants, A. B., Phillips, E. S., Beedle, A. S., Burdge, G. C. and Hanson, M. A. (2007). Metabolic plasticity during mammalian development is directionally dependent on early nutritional status. *Proceedings of the National Academy of Sciences* **104**(31): 12796-12800.

Godfrey, K. M., Forrester, T., Barker, D. J. P., Jackson, A. A., Landman, J. P., Hall, J. S. E., Cox, V. and Osmond, C. (1994). Maternal nutritional status in pregnancy and blood pressure in childhood. *BJOG: An International Journal of Obstetrics & Gynaecology* **101**(5): 398-403.

Godin, D., Ivan, E., Johnson, C., Magid, R. and Galis, Z. S. (2000). Remodeling of carotid artery is associated with increased expression of matrix metalloproteinases in mouse blood flow cessation model. *Circulation* **102**(23): 2861-6.

Goland, R. S., Jozak, S., Warren, W. B., Conwell, I. M., Stark, R. I. and Tropper, P. J. (1993). Elevated levels of umbilical cord plasma corticotropin-releasing hormone in growth-retarded fetuses. *J Clin Endocrinol Metab* **77**(5): 1174-9.

Government, Scottish. (2005). Scottish health survey 2003, http://www.scotland.gov.uk/Resource/Doc/924/0019811.pdf

Grant, I., Fischbacher, C. and Whyte, B. (2007). Obesity in scotland: An epidemiology briefing.

Greenberg, A. S., Kraemer, F. B., Soni, K. G., Jedrychowski, M. P., Yan, Q.-W., Graham, C. E., Bowman, T. A. and Mansur, A. (2011). Lipid droplet meets a mitochondrial protein to regulate adipocyte lipolysis. *EMBO J* **30**(21): 4337-4339.

Gregoire, F. M., Johnson, P. R. and Greenwood, M. R. (1995). Comparison of the adipoconversion of preadipocytes derived from lean and obese zucker rats in serum-free cultures. *Int J Obes Relat Metab Disord* **19**(9): 664-70.

Gregoire, F. M., Smas, C. M. and Sul, H. S. (1998). Understanding adipocyte differentiation. *Physiol Rev* **78**(3): 783-809.

Griffin, M. E., Marcucci, M. J., Cline, G. W., Bell, K., Barucci, N., Lee, D., Goodyear, L. J., Kraegen, E. W., White, M. F. and Shulman, G. I. (1999). Free fatty acid-induced insulin resistance is associated with activation of protein kinase c theta and alterations in the insulin signaling cascade. *Diabetes* **48**(6): 1270-1274.

Grigore, D., Ojeda, N. B. and Alexander, B. T. (2008). Sex differences in the fetal programming of hypertension. *Gender Medicine* **5**(Supplement 1): S121-S132.

Grumbach, M. M. and Auchus, R. J. (1999). Estrogen: Consequences and implications of human mutations in synthesis and action. *Journal of Clinical Endocrinology & Metabolism* **84**(12): 4677-4694.

Grunfeld, J. P. (1990). Glucocorticoids in blood pressure regulation. *Horm Res* **34**(3-4): 111-3.

Guelinckx, I., Devlieger, R., Beckers, K. and Vansant, G. (2008). Maternal obesity: Pregnancy complications, gestational weight gain and nutrition. *Obes Rev* **9**(2): 140-50.

Guh, D., Zhang, W., Bansback, N., Amarsi, Z., Birmingham, C. L. and Anis, A. (2009). The incidence of co-morbidities related to obesity and overweight: A systematic review and meta-analysis. *BMC Public Health* **9**(1): 88.

Gulick, T., Cresci, S., Caira, T., Moore, D. D. and Kelly, D. P. (1994). The peroxisome proliferator-activated receptor regulates mitochondrial fatty acid oxidative enzyme gene expression. *Proceedings of the National Academy of Sciences* **91**(23): 11012-11016.

Gunderson, E. P., Jacobs Jr, D. R., Chiang, V., Lewis, C. E., Tsai, A., Quesenberry Jr, C. P. and Sidney, S. (2009). Childbearing is associated with higher incidence of the metabolic syndrome among women of reproductive age controlling for measurements before pregnancy: The cardia study. *American Journal of Obstetrics and Gynecology* **201**(2): 177.e1-177.e9.

Guo, F. and Jen, K. L. (1995). High-fat feeding during pregnancy and lactation affects offspring metabolism in rats. *Physiol Behav* **57**(4): 681-6.

Gustafsson, J.-Å., Eneroth, P., Pousette, Å., Skett, P., Sonnenschhn, C., Stenberg, Å. and Åhlén, A. (1977). Programming and differentiation of rat liver enzymes. *Journal of Steroid Biochemistry* **8**(5): 429-443.

Gustafsson, J.-Å., Ingelman-Sundberg, M. and Stenberg, Å. (1975). Neonatal androgenic programming of hepatic steroid metabolism in rats. *Journal of Steroid Biochemistry* **6**(5): 643-649.

Gustafsson, J. A., Mode, A., Norstedt, G., Eneroth, P. and Hokfelt, T. (1983). Central control of prolactin and estrogen receptors in rat liver--expression of a novel endocrine system, the hypothalamo-pituitary-liver axis. *Annu Rev Pharmacol Toxicol* **23**: 259-78.

Hadoke, P., Wainwright, C. L., Wadsworth, R. M., Butler, K. and Giddings, M. J. (1995). Characterization of the morphological and functional alterations in rabbit subclavian artery subjected to balloon angioplasty. *Coron Artery Dis* **6**(5): 403-15.

Hadoke, P. W., Lindsay, R. S., Seckl, J. R., Walker, B. R. and Kenyon, C. J. (2006a). Altered vascular contractility in adult female rats with hypertension programmed by prenatal glucocorticoid exposure. *J Endocrinol* **188**(3): 435-42.

Hadoke, P. W., Macdonald, L., Logie, J. J., Small, G. R., Dover, A. R. and Walker, B. R. (2006b). Intra-vascular glucocorticoid metabolism as a modulator of vascular structure and function. *Cell Mol Life Sci* **63**(5): 565-78.

Haisenleder, D. J., Schoenfelder, A. H., Marcinko, E. S., Geddis, L. M. and Marshall, J. C. (2011). Estimation of estradiol in mouse serum samples: Evaluation of commercial estradiol immunoassays. *Endocrinology*.

Hales, C. N. and Barker, D. J. (1992). Type 2 (non-insulin-dependent) diabetes mellitus: The thrifty phenotype hypothesis. *Diabetologia* **35**(7): 595-601.

Han, J., Xu, J., Epstein, P. N. and Liu, Y. Q. (2005). Long-term effect of maternal obesity on pancreatic beta cells of offspring: Reduced beta cell adaptation to high glucose and high-fat diet challenges in adult female mouse offspring. *Diabetologia* **48**(9): 1810-8.

Handa, R. J., Burgess, L. H., Kerr, J. E. and O'Keefe, J. A. (1994). Gonadal steroid hormone receptors and sex differences in the hypothalamo-pituitary-adrenal axis. *Hormones and Behavior* **28**(4): 464-476.

Handy, D. E., Castro, R. and Loscalzo, J. (2011). Epigenetic modifications. *Circulation* **123**(19): 2145-2156.

Hansen, P. A., Han, D. H., Nolte, L. A., Chen, M. and Holloszy, J. O. (1997). Dhea protects against visceral obesity and muscle insulin resistance in rats fed a high-fat diet. *American Journal of Physiology - Regulatory, Integrative and Comparative Physiology* **273**(5): R1704-R1708.

Hanson, R. W. (2009). Thematic minireview series: A perspective on the biology of phosphoenolpyruvate carboxykinase 55 years after its discovery. *J Biol Chem* **284**(40): 27021-3.

He, W., Barak, Y., Hevener, A., Olson, P., Liao, D., Le, J., Nelson, M., Ong, E., Olefsky, J. M. and Evans, R. M. (2003). Adipose-specific peroxisome proliferator-activated receptor γ knockout causes insulin resistance in fat and liver but not in muscle. *Proceedings of the National Academy of Sciences* **100**(26): 15712-15717.

Heine, P. A., Taylor, J. A., Iwamoto, G. A., Lubahn, D. B. and Cooke, P. S. (2000). Increased adipose tissue in male and female estrogen receptor-α knockout mice. *Proceedings of the National Academy of Sciences of the United States of America* **97**(23): 12729-12734.

Heinlein, C. A. and Chang, C. (2002). The roles of androgen receptors and androgen-binding proteins in nongenomic androgen actions. *Molecular Endocrinology* **16**(10): 2181-2187.

Herberg, L. and Coleman, D. L. (1977). Laboratory animals exhibiting obesity and diabetes syndromes. *Metabolism* **26**(1): 59-99.

Hermann, G. M., Miller, R. L., Erkonen, G. E., Dallas, L. M., Hsu, E., Zhu, V. and Roghair, R. D. (2009). Neonatal catch up growth increases diabetes susceptibility but improves behavioral and cardiovascular outcomes of low birth weight male mice. *Pediatric Research* **66**(1): 53-58 10.1203/PDR.0b013e3181a7c5fd.

Hermanowski-Vosatka, A., Balkovec, J. M., Cheng, K., Chen, H. Y., Hernandez, M., Koo, G. C., Le Grand, C. B., Li, Z., Metzger, J. M., Mundt, S. S., Noonan, H., Nunes, C. N., Olson, S. H., Pikounis, B., Ren, N., Robertson, N., Schaeffer, J. M., Shah, K., Springer, M. S., Strack, A. M., Strowski, M., Wu, K., Wu, T., Xiao, J., Zhang, B. B., Wright, S. D. and Thieringer, R. (2005). 11beta-hsd1 inhibition ameliorates metabolic syndrome and prevents progression of atherosclerosis in mice. *J Exp Med* **202**(4): 517-27.

Herrington, D. M. (1999). The hers trial results: Paradigms lost? Heart and estrogen/progestin replacement study. *Ann Intern Med* **131**(6): 463-6.

Ho, K. J. and Liao, J. K. (2002). Nonnuclear actions of estrogen. *Arteriosclerosis, Thrombosis, and Vascular Biology* **22**(12): 1952-1961.

Holm, C., Østerlund, T., Laurell, H. and Contreras, J. A. (2000). Molecular mechanisms regulating hormone-sensitive lipase and lipolysis. *Annual Review of Nutrition* **20**(1): 365-393.

Holmes, M. C., Abrahamsen, C. T., French, K. L., Paterson, J. M., Mullins, J. J. and Seckl, J. R. (2006). The mother or the fetus? 11β-hydroxysteroid dehydrogenase type 2 null mice provide evidence for direct fetal programming of behavior by endogenous glucocorticoids. *The Journal of Neuroscience* **26**(14): 3840-3844.

Holzmann, S., Kukovetz, W. R. and Schmidt, K. (1980). Mode of action of coronary arterial relaxation by prostacyclin. *J Cyclic Nucleotide Res* **6**(6): 451-60.

Hong, J., Stubbins, R., Smith, R., Harvey, A. and Nunez, N. (2009). Differential susceptibility to obesity between male, female and ovariectomized female mice. *Nutrition Journal* **8**(1): 11.

Hopkins, S. A., Baldi, J. C., Cutfield, W. S., McCowan, L. and Hofman, P. L. (2010). Exercise training in pregnancy reduces offspring size without changes in maternal insulin sensitivity. *Journal of Clinical Endocrinology & Metabolism* **95**(5): 2080-2088.

Hotamisligil, G., Shargill, N. and Spiegelman, B. (1993). Adipose expression of tumor necrosis factor-alpha: Direct role in obesity-linked insulin resistance. *Science* **259**(5091): 87-91.

Hou, M., Liu, Y., Zhu, L., Sun, B., Guo, M., Burén, J. and Li, X. (2011). Neonatal overfeeding induced by small litter rearing causes altered glucocorticoid metabolism in rats. *PLoS ONE* **6**(11): e25726.

Howie, G. J., Sloboda, D. M., Kamal, T. and Vickers, M. H. (2009). Maternal nutritional history predicts obesity in adult offspring independent of postnatal diet. *J Physiol* **587**(Pt 4): 905-15.

Hwang, L. L., Wang, C. H., Li, T. L., Chang, S. D., Lin, L. C., Chen, C. P., Chen, C. T., Liang, K. C., Ho, I. K., Yang, W. S. and Chiou, L. C. (2010). Sex differences in high-fat diet-induced obesity, metabolic alterations and learning, and synaptic plasticity deficits in mice. *Obesity (Silver Spring)* **18**(3): 463-9.

Iafrati, M. D., Karas, R. H., Aronovitz, M., Kim, S., Sullivan, T. R., Jr., Lubahn, D. B., O'Donnell, T. F., Jr., Korach, K. S. and Mendelsohn, M. E. (1997). Estrogen inhibits the vascular injury response in estrogen receptor alpha-deficient mice. *Nat Med* **3**(5): 545-8.

Imai, E., Stromstedt, P. E., Quinn, P. G., Carlstedt-Duke, J., Gustafsson, J. A. and Granner, D. K. (1990). Characterization of a complex glucocorticoid response unit in the phosphoenolpyruvate carboxykinase gene. *Mol Cell Biol* **10**(9): 4712-9.

Ishibashi, S., Brown, M. S., Goldstein, J. L., Gerard, R. D., Hammer, R. E. and Herz, J. (1993). Hypercholesterolemia in low density lipoprotein receptor knockout mice and its reversal by adenovirus-mediated gene delivery. *J Clin Invest* **92**(2): 883-93.

J. Shelton, D. W. H. G. J. M. W. S. O. C. R. W. (2003). The neointimal response to endovascular injury is increased in obese zucker rats. *Diabetes, Obesity and Metabolism* **5**(6): 415-423.

Jackman, R. W. and Kandarian, S. C. (2004). The molecular basis of skeletal muscle atrophy. *Am J Physiol Cell Physiol* **287**(4): C834-43.

Jamieson, P. M., Nyirenda, M. J., Walker, B. R., Chapman, K. E. and Seckl, J. R. (1999). Interactions between oestradiol and glucocorticoid regulatory effects on liver-specific glucocorticoid-inducible genes: Possible evidence for a role of hepatic 11beta-hydroxysteroid dehydrogenase type 1. *J Endocrinol* **160**(1): 103-9.

Jamieson, P. M., Walker, B. R., Chapman, K. E., Andrew, R., Rossiter, S. and Seckl, J. R. (2000). 11 beta-hydroxysteroid dehydrogenase type 1 is a predominant 11 beta-reductase in the intact perfused rat liver. *J Endocrinol* **165**(3): 685-92.

Jayakody, L., Senaratne, M., Thomson, A. and Kappagoda, T. (1987). Endothelium-dependent relaxation in experimental atherosclerosis in the rabbit. *Circulation Research* **60**(2): 251-264.

Jeong, S., Han, M., Lee, H., Kim, M., Kim, J., Nicol, C. J., Kim, B. H., Choi, J. H., Nam, K.-H., Oh, G. T. and Yoon, M. (2004). Effects of fenofibrate on high-fat diet-induced body weight gain and adiposity in female c57bl/6j mice. *Metabolism* **53**(10): 1284-1289.

Ji, H. and Friedman, M. I. (2007). Reduced capacity for fatty acid oxidation in rats with inherited susceptibility to diet-induced obesity. *Metabolism* **56**(8): 1124-30.

Jones, J. R., Barrick, C., Kim, K.-A., Lindner, J., Blondeau, B., Fujimoto, Y., Shiota, M., Kesterson, R. A., Kahn, B. B. and Magnuson, M. A. (2005). Deletion of pparî³ in adipose tissues of mice protects against high fat diet-induced obesity and insulin resistance. *Proceedings of the National Academy of Sciences* **102**(17): 6207-6212.

Jones, M. E. E., Thorburn, A. W., Britt, K. L., Hewitt, K. N., Misso, M. L., Wreford, N. G., Proietto, J., Oz, O. K., Leury, B. J., Robertson, K. M., Yao, S. and Simpson, E. R. (2001). Aromatase-deficient (arko) mice accumulate excess adipose tissue. *The Journal of Steroid Biochemistry and Molecular Biology* **79**(1-5): 3-9.

Jousilahti, P., Vartiainen, E., Tuomilehto, J. and Puska, P. (1999). Sex, age, cardiovascular risk factors, and coronary heart disease : A prospective follow-up study of 14 786 middle-aged men and women in finland. *Circulation* **99**(9): 1165-1172.

Julius, S., Valentini, M. and Palatini, P. (2000). Overweight and hypertension : A 2-way street? *Hypertension* **35**(3): 807-13.

Kahn, B. B. and Flier, J. S. (2000). Obesity and insulin resistance. *J Clin Invest* **106**(4): 473-81.

Kanagalingam, M. G., Forouhi, N. G., Greer, I. A. and Sattar, N. (2005). Changes in booking body mass index over a decade: Retrospective analysis from a glasgow maternity hospital. *BJOG: An International Journal of Obstetrics & Gynaecology* **112**(10): 1431-1433.

Karas, R. H., Schulten, H., Pare, G., Aronovitz, M. J., Ohlsson, C., Gustafsson, J.-A. and Mendelsohn, M. E. (2001). Effects of estrogen on the vascular injury response in estrogen receptor α,β (double) knockout mice. *Circulation Research* **89**(6): 534-539.

Kauser, K. and Rubanyi, G. M. (1994). Gender difference in bioassayable endothelium-derived nitric oxide from isolated rat aortae. *Am J Physiol* **267**(6 Pt 2): H2311-7.

Kautzky-Willer A., H. A. (2009). Metabolic diseases and associated complications: Sex and gender matter! *European Journal of Clinical Investigation* **39**(8): 631-648.

Kawamura, A., Fujiwara, H., Onodera, T., Wu, D. J., Matsuda, M., Ishida, M., Takemura, G., Fujiwara, Y. and Kawai, C. (1989). Response of large and small coronary arteries of pigs to intracoronary injection of acetylcholine: Angiographic and histologic analysis. *Int J Cardiol* **25**(3): 289-302.

Kaye, S. A., Folsom, A. R., Sprafka, J. M., Prineas, R. J. and Wallace, R. B. (1991). Increased incidence of diabetes mellitus in relation to abdominal adiposity in older women. *J Clin Epidemiol* **44**(3): 329-34.

Keenan, K., Grant, I. and Ramsay, J. (2011). The scottish health survery, obesity. S. National Statistics. Edinburgh, Scottish Government.

Keller, H., Givel, F., Perroud, M. and Wahli, W. (1995). Signaling cross-talk between peroxisome proliferator-activated receptor/retinoid x receptor and estrogen receptor through estrogen response elements. *Molecular Endocrinology* **9**(7): 794-804.

Kershaw, E. E. and Flier, J. S. (2004). Adipose tissue as an endocrine organ. *Journal of Clinical Endocrinology & Metabolism* **89**(6): 2548-2556.

Kersten, S. (2001). Mechanisms of nutritional and hormonal regulation of lipogenesis. *EMBO Rep* **2**(4): 282-6.

Ketonen, J., Shi, J., Martonen, E. and Mervaala, E. (2010). Periadventitial adipose tissue promotes endothelial dysfunction via oxidative stress in diet-induced obese c57bl/6 mice. *Circ J*.

Khan, I., Dekou, V., Hanson, M., Poston, L. and Taylor, P. (2004). Predictive adaptive responses to maternal high-fat diet prevent endothelial dysfunction but not hypertension in adult rat offspring. *Circulation* **110**(9): 1097-102.

Khan, I. Y., Dekou, V., Douglas, G., Jensen, R., Hanson, M. A., Poston, L. and Taylor, P. D. (2005). A high-fat diet during rat pregnancy or suckling induces cardiovascular dysfunction in adult offspring. *Am J Physiol Regul Integr Comp Physiol* **288**(1): R127-33.

Khan, I. Y., Taylor, P. D., Dekou, V., Seed, P. T., Lakasing, L., Graham, D., Dominiczak, A. F., Hanson, M. A. and Poston, L. (2003). Gender-linked hypertension in offspring of lard-fed pregnant rats. *Hypertension* **41**(1): 168-75.

Kibos, A., Campeanu, A. and Tintoiu, I. (2007). Pathophysiology of coronary artery inâ€ • stent restenosis. *Acute Cardiac Care* **9**(2): 111-119.

Kiewe, P., Gueller, S., Komor, M., Stroux, A., Thiel, E. and Hofmann, W. K. (2009). Prediction of qualitative outcome of oligonucleotide microarray hybridization by measurement of rna integrity using the 2100 bioanalyzer capillary electrophoresis system. *Ann Hematol* **88**(12): 1177-83.

Kim, F., Pham, M., Maloney, E., Rizzo, N. O., Morton, G. J., Wisse, B. E., Kirk, E. A., Chait, A. and Schwartz, M. W. (2008). Vascular inflammation, insulin resistance, and reduced nitric oxide production precede the onset of peripheral insulin resistance. *Arterioscler Thromb Vasc Biol*: ATVBAHA.108.169722.

Kim, J. K., Fillmore, J. J., Chen, Y., Yu, C., Moore, I. K., Pypaert, M., Lutz, E. P., Kako, Y., Velez-Carrasco, W., Goldberg, I. J., Breslow, J. L. and Shulman, G. I. (2001). Tissue-specific overexpression of lipoprotein lipase causes tissue-specific insulin resistance. *Proc Natl Acad Sci U S A* **98**(13): 7522-7.

Kino, T. and Chrousos, G. P. (2011a). Acetylation-mediated epigenetic regulation of glucocorticoid receptor activity: Circadian rhythm-associated alterations of glucocorticoid actions in target tissues. *Mol Cell Endocrinol* **336**(1-2): 23-30.

Kino, T. and Chrousos, G. P. (2011b). Circadian clock-mediated regulation of target-tissue sensitivity to glucocorticoids: Implications for cardiometabolic diseases. *Endocr Dev* **20**: 116-26.

Kintscher, U. and Law, R. E. (2005). Pparî³-mediated insulin sensitization: The importance of fat versus muscle. *American Journal of Physiology - Endocrinology And Metabolism* **288**(2): E287-E291.

Kirk, S. L., Samuelsson, A. M., Argenton, M., Dhonye, H., Kalamatianos, T., Poston, L., Taylor, P. D. and Coen, C. W. (2009). Maternal obesity induced by diet in rats permanently influences central processes regulating food intake in offspring. *PLoS ONE* **4**(6): e5870.

Kirkby, N. S., Low, L., Seckl, J. R., Walker, B. R., Webb, D. J. and Hadoke, P. W. (2011). Quantitative 3-dimensional imaging of murine neointimal and atherosclerotic lesions by optical projection tomography. *PLoS One* **6**(2): e16906.

Kissebah, A. H. and Krakower, G. R. (1994). Regional adiposity and morbidity. *Physiological Reviews* **74**(4): 761-811.

Kley, H. K., Solbach, H. G., McKinnan, J. C. and Kruskemper, H. L. (1979). Testosterone decrease and oestrogen increase in male patients with obesity. *Acta Endocrinologica* **91**(3): 553-563.

Kolehmainen, M., Vidal, H., Ohisalo, J. J., Pirinen, E., Alhava, E. and Uusitupa, M. I. (2002). Hormone sensitive lipase expression and adipose tissue metabolism show gender difference in obese subjects after weight loss. *Int J Obes Relat Metab Disord* **26**(1): 6-16.

Koritnik, D. R., Koshy, A. and Hoversland, R. C. (1995). 17î²-estradiol treatment increases the levels of estrogen receptor and its mrna in male rat liver. *Steroids* **60**(8): 519-529.

Kotani, K., Tokunaga, K., Fujioka, S., Kobatake, T., Keno, Y., Yoshida, S., Shimomura, I., Tarui, S. and Matsuzawa, Y. (1994). Sexual dimorphism of age-related changes in whole-body fat distribution in the obese. *Int J Obes Relat Metab Disord* **18**(4): 207-2.

Kotelevtsev, Y., Brown, R. W., Fleming, S., Kenyon, C., Edwards, C. R., Seckl, J. R. and Mullins, J. J. (1999). Hypertension in mice lacking 11beta-hydroxysteroid dehydrogenase type 2. *J Clin Invest* **103**(5): 683-9.

Kotelevtsev, Y., Holmes, M. C., Burchell, A., Houston, P. M., Schmoll, D., Jamieson, P., Best, R., Brown, R., Edwards, C. R., Seckl, J. R. and Mullins, J. J. (1997). 11beta-hydroxysteroid dehydrogenase type 1 knockout mice show attenuated glucocorticoid-inducible responses and resist hyperglycemia on obesity or stress. *Proc Natl Acad Sci U S A* **94**(26): 14924-9.

Kotsis, V. T., Stabouli, S. V., Papamichael, C. M. and Zakopoulos, N. A. (2006). Impact of obesity in intima media thickness of carotid arteries. *Obesity (Silver Spring)* **14**(10): 1708-15.

Koukkou, E., Ghosh, P., Lowy, C. and Poston, L. (1998). Offspring of normal and diabetic rats fed saturated fat in pregnancy demonstrate vascular dysfunction. *Circulation* **98**(25): 2899-2904.

Koupil, I. and Toivanen, P. (2008). Social and early-life determinants of overweight and obesity in 18-year-old swedish men. *Int J Obes (Lond)* **32**(1): 73-81.

Krege, J. H., Hodgin, J. B., Hagaman, J. R. and Smithies, O. (1995). A noninvasive computerized tail-cuff system for measuring blood pressure in mice. *Hypertension* **25**(5): 1111-1115.

Krishnan, A. V., Swami, S. and Feldman, D. (2001). Estradiol inhibits glucocorticoid receptor expression and induces glucocorticoid resistance in mcf-7 human breast cancer cells. *The Journal of Steroid Biochemistry and Molecular Biology* **77**(1): 29-37.

Kumar, A. and Lindner, V. (1997). Remodeling with neointima formation in the mouse carotid artery after cessation of blood flow. *Arterioscler Thromb Vasc Biol* **17**(10): 2238-44.

Kurtz, T. W., Morris, R. C. and Pershadsingh, H. A. (1989). The zucker fatty rat as a genetic model of obesity and hypertension. *Hypertension* **13**(6): 896-901.

Kushwaha, R. S. and Hazzard, W. R. (1981). Exogenous estrogens attenuate dietary hypercholesterolemia and atherosclerosis in the rabbit. *Metabolism* **30**(4): 359-366.

Laffitte, B. A., Repa, J. J., Joseph, S. B., Wilpitz, D. C., Kast, H. R., Mangelsdorf, D. J. and Tontonoz, P. (2001). Lxrs control lipid-inducible expression of the apolipoprotein e gene in macrophages and adipocytes. *Proc Natl Acad Sci U S A* **98**(2): 507-12.

Laitinen, J., Power, C. and Jarvelin, M. R. (2001). Family social class, maternal body mass index, childhood body mass index, and age at menarche as predictors of adult obesity. *Am J Clin Nutr* **74**(3): 287-94.

Langley-Evans, S. C., Phillips, G. J., Benediktsson, R., Gardner, D. S., Edwards, C. R., Jackson, A. A. and Seckl, J. R. (1996). Protein intake in pregnancy, placental glucocorticoid metabolism and the programming of hypertension in the rat. *Placenta* **17**(2-3): 169-72.

Langley-Evans, S. C., Phillips, G. J. and Jackson, A. A. (1994). In utero exposure to maternal low protein diets induces hypertension in weanling rats, independently of maternal blood pressure changes. *Clin Nutr* **13**(5): 319-24.

Langley, S. C., Browne, R. F. and Jackson, A. A. (1994). Altered glucose tolerance in rats exposed to maternal low protein diets in utero. *Comp Biochem Physiol Physiol* **109**(2): 223-9.

Lavery, G. G., Walker, E. A., Draper, N., Jeyasuria, P., Marcos, J., Shackleton, C. H., Parker, K. L., White, P. C. and Stewart, P. M. (2006). Hexose-6-phosphate dehydrogenase knock-out mice lack 11 beta-hydroxysteroid dehydrogenase type 1-mediated glucocorticoid generation. *J Biol Chem* **281**(10): 6546-51.

Le May, C. d., Pineau, T., Bigot, K., Kohl, C., Girard, J. and PÃ©gorier, J.-P. (2000). Reduced hepatic fatty acid oxidation in fasting pparî± null mice is due to impaired mitochondrial hydroxymethylglutaryl-coa synthase gene expression. *FEBS Letters* **475**(3): 163-166.

Lehrke, M. and Lazar, M. A. (2005). The many faces of ppargamma. *Cell* **123**(6): 993-9.

Leonardsson, G. r., Steel, J. H., Christian, M., Pocock, V., Milligan, S., Bell, J., So, P.-W., Medina-Gomez, G., Vidal-Puig, A., White, R. and Parker, M. G. (2004). Nuclear receptor corepressor rip140 regulates fat accumulation. *Proceedings of the National Academy of Sciences of the United States of America* **101**(22): 8437-8442.

Levin, B. E., Dunn-Meynell, A. A., Balkan, B. and Keesey, R. E. (1997). Selective breeding for diet-induced obesity and resistance in sprague-dawley rats. *Am J Physiol Regul Integr Comp Physiol* **273**(2): R725-730.

Levin, B. E. and Keesey, R. E. (1998). Defense of differing body weight set points in diet-induced obese and resistant rats. *Am J Physiol* **274**(2 Pt 2): R412-9.

Levitt, N. S., Lindsay, R. S., Holmes, M. C. and Seckl, J. R. (1996). Dexamethasone in the last week of pregnancy attenuates hippocampal glucocorticoid receptor gene expression and elevates blood pressure in the adult offspring in the rat. *Neuroendocrinology* **64**(6): 412-8.

Lewis, J. G., Bagley, C. J., Elder, P. A., Bachmann, A. W. and Torpy, D. J. (2005). Plasma free cortisol fraction reflects levels of functioning corticosteroid-binding globulin. *Clinica Chimica Acta* **359**(1â€“2): 189-194.

Liang, C., Oest, M. E. and Prater, M. R. (2009). Intrauterine exposure to high saturated fat diet elevates risk of adult-onset chronic diseases in c57bl/6 mice. *Birth Defects Research Part B: Developmental and Reproductive Toxicology* **86**(5): 377-384.

Liao, J. K. (1994). Inhibition of gi proteins by low density lipoprotein attenuates bradykinin-stimulated release of endothelial-derived nitric oxide. *Journal of Biological Chemistry* **269**(17): 12987-92.

Libby, P. (2002). Inflammation in atherosclerosis. *Nature* **420**(6917): 868-74.

Licinio, J., Caglayan, S., Ozata, M., Yildiz, B. O., de Miranda, P. B., O'Kirwan, F., Whitby, R., Liang, L., Cohen, P., Bhasin, S., Krauss, R. M., Veldhuis, J. D., Wagner, A. J., DePaoli, A. M., McCann, S. M. and Wong, M.-L. (2004). Phenotypic effects of leptin replacement on morbid obesity, diabetes mellitus, hypogonadism, and behavior in leptin-deficient adults. *Proceedings of the National Academy of Sciences of the United States of America* **101**(13): 4531-4536.

Lillycrop, K. A., Phillips, E. S., Jackson, A. A., Hanson, M. A. and Burdge, G. C. (2005). Dietary protein restriction of pregnant rats induces and folic acid supplementation prevents epigenetic modification of hepatic gene expression in the offspring. *The Journal of Nutrition* **135**(6): 1382-1386.

Lillycrop, K. A., Slater-Jefferies, J. L., Hanson, M. A., Godfrey, K. M., Jackson, A. A. and Burdge, G. C. (2007). Induction of altered epigenetic regulation of the hepatic glucocorticoid receptor in the offspring of rats fed a protein-restricted diet during pregnancy suggests that reduced DNA methyltransferase-1 expression is involved in impaired DNA methylation and changes in histone modifications. *Br J Nutr* **97**(6): 1064-73.

Lindsay, R. S., Lindsay, R. M., Waddell, B. J. and Seckl, J. R. (1996). Prenatal glucocorticoid exposure leads to offspring hyperglycaemia in the rat: Studies with the 11 beta-hydroxysteroid dehydrogenase inhibitor carbenoxolone. *Diabetologia* **39**(11): 1299-305.

Liu, Y.-J., Nakagawa, Y., Toya, K., Saegusa, H., Nasuda, K., Endoh, A. and Ohzeki, T. (1998). Effects of thyroid hormone (thyroxine) and testosterone on hepatic 11β-hydroxysteroid dehydrogenase mrna and activity in pubertal hypothyroid male rats. *Metabolism* **47**(4): 474-477.

Liu, Y., Nakagawa, Y., Wang, Y., Li, R., Li, X., Ohzeki, T. and Friedman, T. C. (2003). Leptin activation of corticosterone production in hepatocytes may contribute to the reversal of obesity and hyperglycemia in leptin-deficient ob/ob mice. *Diabetes* **52**(6): 1409-1416.

Livingstone, D. E. (2009). Susceptibility to hyperinsulinaemia and fatty liver with loss of 5alpha-reductase 1 occurs in rats and mice and is not androgen dependent. *Endocrine Abstracts* **19**: P136.

Livingstone, D. E., Jones, G. C., Smith, K., Jamieson, P. M., Andrew, R., Kenyon, C. J. and Walker, B. R. (2000a). Understanding the role of glucocorticoids in obesity: Tissue-specific alterations of corticosterone metabolism in obese zucker rats. *Endocrinology* **141**(2): 560-3.

Livingstone, D. E., Kenyon, C. J. and Walker, B. R. (2000b). Mechanisms of dysregulation of 11 beta-hydroxysteroid dehydrogenase type 1 in obese zucker rats. *J Endocrinol* **167**(3): 533-9.

Livingstone, D. E. W., Grassick, S. L., Currie, G. L., Walker, B. R. and Andrew, R. (2009). Dysregulation of glucocorticoid metabolism in murine obesity: Comparable effects of leptin resistance and deficiency. *J Endocrinol* **201**(2): 211-218.

Livingstone, D. E. W., McInnes, K. J., Walker, B. R. and Andrew, R. (2005). Increased a-ring reduction of glucocorticoids in obese zucker rats: Effects of insulin sensitization[ast][ast]. *Obesity* **13**(9): 1523-1526.

Lohn, M., Dubrovska, G., Lauterbach, B., Luft, F. C., Gollasch, M. and Sharma, A. M. (2002). Periadventitial fat releases a vascular relaxing factor. *FASEB J.* **16**(9): 1057-1063.

Loos, R. J. and Bouchard, C. (2003). Obesity--is it a genetic disorder? *J Intern Med* **254**(5): 401-25.

Lorenz, M. W., Markus, H. S., Bots, M. L., Rosvall, M. and Sitzer, M. (2007). Prediction of clinical cardiovascular events with carotid intima-media thickness. *Circulation* **115**(4): 459-467.

Low, S. C., Assaad, S. N., Rajan, V., Chapman, K. E., Edwards, C. R. W. and Seckl, J. R. (1993). Regulation of 11{beta}-hydroxysteroid dehydrogenase by sex steroids in vivo: Further evidence for the existence of a second dehydrogenase in rat kidney. *J Endocrinol* **139**(1): 27-35.

Low, S. C., Chapman, K. E., Edwards, C. R., Wells, T., Robinson, I. C. and Seckl, J. R. (1994). Sexual dimorphism of hepatic 11 beta-hydroxysteroid dehydrogenase in the rat: The role of growth hormone patterns. *J Endocrinol* **143**(3): 541-8.

Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951). Protein measurement with the folin phenol reagent. *Journal of Biological Chemistry* **193**(1): 265-275.

Lu, D., Willard, D., Patel, I. R., Kadwell, S., Overton, L., Kost, T., Luther, M., Chen, W., Woychik, R. P., Wilkison, W. O. and et al. (1994). Agouti protein is an antagonist of the melanocyte-stimulating-hormone receptor. *Nature* **371**(6500): 799-802.

Ludmer, P. L., Selwyn, A. P., Shook, T. L., Wayne, R. R., Mudge, G. H., Alexander, R. W. and Ganz, P. (1986). Paradoxical vasoconstriction induced by acetylcholine in atherosclerotic coronary arteries. *New England Journal of Medicine* **315**(17): 1046-1051.

Lundberg, M. S. and Crow, M. T. (1999). Age-related changes in the signaling and function of vascular smooth muscle cells. *Experimental Gerontology* **34**(4): 549-557.

Luscher, T., Boulanger, C., Dohi, Y. and Yang, Z. (1992). Endothelium-derived contracting factors. *Hypertension* **19**(2): 117-130.

Ma, H., Sprecher, H. W. and Kolattukudy, P. E. (1998). Estrogen-induced production of a peroxisome proliferator-activated receptor (ppar) ligand in a pparî³-expressing tissue. *Journal of Biological Chemistry* **273**(46): 30131-30138.

Ma, X.-M., Camacho, C. and Aguilera, G. (2001). Regulation of corticotropin-releasing hormone (crh) transcription and crh mrna stability by glucocorticoids. *Cellular and Molecular Neurobiology* **21**(5): 465-475.

Macfarlane, D. P., Forbes, S. and Walker, B. R. (2008). Glucocorticoids and fatty acid metabolism in humans: Fuelling fat redistribution in the metabolic syndrome. *J Endocrinol* **197**(2): 189-204.

Maher, M. A., Banz, W. J., Truett, G. E. and Zemel, M. B. (1996). Dietary fat and sex modify heterozygote effects of the rat fatty (fa) allele. *J. Nutr.* **126**(10): 2487-2493.

Majmudar, N. G., Robson, S. C. and Ford, G. A. (2000). Effects of the menopause, gender, and estrogen replacement therapy on vascular nitric oxide activity. *J Clin Endocrinol Metab* **85**(4): 1577-83.

Mammi, C., Calanchini, M., Antelmi, A., Cinti, F., Rosano, G. M., Lenzi, A., Caprio, M. and Fabbri, A. (2012). Androgens and adipose tissue in males: A complex and reciprocal interplay. *Int J Endocrinol* **2012**: 789653.

Mangos, G. J., Walker, B. R., Kelly, J. J., Lawson, J. A., Webb, D. J. and Whitworth, J. A. (2000). Cortisol inhibits cholinergic vasodilatation in the human forearm. *Am J Hypertens* **13**(11): 1155-1160.

Manson, J. E., Hsia, J., Johnson, K. C., Rossouw, J. E., Assaf, A. R., Lasser, N. L., Trevisan, M., Black, H. R., Heckbert, S. R., Detrano, R., Strickland, O. L., Wong, N. D., Crouse, J. R., Stein, E. and Cushman, M. (2003). Estrogen plus progestin and the risk of coronary heart disease. *New England Journal of Medicine* **349**(6): 523-534.

Marin, P., Holmang, S., Gustafsson, C., Jonsson, L., Kvist, H., Elander, A., Eldh, J., Sjostrom, L., Holm, G. and Bjorntorp, P. (1993). Androgen treatment of abdominally obese men. *Obes Res* **1**(4): 245-51.

Marin, P., Krotkiewski, M. and Bjorntorp, P. (1992). Androgen treatment of middle-aged, obese men: Effects on metabolism, muscle and adipose tissues. *Eur J Med* **1**(6): 329-36.

Mark, A. L., Shaffer, R. A., Correia, M. L., Morgan, D. A., Sigmund, C. D. and Haynes, W. G. (1999). Contrasting blood pressure effects of obesity in leptin-deficient ob/ob mice and agouti yellow obese mice. *J Hypertens* **17**(12 Pt 2): 1949-53.

Marmorston, J., Moore, F. J., Hopkins, C. E., Kuzma, O. T. and Weiner, J. (1962). Clinical studies of long-term estrogen therapy in men with mvocardial infarction. *Proc Soc Exp Biol Med* **110**: 400-8.

Marsh, A. J., Fontes, M. A., Killinger, S., Pawlak, D. B., Polson, J. W. and Dampney, R. A. (2003). Cardiovascular responses evoked by leptin acting on neurons in the ventromedial and dorsomedial hypothalamus. *Hypertension* **42**(4): 488-93.

Martin-Gronert, M. S., Fernandez-Twinn, D. S., Poston, L. and Ozanne, S. E. (2010). Altered hepatic insulin signalling in male offspring of obese mice. *Journal of Developmental Origins of Health and Disease* **1**(03): 184-191.

Martin-Gronert, M. S. and Ozanne, S. E. (2010). Mechanisms linking suboptimal early nutrition and increased risk of type 2 diabetes and obesity. *J Nutr* **140**(3): 662-6.

Masuzaki, H., Paterson, J., Shinyama, H., Morton, N. M., Mullins, J. J., Seckl, J. R. and Flier, J. S. (2001). A transgenic model of visceral obesity and the metabolic syndrome. *Science* **294**(5549): 2166-70.

Matoba, T., Shimokawa, H., Nakashima, M., Hirakawa, Y., Mukai, Y., Hirano, K., Kanaide, H. and Takeshita, A. (2000). Hydrogen peroxide is an endothelium-derived hyperpolarizing factor in mice. *J Clin Invest* **106**(12): 1521-30.

Mattsson, C., Lai, M., Noble, J., McKinney, E., Yau, J. L., Seckl, J. R. and Walker, B. R. (2003). Obese zucker rats have reduced mineralocorticoid receptor and 11β-hydroxysteroid dehydrogenase type 1 expression in hippocampus; implications for dysregulation of the hypothalamic-pituitary-adrenal axis in obesity. *Endocrinology* **144**(7): 2997-3003.

Mattsson, C., Rask, E., Carlstrom, K., Andersson, J., Eliasson, M., Ahren, B., Soderberg, S. and Olsson, T. (2007). Gender-specific links between hepatic 11β reduction of cortisone and adipokines. *Obesity* **15**(4): 887-894.

Matute, M. L. and Kalkhoff, R. K. (1973). Sex steroid influence on hepatic gluconeogenesis and glycogen formation. *Endocrinology* **92**(3): 762-768.

Mayes, J. S. and Watson, G. H. (2004). Direct effects of sex steroid hormones on adipose tissues and obesity. *Obesity Reviews* **5**(4): 197-216.

McCulloch, A. I. and Randall, M. D. (1998). Sex differences in the relative contributions of nitric oxide and edhf to agonist-stimulated endothelium-dependent relaxations in the rat isolated mesenteric arterial bed. *Br J Pharmacol* **123**(8): 1700-6.

McCurdy, C., Bishop, J., Williams, S., Grayson, B., Smith, M., Friedman, J. and Grove, K. (2009). Maternal high-fat diet triggers lipotoxicity in the fetal livers of nonhuman primates. *J Clin Invest* **119**(2): 323 - 335.

McElroy, J. F. and Wade, G. N. (1987). Short- and long-term effects of ovariectomy on food intake, body weight, carcass composition, and brown adipose tissue in rats. *Physiol Behav* **39**(3): 361-5.

McGill, H. C., McMahan, C. A., Herderick, E. E., Zieske, A. W., Malcom, G. T., Tracy, R. E., Strong, J. P. and Group, f. t. P. D. o. A. i. Y. R. (2002). Obesity accelerates the progression of coronary atherosclerosis in young men. *Circulation* **105**(23): 2712-2718.

McInnes, K. J. (2010). Adipose-specific knockout of androgen receptors in mice results in hyperinsulinaemia without obesity. Society for Endocrinology. Manchester.

McInnes, K. J., Corbould, A., Simpson, E. R. and Jones, M. E. (2006). Regulation of adenosine 5',monophosphate-activated protein kinase and lipogenesis by androgens contributes to visceral obesity in an estrogen-deficient state. *Endocrinology* **147**(12): 5907-13.

McInnes, K. J., Kenyon, C. J., Chapman, K. E., Livingstone, D. E. W., Macdonald, L. J., Walker, B. R. and Andrew, R. (2004). 5{alpha}-reduced glucocorticoids, novel endogenous activators of the glucocorticoid receptor. *J. Biol. Chem.* **279**(22): 22908-22912.

McMillen, I. C. and Robinson, J. S. (2005). Developmental origins of the metabolic syndrome: Prediction, plasticity, and programming. *Physiol Rev* **85**(2): 571-633.

Medina-Gomez, G., Gray, S. and Vidal-Puig, A. (2007). Adipogenesis and lipotoxicity: Role of peroxisome proliferator-activated receptor γ (pparγ) and pparγ coactivator-1 (pgc1). *Public Health Nutrition* **10**(10A): 1132-1137.

Mehta, K., Van Thiel, D. H., Shah, N. and Mobarhan, S. (2002). Nonalcoholic fatty liver disease: Pathogenesis and the role of antioxidants. *Nutrition Reviews* **60**(9): 289-293.

Mendelsohn, M. E. and Karas, R. H. (2005). Molecular and cellular basis of cardiovascular gender differences. *Science* **308**(5728): 1583-1587.

Merialdi, M., Carroli, G., Villar, J., Abalos, E., Gulmezoglu, A. M., Kulier, R. and de Onis, M. (2003). Nutritional interventions during pregnancy for the prevention or treatment of impaired fetal growth: An overview of randomized controlled trials. *J Nutr* **133**(5 Suppl 2): 1626S-1631S.

Mesman, I., Roseboom, T. J., Bonsel, G. J., Gemke, R. J., van der Wal, M. F. and Vrijkotte, T. G. (2009). Maternal pre-pregnancy body mass index explains infant's weight and bmi at 14 months: Results from a multi-ethnic birth cohort study. *Arch Dis Child* **94**(8): 587-95.

Meyer, M. R., Clegg, D. J., Prossnitz, E. R. and Barton, M. (2011). Obesity, insulin resistance and diabetes: Sex differences and role of oestrogen receptors. *Acta Physiol (Oxf)* **203**(1): 259-69.

Meziane, H., Ouagazzal, A. M., Aubert, L., Wietrzych, M. and Krezel, W. (2007). Estrous cycle effects on behavior of c57bl/6j and balb/cbyj female mice: Implications for phenotyping strategies. *Genes Brain Behav* **6**(2): 192-200.

Michailidou, Z., Jensen, M. D., Dumesic, D. A., Chapman, K. E., Seckl, J. R., Walker, B. R. and Morton, N. M. (2007). Omental 11beta-hydroxysteroid dehydrogenase 1 correlates with fat cell size independently of obesity. *Obesity (Silver Spring)* **15**(5): 1155-63.

Miller, T. B. and Larner, J. (1973). Mechanism of control of hepatic glycogenesis by insulin. *Journal of Biological Chemistry* **248**(10): 3483-3488.

Miller, W. H., Jr., Faust, I. M. and Hirsch, J. (1984). Demonstration of de novo production of adipocytes in adult rats by biochemical and radioautographic techniques. *J Lipid Res* **25**(4): 336-47.

Mingrone, G., Manco, M., Mora, M. E., Guidone, C., Iaconelli, A., Gniuli, D., Leccesi, L., Chiellini, C. and Ghirlanda, G. (2008). Influence of maternal obesity on insulin sensitivity and secretion in offspring. *Diabetes Care* **31**(9): 1872-6.

Minnich, A., Tian, N., Byan, L. and Bilder, G. (2001). A potent pparî± agonist stimulates mitochondrial fatty acid î²-oxidation in liver and skeletal muscle. *American Journal of Physiology - Endocrinology And Metabolism* **280**(2): E270-E279.

Mitev, Y. A., Darwish, M., Wolf, S. S., Holsboer, F., Almeida, O. F. X. and Patchev, V. K. (2003). Gender differences in the regulation of 3î±-hydroxysteroid dehydrogenase in rat brain and sensitivity to neurosteroid-mediated stress protection. *Neuroscience* **120**(2): 541-549.

Molnar, J., Yu, S., Mzhavia, N., Pau, C., Chereshnev, I. and Dansky, H. M. (2005). Diabetes induces endothelial dysfunction but does not increase neointimal formation in high-fat diet fed c57bl/6j mice. *Circ Res* **96**(11): 1178-1184.

Monassier, L., Combe, R. and Fertak, L. E. (2006). Mouse models of hypertension. *Drug Discovery Today: Disease Models* **3**(3): 273-281.

Montano, M. M., Wang, M.-H., Even, M. D. and Vom Saal, F. S. (1991). Serum corticosterone in fetal mice: Sex differences, circadian changes, and effect of maternal stress. *Physiology & Behavior* **50**(2): 323-329.

Moran, A., Jacobs, D. R., Jr., Steinberger, J., Steffen, L. M., Pankow, J. S., Hong, C. P. and Sinaiko, A. R. (2008). Changes in insulin resistance and cardiovascular risk during adolescence: Establishment of differential risk in males and females. *Circulation* **117**(18): 2361-8.

Morton, N. M., Densmore, V., Wamil, M., Ramage, L., Nichol, K., Bunger, L., Seckl, J. R. and Kenyon, C. J. (2005). A polygenic model of the metabolic syndrome with reduced circulating and intra-adipose glucocorticoid action. *Diabetes* **54**(12): 3371-3378.

Morton, N. M., Holmes, M. C., Fievet, C., Staels, B., Tailleux, A., Mullins, J. J. and Seckl, J. R. (2001). Improved lipid and lipoprotein profile, hepatic insulin sensitivity, and glucose tolerance in 11beta-hydroxysteroid dehydrogenase type 1 null mice. *J Biol Chem* **276**(44): 41293-300.

Morton, N. M., Ramage, L. and Seckl, J. R. (2004). Down-regulation of adipose 11beta-hydroxysteroid dehydrogenase type 1 by high-fat feeding in mice: A potential adaptive mechanism counteracting metabolic disease. *Endocrinology* **145**(6): 2707-12.

Muller-Riemenschneider, F., Reinhold, T., Berghofer, A. and Willich, S. (2008). Health-economic burden of obesity in europe. *European Journal of Epidemiology* **23**(8): 499-509.

Mundy, A. L., Haas, E., Bhattacharya, I., Widmer, C. C., Kretz, M., Hofmann-Lehmann, R., Minotti, R. and Barton, M. (2007). Fat intake modifies vascular responsiveness and receptor expression of vasoconstrictors: Implications for diet-induced obesity. *Cardiovascular Research* **73**(2): 368-375.

Muniyappa, R., Montagnani, M., Koh, K. K. and Quon, M. J. (2007). Cardiovascular actions of insulin. *Endocr Rev* **28**(5): 463-491.

Muntzel, M. and Drueke, T. (1992). A comprehensive review of the salt and blood pressure relationship. *Am J Hypertens* **5**(4 Pt 1): 1S-42S.

Nakae, J., Kitamura, T., Silver, D. L. and Accili, D. (2001). The forkhead transcription factor foxo1 (fkhr) confers insulin sensitivity onto glucose-6-phosphatase expression. *The Journal of Clinical Investigation* **108**(9): 1359-1367.

Napoli, C., de Nigris, F., Welch, J. S., Calara, F. B., Stuart, R. O., Glass, C. K. and Palinski, W. (2002). Maternal hypercholesterolemia during pregnancy promotes early atherogenesis in ldl receptor-deficient mice and alters aortic gene expression determined by microarray. *Circulation* **105**(11): 1360-7.

Napoli, C., Glass, C. K., Witztum, J. L., Deutsch, R., D'Armiento, F. P. and Palinski, W. (1999). Influence of maternal hypercholesterolaemia during pregnancy on progression of early atherosclerotic lesions in childhood: Fate of early lesions in children (felic) study. *Lancet* **354**(9186): 1234-41.

Napoli, C., Witztum, J. L., Calara, F., de Nigris, F. and Palinski, W. (2000). Maternal hypercholesterolemia enhances atherogenesis in normocholesterolemic rabbits, which is inhibited by antioxidant or lipid-lowering intervention during pregnancy: An experimental model of atherogenic mechanisms in human fetuses. *Circ Res* **87**(10): 946-52.

Nechushtan, H., Benvenisty, N., Brandeis, R. and Reshef, L. (1987). Glucocorticoids control phosphoenolpyruvate carboxykinase gene expression in a tissue specific manner. *Nucleic Acids Research* **15**(16): 6405-6417.

Ng, S.-F., Lin, R. C. Y., Laybutt, D. R., Barres, R., Owens, J. A. and Morris, M. J. (2010). Chronic high-fat diet in fathers programs [bgr]-cell dysfunction in female rat offspring. *Nature* **467**(7318): 963-966.

Nikolsky, E., Kosinski, E., Mishkel, G. J., Kimmelstiel, C., McGarry, J. T. F., Mehran, R., Leon, M. B., Russell, M. E., Ellis, S. G. and Stone, G. W. (2005). Impact of obesity on revascularization and restenosis rates after bare-metal and drug-eluting stent implantation (from the taxus-iv trial). *The American Journal of Cardiology* **95**(6): 709-715.

Nishikawa, S., Yasoshima, A., Doi, K., Nakayama, H. and Uetsuka, K. (2007). Involvement of sex, strain and age factors in high fat diet-induced obesity in c57bl/6j and balb/ca mice. *Exp Anim* **56**(4): 263-72.

Nivoit, P., Morens, C., Van Assche, F. A., Jansen, E., Poston, L., Remacle, C. and Reusens, B. (2009). Established diet-induced obesity in female rats leads to offspring hyperphagia, adiposity and insulin resistance. *Diabetologia* **52**(6): 1133-42.

Nolten, W. E., Lindheimer, M. D., Rueckert, P. A., Oparil, S. and Ehrlich, E. N. (1980). Diurnal patterns and regulation of cortisol secretion in pregnancy. *Journal of Clinical Endocrinology & Metabolism* **51**(3): 466-472.

Noonan, W. T. and Banks, R. O. (2000). Renal function and glucose transport in male and female mice with diet-induced type ii diabetes mellitus. *Experimental Biology and Medicine* **225**(3): 221-230.

Norman, J. F. and LeVeen, R. F. (2001). Maternal atherogenic diet in swine is protective against early atherosclerosis development in offspring consuming an atherogenic diet post-natally. *Atherosclerosis* **157**(1): 41-7.

Normington, K. and Russell, D. W. (1992). Tissue distribution and kinetic characteristics of rat steroid 5 alpha-reductase isozymes. Evidence for distinct physiological functions. *J Biol Chem* **267**(27): 19548-54.

Noronha, B. T., Li, J. M., Wheatcroft, S. B., Shah, A. M. and Kearney, M. T. (2005). Inducible nitric oxide synthase has divergent effects on vascular and metabolic function in obesity. *Diabetes* **54**(4): 1082-9.

Nussey, S. and Whitehead, S. (2001). Endocrinology: An integrated approach.

Nuutila, P., Knuuti, M. J., MÃ¤ki, M., Laine, H., Ruotsalainen, U., TerÃ¤s, M., Haaparanta, M., Solin, O. and Yki-JÃ¤rvinen, H. (1995). Gender and insulin sensitivity in the heart and in skeletal muscles. Studies using positron emission tomography. *Diabetes* **44**(1): 31-36.

Nwe, K. H., Hamid, A., Morat, P. B. and Khalid, B. A. (2000). Differential regulation of the oxidative 11beta-hydroxysteroid dehydrogenase activity in testis and liver. *Steroids* **65**(1): 40-5.

Nyirenda, M. J., Lindsay, R. S., Kenyon, C. J., Burchell, A. and Seckl, J. R. (1998). Glucocorticoid exposure in late gestation permanently programs rat hepatic phosphoenolpyruvate carboxykinase and glucocorticoid receptor expression and causes glucose intolerance in adult offspring. *J Clin Invest* **101**(10): 2174-81.

O'Brien, R., Lucas, P., Forest, C., Magnuson, M. and Granner, D. (1990). Identification of a sequence in the pepck gene that mediates a negative effect of insulin on transcription. *Science* **249**(4968): 533-537.

O'Leary, D. H., Polak, J. F., Kronmal, R. A., Manolio, T. A., Burke, G. L. and Wolfson, S. K., Jr. (1999). Carotid-artery intima and media thickness as a risk factor for myocardial infarction and stroke in older adults. Cardiovascular health study collaborative research group. *N Engl J Med* **340**(1): 14-22.

O'Meara, J. G., Kardia, S. L. R., Armon, J. J., Brown, C. A., Boerwinkle, E. and Turner, S. T. (2004). Ethnic and sex differences in the prevalence, treatment, and control of dyslipidemia among hypertensive adults in the genoa study. *Arch Intern Med* **164**(12): 1313-1318.

O'Regan, D., Kenyon, C. J., Seckl, J. R. and Holmes, M. C. (2004). Glucocorticoid exposure in late gestation in the rat permanently programs gender-specific differences in adult cardiovascular and metabolic physiology. *Am J Physiol Endocrinol Metab* **287**(5): E863-870.

O'Regan, D., Kenyon, C. J., Seckl, J. R. and Holmes, M. C. (2008). Prenatal dexamethasone 'programmes' hypotension, but stress-induced hypertension in adult offspring. *J Endocrinol* **196**(2): 343-352.

O'Sullivan, A. J. and Ho, K. K. (1995). A comparison of the effects of oral and transdermal estrogen replacement on insulin sensitivity in postmenopausal women. *Journal of Clinical Endocrinology & Metabolism* **80**(6): 1783-8.

Ogden, C. L., Carroll, M. D., Curtin, L. R., McDowell, M. A., Tabak, C. J. and Flegal, K. M. (2006). Prevalence of overweight and obesity in the united states, 1999-2004. *JAMA* **295**(13): 1549-55.

Ohlsson, C., Hellberg, N., Parini, P., Vidal, O., Bohlooly, M., Rudling, M., Lindberg, M. K., Warner, M., Angelin, B. and Gustafsson, J.-Ã. k. (2000). Obesity and disturbed lipoprotein profile in estrogen receptor-î±-deficient male mice. *Biochemical and Biophysical Research Communications* **278**(3): 640-645.

Olswang, Y., Blum, B., Cassuto, H., Cohen, H., Biberman, Y., Hanson, R. W. and Reshef, L. (2003). Glucocorticoids repress transcription of phosphoenolpyruvate carboxykinase (gtp) gene in adipocytes by inhibiting its c/ebp-mediated activation. *Journal of Biological Chemistry* **278**(15): 12929-12936.

Olswang, Y., Cohen, H., Papo, O., Cassuto, H., Croniger, C. M., Hakimi, P., Tilghman, S. M., Hanson, R. W. and Reshef, L. (2002). A mutation in the peroxisome proliferator-activated receptor î³-binding site in the gene for the cytosolic form of phosphoenolpyruvate carboxykinase reduces adipose tissue size and fat content in mice. *Proceedings of the National Academy of Sciences of the United States of America* **99**(2): 625-630.

Oltman, C. L., Coppey, L. J., Gellett, J. S., Davidson, E. P., Lund, D. D. and Yorek, M. A. (2005). Progression of vascular and neural dysfunction in sciatic nerves of zucker diabetic fatty and zucker rats. *American Journal of Physiology - Endocrinology And Metabolism* **289**(1): E113-E122.

Oltman, C. L., Richou, L. L., Davidson, E. P., Coppey, L. J., Lund, D. D. and Yorek, M. A. (2006). Progression of coronary and mesenteric vascular dysfunction in zucker obese and zucker diabetic fatty rats. *American Journal of Physiology - Heart and Circulatory Physiology* **291**(4): H1780-H1787.

Oosterveer, M. H., van Dijk, T. H., Tietge, U. J., Boer, T., Havinga, R., Stellaard, F., Groen, A. K., Kuipers, F. and Reijngoud, D. J. (2009). High fat feeding induces hepatic fatty acid elongation in mice. *PLoS One* **4**(6): e6066.

Ozaki, T., Nishina, H., Hanson, M. A. and Poston, L. (2001). Dietary restriction in pregnant rats causes gender-related hypertension and vascular dysfunction in offspring. *J Physiol* **530**(Pt 1): 141-52.

Ozanne, S. E., Smith, G. D., Tikerpae, J. and Hales, C. N. (1996a). Altered regulation of hepatic glucose output in the male offspring of protein-malnourished rat dams. *Am J Physiol* **270**(4 Pt 1): E559-64.

Ozanne, S. E., Wang, C. L., Coleman, N. and Smith, G. D. (1996b). Altered muscle insulin sensitivity in the male offspring of protein-malnourished rats. *Am J Physiol* **271**(6 Pt 1): E1128-34.

Ozols, J. (1995). Lumenal orientation and post-translational modifications of the liver microsomal 11-hydroxysteroid dehydrogenase. *Journal of Biological Chemistry* **270**(5): 2305-2312.

Pagliassotti, M. J., Knobel, S. M., Shahrokhi, K. A., Manzo, A. M. and Hill, J. O. (1994). Time course of adaptation to a high-fat diet in obesity-resistant and obesity-prone rats. *Am J Physiol Regul Integr Comp Physiol* **267**(3): R659-664.

Pagliassotti, M. J., Prach, P. A., Koppenhafer, T. A. and Pan, D. A. (1996). Changes in insulin action, triglycerides, and lipid composition during sucrose feeding in rats. *Am J Physiol Regul Integr Comp Physiol* **271**(5): R1319-1326.

Paik, S. G., Michelis, M. A., Kim, Y. T. and Shin, S. (1982). Induction of insulin-dependent diabetes by streptozotocin. Inhibition by estrogens and potentiation by androgens. *Diabetes* **31**(8): 724-729.

Palermo, M., Marazzi, M. G., Hughes, B. A., Stewart, P. M., Clayton, P. T. and Shackleton, C. H. (2008). Human delta4-3-oxosteroid 5beta-reductase (akr1d1) deficiency and steroid metabolism. *Steroids* **73**(4): 417-23.

Palinski, W., D'Armiento, F. P., Witztum, J. L., de Nigris, F., Casanada, F., Condorelli, M., Silvestre, M. and Napoli, C. (2001). Maternal hypercholesterolemia and treatment during pregnancy influence the long-term progression of atherosclerosis in offspring of rabbits. *Circ Res* **89**(11): 991-6.

Park, S.-H., Marso, S. P., Zhou, Z., Foroudi, F., Topol, E. J. and Lincoff, A. M. (2001). Neointimal hyperplasia after arterial injury is increased in a rat model of non-insulin-dependent diabetes mellitus. *Circulation* **104**(7): 815-819.

Pasquali, R. (2006). Obesity and androgens: Facts and perspectives. *Fertil Steril* **85**(5): 1319-40.

Paterson, J. M., Morton, N. M., Fievet, C., Kenyon, C. J., Holmes, M. C., Staels, B., Seckl, J. R. and Mullins, J. J. (2004). Metabolic syndrome without obesity: Hepatic overexpression of 11Î²-hydroxysteroid dehydrogenase type 1 in transgenic mice. *Proceedings of the National Academy of Sciences of the United States of America* **101**(18): 7088-7093.

Paulsen, S. K., Nielsen, M. P., Richelsen, B., Bruun, J. M., Flyvbjerg, A. and Pedersen, S. B. (2008). Upregulation of adipose 11-beta-hydroxysteroid dehydrogenase type 1 expression in ovariectomized rats is due to obesity rather than lack of estrogen. *Obesity (Silver Spring)* **16**(4): 731-5.

Paulsen, S. K., Pedersen, S. B., Fisker, S. and Richelsen, B. (2007). 11beta-hsd type 1 expression in human adipose tissue: Impact of gender, obesity, and fat localization. *Obesity (Silver Spring)* **15**(8): 1954-60.

Pedersen, S. B., Fuglsig, S., Sjogren, P. and Richelsen, B. (1996). Identification of steroid receptors in human adipose tissue. *Eur J Clin Invest* **26**(12): 1051-6.

Phillips, M. S., Liu, Q., Hammond, H. A., Dugan, V., Hey, P. J., Caskey, C. J. and Hess, J. F. (1996). Leptin receptor missense mutation in the fatty zucker rat. *Nat Genet* **13**(1): 18-9.

Piatti, P., Di Mario, C., Monti, L. D., Fragasso, G., Sgura, F., Caumo, A., Setola, E., Lucotti, P., Galluccio, E., Ronchi, C., Origgi, A., Zavaroni, I., Margonato, A. and Colombo, A. (2003). Association of insulin resistance, hyperleptinemia, and impaired nitric oxide release with in-stent restenosis in patients undergoing coronary stenting. *Circulation* **108**(17): 2074-2081.

Picard, F., Boivin, A., Lalonde, J. and Deshaies, Y. (2002). Resistance of adipose tissue lipoprotein lipase to insulin action in rats fed an obesity-promoting diet. *American Journal of Physiology - Endocrinology And Metabolism* **282**(2): E412-E418.

Pilkis, S. J. and Granner, D. K. (1992). Molecular physiology of the regulation of hepatic gluconeogenesis and glycolysis. *Annual Review of Physiology* **54**(1): 885-909.

Plagemann, A., Harder, T., Brunn, M., Harder, A., Roepke, K., Wittrock-Staar, M., Ziska, T., Schellong, K., Rodekamp, E., Melchior, K. and Dudenhausen, J. W. (2009). Hypothalamic proopiomelanocortin promoter methylation becomes altered by early overfeeding: An epigenetic model of obesity and the metabolic syndrome. *The Journal of Physiology* **587**(20): 4963-4976.

Poehlman, E. T., Toth, M. J. and Gardner, A. W. (1995). Changes in energy balance and body composition at menopause: A controlled longitudinal study. *Annals of Internal Medicine* **123**(9): 673-675.

Pouliot, M. C., Despres, J. P., Nadeau, A., Moorjani, S., Prud'Homme, D., Lupien, P. J., Tremblay, A. and Bouchard, C. (1992). Visceral obesity in men. Associations with glucose tolerance, plasma insulin, and lipoprotein levels. *Diabetes* **41**(7): 826-34.

Power, M. L. and Schulkin, J. (2008). Sex differences in fat storage, fat metabolism, and the health risks from obesity: Possible evolutionary origins. *Br J Nutr* **99**(5): 931-40.

Preiss-Landl, K., Zimmermann, R., Hammerle, G. and Zechner, R. (2002). Lipoprotein lipase: The regulation of tissue specific expression and its role in lipid and energy metabolism. *Curr Opin Lipidol* **13**(5): 471-81.

Prentice, A., Cole, T., Foord, F., Lamb, W. and Whitehead, R. (1987). Increased birthweight after prenatal dietary supplementation of rural african women. *The American Journal of Clinical Nutrition* **46**(6): 912-925.

Puah, J. A. and Bailey, C. J. (1985). Effect of ovarian hormones on glucose metabolism in mouse soleus muscle. *Endocrinology* **117**(4): 1336-1340.

Pugeat, M., Moulin, P., Cousin, P., Fimbel, S., Nicolas, M. H. l. n., Crave, J. C. and Lejeune, H. (1995). Interrelations between sex hormone-binding globulin (shbg), plasma lipoproteins and cardiovascular risk. *The Journal of Steroid Biochemistry and Molecular Biology* **53**(1-6): 567-572.

Purkayastha, S., Zhang, G. and Cai, D. (2011). Uncoupling the mechanisms of obesity and hypertension by targeting hypothalamic ikk-[beta] and nf-[kappa]b. *Nat Med* **17**(7): 883-887.

Rabe, K., Lehrke, M., Parhofer, K. G. and Broedl, U. C. (2008). Adipokines and insulin resistance. *Mol Med* **14**(11-12): 741-51.

Rahmouni, K., Morgan, D. A., Morgan, G. M., Mark, A. L. and Haynes, W. G. (2005). Role of selective leptin resistance in diet-induced obesity hypertension. *Diabetes* **54**(7): 2012-2018.

Ramsay, J. E., Ferrell, W. R., Crawford, L., Wallace, A. M., Greer, I. A. and Sattar, N. (2002). Maternal obesity is associated with dysregulation of metabolic, vascular, and inflammatory pathways. *J Clin Endocrinol Metab* **87**(9): 4231-7.

Rankinen, T., Zuberi, A., Chagnon, Y. C., Weisnagel, S. J., Argyropoulos, G., Walts, B., Perusse, L. and Bouchard, C. (2006). The human obesity gene map: The 2005 update. *Obesity (Silver Spring)* **14**(4): 529-644.

Rask, E., Olsson, T., Soderberg, S., Andrew, R., Livingstone, D. E., Johnson, O. and Walker, B. R. (2001). Tissue-specific dysregulation of cortisol metabolism in human obesity. *J Clin Endocrinol Metab* **86**(3): 1418-21.

Rask, E., Walker, B. R., Soderberg, S., Livingstone, D. E., Eliasson, M., Johnson, O., Andrew, R. and Olsson, T. (2002). Tissue-specific changes in peripheral cortisol metabolism in obese women: Increased adipose 11beta-hydroxysteroid dehydrogenase type 1 activity. *J Clin Endocrinol Metab* **87**(7): 3330-6.

Ravelli, A. C., van der Meulen, J. H., Michels, R. P., Osmond, C., Barker, D. J., Hales, C. N. and Bleker, O. P. (1998). Glucose tolerance in adults after prenatal exposure to famine. *Lancet* **351**(9097): 173-7.

Rebholz, S. L., Jones, T., Burke, K. T., Jaeschke, A., Tso, P., D'Alessio, D. A. and Woollett, L. A. (2011). Multiparity leads to obesity and inflammation in mothers and obesity in male offspring. *American Journal of Physiology - Endocrinology And Metabolism* **302**(4): E449-E457.

Rebuffe-Scrive, M., Surwit, R., Feinglos, M., Kuhn, C. and Rodin, J. (1993). Regional fat distribution and metabolism in a new mouse model (c57bl/6j) of non-insulin-dependent diabetes mellitus. *Metabolism* **42**(11): 1405-9.

Reckelhoff, J. F. (2001). Gender differences in the regulation of blood pressure. *Hypertension* **37**(5): 1199-1208.

Reddy, T. E., Pauli, F., Sprouse, R. O., Neff, N. F., Newberry, K. M., Garabedian, M. J. and Myers, R. M. (2009). Genomic determination of the glucocorticoid response reveals unexpected mechanisms of gene regulation. *Genome Res* **19**(12): 2163-71.

Reid, B. N., Ables, G. P., Otlivanchik, O. A., Schoiswohl, G., Zechner, R., Blaner, W. S., Goldberg, I. J., Schwabe, R. F., Chua, S. C., Jr. and Huang, L. S. (2008). Hepatic overexpression of hormone-sensitive lipase and adipose triglyceride lipase promotes fatty acid oxidation, stimulates direct release of free fatty acids, and ameliorates steatosis. *J Biol Chem* **283**(19): 13087-99.

Renato, P. (2006). Obesity and androgens: Facts and perspectives. *Fertility and Sterility* **85**(5): 1319-1340.

Renoir, J. M., Mercier-Bodard, C. and Baulieu, E. E. (1980). Hormonal and immunological aspects of the phylogeny of sex steroid binding plasma protein. *Proc Natl Acad Sci U S A* **77**(8): 4578-82.

Reshef, L., Olswang, Y., Cassuto, H., Blum, B., Croniger, C. M., Kalhan, S. C., Tilghman, S. M. and Hanson, R. W. (2003). Glyceroneogenesis and the triglyceride/fatty acid cycle. *J Biol Chem* **278**(33): 30413-6.

Reynolds, R. M., Osmond, C., Phillips, D. I. and Godfrey, K. M. (2010). Maternal bmi, parity, and pregnancy weight gain: Influences on offspring adiposity in young adulthood. *J Clin Endocrinol Metab* **95**(12): 5365-9.

Reynolds, R. M., Walker, B. R., Syddall, H. E., Andrew, R., Wood, P. J., Whorwood, C. B. and Phillips, D. I. W. (2001). Altered control of cortisol secretion in adult men with low birth weight and cardiovascular risk factors. *Journal of Clinical Endocrinology & Metabolism* **86**(1): 245-250.

Riant, E., Waget, A., Cogo, H., Arnal, J.-F., Burcelin, R. and Gourdy, P. (2009). Estrogens protect against high-fat diet-induced insulin resistance and glucose intolerance in mice. *Endocrinology*: en.2008-0971.

Rinaldo, P., Matern, D. and Bennett, M. J. (2002). Fatty acid oxidation disorders. *Annu Rev Physiol* **64**: 477-502.

Rivas, A., Fisher, J. S., McKinnell, C., Atanassova, N. and Sharpe, R. M. (2002). Induction of reproductive tract developmental abnormalities in the male rat by lowering androgen production or action in combination with a low dose of diethylstilbestrol: Evidence for importance of the androgen-estrogen balance. *Endocrinology* **143**(12): 4797-4808.

Rizner, T. L. n., Lin, H. K., Peehl, D. M., Steckelbroeck, S., Bauman, D. R. and Penning, T. M. (2003). Human type 3 α-hydroxysteroid dehydrogenase (aldo-keto reductase 1c2) and androgen metabolism in prostate cells. *Endocrinology* **144**(7): 2922-2932.

Rodrigues, A. L., de Moura, E. G., Fonseca Passos, M. C., Potente Dutra, S. C. and Lisboa, P. C. (2009). Postnatal early overnutrition changes the leptin signalling pathway in the hypothalamic-pituitary-thyroid axis of young and adult rats. *The Journal of Physiology* **587**(11): 2647-2661.

Rodriguez-Cuenca, S., Monjo, M., Proenza, A. M. and Roca, P. (2005). Depot differences in steroid receptor expression in adipose tissue: Possible role of the local steroid milieu. *American Journal of Physiology - Endocrinology And Metabolism* **288**(1): E200-E207.

Rolls, B. A., Gurr, M. I., Van Duijvenvoorde, P. M., Rolls, B. J. and Rowe, E. A. (1986). Lactation in lean and obese rats: Effect of cafeteria feeding and of dietary obesity on milk composition. *Physiology & Behavior* **38**(2): 185-190.

Rolls, B. J., Rowe, E. A. and Turner, R. C. (1980). Persistent obesity in rats following a period of consumption of a mixed, high energy diet. *J Physiol* **298**: 415-27.

Roseboom, T. J., van der Meulen, J. H., Ravelli, A. C., van Montfrans, G. A., Osmond, C., Barker, D. J. and Bleker, O. P. (1999). Blood pressure in adults after prenatal exposure to famine. *J Hypertens* **17**(3): 325-30.

Rosella, G., Zajac, J. D., Kaczmarczyk, S. J., Andrikopoulos, S. and Proietto, J. (1993). Impaired suppression of gluconeogenesis induced by overexpression of a noninsulin-responsive phosphoenolpyruvate carboxykinase gene. *Molecular Endocrinology* **7**(11): 1456-62.

Rosen, E. D., Sarraf, P., Troy, A. E., Bradwin, G., Moore, K., Milstone, D. S., Spiegelman, B. M. and Mortensen, R. M. (1999). Ppar gamma is required for the differentiation of adipose tissue in vivo and in vitro. *Mol Cell* **4**(4): 611-7.

Rosenstock, J., Banarer, S., Fonseca, V. A., Inzucchi, S. E., Sun, W., Yao, W., Hollis, G., Flores, R., Levy, R., Williams, W. V., Seckl, J. R. and Huber, R. (2010). The 11-beta-hydroxysteroid dehydrogenase type 1 inhibitor incb13739 improves hyperglycemia in patients with type 2 diabetes inadequately controlled by metformin monotherapy. *Diabetes Care* **33**(7): 1516-22.

Ross, R. (1999). Atherosclerosis--an inflammatory disease. *N Engl J Med* **340**(2): 115-26.

Ross, R., Aru, J., Freeman, J., Hudson, R. and Janssen, I. (2002a). Abdominal adiposity and insulin resistance in obese men. *Am J Physiol Endocrinol Metab* **282**(3): E657-63.

Ross, R., Freeman, J., Hudson, R. and Janssen, I. (2002b). Abdominal obesity, muscle composition, and insulin resistance in premenopausal women. *J Clin Endocrinol Metab* **87**(11): 5044-51.

Rotteveel, J., van Weissenbruch, M. M., Twisk, J. W. and Delemarre-Van de Waal, H. A. (2008). Infant and childhood growth patterns, insulin sensitivity, and blood pressure in prematurely born young adults. *Pediatrics* **122**(2): 313-21.

Rueda-Clausen, C. F., Lahera, V., Calderón, J., Bolivar, I. C., Castillo, V. R., Gutiérrez, M., Carreño, M., Oubiña, M. d. P., Cachofeiro, V. and López-Jaramillo, P. (2010). The presence of abdominal obesity is associated with changes in vascular function independently of other cardiovascular risk factors. *International Journal of Cardiology* **139**(1): 32-41.

Rupprecht, R., Arriza, J. L., Spengler, D., Reul, J. M., Evans, R. M., Holsboer, F. and Damm, K. (1993). Transactivation and synergistic properties of the mineralocorticoid receptor: Relationship to the glucocorticoid receptor. *Mol Endocrinol* **7**(4): 597-603.

Russell, D. W. and Wilson, J. D. (1994). Steroid 5 alpha-reductase: Two genes/two enzymes. *Annu Rev Biochem* **63**: 25-61.

Sader, M. A. and Celermajer, D. S. (2002). Endothelial function, vascular reactivity and gender differences in the cardiovascular system. *Cardiovascular Research* **53**(3): 597-604.

Saito, T., Ciobotaru, A., Bopassa, J. C., Toro, L., Stefani, E. and Eghbali, M. (2009). Estrogen contributes to gender differences in mouse ventricular repolarization. *Circulation Research* **105**(4): 343-352.

Salpeter, S. R., Walsh, J. M. E., Ormiston, T. M., Greyber, E., Buckley, N. S. and Salpeter, E. E. (2006). Meta-analysis: Effect of hormone-replacement therapy on components of the metabolic syndrome in postmenopausal women. *Diabetes, Obesity and Metabolism* **8**(5): 538-554.

Samuelsson, A.-M., Morris, A., Igosheva, N., Kirk, S. L., Pombo, J. M. C., Coen, C. W., Poston, L. and Taylor, P. D. (2010). Evidence for sympathetic origins of hypertension in juvenile offspring of obese rats. *Hypertension* **55**(1): 76-82.

Samuelsson, A. M., Matthews, P. A., Argenton, M., Christie, M. R., McConnell, J. M., Jansen, E. H., Piersma, A. H., Ozanne, S. E., Twinn, D. F., Remacle, C., Rowlerson, A., Poston, L. and Taylor, P. D. (2008). Diet-induced obesity in female mice leads to offspring hyperphagia, adiposity, hypertension, and insulin resistance: A novel murine model of developmental programming. *Hypertension* **51**(2): 383-92.

Sandeep, T. C., Andrew, R., Homer, N. Z., Andrews, R. C., Smith, K. and Walker, B. R. (2005). Increased in vivo regeneration of cortisol in adipose tissue in human obesity and effects of the 11beta-hydroxysteroid dehydrogenase type 1 inhibitor carbenoxolone. *Diabetes* **54**(3): 872-9.

Sasaki, K., Cripe, T. P., Koch, S. R., Andreone, T. L., Petersen, D. D., Beale, E. G. and Granner, D. K. (1984). Multihormonal regulation of phosphoenolpyruvate carboxykinase gene transcription. The dominant role of insulin. *J Biol Chem* **259**(24): 15242-51.

Sata, M., Maejima, Y., Adachi, F., Fukino, K., Saiura, A., Sugiura, S., Aoyagi, T., Imai, Y., Kurihara, H., Kimura, K., Omata, M., Makuuchi, M., Hirata, Y. and Nagai, R. (2000). A mouse model of vascular injury that induces rapid onset of medial cell apoptosis followed by reproducible neointimal hyperplasia. *Journal of Molecular and Cellular Cardiology* **32**(11): 2097-2104.

Sato, T., Matsumoto, T., Yamada, T., Watanabe, T., Kawano, H. and Kato, S. (2003). Late onset of obesity in male androgen receptor-deficient (ar ko) mice. *Biochemical and Biophysical Research Communications* **300**(1): 167-171.

Savage, D., Perkins, J., Hong Lim, C. and Bund, S. J. (2003). Functional evidence that k+ is the non-nitric oxide, non-prostanoid endothelium-derived relaxing factor in rat femoral arteries. *Vascul Pharmacol* **40**(1): 23-8.

Sayer, A. A., Cooper, C. and Barker, D. J. P. (1997). Is lifespan determined in utero? *Archives of Disease in Childhood - Fetal and Neonatal Edition* **77**(3): F162-F164.

Schafer, K., Halle, M., Goeschen, C., Dellas, C., Pynn, M., Loskutoff, D. J. and Konstantinides, S. (2004). Leptin promotes vascular remodeling and neointimal growth in mice. *Arterioscler Thromb Vasc Biol* **24**(1): 112-117.

Schlender, K. K. and Reimann, E. M. (1977). Glycogen synthase kinases. Distribution in mammalian tissues of forms that are independent of cyclic amp. *J Biol Chem* **252**(7): 2384-9.

Schroeder, A., Mueller, O., Stocker, S., Salowsky, R., Leiber, M., Gassmann, M., Lightfoot, S., Menzel, W., Granzow, M. and Ragg, T. (2006). The rin: An rna integrity number for assigning integrity values to rna measurements. *BMC Molecular Biology* **7**(1): 3.

Schultz, K. and Schultz, G. (1977). Sodium nitroprusside and other smooth muscle-relaxants increase cyclic gmp levels in rat ductus deferens. *Nature* **265**(5596): 750-1.

Schweiger, M., Schreiber, R., Haemmerle, G., Lass, A., Fledelius, C., Jacobsen, P., Tornqvist, H., Zechner, R. and Zimmermann, R. (2006). Adipose triglyceride lipase and hormone-sensitive lipase are the major enzymes in adipose tissue triacylglycerol catabolism. *Journal of Biological Chemistry* **281**(52): 40236-40241.

SDSM (Scottish Diabetes Survery Monitoring) Group, (2011). Scottish diabetes survey 2010, http://www.diabetesinscotland.org.uk/Publications/SDS%202010.pdf

Seckl, J. R., Morton, N. M., Chapman, K. E. and Walker, B. R. (2004). Glucocorticoids and 11beta-hydroxysteroid dehydrogenase in adipose tissue. *Recent Prog Horm Res* **59**: 359-93.

Seckl, J. R. and Walker, B. R. (2001). Minireview: 11beta-hydroxysteroid dehydrogenase type 1- a tissue-specific amplifier of glucocorticoid action. *Endocrinology* **142**(4): 1371-6.

Shankar, K., Harrell, A., Liu, X., Gilchrist, J. M., Ronis, M. J. and Badger, T. M. (2008). Maternal obesity at conception programs obesity in the offspring. *Am J Physiol Regul Integr Comp Physiol* **294**(2): R528-38.

Sharpe, J., Ahlgren, U., Perry, P., Hill, B., Ross, A., Hecksher-Sorensen, J., Baldock, R. and Davidson, D. (2002). Optical projection tomography as a tool for 3d microscopy and gene expression studies. *Science* **296**(5567): 541-5.

Shelley, P., Martin-Gronert, M. S., Rowlerson, A., Poston, L., Heales, S. J., Hargreaves, I. P., McConnell, J. M., Ozanne, S. E. and Fernandez-Twinn, D. S. (2009). Altered skeletal muscle insulin signaling and mitochondrial complex ii-iii linked activity in adult offspring of obese mice. *Am J Physiol Regul Integr Comp Physiol* **297**(3): R675-81.

Shiell, A. W., Campbell, D. M., Hall, M. H. and Barker, D. J. (2000). Diet in late pregnancy and glucose-insulin metabolism of the offspring 40 years later. *BJOG* **107**(7): 890-5.

Shimomura, Y., Bray, G. A. and Lee, M. (1987). Adrenalectomy and steroid treatment in obese (ob/ob) and diabetic (db/db) mice. *Horm Metab Res* **19**(7): 295-9.

Short, M. K., Clouthier, D. E., Schaefer, I. M., Hammer, R. E., Magnuson, M. A. and Beale, E. G. (1992). Tissue-specific, developmental, hormonal, and dietary regulation of rat phosphoenolpyruvate carboxykinase-human growth hormone fusion genes in transgenic mice. *Mol Cell Biol* **12**(3): 1007-20.

Shughrue, P. J., Lane, M. V. and Merchenthaler, I. (1997). Comparative distribution of estrogen receptor-α and -β mrna in the rat central nervous system. *The Journal of Comparative Neurology* **388**(4): 507-525.

Siiteri, P. K. and Wilson, J. D. (1974). Testosterone formation and metabolism during male sexual differentiation in the human embryo. *Journal of Clinical Endocrinology & Metabolism* **38**(1): 113-125.

Simonyte, K., Rask, E., Naslund, I., Angelhed, J. E., Lonn, L., Olsson, T. and Mattsson, C. (2009). Obesity is accompanied by disturbances in peripheral glucocorticoid metabolism and changes in fa recycling. *Obesity (Silver Spring)* **17**(11): 1982-7.

Simpson, E. R. and Waterman, M. R. (1988). Regulation of the synthesis of steroidogenic enzymes in adrenal cortical cells by acth. *Annu Rev Physiol* **50**: 427-40.

Sinning, C., Wild, P. S., Echevarria, F. M. O., Wilde, S., Schnabel, R., Lubos, E., Herkenhoff, S., Bickel, C., Klimpe, S., Gori, T., Mänzel, T. F., Blankenberg, S. and Espinola-Klein, C. (2011). Sex differences in early carotid atherosclerosis (from the community-based gutenberg-heart study). *The American Journal of Cardiology* **107**(12): 1841-1847.

Skolleborg, K. C., Gronbech, J. E., Grong, K., Abyholm, F. E. and Lekven, J. (1990). Distribution of cardiac output during pentobarbital versus midazolam/fentanyl/fluanisone anaesthesia in the rat. *Lab Anim* **24**(3): 221-7.

Smoak, K. A. and Cidlowski, J. A. (2004). Mechanisms of glucocorticoid receptor signaling during inflammation. *Mechanisms of Ageing and Development* **125**(10â€“11): 697-706.

Sorensen, M. B., Rosenfalck, A. M., Hojgaard, L. and Ottesen, B. (2001). Obesity and sarcopenia after menopause are reversed by sex hormone replacement therapy. *Obesity* **9**(10): 622-626.

Soukas, A., Socci, N. D., Saatkamp, B. D., Novelli, S. and Friedman, J. M. (2001). Distinct transcriptional profiles of adipogenesis in vivo and in vitro. *J Biol Chem* **276**(36): 34167-74.

Spagnoli, L. G., Orlandi, A., Mauriello, A., Santeusanio, G., de Angelis, C., Lucreziotti, R. and Ramacci, M. T. (1991). Aging and atherosclerosis in the rabbit : 1. Distribution, prevalence and morphology of atherosclerotic lesions. *Atherosclerosis* **89**(1): 11-24.

Srinivasan, M., Katewa, S. D., Palaniyappan, A., Pandya, J. D. and Patel, M. S. (2006). Maternal high-fat diet consumption results in fetal malprogramming predisposing to the

onset of metabolic syndrome-like phenotype in adulthood. *Am J Physiol Endocrinol Metab* **291**(4): E792-9.

Stahn, C. and Buttgereit, F. (2008). Genomic and nongenomic effects of glucocorticoids. *Nat Clin Pract Rheumatol* **4**(10): 525-33.

Stampfer, M. J., Colditz, G. A., Willett, W. C., Manson, J. E., Rosner, B., Speizer, F. E. and Hennekens, C. H. (1991). Postmenopausal estrogen therapy and cardiovascular disease. *New England Journal of Medicine* **325**(11): 756-762.

Stanner, S. A., Bulmer, K., Andres, C., Lantseva, O. E., Borodina, V., Poteen, V. V. and Yudkin, J. S. (1997). Does malnutrition in utero determine diabetes and coronary heart disease in adulthood? Results from the leningrad siege study, a cross sectional study. *BMJ* **315**(7119): 1342-8.

Stapleton, P. A., James, M. E., Goodwill, A. G. and Frisbee, J. C. (2008). Obesity and vascular dysfunction. *Pathophysiology* **15**(2): 79-89.

Steinberg, H. O. and Baron, A. D. (2002). Vascular function, insulin resistance and fatty acids. *Diabetologia* **45**(5): 623-34.

Steinberg, H. O., Brechtel, G., Johnson, A., Fineberg, N. and Baron, A. D. (1994). Insulin-mediated skeletal muscle vasodilation is nitric oxide dependent. A novel action of insulin to increase nitric oxide release. *J Clin Invest* **94**(3): 1172-9.

Steinberg, H. O., Chaker, H., Leaming, R., Johnson, A., Brechtel, G. and Baron, A. D. (1996). Obesity/insulin resistance is associated with endothelial dysfunction. Implications for the syndrome of insulin resistance. *J Clin Invest* **97**(11): 2601-10.

Stephenson, K., Tunstead, J., Tsai, A., Gordon, R., Henderson, S. and Dansky, H. M. (2003). Neointimal formation after endovascular arterial injury is markedly attenuated in db/db mice. *Arterioscler Thromb Vasc Biol* **23**(11): 2027-2033.

Stevenson, J. C. (2004). The metabolic basis for the effects of hrt on coronary heart disease. *Endocrine* **24**(3): 239-44.

Study Cooperative Research Group, H. (2008). Hyperglycemia and adverse pregnancy outcomes. *New England Journal of Medicine* **358**(19): 1991-2002.

Stuebe, A. M., Forman, M. R. and Michels, K. B. (2009). Maternal-recalled gestational weight gain, pre-pregnancy body mass index, and obesity in the daughter. *Int J Obes (Lond)* **33**(7): 743-52.

Su, C.-L., Sztalryd, C., Contreras, J. A., Holm, C., Kimmel, A. R. and Londos, C. (2003). Mutational analysis of the hormone-sensitive lipase translocation reaction in adipocytes. *Journal of Biological Chemistry* **278**(44): 43615-43619.

Sumino, H., Ichikawa, S., Yoshida, A., Murakami, M., Kanda, T., Mizunuma, H., Sakamaki, T. and Kurabayashi, M. (2003). Effects of hormone replacement therapy on weight, abdominal fat distribution, and lipid levels in japanese postmenopausal women. *Int J Obes Relat Metab Disord* **27**(9): 1044-1051.

Sun, K., Yang, K. and Challis, J. R. (1997). Differential expression of 11 beta-hydroxysteroid dehydrogenase types 1 and 2 in human placenta and fetal membranes. *J Clin Endocrinol Metab* **82**(1): 300-5.

Surwit, R. S., Feinglos, M. N., Rodin, J., Sutherland, A., Petro, A. E., Opara, E. C., Kuhn, C. M. and Rebuffe-Scrive, M. (1995). Differential effects of fat and sucrose on the development of obesity and diabetes in c57bl/6j and a/j mice. *Metabolism* **44**(5): 645-51.

Surwit, R. S., Kuhn, C. M., Cochrane, C., McCubbin, J. A. and Feinglos, M. N. (1988). Diet-induced type ii diabetes in c57bl/6j mice. *Diabetes* **37**(9): 1163-7.

Surwit, R. S., Seldin, M. F., Kuhn, C. M., Cochrane, C. and Feinglos, M. N. (1991). Control of expression of insulin resistance and hyperglycemia by different genetic factors in diabetic c57bl/6j mice. *Diabetes* **40**(1): 82-7.

Symons, J. D., McMillin, S. L., Riehle, C., Tanner, J., Palionyte, M., Hillas, E., Jones, D., Cooksey, R. C., Birnbaum, M. J., McClain, D. A., Zhang, Q.-J., Gale, D., Wilson, L. J. and Abel, E. D. (2009). Contribution of insulin and akt1 signaling to endothelial nitric oxide synthase in the regulation of endothelial function and blood pressure. *Circ Res* **104**(9): 1085-1094.

Szyf, M., Weaver, I. C. G., Champagne, F. A., Diorio, J. and Meaney, M. J. (2005). Maternal programming of steroid receptor expression and phenotype through DNA methylation in the rat. *Frontiers in Neuroendocrinology* **26**(3-4): 139-162.

Tagawa, H., Shimokawa, H., Tagawa, T., Kuroiwa-Matsumoto, M., Hirooka, Y. and Takeshita, A. (1997). Short-term estrogen augments both nitric oxide-mediated and non-nitric oxide-mediated endothelium-dependent forearm vasodilation in postmenopausal women. *J Cardiovasc Pharmacol* **30**(4): 481-8.

Tagawa, N., Yuda, R., Kubota, S., Wakabayashi, M., Yamaguchi, Y., Kiyonaga, D., Mori, N., Minamitani, E., Masuzaki, H. and Kobayashi, Y. (2009). 17beta-estradiol inhibits 11beta-hydroxysteroid dehydrogenase type 1 activity in rodent adipocytes. *J Endocrinol* **202**(1): 131-9.

Takeda, K., Toda, K., Saibara, T., Nakagawa, M., Saika, K., Onishi, T., Sugiura, T. and Shizuta, Y. (2003). Progressive development of insulin resistance phenotype in male mice with complete aromatase (cyp19) deficiency. *J Endocrinol* **176**(2): 237-46.

Talbott, E., Guzick, D., Clerici, A., Berga, S., Detre, K., Weimer, K. and Kuller, L. (1995). Coronary heart disease risk factors in women with polycystic ovary syndrome. *Arteriosclerosis, Thrombosis, and Vascular Biology* **15**(7): 821-826.

Tamashiro, K. L., Terrillion, C. E., Hyun, J., Koenig, J. I. and Moran, T. H. (2009). Prenatal stress or high-fat diet increases susceptibility to diet-induced obesity in rat offspring. *Diabetes* **58**(5): 1116-25.

Tang, J. J., Srivastava, R. A., Krul, E. S., Baumann, D., Pfleger, B. A., Kitchens, R. T. and Schonfeld, G. (1991). In vivo regulation of apolipoprotein a-i gene expression by estradiol and testosterone occurs by different mechanisms in inbred strains of mice. *Journal of Lipid Research* **32**(10): 1571-85.

Taylor, A. and Al-Azzawi, F. (2000). Immunolocalisation of oestrogen receptor beta in human tissues. *Journal of Molecular Endocrinology* **24**(1): 145-155.

Taylor, P. D., Khan, I. Y., Hanson, M. A. and Poston, L. (2004). Impaired edhf-mediated vasodilatation in adult offspring of rats exposed to a fat-rich diet in pregnancy. *J Physiol* **558**(Pt 3): 943-51.

Taylor, P. D., McConnell, J., Khan, I. Y., Holemans, K., Lawrence, K. M., Asare-Anane, H., Persaud, S. J., Jones, P. M., Petrie, L., Hanson, M. A. and Poston, L. (2005). Impaired glucose homeostasis and mitochondrial abnormalities in offspring of rats fed a fat-rich diet in pregnancy. *Am J Physiol Regul Integr Comp Physiol* **288**(1): R134-9.

Tchernof, A., Calles-Escandon, J., Sites, C. K. and Poehlman, E. T. (1998). Menopause, central body fatness, and insulin resistance: Effects of hormone-replacement therapy. *Coron Artery Dis* **9**(8): 503-11.

Tchernof, A. and Poehlman, E. T. (1998). Effects of the menopause transition on body fatness and body fat distribution. *Obes Res* **6**(3): 246-54.

Tequeanes, A. L., Gigante, D. P., Assuncao, M. C., Chica, D. A. and Horta, B. L. (2009). Maternal anthropometry is associated with the body mass index and waist:Height ratio of offspring at 23 years of age. *J Nutr* **139**(4): 750-4.

Thompson, J. and Khalil, R. A. (2003). Gender differences in the regulation of vascular tone. *Clin Exp Pharmacol Physiol* **30**(1-2): 1-15.

Toda, N. and Okamura, T. (1990). Comparison of the response to 5-carboxamidotryptamine and serotonin in isolated human, monkey and dog coronary arteries. *Journal of Pharmacology and Experimental Therapeutics* **253**(2): 676-682.

Tomlinson, J. W., Sinha, B., Bujalska, I., Hewison, M. and Stewart, P. M. (2002). Expression of 11î²-hydroxysteroid dehydrogenase type 1 in adipose tissue is not increased in human obesity. *Journal of Clinical Endocrinology & Metabolism* **87**(12): 5630-5635.

Traupe, T., D'Uscio, L. V., Muenter, K., Morawietz, H., Vetter, W. and Barton, M. (2002). Effects of obesity on endothelium-dependent reactivity during acute nitric oxide synthase inhibition: Modulatory role of endothelin. *Clin Sci (Lond)* **103 Suppl 48**: 13S-15S.

Trogan, E., Fayad, Z. A., Itskovich, V. V., Aguinaldo, J.-G. S., Mani, V., Fallon, J. T., Chereshnev, I. and Fisher, E. A. (2004). Serial studies of mouse atherosclerosis by in vivo magnetic resonance imaging detect lesion regression after correction of dyslipidemia. *Arteriosclerosis, Thrombosis, and Vascular Biology* **24**(9): 1714-1719.

Vachharajani, V., Russell, J. M., Scott, K. L., Conrad, S., Stokes, K. Y., Tallam, L., Hall, J. and Granger, D. N. (2005). Obesity exacerbates sepsis-induced inflammation and microvascular dysfunction in mouse brain. *Microcirculation* **12**(2): 183-94.

Van Guilder, G. P., Hoetzer, G. L., Dengel, D. R., Stauffer, B. L. and DeSouza, C. A. (2006). Impaired endothelium-dependent vasodilation in normotensive and normoglycemic obese adult humans. *Journal of Cardiovascular Pharmacology* **47**(2): 310-313 10.1097/01.fjc.0000205097.29946.d3.

Van Heek, M., Compton, D. S., France, C. F., Tedesco, R. P., Fawzi, A. B., Graziano, M. P., Sybertz, E. J., Strader, C. D. and Davis, H. R., Jr. (1997). Diet-induced obese mice develop peripheral, but not central, resistance to leptin. *J Clin Invest* **99**(3): 385-90.

van Schaftingen, E. and Gerin, I. (2002). The glucose-6-phosphatase system. *Biochem J* **362**(Pt 3): 513-32.

van Weerden, W. M., Bierings, H. G., Van Steenbrugge, G. J., De Jong, F. H. and Schröder, F. H. (1992). Adrenal glands of mouse and rat do not synthesize androgens. *Life Sciences* **50**(12): 857-861.

Veilleux, A., Rheaume, C., Daris, M., Luu-The, V. and Tchernof, A. (2009). Omental adipose tissue type 1 11 beta-hydroxysteroid dehydrogenase oxoreductase activity, body fat distribution, and metabolic alterations in women. *J Clin Endocrinol Metab* **94**(9): 3550-7.

Verlohren, S., Dubrovska, G., Tsang, S. Y., Essin, K., Luft, F. C., Huang, Y. and Gollasch, M. (2004). Visceral periadventitial adipose tissue regulates arterial tone of mesenteric arteries. *Hypertension* **44**(3): 271-6.

Vilaro, S., Llobera, M., Bengtsson-Olivecrona, G. and Olivecrona, T. (1988). Lipoprotein lipase uptake by the liver: Localization, turnover, and metabolic role. *American Journal of Physiology - Gastrointestinal and Liver Physiology* **254**(5): G711-G722.

Villar, I. C., Hobbs, A. J. and Ahluwalia, A. (2008). Sex differences in vascular function: Implication of endothelium-derived hyperpolarizing factor. *J Endocrinol* **197**(3): 447-462.

Virdis, A. and Schiffrin, E. L. (2003). Vascular inflammation: A role in vascular disease in hypertension? *Curr Opin Nephrol Hypertens* **12**(2): 181-7.

Wake, D. J., Rask, E., Livingstone, D. E., Soderberg, S., Olsson, T. and Walker, B. R. (2003). Local and systemic impact of transcriptional up-regulation of 11beta-hydroxysteroid dehydrogenase type 1 in adipose tissue in human obesity. *J Clin Endocrinol Metab* **88**(8): 3983-8.

Walker, B. R. and Andrew, R. (2006). Tissue production of cortisol by 11beta-hydroxysteroid dehydrogenase type 1 and metabolic disease. *Ann N Y Acad Sci* **1083**: 165-84.

Walker, E. A. and Stewart, P. M. (2003). 11beta-hydroxysteroid dehydrogenase: Unexpected connections. *Trends Endocrinol Metab* **14**(7): 334-9.

Wang, H. and Eckel, R. H. (2009). Lipoprotein lipase: From gene to obesity. *American Journal of Physiology - Endocrinology And Metabolism* **297**(2): E271-E288.

Wang, X. and Kilgore, M. W. (2002). Signal cross-talk between estrogen receptor alpha and beta and the peroxisome proliferator-activated receptor gamma1 in mda-mb-231 and mcf-7 breast cancer cells. *Molecular and Cellular Endocrinology* **194**(1-2): 123-133.

Warden, C. H. and Fisler, J. S. (2008). Comparisons of diets used in animal models of high-fat feeding. *Cell Metabolism* **7**(4): 277-277.

Wardle, J., Waller, J. and Jarvis, M. J. (2002). Sex differences in the association of socioeconomic status with obesity. *Am J Public Health* **92**(8): 1299-304.

Waterland, R. A. and Jirtle, R. L. (2004). Early nutrition, epigenetic changes at transposons and imprinted genes, and enhanced susceptibility to adult chronic diseases. *Nutrition* **20**(1): 63-68.

Weaver, I. C., Cervoni, N., Champagne, F. A., D'Alessio, A. C., Sharma, S., Seckl, J. R., Dymov, S., Szyf, M. and Meaney, M. J. (2004). Epigenetic programming by maternal behavior. *Nat Neurosci* **7**(8): 847-54.

Weiser, M. J. and Handa, R. J. (2009). Estrogen impairs glucocorticoid dependent negative feedback on the hypothalamic-pituitary-adrenal axis via estrogen receptor alpha within the hypothalamus. *Neuroscience* **159**(2): 883-895.

Welberg, L. A., Seckl, J. R. and Holmes, M. C. (2001). Prenatal glucocorticoid programming of brain corticosteroid receptors and corticotrophin-releasing hormone: Possible implications for behaviour. *Neuroscience* **104**(1): 71-9.

Wersinger, S., Haisenleder, D., Lubahn, D. and Rissman, E. (1999). Steroid feedback on gonadotropin release and pituitary gonadotropin subunit mrna in mice lacking a functional estrogen receptor α. *Endocrine* **11**(2): 137-143.

West, D. B., Boozer, C. N., Moody, D. L. and Atkinson, R. L. (1992). Dietary obesity in nine inbred mouse strains. *Am J Physiol* **262**(6 Pt 2): R1025-32.

West, D. B., Waguespack, J. and McCollister, S. (1995). Dietary obesity in the mouse: Interaction of strain with diet composition. *Am J Physiol* **268**(3 Pt 2): R658-65.

Westerbacka, J., CornÃ©r, A., Tiikkainen, M., Tamminen, M., Vehkavaara, S., HÃ¤kkinen, A. M., Fredriksson, J. and Yki-JÃ¤rvinen, H. (2004). Women and men have similar amounts of liver and intra-abdominal fat, despite more subcutaneous fat in women: Implications for sex differences in markers of cardiovascular risk. *Diabetologia* **47**(8): 1360-1369.

WHIS (Women's Health Initiative Steering) Committee. (2004). Effects of conjugated equine estrogen in postmenopausal women with hysterectomy. *JAMA: The Journal of the American Medical Association* **291**(14): 1701-1712.

Whitaker, R. C. (2004). Predicting preschooler obesity at birth: The role of maternal obesity in early pregnancy. *Pediatrics* **114**(1): e29-36.

White, C. L., Purpera, M. N. and Morrison, C. D. (2009). Maternal obesity is necessary for the programming effect of a high-fat diet on offspring. *Am J Physiol Regul Integr Comp Physiol*: 91015.2008.

White, R. E. (2002). Estrogen and vascular function. *Vascular Pharmacology* **38**(2): 73-80.

WHO. (2011). "Obesity and overweight fact sheet." Retrieved November 25th, 2011, from http://www.who.int/mediacentre/factsheets/fs311/en/index.html.

Widmer, C. C., Mundy, A. L., Kretz, M. and Barton, M. (2006). Marked heterogeneity of endothelin-mediated contractility and contraction dynamics in mouse renal and femoral arteries. *Experimental Biology and Medicine* **231**(6): 777-781.

Wigg, S. J., Tare, M., Tonta, M. A., O'Brien, R. C., Meredith, I. T. and Parkington, H. C. (2001). Comparison of effects of diabetes mellitus on an edhf-dependent and an edhf-independent artery. *Am J Physiol Heart Circ Physiol* **281**(1): H232-40.

Williams, I. L., Wheatcroft, S. B., Shah, A. M. and Kearney, M. T. (2002). Obesity, atherosclerosis and the vascular endothelium: Mechanisms of reduced nitric oxide bioavailability in obese humans. *Int J Obes Relat Metab Disord* **26**(6): 754-64.

Williams, T. D., Chambers, J. B., Roberts, L. M., Henderson, R. P. and Overton, J. M. (2003). Diet-induced obesity and cardiovascular regulation in c57bl/6j mice. *Clinical and Experimental Pharmacology and Physiology* **30**(10): 769-778.

Wimmer, M. (1989a). The effect of sex hormones on the acinar distribution pattern of phosphoenolpyruvate carboxykinase activity in rat liver. *Biological Chemistry Hoppe-Seyler* **370**(2): 683-690.

Wimmer, M. (1989b). Effects of starvation and refeeding a high carbohydrate diet on the intra-acinar distribution pattern of phosphoenolpyruvate carboxykinase activity in the liver of male and female rats. *Histochemistry and Cell Biology* **92**(4): 331-336.

Windle, R. J., Wood, S. A., Shanks, N., Lightman, S. L. and Ingram, C. D. (1998). Ultradian rhythm of basal corticosterone release in the female rat: Dynamic interaction with the response to acute stress. *Endocrinology* **139**(2): 443-50.

Witte, M. M., Resuehr, D., Chandler, A. R., Mehle, A. K. and Overton, J. M. (2010). Female mice and rats exhibit species-specific metabolic and behavioral responses to ovariectomy. *Gen Comp Endocrinol* **166**(3): 520-8.

Wong, W. P., Tiano, J. P., Liu, S., Hewitt, S. C., Le May, C., Dalle, S., Katzenellenbogen, J. A., Katzenellenbogen, B. S., Korach, K. S. and Mauvais-Jarvis, F. (2010). Extranuclear estrogen receptor-alpha stimulates neurod1 binding to the insulin promoter and favors insulin synthesis. *Proc Natl Acad Sci U S A* **107**(29): 13057-62.

Wu, Z., Rosen, E. D., Brun, R., Hauser, S., Adelmant, G., Troy, A. E., McKeon, C., Darlington, G. J. and Spiegelman, B. M. (1999). Cross-regulation of c/ebp alpha and ppar gamma controls the transcriptional pathway of adipogenesis and insulin sensitivity. *Mol Cell* **3**(2): 151-8.

Xu, Y., Nedungadi, T. P., Zhu, L., Sobhani, N., Irani, B. G., Davis, K. E., Zhang, X., Zou, F., Gent, L. M., Hahner, L. D., Khan, S. A., Elias, C. F., Elmquist, J. K. and Clegg, D. J. (2011). Distinct hypothalamic neurons mediate estrogenic effects on energy homeostasis and reproduction. *Cell Metab* **14**(4): 453-65.

Yacoub, L. K., Vanni, T. M. and Goldberg, I. J. (1990). Lipoprotein lipase mrna in neonatal and adult mouse tissues: Comparison of normal and combined lipase deficiency (cld) mice assessed by in situ hybridization. *J Lipid Res* **31**(10): 1845-52.

Yan, X., Zhu, M. J., Xu, W., Tong, J. F., Ford, S. P., Nathanielsz, P. W. and Du, M. (2010). Up-regulation of toll-like receptor 4/nuclear factor-kappab signaling is associated with enhanced adipogenesis and insulin resistance in fetal skeletal muscle of obese sheep at late gestation. *Endocrinology* **151**(1): 380-7.

Yeh, S., Miyamoto, H., Shima, H. and Chang, C. (1998). From estrogen to androgen receptor: A new pathway for sex hormones in prostate. *Proceedings of the National Academy of Sciences* **95**(10): 5527-5532.

Yeh, S., Tsai, M. Y., Xu, Q., Mu, X. M., Lardy, H., Huang, K. E., Lin, H., Yeh, S. D., Altuwaijri, S., Zhou, X., Xing, L., Boyce, B. F., Hung, M. C., Zhang, S., Gan, L. and Chang, C. (2002). Generation and characterization of androgen receptor knockout (arko) mice: An in vivo model for the study of androgen functions in selective tissues. *Proc Natl Acad Sci U S A* **99**(21): 13498-503.

Yki-Jarvinen, H. (2004). Thiazolidinediones. *New England Journal of Medicine* **351**(11): 1106-1118.

Yoon, M. (2009). The role of pparalpha in lipid metabolism and obesity: Focusing on the effects of estrogen on pparalpha actions. *Pharmacol Res* **60**(3): 151-9.

Yoon, M., Jeong, S., Nicol, C. J., Lee, H., Han, M., Kim, J. J., Seo, Y. J., Ryu, C. and Oh, G. T. (2002). Fenofibrate regulates obesity and lipid metabolism with sexual dimorphism. *Exp Mol Med* **34**(6): 481-8.

Yukimura, Y., Bray, G. A. and Wolfse, A. R. (1978). Some effects of adrenalectomy in the fatty rat. *Endocrinology* **103**(5): 1924-1928.

Zhang, S. H., Reddick, R. L., Burkey, B. and Maeda, N. (1994a). Diet-induced atherosclerosis in mice heterozygous and homozygous for apolipoprotein e gene disruption. *J Clin Invest* **94**(3): 937-45.

Zhang, S. H., Reddick, R. L., Piedrahita, J. A. and Maeda, N. (1992). Spontaneous hypercholesterolemia and arterial lesions in mice lacking apolipoprotein e. *Science* **258**(5081): 468-71.

Zhang, X., Zhang, G., Zhang, H., Karin, M., Bai, H. and Cai, D. (2008). Hypothalamic ikκ/nf-κb and er stress link overnutrition to energy imbalance and obesity. *Cell* **135**(1): 61-73.

Zhang, Y., Proenca, R., Maffei, M., Barone, M., Leopold, L. and Friedman, J. M. (1994b). Positional cloning of the mouse obese gene and its human homologue. *Nature* **372**(6505): 425-32.

Zhao, H., Tian, Z., Hao, J. and Chen, B. (2005). Extragonadal aromatization increases with time after ovariectomy in rats. *Reprod Biol Endocrinol* **3**: 6.

Zhu, L., Hou, M., Sun, B., Buren, J., Zhang, L., Yi, J., Hernell, O. and Li, X. (2010). Testosterone stimulates adipose tissue 11beta-hydroxysteroid dehydrogenase type 1 expression in a depot-specific manner in children. *J Clin Endocrinol Metab* **95**(7): 3300-8.

Zhu, L. J. and Altmann, S. W. (2005). Mrna and 18s-rna coapplication-reverse transcription for quantitative gene expression analysis. *Anal Biochem* **345**(1): 102-9.

Zygmunt, P. M., Ryman, T. and Hogestatt, E. D. (1995). Regional differences in endothelium-dependent relaxation in the rat: Contribution of nitric oxide and nitric oxide-independent mechanisms. *Acta Physiol Scand* **155**(3): 257-66.