Molecular Mechanisms of Ovulation and Luteinisation

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Declaration

The composition of this thesis and the experiments described within are the unaided work of the author. No part of this work has previously been accepted for any other degree, nor is any part of it being submitted concurrently in candidature for another degree.

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Table of Contents

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Publications

Abstracts

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Peer-reviewed research papers

Tetsuka, M., Milne, M. **Simpson, G.E.** and Hillier, S.G. (1999) Expression of 11β-hydroxysteroid dehydrogenase, glucocorticoid receptor, and mineralocorticoid receptor genes in rat ovary. *J. Endocrinol.* 60, 330-335

Tetsuka, M., Haines, L.C., Milne, M., **Simpson, G.E.** and Hillier, S.G. (1999) Regulation of 11β-hydroxysteroid dehydrogenase type 1 gene expression by luteinising hormone and interleukin-1β in cultured rat granulosa cells. (in press)

Abstract

Progesterone (P) synthesis is regulated by gonadotrophins and is the major steroid hormone produced by the corpus luteum. It is also present in the follicle, in lower concentrations, before ovulation and luteinisation and is known to be essential for follicular rupture and granulosa cell differentiation.

P is synthesised in the ovary by the actions of the steroidogenic enzymes cytochrome P450 side-chain cleavage (P450scc) and 3β-hydroxysteroid dehydrogenase (3βHSD) on cholesterol with Steroidogenic Acute Regulatory (StAR) protein aiding the delivery of cholesterol to the mitochondria where P450scc resides. P exhibits its effects through the genomic receptor PR, which is only transiently expressed in rat granulosa cells (GC) after the mid-cycle LH surge and before ovulation. Therefore intrafollicular P action depends on P synthesis and PR expression.

However, P is not the only steroid known to act through PR. Glucocorticoids (G) and P cross-react with each other's genomic receptors (PR and GR). Although G is not synthesised within the ovary itself, the enzyme 11β hydroxysteroid dehydrogenase (11βHSD), found in the ovary, modulates its activity. The bi-directional 11βHSD type 1 is predominantly a reductase, producing active forms of G (cortisol and corticosterone) and type 2 is a dehydrogenase producing inactive forms (cortisone and 11-dehydrocorticosterone). P may also exert an effect through a non-genomic receptor (NGPR) which has been identified in tissues of other species. Expression of NGPR in the ovary would suggest another mode of P paracrine/autocrine action.

Both G and P exhibit anti-inflammatory characteristics in the uterus and since ovulation has been likened to an inflammatory reaction, it is my hypothesis that P and G work in synergy through genomic and nongenomic receptors to regulate ovulation. Therefore, RNase protection assays and in situ hybridisation were developed to examine spatio-temporal expression of genes believed to be crucial to P and G production, metabolism and reception in the rat ovary.

Gonadotrophins were shown to up-regulate the expression of StAR, P450scc, 3βHSD and 11βHSD1 and down-regulate 11βHSD2 expression in whole rat ovary and in GC cultures.

PR mRNA was expressed transiently 6h after the LH surge whereas GR mRNA was expressed throughout the cycle. Therefore, genes that regulate the synthesis of P, the activation of G and both genomic receptors were developmentally regulated, being induced by gonadotrophins.

In GC cultures, after priming with FSH to induce functional maturity, concurrent addition of the antiprogestin, RU486, inhibited the stimulatory effects of LH on 11βHSD1, StAR, P450scc and 3βHSD mRNA levels, 6h after treatment began. PR gene expression was unaltered. However, with 12h treatment, 11βHSD gene expression had increased and StAR, P450scc and 3βHSD mRNA levels had decreased with RU486. The previously recognised ability of RU486 to halt ovulation and luteinisation may therefore be due to its effect on the expression of genes regulating P and G. However, nongenomic P action was not ruled out since P bound to a cytosolic protein which showed characteristics of NGPR identified in other species in both mature and immature ovaries.

In summary, gonadotrophins act on ovarian GC to induce the expression of genes which aid in the synthesis and activation of P and G. These steroids may act locally through genomic or non-genomic receptors mediating events which lead to follicle rupture. The presence of P, G and their receptors in the ovary after ovulation suggests a role for these anti-inflammatory steroids in the corpus luteum, perhaps remodelling the ruptured ovarian surface.

Abbreviations

3βHSD	3β-hydroxysteroid dehydrogenase/Δ^{5-4} isomerase
5αR	5α reductase
11βHSD	11β-hydroxysteroid dehydrogenase
17βHSD	17β-hydroxysteroid dehydrogenase
20αHSD	20α-hydroxysteroid dehydrogenase
apo	apolipoprotein
ATP	adenosine triphosphate
bp	base pair(s)
BSA	bovine serum albumin
cAMP	cyclic adenosine 3',5'-monophosphate
cDNA	complementary deoxyribonucleic acid
CRE	cAMP response element
CREB	cAMP response element binding protein
CTP	cytosine triphosphate
dATP	(2'-deoxy-) adenosine 5'-triphosphate
dNTP	deoxyribonucleotide triphosphate
DCS	donor calf serum
DES	diethylstilboestrol
DHT	5α dihydrotestosterone
DNase	deoxyribonuclease
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
FGF	fibroblast growth factor
ER	oestrogen receptor
FSH	follicle stimulating hormone
GnRH	gonadotrophin releasing hormone
G-protein	guanyl-nucleotide-binding protein
GDP	guanosine diphosphate
GH	growth hormone

GnRH	gonadotrophin-releasing hormone
GR	glucocorticoid receptor
GTP	guanosine triphosphate
GTPase	guanosine triphosphatase
hCG	human chorionic gonadotrophin
HDL	high-density lipoprotein
HRE	hormone response element
hsp	heat shock protein
ICI 164,275	N-n-butyl-11-(1,3,5[10]-oestratriene-3,17β-diol-7β-yl)-undecanamide
ICI 164,384	N-n-butyl-11-(1,3,5[10]-oestratriene-3,17β-diol-7β-yl)-N-methylundecanamide
IGF	insulin-like growth factor
IL	interleukin
IMM	inner mitochondrial membrane
IPTG	isopropyl β-D-thiogalactopyranoside
kb	kilobase(s)
LB	Luria-Bertani
LDL	low-density lipoprotein
LH	luteinising hormone
LHR	luteinising hormone receptor
MOPS	3-[N-Morpholino]propane-sulphonic acid
NADH	β-nicotinamide adenine dinucleotide, reduced
NADPH	β-nicotinamide adenine dinucleotide phosphate, reduced
NGPR	non genomic progesterone receptor
P450arom	cytochrome-P450 aromatase
P450scc	cytochrome-P450 side-chain cleavage
(D)PBS	(Dulbecco's) phosphate buffered saline
PCR	polymerase chain reaction
PI	phosphoinostide
PKA	protein kinase A
PKC	protein kinase C
PMSG	pregnant mare's serum gonadotrophin
PR	progesterone receptor

PRE	progesterone response element
RIA	radioimmunoassay
rh	recombinant human
RNase	ribonuclease
(m or t)RNA	(messenger or transfer) ribonucleic acid
rNTP	ribonucleotide triphosphate
RPA	RNase protection assay
RT-PCR	reverse transcription polymerase chain reaction
SDS	sodium dodecyl sulphate
SRE	steroid response element
StAR	steroidogenic acute regulatory (protein)
TEA	triethanolamine
TEMED	N,N,N',N'-Tetramethylethylenediamine
TESPA	3-aminopropyltriethoxysilane
TNF	tumour growth factor
TPA	tissue plasminogen activator
TRIS	tris(hydroxymethyl)aminomethane
TTP	thymidine triphosphate
UTP	uridine triphosphate
UV	ultraviolet
X-gal	5-Bromo-4-chloro-3-indolyl-β-D-galatoctopyraoside

Chapter 1 Literature Review

1 Introduction

The mammalian ovary serves a dual function, being responsible for the release of viable oocytes at the appropriate time to allow fertilisation, and secondly to secrete steroids that regulate female sexual behaviour and prepare the body for implantation and lactation.

Follicular development, ovulation and maintenance of the corpus luteum are under control of the gonadotrophic hormones, follicle stimulating hormone (FSH) and luteinising hormone (LH), released by the pituitary which, in turn, is regulated by gonadotrophin-releasing hormone (GnRH) from the hypothalamus. Under gonadotrophic control, granulosa and thecal cells of the ovary mature and acquire the capability of synthesising oestrogen. This oestrogen has local effects within the ovary, but also has a negative feedback function on the pituitary and hypothalamus. As oestrogen concentrations increase, the feedback adopts a positive effect, resulting in a mid-cycle LH surge inducing ovulation. The cells of the ruptured, empty follicle luteinise and form a corpus luteum, secreting progesterone until luteolysis is initiated.

This thesis concentrates on the local ovarian regulatory functions of steroids, steroid metabolising enzymes and receptors for steroid hormones in the mediation of follicular maturation and ovulation. As a prelude to the experimental work, I shall review current knowledge of the endocrine and paracrine regulation of ovarian function, emphasising steroid production and action.

2 Structural and cellular composition of the ovary

The mammalian ovaries are paired organs situated in the peritoneal cavity. Blood, lymph and nerve supplies travel through a ligament known as the mesovarium and join the ovary at the ovarian hilus. Each ovary is surrounded by a capsule called the bursa which completely encloses the ovaries of rats and mice and allows very little contact

with the peritoneum. The ovary itself is covered in a smooth epithelium called the surface epithelium. During embryonic development, this epithelium proliferates to give rise to the underlying cortex, in which germ cells are embedded (Franchi *et al.*, 1962).

During foetal life the germ cells in the ovary proliferate by mitosis to form oogonia. They enter the first meiotic division but become arrested at the diplotene stage. At this point the germ cells are termed primary oocytes. Therefore, the number of germ cells in the ovary is fixed at birth (or just after birth in the rat) and they are not replaced as they are released. The primordial follicle is composed of a single layer of precursor granulosa cells and a single immature oocyte. These granulosa cell precursors form gap junctions with the oocyte and are separated from the surrounding stroma by a basement membrane, the membrana propria. The oocyte and primordial granulosa cells have no vascular supply and exist in a micro-environment enclosed by the basement membrane. Little is known about the influence of the oocyte on follicle development, although it is essential because the follicle does not form in its absence (Buccione *et al.*, 1990).

Every day during the reproductive lifespan of a female, a number of follicles will grow, with spindle-shaped primordial (granulosa) cells becoming cuboidal and proliferative. These are now known as granulosa cells and the follicle is now a primary follicle. The oocyte, still arrested in diplotene, approximately doubles in size. Oocyte growth is arrested but RNA and protein are still synthesised (Lintern-Moore and Moore, 1979). Also, an opaque layer between the granulosa cells and the oocyte, called the zona pellucida is formed from glycoproteins secreted by the oocyte (Philpott *et al.*, 1987; Lira *et al.*, 1990). Re-initiation of meiosis occurs spontaneously in isolated oocytes from primary follicles (Eppig and Downs, 1984), therefore the growth of the oocyte is associated with the development of the ability to resume meiosis. Resumption of meiosis occurs just prior to ovulation through an unknown mechanism (Freeman, 1988). Cyclic AMP can maintain oocytes in meiotic arrest, therefore it is thought that FSH-stimulated granulosa cells pass cAMP through gap junctions (Eppig and Downs, 1984).

Further granulosa cell proliferation and growth leads to secondary follicle growth. Coincident with granulosa cell proliferation, stromal cells outside the basement membrane differentiate and arrange in concentric cell layers to form the theca interna and the theca externa. The theca interna is the steroidogenic layer adjacent to the

basement membrane and the theca externa is the fibrous wall of the follicle which merges with the surrounding stroma. The secondary follicle acquires an independent vascular supply which terminates in a capillary bed at the basement membrane. The granulosa cells and oocyte thus remain avascular, but steroids produced in the theca can be exported from the ovary.

Tertiary follicle formation is accompanied by further hypertrophy of theca cells and the appearance of a fluid-filled atrium among the granulosa cells. The antral fluid consists of a plasma transudate and secretory products of granulosa cells. In association with antrum formation, the granulosa and theca cells develop intercellular gap junctions (Albertini and Anderson, 1974) which allow cell-cell communication and synchronised co-ordination of follicular function. Up to this stage, follicular growth is relatively gonadotrophin independent, since follicles of hypophysectomized animals grow to early antral stage (Hirshfield, 1985), although this does not mean that these follicles are not gonadotrophin-responsive (Greenwald and Terranova, 1988). In the absence of gonadotrophins, many follicles become atretic, and healthy follicles will proceed no further than the early antral stage.

Under the influence of gonadotrophins, the follicle rapidly increases in size to form a Graafian follicle. The antral fluid increases in volume and the oocyte, surrounded by the cumulus oophorus (granulosa cells in direct contact with the oocyte), assumes a polar, eccentric position within the follicle (Gougeon, 1982). The mature Graafian follicle is ready to release the ovum.

Recruited primordial follicles develop independently of each other, either becoming dominant follicles destined to ovulate, or degenerating as a result of atresia (Byskov, 1978). In the case of atresia, the oocyte and granulosa cells degenerate and are replaced by fibrous tissue, and the theca cells return to the pool of ovarian interstitial cells (Erickson *et al.*, 1985).

3 Ovarian Function

Ovarian follicular development is dependent upon the secretion of gonadotrophins; follicle stimulating hormone (FSH) and luteinising hormone (LH), which act through receptors on the somatic cells of the follicle. Gonadotrophins are released from the

anterior pituitary under the control of the hypothalamo-pituitary-ovarian axis involving a complex feedback mechanism (Knobil *et al.*, 1980). FSH and LH are heterodimeric glycoproteins which share a common α-subunit consisting of 89 amino acids and two glycosylation sites. The β-subunit confers specificity and the β-subunit of LH and FSH only have 7 homologous amino acids. Also, the FSH β-subunit has two glycosylation sites compared to one for the LH β-subunit. FSH has a molecular weight of 32.6kDa compared to 34kDa for LH (Johnson and Everitt, 1995).

3.1 Endocrine regulation (FSH, LH)

3.1.1. Control of gonadotrophin secretion

FSH and LH are secreted by gonadotroph cells in the anterior pituitary in response to gonadotrophin-releasing hormone (GnRH). GnRH is a decapeptide produced by a small number of GnRH-secreting neurones in the forebrain and defined centres in the hypothalamus. It is transported to the median eminence and is released in a pulsatile manner into the portal system where it binds to specific, high affinity G-protein receptors on the cell surface of the gonadotrophs (Clayton and Catt, 1981). Activation of the GnRH receptors stimulates a calcium-dependent release of gonadotrophins from storage granules, and during states of enhanced gonadotrophin secretion there is evidence for increased synthesis of α- and β-subunits. LH is released from intracellular stores in pulses in response to GnRH pulses, whereas FSH release is dependent on its rate of synthesis (McNeilly, 1988). GnRH also enhances pituitary responsiveness to subsequent stimulation by GnRH by increasing the number of its own receptors. All these actions of GnRH are mediated by a calcium-dependent mechanism involving protein kinase C activation (Conn, 1989).

FSH, LH, oestrogens and progestins all have an effect on GnRH secretion with positive and negative feedback mechanisms. These will be discussed fully in Section 4 of this chapter.

3.1.2 The role of FSH in the regulation of ovarian function

Follicle stimulating hormone (FSH), as its name suggests, is responsible for the maturation of the follicle (Greep *et al.*, 1942), its actions being localised to the granulosa cells where FSH receptors are present. The FSH receptor has been observed throughout follicle development, except in primordial follicles and old corpora lutea (Oxberry and Greenwald, 1982; Camp *et al.*, 1991). FSH is known to induce the expression of several genes in granulosa cells (see Fig. 1), which are potentially involved in autocrine and paracrine control of granulosa and thecal proliferation and differentiation. This includes the up-regulation of the expression of its own receptor (Richards *et al.*, 1976), which sensitises granulosa cells to further stimulation by FSH. LH receptor expression has been shown to be induced in granulosa cells by FSH stimulation both *in vivo* and *in vitro* (Erickson *et al.*, 1979) and thus granulosa cells are LH responsive during the later stages of follicular development.

FSH also induces P450arom mRNA (Steinkampf *et al.*, 1987) which leads to an increased conversion of thecal androgen to oestrogen (Dorrington *et al.*, 1975). Oestrogen augments the ability of FSH to induce LH receptors (Rani *et al.*, 1981) and also increases proliferation of granulosa cells *in vivo* (Hsueh *et al.*, 1984), but not *in vitro* (Hammond *et al.*, 1985) suggesting other additional paracrine factors control this mitogenic effect. Increased oestrogen production leads to a pituitary feedback mechanism which allows increased secretion of LH. At late follicular phase, when the granulosa cells express LH receptors, the positive feedback mechanism brings about the LH surge, inducing ovulation, corpus luteum formation and luteal progesterone production (Yen, 1986).

Inhibin, activin and follistatin are probably involved in the feedback control of gonadotrophin secretion and are possible regulators of follicular development (Bicsak *et al.*, 1986; Hillier *et al.*, 1989; Michel *et al.*, 1990; Michel *et al.*, 1991), production of insulin-like growth factors (Oliver *et al.*, 1989), and many morphological changes associated with granulosa cell differentiation such as the production of gap junctions (Amsterdam and Rotmensch, 1987).

FSH also induces the StAR (Steroidogenic Acute Regulatory) protein (Pescador *et al.*, 1997) which is involved in the transportation of cholesterol across the inner

mitochondrial membrane where enzymes essential to steroidogenesis are situated. The importance of StAR protein will be discussed fully in section 3.2.

Previously, studies of the effects of FSH have had to take into account LH contamination due to the methods of extracting gonadotrophins from urine. Now genetically engineered recombinant gonadotrophins are manufactured which produce pure FSH or LH and therefore the exact contribution each gonadotrophin makes to follicular development can be established. This thesis is based on experiments using such 'pure' gonadotrophins enabling a more accurate determination of each hormone's effect.

Fig 1.1
FSH-inducible genes in ovarian granulosa cells.
(From S.G. Hillier (Ed.) 'Cellular Basis of Follicular Endocrine Function', 1990a)

mRNA	Reference
P450scc	(Richards *et al.*, 1987)
P450arom	(Steinkampf *et al.*, 1987; Hickey *et al.*, 1988)
RIIβ subunit of type II cAMP-dependent Protein kinase	(Hedin *et al.*, 1987)
Tissue plasminogen activator	(O'Connell *et al.*, 1987; Ohlsson *et al.*, 1988)
Renin	(Kim *et al.*, 1987)
Apolipoprotein E	(Wyne *et al.*, 1989)
Prostaglandin endoperoxidase synthase	(Richards *et al.*, 1987)
Inhibin α subunit	(Woodruff *et al.*, 1987)
Inhibin βA and βB subunits	(Turner *et al.*, 1989)
Pro-opiomelanocortin (POMC)	(Young *et al.*, 1989)
Heat shock protein (hsp90)	(Ben-Ze'ev and Amsterdam, 1989)
Follistatin	(Shimasaki *et al.*, 1988)
LH receptor	(Segaloff *et al.*, 1990)

3.1.3 The role of LH in the regulation of ovarian function

As described in the previous section, FSH, through the induction of follicular oestrogen synthesis, brings about a surge in LH which triggers ovulation and subsequent maintenance of corpora lutea. LH administered *in vivo* in the absence of FSH leads to growth of follicles (Greep *et al.*, 1942), but this appears to be due largely to thecal hypertrophy (Erickson *et al.*, 1985).

Until the later stages of follicular development, LH receptors are limited to the thecal cells where the ligand binds to stimulate the production of a number of factors, in particular androgens and progestogens (Erickson *et al.*, 1985). Therefore, LH acts on the thecal cells of the developing follicle providing granulosa cells with androgen substrate for the synthesis of oestrogens. As the follicle matures, granulosa cells mature in response to FSH and express receptors for LH. Low levels of LH act in the same way as FSH to stimulate granulosa cells to produce oestrogen, progesterone, inhibin and other factors (Hillier, 1985). High levels of LH cause ovulation and luteinisation of granulosa cells. These granulosa-lutein cells then secrete progesterone (Moor, 1974).

3.1.4 Intracellular Action

Gonadotrophins and peptide factors act through membrane-bound receptors which trigger a complex system of intracellular signalling pathways.

Adenylate cyclase

FSH and LH receptors belong to a large family of structurally-related receptors which contain a seven transmembrane domain, common to guanyl-nucleotide-binding protein (G-protein) receptors. These receptors are long proteins with seven hydrophobic helices forming the transmembrane domain. The amino-terminus contains N-linked oligosaccharides lying on the extracellular side, and the carboxyl-terminus resides on the cytosolic side. The hormone-binding site is located in a pocket formed by the transmembrane helices. Binding of the gonadotrophin to its receptor causes a conformational change in the associated stimulatory G-protein (Gs) which, in its activated state, releases the commonly bound GDP and binds instead to GTP. The α-subunit of the activated G-protein, bearing GTP, dissociates from the $\beta\gamma$ subunits of the G-protein and activates the membrane-bound adenylate cyclase. GTP is converted back to the inactive GDP form by the GTPase action of the α-subunit, effectively harnessing the reaction. Activated adenylate cyclase induces the formation of cytosolic cAMP

from ATP which consequently binds to protein kinase A (PKA). Therefore the addition of cyclic AMP to a cellular system can be used to imitate the effect of gonadotrophins binding to their receptors.

PKA is a tetramer consisting of two regulatory subunits which bind cAMP and two catalytic subunits which possess phosphotransferase activity (Beebe *et al.*, 1989). Binding of cAMP to the regulatory subunits suppresses phosphotransferase activity, and therefore allows phosphorylation of protein substrates which either immediately stimulate steroidogenesis or exert long term effects on cAMP-regulated genes through control of transcription rates (Kurten and Richards, 1989). It is likely that PKA stimulates a cAMP response element binding protein (CREB) which binds as a dimer to an enhancer element and thus the cAMP response element (CRE) mediates cAMP responsive gene expression. Intracellular cAMP is responsible for the regulation of several genes including StAR (Payne and Youngblood, 1995), P450scc (Clemens *et al.*, 1994) and 3βHSD (Chedrese *et al.*, 1990b). The concentration of many circulating hormones can be as low as 10^{-10}M. The adenylate cyclase cascade greatly amplifies this effect by:

1) Each hormone-receptor complex catalyses the formation of many Gα-GTPs,
2) Many molecules of cAMP are formed by the activation of adenylate cyclase, and
3) Each cAMP-activated protein kinase can alter the activity of many molecules of each target protein (Stryer, 1988).

Phosphoinositide (PI) Hydrolysis

Protein kinase C activity has been observed to be stimulated by LH in granulosa and thecal/interstitial cells of varying maturity, showing a development-related response. This action is thought to occur through different G-protein intermediates involving the hydrolysis of phosphatidyl inositol 4,5-bisphosphate (PIP_2). In immature granulosa cells (Kasson *et al.*, 1985) and thecal/interstitial cells (Hofeditz *et al.*, 1988), protein kinase C brings about the inhibition of gonadotrophin-stimulated steroidogenesis. However, protein kinase C acutely increases cAMP levels and hence steroidogenesis in luteal cells (Davis *et al.*, 1989).

PIP_2 metabolism signalling can also be activated by non-steroidal factors including various growth factors, vasopressin, GnRH-like peptides, angiotensin II and prostaglandin F2α (Michell, 1989).

Tyrosine Kinase

Receptors for insulin, insulin-like growth factor (IGF)-I (Adashi *et al.*, 1985), epidermal growth factor and tumour growth factor (Feng *et al.*, 1987) have all been identified in granulosa cells. The ligand binding site of these transmembrane molecules is located on the outer side of the membrane and the tyrosine kinase portion is found in the cytosol. When activated by the ligand domain, the intracellular tyrosine kinase promotes phosphorylation of tyrosine residues in proteins involved in cell growth and/or differentiation (Michell, 1989).

3.2 Steroidogenesis

The ovary is the principal site of steroid hormone production in the female, although a number of organs are steroidogenic. A biochemical pathway, regulated by a number of enzymes, enables the ovary to metabolises cholesterol to produce progestogens, androgens and oestrogens. Glucocorticoids are synthesised in the adrenal cortex, but can be metabolised within the ovary (see figure 1.2). These ovarian enzymes are expressed in a cell-specific manner and are under the control of gonadotrophins and locally produced paracrine factors. The precise co-ordination of these enzymes and steroids is essential for follicle development and the ovulation of a healthy oocyte.

Cholesterol Uptake

Progesterone and oestradiol, derived from cholesterol, are the two major steroids produced in the ovary. Cholesterol is present in the ovary from *de novo* synthesis, low density lipoproteins (LDL) uptake or high density lipoproteins (HDL) delivery. The method in which the lipoprotein is utilised varies according to species and cell type.

LDL in the form of apolipoprotein B (apo B) and apolipoprotein E (apo E), bind to receptors, situated in clathrin-coated pits, on the cell surface of steroidogenic cells of the ovary. These pits are internalised and cholesterol esters are released from the complex by the action of cholesterol ester hydrolase, to give a reservoir of cholesterol. HDL can bind apo E and be internalised by the LDL receptor. In the rat ovary, a HDL binding protein has been described that 'delivers' cholesterol to the cell without the

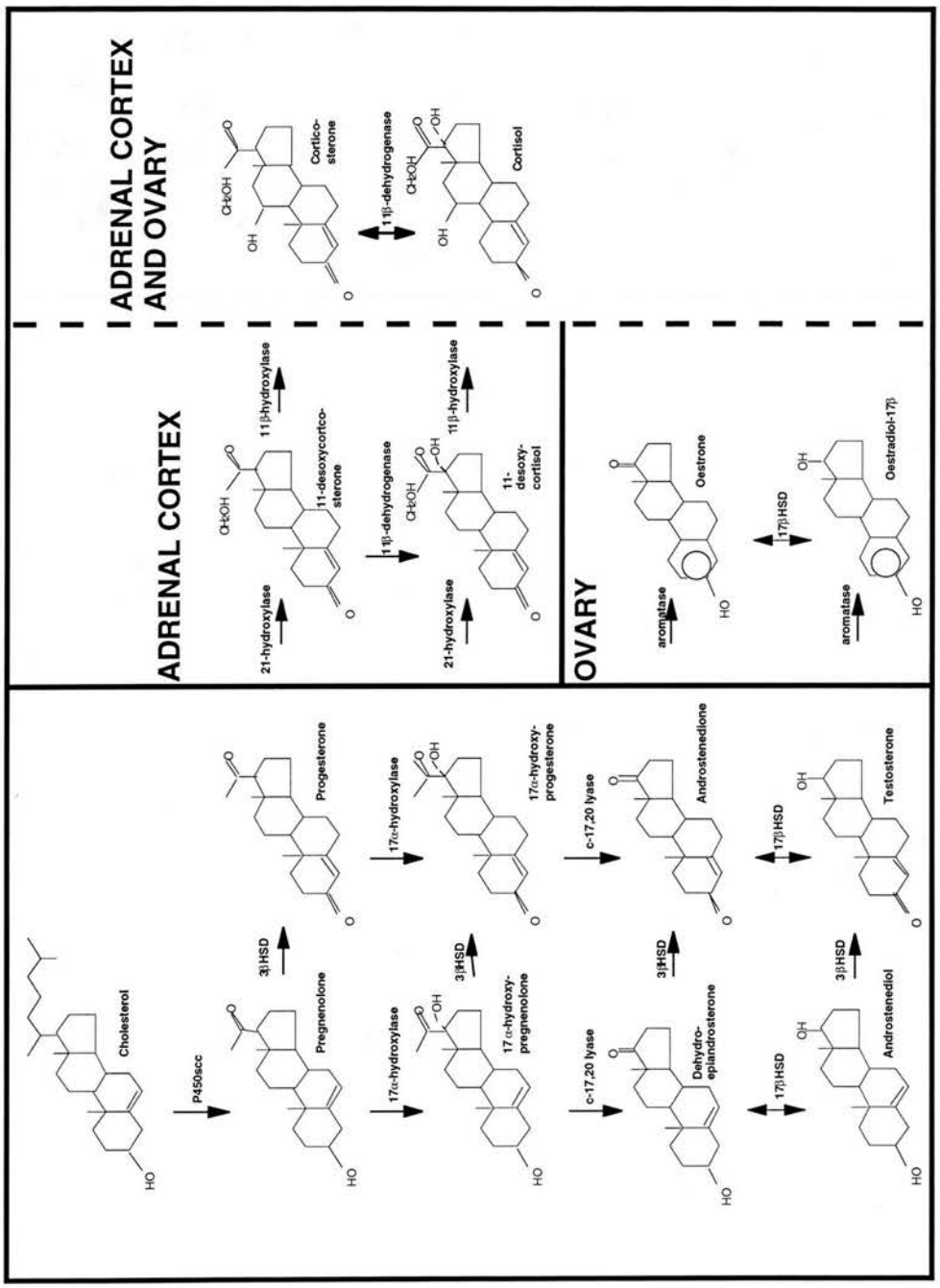


Fig 1.2 Pathways of steroid synthesis

complex being internalised and degraded. Therefore, there appears to be several different routes for cholesterol uptake.

Once internalised, cholesterol is delivered to the outer mitochondrial membrane via sterol carrier protein 2 (SCP2) and must cross the aqueous space between the inner and outer mitochondrial membranes to access P450scc located on the inner mitochondrial membrane. Aqueous diffusion of cholesterol is very slow (Phillips *et al.*, 1987) and would not supply sufficient quantities of substrate for the rapid increase in steroidogenesis associated with gonadotrophic stimulation. Facilitated transport of cholesterol is therefore required.

Although P450scc has previously been regarded as the rate-limiting step in steroidogenesis, recent investigations show that steroidogenic acute regulatory (StAR) protein, controlling mitochondrial internalisation of cholesterol, is the rate-limiting factor (Simpson *et al*, 1972). Cholesterol side chain cleavage is catalysed by cytochrome P450scc to produce pregnenolone in the inner mitochondrial membrane (IMM) and is the first committed step of steroidogenesis. The mobilisation of cholesterol to the IMM is regulated by the hormone-induced family of 30kDa mitochondrial StAR proteins (Clark *et al.*, 1994). It has been established that StAR expression, in the absence of hormone stimulation, induces steroid production, indicating its importance in steroidogenesis.

StAR

Immunoelectron microscopy has localised StAR to the intramembranous face of cristae and the intramembranous space (King *et al.*, 1995). The nascent 37kDa StAR protein has an N-terminal sequence that directs it into the mitochondria where it is processed to yield a mature 30kDa protein (Stocco and Clark, 1996). However, this N-terminal sequence is not essential for steroidogenic activity (Arakane *et al.*, 1998) suggesting StAR can also act on the outside of the mitochondria. After this sequence has been cleaved, membranes dissociate and cholesterol can no longer pass across the inter-membrane space. However, the mode of transport StAR facilitates is not yet known. Stocco and Clark suggest:

1) cholesterol may transfer in a passive manner when contact sites are formed,

2) StAR may bind to cholesterol and be carried to the inner mitochondrial membrane, or

3) a hydrophobic core is formed through which cholesterol can pass (Stocco and Clark, 1996).

StAR requires no receptor or cofactor for its action leading to the theory that the protein's C-terminus interacts with the outer mitochondrial membrane lipids, promoting cholesterol desorption from the outer membrane and its transfer to the inner membrane where it can then access P450scc (Kallen *et al.*, 1998).

Regulation of StAR expression is, as yet, unknown. The 5' promoter regions of the human and mouse StAR gene have been sequenced and do not appear to have putative cAMP response elements (CREs), despite being under cAMP regulation (Waterman, 1994). However a binding site for the orphan nuclear receptor SF-1/AdBP4 which is expressed in a cAMP-dependent manner has been found upstream of the murine and human StAR transcription start site, indicating a possible control mechanism (Stocco and Clark, 1996). The short half-life and the inactivation of the StAR protein would explain the need for its continuous production throughout steroidogenesis. In view of the importance of StAR in all steroid hormone synthesis, a major part of the work described herein is dedicated to determining the site and time of StAR expression in the ovulatory follicle.

Steroidogenic P450 Enzymes

Three members of the cytochrome P450 enzyme superfamily are involved in ovarian steroidogenesis. All mammalian members of this group of enzymes are membrane-bound proteins with a haem prosthetic group and they require transport of electrons from NADPH via intermediates, including one flavoprotein (Strauss and Miller, 1991).

Cholesterol side chain cleavage

Once inside the mitochondrion, a C_6 unit from the side chain of cholesterol is removed to reduce the number of carbon atoms from 27 to 21, by an enzyme complex situated on the inner mitochondrial membrane, thus forming pregnenolone. This complex consists of the cytochrome P450 side chain cleavage enzyme and the electron transport proteins adrenodoxin and adrenodoxin reductase (Waterman and Simpson, 1989). P450scc mRNA and protein are found in both theca and granulosa cells of antral follicles, consistent with the progesterone production capabilities of both cell types (Zlotkin *et al.*, 1986; Goldring *et al.*, 1987; Hedin *et al.*, 1987). The enzyme is regulated by gonadotrophins, via cAMP-dependent kinase, which increase P450scc, adrenodoxin and adrenodoxin reductase (Tuckey and Stevenson, 1986). P450scc

mRNA is also known to be up-regulated by the action of FSH, with the addition of oestrogen augmenting this stimulatory effect (Richards *et al.*, 1987). Because of the essential role of P450scc in progesterone biosynthesis, work described in this thesis examines the expression of the P450scc gene in the maturing follicle.

17α-hydroxylase/C17-20 lyase

17α-hydroxylase/C17-20 lyase (Hedin *et al.*, 1987) (P45017α) is the enzyme essential for the conversion of progestogens to androgens. Electrons are supplied by NADPH via P450 reductase for the first reaction and via P450 reductase or cytochrome b_5 for the second reaction (Takemori and Kominami, 1984). P45017α can utilise either pregnenolone (Δ5-progestogen) or progesterone (Δ4–progestogen) as a substrate to produce either dehydroepiandrosterone or androstenedione, respectively. Ovarian P45017α expression is restricted to the thecal cells, the major androgen producing cells of the ovary (Human, [Voutilainen *et al.*, 1986]; Bovine, [Rodgers *et al.*, 1986]). Small increases in LH concentration are known to stimulate P45017α activity (Bogovich *et al.*, 1981), however mRNA, protein content and bioactivity drop sharply after the LH surge (Hedin *et al.*, 1987).

P450 aromatase

The aromatase enzyme complex is located on the smooth endoplasmic reticulum and is responsible for the conversion of C-19 androgens to C-18 oestrogens. The complex consists of P450 aromatase and NADPH cytochrome P450 reductase, which supplies the electrons required for the reaction (Osawa *et al.*, 1987). The two major substrates for this enzyme are androstenedione and testosterone (17β-hydroxylated androstenedione) which yield oestrone and 17β-oestradiol, respectively. Aromatase activity has been localised to granulosa cells (Sasano *et al.*, 1989). The aromatase gene is known to be induced by FSH (Steinkampf *et al.*, 1987) and its transcription, translation and activity is enhanced by oestradiol (Hickey *et al.*, 1988). Other potential stimulators of aromatase include IGF-1 and cAMP analogues (Steinkampf *et al.*, 1987).

Hydroxysteroid Dehydrogenases

In steroidogenic tissues, hydroxysteroid dehydrogenases (HSDs) catalyse the final steps in androgen, oestrogen and progesterone biosynthesis. In peripheral tissues, including steroid target tissues, they convert potent steroid hormones into inactive metabolites hence regulating the amount of active steroid hormone that can bind to the

target receptors. HSDs catalyse bi-directional reactions and each HSD exists in multiple isoforms, showing tissue specificity (for reveiw see Penning, 1997).

3β-hydroxysteroid dehydrogenase

3βHSD interconverts Δ^5 and Δ^4-steroids. In the adrenal cortex, it plays a pivotal role in the formation of glucocorticoids and mineralocorticoids. In the ovary, 3βHSD was shown by immunohistochemical staining to be present in thecal cells throughout follicular development and during atresia. However, granulosa cells require gonadotrophin stimulation to stain positive (Bogovich and Richards, 1984). In the rat, 4 isoforms of 3βHSD are present, type 1 being the most prevalent in the ovary with its expression and enzyme activity being up-regulated by hCG and decreased by prolactin (Chedrese *et al.*, 1990a; Chedrese *et al.*, 1990b). These responses are physiologically relevant because hCG is luteotrophic and prolactin stimulates luteolysis. The inhibitory effect of prolactin on 3βHSD expression and activity during luteolysis is reflected in the progressive decrease in progesterone concentration, whilst serum pregnenolone levels are elevated. These findings indicate that inhibition of 3βHSD gene expression in the corpus luteum occurs early in the luteolytic process induced by prolactin, and could well play a role in prolactin-induced luteolysis (Chedrese et al, 1990b). Rat *in vivo* experiments, have shown no significant rise in 3βHSD mRNA after PMSG treatment alone, but 24h after the addition of hCG, an increase of 187% in the message of 3βHSD was observed. This high expression continued throughout the pseudopregnancy (Kaynard *et al.*, 1992). In contrast, Tanaka and colleagues showed 3βHSD activity to be elevated 4h after hCG injection in PMSG-primed animals (Tanaka *et al.*, 1993). These increases were inhibited by the antiprogestin, RU486, or amplified by progesterone. These results indicate that progesterone concentrations regulate 3βHSD activity, suggesting an autocrine regulation of progesterone production during ovulation in immature rat ovaries stimulated with PMSG and hCG. Because of the crucial role of 3βHSD in progesterone activation, this thesis studies its expression in the ovary.

17-β hydroxysteroid dehydrogenase

17-β hydroxysteroid dehydrogenase (17βHSD) isozyme catalyses the final steps in androgen and oestrogen biosynthesis. Eight isozymes have been identified. In the ovary and placenta, 17βHSD type 1 converts oestrone (a weak oestrogen) to 17β-oestradiol (a potent oestrogen) using NADPH as a cofactor (for review see Peltoketo *et al*, 1999). Immunocytochemical studies have shown type 1 to be present in granulosa

cells of the human ovary (Sawetawan *et al.*, 1994). It also appears to require the presence of the oestrogen receptor for optimal mRNA expression and activity as shown in ER negative cell lines (Penning, 1997). Protein kinase C and a cAMP analogue were shown to elevate 17βHSD type 1 mRNA expression. However, further experiments have shown the enzyme is regulated by a non-classic cAMP-dependent mechanism that remains unclear (Penning, 1997). Type 2 uses NAD^+ as a cofactor and catalyses the oxidation of testosterone and 17β-oestradiol to form androstenedione and oestrone, respectively (i.e. inactivates androgens and oestrogens). To date, type 2 has not been identified in the ovary.

11β hydroxysteroid dehydrogenase

Although the ovaries do not undertake *de novo* glucocorticoid synthesis because they do not express P450C21 and P450C11β enzymes (Omura and Morohashi, 1995), they do express 11β hydroxysteroid dehydrogenase (11βHSD), which catalyses the conversion of the bio-active glucocorticoids, cortisol and corticosterone, to the inactive forms, cortisone and 11-dehydrocorticosterone, in humans and rodents respectively. This enzyme exists as two isoforms, the $NADP^+$-dependent bi-directional 11βHSD type 1 with predominant reductase activity and low binding affinity for cortisol and corticosterone (Stewart and Mason, 1995) and the NAD^+-dependent dehydrogenase type 2 with high binding affinity for cortisol and corticosterone (Stewart *et al.*, 1994; Albiston *et al.*, 1994). There are two known corticosteroid hormones, glucocorticoid (G) and mineralocorticoid (M), both of which have distinct receptors, GR and MR, respectively. MR shows non-selective, high affinity binding to the active glucocorticoid and mineralocorticoid, aldosterone, and both the GR and the MR bind to identical hormone response elements on regulated genes (Arriza *et al.*, 1987). Therefore, mineralocorticoid target organs must be protected from non-specific binding of active glucocorticoid to the MR. This has been shown to occur in mineralocorticoid target organs such as the kidney and colon, where 11βHSD2 catalyses active glucocorticoid to inactive forms, thereby protecting non-selective MR from inappropriate stimulation by active glucocorticoid (Shimojo *et al.*, 1997).

The ovary is known to be a glucocorticoid target organ, with glucocorticoid sites identified in rat granulosa cells (Schreiber *et al.*, 1982), and 11βHSD found in human granulosa cells (Michael *et al.*, 1993; Michael *et al.*, 1995). 11βHSD1 mRNA is up-regulated and 11βHSD2 down-regulated in human granulosa cells after hCG stimulation (Tetsuka *et al.*, 1997). Because of the importance of glucocorticoid

metabolism in regulating glucocorticoid action, this thesis examines 11βHSD gene expression in the ovary.

20α hydroxysteroid dehydrogenase

20α hydroxysteroid dehydrogenase (20αHSD) plays an important role in converting progesterone (a potent progestin) to the inactive progestin 20α-hydroxyprogesterone, thus regulating the amount of active progestin that can bind to the progesterone receptor. Due to its ability to inactivate progesterone, it may play a significant role in luteolysis. Progesterone itself was shown to down-regulate the expression of 20αHSD in the rat corpus luteum, even in the absence of the progesterone receptor (Sugino *et al.*, 1997). It is thought progesterone acts through the glucocorticoid receptor to reduce progesterone secretion from the corpus luteum.

5α reductase

5α reductase (5αR) is involved in steroid catabolism (progesterone to the biologically inactive 5α-pregnane-3,20-dione and cortisol to tetrahydrocortisol), and also generates the potent androgen, 5α dihydrotestosterone (DHT), from testosterone. 5αR activity is inhibited by FSH (Payne *et al.*, 1989), an effect augmented by competitive substrates progesterone and testosterone, and the non-competitive inhibitor, oestradiol. 5α-reduced androgens from stromal cells competitively inhibit granulosa cell aromatase (Hillier *et al.*, 1980) and thus act to modulate oestrogen synthesis.

3.3 Local regulation by steroids

The principal ovarian steroid hormones, progesterone and oestrogen, control or influence all aspects of reproduction. Androgens and glucocorticoids also play important roles in ovarian function and together constitute a complex interaction essential for follicular growth, ovulation, pregnancy and parturition.

These steroid hormones classically act through nuclear receptors which belong to a large ligand-activated transcription factor family. A schematic diagram of the structural features of PR can be seen in fig 1.3. These receptors share a common structure including a variable N-terminal domain (A/B region), highly conserved DNA binding domains (C region) and an hydrophilic D region believed to hinge the DNA binding

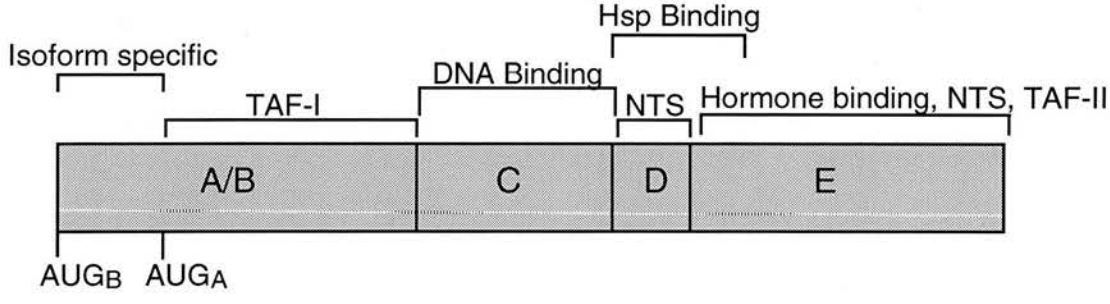


Fig 1.3 Schematic representation of the structural features of the progesterone receptor. The structure of the gene encoding both isoforms (PRA and PRB) of the progesterone receptor is shown, along with the location of the n-terminal initiation codon for each isoform (AUG_B and AUG_A). The basic structure of this gene is shared by all members of the steroid, thyroid, vitamin D, retinoic acid and orphan receptor superfamily, with five functional domains: an N-terminal transactivation domain (A/B), a DNA-binding domain (C), a hinge region (D), and a hormone-binding domain (E). Regions important for heat shock protein (Hsp) binding, nuclear translocation (NTS) and transcriptional activation (TAF-I, II) are also indicated. (Adapted from Pinter *et al*, 1996).

and the steroid binding domain (region E/F). In their 'inactive' state, they are bound to a number of proteins, in particular a heat shock protein, hsp90 (Ziemieki *et al.*, 1986). This prevents activation of the receptor in the absence of steroid. Free steroid passes through the plasma membrane by passive diffusion, and then across the nuclear membrane, where it binds to its specific receptor. Some steroid receptors are found in an inactive state in the cytosol, and only when bound to ligand do they travel to the nucleus. The steroid-ligand complex undergoes a conformational change involving dissociation of hsp90, receptor dimerisation and phosphorylation enabling binding of the complex to the steroid response elements (SRE). These elements exist adjacent to target genes on genomic DNA and binding of the ligand-receptor complex induces transcription modulation (Funder, 1993).

Steroid hormone receptors do not show complete specificity for their ligands. For example, oestrogen and androgen receptors show some affinity for all steroids, and high concentrations of progesterone bind to both androgen and glucocorticoid receptors (Clark and Mani, 1994). Likewise, glucocorticoids at high concentrations are known to bind to progesterone receptors (Walters and Clark, 1977). It should also be noted that steroid action is tissue and species specific, demonstrating different effects in different cells and species.

However, not all steroid action can be explained by this classic receptor model. Some steroid effects are too rapid, or occur in cells that do not, or are prevented from transcription or translation of mRNA. Also, their action may not be blocked by antagonists of the classic genomic steroid receptors. These receptors have been called nongenomic steroid receptors (Revelli *et al.*, 1998).

Progesterone

Progesterone is the hormone of pregnancy, initially being produced by the corpus luteum, preparing the uterus for implantation. It can act as a classic hormone, being transported to target organs such as the uterus or the hypothalamic-pituitary axis, or locally within the ovary. Progesterone is essential for luteinisation (Natraj and Richards, 1993) and inhibitors of progesterone synthesis (Tanaka *et al.*, 1993) have all been reported to inhibit ovulation. Indeed, mice lacking the PR gene show normal follicular growth, but never ovulate (Lydon *et al.*, 1995).

Progesterone has been shown to inhibit antral follicular growth (Buffler and Roser, 1974; Goodman and Hodgen, 1983) suggesting it has an inhibitory effect on follicular growth (Goodman and Hodgen, 1983). These results have been explained in terms of negative feedback of gonadotrophin secretion by progesterone, rather than a direct effect of progesterone on the ovary itself (Taya *et al.*, 1981; Garza and Terranova, 1984). However, progesterone was shown to inhibit follicular growth induced by oestrogen (Smith and Bradbury, 1966; Payne *et al.*, 1956) and gonadotrophin (Chiras and Greenwald, 1978). In other reports, elevated progesterone levels have been shown to augment the effect of hCG on the growth of small follicles (Bogovich *et al.*, 1981; Richards and Bogovich, 1982). Reasons for this discrepancy may be differences in concentrations of progesterone and/or follicle maturity.

Progesterone is thought to act during the later stages of follicular development, when enzymes responsible for progesterone production and the genomic progesterone receptor are expressed. 3βHSD is not induced until after the LH surge (Kaynard *et al.*, 1992; Tanaka *et al.*, 1993) and PR is expressed only in the late follicular phase and luteal cells of the human and monkey (Press and Greene, 1988; Hild-Petito *et al.*, 1988). In the rat, PR is expressed for a short transient period approximately 6h after LH stimulation, *in vivo* (Park-Sarge and Mayo, 1994; Park and Mayo, 1991) and *in vitro* (Park-Sarge and Mayo, 1994; Natraj and Richards, 1993) suggesting progesterone may exert its effects during a limited time frame between the preovulatory surge and ovulation. PR has also been identified in the corpus luteum of the pig (Iwai *et al.*, 1991), the monkey (Press and Greene, 1988; Chandrasekher *et al.*, 1994) and the cow (Jacobs and Smith, 1981), but not in the rat (Park-Sarge *et al.*, 1995). However, evidence of a non-genomic PR in bovine luteinised granulosa and thecal cells (Rae *et al.*, 1998a) suggests another method of progesterone action.

Chaffkin *et al* (1993) identified progesterone's autocrine effect on human granulosa cells in culture, showing that cells plated at high concentrations produced progesterone which halted proliferation and induced differentiation to luteal cells. This effect was inhibited by RU486 or aminoglutethimide, which blocks progesterone synthesis. Duffy and Stouffer report up-regulation of progesterone receptor mRNA in the corpus luteum of the macaque with increased concentrations of progesterone and receptor protein in luteal cells throughout the luteal phase. However, high levels of PR mRNA continues while progesterone and the receptor protein concentrations decrease as the corpus luteum regresses, suggesting PR translation and PR transcription are regulated

in an asynchronous manner during the life span of the corpus luteum (Duffy and Stouffer, 1995). On the contrary, the rat corpus luteum expresses undetectable levels of PR mRNA, and inhibition of progesterone, with or without oestrogen, did not increase PR expression (Park-Sarge *et al.*, 1995). The lack of PR could not be attributed to progesterone-induced down-regulation of PR mRNA or lack of oestrogen-induced up-regulation of PR mRNA.

The progesterone receptor exists in two isoforms in all species examined except the rabbit (Loosefelt *et al.*, 1984). Isoform A is a N-terminally truncated form of the B isoform and both are expressed in response to gonadotrophins (Natraj and Richards, 1993). Kraus and colleagues (1993) cloned the 5'-region of the rat PR gene and found two functionally distinct promoters. The promoter region for the B isoform incorporated an oestrogen response element (ERE), but the A isoform promoter region showed no such sequence. However, there is much evidence to indicate that ovarian PR is not under oestrogenic control (for review see Pinter *et al.*, 1996), unlike that of the uterus. Expression of rat ovarian PRB is induced by promoters of the PKA pathway, despite no known cAMP-responsive sequences being housed in the 5' DNA flanking region of the PRB gene. Mutation studies have shown specific consensus sequences upstream of the PRB transcription start site are necessary for cAMP-induced transcription of the gene (Park-Sarge and Sarge, 1995). Agents such as insulin-like growth factor-I which act through tyrosine kinase pathways have also been shown to increase PR expression in uterine epithelial cells and breast cancer cells (Aronica and Katzenellenbogen, 1991) but the effects of intermediates of this pathways have not been examined.

Takimoto and colleagues showed that binding of progesterone to its receptor activates the PKA pathway and therefore substrates of these intracellular phosphorylation pathways e.g. cAMP, its derivatives and agents that elevate cAMP (forskolin and 8-bromo-cAMP) can also mimic the effects of gonadotrophins (Takimoto *et al.*, 1992; Denner *et al.*, 1990), showing a ligand-independent trans-activation by steroid receptors. Differential action of the two isoforms has been displayed by induction of the human PR gene by 8-bromo-cAMP in the presence and absence of ligand. Binding of the progestin agonist, R5020, acted in synergy with 8-bromo-cAMP to amplify hPRA-mediated activity to a higher degree than that of hPRB (Kazmi *et al.*, 1993). PRB has been reported to be a stronger transcriptional activator than PRA (Vegeto *et al.*, 1993). Vegeto and colleagues (1993) also demonstrated that although PRA was

inactive as a transcriptional activator, it functioned as a potent ligand-dependent repressor of PRB transcriptional activity. PRA also functioned as a repressor of oestrogen (McDonnell and Goldman, 1994), mineralocorticoid, androgen and glucocorticoid (Vegeto *et al.*, 1993) receptors. PRA can also act as a transcriptional activator by stimulating the tyrosine amino transferase promoter in HeLa cells. Therefore progesterone receptors can act as a repressor or activator of transcription in a cell specific manner (Vegeto *et al.*, 1993). Because of the mandatory role for genomic or non-genomic reception in mediating progesterone action, the work contained herein examines the expression of PR and the presence of a possible non-genomic PR in the ovary.

Antiprogestins

Antiprogestins or antigestagens act by binding to PR to antagonise the effects of progesterone. The most commonly used antiprogestin is RU486 (mifepristone) which has undergone clinical trials and is now used routinely to induce abortion in the first trimester of pregnancy. Its continuous administration impairs follicular development and inhibits ovulation in women although the location of its action is not known. Heikinheimo and colleagues concluded the inhibition of ovulation in monkeys using RU486 occurred mainly at the level of the hypothalamus, but also possibly through the granulosa cells (Heikinheimo *et al.*, 1995). Studies done in rat, directed at the ovarian level of control, indicate RU486 regulates the activity of 3β-HSD, possibly via its action on PR as opposed to a direct action (Telleria and Deis, 1994).

RU486 has a high affinity for PR and glucocorticoid receptor (GR). After binding with the receptor, hsp90 does not dissociate, as with normal ligand-receptor activation, therefore interfering with DNA interaction at the hormone response element (for review see Mahajan and London, 1997).

The antiprogestins RU486 and ZK98299 do not block the LH-induced expression and translation of PR in rat granulosa culture, but do block luteinisation (Natraj and Richards, 1993), implying progesterone action is necessary for luteinisation. *In vivo* studies have shown mifepristone and onapristone disrupt folliculogenesis and inhibit the LH surge when administered during the follicular phase in rats (Brannstrom, 1993) and human (Croxatto *et al.*, 1994), leading to the development of a possible once-a-month contraceptive pill. Interestingly, RU486 can block ovulation in the rat only if

administered 2h before LH. If injected concomitant or after LH treatment, there is no effect on ovulation. The preovulatory progesterone surge is also blocked, probably due to the decrease in 3βHSD expression (Tanaka *et al.*, 1993). This indicates that progesterone is acting immediately before the LH surge.

Another method by which antiprogestins can act as a contraceptive is the treatment of low doses of ZK98299 at weekly intervals, which does not block folliculogenesis or ovulation, but has an inhibitory effect on the endometrium, which would not allow proper implantation of the fertilised egg (Ishwad *et al.*, 1993). Blocking the effects of progesterone via an antiprogestin opens up a great number of opportunities for novel contraceptives.

Because of the ability of RU486 to block both PR and GR, this thesis examines the effects of RU486 and Org31710, an antiprogestin with little affinity to GR, on the expression of gonadotrophically-regulated genes in the rat ovary. Figure 8.2 shows the molecular structures of both of these antigestagens.

Oestrogens

Oestrogens have an autocrine effect on granulosa cells, paracrine effects on other ovarian cells and multiple systemic effects throughout the body. Both granulosa cells and thecal cells appear to be sites of oestrogen action (Clark and Mani, 1994). In rats, in the absence of gonadotrophins, oestrogens display an autocrine effect by increasing proliferation and up-regulating gonadotrophin receptor expression (Hsueh *et al.*, 1984). The administration of oestrogen *in vivo* is used to increase yields of granulosa cells in *in vitro* cell culture (Dorrington *et al.*, 1975). Also at this level, they stimulate transcription of inhibin α- and β-subunit mRNA (Turner *et al.*, 1989) and exert a direct anti-atretic effect (Harman *et al.*, 1975). Oestrogen acts to enhance the stimulatory effect of FSH on P450arom and P450scc enzyme expression, thereby stimulating its own production, and the expression of RIIβ subunit of type II cAMP-dependent protein kinase (Richards *et al.*, 1987). This may indicate an increased intracellular capacity to respond to cAMP, which may be responsible for the up-regulation of many of the oestrogen and FSH functions displayed in granulosa cells. The acquisition of LH receptors by FSH in granulosa cells is also augmented by oestrogen (Richards *et al.*, 1976).

The classic oestrogen receptor (ER) has been identified in the ovaries of all animals studied. However, expression is not consistent between cell types and species. ER have been found in the nuclei of rat granulosa cells (Richards, 1975). In an immunocytochemical study in primates, granulosa cells appeared not to express ER, but the surface epithelium stained positive (Hild-Petito *et al.*, 1988) whereas in human ovaries ER was found in the granulosa cells of large follicles prior to the LH surge (Iwai *et al.*, 1990). Nongenomic oestrogen receptors have been found throughout the ovary, in granulosa cells, endometrial cells and oocytes (Revelli *et al.*, 1998).

Androgens

The steroidogenic pathway to androgens can take two routes, depending on the species. Mono-ovulatory species such as the cow and primates follow the Δ5 pathway, involving pregnenolone to 17-OH-pregnenolone to DHEA to aromatisable androgens. However, species such as rat, pig and rabbit appear to prefer a route through progesterone, known as the Δ4 pathway.

Androgens not only serve as a substrate for oestrogen synthesis, but also have a direct hormonal role in local control of follicular development. The somatic cells surrounding the oocyte form highly differentiated layers involving predominantly granulosa cells (GC) and thecal cells (TC). The latter are responsive to LH (Zeleznik *et al.*, 1974) and GC are FSH responsive, only acquiring receptors to LH later in follicular maturation (Erickson *et al.*, 1979). The two-cell, two-gonadotrophin model proposed by Armstrong *et al* (1978) describes the LH and FSH action on the synthesis of androgens by theca cells and the metabolism of these androgens to oestrogens by granulosa cells. Hillier suggests paracrine signalling (granulosa to thecal) to explain amounts of precursor androgen in the thecal cells being similar to that aromatised in the granulosa cells of preovulatory follicles (Hillier, 1991a). Androgens are capable of promoting gonadotrophin-stimulated granulosa cell aromatase activity (Hillier and deZwart, 1981) and development of LH responsiveness (Shaw *et al.*, 1989). Their action in granulosa cells augments FSH-induced mechanisms, for example, progesterone and oestrogen production *in vitro* is greatly enhanced by the addition of androgens in many species, including the rat (Armstrong and Dorrington, 1976).

Androgens exhibit a paracrine effect regulating the FSH-induced differentiation of granulosa cells *in vivo* (Hillier, 1985), and administration of excess androgens results in follicular atresia or granulosa cell apoptosis (Conway *et al.*, 1990). Tetsuka and Hillier

(1996) observed that androgens down-regulate the gene expression of their own receptors (AR) in cultured rat granulosa cells, and FSH dose-dependently reverses this effect, suggesting that auto regulation of AR mRNA might be a protective mechanism against premature atresia in immature follicles. The function of androgens *in vivo* is not certain, since administration of anti-androgens does not disrupt rat oestrous cycles (Neumann *et al.*, 1970), nor does it affect FSH-responsiveness in rats (Zeleznik *et al.*, 1979), and androgen-resistant mice undergo apparently normal cycles (Lyon and Glenister, 1974).

Glucocorticoids and mineralocorticoids

Glucocorticoids are found in a variety of tissues and are involved in nutrition through caloric storage, tissue repair and stress response. Their role in the immune system is controlling inflammation with an immunosuppressive action. The presence of GR (Schreiber *et al.*, 1982) and 11βHSD (Michael *et al.*, 1993) in the ovary has increased interest in glucocorticoids' involvement in reproduction at the ovarian level. Ovulation has been likened to an immune reaction (Espey, 1990), which is why glucocorticoid activation is a topic of this thesis.

Mineralocorticoids regulate fluid dynamics in target tissues. However, MR and GR bind to identical sequences on regulated genes and MR *in vitro* is non-selective, binding aldosterone and cortisol/corticosterone with equal affinity (Arriza *et al.*, 1987). Therefore, mineralocorticoid targets are protected from inappropriate stimulation by inactivating glucocorticoids via 11βHSD action.

Corticosteroids can inhibit aromatase activity (and hence oestrogen production) whilst stimulating tissue plasminogen activator (TPA) (Wang and Leung, 1989) and progesterone secretion by granulosa cells *in vitro* (Hsueh and Erickson, 1978). This TPA is thought to increase proteases to aid in the breakdown of the follicle wall at ovulation. It has been suggested that a reduced concentration of corticosteroids may be responsible for the development of polycystic ovarian syndrome (PCO), the reduced oestrogen causing LH hypersecretion which is thought to be a factor contributing to anovulation, and typical of PCO.

Although it can be deduced that glucocorticoids play a role in follicular development and ovulation, they are not essential for fertility as shown by 11βHSD knock-out mice (Kotelevtsev *et al.*, 1996). However in view of the inflammatory nature of ovulation, the

potential ability of the ovaries to metabolise glucocorticoid, and the presence of ovarian GR, a major emphasis of this thesis is the regulation of 11βHSDs and GR.

3.4 Local, Non-steroidal Regulation

The ovary produces various non-steroidal factors, such as growth factors (e.g. IGF-I), cytokines (e.g. prostaglandins), and developmental factors (e.g. inhibin and activin) that are involved in regulating tissue homeostasis and steroidogenesis. These agents are locally produced, and act within the ovary. (For a review see Tsafriri and Adashi, 1994). Because of their potential to influence steroid production, metabolism and action in the ovary, examples of these factors and their known effects will now be briefly reviewed.

Growth factors

Insulin-like growth factors (IGFs) are believed to play a role in cyclic ovarian development through mediation of some of the effects of FSH. IGFs have a close homology with insulin, and IGF-I, IGF-II and insulin cross react with each other's receptors. IGFs are produced in the liver under the control of growth hormone (GH), and in the ovary, exerting local action. The granulosa cells are the principal site of IGF-I gene expression and protein synthesis in the rat and the pig. Porcine granulosa cell IGF-I production is stimulated by FSH, LH, GH and oestradiol via the cAMP second messenger pathway. Expression of granulosa cell IGF-I receptors is also stimulated by FSH, LH and GH. IGF-I acts to amplify gonadotrophin action by augmenting FSH-stimulated progesterone production, increasing P450scc levels, oestrogen synthesis and up-regulating P450arom gene expression. IGF-II is expressed by thecal cells and is found in follicular fluid of humans. Similarly, granulosa cells produce IGF-II *in vitro* in response to FSH, LH, GH and dibutyryl cAMP, indicating a potential local role for IGFs during follicular development.

Cytokines

Cytokines are of particular relevance because of their involvement in inflammatory modulation. The ovary contains immune cells, including macrophages, mast cells and T lymphocytes which are a potential source of cytokines. During follicular development, cytokines aid granulosa cell proliferation while inhibiting differentiation. As mentioned

earlier, follicle rupture is likened to an inflammatory reaction (Espey, 1990) and IL-1 and TNFα are the main cytokines involved in this process. Cytokines are also involved in remodelling of the corpus luteum at luteolysis. (For reviews see Vinatier *et al*, 1995 and Adashi, 1997).

Interleukin-1 (IL-1) is a system composed of ligands (IL-1α and IL-1β), receptors (IL-1R), a regulator of the system, IL-1ra (IL-1 receptor antagonist) and extracellular binding proteins. *In vivo* studies have shown IL-1 to have antigonadotrophic or antisteroidogenic properties, possibly from either ovarian macrophages or somatic ovarian cells. Because IL-1 is known to be a mediator of inflammation and because ovulation has been likened to an inflammatory reaction, IL-1 may play a role in the preovulatory cascade terminating in the breakdown of the follicle wall and ovulation. Indeed, IL-1ra suppresses hCG-induced ovulation in the rat (Simon *et al.*, 1994). In addition, IL-1 has been shown to increase prostaglandin biosynthesis, plasminogen activator production, collagenase activation and vascular permeability enhancement, which are all involved in follicular rupture. Expression of IL-1 can be regulated by progesterone or oestrogen, with stimulation from low steroid concentrations and inhibition with high levels. IL-1 is also known to block basal and LH-induced progesterone secretion in granulosa cells which may explain its ability to suspend luteinisation.

TNFα is produced in the ovary by macrophages, granulosa cells and thecal cells. It too is a powerful inhibitor of gonadotrophin-induced steroidogenesis and responds to differing concentrations of steroids in the same manner as IL-1.

Prostaglandins

Prostaglandins (PGs) are know to be autocrine/paracrine regulators at various stages of follicular development. They are induced by cytokines and because of their essential role in the inflammatory process, they are of central importance to ovulation. A prostaglandin synthesis inhibitor, indomethacin, was shown to prevent ovulation (Armstrong and Grinwich, 1972). This effect was reversed with the addition of exogenous PGE_2 (Tsafriri *et al.*, 1972). Measurements of prostaglandins in follicular fluid show that concentrations of PGE_2, $PGF_{2\alpha}$ and PGI_2 -metabolite (6-keto-PGF1α) markedly increase in follicles prior to ovulation (Le Maire *et al.*, 1973; Hinricks and Le Lannou, 1988); Murdoch *et al.*, 1986). Prostaglandins have also been identified in the

corpus luteum where they are thought to modulate LH-induced progesterone production (Maas *et al.*, 1992).

The expression of an enzyme that synthesises prostaglandins (prostaglandin synthase or cyclooxygenase-2 [COX-2]) occurs transiently approximately 6h after the LH surge in rats, the same time as the transcription of PR. The prostaglandin synthase-2 gene (PGS-2 or COX-2) is an isoform of PGS, regulated by LH, GnRH and EGF, IL-1β, TNFα and involves multiple cellular pathways including protein kinase-A, C and tyrosine kinase (Brannstrom *et al.*, 1993; Sirois *et al.*, 1992; Wong and Richards, 1992; Morris and Richards, 1992). Experiments regarding progesterone's effect on prostaglandin have been conflicting. In some cases, progesterone is seen to inhibit PGS-2 in granulosa cells (Hellberg *et al.*, 1996), and in others the withdrawal of progesterone is seen to reduce prostaglandin production (Lipner and Greep, 1971).

Inhibin and activin

Inhibin and activin are known to exert local actions in folliculogenesis, luteinisation and atresia and are named according to their inhibitory (inhibin) or stimulatory (activin) action on FSH production by pituitary cells *in vitro*.

Inhibin is a heterodimer glycoprotein consisting of an α-subunit and one of two β-subunits, β_A (inhibin-A) and β_B (inhibin-B). Activins are homodimeric, composed of only β -subunits, giving activin-A ($\beta_A\beta_A$) and activin-AB ($\beta_A\beta_B$). The principal site of ovarian inhibin production is the granulosa cell (Meunier *et al.*, 1988). The expression of the inhibin and activin subunits varies with development of the follicle. During early stages, β -subunit mRNAs are abundant (Schwall *et al.*, 1990; Meunier *et al.*, 1988), but as the follicle develops, inhibin α-subunit mRNA expression increases (Schwall *et al.*, 1990) and is maintained after ovulation in the human corpus luteum where it reaches maximum levels (Davis *et al.*, 1987). This expression is under the control of FSH and indicates activin to be more prominent in early follicle development, decreasing as follicular inhibin levels rise.

In vitro, both activin and inhibin affect androgen synthesis in the theca interna (Hsueh *et al.*, 1987). Picomolar concentrations of inhibin increase the LH/IGF-1 stimulated androgen production, whereas activin has a converse effect (Hillier, 1991b). Inhibin can over-ride the effects of activin in a dose-dependent manner in rats (Hsueh *et al.*,

1987) and humans (Hillier, 1991b). Both proteins selectively affect C19 steroids *in vitro*, suggesting that activin and inhibin act on cytochrome $P450_{C17}$ expression and/or activity.

In granulosa cells, activin augments FSH-induced aromatase activity and stimulates FSH-induced progesterone and inhibin production, LH and FSH receptor expression and cAMP formation in immature granulosa cells. However, in mature, LH-responsive cells and granulosa-lutein cells, progesterone synthesis is inhibited (Miro *et al.*, 1991; Li *et al.*, 1992; Miro and Hillier, 1992). Inhibin action on granulosa cells shows conflicting results, some indicating inhibition of aromatase activity (Ying *et al.*, 1986) whilst others show no effect (Hutchinson *et al.*, 1987).

In vitro regulation of thecal cell androgen production by activin and inhibin has been examined in the rat (Hsueh *et al.*, 1987) and human (Hillier *et al.*, 1991b). In both species inhibin augments LH-stimulated androgen production *in vitro* by thecal cells, whereas activin inhibits LH-stimulated androgen production; these two factors antagonising each other when incubated together with LH. Therefore, both activin and inhibin exert potentially significant autocrine and paracrine effects on the regulation of follicular function, although the indispensability of either of these factors to normal follicular development has yet to be demonstrated.

Follistatin

Follistatin is a single chain polypeptide that suppresses FSH release from pituitary cells, and is known to be present in porcine follicular fluid (Ueno *et al.*, 1987). It binds to activin, antagonising its action (Nakamura *et al.*, 1990). Expression of follistatin in the ovary is similar to the pattern of inhibin-α; it is confined to granulosa cells of developing follicles from early antral stages, with a maximum being reached in the preovulatory follicle. Expression then declines after the LH surge and is completely absent by the time of luteal regression (Nakatani *et al.*, 1991). Follistatin expression is induced by FSH or PMSG (Simasaki *et al.*, 1989; Michel *et al.*, 1990; Saito *et al.*, 1991).

Follistatin is known to inhibit aromatase activity and inhibin production, and augment FSH-stimulated progesterone production (Xiao *et al.*, 1990), but has little effect on progesterone secretion by cultured preovulatory follicles (Tsafriri *et al.*, 1989). These

effects of follistatin seem to be mediated through a suppression of cAMP accumulation (Xiao and Findlay, 1991).

GnRH

Gonadotrophin releasing hormone (GnRH) has been shown to regulate the ovary directly, with high affinity membrane receptors being located on the granulosa cells and luteal cells of the rat. It is thought the half-life and low circulating concentrations of GnRH excludes the possibility of hypothalamic GnRH acting on the ovary itself; however, GnRH-like peptides have been identified in the ovary. GnRH exerts both stimulatory and inhibitory actions on the ovary and ovarian cells. Stimulatory effects, thought to involve PKC, include granulosa cell production of oestrogen, progesterone, 20α-hydroxyprogesterone and prostaglandins. It can also mimic the ability of LH to induce ovulation, thought to be due to its capability of stimulating *in vitro* prostaglandin and plasminogen activator synthesis. GnRH's inhibitory effects include suppression of FSH-induced steroidogenesis and LH receptors on primary cultures of granulosa cells. This inhibition is thought to occur at many sites including cAMP production, protein kinase activation, progesterone and oestrogen synthesis and inhibition of P450arom, 3βHSD and P450scc. It is also believed GnRH-like peptides may have a role in follicular atresia. For review see (Tsafriri and Adashi, 1994).

4 Ovulation (humans and rats)

Ovulation is the consequence of orchestrated endocrine (FSH and LH) and paracrine (steroids, growth factors and cytokines) action on ovarian cells, giving rise to the ovarian cycle, compiling follicular recruitment, selection and rupture, followed by luteinisation and luteolysis. The ovarian cycle is controlled by a feedback system involving hormones produced by the hypothalamus, pituitary gland and ovaries. GnRH secreted from the hypothalamus induces the production of gonadotrophins in the pituitary which stimulate follicular growth and differentiation in the ovaries, resulting in the release of a mature ovum. The mammalian ovarian cycle consists of a repeated process of follicular recruitment, selection, ovulation and corpus luteum formation, then degeneration. These processes constitute a follicular or proliferative phase and a luteal phase.

During the proliferative phase, LH pulse frequency increases and mean tonic FSH concentrations rise, encouraging the growth of small follicles. Consequently, oestradiol concentrations rise and FSH levels fall due to negative feedback, allowing selection of the dominant follicle(s). The transition to the luteal phase is marked by a gonadotrophin surge bringing about ovulation. The corpus luteum forms from the ruptured follicle, which in humans secretes progesterone, oestradiol and inhibin. The life-span of the corpus luteum during a non-fertilised cycle dictates the length of the luteal phase. Luteolysis removes the negative feedback on gonadotrophin levels and another cycle begins.

The length of cycle, the follicular and luteal phases within that cycle and the number of eggs ovulated are species dependent. The differences in lengths of the two phases between species reflect different ovarian cycles.

In humans and primates, the follicular phases are approximately the same duration and gonadotrophin-dependent development of small follicles does not begin until the degeneration of the corpus luteum. This is probably due to oestrogen and inhibin being secreted by the corpus luteum as well as progesterone (Baird, 1991), resulting in the suppression of gonadotrophin secretion from the pituitary.

In other species, such as cows, pigs and sheep, the luteal phase is of similar length to humans, but their follicular phase is much shorter. The corpus luteum of these species does not secrete oestrogen or inhibin (Greenwald and Terranova, 1988), and thus FSH levels are much higher during the luteal phase, which allows follicular development to occur despite the presence of the corpus luteum.

In rodents, including rats, mice and hamsters, the corpus luteum is not functional, (i.e. secretes little progesterone) and regresses within 2-3 days, unless coitus takes place. Follicular development in rats is triggered by a second FSH surge on the morning of oestrus, at about the same time as ovulation (Barraclough and Wise, 1979). As a result, rats have a very short oestrous cycle of four to five days. A functional luteal phase can be induced by mating the female with an infertile male to cause pseudopregnancy.

In rabbits and cats, ovulation does not take place until the cervix has been stimulated. These species appear to have permanent follicular phases, with repeated waves of antral

follicular development and atresia occurring until mating, which triggers the LH surge, leading to ovulation followed by a luteal phase.

Humans have menstrual cycles in which the endometrium is shed at the end of the luteal phase of each infertile cycle. This endometrium must be regenerated before the next ovulation in preparation for embryo implantation and therefore the follicular phase is extended by suppressing gonadotrophin-dependent follicular development until the corpus luteum has degenerated.

4.1 Recruitment

Throughout life there is a continuous number of follicles that begin gonadotrophin-independent growth in response to unknown cues. Follicles do not respond to gonadotrophin until they have developed a thecal layer (Goldenberg *et al.*, 1973). By antral stages of development these follicles have granulosa cell FSH receptors and thecal cell LH receptors (Oxberry and Greenwald, 1982; Uilenbroek and Richards, 1979). In rats and sheep, the LH surge results in a preovulatory follicle secreting negligible amounts of oestradiol and inhibin (Barraclough and Wise, 1979; Watnabe *et al.*, 1990; Campbell *et al.*, 1990) and therefore FSH levels are allowed to peak for a second time (Butcher *et al.*, 1974; Pant *et al.*, 1977). It is this FSH surge that is responsible for the recruitment of the small antral follicles present in the ovary at that time. In most species therefore, the development of follicles in a cycle commences at the beginning of the luteal phase of the previous cycle. In primates, the situation is different. The corpus luteum secretes oestradiol and inhibin and therefore gonadotrophin levels are suppressed due to negative feedback by these factors. It is only when the corpus luteum regresses that FSH levels are allowed to rise and the next wave of small follicles is recruited.

The recruited follicle contains a heterogenous population of granulosa cells. Mural granulosa cells are the most differentiated and express P450scc (Goldschmit *et al.*, 1989) and LH receptors (Oxberry and Greenwald, 1982; Richards *et al.*, 1987). Granulosa cells surrounding the oocyte and lining the antrum are mainly proliferative and express the greatest amounts of mRNA for IGF-I (Oliver *et al.*, 1989) which is implicated in the control of granulosa cell proliferation. There appears to be a gradient of differentiation, at its highest near the thecal layer and the blood supply, and a

gradient of proliferation, at its highest in the opposite direction nearest the oocyte. It is likely that this is due to the mural granulosa cells having better access to FSH and other factors from blood, and to regulatory factors such as androgen from the theca. Although cAMP produced by FSH-stimulated (or LH-stimulated) cells can be passed through cells by gap junctions, it appears that it does not penetrate the most distant cells, possibly being broken down by phosphodiesterases *en route.*

4.2 Selection

As the follicle develops, the steroidogenic capacity of its granulosa cells increase in response to FSH. As a result, towards the end of the follicular phase of the cycle, concentrations of oestradiol and inhibin in the blood increase which feed back to the pituitary, to suppress the secretion of FSH. Therefore, trophic support decreases and a number of growing follicles are unable to withstand this decline and become atretic. However, some follicles are more advanced and have high gonadotrophic responsiveness. These continue to grow despite the declining trophic support. Such follicles are called 'dominant' and the number selected varies with species. An important factor in the selection of a dominant follicle is oestradiol synthesis which decreases pituitary FSH production by negative feedback. Since all follicles are exposed to the same concentrations of gonadotrophins, it seems likely that this increased sensitivity to FSH is due to the influence of locally produced substances. In addition, one of the most important effects of FSH is its ability to induce the expression of LH receptors on granulosa cell membranes. This enables these cells to respond to tonic levels of LH in the same way they would to FSH, since both receptors are coupled to the cAMP intracellular signalling system. Hence, in mature granulosa cells, LH can stimulate cAMP accumulation and act as a substitute for the lowered FSH levels (see Johnson and Everitt (1995) *Essential Reproduction*).

4.3 Rupture

As the dominant follicle develops, it produces increasing amounts of oestradiol which eventually switch from negative to positive feedback control over the pituitary. This leads to a massive release of FSH, and more importantly LH. This LH surge stimulates resumption of meiosis as well as production of progesterone (Moor, 1974) and prostaglandins (Morioka *et al.*, 1989; Wong *et al.*, 1989) by the granulosa cells of the preovulatory follicle. Progesterone promotes ovulation in perfused ovaries (Baranczuk and Fainstat, 1976; Greenwald, 1977; Tsafriri *et al.*, 1987) possibly by interfering with collagen synthesis in the follicle wall (Tjugum *et al.*, 1984). The action of prostaglandin may be exerted at several levels. Prostaglandins produced in response to the gonadotrophin surge lead to increased blood flow to the follicle and an increase in vascular permeability (Brannstrom and Janson, 1991), which increases the volume of the follicular fluid. The last phase of follicular growth is not due to cell proliferation, but an increase in follicular fluid volume.

Proteases, such as plasminogen activators, are known to be produced by granulosa and thecal cells in response to the gonadotrophin surge and are maximal at the time of ovulation (Curry *et al.*, 1989). hCG has been shown to stimulate the expression of collagenases (Reich *et al.*, 1991) which degrade connective tissue of the follicle wall aiding oocyte expulsion. Both prostaglandins and progesterone can stimulate granulosa cell plasminogen activator synthesis (Canapari and Strickland, 1986; Ny *et al.*, 1985).

Prior to ovulation, gap junctions between cumulus cells and between the oocyte and cumulus cells break down (Linder *et al.*, 1977), and the cumulus mass expands due to intracellular accumulation of hyaluronic acid (Eppig, 1991). The oocyte then re-initiates meiosis, possibly due to the removal of inhibitory factors such as cAMP or purines produced by the granulosa cells. The prostaglandin-induced increased follicular fluid causes high pressure and provides the driving force for extrusion of the oocyte. The follicular fluid bursts through and the cumulus-surrounded oocyte follows in a stream onto the ovarian surface. It is the inflammatory nature of ovulation that is addressed in this thesis.

4.4 Corpus Luteum

After ovulation, the follicle collapses, the basement membrane fragments and the cells of the follicle wall fill the antrum to form the corpus luteum. Blood vessels invade the new structure due to the release of angiogenic factors such as fibroblast growth factors (FGFs) (Findlay, 1986). The corpus luteum secretes progesterone and in primates inhibin and oestradiol are also produced (Savard *et al.*, 1965; McLachlan *et al.*, 1987). The function of progesterone is to provide an endometrium capable of supporting pregnancy until the placenta develops and takes over the role.

The granulosa cells of the former follicle cease to proliferate, undergo hypertrophy and accumulate cytoplasmic lipid droplets and large numbers of mitochondria; all characteristics of steroid-secreting cells. This luteinisation is the final stage of differentiation for granulosa cells. The granulosa-lutein cells lose their FSH receptors (Richards *et al.*, 1976; Oxberry and Greenwald, 1982), but remain LH responsive. The thecal-lutein cells remain LH responsive and secrete progesterone. In rats and mice, prolactin also stimulates luteal steroidogenesis (Hilliard, 1973), but its luteotrophic action is less clear in other species. The steroidogenic capacity of the luteal cells is at its greatest at the beginning of the luteal phase and declines thereafter, although LH responsiveness may increase (Fisch *et al.*, 1989). Progesterone levels continue to rise during the luteal phase, probably due to the increasing vascularisation of the corpus luteum during the first half of the luteal phase (Niswender *et al.*, 1976). During the second half of a non-fertile cycle, progesterone secretion by the corpus luteum falls until the gland degenerates completely. The life-span of the corpus luteum is fixed in different species; in sheep, the luteal phase occupies most of the cycle, whereas in the rat, there is no real luteal phase unless coitus takes place (Barraclough and Wise, 1979). The degeneration of the corpus luteum is independent of LH levels and is thought to be pre-programmed (Zeleznik, 1991). In the event of pregnancy, chorionic gonadotrophin (CG) is secreted by the placenta of some species such as human. This hormone acts via the LH receptor to rescue the corpus luteum and prolong its lifespan. The greater half-life of CG compared to LH (Rizkallah *et al.*, 1969) and slower internalisation of CG-receptor complexes by luteal cells (Mock and Niswender, 1983) are believed to be why CG is able to rescue the corpus luteum when LH is not.

5 Scope of thesis

The work detailed in this thesis was undertaken to examine progesterone synthesis, reception and action on the rat ovary, and more specifically, on rat granulosa cells. Because progesterone can act through GR and glucocorticoid through PR, the role of glucocorticoid in the system was also studied. Glucocorticoids are not synthesised in the ovary due to lack of appropriate steroidogenic enzymes, but metabolism does occur. Regulation of this metabolism and the effects of the antiprogestin/antiglucocorticoid, RU486, on steroidogenesis and steroid metabolism was examined. The possible presence of a non-genomic PR was also addressed. The aim of this study was to elucidate the paracrine effect on progesterone and glucocorticoid action in the rat ovary.

Chapter 2 Materials and Methods

The methodologies detailed within this chapter are general methodologies, used throughout the project. More specialised methods are described in the relevant chapters.

All chemicals were purchased from Sigma Chemical Co., Poole, UK, unless otherwise stated.

1. Animals and Treatments

All animal handling and treatments were performed according to the guidelines issued by the Home Office, UK, and appropriate project licences were in place. Injections were undertaken by trained animal house staff.

Immature female Wistar rats were used for the experiments presented throughout this thesis. These animals were either bred in-house or purchased from Charles River UK Limited (Margate, Kent, UK) and were between 21-26 days old at the beginning of the experiments. They were housed in a temperature and light-controlled room and fed on proprietary rat food *ad libitum*.

In vivo treatments
Experimental groups ($n \geq 5$) were subcutaneously injected with 10IU of pregnant mare's serum gonadotrophin (PMSG). Where human chorionic gonadotrophin (hCG) was administered *in vivo*, to mimic the LH surge, 10IU hCG were injected subcutaneously 48 hours post PMSG treatment.

Animals to be used for granulosa cell culture were injected with diethylstilboestrol (DES). 2mg DES was dissolved in 10µl ethanol and 90µl glycol (BDH, Lutterworth, Leics, UK). Two 2mg doses were administered subcutaneously 24h apart.

The animals were killed by carbon dioxide asphyxiation and the ovaries were removed and dissected free from fat and other surrounding tissues. Tissues for *in vivo* studies were snap frozen in liquid nitrogen (-196°C).

Cell culture method

Granulosa cells from preantral and early antral follicles were harvested from the DES-treated animals' ovaries by gentle puncture with a hypodermic needle in culture medium. Cells were then centrifuged at 1500rpm for 10min and resuspended in 1ml of medium. To disperse the cells and eliminate clumps, the suspension was drawn through a pipette tip several times, made up to 10ml and allowed to settle for 1min. The cells remaining in suspension were then centrifuged, resuspended and titrated as before. An aliquot of the cell suspension was removed and mixed with an equal volume of trypan blue solution, and the cells in this mixture were counted using a haemocytometer. Viability was assessed based on the exclusion of the dye and was typically >40%. Yields were approximately $1x10^6$ granulosa cells per DES-treated animal. Cells were cultured in Medium 199 (M199) (GibcoBRL Ltd, Paisley, UK) containing 25mM HEPES buffer, extra (2mM) L-glutamine, penicillin (50 IU/ml) and streptomycin (50mg/ml) with 1% (w/v) BSA . The term 'medium' from here on refers to M199 with supplements added. Multiwell plastic dishes (Corning, New Jersey, USA) were precoated with donor calf serum (GibcoBRL) and washed twice with 500µl Dulbecco's phosphate buffered saline (DPBS) (GibcoBRL) before use. 250µl medium 199 containing the appropriate hormone treatment was added to each well and to this, 250µl of the cell suspension containing the appropriate number of cells was added. The cultures were incubated at 37°C in a humidified incubator gassed with 95% air/5% CO_2 for the given time and hormonal conditions according to each experiment. Gonadotrophins used were pituitary FSH (LER 8/116; FSH bioactivity 900IU/mg, LH bioactivity 0.5IU/mg, kindly donated by Prof. L.E. Reichert, Jr.), rhFSH (Serono Laboratories UK Ltd, Welwyn Garden City, Herts. UK; batch BFBA 9612, kindly provided by Dr Norah Spears, University of Edinburgh) and rhLH (Serono, batch R-HLH C22, ECH Z, ech1, biological activity 82,269IU/ml (Van Hell method), LH concentration 4.7mg/ml (optical density)).

2. Extraction, purification and quantification of RNA

Total RNA was extracted from tissues according to the method of Chomczynski and Sacchi (Chomczynski and Sacchi, 1987). Medium was removed from granulosa cell cultures and the cells were lysed on ice with cold "solution D" (4M guanidinium thiocyanate (Fluka, Glossop, Derbyshire), 25mM sodium citrate, 0.5%[w/v] sarcosyl, 100mM β-mercaptoethanol; 200µl/well). The cell lysate was transferred to

polypropylene tubes (Falcon 2059, Becton Dickinson, Cowley, Oxford). Alternatively, frozen whole ovaries were homogenised in ice-cold solution D in corex tubes (Corning Costar UK Ltd, High Wycombe, Bucks). Cellular DNA was sheared by passing the solution through a hypodermic syringe fitted with a 21-gauge needle several times. One tenth of a volume of 2M sodium acetate (pH 4), one volume of water-saturated phenol and one fifth chloroform : isoamyl alcohol (49:1 v/v) were added, the tubes being vortexed between additions. The tubes were placed on ice for 15min and centrifuged at 10,000 x g for 20min at 4°C to separate the phases. The aqueous (top) phases were transferred to fresh tubes and an equal volume of isopropanol was added and mixed thoroughly. The tubes were left at -20°C for at least 1h to allow the RNA to precipitate, then spun at 10,000 x g for 20min at 4°C. The supernatants were poured off, and the pellets dissolved in 600µl of solution D and transferred to microcentrifuge tubes. RNA was reprecipitated with an equal volume of isopropanol at -20°C for at least 1h, and recovered by centrifugation at 13,000 x g for 15min at 4°C. RNA pellets were washed with 70% (v/v) ethanol (BDH) and allowed to dry at room temperature, before being dissolved in deionised formamide.

Concentration and purity of total RNA preparations were determined by spectrophotometry. 2µl of each preparation was diluted in 200µl of water and transferred to a spectrophotometry cuvette. The purity of the RNA sample was calculated from the ratio of the absorbance of the solution at 260nm (A_{260}) to its absorbance at 280nm (A_{280}). A ratio of 2.0 indicated the degree of purity. The concentration of RNA was calculated from the A_{260} value where an optical density of 1.0 is equal to 40µg/ml RNA.

The RNA samples dissolved in formamide were stored at -20°C until assayed.

3. Reverse-Transcription Polymerase Chain Reaction (RT-PCR)

Reverse transcription polymerase chain reaction (RT-PCR) was used to amplify cDNA which could then be cloned into pCR-Script™ Amp SK(+) plasmid (Stratagene, Cambridge, UK) and used to make labelled riboprobes for RPA and in situ hybridisation.

Primers were designed using the software package GeneJockey II (Biosoft, Cambridge, UK). All the genes to be examined, except the rat steroidogenic acute regulatory protein gene, had previously been sequenced. All primers were of suitable melting temperatures (T_m) and checked for internal stability (GC/AT ratio) and specificity, avoiding self and false priming. Primer length was between 16 and 24 nucleotides for optimal amplification of less than 100bp (Rychlik, 1993).

Reverse transcription

The sample RNA (suspended in water) was denatured at 65°C for 5min in a 10µl reaction, containing 1µl antisense (3') primer (Cruachem, Glasgow, UK). The following components were added to give a 20µl reaction mix (final concentrations are given). 1x RT reaction buffer (50mM Tris-HCl [pH 8], 75mM KCl, 30mM $MgCl_2$) supplied with RT enzyme; 200µM each dNTP (Promega); 40U RNaseOUT (Gibco BRL) - a recombinant RNase inhibitor; and 50U MMLV-reverse transcriptase (Stratagene Cloning Systems, La Jolla, CA, USA).

The RT mixture was incubated at 37°C for 1h, followed by 72°C for 10min to inactivate the enzyme and cooled on ice prior to PCR.

Polymerase Chain Reaction (PCR)

The following components were mixed on ice and added to 5µl of the RT sample to give a 50µl reaction mix. 1x PCR reaction buffer (20mM Tris-HCl [pH 8.8], 2mM $MgSO_2$, 10mM KCl, 10mM $(NH_4)_2SO_4$, 0.1% Triton X-100, 100µg/ml BSA) supplied with *Pfu* enzyme; 200µM each dNTP (Promega); 0.5µM sense (5') primer; 0.5µM antisense (3') primer; and 2.5U *Pfu* DNA polymerase (Stratagene). *Pfu* DNA polymerase was used because it has a 3'->5' proof-reading activity to generate DNA fragments of high fidelity which is important when constructing templates to be used for RNase protection assay. *Pfu* also produces blunt-ended fragments which is useful when cloning into the plasmid vector pCR-Script™ Amp SK(+) (see section 5).

PCR temperature cycling was carried out in a Hybaid Omn-E Thermal Cycler. One cycle consisted of 45 sec at 94°C for nucleic acid denaturation; 45 sec between 55-70°C for primer annealing and 45 sec at 72°C for product extension. This was repeated for 30 cycles for DNA amplification. Extension time in the final stage was prolonged to

10 min at 72°C to allow completion of product synthesis. (PCR optimisation involved the increase of primer annealing temperatures to enhance stringency).

Assessing the PCR products

2% agarose gel electrophoresis was used to assess PCR product formation. When DNA purification from the gel was necessary, 2% low melting temperature agarose (Flowgen, FMC BioProducts, Rockland, ME, USA) gels were used. Each gel was made up with TAE buffer and 0.04% ethidium bromide for DNA visualisation under UV light. Molecular weight markers (GibcoBRL) were used to determine product size.

DNA purification from agarose gels

DNA was purified using the Promega Wizard kit with vacuum manifold. The desired band was cut from the low melting point gel and incubated at 70°C until the agarose had completely melted. 1ml DNA purification resin was used for DNA extraction under vacuum through the minicolumn. The column was washed with 2ml 80% isopropanol under vacuum and the residue was removed by centrifugal force. DNA was eluted with 50µl TE buffer.

Identification of the PCR product

To verify the authenticity of the PCR fragments, 2-5µl was subjected to enzyme digestion. Restriction sites were located using GeneJockey. Specific buffer conditions and optimal incubation temperatures were outlined in the Promega laboratory manual. 1U of enzyme/µg DNA was used with 1x buffer in a 20µl reaction. Incubation was for 1h and the digest analysed by 2% gel electrophoresis.

4. RNA templates

18S

18S is ribosomal RNA which is constitutively expressed throughout the cell cycle and was used as an internal control to show equal loading. The plasmid used contains a 80bp cDNA insert complementary to human ribosomal RNA and is under the control of T7 RNA polymerase. This was purchased from Ambion (ams Biotechnology, Witney Oxon, England).

Aromatase

Rat P450arom riboprobe was synthesised from a 1.0kb cDNA cloned into pGEM3Zf(+) (Promega) generously donated by Dr. J. S. Richards, Baylor College of Medicine, Houston, Texas, USA (Hickey *et al.*, 1990). When cut with AccIII, SP6 RNA polymerase generates an antisense riboprobe of approximately 300bp.

LHR

Luteinising hormone receptor (LHR) riboprobe template was kindly donated by Dr. Aaron Hsueh (LaPolt *et al.*, 1990). The clone corresponds to bases 249 to 849 of the rat LHR cDNA reported by (McFarland *et al.*, 1989), subcloned into pGEM-4Z plasmid (Promega). When linearised with BglII and transcribed with T7 RNA polymerase, an antisense probe of 408bp was produced.

PR total

The plasmid containing the rat progesterone receptor (PR) cDNA encoding the hormone-binding domain (Park and Mayo, 1991) was kindly donated by Dr O-K. Park-Sarge, Department of Physiology, University of Kentucky, Lexington, Kentucky. This cDNA was cloned into pGEM-4Z. When linearised with BamI, T7 RNA polymerase generated a riboprobe of 550bp.

PRB and PR(A+B)

Construction of the template cDNA used to transcribe riboprobes for PRB and PR(A+B) is described in chapter 3, section 2.3.

StAR

At the time of constructing this template, the rat StAR sequence was unknown. However, sequences from human, mouse, cow and pig were known and homology between them was examined using GeneJockey. Areas of high homology between species were chosen to use as primers for RT-PCR. Using primers that were complementary to the human sequence, RT-PCR was performed, as described in section 3 with an annealing temperature of 50°C. The products were run on a gel, a band of correct length was cut and eluted from the gel and cloned and sequenced as described in sections 5 and 6. When linearised with NcoI, transcription with T7 RNA polymerase gives a riboprobe of 351bp. The (human) primers used and the corresponding area later identified in the rat sequence are shown below. (Underlined letters represent non-homologous nucleotides).

StAR primers

	5'	3'	
antisense	TGGAAGAAGG	AGAGTCAGCA GG	human
	TGGAAGAAGG	AAAGCCAGCA GG	rat
sense	ACCTGGTTGA	TGATGCTCTT GG	human
	ACTTGGTTGA	TGATGGTCTT TG	rat

P450scc

Primers (as shown below) for RT-PCR were chosen from the sequenced rat P450scc gene using GeneJockey. A PCR annealing temperature of 65°C was used for 30 cycles to produce a product of approximately 730bp. This was cloned and sequenced, and if linearised with EcoRI, T3 RNA polymerase produced an antisense riboprobe.

P450scc primers

	5'	3'
antisense	ACCTTCAAGT	TGTGTGCCAT TTCAT
sense	GACCCTAAGG	ACGCAGCGAC TCTCTT

3βHSD

The plasmid containing the cDNA encoding the rat 3βHSD type 1 (Lorence *et al.*, 1991) was generously donated by Prof. J. Ian Mason, presently at The University of Edinburgh, Scotland, UK. The pCMV5R3β-HSD vector described was cut with PstI and PvuII to release a 479bp fragment corresponding to 453-932bp of the original vector. This was subcloned into pBluescript SK+ (Promega). The orientation of the insert was such that when linearised with EcoRI, T3 RNA polymerase could be used to transcribe antisense RNA.

GR

cDNA template was prepared by Dr Masa Tetsuka (Tetsuka *et al.*, 1999). When cut with EcoRI and transcribed with T3 RNA polymerase, an antisense riboprobe of 770bp was produced. The primers used for creating the cDNA template from rat RNA are shown below.

GR primers

	5'	3'
antisense	CCTTAGGAAC TGAGGAGAGA AGCA	
sense	CTCTGCCTGG TGTGCTCCGA TGAA	

11βHSD1

cDNA template was prepared by Dr Masa Tetsuka (Tetsuka *et al.*, 1999). When cut with EcoRV and transcribed with T3 RNA polymerase, an antisense riboprobe of 620bp was produced.

11βHSD2

cDNA template was prepared by Dr Masa Tetsuka (Tetsuka *et al.*, 1999). When cut with NotI and transcribed with T7 RNA polymerase, an antisense riboprobe of 410bp was produced.

5. Cloning, plasmid preparation and analysis

Cloning DNA

Plasmids containing cDNA inserts were either kindly donated, obtained from colleagues or were prepared by RT-PCR from RNA from the appropriate tissues and subcloned into a plasmid vector. All cloning was done using pCR-Script™ Amp SK(+) Cloning Kit from Stratagene. pCR-Script™ Amp SK(+) is derived from the pBluescript ® II SK(+) phagemid. This cloning vector includes an ampicillin-resistance gene, a *lac* promoter for gene expression, T3 and T7 RNA polymerase promoters for *in vitro* production of RNA and a multiple cloning site.

Ligation and transformation of the RT-PCR product was undertaken as briefly described below.

Ligation

The following components were added together in order in a microcentrifuge tube. 1µl pCR-Script™ Amp SK(+) cloning vector, 1µl pCR-Script 10x reaction buffer, 0.5µl 10mM rATP, 2-4µl PCR product, 1µl *Srf* I restriction enzyme, 1µl T4 DNA ligase and distilled water to a final volume of 10µl. This was mixed gently and incubated for 1 h at room temperature before the reaction was stopped by heating at 65°C for 10min. The cloning reaction was stored on ice until transformation into Epicurian Coli® XL1-Blue MRF' Kan supercompetent cells.

Transformation

40µl Epicurian Coli® XL1-Blue MRF' Kan supercompetent cells were thawed on ice and transferred into a prechilled 15ml Falcon polypropylene tube (Falcon). β-mercaptoethanol was added to give a final concentration of 25mM and this was incubated on ice for 10 min, being swirled gently every 2 min. 2µl of the cloning reaction (from above) was added, swirled gently and incubated on ice for 30 min. The reaction was then heat pulsed for exactly 45 sec at 42°C and then returned to ice. 0.45ml of SOC medium (GibcoBRL) was added and the reaction was incubated at 37°C for 1h with shaking at 225rpm. 100µl of this mixture was then spread onto a LB-ampicillin agar plate containing X-gal and IPTG. The agar plates were incubated overnight at 37°C. Colonies appearing white in colour were picked and grown for plasmid extraction.

Plasmid preparation from bacterial cultures

3ml LB broth (section 10) containing 50µg/ml ampicillin was inoculated with a white bacterial colony from above. This was incubated overnight at 37°C at 225 rpm.

Plasmid DNA was isolated from 3ml bacterial cultures by the alkaline lysis method using the 'Wizard Minipreps' DNA purification system from Promega. Briefly, suspensions were centrifuged at 1600g and the supernatant discarded, cells resuspended in buffer containing 50mM Tris/HCl, pH 7.5, 10mM EDTA and 100µg/ml RNase and lysed with an equal volume of 0.2M NaOH and 1% SDS. The suspension was neutralised with 2.55M potassium acetate and centrifuged for 5min at 12,000g resulting in sedimentation of bacterial genomic DNA. The supernatant

(approximately 600µl) was removed, mixed with 1ml DNA purification resin and passed down a miniprep column which retarded only the plasmid DNA. The column was washed with an ethanol based solution, 50µl pure water added and incubated at room temperature for 1min. DNA was eluted from the column by centrifugation at 12,000g for 20 sec.

Plasmid sizes were checked and restriction analysed by digesting with restriction enzymes to linearise or cut fragments from the circular plasmid. The sizes were checked on 2% agarose gels.

6. DNA sequencing

DNA sequencing was undertaken using the chain-termination DNA sequencing method (Sanger *et al.*, 1977) utilising the Sequenase Version 2.0 DNA Sequencing Kit (United States Biochemical, Amersham Life Science, Buckinghamshire). The DNA cloned into the plasmid pCR-Script™ Amp SK(+) was sequenced from both 3' and 5' ends using oligonucleotide primers corresponding to T7 and T3 RNA polymerase sites at either side of the insert.

	5'	3'
T3 primer	AATTAACCCT CACTAAAGGG	
T7 primer	GTAATACGAC TCACTATAGG GC	

A brief description of the methodology follows.

The DNA plasmid was denatured (i.e. made single-stranded) by the addition of 250mM NaOH to the plasmid DNA (2.5µg) and sequencing primer (0.15pmol) mix. This was precipitated in the presence of 0.3M sodium acetate and ethanol and resuspended in Sequenase reaction buffer and 10% DMSO. The primers were then annealed to the DNA.

[^{35}S] dATP (400Ci/mmol, Amersham International, Aylesbury, UK) was used to label the reactions and was added to individual mixes containing the unlabelled dGTP, dCTP and dTTP. A chain-terminating 2',3'-dideoxynucleoside triphosphate (ddNTP) and Sequenase Polymerase were also added to each of the reaction mixes. The labelled

mixes were each added to the DNA mix and incubated at 37°C for exactly 5min. Stop solution was added to each mix and after denaturing at 75°C for 2min the reactions were run on a 6% acrylamide denaturing gel (as described in RPA methodology) at 40W, ~50°C for several hours. The gel was then dried and exposed to autoradiographic film (X-Omat XAR5, Kodak).

7. Progesterone measurement of culture medium

Progesterone (4-pregnene-3,20-dione) was measured in culture media by specific radioimmunoassay. The assay buffer was PBS/gelatine (0.05M sodium phosphate, 0.15M NaCl, 0.1% gelatin; all BDH). The antibody, anti-progesterone-11α-hemisuccinate-BSA (R31/12) was provided by the Medical Research Council, Mill Hill, London and was used at a working dilution of 1:4,500. Cross-reaction of the antibody with oestradiol-17β, ICI 164,384, ICI 164,275, or SCH 16,423 was <0.01% (Turner, 1992). Cross-reaction with testosterone, 5α-dihydrotestosterone and 2-hydroxyoestradiol was 0.04%, 0.05% and 0.02% respectively. Tracer, [1,2,6,7-^{3}H]-progesterone (80-110 Ci/nmol, Amersham), was stored at -20°C in ethanol, at a concentration of 10μCi/ml. Before use, this stock was diluted 1:400 in assay buffer to give 10,000 cpm/500μl. A standard curve covering the range of 0.04 to 5.1 pmol progesterone/tube, made up in culture medium, was used. Samples (1 - 200μl) or standards were placed in tubes and the volume made up to 200μl. Quality controls (0.26 and 2.7 pmol/tube) were also included at the beginning and end of each assay. Antibody (300μl) and tracer (500μl) were added, and the tubes vortexed and incubated overnight at 4°C. Tubes were placed on ice for 30min before the addition of 200μl of cold dextran-coated charcoal (1.25% (w/v) dextran, 0.12% (w/v) charcoal in assay buffer) to separate bound and free progesterone. Tubes were mixed and incubated for 10min on ice, then centrifuged at 3,000rpm for 10min at 4°C. Supernatants were decanted and bound tracer was measured by liquid scintillation counting (RackBeta). Standard curves were calculated using a commercial computer program (AssayZap, Biosoft, Cambridge, UK). Samples which were outside the detection limits were re-assayed at the appropriate volume. The detection limit of the assay was within the range 0.05-0.06pg/tube. Inter- and intra-assay coefficients of variation were <15%.

8. Ribonuclease protection assay

Preparation of radiolabelled cRNA probes

Radiolabelled antisense cRNA probes were prepared as follows. Transcription buffer (Promega), DTT, Rnasin, ATP, GTP and CTP (0.5mM each), α^{32}P-UTP (800Ci/mmol, Amersham International, Aylesbury, UK), plasmid template DNA containing the appropriate cDNA insert of the probe to be transcribed and the corresponding RNA polymerase to produce antisense RNA. As a control for sample RNA loading, 18S antisense RNA probe (Ambion, Austin, TX) was prepared. This mix was incubated at 37°C for approximately 2h. RQ1 RNase-free DNase (Promega) was added and placed at 37°C for 30min to digest the DNA template. In order to purify full length cRNA transcripts, radiolabelled RNA probes were gel-purified. RNA probes were mixed with 80% gel loading dye (80% deionised formamide, 1mM EDTA, 0.1% xylene cyanol, 0.1% bromophenol blue, 0.1% SDS), heated at 95°C for 3min and loaded onto a 5% denaturing polyacrylamide gel and separated by electrophoresis at 3W (constant power) for 1h. After electrophoresis, the gel was wrapped in cling film and exposed briefly (2-3 minutes) to an autoradiographic film (X-Omat XAR5, Kodak). The segment of the gel containing the full length transcript was excised and the probe eluted in elution buffer (0.5M ammonium acetate, 1mM EDTA, 0.2% SDS) at 37°C for at least 2h. The solution was centrifuged at 10,000rpm for 5min and the activity of the supernatant was measured by liquid scintillation counting.

RNase protection assay

3-10μg of total RNA was combined with the ^{32}P-labelled riboprobes (approximately 2x 10^4 cpm of the highly specific probes and 1x 10^4 cpm of 18S) in an Eppendorf tube and the RNA was precipitated by adding 0.1 volumes of 3M sodium acetate and 2.5 total volume of 100% molecular grade ethanol. This was placed at -20°C for at least 15min and centrifuged at 13,000 rpm for 15min at 4°C. The pellet was washed with 70% ethanol and was allowed to dry at room temperature. The pellet was resuspended in 20μl formamide hybridisation buffer (80% deionisied formamide, 1mM EDTA, 40mM PIPES [pH 6.4], 0.2M sodium acetate), denatured at 90°C for 5min and incubated at 52°C for 16h in a water bath.

After hybridisation of probes to RNA, non-hybridised, single-stranded RNA was selectively digested by incubating with 200μl RNase A/T1 digestion buffer (10 mM Tris-HCl [pH 7.5], 5mM EDTA, and 300mM NaCl containing 10μg/ml RNase A and

200U/ml RNase T1; both enzymes from Boeringer Mannheim, Mannheim, Germany) for 30min at 37°C. The digestion reaction was terminated by the addition of 1% SDS and 0.5μg/μl of proteinase K. The RNA was then isolated by the addition of 1 volume of phenol : chloroform : isoamyl alcohol (25:24:1), vortexed and centrifuged at 13,000 rpm for 5min. The upper aqueous layer was transferred to a fresh tube and the reactions were precipitated in the presence of 0.1 volume of 3M sodium acetate and 2.5 volumes of 100% ethanol for 30 minutes at -20°C and centrifuged at 13,000 rpm for 20min at 4°C. The supernatant was carefully removed and the pellet redissolved in 10μl of 80% gel loading dye, heated to 95°C for 3min and loaded on to a 5% denaturing acrylamide gel. Undigested controls were included to check for full RNase digestion. Samples were run for approximately 1h at 35W before drying the gel at 80°C and exposing to an autoradiographic film (X-Omat XAR5, Kodak), and quantified by electronic autoradiography (Instant Imager; Packard, Downers Grove, IL, USA).

Denaturing acrylamide RNase protection assay gel

A 40ml gel mix containing 8M urea, 5% acrylamide (19:1 acrylamide: bisacrylamide [Bio-Rad Laboratories, Hercules, CA, USA]) and 1x TBE was polymerised using 40μl TEMED and 200μl fresh 10% ammonium persulphate and before complete polymerisation was poured carefully between clean glass plates. A comb was inserted to form wells for sample loading. The gel was run in 1x TBE buffer.

9. In Situ hybridisation

Tissues were fixed by submerging in 4% paraformaldehyde overnight and then transferring to 70% ethanol for storage before processing and wax-embedding.

Tissues were processed through a graded series of alcohols in an automatic 2LE processor (Shandon Scientific Limited, Cheshire, UK) using a standard 20h cycle, and embedded in paraffin wax. Tissue processing was kindly performed by Sheila MacPherson.

Glass microscope slides were soaked in 10% Decon 90 (Decon Laboratories Ltd, Sussex) for an hour and washed in running tap water, again for one hour. These were rinsed in distilled water and allowed to dry overnight at 60°C to prevent RNase

contamination. All slides were subsequently washed in a 0.25% solution of 3-aminopropyl triethoxysilane (TESPA) in acetone (BDH Ltd, Poole, UK), followed by a wash in acetone, and finally rinsed in filtered distilled water and dried.

Paraffin wax embedded tissue was sectioned to a thickness of 5μm using a hand operated "820" Spencer Microtome (American Optical Corporation) and a D-profile knife. Sections were floated on RNase-free water, transferred on to the treated slides and dried at 37°C overnight before use.

Radioactive in situ hybridisation

cDNA template for StAR was constructed as described in section 4 of this chapter. The riboprobe was made by the same methods as those described in section 8 for RNase protection assays, only using α^{32}P-UTP (1000Ci/mmol).

Paraffin wax embedded tissue was cleared in histoclear for 10 min and rehydrated in a series of alcohols with decreasing concentrations. Tissue was placed in 0.2N HCl for 20 min followed by two 5 min washes in distilled water. Sections were then treated with 2 μg/ml proteinase K (Sigma) in buffer containing 100mM Tris/HCl pH 7.8 and 50mM EDTA at 37°C for 20 min followed by distilled water for 2 min. Sections were washed briefly in 0.1M triethanolamine (TEA) pH 8.0 and acetylated in 0.25% acetic anhydride in 0.1M TEA pH 8.0 for 10 min. Finally, sections were rinsed in distilled water and prehybridised in buffer containing 4 x STE (section 10), 1 x Denhardt's solution, 10mM DTT, 125μg/ml salmon sperm DNA, 125μg/ml yeast transfer RNA in 50% deionised formamide for 2-4h at 60°C.

Hybridisation was continued overnight with a probe concentration of 1 x 10^6 cpm in 40μl buffer/slide. Hybridisation buffer was prehybridisation buffer with 10% dextran sulphate added. The incubation was carried out beneath coverslips prepared from gel bond film (Flowgen), in a humidified chamber at 60°C.

After incubation, sections were washed in two changes of 4 x SSC for 5min each to remove the coverslip, and treated with RNase A at a concentration of 20μg/ml in 0.5M NaCl, 10mM Tris pH 8.0 and 1mM EDTA for 30min at 37°C. Sections were washed in RNase buffer alone for 30min at 37°C followed by 30min washes in 4 x SSC and 2 x SSC at room temperature and a final wash in 0.1 x SSC at room temperature for

30min. Sections were then dehydrated in alcohols of increasing concentration all containing 300mM ammonium acetate.

After air drying, slides were warmed to 45°C and dipped in prewarmed NTB3 emulsion (Kodak) at 45°C in the dark. Emulsion-coated slides were stored in a humidified, lightproof box overnight before transfer to a lightproof polyacetyl black trough (Lamb's Laboratory Supplies, London, UK) with silica gel, and stored at 4°C for 5 weeks.

Silver grains formed by reaction of the hybridised, radiolabelled probe with the emulsion were developed using Kodak D19 developer as follows. Slides were placed in developer cooled to 15°C for 4 min, followed by a 20sec wash in pure water and grains were fixed by incubation for 10min in Kodak polymax at 14°C. Sections were washed in pure water for 20min, rinsed in running tap water for 20min, stained with haematoxylin, dehydrated and mounted in Pertex (O. Kindler GmbH & Co. supplied by Laboratory Sales Ltd., Rochdale, England).

Slides were analysed under dark field using an Olympus BH2 microscope (Leitz, Wetzlar, Germany) to visualise the silver grains indicating areas of hybridisation. Bright field microscopy was used to examine the morphology of the ovaries.

10. Buffers and solutions

LB Medium (Luria-Bertani Medium)
(per litre)
10g bacto-trypton
5g bacto-yeast extract
10g NaCl
Adjust to pH 7.0 with NaOH, and autoclave

Sodium chloride, Tris, EDTA (STE)
0.1M NaCl
10mM Tris-HCl (pH 8.0)
1mM EDTA (pH 8.0)

1 x Tris-acetate (TAE)
0.04M Tris-acetate
0.001M EDTA

Tris-EDTA (TE) pH 7.4
10mM Tris-HCl (pH 7.4)
1mM EDTA (pH 8.0)

0.5 x Tris-borate (TBE)
0.045M Tris-borate
0.001M EDTA

Chapter 3 Progesterone in the Rat Ovary

1 Introduction

Progesterone is the hallmark of ovulation. The massive concentrations, required to sustain the uterine lining in preparation for implantation, are produced by the granulosa-lutein cells of the corpus luteum. However, this is not the only function of progesterone.

It has previously been shown that pharmacological (Loutradis *et al.*, 1991; Brannstrom, 1993; Batista *et al.*, 1994) or biochemical (Hibbert *et al.*, 1996) blockade of progesterone synthesis or action is associated with anvolution. Progesterone acts in an endocrine fashion on the hypothalamic-pituitary axis, as described, but it also exerts an essential local action within the ovary (Lipner and Wendelken, 1971; Brannstrom and Janson, 1989; Loutradis *et al.*, 1991; Lydon *et al.*, 1995; Hibbert *et al.*, 1996). This local action may be autocrine or paracrine, i.e. it may be produced by and may act upon (traditionally through a nuclear receptor) the same cell or local surrounding cells, respectively. This paracrine/autocrine feedback mechanism may exist within the mature follicle whereby locally produced progesterone stimulates the cascade of events leading to luteinisation and ovulation (Fanjul *et al.*, 1983; Iwamasa *et al.*, 1992; Morgan *et al.*, 1994; Donath *et al.*, 1997; Lioutas *et al.*, 1997). Conclusive evidence that progesterone has an essential local (ovarian) action comes from PR knockout mice which fail to ovulate with exogenous gonadotrophin treatment (Lydon *et al.*, 1995). Effectively, interference with progesterone production, metabolism or reception can potentially be used to bring about anovulation.

Before ovulation can occur, a follicle must be mature and responsive to LH. FSH controls the production of a variety of hormones, peptides/proteins and co-factors essential for the maturation of the preovulatory follicle. A complex endocrine feedback mechanism brings about a surge in LH concentrations which instigates the expression of ovarian genes involved in ovulation. Among these FSH- and LH-induced genes are those responsible for progesterone production and progesterone receptor expression (Iwai *et al.*, 1990; Park and Mayo, 1991). In humans and many primates, ovulation takes place 36-38h after the LH surge (Weick *et al.*, 1973; Hoff *et al.*, 1983). In rats,

this period is approximately 10-12h (Blandau, 1955). Therefore, progesterone and its receptor are expressed following the LH surge, yet before ovulation.

Progesterone is made de novo from cholesterol. Pregnenolone is derived from cholesterol via the P450 side chain cleavage (P450scc) enzyme complex situated on the inner mitochondrial membrane. Steroid acute regulatory (StAR) protein is responsible for this mitochondrial internalisation. Pregnenolone is then converted to progesterone by 3β-hydroxysteroid dehydrogenase (3βHSD) in the cell cytoplasm. The progesterone receptor (PR) exists as two known isoforms, PRB and the transcriptionally truncated PRA. The functions of these isoforms are not yet fully understood, but either one or both of them are present at the same time as progesterone synthesis takes place. The binding of progesterone to its receptor then may induce the ligand activated transcription associated with PR activation. This scheme is represented in fig.3.1.

The precise sequence of gene expression underpinning production, metabolism and reception of progesterone during follicular maturation in vivo is not known. For progesterone to fulfil a physiologically significant paracrine or autocrine mode of action, these genes would need to be subject to co-ordinate regulation by gonadotrophins. Here, this hypothesis is tested by using Rnase protection assays and *in situ* hybridisation to localise the expression of these genes in the ovaries of immature female rats treated with exogenous gonadotrophins to promote graded stages of preovulatory follicular maturation and luteinisation.

2 Materials and methods

2.1 Animals

Animals and chemicals were as described in Chapter 2.1. A preliminary experiment was undertaken to optimise the conditions of the RNase protection assay (RPA) technique. Immature rats were injected subcutaneously with 10IU PMSG, and after 48h the appropriate animals then received 10IU hCG to simulate the LH surge. PMSG was used due to its longer half-life which enabled the animals to be injected only once and not 4 times as is necessary with recombinant human FSH (rhFSH) (Miro *et al.*, 1995). The rats were then killed at 0h, 6h and 12h after the hCG injection. A control group of animals received no injections and were killed at the same time as the 0h

group. Each group contained 3 animals. When the RPA technique was established, and positive results were obtained, an experiment was set up similar to that described above, but with more time points. Each group contained 5-6 animals. Treatment groups were injected with 10IU of PMSG then animals were killed at 0h, 3h, 6h, 9h, 12h, 24h and 5days after the hCG injection. The control group was killed at the same time as the 0h group. One ovary was snap frozen and RNA was extracted and the other was fixed in 4% paraformaldehyde for in situ hybridisation analysis as described in chapter 2.9. This experiment was repeated 3 times to give triplicate samples.

2.2 Analysis of StAR, P450scc, 3βHSD gene expression

The StAR, P450scc and 3βHSD riboprobes used for RPA and in situ analysis were constructed as described in chapter 2.4. These probes, together with 18S could be hybridised with sample RNA for RPA because there were sufficient differences in their sizes to allow them all to be run on the same denaturing gel.

2.3 Identification of PR isoforms

A probe used to measure the combined expression of both isoforms was kindly donated by Ok-Kyong Park-Sarge (Park and Mayo, 1991). In order to analyse the differential expression of the two PR isoforms, probes were constructed to hybridise to different areas of the relative mRNAs. Fig.3.2A shows the areas of hybridisation of the riboprobes to the target mRNA. Probe PRB should hybridise to the amino terminal of the PRB mRNA, specific only to the B isoform. Probe PR(A+B) should hybridise, in part, to both PRA and PRB, but RNase treatment should result in a full-length protected fragment of B and a shorter protected fragment for A as demonstrated in fig.3.3.

Kraus et al (1993) described the promoter regions identified with the two PR isoforms. The exact transcription start site of the A isoform had not been identified: however it was known to be downstream of the PRB translation start site. Primers were designed for RT-PCR to amplify an area of approximately 620bp, downstream of the PRB translation start site, within the PRA promoter region thought to incorporate the PRA transcription start site. RT-PCR was carried out as described in Chapter 2, section 3 with an annealing temperature of 65°C and for 30 cycles for both sets of primers using total RNA extracted from rat uterus. The probes were cloned, sequenced and RPAs were carried out as previously described.

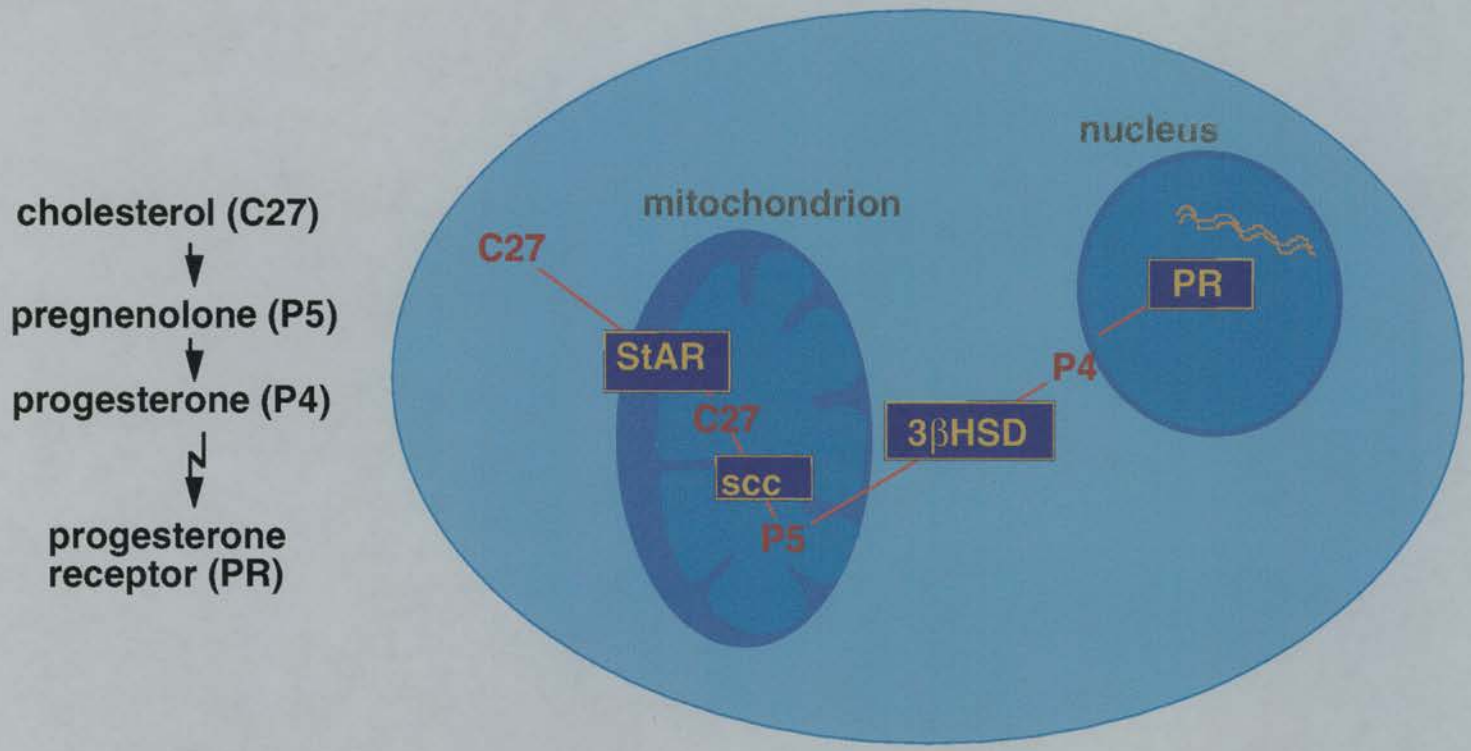
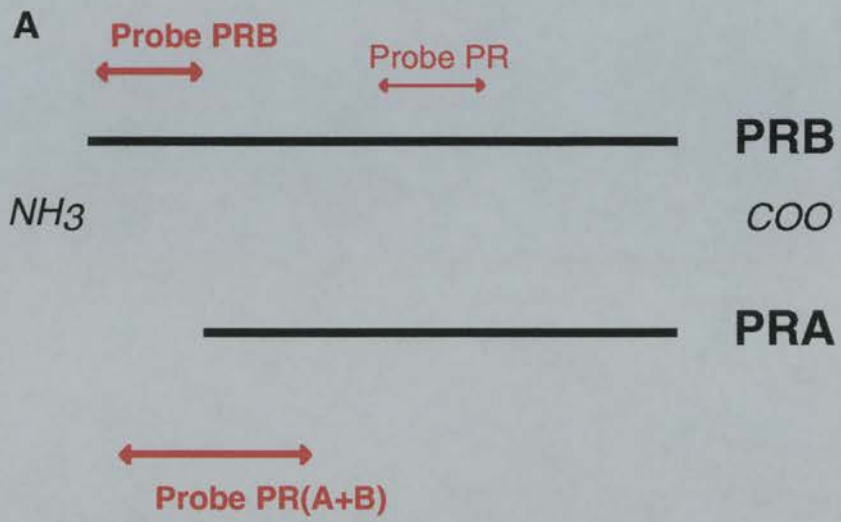


FIG. 3.1 A scheme for progesterone synthesis and reception



B

PRB (175bp)

sense	GTGTGAGGAT TCTGCCTTTC TGCT	(40)
antisense	AGGTTTACAT CACAGTCCTC ACCA	(215)

PR(A+B) (618bp)

sense	GACTCTCCAC ACATCTGG	(653)
antisense	TGCTGAGATG GCTTCACC	(1271)

FIG. 3.2 Probe design to identify rat PR isoforms A and B.
A. Probe PRB hybridises to the amino terminus of PRB, distinct to this isoform. Probe PR(A+B) hybridises with a larger area of PRB, but only a small area of PRA would hybridise to this probe. Probe PR hybridised to an area common to both isoforms.
B. Primers were designed using GeneJockey and used in RT-PCR to construct a vector containing the specified areas of PR cDNA.

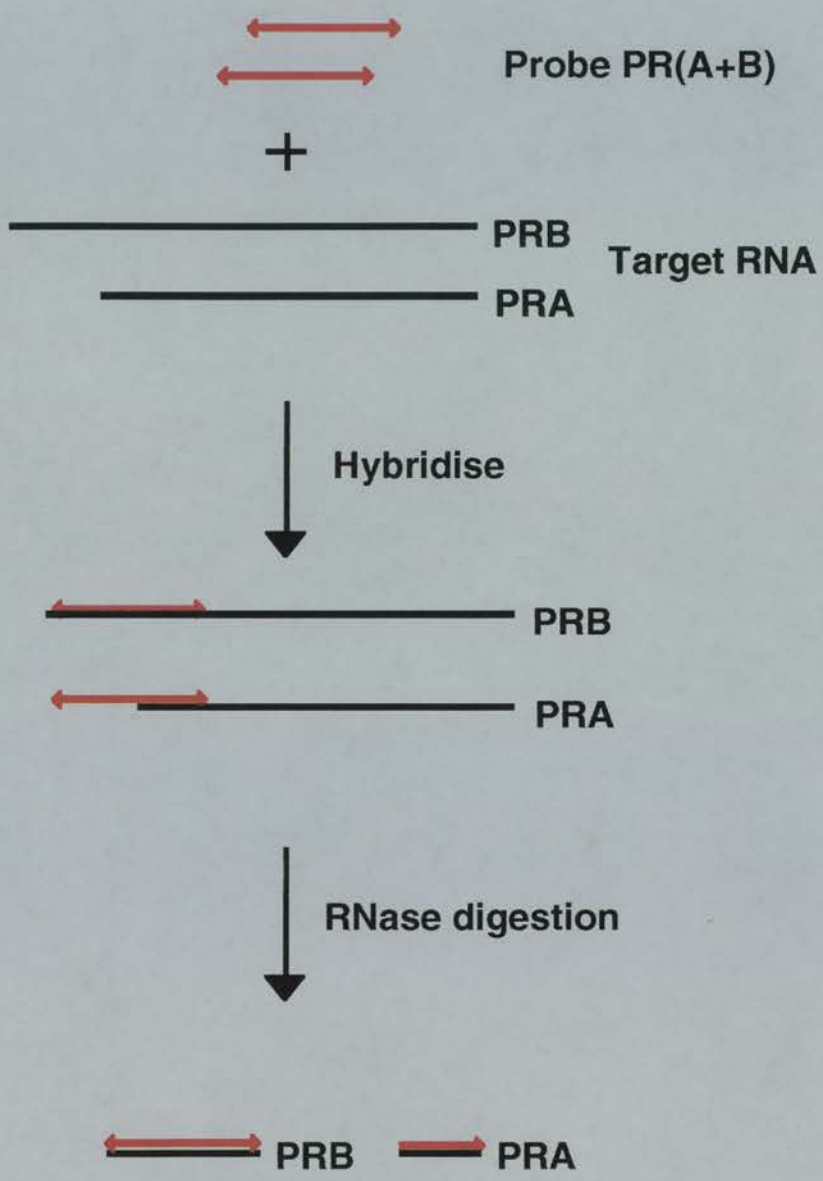


FIG. 3.3 RNase Protection Assay using probe PR(A+B).
Probe (A+B) hybridises fully to PRB, but only partially to PRA. Single stranded RNA is digested with RNase treatment, leaving only double stranded duplexes. When run on a denaturing gel, these two fragments will separate due to their different sizes.

2.4 Blood serum progesterone concentration measurement

Blood serum progesterone was assayed using Coat-A-Count Progesterone (DPC, Los Angeles, USA) according to the manufacturers' instructions. This is a direct assay and blood separation is not necessary. The antiserum is highly specific to progesterone, with a low cross reactivity (≤9%) to other naturally occurring steroids.

2.5 Statistics

Statistical analysis was carried out using commercial software (CLR ANOVA; Clear Lake Research Inc., Houston, TX, USA). Analysis of variance with the t-test was used to analyse differences between experimental and control observations. Differences assigned a P value of <0.05 were regarded as statistically significant.

3 Results

3.1 Optimising progesterone receptor RPA

Measurement of progesterone receptor (PR) was done by RNase protection assay (RPA), a sensitive and relatively accurate technique which allows small quantities of RNA to be analysed. Each RPA must contain a positive control (an RNA sample known to contain the target mRNA) and a negative control (an RNA sample known to be devoid of target mRNA). 18S was used as an internal control for each RNA sample. 18S is a ribosomal 'house-keeping' gene which is constitutively expressed throughout the cell cycle. It is used to check equal gel loading and the quantity of the target gene is standardised against the amount of 18S. A radio-labelled 18S probe and the target gene probe are hybridised to each RNA sample. Also, each gel has a lane containing full length probe, unhybridised and undigested. This is usually approximately 20-80bp longer than the protected fragment due to the inclusion of plasmid cloning sites. Fig.3.4 shows a photograph of an autoradiographic film of an RPA of PR.

To test the accuracy of the technique, a dose-dependent experiment was undertaken with increasing concentrations of the positive control, total RNA extracted from rat uterus. 2.5μg, 5μg, 7.5μg, 10μg and 20μg of total RNA was hybridised with PR and 18S probes. Fig.3.5 shows the expression of both PR and 18S RNA with increasing amounts of total RNA. The counts per minute of the PR and 18S protected bands were plotted against the quantity of RNA in the sample (fig.3.6). Both gave

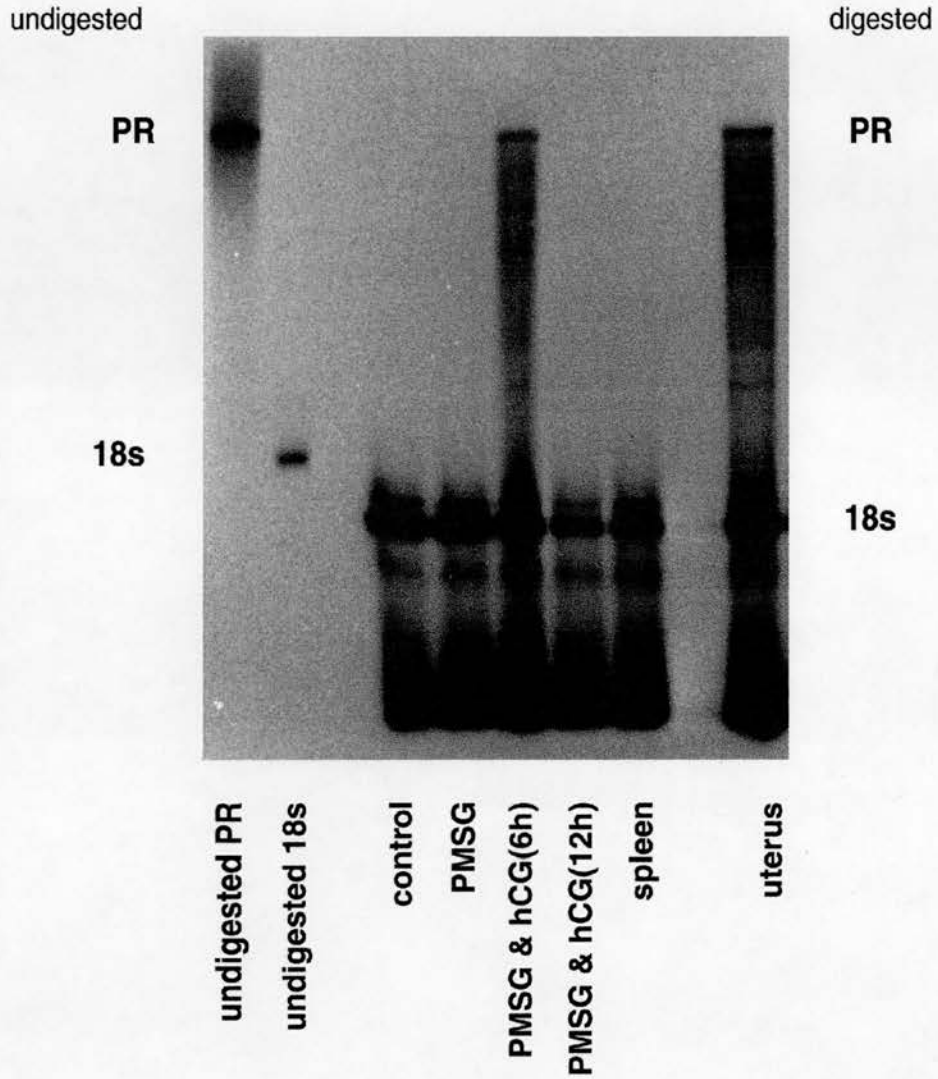


FIG. 3.4 RNase protection assay using probe PR.
Immature rats were treated with 10IU of PMSG, followed by 10IU of hCG. The 3 animals in each group were killed at 0h, 6h and 12h post hCG. The control group received no injections and were killed with group 0h. The uteri and spleens were removed from the 12h treatment group. 10μg of total RNA from each sample were hybridised with probes PR and 18S. The RPA gel was exposed to a Kodak X-Omat AR-5 autoradiogram for 2 days at -70°C using an intensifying screen.

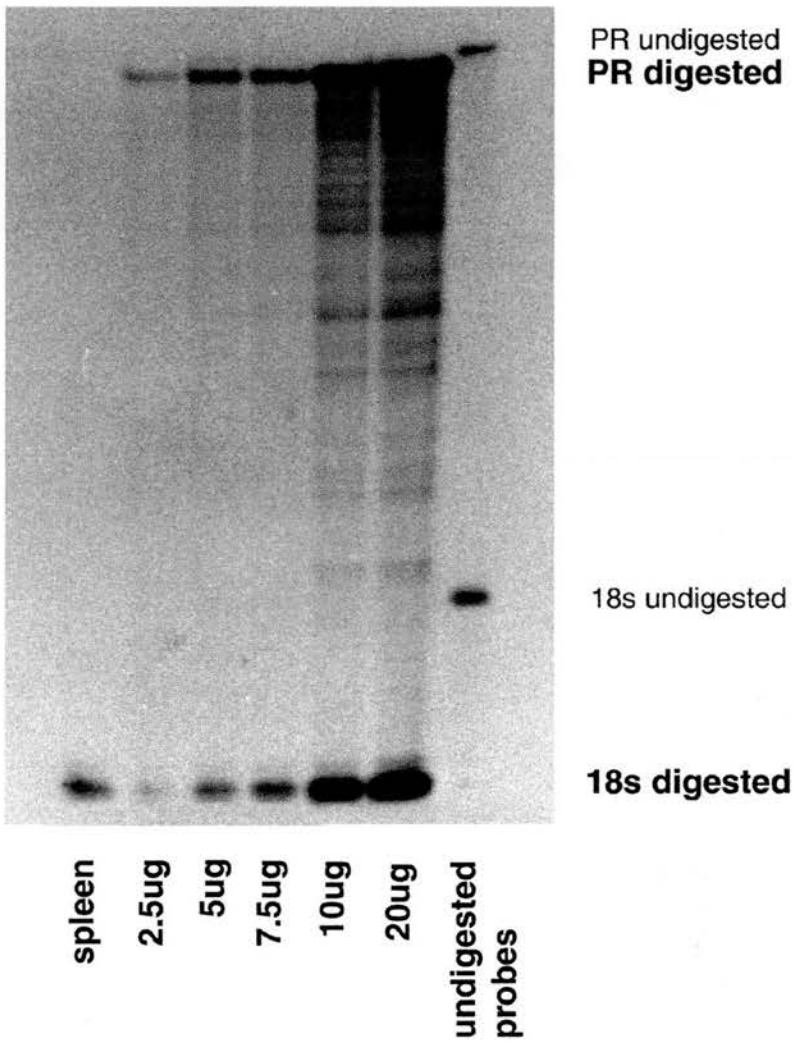


FIG. 3.5 RNase protection assay using probe PR with increasing concentrations of rat uterus total RNA.

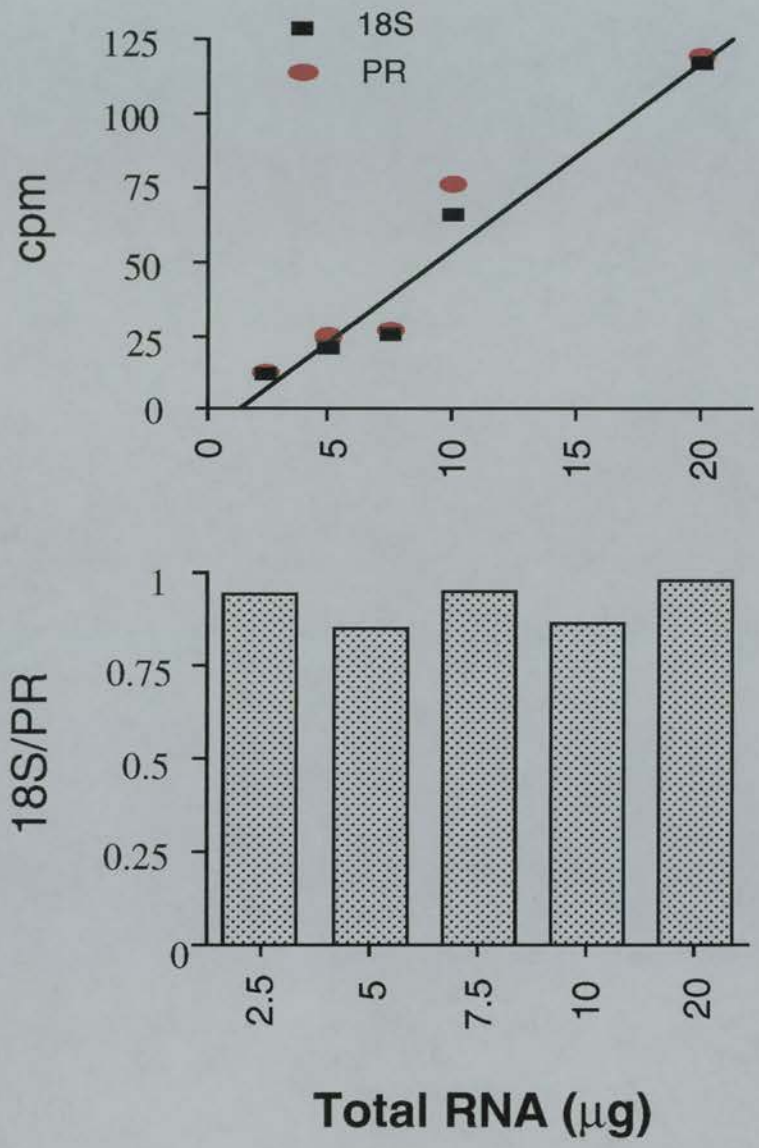


FIG. 3.6 Analysis of probe PR's dose dependency.
Quantification on an instant imager of the RPA shown in fig.3.5 gave the cpm of each band. A. The cpm of 18S and PR were plotted against total RNA concentration to give an approximate linear relationship. B. 18S/PR was plotted against total RNA to demonstrate the constant relationship between the intensity of the two probes and the RNA concentration.

approximately linear lines of the same degree. 18S/PR ratio demonstrated a small range between 0.85 and 0.98 indicating they were not significantly different from one another and 18S was a reliable internal control.

3.2 Quantifying the expression of PRA and PRB isoforms

The PR(A+B) probe was hybridised with 10μg rat uterus RNA. Fig.3.7 shows no protected bands with PR(A+B) probe. It was expected to observe several bands, relating to the number of PRA transcription start sites, as occurs in the B isoform. A variety of hybridisation temperatures and RNase conditions were tried, but no positive results were obtained.

3.3 Quantifying StAR, P450scc and gene expression

10μg of ovarian total RNA samples were hybridised with StAR, P450scc, 3βHSD and 18S and an autoradiogram of the gel is shown in fig.3.8. Non-equal loading was adjusted for by quantifying 18S and each sample was standardised to an arbitrary scale where the control sample showed 100% expression (fig.3.9).

StAR gene expression rose with 48h of PMSG treatment. hCG treatment dramatically increased this to over twice its expression in the immature ovary. By 9h after the hCG injection StAR expression had reached its maximum, only to decrease again slightly after this. The CL showed expression of over two-fold greater than the control ovary.

P450scc gene expression showed an increase of over 100% with 48h treatment of PMSG compared to the control sample. hCG induced a further increase to approximately 3 times the gene expression in the control. P450scc mRNA reached peak expression by 9h after hCG treatment and remained high throughout the rest of the experiment. Gonadotrophins induced a greater increase in P450scc expression than either of the other enzymes.

3βHSD expression was increased approximately 50% by PMSG treatment, with a further slight increase with hCG treatment, reaching a maximum of 1.5 times the control values by 9h after hCG, and remaining high for the rest of the time course.

3.4 Measurement of progesterone in circulating blood

The ovary is the major progesterone producing gland of the non-pregnant female. Thus progesterone concentration in circulating blood is a good indication of ovarian

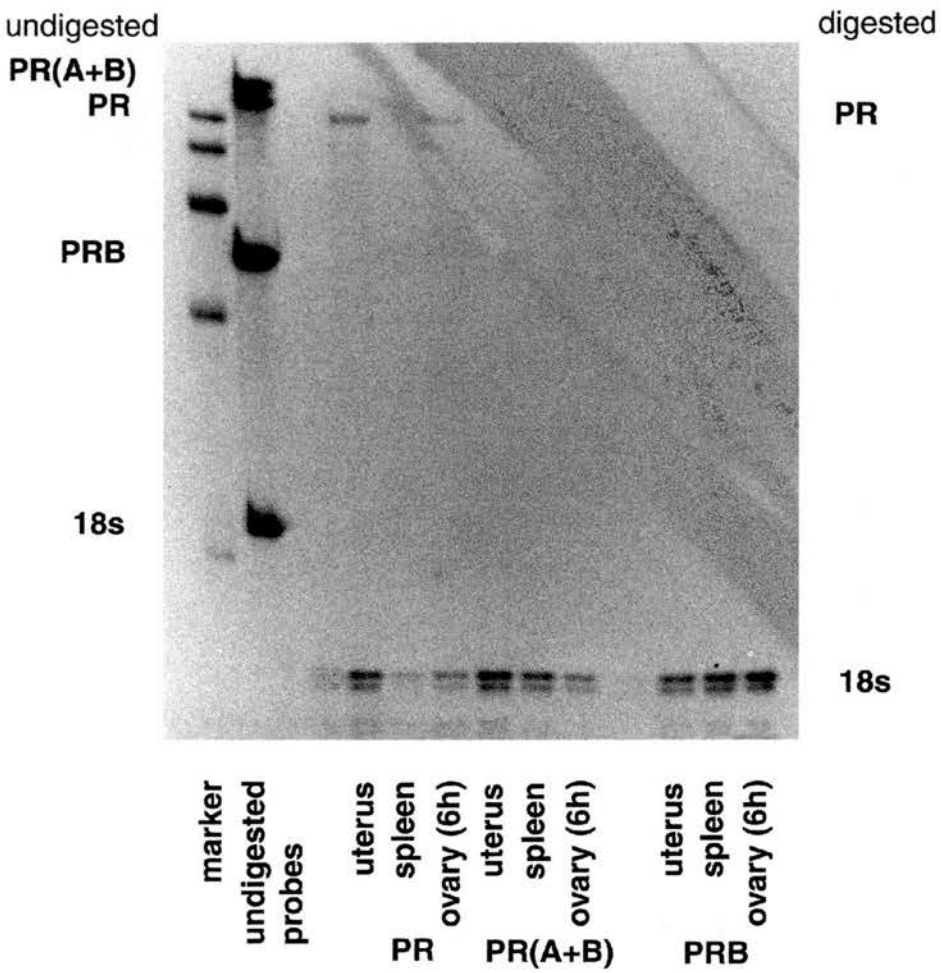


FIG. 3.7 RNase protection assay using probes PR, PR(A+B) and PRB.
Tissue was collected from treated rats as previously described. Uterus and ovary, 6h after hCG treatment, were used as positive controls and spleen was used as a negative control. 10μg of sample were hybridised to each probe. The lane of undigested probes shows full length riboprobes. The RNA marker sizes are 500bp, 400bp, 300bp, 200bp and 100bp.

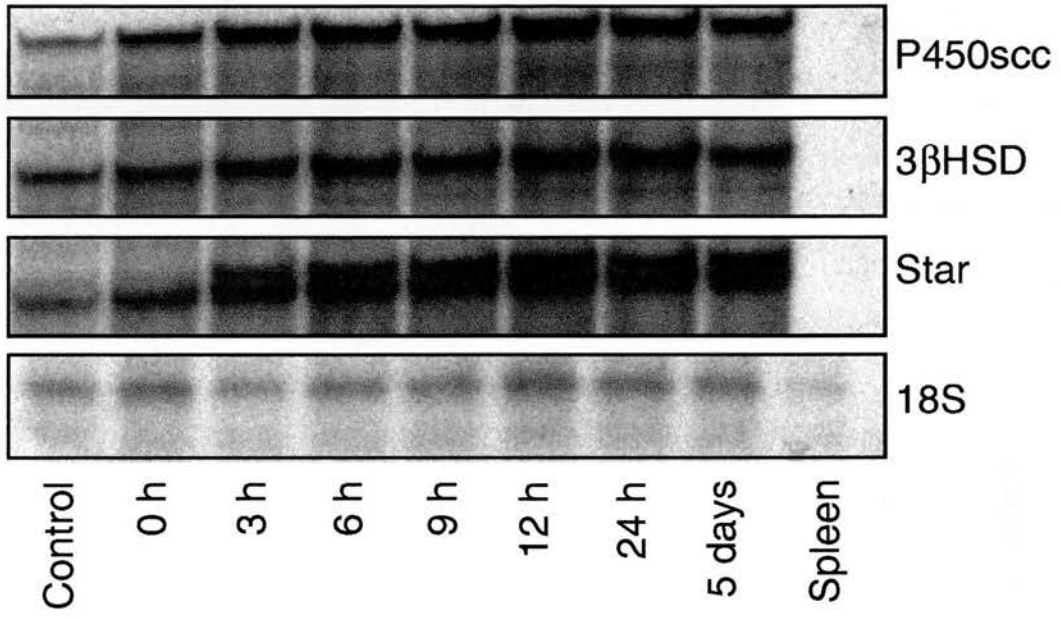


FIG. 3.8 RNase protection assay showing the induction of the steroidogenic enzymes StAR, P450scc and 3βHSD in the immature rat ovary with PMSG and hCG administration. Animals were injected with 10IU of PMSG, followed by 10IU of hCG and killed and at 0h, 3h, 6h, 9h, 12h, 24h and 5 days post hCG. The control group received no injections and were killed with group 0h. The intensity of each band was quantified using an instant imager and then exposed to a Kodak X-Omat AR-5 autoradiogram for 2 days at -70°C using an intensifying screen.

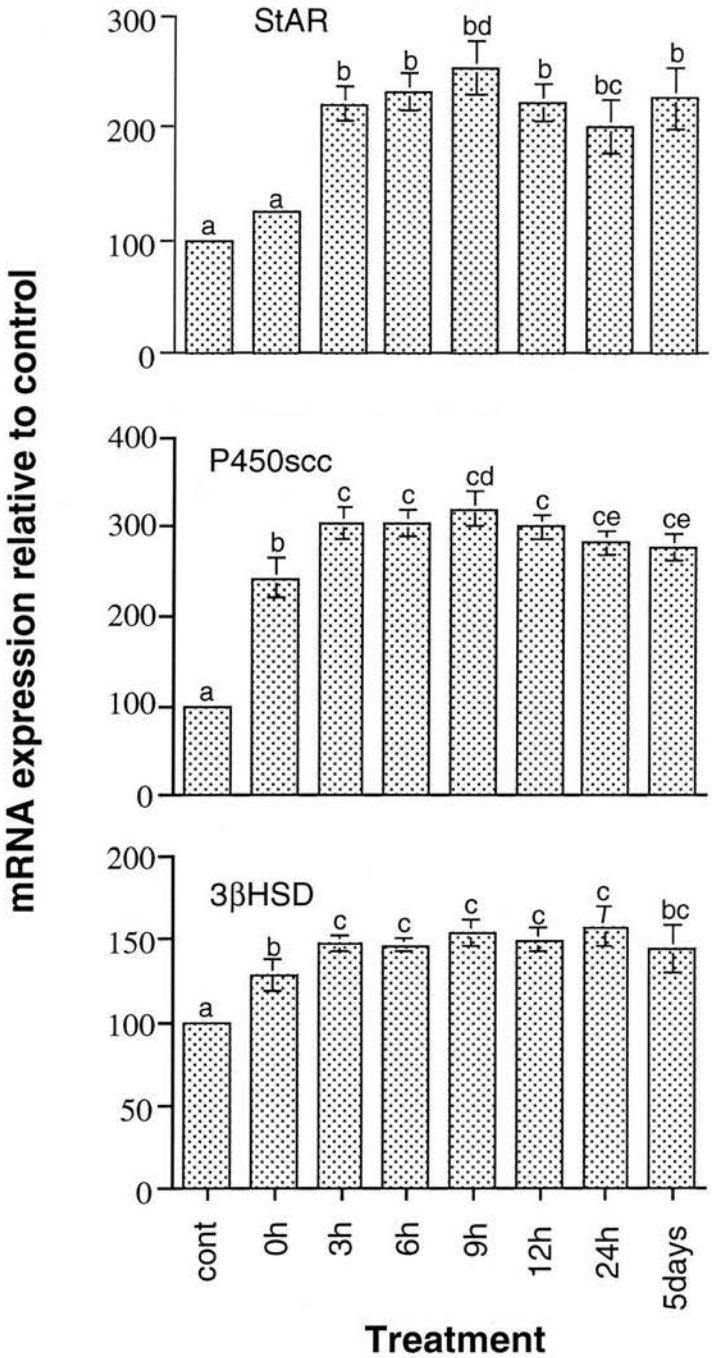


FIG. 3.9 Quantification of the induction of the steroidogenic enzymes StAR, P450scc and 3βHSD in the immature rat ovary with PMSG and hCG administration (from the RPA in fig.3.8). Each sample was standardised against 18S and the control sample was assigned an arbitrary value of 100%. Values represent the mean (±SE) from triplicate experiments. In each graph, histograms with different superscripts are significantly different from one another ($p<0.05$).

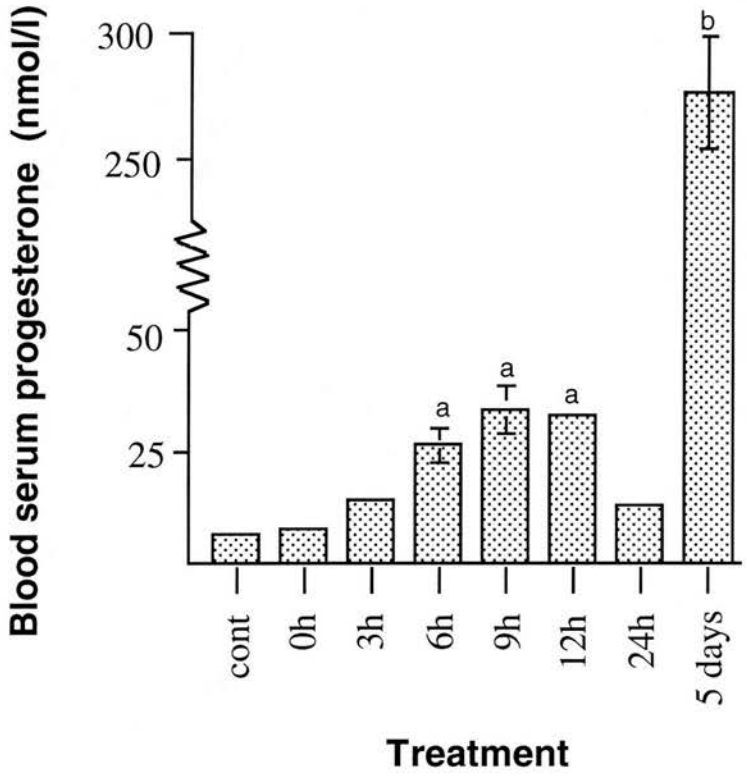


FIG. 3.10 Blood serum progesterone concentrations in the immature rat with PMSG and hCG administration. Animals were injected with 10IU of PMSG, followed by 10IU of hCG and killed and blood samples removed at 0h, 3h, 6h, 9h, 12h, 24h and 5 days post hCG. The control group received no injections and were killed with group 0h. Values represent the mean (±SE) from triplicate experiments. In each graph, histograms with different superscripts are significantly different from one another ($p<0.05$).

steroidogenic activity (fig.3.10). The immature rat showed a very low progesterone concentration of approximately 7nmol/l with only a very slight but not significant rise after PMSG treatment. After hCG treatment the levels of progesterone increased gradually, peaking at 9h post hCG with a concentration of 38nmol/l. This declined again by 24h post hCG to 14.4nmol/l. With the formation of the corpus luteum, progesterone levels increased by almost 40-fold (to 277nmol/l) by day 5 after the hCG injection.

3.5 Quantifying PR expression

RPA was used to detect PR gene expression. PR probe hybridised to both A and B PR isoforms giving a band of the same length, i.e. total PR expression could be quantified with PR probe. Fig.3.11 and 3.12 show the transient expression of the PR in the rat ovary. The immature female did not express PR and PMSG had no effect on expression. PR mRNA appeared 6h after the hCG injection of PMSG primed rats, only to decrease sharply and was undetectable again by 12h post hCG. PR gene expression was absent in the corpus luteum of the rat.

3.6 Analysis of the PRB isoform gene expression

Probe PRB was used to analyse which PR isoform was being expressed during the transient phase (i.e. 6h after the hCG injection). Due to the small size of the PR probe (175bp) and the low expression of the PRB gene, 20µg of total RNA was used for each sample. Fig.3.13 shows PRB gene expression at 3h, increasing at 6h and disappearing by 9h after the hCG injection. This result could not be quantified because the signal was so low, and was not repeated due to the large amount of RNA required to visualise the protected band. Theoretically PRA could be quantified by subtracting the PRB quantity from the total PR. This was not done due to the inaccuracies this would incur since each probe is of a different length, leading to more radioisotope in the longer probe, due only to the size of the probe. ^{32}P-UTP was used to label the riboprobes. Therefore the number of labelled nucleotides incorporated within the length of probe would have to be counted and the cpm adjusted for the incorporation of these nucleotides. It was therefore decided this method of quantification was not sufficiently accurate.

3.7 In situ hybridisation analysis of StAR mRNA

In situ hybridisation using StAR probe on rat ovaries of varying maturity is shown in figures 3.14 to 3.17. Light field photographs were taken to aid identification of

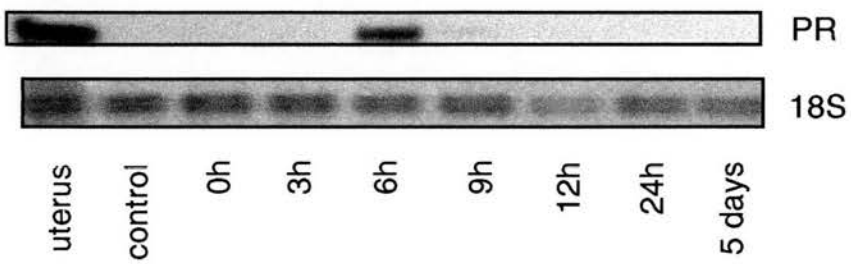


FIG. 3.11 RNase protection assay showing the gene expression of total PR in the immature rat ovary with PMSG and hCG administration. Animals were injected with 10IU of PMSG, followed by 10IU of hCG and killed at 0h, 3h, 6h, 9h, 12h, 24h and 5 days post hCG. The control group received no injections and were killed with group 0h. The intensity of each band was quantified using an instant imager and then exposed to a Kodak X-Omat AR-5 autoradiogram for 2 days at -70°C using an intensifying screen.

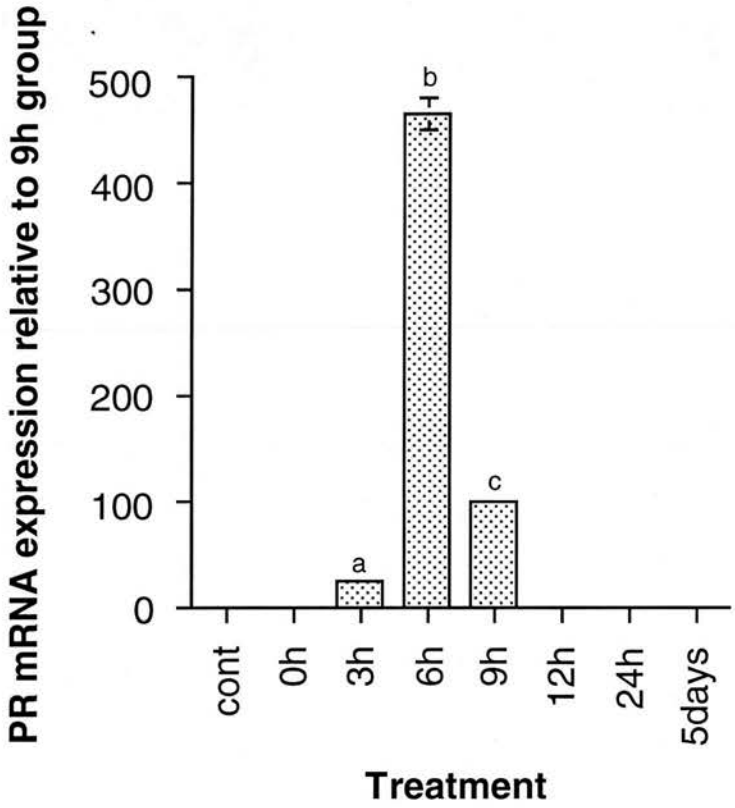


FIG. 3.12 Quantification of the induction of total PR in the immature rat ovary with PMSG and hCG administration (from the RPA in fig.3.11). Each sample was standardised against 18S and the 9h sample was assigned an arbitrary value of 100%. Values represent the mean (±SE) from triplicate experiments. Histograms with different superscripts are significantly different from one another ($p<0.05$).

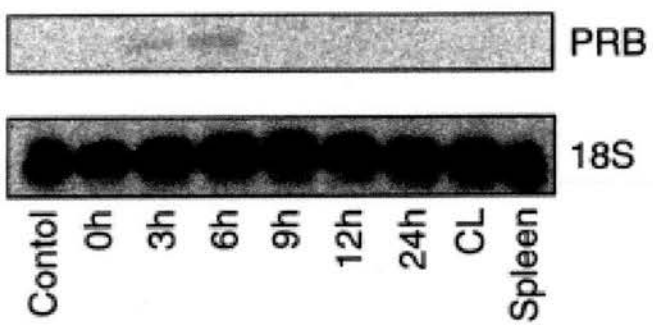


FIG. 3.13 RNase protection assay showing the gene expression of PRB isoform in the immature rat ovary with PMSG and hCG administration. Animals were injected with 10IU of PMSG, followed by 10IU of hCG and killed at 0h, 3h, 6h, 9h, 12h, 24h and 5 days post hCG. The control group received no injections and were killed with group 0h. 20mg of total RNA was hybridised to the probes. The intensity of each band was quantified using an instant imager and then exposed to a Kodak X-Omat AR-5 autoradiogram at -70°C using an intensifying screen. The bottom half of the gel containing the area of 18S was exposed to the autoradiogram overnight, and the top half containing the PRB section was exposed for 2 weeks.

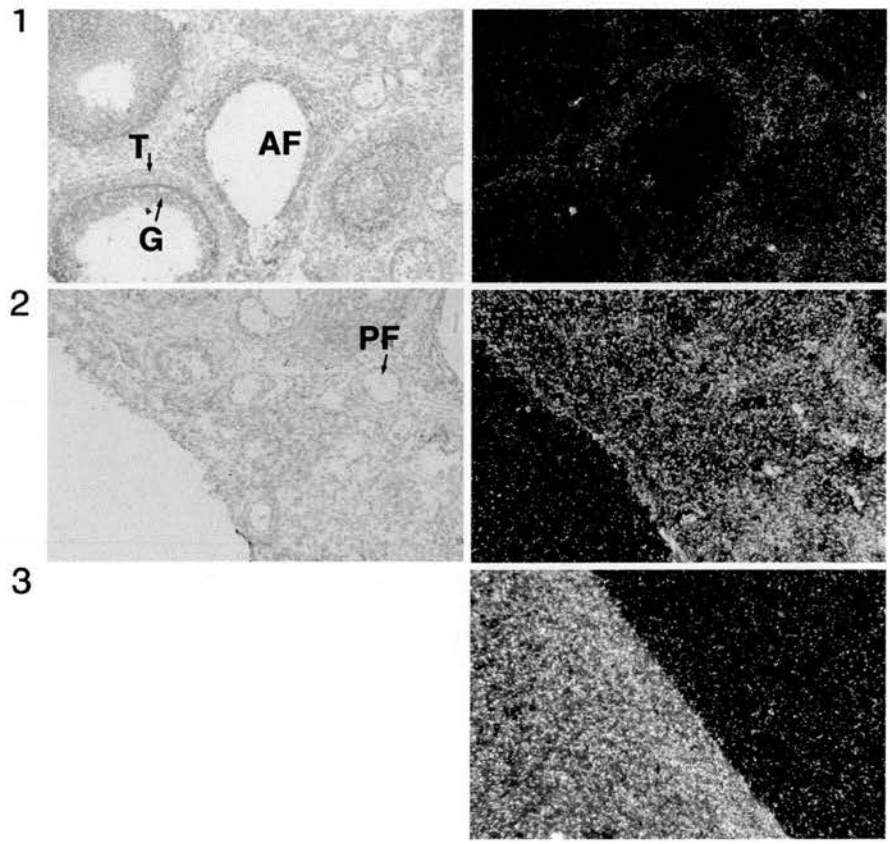


Fig. 3.14 In situ hybridisation using StAR probe on rat ovaries (x20 magnification).
1. Immature ovary probed with sense StAR probe (light and dark field).
2. Immature ovary probed with antisense StAR probe (light and dark field).
3. Spleen probed with antisense probe (dark field)

AF=atretic follicle
G=granulosa cells
T=thecal cells
PF=primary follicle

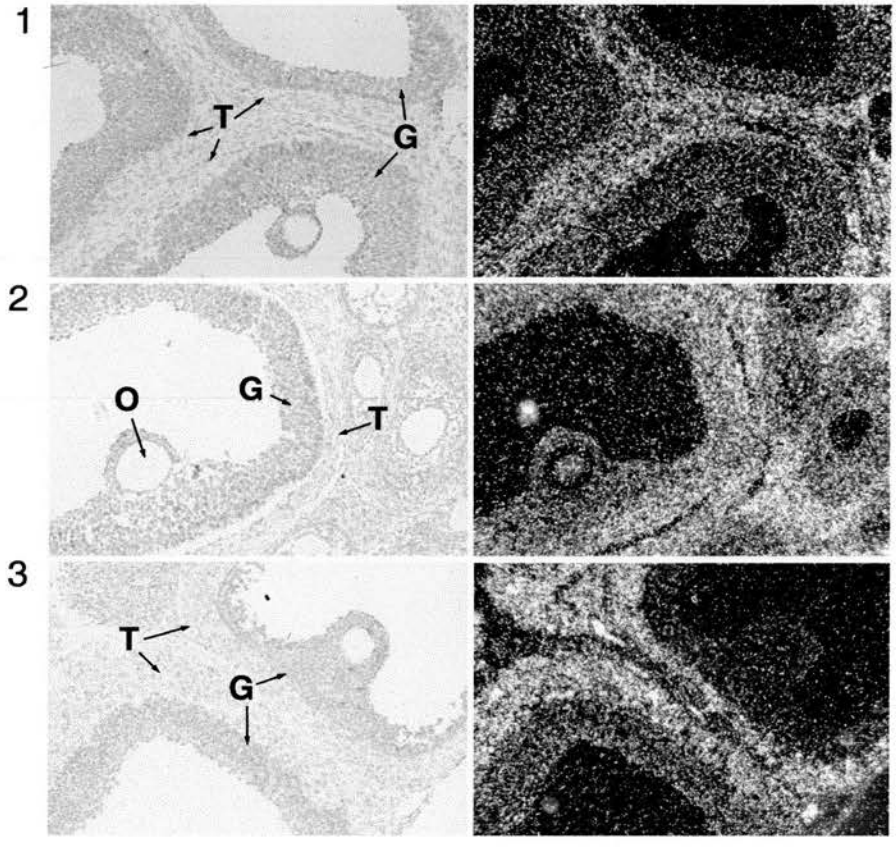


Fig 3.15 In situ hybridisation using StAR antisense probe on rat ovaries (x20 magnification, light and dark field).
1. Treated with PMSG for 48h.
2. Treated with PMSG for 48h, followed by hCG for 3h.
3. Treated with PMSG for 48h, followed by hCG for 6h.

G=granulosa cells
O=oocyte
T=thecal cells

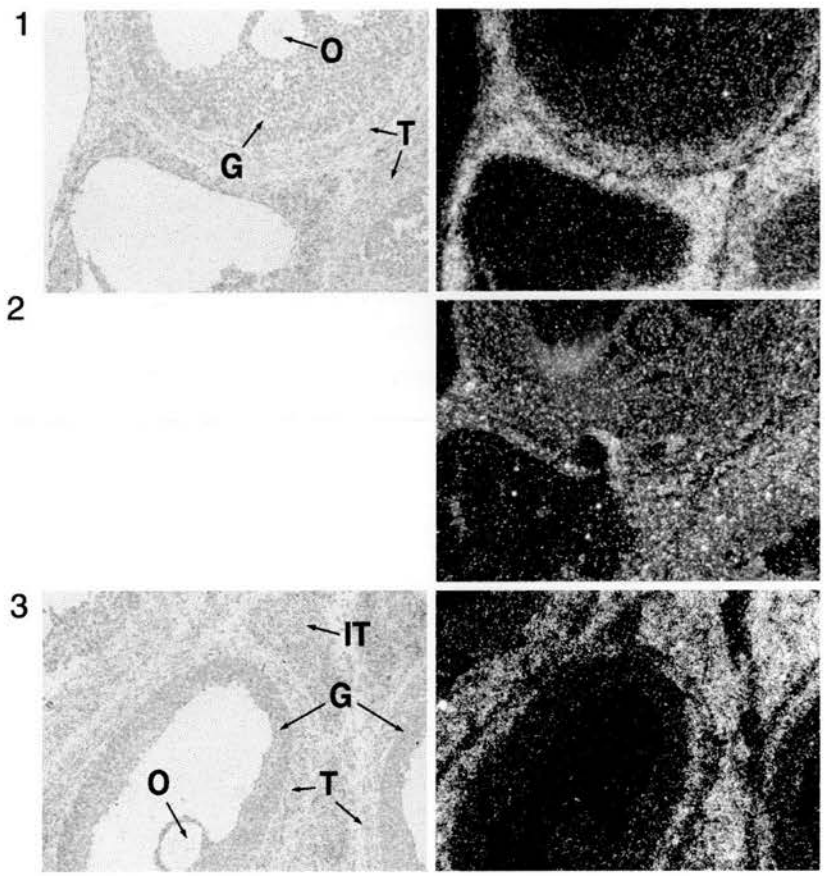


Fig 3.16 In situ hybridisation using StAR probe on rat ovaries (x20 magnification).
1. Treated with PMSG for 48h, follwed by 9h hCG probed with antisense probe (light and dark field).
2. Treated with PMSG for 48h, followed by hCG for 9h probed with sense probe (dark field).
3. Treated with PMSG for 48h, followed by hCG for 12h, probed with antisense probe (light and dark field).

G=granulosa cells
IT=interstitial tissue
O=oocyte
T=thecal cells

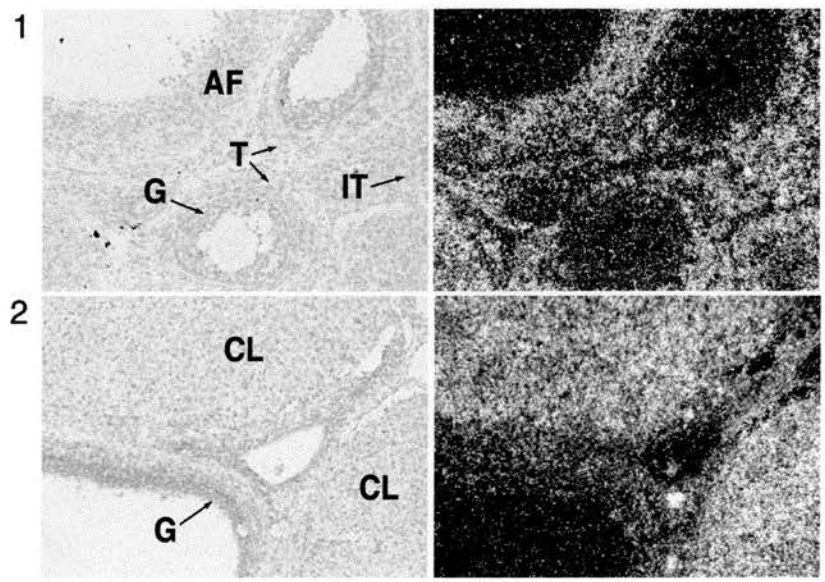


Fig 3.17 In situ hybridisation using StAR antisense probe on rat ovaries (x20 magnification, light and dark field).
1. Treated with PMSG for 48h, followed by hCG for 24h.
2. Treated with PMSG for 48h, followed by hCG for 5d.

AF=ateric follicle
CL=corpus luteum
G=granulosa cells
IT=interstial tissue
T=thecal cells

ovarian morphology. Silver staining of radiolabelled StAR riboprobe could be seen by dark field microscopy. Non-specific binding was shown by using controls of sense probe on ovarian tissue and antisense probes on liver. Immature ovaries showed some StAR expression in interstitial cells, but PMSG treatment for 48h greatly increased StAR expression in theca cells and interstitial tissue. hCG treatment further increased StAR expression in these tissues, intensifying over time. Granulosa cells showed some StAR expression after hCG treatment, but the thecal cells and interstitial tissue remained the main tissue of StAR mRNA expression.

4 Discussion

These data show that the genes for the steroidogenic enzymes/proteins studied were all up-regulated by gonadotrophin stimulation (except for PR A isoform, which could not be established using these methods). Therefore gene expression responsible for progesterone receptor and for the synthesis of progesterone was present in the ovary simultaneously. Serum concentrations of progesterone increased significantly with hCG stimulation, showing that the progesterone produced was secreted into the blood stream in an endocrine fashion. Ovulation was observed in approximately 90% of all ovaries recovered 12h after hCG administration, but not in the 9h group. Therefore all genes studied were maximally expressed before ovulation took place, suggesting a possible paracrine/autocrine role for progesterone in ovulation.

The control group (which received no gonadotrophins) showed some steroidogenic activity, with StAR, P450scc and 3βHSD expression at low levels and only minimal concentrations of progesterone measured in circulating blood. No PR expression was observed, implying progesterone does not act within the immature ovary (at least through the putative genomic receptor).

The theca interna appears to be the primary steroidogenic compartment of the follicle during the later stages of development. Thecal cells are the first to acquire steroidogenic enzymes after gonadotrophic stimulation, with granulosa cells acquiring the steroidogenic apparatus later. The physiological relevance of this difference in timing is not yet known. Perhaps thecal cells are producing androgens for oestrogen production in granulosa cells (although P450arom is not expressed at this time

[Richards, 1994]). Alternatively, this may augment the actions of FSH on granulosa cells, namely the induction P450scc, P450arom, LH receptors and prolactin receptors (Hillier and deZwart, 1981; Fitzpatrick and Richards, 1991).

The synthesis of StAR protein is now regarded as the key regulatory step in steroidogenesis. Its presence is thought to enhance steroid production by delivering cholesterol to the mitochondria. Therefore its acute expression is essential for maximal steroid synthesis. These data show no significant increase in StAR 48h after PMSG treatment, but hCG more than doubled its expression within 3h. Studies by Ronen-Fuhrmann and colleagues (1998) included many more time-points over the period of induced follicle maturation. Their data showed StAR transcripts and protein increased with PMSG, peaking by 8h and declining to near basal levels by 24h. hCG induced a second wave of StAR gene expression and translation which was maximal by 8h after injection. Interestingly, the first wave of StAR expression was solely confined to the non-follicular theca-interstitial cells. Only with hCG did the granulosa and theca interna compartments of the dominant follicle begin to express. The *in situ* hybridisation data in figs 3.14 to 3.17 show slight staining in the interstitial cells of the immature ovary, spreading into the thecal cells after 48h treatment of PMSG. After hCG treatment, the staining becomes more intense in the thecal cells, with the granulosa cells also showing some expression. These data illustrate that granulosa cells acquire some StAR gene expression, but it is the thecal and interstitial cells which appear to be responsible for the majority of transcription.

A similar situation was seen with P450scc and 3βHSD expression. Thecal and interstitial cells (but not granulosa cells of preovulatory follicles) are known to express the P450scc protein. PMSG treatment leads to interstitial thecal cells enriching their P450scc content within 24h, with granulosa cells acquiring the enzyme between 30 and 48h after administration (Zlotkin *et al.*, 1986). These data (fig 3.9) show a large increase in expression of P450scc mRNA by 48h after treatment with PMSG. This increased further following an ovulatory dose of hCG. P450scc mRNA reached its maximal expression by 9h after the hCG injection, a pattern similar to other studies (Goldring *et al.*, 1987; Ronen-Fuhrmann *et al.*, 1998). Zlotkin et al (1986) report follicular P450scc enzyme content to be 15 times higher in the follicle after 48h treatment with PMSG than in the control ovaries of immature rats (Zlotkin *et al.*, 1986). This large increase is not reflected in the level of mRNA expression, since P450scc mRNA is up-regulated only approximately 2.75 times the level of expression in the

control ovary. This confirms previous studies which have shown preovulatory follicles to contain low amounts of P450scc mRNA, yet exhibit substantial amounts of enzyme by Western blotting (Hedin *et al.*, 1987) and immunofluorescent analysis (Goldring *et al.*, 1987). This discrepancy may be partly attributable to the higher dose of PMSG injected in the Zlotkin study (15IU cf. 10IU). hCG has a small but significant effect on increasing P450scc transcript levels, as confirmed by Doody *et al.* (1991).

3βHSD mRNA is up-regulated by PMSG with little further increase observed with hCG treatment. These findings confirm data of Kaynard *et al.* (1992). Expression is observed in the theca throughout development, but it is not until the formation of the preovulatory follicle that the granulosa cells synthesis 3βHSD mRNA (Teerds and Dorrington, 1993).

The progression of steroidogenic gene expression (from the thecal cells to the granulosa cells) is particularly relevant with regard to the transient appearance of PR mRNA in granulosa cells 6h after hCG stimulation. PR expression has been localised to the granulosa cells by in situ hybridisation (Park and Mayo, 1991) and immunocytochemistry (Natraj and Richards, 1993), at the same time as StAR, P450scc and 3βHSD. Therefore, progesterone may be exhibiting an autocrine action in the granulosa cells just prior to ovulation.

3βHSD expression in the ovary is itself under the control of progesterone. Tanaka and colleagues (1993) demonstrated that injecting RU486 2h before hCG reduced the levels of 3βHSD mRNA in granulosa cells, but not in thecal cells. PR is not present in the ovary before the LH surge, and therefore it may be that RU486 is acting elsewhere e.g. the pituitary, to regulate 3βHSD expression in granulosa cells. Alternatively, RU486 may be acting through the glucocorticoid receptor, suppressing 3βHSD transcription. The effect of this decrease in 3βHSD expression on ovulation was not analysed, although a drop in serum progesterone was observed. It is thought that the early, preovulatory acquisition of P450scc activity is an essential feature in the rise of progesterone associated with luteinisation. The mid-cycle gonadotrophin surge does not greatly induce granulosa cell 3βHSD activity to the same degree as P450scc (Chaffin *et al.*, 1999). The dramatic increase in StAR may also aid the preovulatory progesterone rise.

After ovulation, expression of StAR, P450scc and 3βHSD is maintained in luteal tissue and the potential for steroidogenesis remains high. However, this is not reflected in

blood serum progesterone concentrations during this early luteal phase. It should be noted that the rise in progesterone may be associated with an increase in the number of steroidogenic cells due to PMSG-induced proliferation, with the steroidogenic capabilities of those cells remaining constant. A decrease in progesterone may be associated with an increase in its metabolism, although enzymes such as 17α-hydroxylase and c-17,20 lyase which metabolise progesterone to androgens are not present (Eckstein *et al.*, 1985). Indeed progesterone is an effective inhibitor of C-17,20-lyase (Mahajan and Samuels, 1975) and its expression is inhibited by the LH surge (Eckstein *et al.*, 1985). Alternatively, progesterone produced may be metabolised to 20α-hydroxyprogesterone by 20α-HSD which increases in rat preovulatory follicles after hCG stimulation (Tsafriri and Eckstein, 1986). However progesterone itself can down-regulate the expression of 20α-HSD in the corpus luteum (Sugino *et al.*, 1997). 20α-hydroxyprogesterone does not cross react with the antiprogesterone antibody used in the assay and was not quantified by any other method in those studies.

The late-luteal phase corpus luteum secretes a massive quantity of progesterone, despite no further increase in the enzymes present. This may be due to the elaborate vascular system of the late corpus luteum which allows more efficient substrate delivery and product removal.

FSH and LH, when bound to their respective receptors, activate an intracellular cascade involving the PKA pathway which ultimately results in increased intracellular levels of the second messenger cAMP as described in chapter 1, section 3.1.4. Consequently, gene expression induced by gonadotrophins can also occur through stimulation by cAMP or its analogues. Transcription is promoted through the binding of regulatory elements such as cAMP to areas upstream of the target gene. cAMP is known to induce the transcription of StAR (Payne and Youngblood, 1995), P450scc (Clemens *et al.*, 1994) and 3βHSD (Chedrese *et al.*, 1990a) and sites in the PRB promoter region have been identified as essential for cAMP-induced transcription (Park-Sarge and Sarge, 1995). PR mRNA is also induced by activators of the PKC pathway (Natraj and Richards, 1993) and EGF, which is known to activate tyrosine kinase (Wong and Richards, 1992). This induction was not due to increased cAMP and it may be that all these pathways are involved in the regulation of PR mRNA. In contrast to other PR expressing tissues, granulosa cell PR is not under oestrogenic control, shown *in vivo* and *in vitro* (Park-Sarge and Mayo, 1994) demonstrating tissue specificity.

In conclusion, the major findings of this chapter suggest that steroidogenesis is up-regulated by PMSG and hCG treatment in the immature rat. StAR, P450scc and 3βHSD are all expressed maximally by 9h after hCG treatment when progesterone concentrations have also increased. In contrast, the progesterone receptor is transiently expressed at approximately 6h after hCG. This suggests progesterone is acting locally through the genomic receptor for a very short period only, despite the continuous production of progesterone throughout the cycle.

This *in vivo* experiment illustrates a realistic physiological scenario, but it is not possible to isolate the cell types or even organs involved. Many proteins and steroids from outside the ovary, particularly from the hypothalamus and pituitary, act on the ovarian follicles. Therefore ovarian cell culture may be required to deduce to what extent progesterone acts in an autocrine/paracrine fashion. These studies are described in the next chapter.

Chapter 4 Progesterone in Rat Granulosa Cell Culture

1 Introduction

Granulosa cells transiently express PR mRNA in the rat *in vivo* and *in vitro* with gonadotrophin treatment (Natraj and Richards, 1993; Park-Sarge and Mayo, 1994). Thecal cells are thought to be the major producers of steroids, as illustrated by StAR and 3βHSD expression, but *in situ* studies have shown granulosa cells to acquire steroidogenic activity in the presence of FSH and LH (Ronen-Fuhrmann *et al.*, 1998; Teerds and Dorrington, 1993). Cell culture allows the isolation of granulosa cells which can then be treated and studied without the influences of other cells in the physiological system. The aim of this chapter is to examine progesterone synthesis and receptor expression induced by gonadotrophins in isolated granulosa cells.

The previous chapter described the effects of gonadotrophins on the immature ovary of the rat, in particular, on progesterone synthesis and progesterone receptor (PR) expression *in vivo*. In order to study the regulation of the genes involved, an isolated cell culture model was employed to imitate the *in vivo* system of chapter 3.

The granulosa cell culture model selected for this purpose has been widely used in many laboratories (Hillier *et al.*, 1978). Granulosa cells were isolated from immature rats pre-treated *in vivo* with diethylstilboestrol (DES). This treatment is known to stimulate proliferation (Simpson *et al.*, 1941), and yields large numbers of granulosa cells that can be harvested in an undifferentiated state from preantral and small antral follicles.

Factors known to affect progesterone synthesis *in vitro* include extracellular matrix and cell density, as well as hormonal stimulation. In this phase of the research, cells were cultured in wells previously plated with donor calf serum (See chapter 2.1). Serum aids anchorage of the cells to the bottom of the plastic well. Each well also received testosterone supplement which is known to augment FSH's ability to induce progesterone synthesis (Armstrong and Dorrington, 1976).

Plating density is also important in that it appears to affect progesterone synthesis. Del Vecchio and colleagues (1995) showed that bovine luteal cells produced more progesterone (a marker of granulosa cell differentiation) if in contact with each other.

In this study, a high cell density was used to aid analysis of RNA, as RNA recovery from cells would decrease if spread over a larger number of wells. Therefore cell density was higher than that of other studies in this laboratory.

Previous studies of the effects of FSH have had to take account of LH contamination due to the methods of extracting gonadotrophins from urine. Now, genetically engineered recombinant FSH and LH are manufactured commercially. The experiments described in this chapter exploited these 'pure' gonadotrophins, enabling a more specific determination of the effect of each hormone.

2 Methods

2.1 Preliminary cell culture

The animals and the cell culture method were described in chapter 2, section 1. Cells were plated at 200,000 cells/well (0.5ml medium/well). All groups received testosterone (10^{-6}M final concentration). The control group received no other treatments and three other groups were treated with pituitary FSH (batch LER 8/116, see chapter 2.1) to a final concentration of 50ng/ml. After 48h in the incubator (conditions described in chapter 2.1), the medium was carefully poured from all the groups and the wells washed once with 0.5ml Dulbecco's phosphate buffered saline (DPBS) (Gibco Ltd, BRL, Paisley, Renfrewshire, UK). To the control group and the FSH group 0.5ml of medium was added. To the remaining two groups, 0.5ml medium containing 500ng/ml rhLH was added. These experiments were stopped immediately (0h) for the control and FSH groups, and at 6h for the FSH+LH(6h) group and after 12h for the FSH+LH(12h) group. The plates were placed on ice and the medium was gently removed and stored at -20°C for further analysis. 250µl 'solution D' was added to the cells remaining in the wells and the plates were frozen until RNA extraction (described in chapter 2, section 2).

2.2 Cell culture analysis

RNA protection assays were performed on 5µg of the extracted RNA to quantify StAR, P450scc and 3βHSD expression. Progesterone produced by the cells was measured in the collected medium by radioimmunoassay as described in chapter 2.7.

These experiments gave unexpected results (section 3.1) and will be discussed in detail later. Experiments were designed to optimise cell culture conditions.

2.3 Optimising FSH treatment

Increasing concentrations of recombinant human (rh) rhFSH were added to cells plated at 200,000 cells/well with 10^{-6}M testosterone. The final rhFSH concentrations in the medium were 0, 0.03, 0.1, 0.3, 1.0 and 3.0ng/ml in one experiment and 0, 1, 3, 10, 30 and 100ng/ml in a second experiment. Cells were incubated for 48h and the medium removed and RNA extracted. RNase protection assays (RPAs) using probes for LH receptor (LHR) and P450aromatase (P450arom) were carried out on the extracted RNA. The optimum rhFSH concentration of 1ng/ml was chosen for further experiments.

2.4 Optimising LH treatment

Cells were plated at 200,000 cells/well and primed with 1ng/ml rhFSH and 10^{-6}M testosterone for 48h, as described above. The medium was poured off and the wells were washed once with 0.5ml DPBS. Medium containing increasing concentrations of rhLH was added to the wells and incubated for 12h. rhLH concentrations were 0, 0.03, 0.1, 0.3, 1.0 and 3.0ng/ml in one experiment and 0, 1, 3, 10, 30 and 100ng/ml in a second experiment. They also contained control groups of 0ng rhFSH followed by 0ng of rhLH for 12h. The second experiment contained a further control group of 1ng/ml FSH for 48h and was terminated at that point. The medium was removed and the RNA was extracted. RPAs were undertaken using probes for StAR and P450scc.

2.5 Effect of testosterone concentration

An experiment as described in section 2.2 was carried out using varying concentrations of testosterone. Wells with final concentrations of 10^{-6}M, 10^{-8}M and 0M testosterone were cultured with increasing concentrations of FSH and the medium collected was assayed for progesterone.

2.6 Effects of cell density

Wells were plated at 50,000 cells/well and 200,000 cells/well with 10^{-6}M testosterone, and increasing concentrations of rhFSH (as described in section 2.2) were added. A second experiment with rhFSH-primed granulosa cells receiving increasing concentrations of rhLH for 12h was also performed (as described in section 2.3). The medium was collected after 48h incubation and assayed for progesterone.

2.7 Time course in vitro *using optimal culture conditions*

Optimised conditions established from the experiments above were used to study gene expression in isolated granulosa cells over a period of induced maturity and luteinisation. Cells were plated at 160,000 cells/well and nine different treatments were tested. In the first treatment, cells were incubated for 48h, the wells were washed with 0.5ml DPBS and 0.5ml medium with the second treatment was added back to the wells. All groups received 10^{-6}M testosterone during the first 48h. The control group received no additional hormones for the first 48h, was then washed, fresh medium replaced, and the culture stopped at 0h after the addition of the second treatment. All other groups received 1ng/ml rhFSH for 48h and after the addition of the second treatment, were stopped and medium was added back to the wells the second treatment. All groups received 10-6 M testosterone during the first 48h. The control group received no additional hormones for the first 48h, was then washed, fresh medium replaced, and the culture stopped at 0h after the addition of the second treatment. All other groups received 1ng/ml rhFSH for 48h and after the addition of the second treatment were stopped at 0h, 6h, 12h or 24h. Each time point had a group with either no hormone or 3ng/ml rhLH. The medium was assayed for progesterone and RPA was performed on the RNA extracted from the cells.

3 Results

3.1 Preliminary experimental results

Fig 4.1 shows an RPA of StAR, P450scc and 3_HSD expression from the cell culture. This was quantified relative to the control sample and data are presented in fig 4.2. FSH treatment did not alter the expression of these genes and LH only slightly increased mRNA expression. Progesterone concentrations are also shown in fig 4.2. FSH had no effect on progesterone production but LH increased progesterone slightly after 6h and 2-fold by 12h.

These results were unexpected. In vivo, as shown in the previous chapter, gonadotrophins greatly increased the expression of these genes and progesterone production and other studies have reported a larger response in vitro (Armstrong and Dorrington, 1976; McMasters et al., 1987). For these reasons experiments were set up to optimise the in vitro conditions.

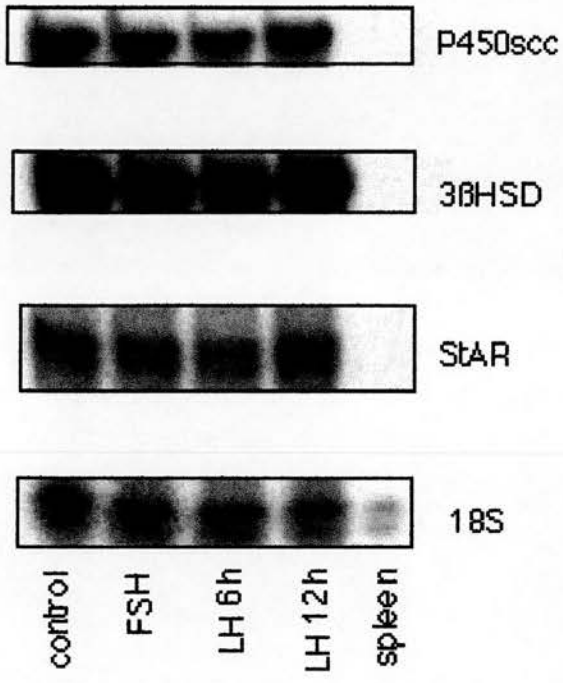


Fig 4.1 RNase protection assay showing the gene expression of StAR, P450scc and 3βHSD in granulosa cells with gonadotrophin treatment. Granulosa cells from immature rats were isolated and cultured in 0.5ml wells. 50ng/ml rhFSH was added to all wells except the control and incubated for 48h. Wells were washed with 0.5ml DPBS and medium containing 500ng/ml rhLH was added to the LH groups. Control and FSH groups received medium with no hormone treatment, and the medium immediately removed and the culture stopped. The LH groups were incubated for a further 6h and 12h. All genes were expressed in all the groups. Spleen was used as a negative control.

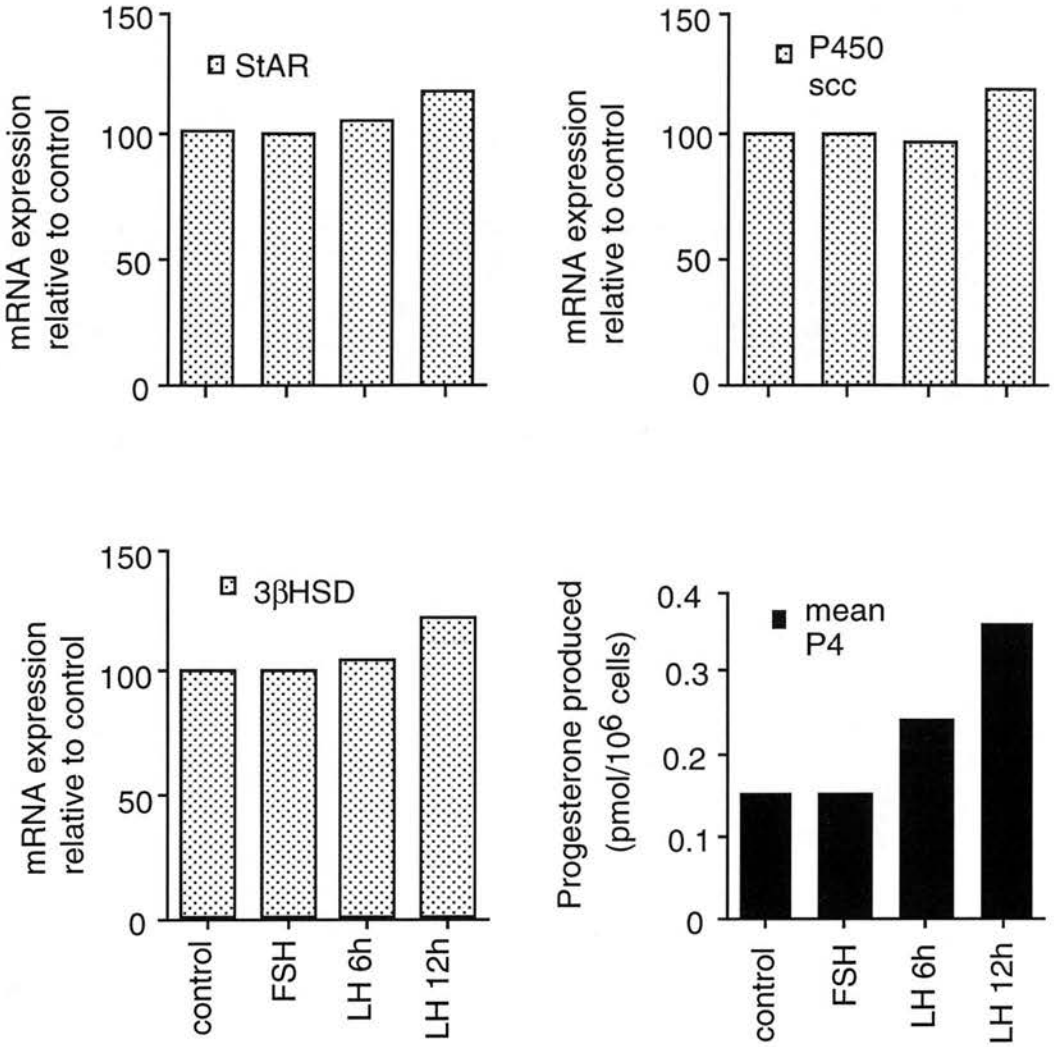


Fig 4.2 Graphs quantifying the expressions of StAR, P450scc and 3βHSD relative to the control group and progesterone concentration in the collected media. mRNA expression showed little increase with gonadotrophin treatment. Progesterone synthesis increased over time with the addition of rhLH. Each RPA sample was standardised against 18S and the control sample was assigned an arbitrary value of 100%.

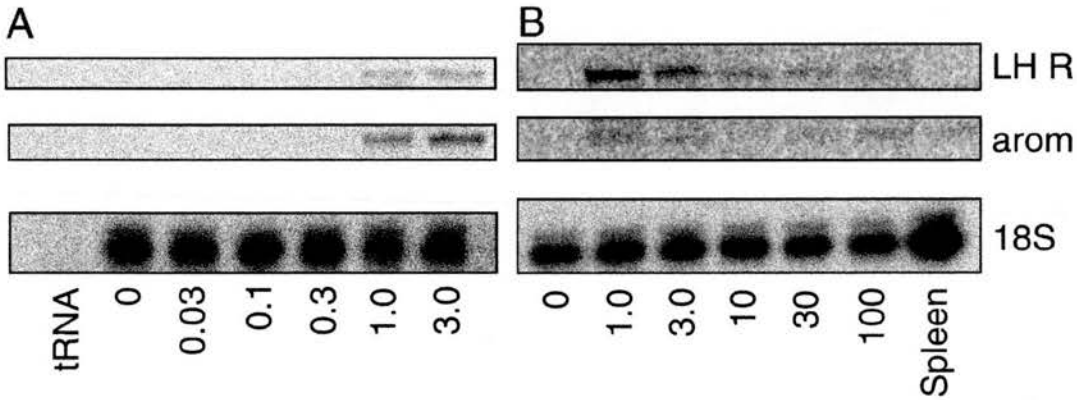


Fig 4.3 RNase protection assays showing the gene expression of luteinising hormone receptor (LHR) and P450arom in cultured granulosa cells after 48h with increasing rhFSH concentrations.
A. LHR and P450arom were expressed with rhFSH concentration of 1-3ng/ml. No expression can be seen below this concentration.
B. LHR probe produced the darkest protected bands with the addition of 1-3ng/ml rhFSH, decreasing with increased concentrations. A similar pattern occurred with P450arom expression.

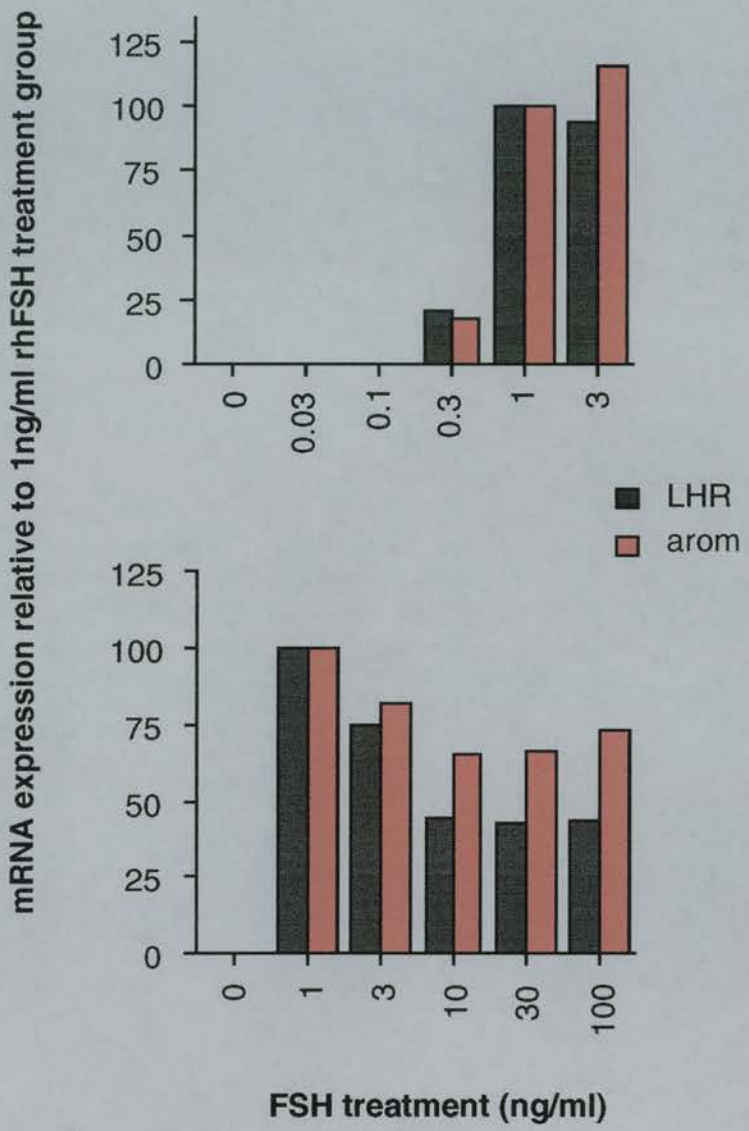


Fig 4.4 Graphs representing LHR and P450arom expression in cultured granulosa cells after 48h incubation with increasing concentrations of rhFSH.
Both LHR and P450arom mRNA increased with rhFSH concentrations and peaked at approximately 1ng/ml. rhFSH concentrations higher than this lead to a decrease in gene expression.

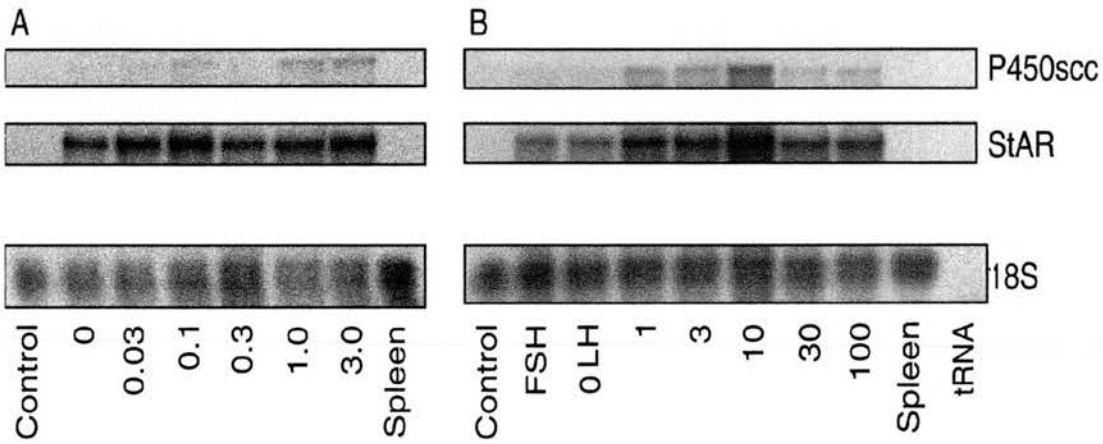


Fig 4.5 RNase protection assays showing the gene expression of StAR and P450scc in cultured granulosa cells with increasing rhLH concentrations. The cultures were previously primed with 1ng/ml rhFSH for 48h and washed with DPBS. Medium containing different rhLH concentrations were added back and incubated for 12h.

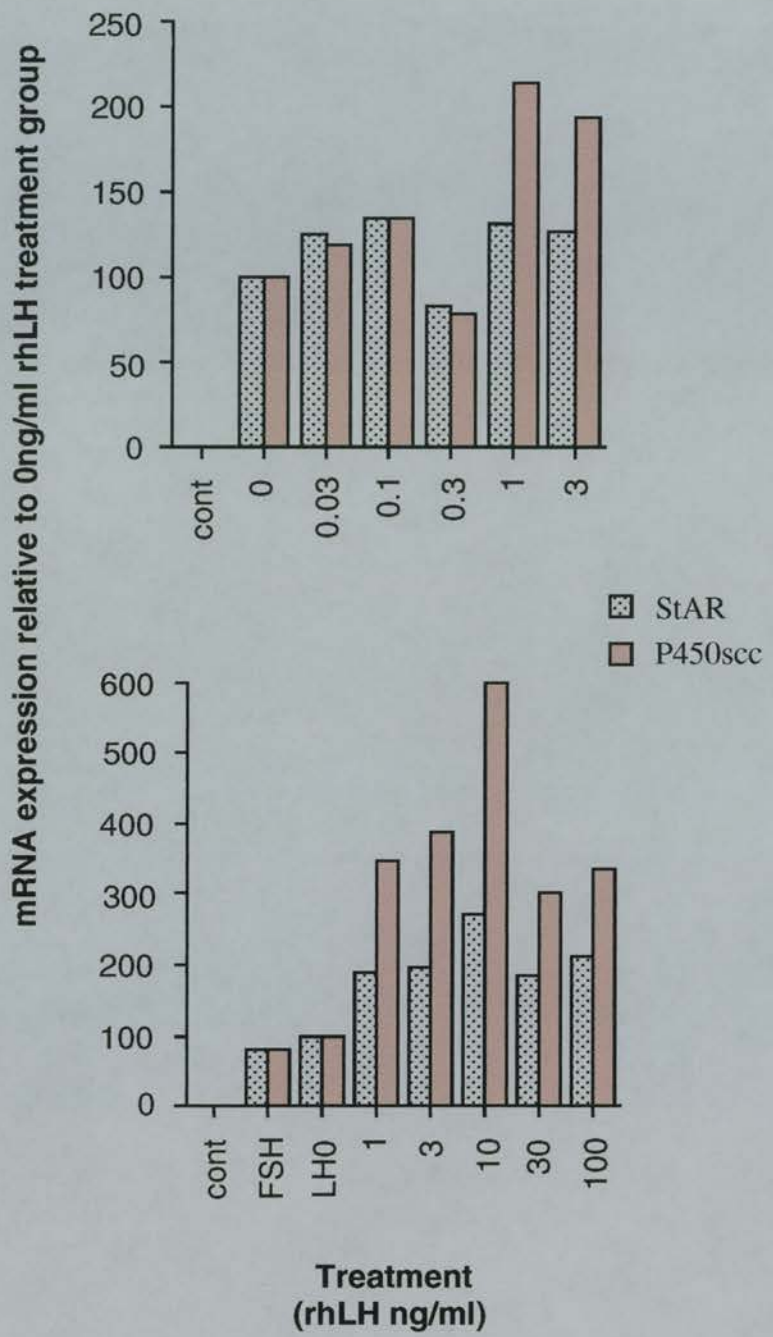


Fig 4.6 Graphs representing gene expression of StAR and P450scc in cultured granulosa cells with increasing rhLH concentrations. Values were standardised against rhLH 0ng/ml control group. Gene expression is maximal at 10ng/ml, showing a dose responsive increase with rhLH concentrations less than this. Gene expression is reduced at concentrations beyond 10ng/ml. Each sample was standardised against 18S and the 0ng/ml rhLH sample was assigned an arbitrary value of 100%.

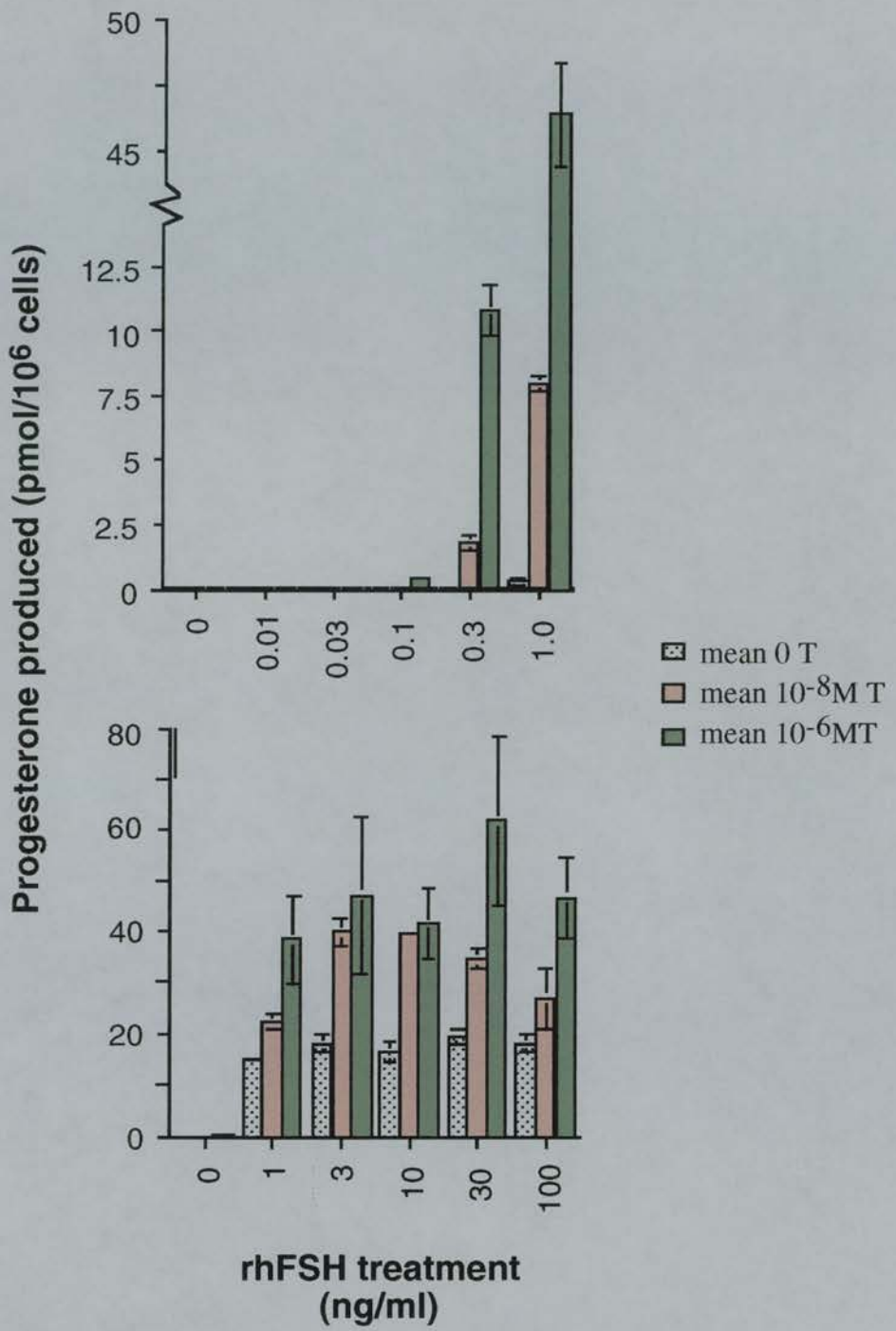


Fig 4.7 Graphs representing progesterone produced by granulosa cells in culture with increasing concentrations of FSH and varying testosterone concentrations in the culture medium. No testosterone in the culture led to little progesterone production. Testosterone at 10^{-8}M shows a greater response to rhFSH treatment, with progesterone peaking at approximately 3ng/ml and declining with concentrations above that. Testosterone at 10^{-6}M shows the greatest response to rhFSH treatment with maximal progesterone synthesis at approximately 30ng/ml rhFSH. Values represent the mean (±SE).

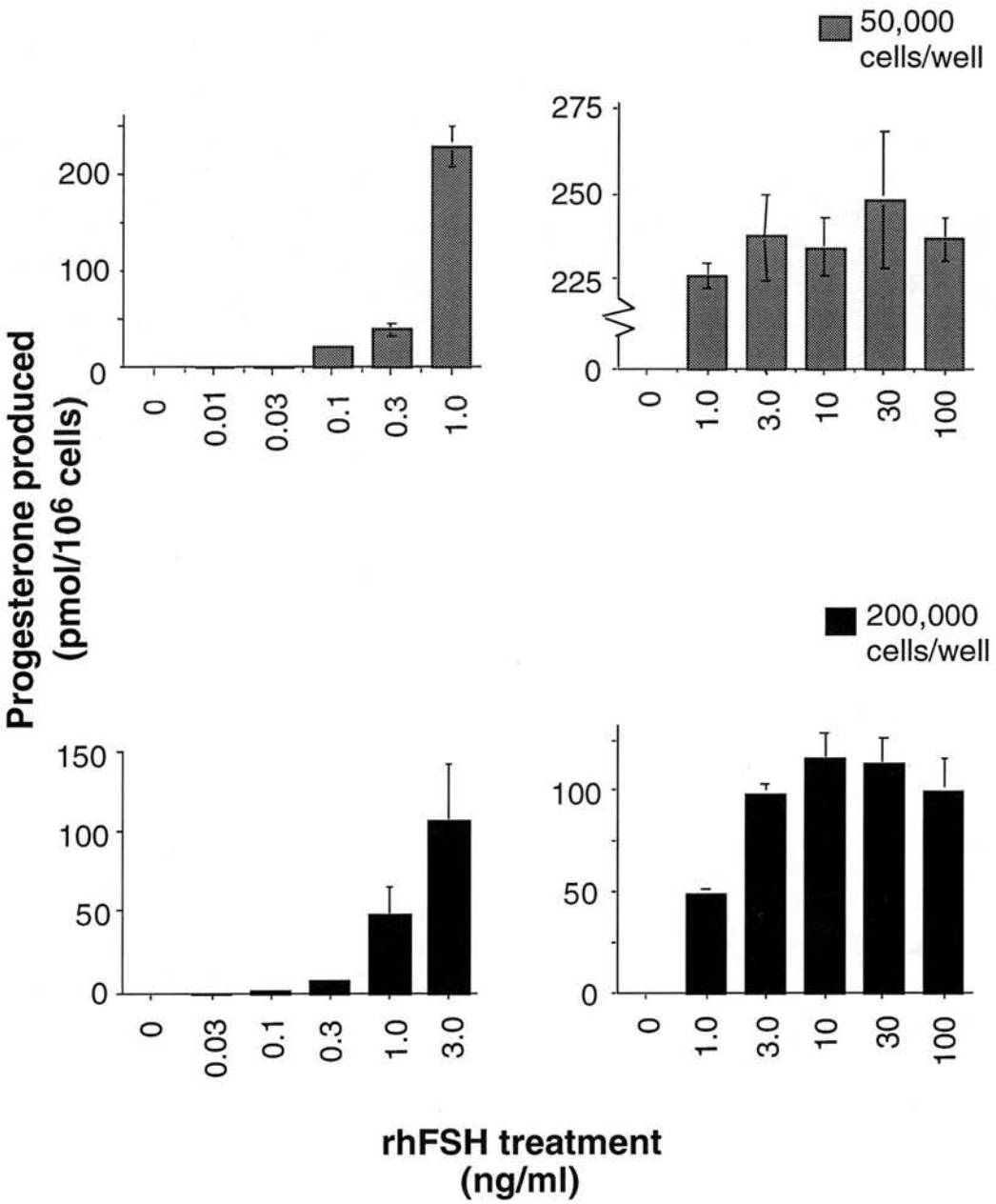


Fig 4.8 Graphs representing the effects of granulosa cell density in vitro on progesterone production with increasing rhFSH concentrations. Both densities show a dose dependent increase in progesterone synthesis with the addition of rhFSH, peaking at approximately 30ng/ml rhFSH. Cells plated at 50,000 cells/well produced approximately 2-fold the progesterone measured in pmol/10^6 cells of those cells plated at 200,000 cells/well. Values represent the mean (±SE).

3.2 Optimisation of rhFSH concentration

In vivo, FSH causes granulosa cells to develop, become LH responsive and produce oestrogen, i.e. express LH receptors (Segaloff *et al*, 1990) and P450arom (Steinkampf *et al*, 1987). Therefore, the expression of these genes indicates that granulosa cells are mature and primed for the LH surge. Fig 4.3 shows RPAs of the FSH dose-response experiments. Protected fragments of aromatase and LH receptor can be seen most clearly with the addition of 1-3ng/ml (see fig 4.4). Both LHR and P450arom require a minimum of 0.3ng/ml rhFSH to induce their expression and peak around 1ng-3ng/ml. Gene expression decreases with high concentrations of rhFSH. An optimum concentration of 1ng/ml rhFSH was adopted for further experiments.

3.3 Optimising LH treatment

StAR and P450scc were chosen as markers of LH action since high levels of LH induce luteinisation and hence the synthesis of progesterone (Moor, 1974). Figs 4.5 and 4.6 show RPAs of StAR and P450scc expression in the rhLH-treated granulosa cells. Both StAR and P450scc were expressed in control groups, but at relatively low concentrations. Expressions of both genes increased with rhLH treatment, peaking at 10ng/ml and decreasing again with higher concentrations. 3ng/ml was chosen as the optimal rhLH concentration for further experiments.

3.4 Effect of testosterone concentration

Fig 4.7 shows the effects of rhFSH and testosterone concentrations on progesterone production. Little progesterone was synthesised with no testosterone present, even at high rhFSH concentrations. There was no significant rhFSH dose dependency. Testosterone at 10^{-8}M increased progesterone synthesis with rising rhFSH concentrations. This peaked at 3ng/ml FSH and declined at concentrations above that. A testosterone concentration of 10^{-6}M induced the greatest amount of progesterone synthesis, which peaked with 30ng/ml rhFSH and declined at 100ng/ml. At the previously appointed optimum FSH concentration of 1ng/ml, progesterone synthesis is approximately 2.5-fold higher in the culture containing 10^{-6}M testosterone that in cultures with no testosterone.

3.5 Effects of cell density

Fig 4.8 shows that cell density did not appear to have any effect on the pattern of progesterone produced, i.e. synthesis was rhFSH dose dependent at both densities (50,000 and 200,000 cells/well), plateauing at approximately 10-30ng/ml. However

cells plated at 50,000 cells/well produced as much as 6 times the amount of progesterone at lower rhFSH concentrations than cells plated at 200,000 cells/well. At higher rhFSH concentrations, this decreased to approximately twice the amount of progesterone synthesised by the lower cell density.

Similar results were obtained with rhLH. At both densities, progesterone synthesis reached a plateau of approximately 1-3ng/ml rhLH (fig 4.9). However, when plated at 50,000 cells/well, granulosa cells produced 10 times more progesterone than when plated at 200,000 cells/well.

3.6 Time course in vitro under optimal conditions

RNA collected from the experiment described in section 2.7 was analysed by RPA to measure the expression of StAR, P450scc and 3βHSD. Fig 4.10 shows a photograph of this RPA gel, and this data was quantified (fig 4.11). This RPA was performed using two sets of samples due to a shortage of RNA.

rhFSH treatment induced the expression of all the genes studied, with no expression in the absence of gonadotrophin treatment. StAR expression increased with time, despite the lack of gonadotrophin stimulation, but not to the same levels as expression in cells treated with rhLH. Addition of rhLH increased the expression of all genes studied maximally after 6h, but expression decreased thereafter.

rhFSH induced progesterone synthesis which continued at low levels in the absence of continued gonadotrophin treatment. rhLH increased progesterone synthesis by approximately 12-fold after 6h, but the response declined with time to only 5-fold after 24h (fig 4.12).

PR expression was measured by RPA using this RNA. Fig 4.13 shows PR expression only after 6h of rhLH exposure after rhFSH priming. By 12h, this had almost gone (fig 4.14).

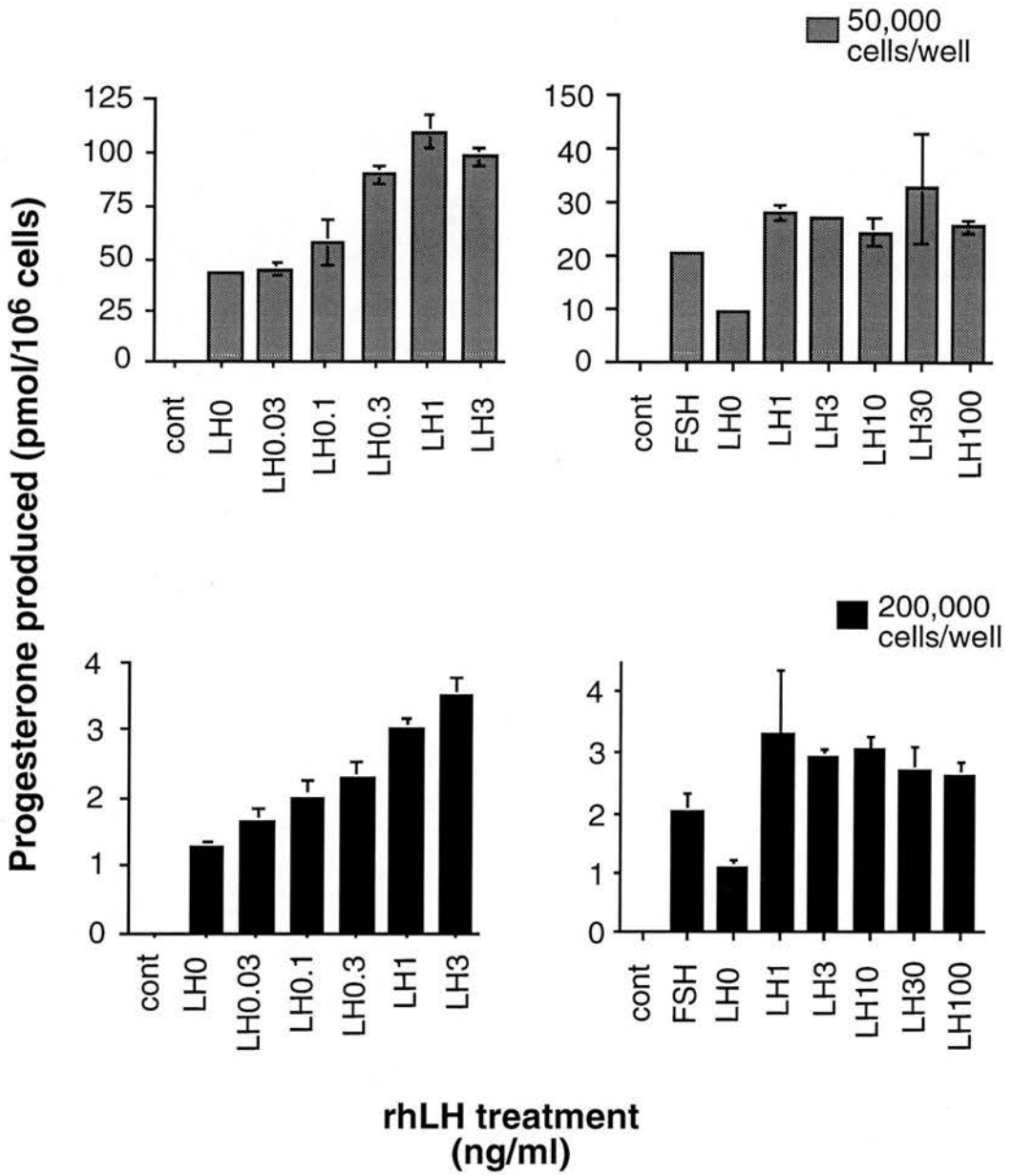


Fig 4.9 Graphs representing the effects of granulosa cell density *in vitro* on progesterone production with increasing rhLH concentrations. Cells were previously primed with 48h of rhFSH, washed with DPBS and medium containing increasing concentration rhLH added back to the culture. Progesterone synthesis showed a dose-dependent relationship in both cell densities with the concentration of rhLH added to the culture until it plateaued at approximately 3ng/ml rhLH. Cells plated at 50,000 cells/well produced approximately 10-fold the progesterone measured in pmol/10^6 cells of those cells plated at 200,000 cells/well. Values represent the mean (±SE).

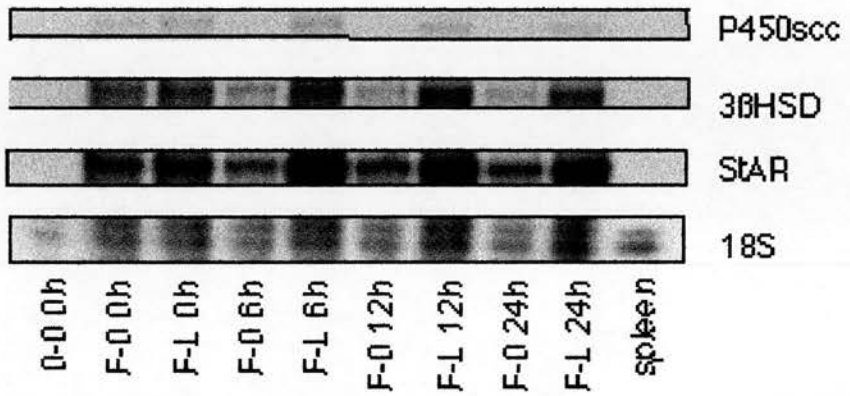


Fig 4.10 RNase protection assay showing the gene expression of StAR, P450scc and 3βHSD in cultured granulosa cells. Control group (0-0 0h) received no gonadotrophins for 48h. All other groups were treated with 1ng/ml rhFSH for 48h. All groups were washed with DPBS and medium containing either no gonadotrophin or 3ng/ml rhLH was added to the cells. The cultures were terminated at 0h, 6h, 12h or 24h after the addition of the second treatment. All genes can be seen to be expressed in all treatments bar the control group 0-0 0h. Spleen was used as a negative control.

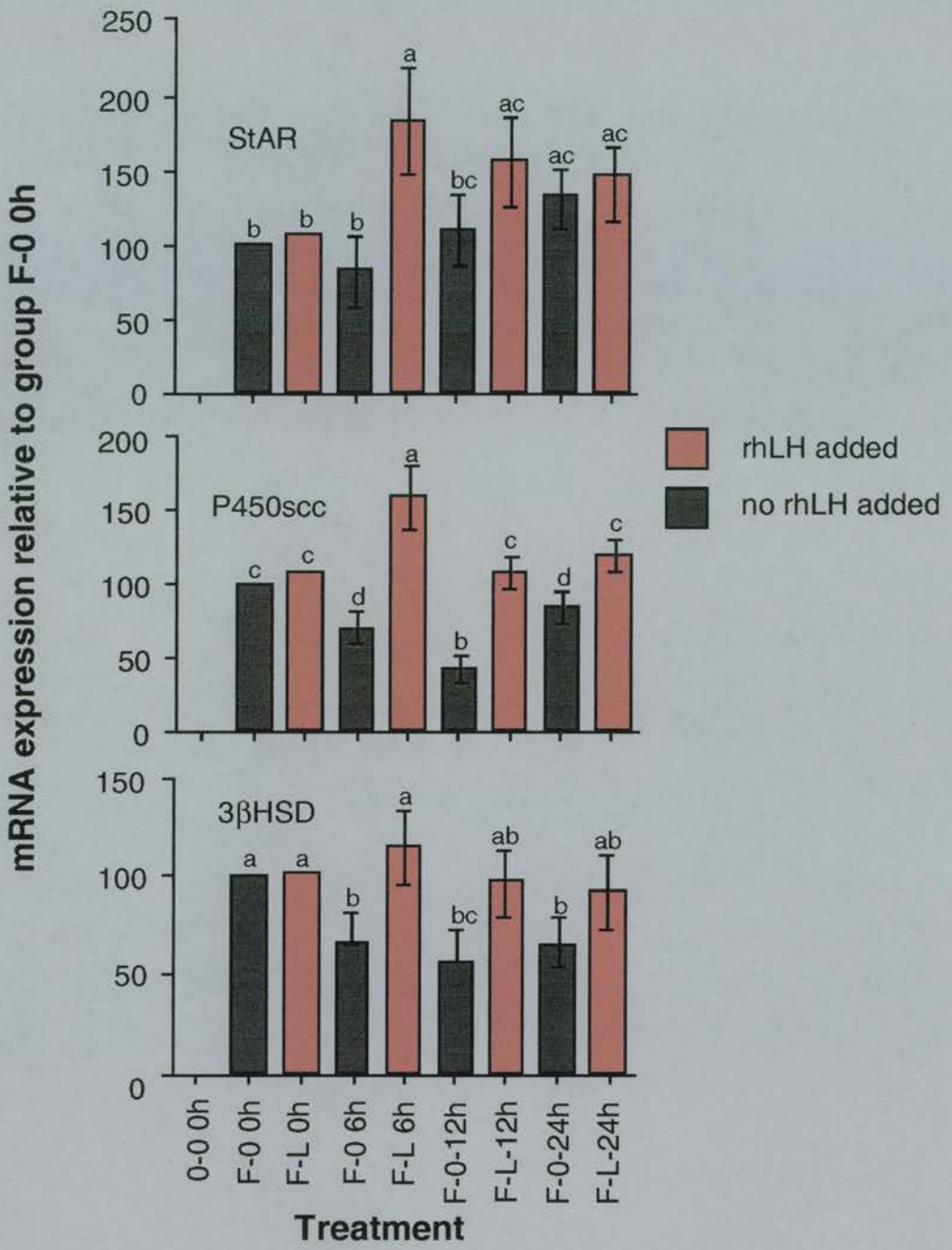


Fig 4.11 Graphs representing gene expression of StAR, P450scc and 3βHSD in cultured granulosa cells.
Control group (0-0 0h) received no gonadotrophins for 48h. All other groups were treated with 1ng/ml rhFSH for 48h. All groups were washed with DPBS and medium containing either no gonadotrophin or 3ng/ml rhLH was added to the cells. The cultures were terminated at 0h, 6h, 12h or 24h after the addition of the second treatment. Each sample was standardised against 18S and the sample F-0 0h was assigned an arbitrary value of 100%. Values represent the mean (±SE) from triplicate experiments. Histograms with different superscripts are significantly different from one another ($p<0.05$).

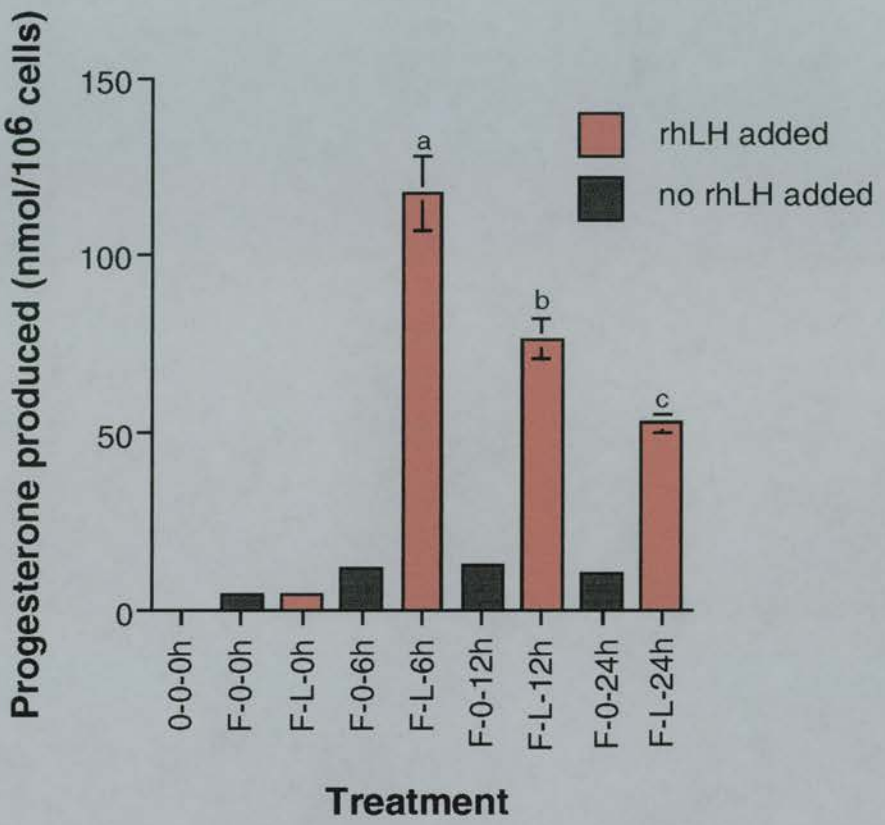


Fig 4.12 Graph representing progesterone synthesis from cultured granulosa cells with varying treatments. Control group (0-0 0h) received no gonadotrophins for 48h. All other groups were treated with 1ng/ml rhFSH for 48h. All groups were washed with DPBS and medium containing either no gonadotrophin or 3ng/ml rhLH was added to the cells. The cultures were terminated at 0h, 6h, 12h or 24h after the addition of the second treatment. Values represent the mean (±SE) from triplicate experiments. Histograms with different superscripts are significantly different from one another ($p<0.01$).

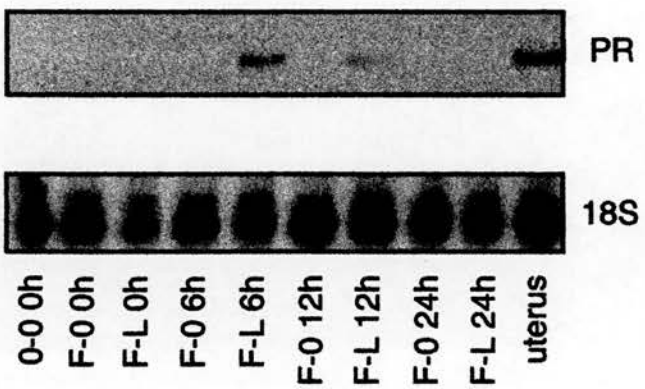


Fig 4.13 RNase protection assay showing the gene expression of total progesterone receptor from cultured granulosa cells with varying treatments. Control group (0-0 0h) received no gonadotrophins for 48h. All other groups were treated with 1ng/ml rhFSH for 48h. All groups were washed with DPBS and medium containing either no gonadotrophin or 3ng/ml rhLH was added to the cells. The cultures were terminated at 0h, 6h, 12h or 24h after the addition of the second treatment. Uterus was used as a positive control.

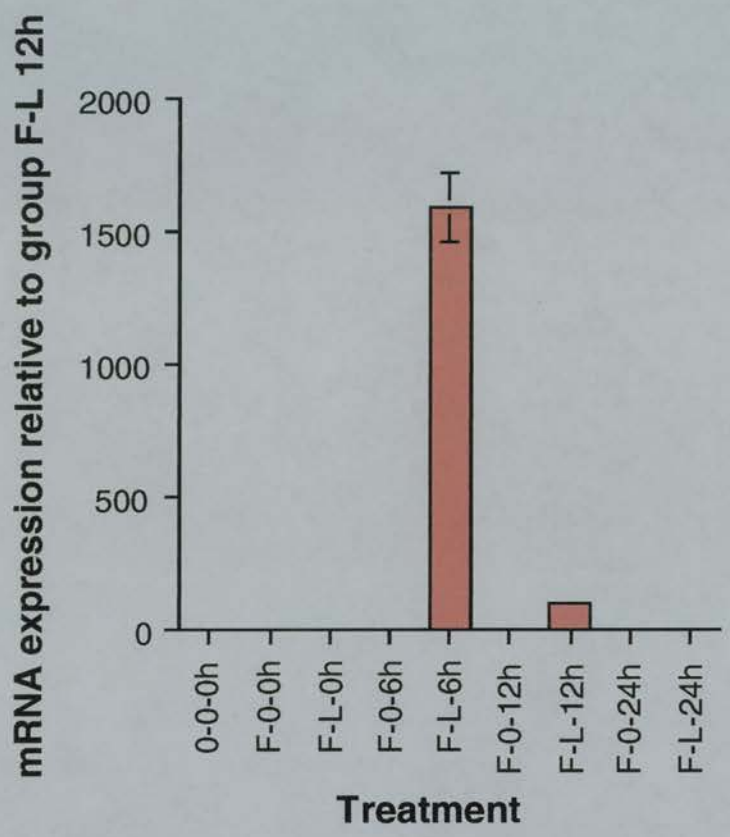


Fig 4.14 Graph representing progesterone receptor mRNA expression by cultured granulosa cells with various treatments. Control group (0-0 0h) received no gonadotrophins for 48h. All other groups were treated with 1ng/ml rhFSH for 48h. All groups were washed with DPBS and medium containing either no gonadotrophin or 3ng/ml rhLH was added to the cells. The cultures were terminated at 0h, 6h, 12h or 24h after the addition of the second treatment. Each sample was standardised against 18S and the F-L-12h sample was assigned an arbitrary value of 100%. Values represent the mean (±SE) from triplicate experiments.

4 Discussion

Granulosa cell culture had been established in this laboratory for many years, but recombinant human gonadotrophins are relatively new, and the correct treatment concentrations for rat ovarian cells had not been optimised. The initial gonadotrophin concentrations were used because they had been successful in inducing PR expression in cultured rat granulosa cells from 17β-oestradiol-primed immature rats (Natraj and Richards, 1993). However, these were purified ovine hormones of unspecified bioactivity.

Pilot experiments with a different batch of rhFSH had been previously done in this laboratory in rat granulosa cell cultures but produced a less pronounced effect than those observed in fig 4.1. Curves of progesterone synthesis suggested that concentrations of 30-100ng/ml rhFSH (batch LER 8/116) would be appropriate for stimulation of steroidogenic enzyme expression (Smyth, 1993). When comparing bioactivity, 50ng/ml corresponds to 0.65IU of LER 8/116 compared to 0.192IU for the rhFSH preparation (BFBA 9612) used in these experiments. The huge difference in progesterone production with rhFSH stimulation may be due to our rats being DES primed *in vivo* prior to culturing. Pre-treatment of animals with oestrogen increases the subsequent effects of FSH *in vitro* on cAMP formation (Jonnassen *et al.*, 1982), P450scc expression (Goldring *et al.*, 1987) and LH receptor formation (Richards *et al.*, 1976; Segaloff *et al.*, 1990).

Optimising the culture conditions enabled the establishment of a more physiological system in which to study cellular behaviour. LH receptor (LHR) and P450arom expression were used as end points to establish an optimal rhFSH concentration. FSH is known to induce LHR in granulosa cells *in vivo* and *in vitro* (Erickson *et al.*, 1979), priming the cells for the LH surge. P450arom is also induced by FSH stimulation (Steinkampf *et al.*, 1987) and is required for the synthesis of oestrogen which augments the effect of FSH on up-regulation of LHR expression (Rani *et al.*, 1981). It appears that rhFSH concentrations above 3ng/ml led to over-stimulation. rhLH also appeared to have a maximal threshold. Above 10ng/ml, expression of genes normally induced *in vivo* at physiological concentrations were down-regulated. StAR and P450scc are essential for the synthesis of progesterone and the process of luteinisation *in vivo* and therefore their expression signifies conditions closer to a normal physiology. The optimal concentrations established here (1ng/ml rhFSH and 3ng/ml

rhLH) were used in all future culture experiments, despite differences from those suggested by the literature.

The presence of androgens enhanced response to rhFSH. This correlates with the situation *in vivo*. Androgens are produced by thecal cells which are the first cell type in the ovary to become steroidogenically active. It follows that it may be necessary to supplement granulosa cells with a steroid that would be present *in vivo*. Androgens, as well as acting as an aromatase substrate for oestradiol synthesis, enhance FSH-stimulated progesterone production in immature (Armstrong and Dorrington, 1976) and mature rat granulosa cells (Zeleznik *et al.*, 1979). These results were verified by our findings that testosterone enhances FSH-stimulated progesterone production by granulosa cells of all stages of maturity *in vitro*. Androgen receptors are expressed in granulosa cells and receptor-mediated androgen action in granulosa cells leads to increased generation of extracellular cAMP and amplification of cAMP-dependent processes initiated by FSH (Hillier and deZwart, 1982).

Plating density was important since increased progesterone synthesis has been reported with high cell concentrations. Chaffkin et al (1993) reported that granulosa-lutein cells plated at low density ($5x10^3$ cells/ml) proliferated, whereas cells plated at a higher density ($50x10^3$ cells/ml) did not proliferate, but differentiated. This may explain the difference in progesterone synthesis with different plating densities. Cells plated at 50,000 cells/well ($25x10^3$ cells/ml) may proliferate to beyond the original plating density, thus increasing the number of cells and rendering the unit of measurement (pmol/10^6 cells) inaccurate. However, it has also been suggested that prolonged exposure to high levels of oestrogen *in vivo* can reduce the capacity of granulosa cells to proliferate *in vitro* (Chakravorty *et al.*, 1991). In any case, the pattern of progesterone production was similar at both cell concentrations analysed and a density between the two of 160,000cells/well was adopted which was enough to give high RNA recovery.

Having established optimum conditions, it was then possible to create *in vitro* conditions similar to those in the *in vivo* experiments described in chapter 3. These results verify that granulosa cells require FSH stimulation to induce steroidogenesis as discussed in chapter 3, section 4. The whole immature ovary displays low levels of steroidogenesis through the basal expression of StAR, P450scc and 3βHSD (see

fig.3.8), but granulosa cell culture experiments demonstrate these genes are expressed in cells other than granulosa cells.

Interestingly, after rhFSH treatment, StAR expression continued to increase with time, despite the lack of gonadotrophin stimulation. It may be that FSH-primed granulosa cells remained prepared for an acute steroidogenic response for some time. Alternatively, the rhFSH dose used may have overstimulated the granulosa cells (despite optimisation) inducing them to slowly differentiate. P450scc, the enzyme responsible for the first step of *de novo* synthesis of all steroids, apparently required continued gonadotrophin stimulation for its expression, but mRNA levels did recover 24h after the removal of rhFSH. Over-stimulation may account for continued P450scc expression, since reports have shown that P450scc expression by immature granulosa cells is cAMP-dependent, but once luteinised, expression becomes cAMP-independent (Oonk, 1989). No significant increase was seen in 3βHSD expression and this was reflected in progesterone synthesis. Perhaps granulosa cells retain their steroidogenic capabilities (StAR and P450scc expression remaining high) but synthesise a steroid other than progesterone.

Similar patterns of gene expression to those seen *in vivo* were observed in culture with rhLH treatment inducing StAR, P450scc and 3βHSD. However, levels of these messages decreased over the length of the experiment, and progesterone secretion reflected this decline. Many reasons could account for this response. There is no vascular system to remove any products synthesised in this culture system and therefore the conditions surrounding these cells may have become unfavourable for continued progesterone production. Numerous steroids or proteins may be suspected; indeed it may be the accumulation of progesterone itself. StAR, P450scc and 3βHSD expression and progesterone synthesis are all at a maximum 6h after rhLH treatment. PR mRNA is only present for a short time around 6h and it may be that the activation of PR by the high concentrations of progesterone is responsible for the drop in steroidogenesis.

In summary, this study is the first to demonstrate co-ordinated regulation of genes involved in progesterone synthesis (StAR, P450scc and 3βHSD) and reception (PR) *in vitro*. However, progesterone is not the only PR binding steroid, and it may be that glucocorticoids, known to be present in the ovary *in vivo*, may act through the PR. Knowledge of the dynamics between activation and inactivation of glucocorticoids

could answer some of the questions surrounding the importance of the influence of progesterone on ovulation and luteinisation.

Chapter 5 Glucocorticoid Metabolism in the Rat Ovary

1. Introduction

The ovary has characteristics of a glucocorticoid target organ. High affinity glucocorticoid binding sites, presumed to be glucocorticoid receptors (GR) have been identified in granulosa cells and corpora lutea (Schreiber *et al.*, 1982) and 11β-hydroxysteroid dehydrogenase (11βHSD), an enzyme which bi-directionally activates and inactivates glucocorticoids has been localised to the oocyte and corpus luteum of rats (Benediktsson *et al.*, 1992; Roland and Funder, 1996; Waddell *et al.*, 1996) and granulosa cells of humans (Michael *et al.*, 1993; Michael and Cooke, 1994; Michael *et al.*, 1995). Glucocorticoids can also modulate ovarian responsiveness to gonadotrophins (Schoonmaker and Erickson, 1983; Harlow *et al.*, 1987; Wang and Leung, 1989; Jia *et al.*, 1990).

Glucocorticoids are synthesised in the adrenal cortex in response to adrenocorticotrophin (ACTH), but are not synthesised in the ovary, due to the lack of P450C21 and P450C11β enzymes (Omura and Morohashi, 1995). Glucocorticoids are most commonly involved in stress, nutrition and tissue repair and induce cellular differentiation and metabolism. Their anti-inflammatory actions include regulating cytokines and adhesion molecules and modulating the expression of genes involved in inflammation. It is the anti-inflammatory action of glucocorticoid that is thought to effect the ovary. Progesterone shares the same anti-inflammatory potency as glucocorticoids (van der Burg and van der Saag, 1996) and the presence of both steroids and their receptors in the same or adjacent cells opens up the possibility of a number of interactions, since ligands and receptors cross-react. Therefore, spatio-temporal control over receptor expression, progesterone synthesis and metabolism, and glucocorticoid activation and inactivation is critical for correct receptor-ligand activation.

11βHSD exists as two isoforms. Type 1 is a bi-directional $NADP^+$-dependent enzyme with predominant reductase activity and has a high binding affinity for the inactive forms of glucocorticoid, cortisone (in human) and 11-dehydrocorticosterone (in rat) (Stewart and Mason, 1995). Type 2 is an NAD^+-dependent enzyme with dehydrogenase activity and has a high binding affinity for the active forms of cortisol (in humans) and corticosterone (in rats) (Albiston *et al.*, 1994; Stewart *et al.*, 1994).

11βHSD1 is expressed in glucocorticoid target organs such as liver, where glucocorticoids are required to activate the GR (Whorwood *et al.*, 1992). Mineralocorticoid receptors (MR) bind both glucocorticoids and mineralocorticoids with the same affinity. 11βHSD2 occurs in mineralocorticoid target organs, such as the kidney and colon, to inactivate glucocorticoid and protect MR from over-stimulation from active glucocorticoid. Both isozymes exist in human granulosa-lutein cells (Michael and Cooke, 1994) where their relative activity may control the concentration of active and inactive intrafollicular glucocorticoids. Tetsuka et al (1997) showed 11βHSD2 mRNA was high in human granulosa cells and type 1 was the prominent isozyme in luteinised cells. This is reflected in an increased cortisol concentration in follicular fluid following the LH surge (Harlow *et al.*, 1997).

11βHSD may be developmentally regulated, controlling the intrafollicular concentrations of glucocorticoids available to GR, which is thought to be expressed constitutively in granulosa cells and the corpus luteum. Progesterone synthesis and PR expression in response to gonadotrophins has been discussed in chapters 3 (*in vivo*) and 4 (*in vitro*). This chapter makes use of the same models to examine the ovary as a glucocorticoid target organ by studying the expression of 11βHSD and GR.

2. Materials and Methods

2.1 Quantification of 11βHSD isoforms in whole rat ovary

mRNA from the experiment described in chapter 3, section 2.1 was used to identify the gene expression of 11βHSD types 1 and 2 in the maturing rat ovary. RNase protection assay (RPA) using 11βHSD probes described in chapter 2, section 4 was undertaken using 5μg total RNA of each sample.

2.2 Quantification of GR in whole rat ovary

In vivo experiments were undertaken to demonstrate glucocorticoid receptor (GR) mRNA expression in whole ovary. Immature rats were assigned to 3 groups and treated by subcutaneous injection of: 1) hCG 12h group - 10 IU PMSG for 48h and 10 IU hCG for 12h; 2) PMSG group - PMSG for 48h; 3) control group - no treatment. Ovaries were removed, snap frozen and RNA extracted as described in chapter 2, section 2. RPA using GR probe was undertaken using 40μg total RNA of each sample.

2.3 Quantification of 11βHSD isoforms in cultured rat granulosa cells

RNA from experiments described in chapter 4, section 2.6 was analysed using 11βHSD probes as described in chapter 2, section 8.

2.4 Identification of GR mRNA expression

Reverse transcriptase-polymerase chain reaction (RT-PCR) was used to demonstrate GR mRNA expression in RNA samples from chapter 4, section 2.7. PCR primers described in chapter 2, section 4, used to construct a template for making a GR riboprobe, were used to amplify GR mRNA expression. RT-PCR amplification would produce a fragment 772bp long. RNA was precipitated out of formamide by adding 0.1 volume sodium acetate and 2.5 volumes ethanol, placing at -20°C for 30 min and centrifuging for 20 min at 13,000 rpm and redissolving in water. 5μg RNA was used in the RT-PCR reaction as described in chapter 2, section 3. Annealing temperatures of 58°C and 62°C with 30 and 35 cycles were tested with ovarian samples using kidney as a positive control. The DNA RT-PCR products were separated on a 1.2% agarose gel and visualised with ethidium bromide.

3. Results

3.1 Quantification of 11βHSD types 1 and 2 in whole rat ovary

RPAs of *in vivo* time course experiments can be seen in fig 5.1. Data was quantified and plotted in fig 5.2. Both 11βHSD isoforms are present in immature ovaries in very small quantities. 11βHSD1 increased 9-fold with PMSG treatment and continued to rise throughout the cycle and after ovulation. Expression was greatly reduced in the late corpus luteum, 5 days after the hCG injection. 11βHSD2 decreased with gonadotrophin treatment, reaching its lowest concentration at 9h after hCG injection. Levels increased after ovulation at 12h and remained approximately 70% of the mRNA expression in the immature controls.

3.2 Quantification of GR in whole rat ovary

Fig 5.3 shows an RPA and a graph representing GR gene expression in whole ovaries of immature, PMSG-primed and post-ovulatory rats. 20μg of total RNA per group

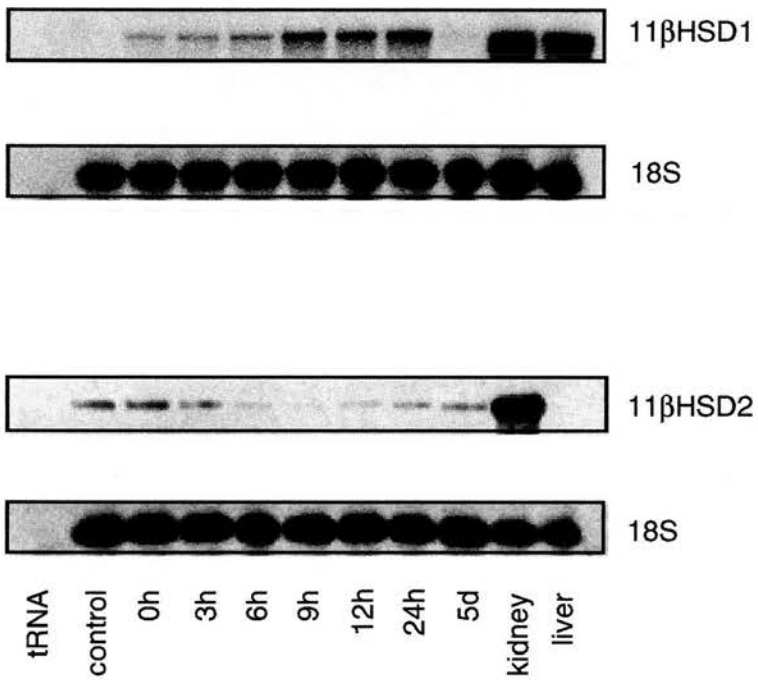


Fig 5.1 RNase protection assay showing the induction of 11βHSD1 and 11βHSD2 in the immature rat ovary with PMSG and hCG administration. Animals were injected with 10IU of PMSG, followed by 10IU of hCG and killed at 0h, 3h, 6h, 9h, 12h, 24h and 5 days post hCG. The control group received no injections and were killed with group 0h. Kidney and liver were used for positive controls. The intensity of each band was quantified using an instant imager and then exposed to a Kodak X-Omat AR-5 autoradiogram for 1 day (18S), 3 days (11βHSD1) or 7 days (11βHSD2) at -70°C using an intensifying screen.

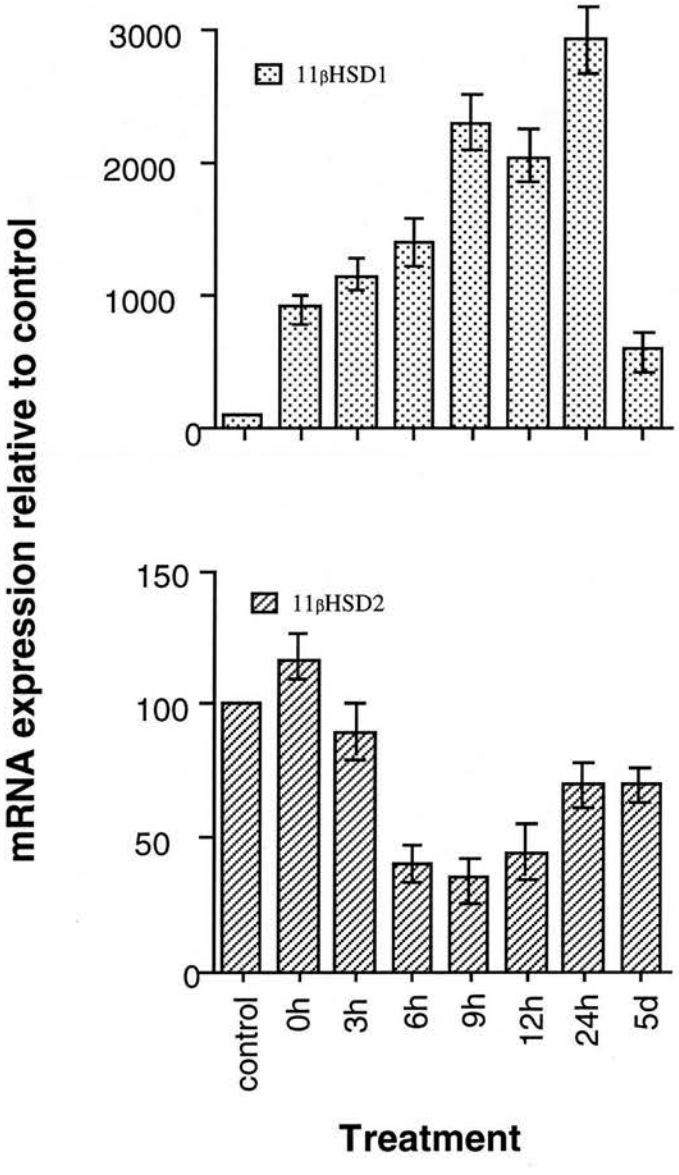


Fig 5.2 Quantification of the induction of 11βHSD1 and 11βHSD2 in the immature rat ovary with PMSG and hCG administration (from the RPA in fig.5.1). Each sample was standardised against 18S and the control sample was assigned an arbitrary value of 100%. Values represent the mean (±SE) from triplicate experiments. In each graph, histograms with different superscripts are significantly different from one another ($p<0.05$).

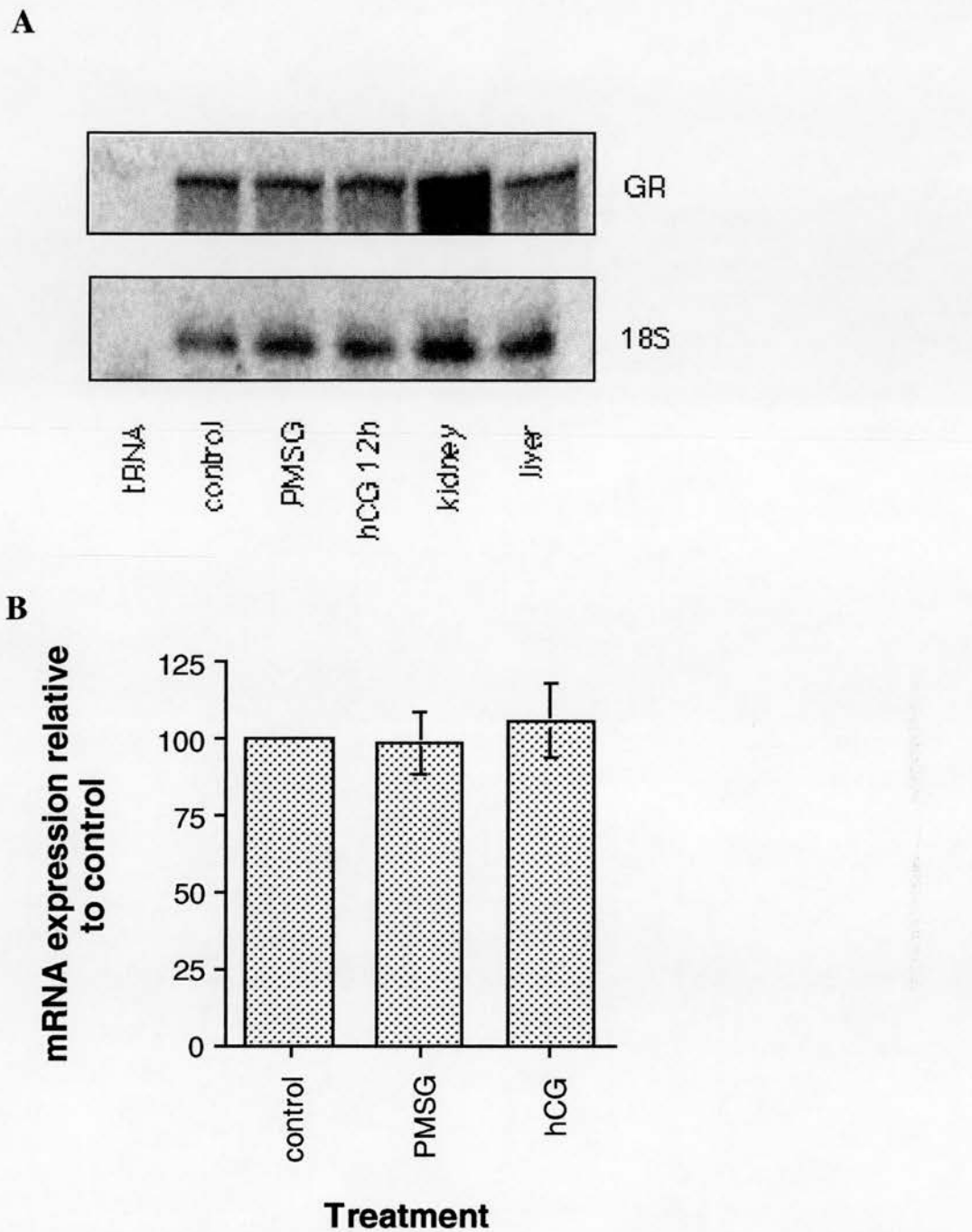


Fig 5.3 Expression of glucocorticoid receptor (GR) in immature rat ovary treated with gonadotrophins. A. RNase protection assay showing the gene expression of GR in immature rat ovary (control), immature rat treated with 10IU PMSG for 48h (PMSG) and immature rat treated with 10IU PMSG for 48h followed by 10IU hCG for 12h (hCG 12h). tRNA was used for a negative control and kidney and liver were used as positive controls. The intensity of each band was quantified using an instant imager and then exposed to a Kodak X-Omat AR-5 autoradiogram for 1 day (18S) and 5 days (GR) at -70°C using an intensifying screen.
B. Graph representing gene expression of GR as described above.

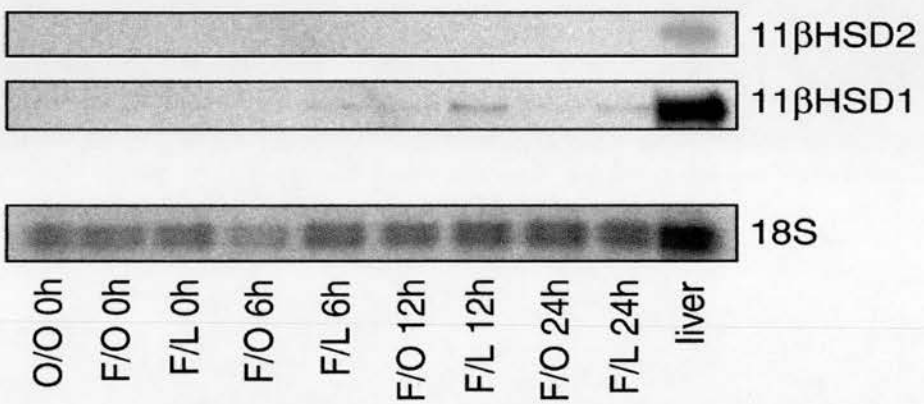


Fig 5.4 RNase protection assay showing the gene expression of 11βHSD1 and 11βHSD2 in cultured granulosa cells. Control group (0-0 0h) received no gonadotrophins for 48h. All other groups were treated with 1ng/ml rhFSH for 48h. All groups were washed with DPBS and medium containing either no gonadotrophin or 3ng/ml rhLH was added to the cells. The cultures were terminated at 0h, 6h, 12h or 24h after the addition of the second treatment. Liver was used as a positive control. The intensity of each band was quantified using an instant imager and then exposed to a Kodak X-Omat AR-5 autoradiogram for 1 day (18S), 5 days (11βHSD1) or 14 days (11βHSD2) at -70°C using an intensifying screen.

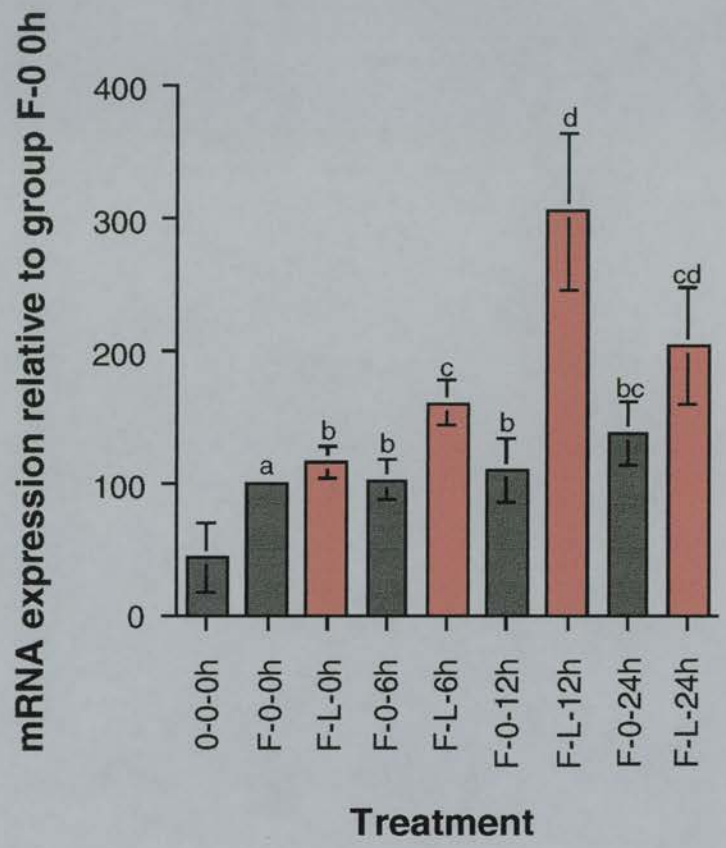


Fig 5.5 Graph representing gene expression of 11βHSD1 in cultured granulosa cells from RPA in fig. 5.4 (11βHSD2 gene expression was negligible). Control group (0-0 0h) received no gonadotrophins for 48h. All other groups were treated with 1ng/ml rhFSH for 48h. All groups were washed with DPBS and medium containing either no gonadotrophin or 3ng/ml rhLH was added to the cells. The cultures were terminated at 0h, 6h, 12h or 24h after the addition of the second treatment. Each sample was standardised against 18S and the F-0-0h sample was assigned an arbitrary value of 100%. Values represent the mean (òSE) from triplicate experiments. Histograms with different superscripts are significantly different from one another ($p<0.05$).

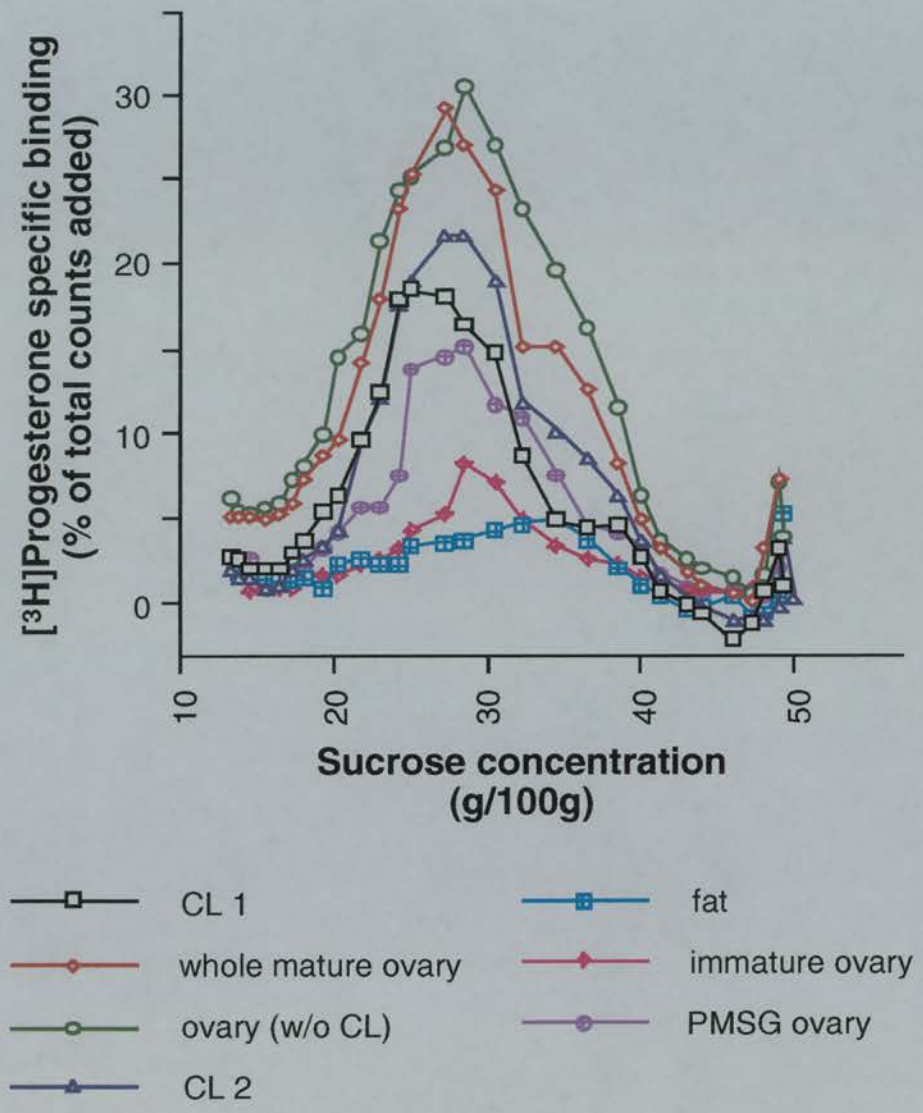


Fig 7.1 Fractionation of rat ovarian tissue homogenates (and adherent adipose tissue) incubated with [³H]-progesterone in the presence of digitonin. Points represent the mean of triplicate analysis.

was used to demonstrate GR was present in all samples in very small quantities, with no significant difference in expression with gonadotrophin treatment.

3.3 Quantification of 11βHSD1 and 2 in rat granulosa cells

Isolated rat granulosa cells were treated with rhFSH for 48h, followed by either no treatment or rhLH for 0h, 6h, 12h or 24h. Fig 5.4 shows an RPA of 11βHSD1 and 2 run on the same gel. Data were quantified and represented in fig 5.5. 5μg total RNA gave no signal with the 11βHSD2 probe, indicating expression is very low or not present in granulosa cells. 11βHSD1 mRNA was present in all groups, being induced by rhFSH treatment. rhLH significantly increased type 1 expression after 6h, and rose to approximately 3-fold by 12h after rhLH treatment before falling again by 24h. rhFSH-primed granulosa cells that received no further treatment showed no significant change in 11βHSD1 gene expression.

3.4 Identifying GR gene expression in cultured rat granulosa cells

RT-PCR on 2.5μg or 5μg for 30 or 35 PCR cycles produced no DNA fragment from ovarian samples. A positive control (kidney) showed the conditions were optimal. A kidney sample with no reverse transcriptase enzyme added in the RT stage showed no bands and therefore amplification was not due to DNA-contaminated RNA. A negative control (water) showed there was no contamination in any of the reagents. These results indicate GR gene expression is not expressed in cultured granulosa cells.

4. Discussion

These results provide evidence that 11βHSD genes are gonadotrophically regulated in the rat ovary. Gene expression of 11βHSD1 (the bi-directional enzyme predominantly exhibiting reductase action to yield active glucocorticoids) is induced by gonadotrophins both in whole ovary *in vivo* and in granulosa cell cultures. Very little expression was seen in the immature ovary or unstimulated granulosa cells, but PMSG/rhFSH treatment induced expression with hCG/rhLH enhancing this effect. In contrast, the expression of mRNA for 11βHSD2 (the enzyme which leads to the inactivation of glucocorticoids) was low in immature ovaries and was down-regulated with gonadotrophin stimulation. *In vitro* experiments did not yield enough RNA per group to allow more than 5-7.5μg per RPA analysis, therefore no 11βHSD2 signal could be identified in *in vitro* experiments. However, studies previously undertaken in

this laboratory have shown 11βHSD2 expression levels to follow a similar pattern to those shown here *in vivo* (Tetsuka *et al.*, 1999). Although glucocorticoid concentrations were not assayed in blood serum or culture medium, the regulation of 11βHSD gene expression, switching from type 2 to type 1 with gonadotrophin stimulation, suggests an increased corticosterone : 11-dehydrocortisterone ratio would occur in maturing rat ovaries. Similarly, increased cortisol concentrations are detected in follicular fluid of human IVF patients after hCG treatment (Harlow *et al.*, 1997).

Glucocorticoid receptor (GR) was shown to be constitutively expressed *in vivo* and was not under gonadotrophic regulation. GR expression *in vitro* could not be determined due to small quantities of RNA recovered from cultured cells. As with 11βHSD2, no GR expression was identified using 7.5μg RNA in an RPA (results not shown). A more sensitive technique of RT-PCR was adopted to attempt to demonstrate the presence of GR mRNA in granulosa cells *in vitro*. However, this too gave negative results, implying GR is not expressed in rat granulosa cells. Other studies performed in this laboratory have shown this not to be the case, as GR mRNA has been identified in immature, PMSG- and hCG-treated rat granulosa cells in culture (Tetsuka *et al.*, 1997).

Glucocorticoids and progesterone bind and activate both PR and GR, suggesting that a complex system must be in operation, requiring tight regulation of genes involved in synthesis, metabolism, activation, inactivation and reception of the ligands and their receptors. Because all genes involved have been shown to be expressed within the same cell type, temporal activation is crucial to this system. PR is transiently expressed, whereas progesterone is present before and after receptor expression. PR expression may be the key to controlling progesterone action. Conversely, GR is expressed constitutively throughout follicular development and luteinisation. Hence, differential expression of 11βHSD1 reductase and 11βHSD2 dehydrogenase may act as the key mechanism in controlling glucocorticoid action.

The timing of 11βHSD gene expression is interesting when compared to that of StAR, P450scc and 3βHSD (described in chapters 3 and 4). Genes responsible for the synthesis of progesterone are maximally expressed 6-9h after hCG injection *in vivo* compared to 12-24h for the gene responsible for the formation of active glucocorticoids. Ovulation in the rat occurs approximately 10-12h after the LH surge as confirmed in this model (chapter 3, section 4). Therefore, genes involved in

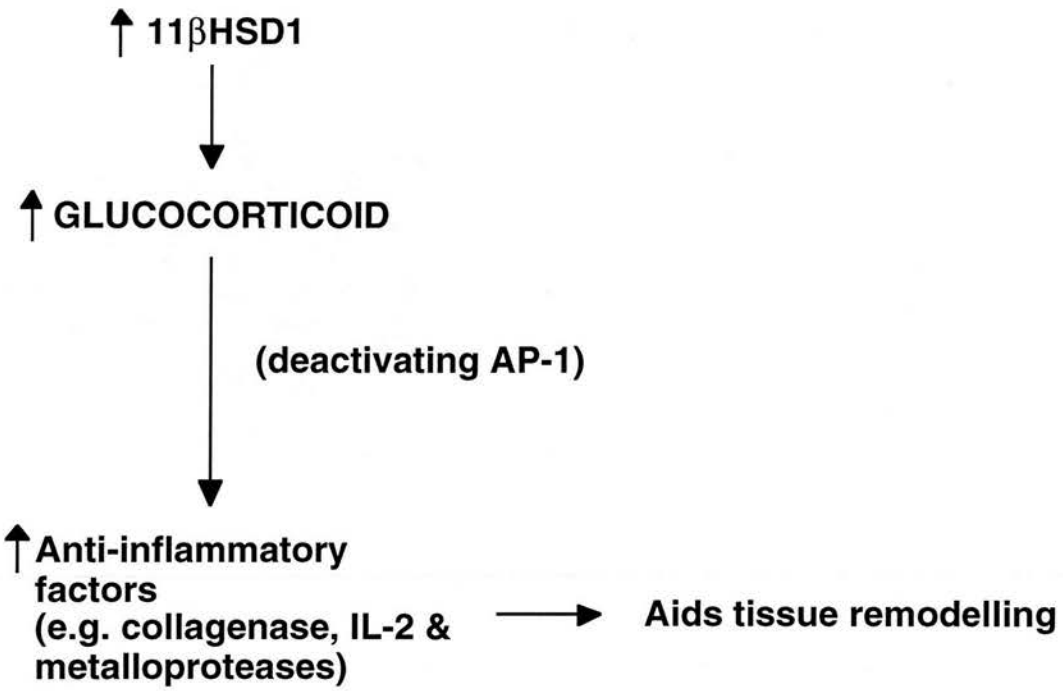


Fig.5.7. Proposed scheme of 11βHSD invovlement in the ovary.

progesterone synthesis (and progesterone concentrations themselves) and glucocorticoid activation are maximal before and after ovulation, respectively. Although progesterone and glucocorticoids cross-react with each other's receptors and both have known anti-inflammatory actions, their temporal regulation would suggest they act at different times, perhaps serving different purposes. PR knock-out mice have shown PR to be essential for ovulation and luteinisation (Lydon *et al.*, 1995). 11βHSD1 knock-out mice are viable and fertile, although the reproductive system of these animals does not appear to have been closely studied (Kotelevtsev *et al.*, 1997). Therefore, 11βHSD1 is not essential for ovarian function, despite gene expression suggesting that the ovary is a glucocorticoid target organ. In the 11βHSD1 knock-out mice, other enzymes may compensate to maintain glucocorticoids levels within the ovary. Alternatively, perhaps ovarian active glucocorticoid concentrations are reduced and the ovary operates with decreased efficiency. If glucocorticoids display an anti-inflammatory action on the ovary after ovulation, as 11βHSD expression suggests, they may act to attenuate the effects of inflammatory factors involved in rupture of the

follicle and remodelling and repair of the ovary wall. Glucocorticoid anti-inflammatory effects involve down-regulating inflammatory factors known to be involved in ovulation (such as collagenase and IL-2) by deactivating activator protein 1 (AP-1) (see general discussion, chapter 8). Inhibition of AP-1 also reduces expression of several metalloproteases (for reviews, see Bamberger *et al.*, 1996; van der Burg and van der Saag, 1996). This is represented in figure 5.7.

Interaction between progesterone and glucocorticoids has been observed in the rat corpus luteum. Progesterone acts through GR and down-regulates the transcription of 20αHSD (Sugino *et al.*, 1997). Hence, progesterone protects itself by down-regulating the enzyme that inactivates it. This action occurs through glucocorticoid response elements (GRE) in the promoter region of the 20αHSD gene (Zhong *et al.*, 1996). Some reports have suggested that glucocorticoids may also affect the synthesis of progesterone by suppressing stimulation induced by FSH and LH, perhaps through inhibiting cAMP production and down-regulating the activity of P450scc and 3βHSD (for review see Michael and Cooke, 1994).

In conclusion, this chapter demonstrates that the rat ovary is a site of 11βHSD expression, consistent with a regulatory function for glucocorticoid. Novelly, it has been shown that the glucocorticoid activating isoform 11βHSD1 is up-regulated in granulosa cells during LH/hCG-induced luteinisation *in vivo* and *in vitro.*

Glucocorticoids and progesterone play important roles in follicular development and ovulation, being regulated by gonadotrophins and, to an extent, by one another. The next chapter aims to examine the effects of progesterone and glucocorticoids on granulosa cell steroidogenic activity by blocking steroid activation through genomic receptors.

Chapter 6 Antiprogestins and Rat Granulosa Cell Culture

1. Introduction

Chapters 4 and 5 confirm granulosa cells are both progesterone and glucocorticoid targets. Genes coding enzymes involved in progesterone synthesis and glucocorticoid activation and inactivation have been shown to be present in granulosa cells and under gonadotrophic regulation. Their respective receptors, PR and GR, have also been identified; PR being transiently expressed 6h after the LH surge and GR being constitutively expressed throughout the ovarian cycle (Tetsuka *et al.*, 1999). However, very little is known about how progesterone and glucocorticoid manifest their paracrine or autocrinc action.

One way of studying progesterone and glucocorticoid action would be to block their receptors. The anti-progestin RU486 also has anti-glucocorticoid properties and binds with strong affinity to both PR and GR (Schreiber *et al.*, 1983). RU486 binding to PR induces different conformational changes in the extreme C-terminus of the receptor compared to agonist binding to PR (Vegeto *et al.*, 1992), but RU486 exerts its action predominantly at a post-DNA (progesterone response elements) binding step. This is possibly through preventing interactions between the receptor/ligand complex and transcription factors (for review see Beck *et al.*, 1993). RU486 exerts its anti-glucocorticoid activity at two levels; prevention of complete GR transformation and alteration of a step subsequent to GR-DNA (glucocorticoid response element) binding. Org31710 is an anti-progestin demonstrating high affinity for PR, but with less than 1% relative binding affinity for GR (Kloosterboer *et al.*, 1994). Since progesterone, glucocorticoid and RU486 cross-react with PR and GR, and Org31710 only binds to PR, it may be possible to deduce the effects of receptor activation by comparing the effects these agonist and antagonists have on granulosa cell gene expression and steroid production. Reports have shown RU486 up-regulates progesterone concentrations (Uilenbroek *et al.*, 1992; Morgan *et al.*, 1994). This effect may occur through PR or GR which leads us to ask whether progesterone acts to alter regulation of genes involved in its own synthesis or reception, and does glucocorticoid play any role in this system?

The ovary is not the only organ to express PR and GR to which antagonist can bind, and effects such as anovulation due to reduced FSH and LH concentrations are probably caused by RU486 binding to receptors in the pituitary and/or hypothalamus (Batista *et al.*, 1994; Heikinheimo *et al.*, 1995). However, many reports have shown that antiprogestins work at an ovarian level. This chapter examines the effects of RU486 and Org31710 on the granulosa cell model optimised and studied in previous chapters to illustrate how progesterone and glucocorticoid act to regulate their own synthesis, activation and reception.

2. Methods and Materials

All culture experiments in this chapter use techniques described in chapter 2, section 2.1 and chapter 4, section 2.6. Rat granulosa cells were plated at 160,000cells/well with 1ng/ml rhFSH (0ng/ml in control groups) and 10^{-6}M testosterone for 48h before washing in 0.5ml DPBS. Further treatments for each experiment are described below.

2.1 Effects of RU486 on granulosa cell culture

3ng/ml rhLH, 1ng/μl rhFSH or no treatment, with or without 10^{-6}M RU486, were added to rhFSH-primed granulosa cells in culture. Control group which received no FSH priming, were plated with no hormone treatment, with or without 10^{-6}M RU486. This was incubated at 37°C for 12h, after which the medium was removed, the cells frozen and RNA extracted. This experiment was repeated 3 times.

The medium was assayed for progesterone and RNA was analysed for 11βHSD1, 11βHSD2, StAR, P450scc and 3βHSD by RNase protection assay as described in previous chapters.

2.2 Effects of RU486 on granulosa cell culture over time

3ng/ml rhLH or no treatment, with or without 10^{-6}M RU486, were added to rhFSH-primed granulosa cells in culture. These groups were stopped either after 6h or 12h. Control groups of FSH-primed or cells with no *in vitro* priming, received no hormone treatment and were stopped immediately (0h). The medium was removed, the cells frozen and RNA extracted. This experiment was repeated 3 times.

The medium was assayed for progesterone, and RNA was analysed for 11βHSD1, 11βHSD2, PR, StAR, P450scc and 3βHSD by RNase protection assay as described in previous chapters.

2.3 Effects of various concentrations of antiprogestins, RU486 and Org31710, on granulosa cell culture

3ng/ml rhLH and 10^{-7}M, 10^{-6}M or 10^{-5}M of RU486 or Org31710 was added to rhFSH primed granulosa cells in culture. Controls of 3ng/ml rhLH alone and no treatment to FSH-primed cells and no treatment to cells with no previous FSH *in vitro* treatment were also included in the study. Cells were incubated at 37°C for 12h, when the medium was removed, the cells frozen and RNA extracted. This experiment was repeated 3 times.

The medium was assayed for progesterone and RNA was analysed for 11βHSD1, 11βHSD2, StAR, P450scc and 3βHSD by RNase protection assay as described in previous chapters.

2.4 Effects of adding exogenous progesterone and glucocorticoid to granulosa cell cultures treated with RU486 and Org31710

3ng/ml rhLH was added to all groups except controls (no further treatment to FSH-primed granulosa cells or cells which received no FSH priming). In addition to LH, the treatments were; RU486 alone, RU486 with progesterone, RU486 with glucocorticoid, Org31710 alone, Org31710 with progesterone, Org31710 with glucocorticoid, progesterone alone or glucocorticoid alone. The concentration of all additional treatments was 10^{-6}M. Cells were incubated at 37°C for 12h, when the medium was removed, the cells frozen and RNA extracted. This experiment was repeated 3 times.

The medium was analysed for 11βHSD1 by RNase protection assay as described in previous chapters.

3. Results

3.1 Effects of RU486 on granulosa cell culture

Gonadotrophins were shown to up-regulate the expression of 11βHSD1, with rhLH for 12h, augmenting the effect of rhFSH-priming as shown in fig 6.2 (confirming results from chapter 5). 11βHSD2 gene expression could not be analysed due to the small amounts of RNA available for quantification. Cells receiving no gonadotrophin stimulation expressed little 11βHSD, but levels were increased 4-fold if rhFSH was added. Further treatment with rhFSH showed no significant rise, contrary to the effects of rhLH. With inclusion of RU486 in conjunction with rhLH in the culture, 11βHSD expression was seen to increase. However, this increase was not statistically significant. RU486 showed no effect on 11βHSD gene expression if rhLH was not present.

Figs 6.3 and 6.4 show that gene expression of StAR, P450scc and 3βHSD by rhFSH-primed rat granulosa cells increases with the consequent addition of gonadotrophins, rhFSH having a greater effect than rhLH. Granulosa cells with no gonadotrophin treatment showed very little StAR, P450scc and 3βHSD expression. RU486 reduced gene expression when administered concurrently with rhLH and increased P450scc and 3βHSD gene expression when added with rhFSH. RPAs to examine StAR, P450scc and 3βHSD were undertaken only once due to the shortage of RNA, therefore these results are not statistically significant.

Gonadotrophin is essential for progesterone synthesis as shown in fig 6.5. rhFSH priming enabled the cells to produce approximately 22pmol progesterone/10^6 cells and continued rhFSH stimulation lead to the synthesis of 73-86pmol/10^6 cells. rhLH increased this to approximately 135pmol/10^6 cells with RU486 having no statistically significant effect on any of these treatments.

3.2 Effects of RU486 on granulosa cell culture over time

Fig 6.6 and 6.7 show rhLH significantly increased 11βHSD1 gene expression after 6h (1.5-fold) and 12h (2.75-fold). The withdrawal of gonadotrophin stimulation lead to a drop in 11βHSD gene expression between 6h and 12h. RU486 did not significantly affect gene expression without continued gonadotrophin stimulation, but concurrent addition of rhLH and RU486 lead to a statistically significant increase after 6h and a non-significant increase after 12h. rhLH induced very little increase in StAR, P450scc

and 3βHSD gene expression after 6h and 12h as illustrated in figs 6.8 and 6.9. However the concurrent addition of RU486 and rhLH lead to a large decrease in StAR, P450scc and 3βHSD gene expression after 6h with little effect after 12h.

PR gene expression was induced after 6h with rhLH treatment, with no expression before that and only a very small amount (150-fold less) at 12h after rhLH addition, as demonstrated in figs 6.10 and 6.11. Concurrent addition of RU486 with rhLH showed no effect on PR gene expression.

Progesterone synthesis required FSH-priming, with little being produced without continuous gonadotrophin stimulation, as illustrated in fig 6.12. The greatest synthesis in progesterone (at approximately 150pmol/10^6 cells) was observed 6h after rhLH, dropping to approximately 105pmol/10^6 cells after 12h. At both these time points, the concurrent addition of RU486 and rhLH resulted in a statistically significant increase in progesterone synthesis.

3.3 Effects of various concentrations of antiprogestins, RU486 and Org31710 on granulosa cell culture

Figs 6.13 and 6.14 show all concentrations of RU486 and Org31710 increased 11βHSD1 gene expression. RNA quantities were too low to measure 11βHSD2 expression. 10^{-7}M RU486 increased 11βHSD1 expression 2.5-fold, 10^{-6}M 2-fold, and 10^{-5}M non-significantly, showing RU486 dose-dependency. Org31710 increased 11βHSD1 gene expression, but not in a dose-dependent fashion.

Figs 6.15 and 6.16 show all concentrations of RU486 and Org31710 decreased gene expression of StAR, P450scc and 3βHSD, with the exception of RU486 at 10^{-7}M. StAR, P450scc and 3βHSD expression decreased with increasing Org31710 concentrations.

Progesterone synthesis increased with all RU486 and Org31710 concentrations, with no significant difference between concentrations of each antiprogestin, as demonstrated in fig 6.17. However, RU486 at 10^{-5}M led to the synthesis of statistically more progesterone than Org31710 at the same concentration.

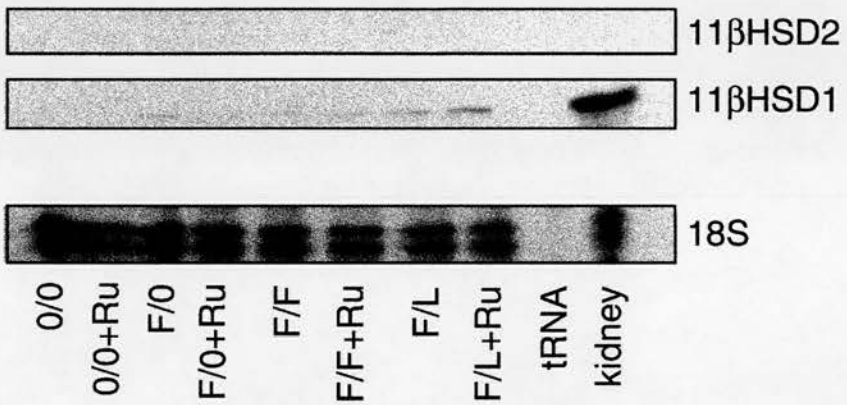


Fig 6.1 RNase protection assay showing the gene expression of 11βHSD1 and 11βHSD2 in cultured granulosa cells. Control groups (0/0) received no gonadotrophin-priming for 48h and no further gonadotrophin treatment. All other groups were treated with 1ng/ml rhFSH for 48h. All groups were washed with DPBS. Medium containing either no gonadotrophin (F/0), 1ng/ml rhFSH (F/F) or 3ng/ml rhLH (F/L) was added to each well. To each of these, RU486 (10^{-6}M) or equivalent volume of ethanol carrier was added. Kidney and tRNA were used as positive and negative controls, respectively. The intensity of each band was quantified by electronic autoradiography and then exposed to a Kodak X-Omat AR-5 autoradiogram for 1 day (18S), 5 days (11βHSD1) or 14 days (11βHSD2) at -70°C using an intensifying screen.

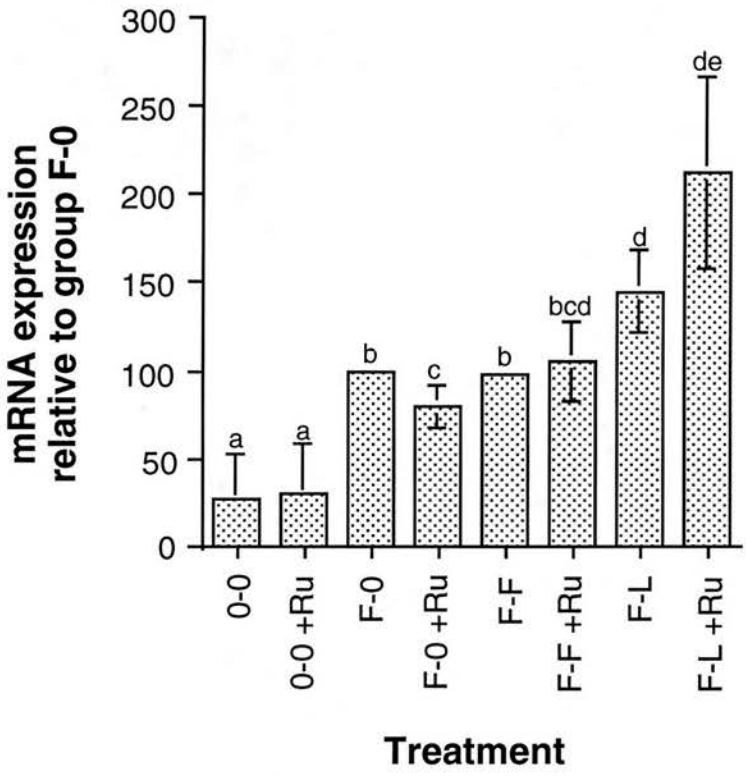


Fig 6.2 Graph representing gene expression of 11βHSD1 in cultured granulosa cells from RPA in fig. 6.1 (11βHSD2 gene expression was negligible). Control groups (0-0) received no gonadotrophin-priming for 48h and no further gonadotrophin treatment. All other groups were treated with 1ng/ml rhFSH for 48h. All groups were washed with DPBS. Medium containing either no gonadotrophin (F-0), 1ng/ml rhFSH (F-F) or 3ng/ml rhLH (F-L) was added to each well. To each of these RU486 (10^{-6}M) or equivalent volume of ethanol carrier was added. Each sample was standardised against 18S and the control sample was assigned an arbitrary value of 100%. Values represent the mean (±SE) from triplicate experiments. Histograms with different superscripts are significantly different from one another ($p<0.05$).

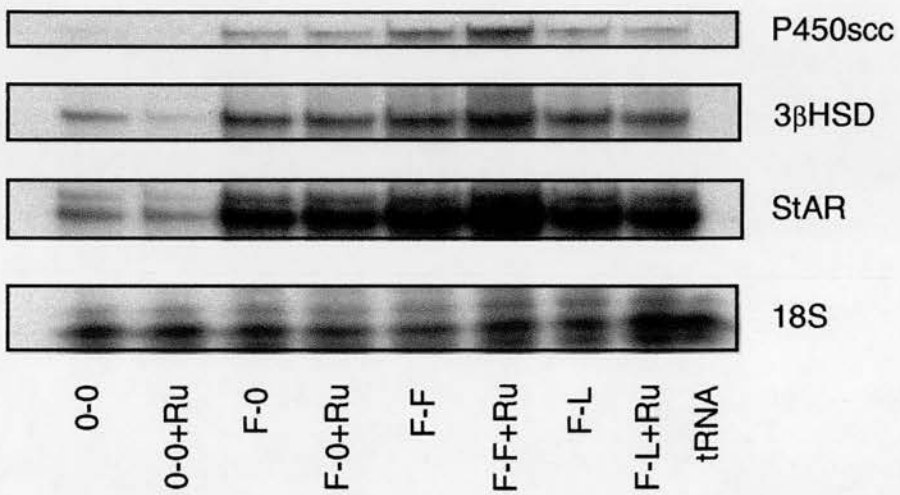


Fig 6.3 RNase protection assay showing the gene expression of StAR, P450scc and 3βHSD in cultured granulosa cells. Control groups (0-0) received no gonadotrophin-priming for 48h and no further gonadotrophin treatment. All other groups were treated with 1ng/ml rhFSH for 48h. All groups were washed with DPBS. Medium containing either no gonadotrophin (F-0), 1ng/ml rhFSH (F/F) or 3ng/ml rhLH (F-L) was added to each well. To each of these RU486 (10^{-6}M) or equivalent volume of ethanol carrier was added. tRNA were used as a negative control. The intensity of each band was quantified by electronic autoradiography and then exposed to a Kodak X-Omat AR-5 autoradiogram for 2 days at -70°C using an intensifying screen.

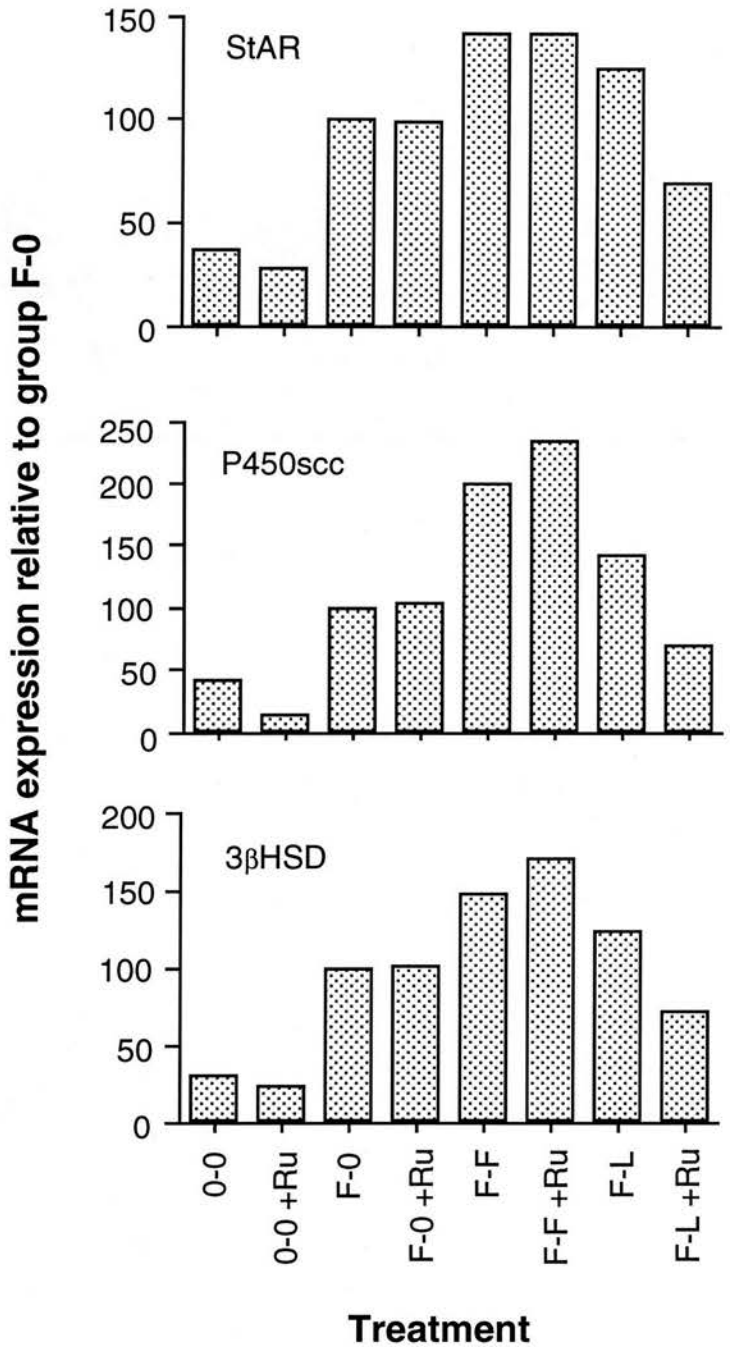


Fig 6.4 Graphs representing gene expression of StAR, P450scc and 3βHSD in cultured granulosa cells. Control groups (0-0) received no gonadotrophin-priming for 48h and no further gonadotrophin treatment. All other groups were treated with 1ng/ml rhFSH for 48h. All groups were washed with DPBS. Medium containing either no gonadotrophin (F-0), 1ng/ml rhFSH (F-F) or 3ng/ml rhLH (F-L) was added to each well. To each of these RU486 (10^{-6}M) or equivalent volume of ethanol carrier was added. Each sample was standardised against 18S and the F-0 sample was assigned an arbitrary value of 100%.

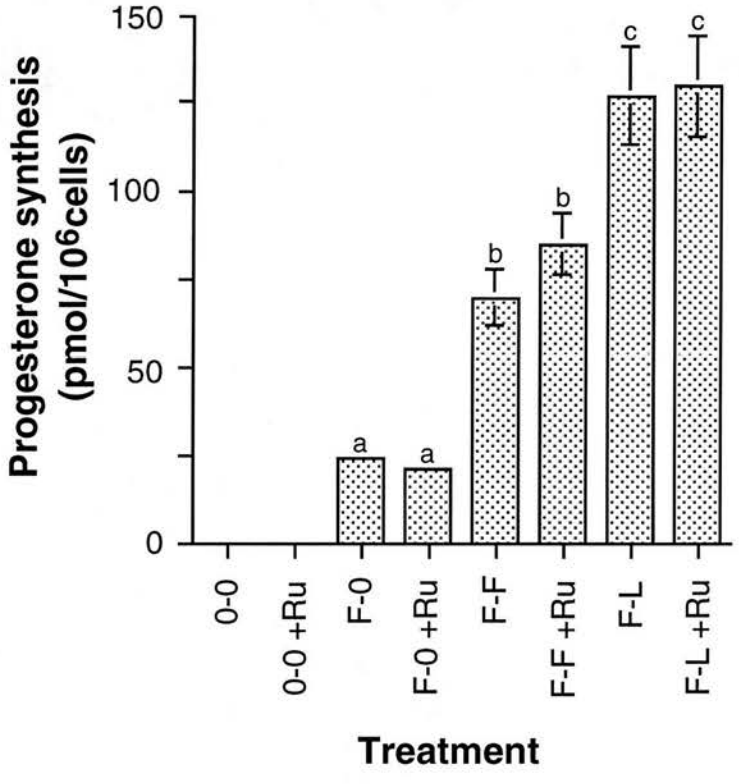


Fig 6.5 Graph representing progesterone synthesis in cultured granulosa cells. Control groups (0-0) received no gonadotrophin-priming for 48h and no further gonadotrophin treatment. All other groups were treated with 1ng/ml rhFSH for 48h. All groups were washed with DPBS. Medium containing either no gonadotrophin (F-0), 1ng/ml rhFSH (F-F) or 3ng/ml rhLH (F-L) was added to each well. To each of these RU486 (10^{-6}M) or equivalent volume of ethanol carrier was added. Values represent the mean (±SE) from triplicate experiments. Histograms with different superscripts are significantly different from one another ($p<0.05$).

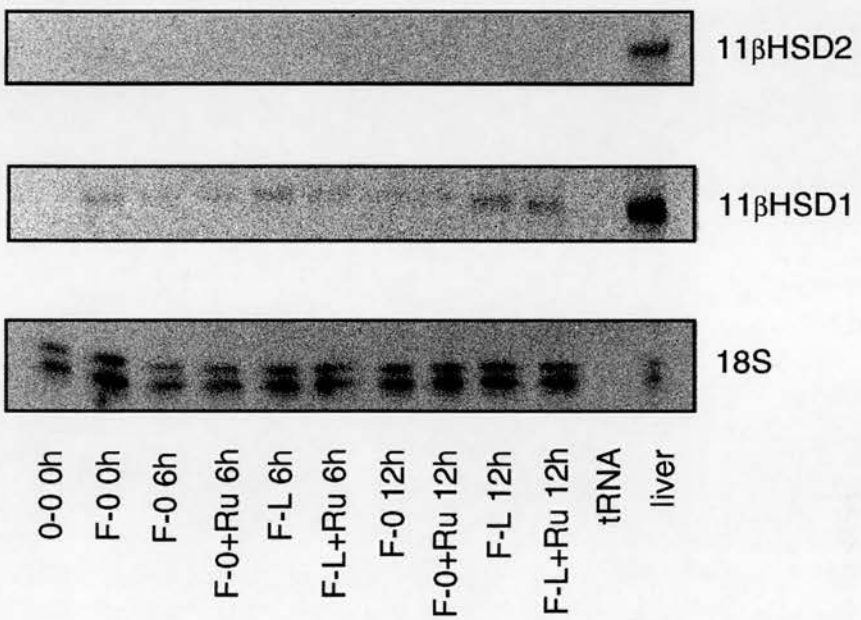


Fig 6.6 RNase protection assay showing the gene expression of 11βHSD1 and 11βHSD2 in cultured granulosa cells. All groups (except 0-0) were treated with 1ng/ml rhFSH for 48h and were washed with DPBS. Medium containing either no gonadotrophin (F-0) or 3ng/ml rhLH (F-L) was added to each well. To each of these RU486 (10^{-6}M) or equivalent volume of ethanol carrier was added. The cultures were stopped at 0h, 6h or 12h after addition of the second treatment. Liver and tRNA were used as a positive and negative controls, respectively. The intensity of each band was quantified by electronic autoradiography and then exposed to a Kodak X-Omat AR-5 autoradiogram for 1 day (18S), 5 days (11βHSD1) or 14 days (11βHSD2) at -70°C using an intensifying screen.

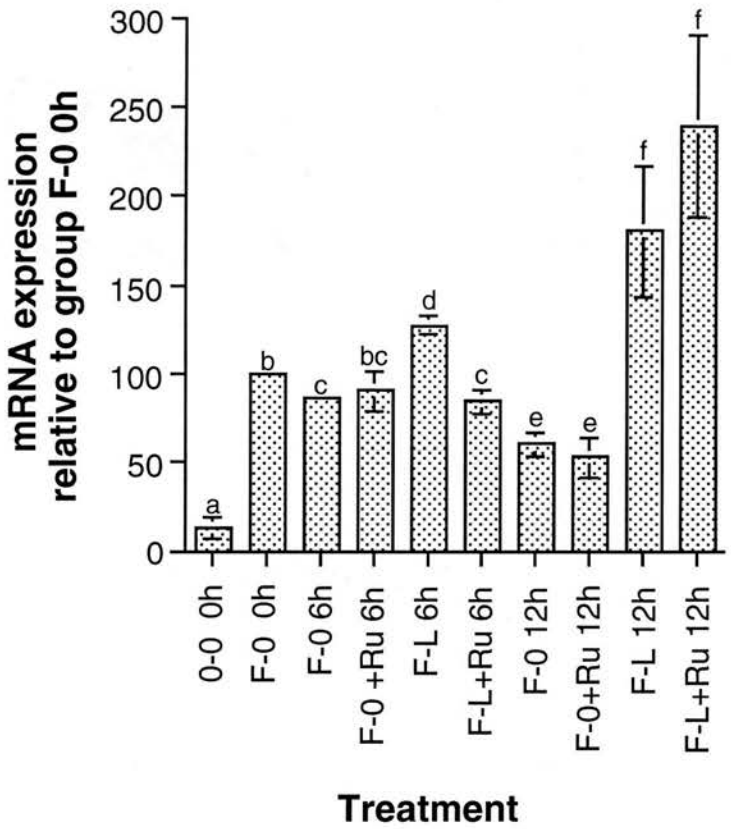


Fig 6.7 Graph representing gene expression of 11βHSD1 in cultured granulosa cells from RPA in fig. 6.1 (11βHSD2 gene expression was negligible). All groups (except 0-0) were treated with 1ng/ml rhFSH for 48h and were washed with DPBS. Medium containing either no gonadotrophin (F-0) or 3ng/ml rhLH (F-L) was added to each well. To each of these RU486 (10^{-6}M) or equivalent volume of ethanol carrier was added. The cultures were stopped at 0h, 6h or 12h after addition of the second treatment. Each sample was standardised against 18S and the F-0 0h sample was assigned an arbitrary value of 100%. Values represent the mean (±SE) from triplicate experiments. Histograms with different superscripts are significantly different from one another ($p<0.05$).

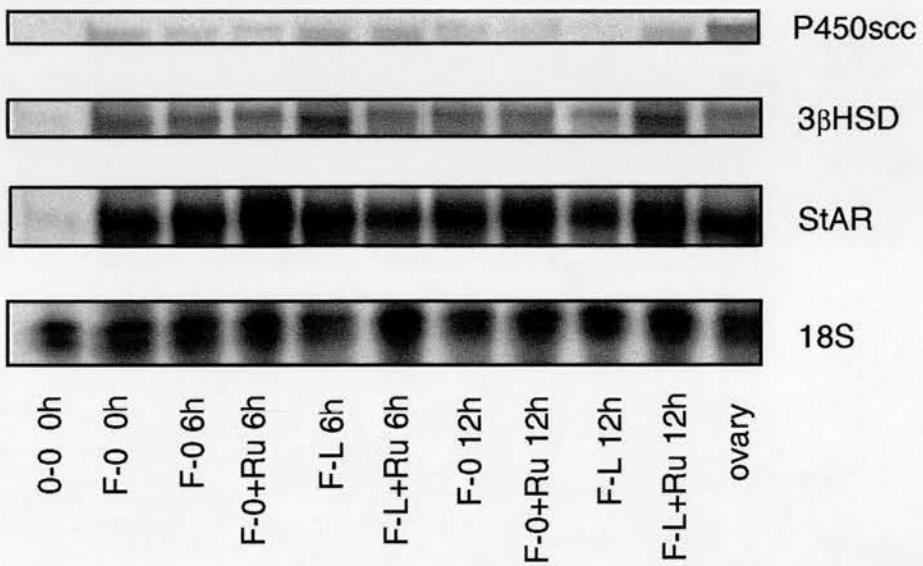


Fig 6.8 RNase protection assay showing the gene expression of StAR, P450scc and 3βHSD in cultured granulosa cells. All groups (except 0-0) were treated with 1ng/ml rhFSH for 48h and were washed with DPBS. Medium containing either no gonadotrophin (F-0) or 3ng/ml rhLH (F-L) was added to each well. To each of these RU486 (10^{-6}M) or equivalent volume of ethanol carrier was added. The cultures were stopped at 0h, 6h or 12h after addition of the second treatment. Ovary was used as a positive control. The intensity of each band was quantified by electronic autoradiography and then exposed to a Kodak X-Omat AR-5 autoradiogram for 2 days at -70°C using an intensifying screen.

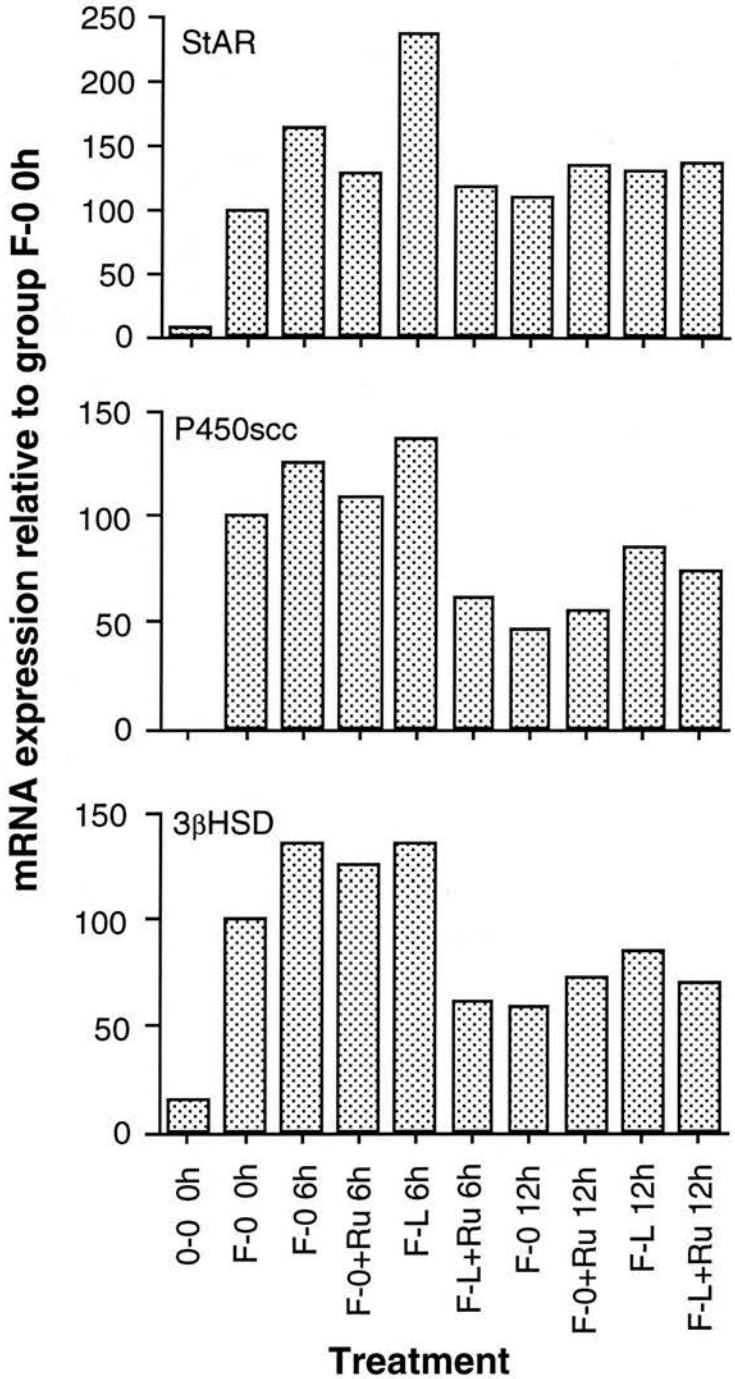


Fig 6.9 Graphs representing gene expression of StAR, P450scc and 3βHSD in cultured granulosa cells. All groups (except 0-0) were treated with 1ng/ml rhFSH for 48h and were washed with DPBS. Medium containing either no gonadotrophin (F-0) or 3ng/ml rhLH (F-L) was added to each well. To each of these RU486 (10^{-6}M) or equivalent volume of ethanol carrier was added. The cultures were stopped at 0h, 6h or 12h after addition of the second treatment. Each sample was standardised against 18S and the F-0 0h group was assigned an arbitrary value of 100%.

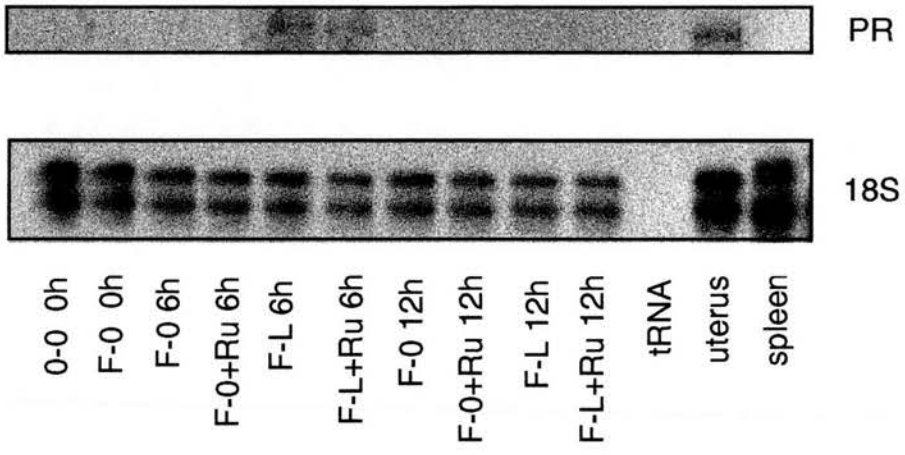


Fig 6.10 RNase protection assay showing the gene expression of total PR in cultured granulosa cells. All groups (except 0-0) were treated with 1ng/ml rhFSH for 48h and were washed with DPBS. Medium containing either no gonadotrophin (F-0) or 3ng/ml rhLH (F-L) was added to each well. To each of these RU486 (10^{-6}M) or equivalent volume of ethanol carrier was added. The cultures were stopped at 0h, 6h or 12h after addition of the second treatment. Uterus was used as a positive control and tRNA and spleen were used as negative controls. The intensity of each band was quantified by electronic autoradiography and then exposed to a Kodak X-Omat AR-5 autoradiogram for 1 day (18S) and 5 days (PR) at -70°C using an intensifying screen.

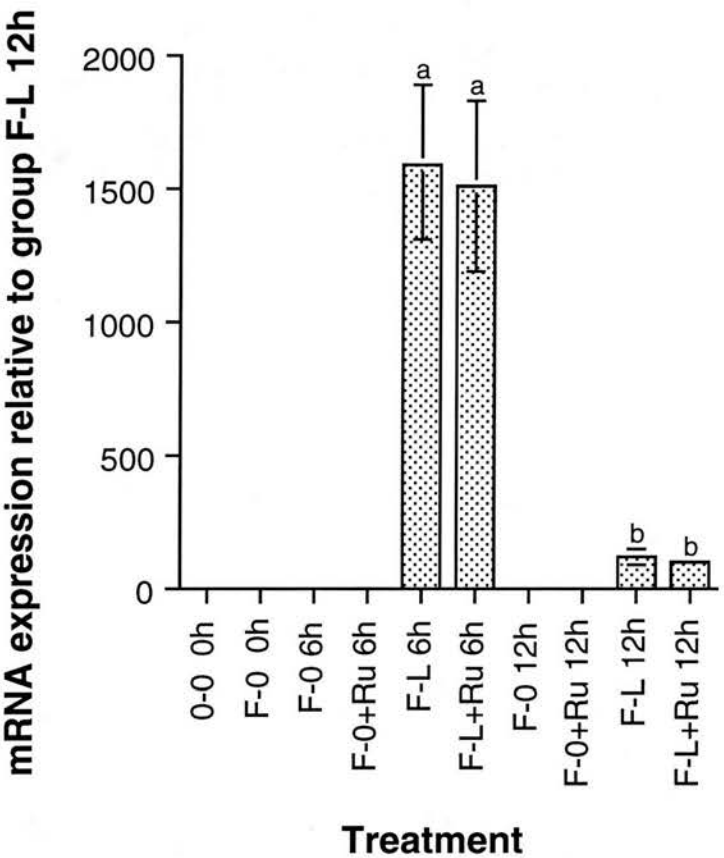


Fig 6.11 Graph representing gene expression of PR in cultured granulosa cells from RPA in fig. 6.10. All groups (except 0-0) were treated with 1ng/ml rhFSH for 48h and were washed with DPBS. Medium containing either no gonadotrophin (F-0) or 3ng/ml rhLH (F-L) was added to each well. To each of these RU486 (10^{-6}M) or equivalent volume of ethanol carrier was added. The cultures were stopped at 0h, 6h or 12h after addition of the second treatment. Each sample was standardised against 18S and the F-L 12h sample was assigned an arbitrary value of 100%. Values represent the mean (±SE) from triplicate experiments. Histograms with different superscripts are significantly different from one another ($p<0.05$).

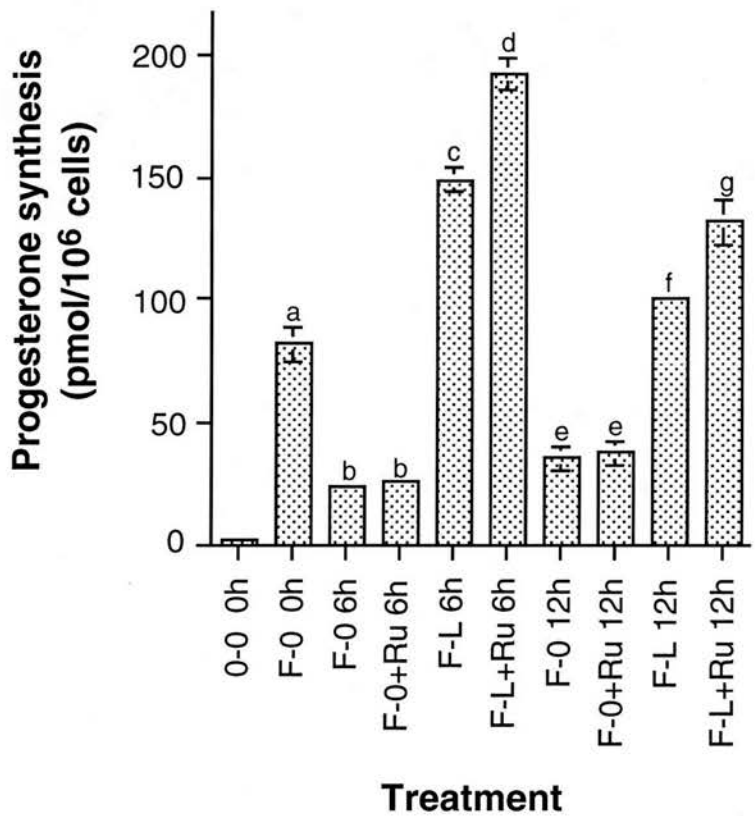


Fig 6.12 Graph representing progesterone synthesis by cultured granulosa cells. All groups (except 0-0) were treated with 1ng/ml rhFSH for 48h and were washed with DPBS. Medium containing either no gonadotrophin (F-0) or 3ng/ml rhLH (F-L) was added to each well. To each of these RU486 (10^{-6}M) or equivalent volume of ethanol carrier was added. The cultures were stopped at 0h, 6h or 12h after addition of the second treatment. Values represent the mean (±SE) from triplicate experiments. Histograms with different superscripts are significantly different from one another ($p<0.05$).

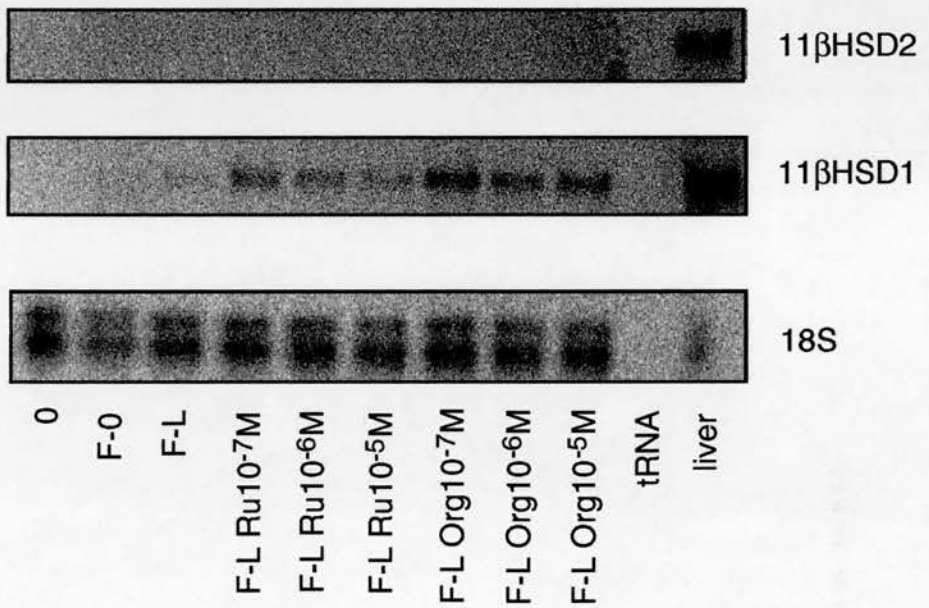


Fig 6.13 RNase protection assay showing the gene expression of 11βHSD1 and 11βHSD2 in cultured granulosa cells. Control group (0-0) received no gonadotrophins for 48h. All other groups were treated with 1ng/ml rhFSH for 48h. All groups were washed with DPBS and medium containing either no gonadotrophin (F-0) or 3ng/ml rhLH was added to the cells. Increasing concentrations of RU486 or Org31710 were added to the LH treatments. Cultures were stopped 12h after the second treatment was added. Liver and tRNA were used as positive and negative controls, respectively. The intensity of each band was quantified by electronic autoradiography and then exposed to a Kodak X-Omat AR-5 autoradiogram for 1 day (18S), 5 days (11βHSD1) or 14 days (11βHSD2) at -70°C using an intensifying screen.

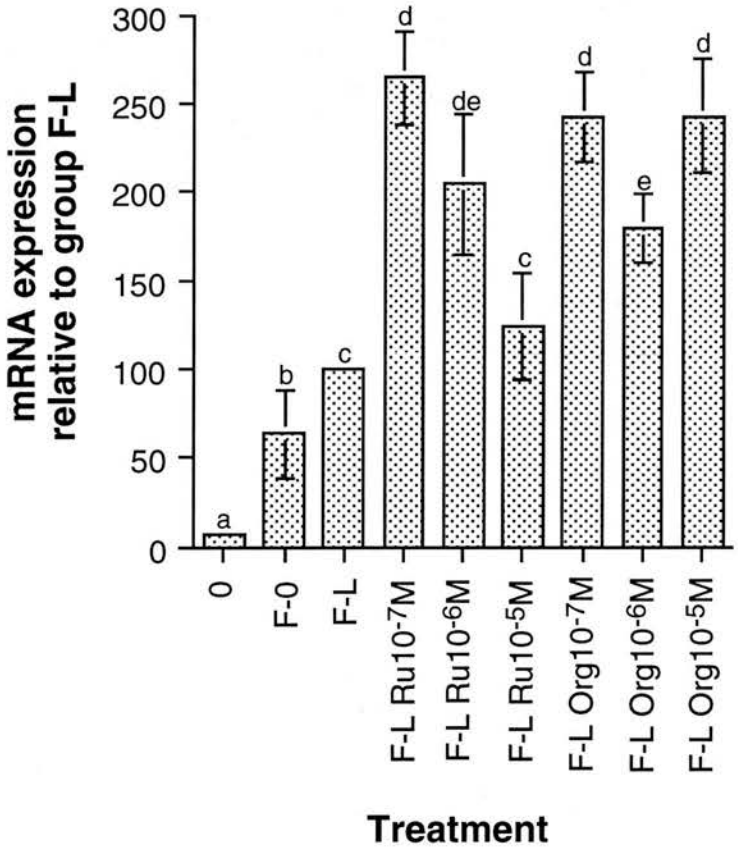


Fig 6.14 Graph representing gene expression of 11βHSD1 in cultured granulosa cells from RPA in fig. 6.1 (11βHSD2 gene expression was negligible). Control group (0-0) received no gonadotrophins for 48h. All other groups were treated with 1ng/ml rhFSH for 48h. All groups were washed with DPBS and medium containing either no gonadotrophin (F-0) or 3ng/ml rhLH was added to the cells. Increasing concentrations of RU486 or Org31710 were added to the LH treatments. Cultures were stopped 12h after the second treatment was added. Each sample was standardised against 18S and the F-L sample was assigned an arbitrary value of 100%. Values represent the mean (±SE) from triplicate experiments. Histograms with different superscripts are significantly different from one another ($p<0.05$).

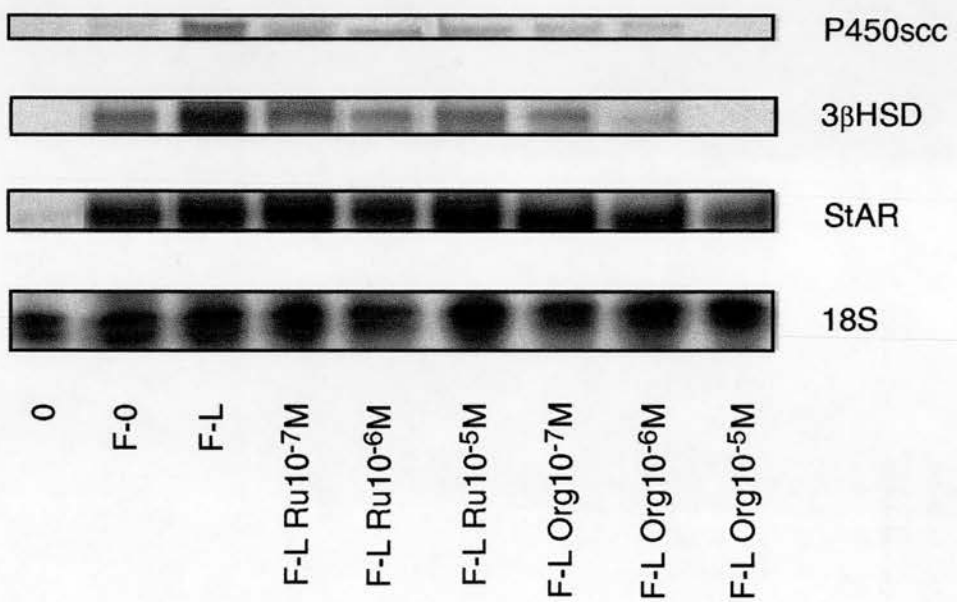


Fig 6.15 RNase protection assay showing the gene expression of StAR, P450scc and 3βHSD in cultured granulosa cells. Control group (0-0) received no gonadotrophins for 48h. All other groups were treated with 1ng/ml rhFSH for 48h. All groups were washed with DPBS and medium containing either no gonadotrophin (F-0) or 3ng/ml rhLH was added to the cells. Increasing concentrations of RU486 or Org31710 were added to the LH treatments. Cultures were stopped 12h after the second treatment was added. The intensity of each band was quantified using an instant imager and then exposed to a Kodak X-Omat AR-5 autoradiogram for 1 day (18S) or 2 days (StAR, P450scc and 3βHSD) at -70°C using an intensifying screen.

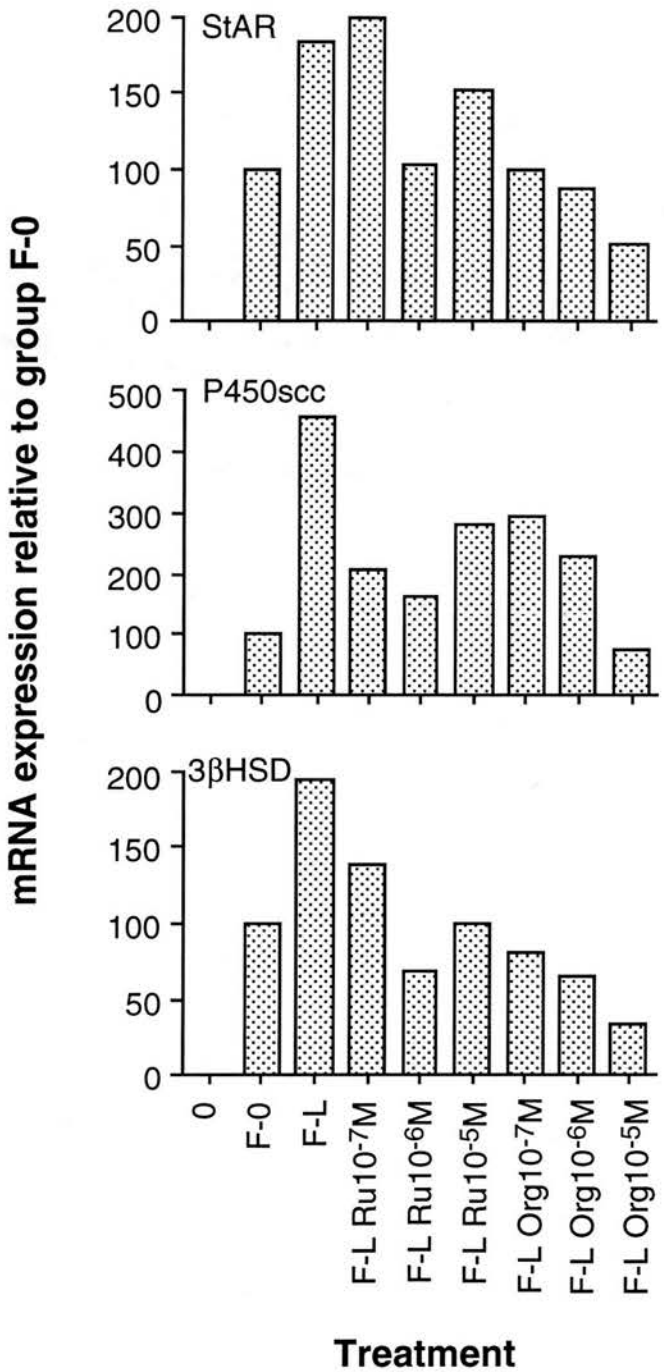


Fig 6.16 Graphs representing gene expression of StAR, P450scc and 3βHSD in cultured granulosa cells. Control group (0-0) received no gonadotrophins for 48h. All other groups were treated with 1ng/ml rhFSH for 48h. All groups were washed with DPBS and medium containing either no gonadotrophin (F-0) or 3ng/ml rhLH was added to the cells. Increasing concentrations of RU486 or Org31710 were added to the LH treatments. Cultures were stopped 12h after the second treatment was added. Each sample was standardised against 18S and the F-0 sample was assigned an arbitrary value of 100%.

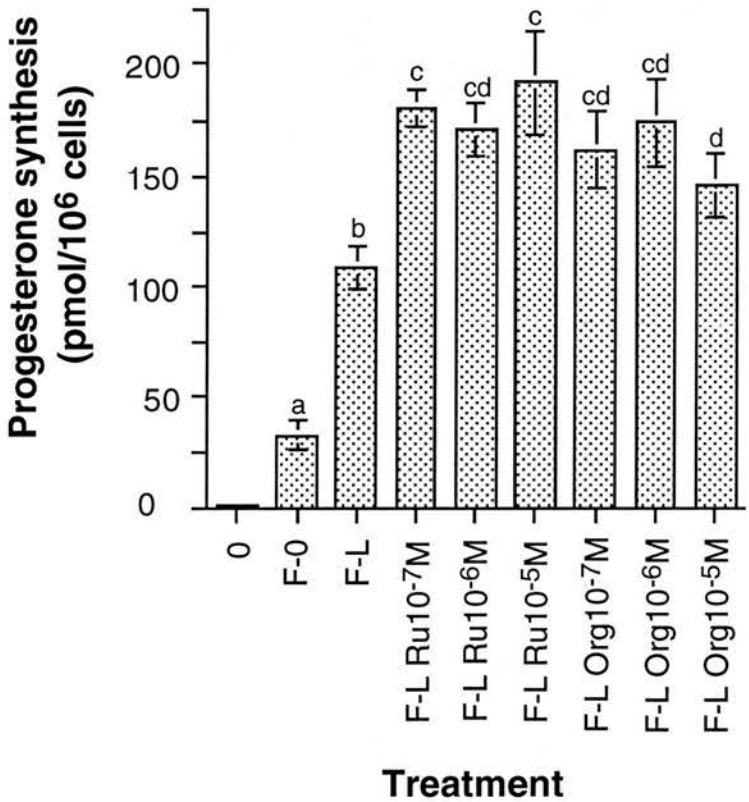


Fig 6.17 Graph representing progesterone synthesis by cultured granulosa cells. Control group (0-0) received no gonadotrophins for 48h. All other groups were treated with 1ng/ml rhFSH for 48h. All groups were washed with DPBS and medium containing either no gonadotrophin (F-0) or 3ng/ml rhLH was added to the cells. Increasing concentrations of RU486 or Org31710 were added to the LH treatments. Cultures were stopped 12h after the second treatment was added. Values represent the mean (±SE) from triplicate experiments. Histograms with different superscripts are significantly different from one another ($p<0.05$).

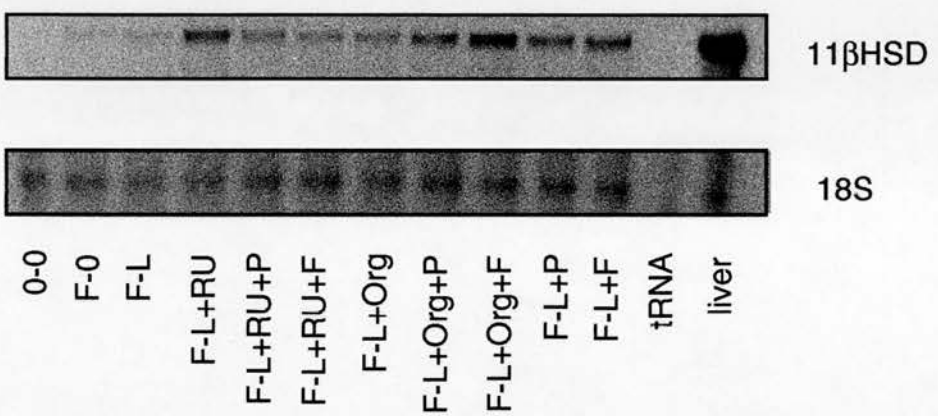


Fig 6.18 RNase protection assay showing the gene expression of 11βHSD1 in cultured granulosa cells. Control group (0-0) received no gonadotrophins for 48h. All other groups were treated with 1ng/ml rhFSH for 48h. All groups were washed with DPBS and medium containing either no gonadotrophin (0-0 and F-0) or 3ng/ml rhLH (F-L) was added to the cells. RU486 (10^{-6}M) alone or in the presence of progesterone (Ru+P) or glucocorticoid (Ru+G), or Org31710 (10^{-6}M) or in the presence of progesterone (Org+P) or glucocorticoid (Org+G) were added to the LH treatments. Groups of rhLH with progesterone or glucocorticoid alone were also included. Steroid concentration was 10^{-6}M. Cultures were stopped 12h after the second treatment was added. Liver and tRNA were used as positive and negative controls, respectively. The intensity of each band was quantified using an instant imager and then exposed to a Kodak X-Omat AR-5 autoradiogram for 1 day (18S) or 5 days (11βHSD1) at -70°C using an intensifying screen.

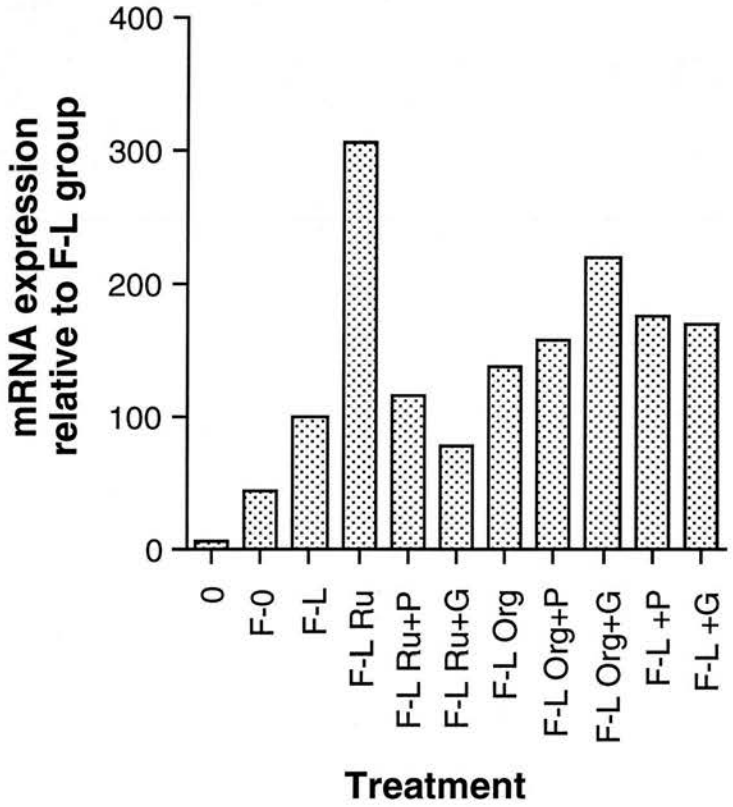


Fig 6.19 Graph representing gene expression of 11βHSD1 in cultured granulosa cells from RPA in fig. 6.18. Control group (0-0) received no gonadotrophins for 48h. All other groups were treated with 1ng/ml rhFSH for 48h. All groups were washed with DPBS and medium containing either no gonadotrophin (0-0 and F-0) or 3ng/ml rhLH (F-L) was added to the cells. RU486 (10^{-6}M) alone or in the presence of progesterone (Ru+P) or glucocorticoid (Ru+G), or Org31710 (10^{-6}M) or in the presence of progesterone (Org+P) or glucocorticoid (Org+G) were added to the LH treatments. Groups of rhLH with progesterone or glucocorticoid alone were also included. Steroid concentration was 10^{-6}M. Cultures were stopped 12h after the second treatment was added. Each sample was standardised against 18S and the F-L sample was assigned an arbitrary value of 100%. Values represent the mean from duplicate experiments.

3.4 Effects of adding exogenous progesterone and glucocorticoid to granulosa cell cultures treated with RU486 and Org31710

Fig 6.19 shows that concurrent addition of rhLH and RU486 to rhFSH-primed granulosa cells increased 11βHSD1 gene expression 3-fold over rhLH treatment alone. This effect was overcome if progesterone or glucocorticoid was added to the culture. Org31710 treatment showed a smaller increase (1.6-fold) in 11βHSD1 expression. Progesterone and glucocorticoid served to further increase this effect. The concurrent addition of rhLH and progesterone or rhLH and glucocorticoid both lead to increases in 11βHSD expression of approximately 1.75-fold above rhLH treatment alone. RNA recovery was too low to allow quantification of 11βHSD2, StAR, P450scc and 3βHSD. This RPA was repeated only twice therefore these results are not staistically significant.

4. Discussion

RU486 and Org31710 affect gene expression of 11βHSD1, StAR, P450scc and 3βHSD and the synthesis of progesterone. RU486 and Org31710 are anti-progestins and RU486 is also known to bind to GR. The enzymes and proteins under anti-progestagenic control activate glucocorticoid and synthesise progesterone. Therefore, progesterone and glucocorticoid are effecting their own synthesis/activation through either PR or GR.

RU486 in conjunction with rhLH was shown to significantly decrease 11βHSD1 gene expression, decrease StAR, P450scc and 3βHSD and significantly increase progesterone synthesis after 6h. RU486 had no effect on total PR expression. However, 12h after the addition of rhLH and RU486, 11βHSD1 gene expression significantly increased, StAR, P450scc and 3βHSD expression decreased and progesterone synthesis increased with the addition of RU486. These results may be physiologically relevant to ovulation which occurs in the rat at approximately 10-12h after the LH surge. RU486 is known to halt follicle rupture and it may be that RU486 acts within the ovary, at least to an extent, by activating glucocorticoid through up-regulating 11βHSD1. Ovulation has been likened to an inflammatory response, the mechanism of follicle rupture being similar to inflammation, and the rebuilding of the follicle wall after ovulation being anti-inflammatory. Glucocorticoids, in excess, have been shown to decrease expression of the inflammatory mediators IL-1 (Knudsen *et al.*, 1987), nuclear factor kappa B (NFκB) (Mukaida *et al.*, 1994) and possibly COX-2 (O'Banion *et al.*, 1992). It may be that this RU486-dependent increase in

glucocorticoid acts to reduce the periovulatory pro-inflammatory response induced by such cytokines and consequently inhibits ovulation. However, glucocorticoid's effect cannot occur through GR or PR since RU486 is blocking receptor sites.

The regulation of gene expression with RU486 and Org31710 in the presence of LH, indicates how activation of either GR or PR affects granulosa cells in culture and suggests how these anti-progestins act within the ovary *in vivo*. StAR, P450scc and 3βHSD gene expression showed a dose-dependent response to Org31710 in the presence of rhLH. Gene expression of StAR, P450scc and 3βHSD dose-dependently decreased with the addition of increasing concentrations of Org31710. 11βHSD1 gene expression showed no correlation to Org31710 concentration. However a dose-dependent relationship was seen with RU486 in the presence of rhLH. In contrast to effects of Org31710 on the steroidogenic enzymes, RU486 increased 11βHSD1 expression, the effect decreasing with increased concentrations of RU486. These results suggest Org31710, which binds very specifically to PR, is acting to regulate expression of genes responsible for the synthesis of progesterone, i.e. progesterone regulates its own synthesis through PR. Progesterone concentrations do not reflect this response in enzyme expression. Progesterone increases with the addition of RU486 and Org31710, but different concentrations do not alter the overall progesterone concentration in the culture. RU486 prevents an increase in 20αHSD activity that normally occurs in rat luteal cells *in vitro* (Uilenbroek *et al.*, 1992), an effect that may also occur with Org31710. This increase in progesterone is in contrast to the human where short term cultures of periovulatory human granulosa-lutein cells showed a decrease in progesterone production in response to RU486 (Dimattina *et al.*, 1986). RU486 increased 11βHSD1 gene expression, and showed a dose-dependent decrease with increased concentrations of RU486. This suggests RU486 is binding to GR in this case, since a different effect was observed with the PR-specific Org31710. This up-regulation of 11βHSD1 expression would lead to an expected increase in glucocorticoid in its active state. Glucocorticoids act on luteal cells to decrease 20αHSD mRNA (Albarracin *et al.*, 1994) and activity (Adashi *et al.*, 1981). This probably occurs directly through GR since the 20αHSD promoter region contains a glucocorticoid response element (GRE) (Zhong *et al.*, 1996). This idea is digramatically represented in fig. 6.20.

The dose-dependency of RU486's effect on 11βHSD1 shows an unusual pattern. The lowest RU486 concentration induced the greatest gene induction, with higher RU486

concentrations being less effective. Indeed, the highest concentration of 10^{-5}M had no significant effect on 11βHSD1 gene expression over rhLH treatment alone. This could be due to a biphasic response, with high concentrations of RU486 having a negative effect. Alternatively, RU486 may bind preferentially to PR (exerting a stimulatory effect), and as RU486 concentrations rise so that RU486 binds to the GR (exerting an inhibitory effect). In this experiment, the anti-progestin Org31710 (binding specifically to PR) induced only a small rise in 11βHSD1 gene expression. Thus inhibition of GR activation by high concentrations of RU486, no longer induces 11βHSD1 expression.

Further investigations have shed some light on this situation. RU486's ability to increase expression of 11βHSD1 was reversed by adding exogenous progesterone or glucocorticoid, with little difference observed between the effect of either steroid. The increase in 11βHSD1 expression induced with Org31710 was not as great as that induced by RU486, but the addition of neither progesterone nor glucocorticoid could reverse the effect. It should be noted that because this analysis was only undertaken twice, the results are not statistically significant, therefore the experiment should be repeated in order to confirm this finding. These results show that both progesterone and glucocorticoid can reverse the effect of RU486 on 11βHSD expression, presumably by competing with RU486 for either GR or PR. Since progesterone and glucocorticoid bind to each other's receptors, these results do not clarify to which receptor RU486 predominantly binds. Statistically significant results with the Org31710 treated cultures may indicate progesterone and PR involvement in glucocorticoid activation.

Progesterone and glucocorticoid play a role in the regulation of 11βHSD1. Both steroids, in the presence of rhLH, statistically increase 11βHSD gene expression. Therefore, it may be that glucocorticoid up-regulates its own synthesis, and progesterone (acting through either PR or GR) up-regulates glucocorticoid activation (see fig 6.20).

Progesterone synthesis was also measured in the experiments involving the addition of exogenous progesterone and glucocorticoid (results not shown). The addition of glucocorticoid induced no significant difference in progesterone measured. However, with the addition of exogenous progesterone, the steroid concentration measured indicated that progesterone was rapidly metabolised.

Regulation of gene expression can also be manifested through receptor expression. GR expression was not studied in these experiments, and no reports of GR expression regulation in the presence of RU486 have been documented. Because PR is not expressed without LH stimulation (Park and Mayo, 1991), any action RU486 has on rhFSH-treated cells must be occurring through blocking of GR, which is constitutively expressed and not under gonadotrophic control (Tetsuka *et al.*, 1999). rhFSH plus RU486 treatment led to a small increase in P450scc and 3βHSD, a non-significant increase in progesterone concentration and no change in 11βHSD1 expression. All StAR, P450scc and 3βHSD RPAs were undertaken only once due to lack of RNA and problems encountered with RPA methodology. This was a problem experienced in many of these experiments and will be discussed further in chapter 8.

PR was shown to be expressed transiently 6h after addition of rhLH, but expression decreased to approximately 1/150 of those levels of expression after 12h. Addition of RU486 caused no significant change in expression confirming the observations of Natraj and Richards (1993). The method of mRNA quantification measures both PRA and PRB isoforms, the expression of which may be differentially regulated by RU486 and Org31710. PRA protein concentration is three times greater than PRB in cultured rat granulosa cells (Natraj and Richards, 1993). When both isoforms are present, PRA is the dominant phenotype and actions of PRB can be overcome in the presence of PRA (Tung *et al.*, 1993). Human PRB bound to RU486 can inappropriately activate transcription, without DNA binding (progesterone response element [PRE]), in the presence of cAMP (Tung *et al.*, 1993). It may be that in this culture system anti-progestins induce a higher ratio of PRB:PRA, and in the presence of rhLH-induced cAMP synthesis, PRB stimulates 11βHSD1 expression, despite the lack of a PRE in the up-stream region of the 11βHSD1 gene.

From these results it can be concluded that progesterone and glucocorticoid are closely linked through their receptors and the enzymes involved in their synthesis, activation and metabolism. Progesterone is essential for ovulation and luteinisation and RU486 and other anti-progestins are known to antagonise this. Progesterone, acting via PR expressed before ovulation, increases collagen degradation, stimulates proteolysis and increases the distensibility of the follicle wall - all mechanisms facilitating follicle rupture (for review see Curry Jr and Nothnick, 1996). RU486 inhibits this proteolytic activity (Iwamasa *et al.*, 1992). In contrast, activation of GR decreases the activity of collagenase and metalloproteases (for review see Cato and Wade, 1996). These

mechanisms contribute to a complex system of gene regulation allowing the tight co-ordination of follicle rupture and repair.

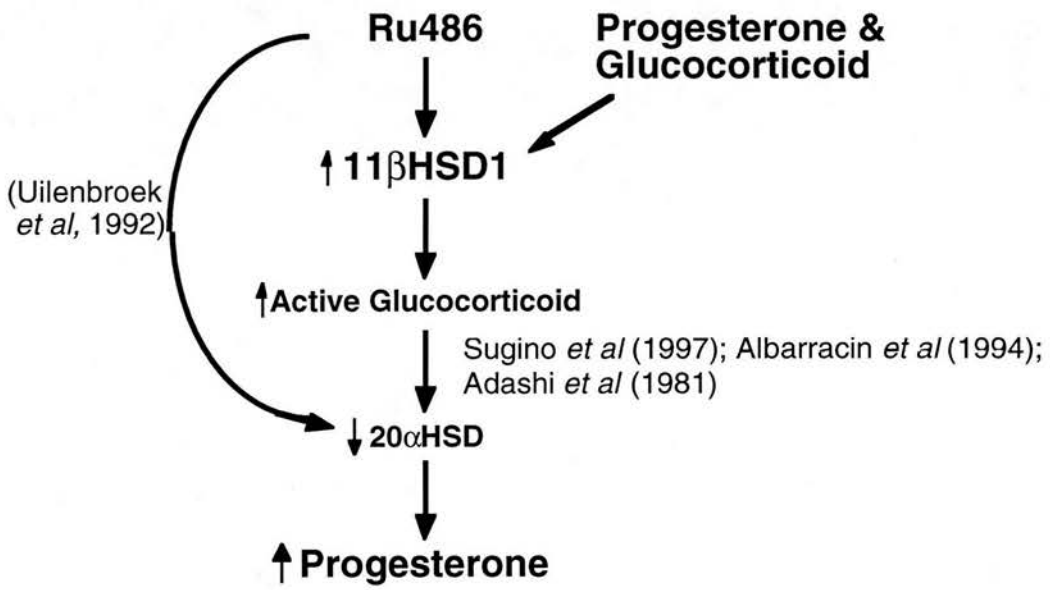


Fig 6.20 Proposed scheme of action in granulosa-lutein cells.

Chapter 7 Non Genomic Progesterone Receptor

1 Introduction

The existence of a genomic PR has been displayed in rat granulosa cells approximately 6h after the LH surge (Park and Mayo, 1991; Natraj and Richards, 1993) and is essential for ovulation and luteinisation (Tanaka *et al.*, 1993; Natraj and Richards, 1993). However, no classic, genomic PR has been identified in the rat ovary at any other time of the cycle, contrary to findings of other species (Park-Sarge *et al.*, 1995). This thesis has demonstrated progesterone affects the regulation of gene expression in granulosa cells, suggesting an autocrine response. However, the lack of PR in the rat corpus luteum, when progesterone synthesis is at its highest, introduces the question of progesterone synthesis control at this time. Progesterone is known to act through GR in the corpus luteum and bring about local regulation in 20αHSD expression (Sugino *et al.*, 1997), but this may not be the only protein involved in the reception of progesterone.

Non-genomic receptors (NGRs) for steroids including oestrogen, androgen and progesterone have been identified in reproductive tissue (for review see Revelli *et al.*, 1998). Activation of these membrane-bound progesterone receptors (NGPRs) can cause an influx of extracellular Ca^{2+} (Blackmore *et al.*, 1990), a decrease in cAMP concentration (Maller *et al.*, 1979) and membrane-bound adenylate cyclase activity, and regulation of the activity of plasma membrane enzymes (Revelli *et al.*, 1998). These responses are too rapid to be explained by genomic receptors (5-60sec) (Machelon *et al.*, 1996). Non-genomic progesterone receptors are thought to be present in oocytes, spermatozoa, granulosa cells and non-reproductive tissues such as brain (Caldwell *et al.*, 1995), intestinal smooth muscle (Bielefeldt *et al.*, 1996) and liver (Meyer *et al.*, 1996) in a variety of species. Due to the rapid response exhibited by NGPR activation, the physiological relevance may be very different to that of the genomic receptor which is known to activate transcription of target genes over a longer period of time. For example, activation of NGPR may acutely influence the protein kinase A pathway.

Specific binding sites for exogenous [^{3}H]-progesterone have been identified in luteal membranes of the human (Bramley and Menzies, 1988a), porcine (Bramley and Menzies, 1988b & 1993; Machelon *et al.*, 1996), bovine (Rae *et al.*, 1998b), ovine

(Bramley and Menzies, 1988c) and equine (Bramley *et al.*, 1995) corpus luteum. These binding sites are highly specific for progesterone and are not blocked by antagonists (RU486) of the classic genomic PR (Rae *et al.*, 1998b). Porcine progesterone-binding membranes are rich in smooth endoplasmic reticulum (SER) marker, NADH-cytochrome C reductase activity, but not other SER markers (Bramley and Menzies, 1988b & 1993). Localisation in bovine corpus luteum was not associated with nuclear, mitochondrial, lysosomal, peroxisomal, Golgi-endoplasmic reticulum-lysosomal and SER markers (Rae *et al.*, 1998a). A progesterone 'receptor' was identified in the cytosolic fraction of immature rat whole ovaries (Schreiber and Hsueh, 1979), and more specifically, granulosa cells (Schreiber and Erickson, 1979), but no other cellular fraction was examined. This binding protein showed many of the characteristics described by Bramley's group, including high specificity binding for progesterone and its analogue, R5020, and was enriched in cytosol fractions.

Bramley's group also demonstrated progesterone-binding was dependent upon the presence of digitonin, a saponin detergent which forms a 1:1 noncovalent complex with steroids with a 3β-hydroxy-Δ5 configuration and a C17 side-chain (Miller, 1984) such as cholesterol. In the absence of digitonin, very little binding was detected, raising doubts as to the physiological relevance of this binding. However, progesterone binding to the non-genomic PR of human sperm membranes which is known to be of high physiological importance, was also stimulated by the presence of digitonin (Ambhaikur and Puri, 1998). Digitonin is used to permeablise cell membranes (Keukens *et al.*, 1996) and as a detergent for selective solubilisation of membrane proteins (Banerjee *et al.*, 1995).

The primary regulation of progesterone action is thought to be through the transient expression of PR. The presence of a non-classical progesterone-binding protein in the rat ovary would reveal a new slant on theories of progesterone action in this tissue. This chapter sought to identify whether a binding protein with characteristics described by Bramley and colleagues was present in particulate fractions of the rat ovary.

2 Materials and Methods

All substrates, fine chemicals, inhibitors, reagents and unlabelled steroids were purchased from Sigma Chemical Co. (Poole, Dorset, UK) or from BDH (Poole, Dorset, UK), unless otherwise stated. [1,2,6,7-^{3}H]-labelled progesterone (100Ci/mmol) was purchased from New England Nuclear Research Products, Du Pont (UK) Ltd (Stevenage, Herts, UK).

2.1 Tissue recovery

Adult rats were used for all tissues except those specified immature (untreated 21 day old) or PMSG (10IU injected subcutaneously then killed 48h later). The animals were killed by carbon dioxide asphyxiation, and the tissues removed, dissected free from fat and snap frozen in liquid nitrogen. Tissues were then homogenised in ice-cold SET medium (100mg tissue/ml SET; 0.3mol/l sucrose, 1mmol/l EDTA 10mmol/l Tris-HCl, pH 7.4) and stored at -20°C until required.

2.2 Sucrose density gradient fractionation

Continuous sucrose density gradients (30 ml; 10-50% w/w) were prepared by the method of Stone (1974). 15ml of 10% w/v sucrose (EDTA, Tris pH 7.4) was layered gently onto 15ml 50% sucrose w/v (EDTA, Tris, pH 7.4) in ultracentrifuge polyallomer tubes. Tubes were capped, then tilted to horizontal and allowed to diffuse for 16 hours at 4°C. Diffused gradients were then returned to vertical, uncapped and stored at 4°C for up to 4 hours before use. Aliquots of tissue homogenate (2.5ml) were mixed with 0.5ml SET. After a 30min incubation on ice, 2.5ml aliquots were gently layered over the sucrose density gradients and centrifuged at 30,000 g_{av} for 2h in a Sorvall VTR 50 vertical tube rotor (4°C). Each gradient was then fractionated using a Buchler-Searle Autodensiflo gradient fractionator equipped with a meniscus-sensitive probe, and fractions (1ml) frozen at -20°C until assay.

2.3 Concentrations of sucrose and protein estimations

Measurement of the sucrose concentration of density gradient fractions was performed using an Abbe refractometer (Atago, Japan). Protein was measured by the method of Lowry *et al* (1951), using bovine serum albumin (BSA) as a standard.

2.4 Steroid binding to tissue fractions in vitro

Based on optimal conditions established in a variety of other species for the measurement of [^{3}H]-progesterone binding to ovarian tissue (Bramley and Menzies, 1988a; Bramley and Menzies, 1988c; Bramley and Menzies, 1993; Bramley and Menzies, 1994), the following binding assay was adopted.

Aliquots (50µl) of tissue fractions from sucrose gradients were incubated at 4°C for 2h in a system containing 40mmol/l Tris-HCl (pH 7.4), 0.1% (w/v) BSA, [^{3}H]-progesterone (50,000cpm) and digitonin (100µg/tube) in a final incubation volume of 0.5ml. Bound tracer was separated from free by adding 1.0ml of dextran-coated charcoal (DCC) (2.5g activated charcoal, (250-350 mesh) and 0.25g Dextran in 500ml Tris-BSA assay buffer). Tubes were centrifuged at 3000g for 10 min (4°C), then supernatants were decanted and mixed with 4ml scintillant (Ecoscint A, National Diagnostics, Atlanta, GA, USA), shaken and counted for tritium by liquid scintillation spectrometry. Tubes with no fraction but with digitonin, and tubes with fraction but no digitonin were routinely included as controls. In binding specificity experiments, unlabelled steroids were dissolved in ethanol, added to the assay tubes, then dried down overnight at room temperature. Assay buffer was then added and the tubes vortexed vigorously (30sec) before proceeding with the addition of the remainder of the assay cocktail and the normal incubation.

2.5 Dose-response to digitonin

Aliquots (20µl) of rat ovarian homogenate were incubated at 4°C for two hours in a system containing 40mmol/l Tris-HCl (pH 7.4), 0.1% (w/v) BSA, [^{3}H]-progesterone (50,000cpm) and increasing concentrations of digitonin (0-50µl of 10mg/ml stock), in a final incubation volume of 0.5 ml. Bound tracer was separated from free as above and counted for tritium by liquid scintillation spectrometry.

2.5 Steroid binding to increasing concentrations of homogenate

Aliquots of tissue homogenate of increasing protein content were incubated at 4°C for two hours in a system containing 40mmol/l Tris-HCl (pH 7.4), 0.1% (w/v) BSA, [^{3}H]-progesterone (50,000cpm) and digitonin (100µg/tube) in a final incubation volume of 0.5 ml. Tubes with no homogenate but with digitonin, and tubes with homogenate but no digitonin were included as controls. Bound tracer was separated from free as above, and counted for tritium by liquid scintillation spectrometry.

3 Results

3.1 Steroid binding to tissue fractions in vitro

Progesterone bound to cellular fractions 12-18 which represented sucrose concentrations of 23-32g/100g as shown in fig 7.1. All tissues tested showed some binding to these fractions, although tissues could not be compared directly due to inequalities in protein content of each sample. Protein concentration of each sample was not quantified. There was little progesterone binding to less dense fractions (cytosol; 12-16g/100g) nor to the very dense fractions (nuclei; 48-50g/100g).

3.2 Dose-response to digitonin

Progesterone binding to homogenate increased dose-dependently with digitonin concentrations. Membranes from both dissected corpus luteum and residual ovary (with corpus luteum removed) showed a similar response. Little binding occurred in the absence of digitonin. However, binding increased 3-fold with increasing digitonin, reaching a plateau at approximately 200-300μg digitonin/tube (fig 7.2).

3.3 Effects of homogenate concentration on steroid binding

In the presence of 100μg digitonin/tube, binding of [^{3}H]-progesterone was dependent on concentration of ovarian tissue (fig 7.3). However, binding plateaued at higher tissue concentrations.

3.4 Specificity of ovarian steroid binding sites

Unlabelled progesterone displaced the binding of [^{3}H]-progesterone to pooled fractions 9-19, greater than any other steroid investigated (fig 7.4). Testosterone, oestradiol benzoate and androstenedione appeared to displace [^{3}H]-progesterone binding to a lesser extent but oestrone, cholesterol, RU486 and cortisol showed no displacement. Similarly, no displacement of [^{3}H]-progesterone binding was observed with 5α- or 5β-hydroxy progesterone, nor with γ amino butyric acid or haloperidol (data not shown).

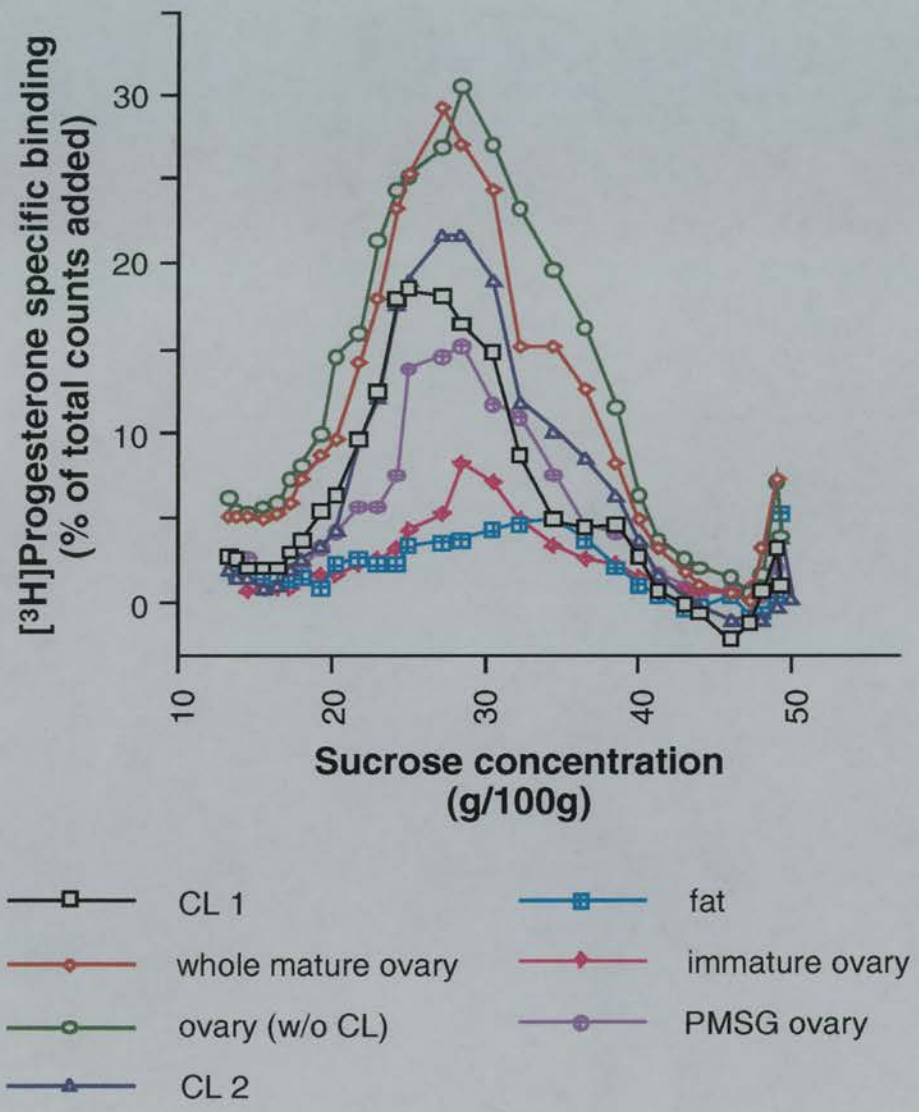


Fig 7.1 Fractionation of rat ovarian tissue homogenates (and adherent adipose tissue) incubated with [³H]-progesterone in the presence of digitonin. Points represent the mean of triplicate analysis.

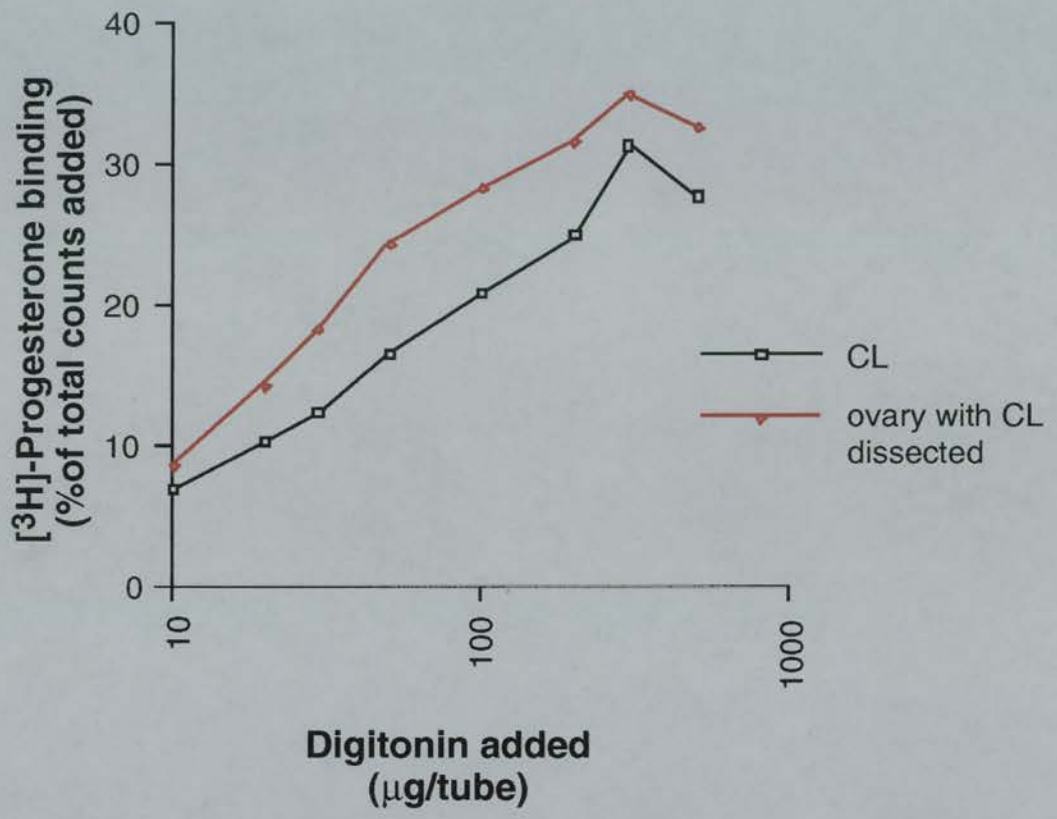


Fig 7.2 Effects of increasing digitonin concentration on [^{3}H]-progesterone binding to pooled rat ovarian sucrose gradient fractions enriched in progesterone binding activity. Points represent the mean of triplicate analysis.

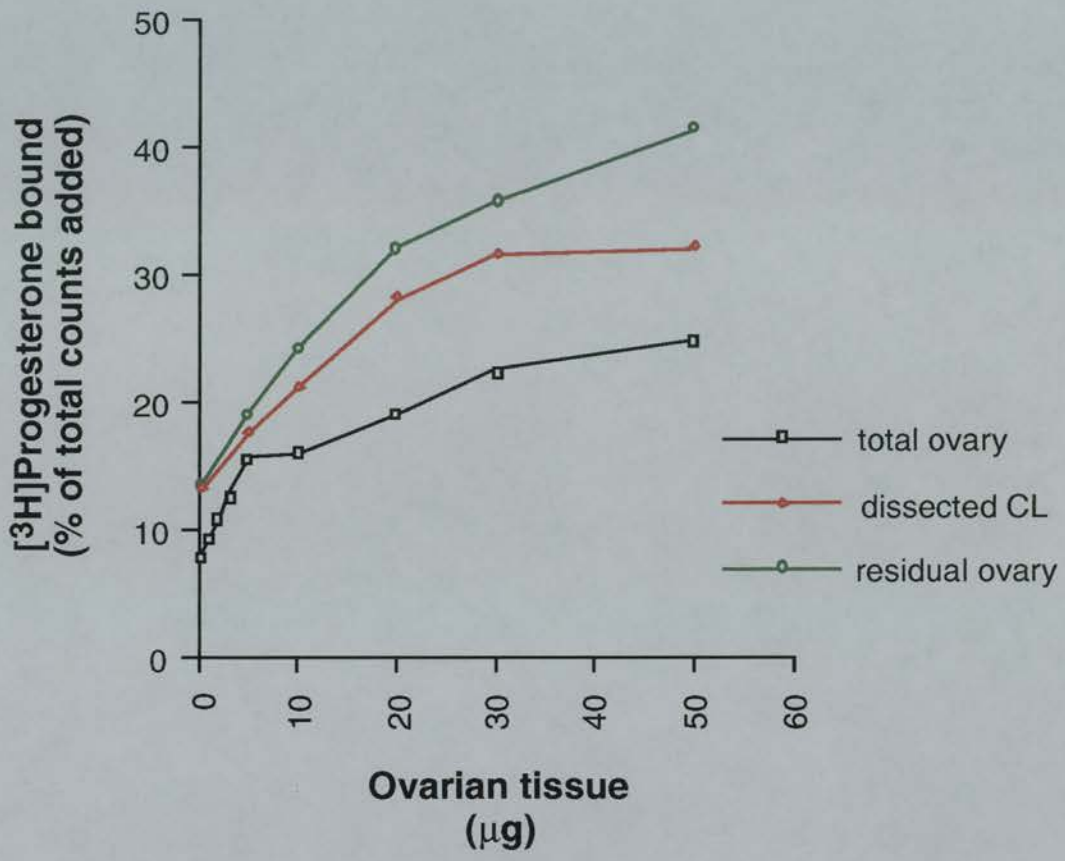


Fig 7.3 [^{3}H]-progesterone binding to increasing concentrations of rat ovarian homogenate sucrose gradient fraction pooled from fractions enriched in progesterone binding activity. Progesterone binding was not corrected for protein content. Points represent the mean of triplicate analysis.

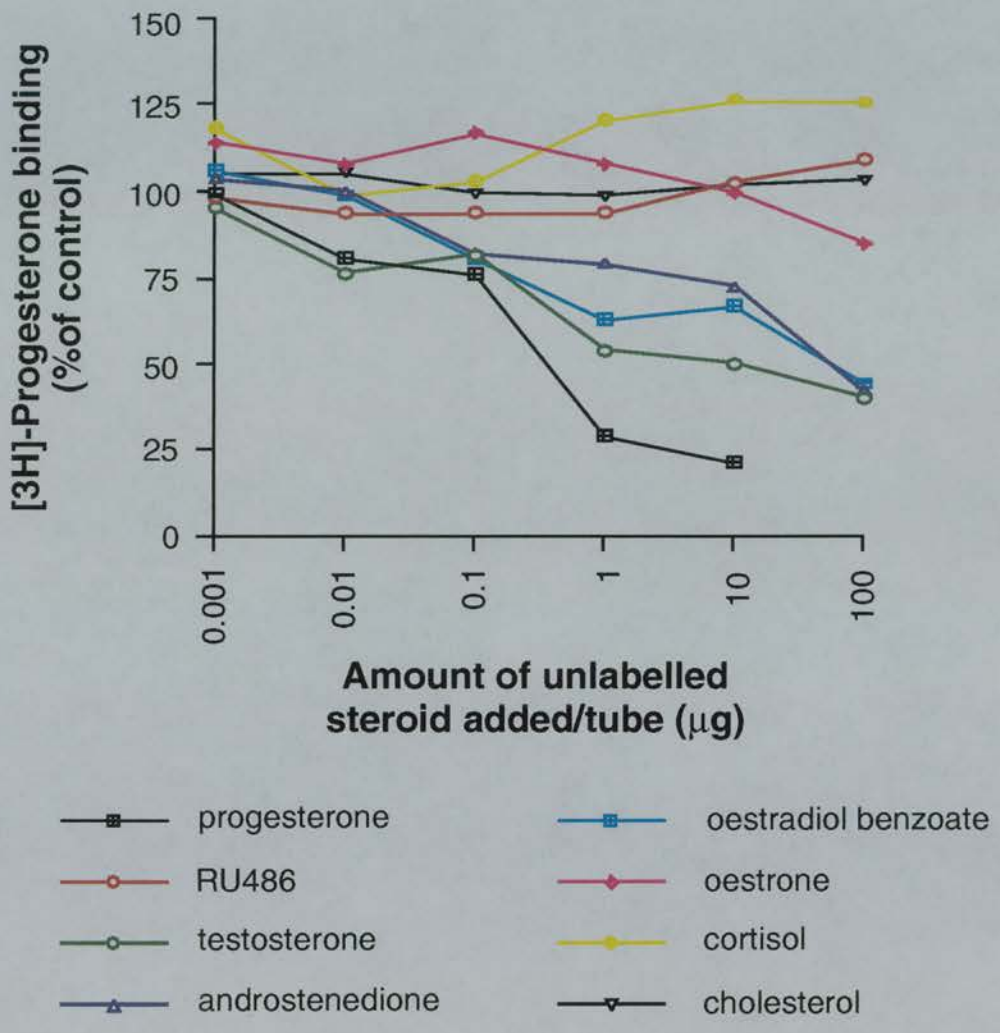


Fig 7.4 Displacement of [^{3}H]-progesterone binding by progesterone, other steroids and the genomic progesterone receptor antagonist RU486. Points represent the mean of triplicate analysis.

4 Discussion

A progesterone-binding site, with characteristics similar to those described by Bramley and colleagues in other species has been identified in rat ovarian tissue. The conditions used in these experiments including pH, temperature and duration of incubation were previously optimised in other species (Bramley and Menzies, 1988a; 1988c; 1993; 1994). Progesterone-binding identified in the rat was associated with a membrane fraction of a similar buoyant density to that previously reported. Binding was dependent upon the presence of digitonin, increased linearly with increasing tissue concentration and was specific for progesterone, with no displacement by the classic PR antagonist, RU486, nor by oestrogens, corticoids or cholesterol.

[^{3}H]-progesterone-binding was maximal in sucrose fractions of approximately 23-32g/100g. This buoyant density is associated with the fractions enriched in plasma membranes and smooth endoplasmic reticulum (SER) membranes (Rae *et al.*, 1998b). Although this subcellular localisation was not confirmed through marker enzyme activity in these studies, Rae *et al* also reported progesterone-binding in the cow corpus luteum to be associated with NADH cytochrome C reductase (a marker of the SER; Rae *et al.*, 1998b) and with other cell surface markers (Rae *et al.*, 1998a).

[^{3}H]-progesterone-binding increased dose-dependently with increasing digitonin concentrations in the incubation. The stimulation of progesterone binding to ovarian membranes is unique to digitonin (Menzies *et al.*, 1999). However, how digitonin acts to stimulate progesterone-binding is not known, though its action does not appear to be related to its detergent properties. Menzies and colleagues (1999) suggest that digitonin complexes specifically with an endogenous steroid which is normally tightly bound to the membrane steroid binding site. This leads to a change in receptor and/or membrane conformation, enabling [^{3}H]-progesterone to gain access to the previously occupied binding site.

Nevertheless, several characteristics of this binding site suggest it to be a receptor. [^{3}H]-progesterone-binding increased linearly with increasing membrane concentrations. These data compared with previous reports which also showed binding to be dependent on pH, temperature and duration of incubation, suggesting steroid binding reached equilibrium, was freely reversible and obeyed mass kinetics, which is behaviour typical of receptor-hormone interactions (Menzies and Bramley, 1994). Binding was

abolished by protease treatment, or boiling of membranes, showing it was a protein (Rae, 1996). However, progesterone binding did not appear to represent tracer binding to the active site of a progesterone-metabolising enzyme (Menzies and Bramley, 1994), nor was it due to classic, genomic PRs, since little binding was observed in cytosolic/nuclear fractions, where the genomic receptor is localised. Similar specificity was also seen in bovine (Rae *et al.*, 1998a; Rae *et al.*, 1998b), porcine (Menzies and Bramley, 1994) and ovine (Bramley and Menzies, 1994) corpus luteum membranes.

The classical, genomic PR is under strict gonadotrophic control, being expressed transiently for a very short period. This allows tight control of local progesterone action. If the membrane progesterone-binding protein is a NGPR, its expression may also be under gonadotrophic regulation. It will be of interest to compare [^{3}H]-progesterone binding in ovarian tissues at different maturity, corrected for membrane protein content. However, Machelon and colleagues (1996) reported progesterone increased Ca^{2+} mobilisation from the endoplasmic reticulum in both luteinised and non-luteinised porcine granulosa cells, but the method of mobilisation differed with states of cell maturation. Developmental regulation of granulosa cell membrane NGPR in the bovine follicle is suggested by decreased [^{3}H]-progesterone-binding with increasing follicle size (Rae *et al.*, 1998a). It should be noted that NGPR expression may differ among species, as with genomic PR expression.

In summary, this study is the first to demonstrate a progesterone-binding protein of this type in the rat. The presence of a NGPR could give rise to a novel method of cellular regulation in rat granulosa cells. Because of its plasma-membrane location, it is unlikely binding of progesterone to NGPRs results in direct regulation of gene transcription. Rather, receptor activation could result in an increase in membrane permeability, allowing an influx of ions that may be involved in intracellular signalling. Further studies to identify whether this protein is indeed a NGPR and whether its expression is under gonadotrophic regulation could lead to a better understanding of local progesterone action within the ovary.

Chapter 8 General Discussion

The results reported in this thesis confirm findings of others, and also introduce novel aspects to progesterone and glucocorticoid synthesis, metabolism, reception and action in the rat ovary. This chapter will summarise these results and expand on the discussions of the preceding chapters.

It is important to note that the data reported within this thesis are measurements of gene transcription and not protein concentration. Changes in mRNA levels do not necessarily reflect the amount of mature protein produced as transcription and translation are under independent control. Likewise, protein levels do not always correspond to bioactivity of the protein, i.e. other regulatory points occur downstream of transcription before the mature protein is finally expressed. However, the mRNA and protein expression of StAR (Ronen-Fuhrmann *et al*, 1998), P450scc (Hedin *et al*, 1987) and 3βHSD (Chedrese *et al*, 1990b) have previously been examined and have shown some correlation between transcription and translation. Protein levels of 11βHSD in the ovary have not been compared to mRNA expression, but 11βHSD1 bioactivity has been demonstrated by an hCG-induced increase in cortisol concentrations in the follicular fluid of human IVF patients (Harlow *et al*, 1997).

In vivo experiments utilised immature rats treated with gonadotrophins to stimulate follicle development, ovulation and the formation of a corpus luteum. Ovarian expression of StAR, P450scc and 3βHSD was shown by RNase protection assay (RPA) to increase with PMSG treatment and rise further with hCG treatment. Maximal expression occurred by 9h after hCG injection and remained high throughout luteinisation. 11βHSD1 mRNA concentration also increased with gonadotrophin stimulation, being maximally expressed 24h after hCG. In contrast, 11βHSD2 expression which was down regulated by hCG. PR was expressed transiently 6h after hCG treatment, with no message being observed before or after this time. GR did not appear to be under gonadotrophic control and was present throughout. Blood serum concentrations of progesterone demonstrated a small, gradual rise at 9h after hCG treatment, but after 5 days, at maximal corpus luteum activity, progesterone concentrations were 100-fold higher. Glucocorticoid concentrations were not assayed. In this model, approximately 90% of rats had ovulated by 12h after hCG treatment.

In order to study paracrine/autocrine effects in the ovary, granulosa cell culture was undertaken using DES-primed immature rats. After demonstrating similar gene expression patterns *in vitro* to those observed *in vivo*, this culture method was employed to study the effects of the antiprogestins, RU486, which binds to both PR and GR, and the PR-specific molecule, Org31710. After 12h in the presence of rhLH, the antiprogestins down-regulated expression of StAR, P450scc and 3βHSD, and increased 11βHSD1 mRNA content and progesterone concentration. PR expression remained unaltered at 6h after concurrent rhLH and RU486 treatment. The addition of exogenous progesterone or glucocorticoid reversed the increase in 11βHSD1 in the presence of RU486. Comparable results with Org31710 were not statistically significant. However, 11βHSD1 did increase significantly in the presence of Org31710, indicating the involvement of PR. The increase in 11βHSD1 associated with Org31710 and RU486 decreased dose-dependently with increased RU486 concentrations, and the highest RU486 concentration resulted in no significant rise in 11βHSD1. Since this dose-dependency was not observed with Org31710 treatment, it is likely this effect occurred through GR. The complexity of GR and PR cross-reacting with both progesterone or glucocorticoid is one of the reasons these results are difficult to interpret. The unknown effects of a possible non-genomic PR should also be acknowledged.

The results described above indicate that both PR and GR are involved in 11βHSD1 regulation, since both Org31710 and RU486 increased 11βHSD1 expression, but only with RU486 was this shown to be dose-dependent. As previously mentioned, the involvement of the two receptors complicates the situation, since each receptor has a different affinity for RU486 and a different response to ligand binding. RU486 exerts its action on GR at more than one step in the receptor activation pathway, preventing (at least some) GR from binding to DNA. However a fraction of the GR bound with RU486 proceeds to the step of DNA-binding and therefore must act as an antiglucocorticoid. RU486 must also affect downstream (post-DNA-binding) steps required for transcriptional enhancement. PR appears to exert its effect primarily at the later step(s) downstream of DNA binding (Beck *et al.*, 1993).

Ligand binding to PR and GR results in different transcriptional responses. PR bound to its ligand activates transcription of target genes. GR acts in a different fashion, being either an activator or repressor of transcription upon ligand binding. Repression occurs through the transcription factors activator protein-1 (AP-1) and nuclear factor- κB (NF-

κB). AP-1 and NF-κB binding sites are found in the promoter regions of many genes involved in inflammation, including cytokines, adhesion molecules and enzymes (for reviews see Cato and Wade, 1996 and Bamberger *et al*, 1996). IL-2 and collagenase are positively regulated by AP-1. Activated GR inhibits binding of AP-1 to its site in promoter regions and therefore blocks transcription of AP-1-regulated genes. A similar method of negative regulation of genes involved in anti-inflammation occurs via NF-κB upon activation of PR or GR (for review see van der Burg and van der Saag, 1996). Negative transcriptional regulation occurs by another method when GR, in the presence of glucocorticoid, binds to a negative glucocorticoid response element (nGRE) upstream of target genes (e.g. POMC gene [Drouin *et al.*, 1993]). This causes inhibition rather than enhancement of transcription.

Another factor to consider is that the antigestagens may be acting as agonists rather than antagonists of GR and PR, and up-regulating 11βHSD1 expression. RU486 acts as an agonist when bound to PRB in the presence of cAMP (Tung *et al.*, 1993) and GR may have similar actions. The addition of progesterone or glucocorticoid *in vitro* significantly increased 11βHSD1 transcription, but this increase was not as great as that shown by RU486. Therefore, if RU486 was acting as a PR or GR agonist, it had a more potent effect on 11βHSD1 expression than either progesterone or glucocorticoid.

The non-physiological nature of conditions employed in cell culture is important when analysing these data. It is unlikely that there is any glucocorticoid present in the medium, since glucocorticoids are not synthesised in the ovary. Therefore 11βHSD1 expression should have not metabolised steroids present in the medium. The addition of cortisol to cultures reversed the increase in 11βHSD1 in response to RU486, indicating glucocorticoids play an active role in this model. Therefore the lack of endogenous glucocorticoid in the culture system may result in an non-physiological response. It should be noted that granulosa cell cultures are contaminated, to a degree, by blood cells such as monocytes and macrophages (Turner, 1992). The presence of other cell types (particularly those concerning the involvement of cytokines) may have influenced these results. *In vitro* experiments, although essential to the understanding of biological functions, do not reflect physiological conditions, and therefore give only a partial picture of events *in vivo*.

The increase in 11βHSD1 transcription associated with RU486 and Org31710 may have relevance to the anovulatory effect of antigestagens *in vivo*. 11βHSD controls the

metabolism of glucocorticoids which are present in follicular fluid *in vivo* (Michael and Cooke, 1994). Although 11βHSD is not essential for fertility (as shown by gene knockout rats [Kotelevtsev *et al.*, 1997]), the cortisol : cortisone ratio is elevated before ovulation (Harlow *et al.*, 1997). If the RU486-dependent increase in 11βHSD1 observed in granulosa cell cultures also happens *in vivo*, RU486 could have the effect of increasing cortisol levels above those normally present in follicular fluid. Progesterone levels are also increased after RU486 treatment, probably due to the decrease in 20αHSD activity (Uilenbroek *et al.*, 1992; Adashi *et al.*, 1981). Although progesterone and cortisol concentrations are increased by LH in normal ovulatory cycles (Harlow *et al.*, 1997), this excess of the anti-inflammatory steroids, progesterone and glucocorticoid, may be responsible, in part, for anovulation in RU486-treated mammals.

Glucocorticoid down-regulates transcription of phospholipase-A2 (PLA2) (Goppelt-Struebe, 1997), prostaglandin synthase (COX-2) (Mitchell *et al.*, 1994; Ishihare *et al.*, 1995) and nitric oxide synthase (NOS) (Radomski *et al.*, 1990), all of which are involved in inflammation and ovulation. This effect may act through NF-κB and AP-1 which are also down-regulated by GR activation (Mukaida *et al.*, 1994; Jonat *et al.*, 1990). The promoter region of the human collagenase gene contains a binding site for AP-1 (Jonat *et al.*, 1990). Therefore GR-activation would induce an associated AP-1 decrease and this would directly down-regulate collagenase transcription. COX-2 activity and PGE and PGF2 synthesis are induced by IL-1 (O'Banion *et al.*, 1992; Brannstrom *et al.*, 1993) and therefore a decrease in IL-1 would lead to decreased synthesis of prostaglandins which have been shown to be essential for ovulation (Tsafriri *et al.*, 1972). A decrease in IL-1 would also down-regulate collagen and augment collagenase expression (Graham *et al.*, 1996).

Excess progesterone also decreases AP-1 (Shemshedini *et al.*, 1991), NF-κB (Kalkhoven *et al.*, 1996), COX-2 (Ishihare *et al.*, 1995) and IL-1β (Mori, 1990); (Polan *et al.*, 1988) activity. IL-1 is also known to block basal and LH-induced progesterone secretion (Barak *et al.*, 1992) Therefore a decrease in IL-1 increases progesterone production, i.e. increases the synthesis of anti-inflammatory steroids. High concentrations of progesterone, similar to those found during the luteal phase, inhibit IL-1 synthesis (Polan *et al.*, 1988). Since RU486 increases progesterone synthesis above that induced by normal LH stimulation, these concentrations may be high enough

to suppress IL-1. All these effects would result in a decrease in pro-inflammatory factors which may cause anovulation.

This decrease in collagenase (by whatever method) may be the outcome of the ability of RU486 to prevent ovulation. Shibata (1990) showed no increase in the collagenolytic enzymes typically associated with gonadotrophic stimulation in the presence of RU486 and hCG.

It may be that progesterone and glucocorticoid concentrations are the keys to the control of ovulation. Progesterone is essential for ovulation as shown by numerous studies, and cortisol also increases before ovulation. Perhaps concentrations of progesterone above a critical threshold prevent ovulation. Many cytokines increase before ovulation, and induce collagenolytic activity, but with RU486 treatment, cytokine levels are reduced, and ovulation does not takes place. The hypothesis described above is illustrated in fig 8.1.

However, there is a problem with this model. GR and PR are presumably occupied, to some extent, by antigestagens. Therefore the resultant increase in glucocorticoid and progesterone cannot act through PR or GR. However, the dose-dependent RU486 response of 11βHSD1 transcription suggests that at lower RU486 concentrations, not all receptors are occupied by the antagonist. Because RU486 binds to both PR and GR, and steroid concentrations are elevated by gonadotrophin and antagonist treatment, the concentration of RU486 (10^{-6}M) used in these experiments may not block all receptor sites. This dose-dependent effect was not seen with the PR-specific antagonist ORG31710, which demonstrated a less impressive up-regulation in 11βHSD1, but did not decrease 11βHSD1 transcription at higher doses. This model demonstrates the importance of antigestagen concentration on the resultant steroid response. The involvement of GR may account for this difference (as previously discussed), or perhaps there are differences in the actions the two antagonists upon binding to their receptors. Structures of both RU486 and ORG31710 can be seen in fig 8.2.

This thesis outlines some of the paracrine and autocrine effects of steroids on the rat ovary and in particular, on cultured rat granulosa cells. It should be noted that the ovary is also an endocrine gland. Complex feedback mechanisms between the hypothalamus, pituitary and the ovary control follicular development. Therefore the increase in progesterone and 11βHSD1 associated with antigestagen treatment in granulosa cell

cultures may not occur in the ovary *in vivo*. If this increase does occur, its effects may not be exerted solely within the ovary. Moreover, the antigestagens administered may act on other target organs, for instance, rats treated with RU486 show a significant decrease in ovulatory LH release (Sanchez-Criado *et al.*, 1990), demonstrating effects outwith the ovary which might inhibit ovulation.

Future Work

Overall, the work described herein has implications for increasing our understanding (and hence being able to manipulate) ovarian and uterine function at two levels, both of which capitalise on the roles of progesterone and glucocorticoids as anti-inflammatory agents.

1. The 'Small' Picture

At an immediate level, the results in this thesis suggest some obvious areas of further work that are likely to be productive. Ovulation has been compared to an inflammatory response: pro-inflammation inducing ovulation and anti-inflammation aiding tissue repair of the punctured ovarian surface after expulsion of the egg. Glucocorticoids are effective in controlling inflammation in a number of tissues. Therefore the LH-induced up-regulation of 11βHSD1 gene expression in the ovary after ovulation would be expected to increase the levels of local active glucocorticoids enabling tissue repair. It is important to note that this thesis studied only gene expression and it is therefore essential to measure protein concentrations and bioactivity to substantiate the suggested hypotheses. This is of particular importance in the case of 11βHSD where the presence of the protein has not been identified in the ovary. By adding exogenous glucocorticoid to the cell culture, and measuring the 11-dehydrocorticosterone : corticosterone ratio, the presence of bioactive 11βHSD protein could be confirmed. An increase in bioactive glucocorticoid at a time after ovulation would concur with findings of human studies (Harlow *et al.*, 1997), augmenting the theory that glucocorticoid action in the ovary is controlled by 11βHSD expression.

Progesterone action has proved essential for ovulation (Tanaka *et al.*, 1993; Lydon *et al.*, 1995) and cross-reaction between PR and GR indicates the importance for tight regulation of genes involved in synthesis, metabolism and reception of progesterone and glucocorticoid. By blocking this action (as demonstrated in this thesis using the

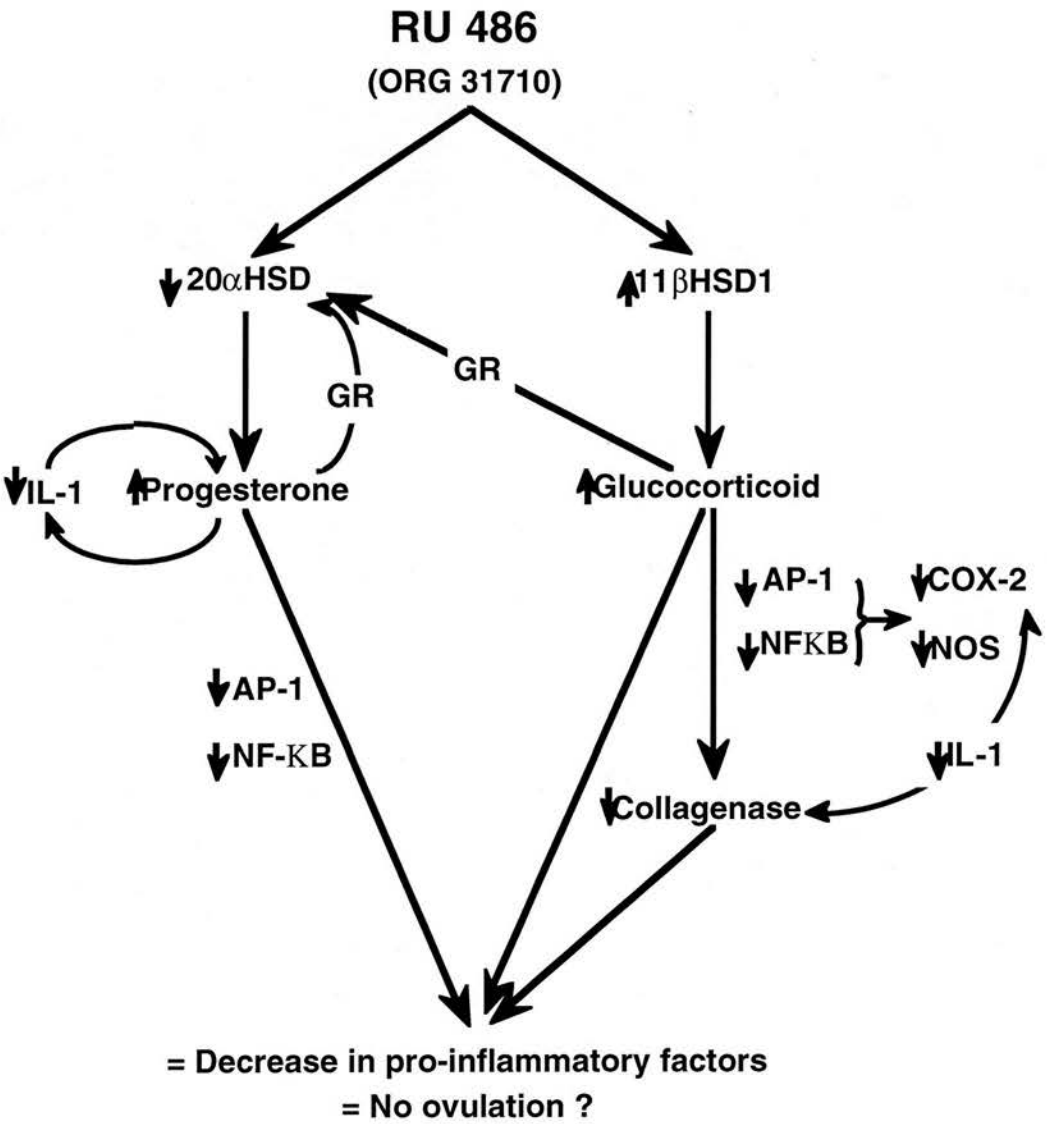
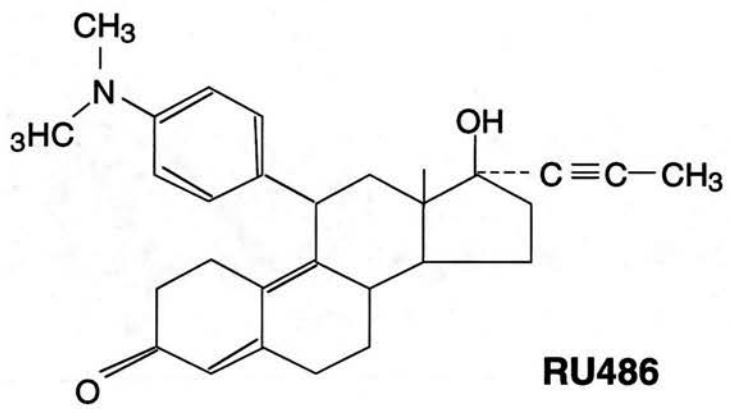
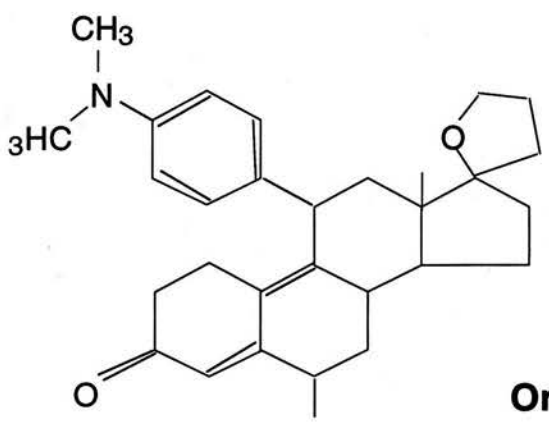


Fig 8.1 Proposed scheme of actions of RU486 and Org31710



RU486



Org31710

Fig 8.2 The molecular structures of the antiprogestins RU486 and Org31710

antigestagens RU486 and Org31710), gene expression of enzymes involved in synthesis and metabolism were regulated, indicating PR and GR (in)activation controls, to some extent, the concentrations of their ligands. Insight into the molecular mechanisms involved in this control could lead to the development of novel antigestagenic contraceptives. Also, to verify glucocorticoid and progesterone's regulation of 20αHSD activity, the culture medium could be assayed for 20α-hydroxyprogesterone concentrations.

In order to examine the plethora of genes and proteins that may be regulated during this inflammatory response, techniques could be optimised further to yield more results from the RNA collected from the treated cells. For example, a minimum of 5 μg total RNA is required for analysis by RNase protection assay (RPA). The cell culture methods used here yielded as little as 10 μg per group, limiting the analysis that could be done. An alternative for the future might be to use quantitative RT-PCR, which would require less RNA and should allow a more extensive study of gene expression. This would allow the examination of transcription patterns of genes encoding multiple cytokines, receptors and enzymes within a single experiment.

This thesis hints at the existence of a non-genomic mode of action for progesterone in the ovary, which could be a potentially important new therapeutic target for contraceptive development. The NGPR should therefore be purified and characterised and the gene studied at the transcriptional and translational level. In the shorter term, NGPR and classic PR binding of progesterone could be quantified and compared in ovaries at different stages of maturity or in ovaries treated with antiprogestins to determine whether NGPR is developmentally regulated and serves a physiologically significant intraovarian function.

2. The 'Big' Picture

More broadly, inflammatory mechanisms appear to underpin many natural (ovulation, menstruation) and disease (endometriosis, painful ovulation, even ovarian cancer) processes in the female reproductive tract. Therefore the ability to target, safely and cheaply, anti-inflammatory therapy to discrete regions of the tract therefore may hold major potential therapeutic benefits. For example, progesterone/glucocorticoids could be locally delivered using a slow release mechanism, raising concentrations above that critical threshold and effectively halting ovulation. Similarly, abnormal

progesterone/glucocorticoid concentrations may be the cause of some forms of infertility. This could be targeted in a number of ways. For example, cortisone (the cortisol precursor) could be administered as a steroid loaded IUD and only in cells expressing 11βHSD1 would it be converted to cortisol and thereby becoming able to bind and activate GR. Likewise, an anti-cortisol antibody could be administered trans-cervically, to block GR-binding, thus reducing GR activation and its downstream actions. Alternatively, cortisol concentrations could be diminished by an enzymatic approach. 11βHSD2 could be targeted towards the ovary, perhaps through gene therapy, to convert cortisol to cortisone, rendering the glucocorticoid inactive and unable to activate GR.

A similar approach could be undertaken to manipulate tissue concentrations of progesterone. For example, a progesterone precursor such as pregnenolone or a synthetic progestin could be used to target cells expressing PR and 3βHSD allowing selectively increased progesterone/progestin synthesis and action at those sites. Gene therapy with steroidogenic enzymes holds similar potential. However, the high cost of developing contraceptive methods based on gene therapy and its unknown reliability safety issues would probably make it commercially unattractive. This is particularly so given that cheap and effective hormonal pills are already available.

An important discovery made during this work was that antiprogestagens have counter-intuitive dose-dependent effects on granulosa cell 11βHSD1 gene expression: high concentrations of RU486 having no significant effect, with lower concentrations strongly inducing gene expression. It remains to be determined if the changes observed are involved in the antiovulatory action of RU486 that have been observed in women. RU486 is already used clinically to inhibit ovulation, but at the doses used, its principal mode of action is likely to be extraovarian. Perhaps targeting of the ovaries with low concentrations of drugs like RU486 (or a synthetic precursor?) holds the key to a completely new style of contraceptive development.

Overall, it emerges that local concentrations of progesterone and glucocorticoid are critical to the functioning of the female reproductive system. There is obvious potential from continuing to study the genes that are involved in their production, metabolism and reception throughout the reproductive tract. The three billion human females living on Earth at the beginning of the third millennium stand to benefit from further research.

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