The Effects of Hypoglycaemia on Vascular Disease and the Brain

Dr Rohana J Wright MBChB MRCP (UK)

Doctor of Medicine Thesis

The University of Edinburgh

2012

Abstract

Hypoglycaemia is the commonest and most feared side-effect of insulin therapy for diabetes, which is increased during strict glycaemic control. It can have detrimental effects on many aspects of cerebral function, but also provokes widespread changes in blood components and vascular flow such that theoretical concerns have been raised regarding its potential effects on the vasculature of people with diabetes. The aims of this thesis were: (a) to further examine the effects of hypoglycaemia on cognitive function, specifically to assess the effects on spatial abilities, and (b) to explore the potential effects of hypoglycaemia on several parameters that influence vascular function.

Spatial ability was assessed using the French and Ekstrom Kit of Factor Referenced (cognitive) Tests in 16 subjects with type 1 diabetes during euglycaemia and hypoglycaemia, which was induced using the hyperinsulinaemic glucose clamp technique. Vascular function was evaluated in a preliminary study by assessing the endothelin response to acute hypoglycaemia in 20 subjects with type 1 diabetes. Subsequently, 16 adult human subjects with type 1 diabetes and 16 subjects without diabetes were studied during hypoglycaemia and euglycaemia with measurement of an extensive battery of tests relevant to vascular function (soluble markers of platelet function, coagulation, and inflammation). Flow cytometry was used to assess the effects of hypoglycaemia and euglycaemia on platelet-monocyte aggregation, CD40 expression on monocytes and CD40 ligand expression on platelets, as these parameters are known to play a role in the initiation and progression of atherogenesis and vascular disease.

Results have indicated that spatial ability was significantly affected during hypoglycaemia, which is relevant to the everyday activities of people with diabetes. In response to hypoglycaemia, significant changes were observed in several intra-vascular indices that could compromise vascular function and exacerbate vascular disease. These findings are of concern given the fact that the main objective of optimal glycaemic control in diabetes is to prevent the development and progression of micro- and macrovascular disease. This research provides a basis for further studies to investigate this concept in greater detail, and adds to the body of evidence demonstrating the importance of avoiding hypoglycaemia while maintaining optimal glycaemic control.

Declaration

This thesis is a presentation of my original research work. Wherever contribution of others is involved, every effort has been made to indicate this clearly. It has not been submitted for any other higher degree or qualification.

Study 1 was researched, conducted, analysed and written by myself.

Study 2 was conducted by Dr Ken MacLeod. I researched the topic, analysed the data and wrote up the study.

Study 3 was researched, conducted, analysed and written by myself. Flow cytometry studies were also performed by me. Soluble marker assays were conducted by Mrs Pamela Dawson at the Royal Infirmary of Edinburgh Haematology laboratory. Counterregulatory hormone assays were conducted at the laboratories of Nottingham University and Royal Victoria Hospital, Belfast.

This work was performed under the guidance of Professor Brian Frier, Department of Diabetes, Royal Infirmary of Edinburgh, UK.

Signed: Date: 26/12/2012

Rohana J Wright

Acknowledgements

I would like to acknowledge the guidance and support of my supervisor, Professor Brian Frier, and also Professor Ian Deary for his support of the cognitive studies, and Professors David Newby and Christopher Ludlam for their support of the vascular studies. My thanks go to the late Dr Ken MacLeod for his practical work on the endothelin study many years ago, which I was then able to analyse and interpret, and to Mrs Pamela Dawson for her hard work on the laboratory assays, of which there were many. I also acknowledge the assistance of Dr David Stirling from the Haematology laboratory at the Royal Infirmary of Edinburgh for his help in the set up of my flow cytometry templates, and for his ongoing support throughout my many hours spent feeding samples into the flow cytometer. I thank Dr Jacqueline Geddes, a fellow researcher, for teaching me the hyperinsulinaemic clamp technique. I would also like to acknowledge the assistance of the laboratories at Nottingham University and Royal Victoria Hospital, Belfast, for performing the counterregulatory hormone assays which we were unable to do in-house.

I would like to acknowledge the patients and staff of the Diabetes Out-Patient clinic and the Clinical Research Facility at the Royal Infirmary of Edinburgh, for being so accommodating and for helping me by participating in my studies.

And finally, I could not have completed this body of work without the support of my husband, Nick, and my daughter, Olivia.

Table of Contents

List of Figures

List of Tables

List of Publications, Abstracts and Presentations

1. **Wright R J**, MacLeod K M, Perros P, Johnston N, Webb D J, Frier B M. Plasma endothelin response to acute hypoglycaemia in adults with type 1 diabetes. *Diabetic Medicine* 2007; 24: 1039-1042.

2. **Wright R J**, Frier B M. Vascular Disease and Diabetes: Is hypoglycaemia an aggravating factor? Review article. *Diabetes Metab Res Revs* 2008; 24: 353-363.

3. **Wright R J**, Frier B M, Deary I J. The effects of acute insulin-induced hypoglycemia on spatial abilities in adults with type 1 diabetes. *Diabetes Care* 2009; 32: 1503-1506.

4. **Wright R J**, Newby D E, Stirling D, Ludlam C A, Macdonald I A, Frier B M. The effects of acute insulin-induced hypoglycemia on indices of inflammation: putative mechanism for aggravating vascular disease in diabetes. *Diabetes Care* 2010; 33: 1591-1597.

5. **Wright R J**, Newby D E, Ludlam C A, Frier B M. The effects of acute insulin-induced hypoglycaemia on platelet-monocyte binding and the CD40-CD40 ligand dyad in non-diabetic subjects (Abstract). *Diabet Med* 2008; 25 (Suppl 1): 46.

   Poster presentation at Diabetes UK APC 2008, Glasgow, UK.

6. **Wright R J**, Newby D E, Ludlam C A, Frier B M. The effects of acute insulin-induced hypoglycaemia on platelet-monocyte binding and the CD40-CD40 ligand dyad in non-diabetic subjects (Abstract). *Diabetes* 2008; 57 (Suppl 1): 2190-PO.

   Published only for American Diabetes Association 68th Scientific Sessions 2008.

7. **Wright R J**, Deary I J, Frier B M. The effects of acute insulin-induced hypoglycaemia on spatial ability in adults with type 1 diabetes (Abstract). *Diabetologia* 2008; 51 (Suppl 1): S272.

Poster presentation at European Association for the Study of Diabetes (EASD) Annual Conference 2008.

8. **Wright R J**, Stirling D, Newby D E, Ludlam C A, Frier B M. The effects of acute insulin-induced hypoglycemia on indices of inflammation: putative mechanism for aggravating vascular disease in diabetes (Abstract). *Diabetes* 2009; 58 (Suppl 1): 635-P.

   Poster presentation at ADA 69th Scientific Sessions 2009.

Keywords

hypoglycaemia, type 1 diabetes, inflammation, cognitive function, vascular complications.

Abbreviations

ACCORD	Action to Control Cardiovascular Risk in Diabetes
ADP	Adenosine diphosphate
ADVANCE	Action in Diabetes and Vascular Disease: Preterax and Diamicron MR Controlled Evaluation
ANOVA	Analysis of variance
BGAT	Blood glucose awareness training
BMI	Body mass index
BOLD	Blood oxygen level-dependent
CD	Cluster of differentiation
CD40L	CD40 ligand
CGMS	Continuous glucose monitoring system
CIMT	Carotid intima-media thickness
CRP	C-reactive protein
CRT	Choice reaction time
CSII	Continuous subcutaneous insulin infusion
CV	Coefficient of variation
DAFNE	Dose Adjustment For Normal Eating
DCCT	Diabetes Control and Complications Trial
DESMOND	Diabetes Education and Self-management for Ongoing and Newly Diagnosed
DIGAMI	Diabetes Mellitus Insulin Glucose Infusion in Acute Myocardial Infarction
DNA	Deoxyribonucleic acid
DSST	Digit symbol substitution task
DVLA	Driver and Vehicle Licensing Authority
ECG	Electrocardiogram
EDIC	Epidemiology of Diabetes Interventions and Complications
EDTA	Ethylene diamine tetra-acetic acid
EEG	Electroencephalogram

ELISA	Enzyme-linked immunosorbent assay
ET	Endothelin
FACS	Fluorescence activated cell sorter
FDP	Fibrinogen degradation products
FIMT	Femoral intima-media thickness
FITC	Fluorescein isothiocyanate
g	grams
GP	General Practice
h	hours
HbA1c	glycated haemoglobin
HR	hazard ratio
IAH	impaired awareness of hypoglycaemia
ICAM	Intercellular adhesion molecule
ICU	Intensive Care Unit
IL	Interleukin
IM	intramuscular
IQ	Intelligence quotient
IQR	Interquartile range
ITU	Intensive Therapy Unit
IV	intravenous
kg	kilograms
l	litre
LVEF	Left ventricular ejection fraction
m^2	metres squared
Mac-1	Macrophage-1
mg	milligrams
min	minute
ml	millilitres
mmHg	millimetres of mercury
mmol	millimoles
MRI	Magnetic resonance imaging

mU	milliunits
NART	National Adult Reading Test
NHS	National Health Service
NICE-SUGAR	Normoglycaemia in Intensive Care Evaluation – Survival Using Glucose Algorithm Regulation
NK	Natural killer
NPH	Neutral Protamine Hagedorn
ns	Not significant
NSE	Neurone-specific endolase
O_2	oxygen
PAI	Plasminogen activator inhibitor
PE	phycoerythrin
PET	Positron emission tomography
pg	Picograms
PMA	Platelet monocyte aggregation
PPACK	D-phenyl-L-prolyl-L-arginine chloromethyl ketone
QOF	Quality and Outcomes Framework
RPE	R-phycoerythrin
sCD40L	Soluble CD40 ligand
SD	Standard deviation
SEM	Standard error of the mean
SH	severe hypoglycaemia
SPSS	Statistical Package for the Social Sciences
SSRI	Selective serotonin reuptake inhibitor
TNF	Tumour necrosis factor
tPA	Tissue plasminogen activator
tPA Ag	Tissue plasminogen activator antigen
UKPDS	UK Prospective Diabetes Study
VACSDM	Veterans Affairs Co-operative Study in Type 2 Diabetes
VADT	Veterans Affairs Diabetes Trial

VCAM-1	Vascular cell adhesion molecule-1
VEGF	Vascular endothelial growth factor
VISEP	Volume and Insulin Therapy in Severe Sepsis and Septic Shock
vWF	von Willebrand factor
WAIS	Wechsler Adult Intelligence Scale
µl	microlitre

Chapter 1:

Introduction

1. Introduction

1.1 Aims of research programme

The aims of this programme of research were to investigate in detail the potential adverse consequences of hypoglycaemia on both spatial abilities and vascular biology. The more information that can be gained about the effects of hypoglycaemia, the more appropriately we can target glycaemic control in the safest possible fashion.

1.2 Epidemiology of hypoglycaemia – The size of the problem

Hypoglycaemia is a fact of life for people with insulin-treated diabetes. Striving to meet glycaemic targets means that it is common; it affects most people with type 1 diabetes at some stage, as well as many people with type 2 diabetes on insulin or insulin secretagogues. It can be unpredictable and there are potentially dangerous sequelae if it is not identified and treated in a timely fashion. Due attention must also be paid to the more chronic effects it can have on a person's ability to lead a normal day-to-day life. It can provoke fear and anxiety for the person with diabetes and for their family, friends and colleagues. Even people with suboptimal glycaemic control who run a higher glycated haemoglobin (HbA1c) can be at risk of hypoglycaemia, as they may exhibit more erratic control with greater swings in blood glucose.

1.2.1 Definitions

There is no current consensus as to the most appropriate way to define hypoglycaemia. It can be defined using the traditional 'Whipple's triad'; symptoms consistent with hypoglycaemia, a low blood glucose concentration, and reversal of the symptoms on correction of the low glucose. However, the alteration of symptom profiles with duration of diabetes or exposure to recurrent hypoglycaemia is a well-recognised phenomenon, leading to the development of impaired hypoglycaemia awareness. In those people, that traditional triad of features may not

be present for every episode (Pramming, 1991). Therefore, using a biochemical cut-off has been recommended, particularly as this makes reporting during clinical trials easier, but again, no consensus exists as to the most appropriate figure to utilise in this regard. The American Diabetes Association definition is of a blood glucose concentration less than 4.0mmol/l (American Diabetes Association, 2005), but as we will go on to discuss when describing the physiology of hypoglycaemia and the provocation of symptoms and cognitive dysfunction that ensues with a declining blood glucose, symptoms will rarely be produced at a level of 4.0mmol/l. As a result, many experts feel this definition is too high, and that a level of 3.5mmol/l may be more practically appropriate as symptoms are usually provoked at this level (Frier, 2009). Many health care professionals involved in diabetes management would believe, however, that advising patients not to treat until their blood glucose is less than 3.5mmol/l could lead to hazardous consequences.

Furthermore, a definition of the severity of an episode of hypoglycaemia is required. It is generally accepted that a mild episode is one that can be self-treated, and a severe episode requires some degree of external assistance for treatment. As a result of the ongoing controversy surrounding definitions, reporting of hypoglycaemia can vary substantially between studies, which makes interpretation and comparison problematic. In addition, self-reporting of hypoglycaemia history is not always reliable. It has been shown that severe hypoglycaemia reporting is reliable up to 1 year after the event (Pederson-Bjergaard, 2003), but recall of mild episodes may only be accurate for up to 1 week (Pramming, 1991). This introduces another problem in analysing hypoglycaemia data.

1.2.2 Frequency of hypoglycaemia

There is no doubt that good glycaemic control can limit the development and progression of microvascular complications. The Diabetes Control and Complications Trial (DCCT), and the long term follow up study, Epidemiology of Diabetes Interventions and Complications

(EDIC), showed that the onset, progression and severity of diabetic complications, in people with type 1 diabetes, can be limited by strict glycaemic control (DCCT Group, 1993, DCCT/EDIC Group, 2005). However, this intensive insulin therapy can increase the risk of severe hypoglycaemia (DCCT Group, 1993).

The annual prevalence of severe hypoglycaemia has been reported as being between 30% and 40% in several large studies including the DCCT (DCCT Group, 1993, EURODIAB Group, 1994, MacLeod, 1993, ter Braak, 2000) with rates increasing with duration of diabetes (UK Hypo Study Group, 2007). The incidence of severe hypoglycaemia ranges from around 1.0 to greater than 3.0 episodes/patient/year, depending on the duration of the disorder, as reported in a collection of studies in type 1 diabetes (Strachan, 2007). The DCCT reported incidences of 0.19 to 0.62 episodes/patient/year in the conventional and intensive treatment groups respectively (DCCT Group, 1993), but subjects were recruited on the basis that they were at relatively low risk of severe hypoglycaemia. Following a feasibility study, patients who had a preceding history of severe hypoglycaemia were excluded from the DCCT, which therefore excluded people with impaired hypoglycaemia awareness, and most people with long duration of diabetes (DCCT Group, 1987). A more recent, inclusive study revealed an incidence of 1.1 episodes/person/year in patients with type 1 diabetes of short duration (<5 years), who are usually well-controlled, and 3.2 episodes/person/year in those with longer duration (>15 years). This study included those people at higher risk of hypoglycaemia. Overall glycaemic control in the study was good, with patients having a mean glycated haemoglobin concentration of 7.8% in the long duration group and 7.3% in the short duration group (UK Hypo Group, 2007).

A summary of the epidemiology of severe hypoglycaemia in type 1 diabetes is shown in Table 1.1 (Pramming, 1991, MacLeod, 1993, ter Braak, 2000, Pedersen-Bjergaard, 2004). From an economic perspective, around 10% of episodes of severe hypoglycaemia affecting

people with type 1 diabetes require some form of emergency medical assistance (Donnelly, 2005); this is not an insignificant cost to the NHS in the UK.

Severe hypoglycaemia in type 2 diabetes has attracted much less attention, but remains a risk in those treated with insulin or sulfonylureas. This population is much larger and includes many elderly people. They may therefore have other co-morbidities such as macrovascular disease, osteoporosis, social isolation and general frailty, making occurrence of severe hypoglycaemia even more hazardous. In the UK Prospective Diabetes Study (UKPDS) (UKPDS Group, 1998), and in several observational studies of people with type 2 diabetes, hypoglycaemia was associated either with intensive treatment with insulin or with sulfonylureas (Zammitt, 2005), but the frequencies may have been underestimated. Hypoglycaemia rates were measured prospectively in the UK Hypoglycaemia Group study, which demonstrated a lower overall incidence of severe hypoglycaemia in people with insulin-treated type 2 diabetes compared to type 1 diabetes, but it increased with duration of insulin therapy (UK Hypo Group Study, 2007, Abraira, 1995, Henderson, 2003, Leese, 2003, Donnelly, 2005; see table 1.2). In the UKPDS, severe hypoglycaemia associated with sulfonylureas was reported to be low with an annual prevalence of 1% (UKPDS Group, 1998), but this was likely to be an underestimate because of the way hypoglycaemic events were recorded; in people with well-controlled type 2 diabetes in the UK Hypoglycaemia Study, the annual prevalence was 7%. Sulfonylurea-induced hypoglycaemia is probably much more common than is appreciated, particularly in an elderly population, although the degree of risk varies with different agents (Amiel, 2008). A third of episodes of severe hypoglycaemia affecting people with type 2 diabetes require emergency medical assistance (Donnelly, 2005). The estimated annual cost to the NHS in the UK of hypoglycaemia in type 2 diabetes is approximately £7.4 million (Amiel, 2008), indicating that it is not an insignificant burden.

Table 1.1: Epidemiology of severe hypoglycaemia in type 1 diabetes

Study	Severe hypoglycaemia (prevalence [%])	Severe hypoglycaemia (incidence [episodes/ patient/year])
Pramming S et al (1991)	36%	1.4
DCCT Research Group (1993)	35% men 31% women	0.19-0.62
MacLeod K M et al (1993)	29.2%	1.6
Ter Braak E W et al (2000)	40.5%	1.5
Pedersen-Bjergaard U et al (2004)	36.7%	1.3
UK Hypoglycaemia Group study (2007)	22% (<5 yrs duration) 46% (>15 yrs duration)	1.1 (<5 yrs duration) 3.2 (>15 yrs duration)

Table 1.2: Epidemiology of severe hypoglycaemia in type 2 diabetes

Study	Severe hypoglycaemia (prevalence [%])	Severe hypoglycaemia (incidence [episodes/patient/year])
Abraira et al (1995)	n/a	0.02
Henderson et al (2003)	15%	0.28
Leese et al (2003)	0.8% (oral agents) 7.3% (insulin)	0.009 (sulfonylurea) 0.005 (metformin) 0.12 (insulin)
Donnelly et al (2005)	3%	0.35
UK Hypoglycaemia Group study (2007)	7% (oral agents) 7% (<2 yrs duration) 25% (>5 yrs duration)	0.1 (oral agents) 0.1 (<2 yrs duration) 0.7 (>5 yrs duration)

1.3 Physiology of Hypoglycaemia

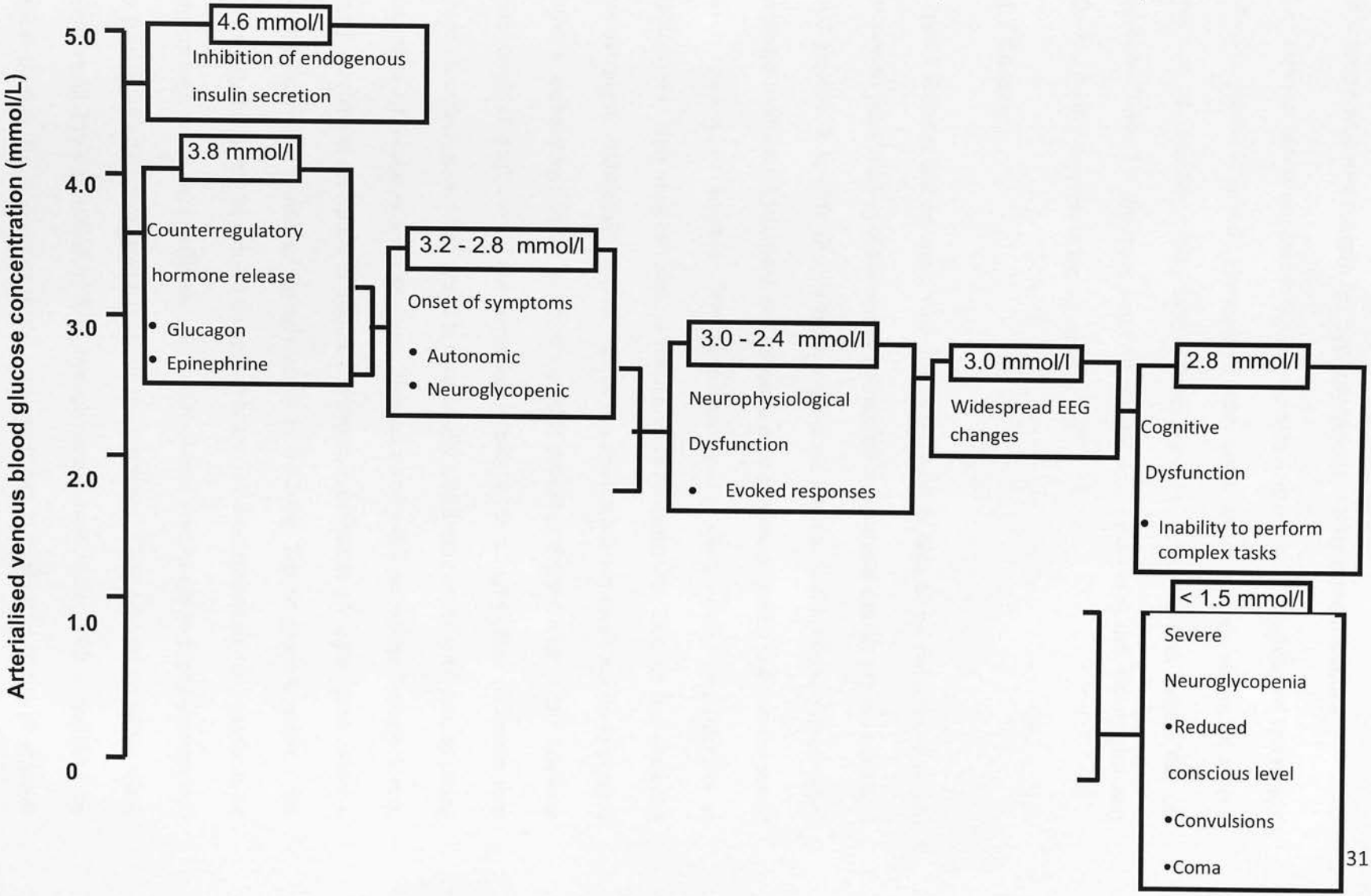
In response to a decreasing blood glucose, a well-described series of events is triggered. This includes physiological, hormonal and neuro-cognitive changes. Some of these responses create symptoms, which may alert the person with diabetes to the impending danger and allow them to take action to restore their blood glucose (figure 1.1).

Counterregulatory hormonal secretion occurs in response to evolving hypoglycaemia. Glucagon and epinephrine are the most prominent in restoring blood glucose to normal, and their secretion follows suppression of endogenous insulin secretion. The glucagon response to hypoglycaemia is attenuated within five years of developing type 1 diabetes, and the epinephrine response may also become attenuated with increasing duration of the disorder (Gerich, 1973, Bolli, 1983). These counterregulatory hormonal deficiencies interfere with normal recovery of blood glucose and can promote prolonged hypoglycaemia in people with diabetes (Thompson, 1993). The same deficiency in glucagon production has been reported in people with advanced type 2 diabetes (Bolli, 1984, Segel, 2002), although there has been some conflicting evidence in this regard (Heller, 1987[1], Levy, 1998). The epinephrine response appears to be preserved in type 2 diabetes (Heller, 1987[2], De Galan, 2001). Secretion of cortisol and growth hormone in response to hypoglycaemia also helps to restore glucose to normal.

Neuroglycopenia is the direct consequence of hypoglycaemia on the brain (Cryer, 2002). It triggers various responses including activation of central autonomic centres. This then stimulates the peripheral sympatho-adrenal system and creates a further series of physiological events. Many of these autonomic responses are manifested as symptoms such as sweating, tremor, palpitation, hunger and feelings of anxiety (McAulay, 2001[1]). Cognitive dysfunction is experienced in a number of ways, including difficulty concentrating, drowsiness and inco-ordination (McAulay, 2001[1]). Most cognitive modalities are impaired

when arterialised blood glucose concentration falls below 2.8 mmol/l. Simple motor function and reaction time tests are preserved, while tests incorporating speed, complexity, or demand careful attention are affected most (Deary, 1993[1]). Perception of any of these symptoms at an early stage can alert the individual to the development of hypoglycaemia and prompt them that immediate action is required to treat the low blood glucose and restore blood glucose to normal.

Figure 1.1: Hierarchy of hormonal and physiological responses to hypoglycaemia in humans. Reproduced from Frier, 2007.



1.4 Causes and risk factors for hypoglycaemia – Why does it occur?

Many different factors can contribute to the development of hypoglycaemia, and several may underlie a particular episode. However, in many severe hypoglycaemic events, no specific cause can be identified (Frier, 1993). There are both causes and risk factors that can contribute. Table 1.3 illustrates many of the potential causes and risk factors that may influence the development of hypoglycaemia.

1.4.1 Causes

In type 1 diabetes and advanced type 2 diabetes, there is little or no endogenous insulin secretion so there is a loss of the natural homeostatic mechanisms usually present to control blood glucose. It is difficult to achieve appropriate plasma insulin concentrations when exogenous insulin is administered, and of course once exogenous insulin is given, the rate of delivery cannot be adjusted. Hypoglycaemia occurs when insulin concentration is inappropriately high when compared to blood glucose concentration. This can be a particular issue overnight. Additionally, hypoglycaemia can result when too much insulin is injected relative to carbohydrate intake, or when a meal is missed or delayed after insulin has been administered. Hypoglycaemia can occur accidentally when a higher dose of insulin than intended is administered in error, and is occasionally a deliberate act of self-harm, by taking an overdose of insulin or a sulfonylurea. Physical activity can precipitate hypoglycaemia, through accelerated absorption of insulin, and depletion of muscle glycogen stores. Alcohol enhances the risk of prolonged hypoglycaemia by inhibiting hepatic gluconeogenesis, and the hypoglycaemia may be delayed for several hours. The development of other autoimmune disorders such as adrenal insufficiency or coeliac disease predisposes to hypoglycaemia and may present with an unexplained increase in its occurrence. There are also occasions when insulin sensitivity is increased, making hypoglycaemia more likely, such as weight loss, physical training, the postpartum period, and sometimes in relation to hormonal changes

during the menstrual cycle. The duration of action of insulin may also be prolonged, as in renal failure, by reducing the rate of clearance of insulin from the circulation.

1.4.2 Risk factors

1.4.2.1 Intensive glycaemic control

The risk of hypoglycaemia is increased when insulin therapy is intensified in an attempt to meet glycaemic targets for type 1 and insulin-treated type 2 diabetes. Severe hypoglycaemia was increased threefold following the institution of intensive insulin regimens in the DCCT (DCCT Group, 1993). Exposure to antecedent episodes of hypoglycaemia can diminish the counterregulatory hormonal response to subsequent hypoglycaemia, and therefore blunt the intensity of symptoms perceived by the patient (Heller, 1991). The glycaemic threshold for epinephrine release is usually at around 3.5 mmol/l (arterialised blood glucose), and 3.0 mmol/l for the onset of autonomic symptoms. However, recurrent episodes of hypoglycaemia can re-set these thresholds at lower blood glucose levels. This leaves a smaller window of opportunity to take corrective action between the onset of symptoms and the development of significant cognitive impairment, so increasing the risk of severe hypoglycaemia. Counterregulatory hormonal deficiencies and impaired awareness of hypoglycaemia often develop when HbA1c declines to near the non-diabetic range (Kinsley, 1995).

Table 1.3: Causes of and risk factors for hypoglycaemia in insulin-treated diabetes

CAUSES	RISK FACTORS
Inadequate dietary carbohydrate a. Unexpected physical exertion b. Social: sport, training, travel, change of occupation c. Dieting /eating disorders d. Breast feeding e. Malabsorption (Coeliac disease) f. Gastroparesis (autonomic neuropathy) Changes in insulin sensitivity/bioavailability a. Acute remission in newly diagnosed diabetes following treatment (honeymoon period) b. Post-delivery in diabetic pregnancy c. Menstruation (variable effects) d. Renal impairment/failure e. Effects of exercise Change in insulin pharmacokinetics a. Change of insulin formulation b. Change of insulin injection site c. Effects of temperature (e.g. hot bath, sauna) Other related conditions a. Endocrine failure (Adrenal insufficiency, hypopituitarism, hypothyroidism) b. Factitious insulin administration	Duration of diabetes Age Strict glycaemic control Impaired awareness of hypoglycaemia History of previous severe hypoglycaemia Sleep Renal impairment C-peptide negativity

1.4.2.2 Duration of diabetes

Increasing duration of diabetes is associated with an increasing frequency of severe hypoglycaemia (SH) (Pramming, 1991). SH is relatively uncommon in the early stages of type 1 and insulin-treated type 2 diabetes because there is usually some residual capacity for endogenous insulin secretion, which is suppressed when blood glucose falls (Davis, 1997). Additionally, in the early stages of type 1 diabetes, a period of remission often occurs, with partial recovery of pancreatic beta cell function, which may necessitate a reduction of insulin dose to avoid hypoglycaemia (the honeymoon period). As progressive beta cell failure ensues, this is accompanied by an increment in the frequency of SH. This is partly because the plasma insulin concentration is then determined by the absorption and dose of exogenous insulin, and is partly associated with reduction of the counterregulatory hormonal mechanisms, which diminish with time; in particular the glucagon response is attenuated within five years of diagnosis in type 1 diabetes (Gerich, 1973, Bolli, 1983).

1.4.2.3 Extremes of age

Severe hypoglycaemia is more common at extremes of age - in very young children and in the elderly. Young children are unable to either recognise or interpret the warning symptoms of hypoglycaemia, and hypoglycaemia may be promoted by irregular eating patterns, erratic physical activity and long periods of sleep. In the ageing population, neurological and non-specific symptoms are prominent (Jaap, 1998), which may cause confusion with the identification of hypoglycaemia. The ageing brain may be more susceptible to the effects of hypoglycaemia, while renal impairment becomes more common with age, and increases the risk of hypoglycaemia (Rabkin, 1984, Muhlhauser, 1991[1]).

1.4.2.4 Impaired awareness of hypoglycaemia

Any condition that reduces the symptomatic awareness of hypoglycaemia and the ability to take early action to treat and reverse hypoglycaemia in response to symptoms will increase the risk of severe hypoglycaemia. This includes sleep when the intensity of warning symptoms is reduced because the sympatho-adrenal response to hypoglycaemia is attenuated and epinephrine secretion is much lower (Jones, 1998). Symptoms are also diminished when lying flat (Hirsch, 1991). Antecedent hypoglycaemia reduces the counterregulatory hormonal response to a subsequent episode of hypoglycaemia (Heller, 1991), and can induce impaired awareness of hypoglycaemia (IAH); recurrent hypoglycaemia during sleep has been implicated in the development of this syndrome (Veneman, 1993). IAH becomes more common with increasing duration of diabetes (Pramming, 1991), and with more frequent exposure to hypoglycaemia, thus creating a vicious circle of recurrent hypoglycaemia. This condition affects 20-25% of people with type 1 diabetes (Hepburn, 1990, Muhlhauser, 1991[2]), but is less common in insulin-treated type 2 diabetes, where the reported prevalence is around 8-9% (Henderson, 2003, Schopman, 2010). Impaired awareness of hypoglycaemia can be very debilitating, with some people being so severely affected that they experience no warning symptoms and can progress rapidly to unconsciousness. Others who are less severely affected have an increasing reliance on neuroglycopenic symptoms or the assistance of relatives and friends to recognise hypoglycaemia. Maintenance of strict glycaemic targets in this group of patients may promote recurrent severe hypoglycaemia and targets should therefore be modified.

1.5 Management of hypoglycaemia

Mild hypoglycaemia is defined by the ability of the person experiencing the episode to self-treat. Successful treatment of hypoglycaemia relies on early detection of a fall in blood glucose to allow prompt self-treatment and prevent progression to more severe hypoglycaemia. Over-treatment of hypoglycaemia will encourage rebound hyperglycaemia and promote weight gain through the consumption of additional carbohydrate. This can also result from prophylactic eating which is undertaken to prevent the initial fall in blood glucose. An algorithm summarising the treatment of acute hypoglycaemia is shown in table 1.4 (MacCuish, 1993).

Table 1.4: Treatment of acute hypoglycaemia. Adapted from MacCuish, 1993.

Duration of hypoglycaemia Minutes → Hours				
By patient	By family	Primary/ paramedical care	Emergency department	In hospital (ITU – cerebral oedema)
Oral carbohydrate (>15g)	Oral carbohydrate (liquid/solid) *or* 1mg glucagon *plus* Oral carbohydrate 20-40g when fully conscious	1mg glucagon IM or IV *or* 25g dextrose IV	25g dextrose IV *or* 1mg glucagon IV *plus* Oral carbohydrate when fully conscious	Mannitol (20% solution, 200ml) Dexamethasone (16-24mg/day) *plus* High flow O2 Anticonvulsants Sedation Dextrose/insulin infusion Potassium infusion

1.5.1 Treatment of acute hypoglycaemia

1.5.1.1 Oral carbohydrate

Many guidelines recommend that 10-15 grams of carbohydrate should be consumed (3 to 5 glucose tablets), followed by some form of starchy carbohydrate to prevent a recurrence of hypoglycaemia (Brodows, 1984). Fifteen grams of oral fast-acting carbohydrate will raise blood glucose by 2.1 mmol/l within 20 minutes (Brodows, 1984). It has been recognised that, while sufficient quantities of orange juice and glucose gel will provide at least 15 grams of carbohydrate, these agents may not provide a very rapid rise in blood glucose, and therefore were not recommended as first-line treatment by the authors of a study assessing available methods of treatment (Slama, 1990). In real life, however, it is generally found that fresh orange juice is effective in treating mild hypoglycaemia. It has been suggested that a supplementary snack to follow the acute treatment should consist of a combination of carbohydrate and protein, but in a comparative study, a protein-enriched snack appeared merely to add calories rather than providing any longer-term protection against a recurrence of hypoglycaemia (Gray, 1996). After consuming the appropriate amount of carbohydrate, the blood glucose should be retested after 15 minutes to confirm that it has risen and by how much, then a further 15 grams of carbohydrate should be consumed if the blood glucose remains below 4 mmol/l (Brodows, 1984). By contrast, when a patient is receiving continuous subcutaneous insulin infusion therapy via a pump, only rapid-acting carbohydrate is required to treat the low blood glucose, and a snack containing complex carbohydrate is not required, as the insulin infusion can be temporarily discontinued.

In severe hypoglycaemia, the nature of the treatment that is required will depend on the conscious level of the individual. If an individual is fully conscious and can swallow, then oral carbohydrate can be administered, but may be refused or resisted if confusion is present. The risk of aspiration is also a concern in a drowsy individual. If oral carbohydrate can be

administered safely the recommended amount is 20 grams. This should produce a rise in blood glucose of approximately 3.6 mmol/l over 45 minutes (Brodows, 1984). If, at 15 minutes, the blood glucose remains below 4.0 mmol/l, a further 15 grams of carbohydrate should be consumed (Brodows, 1984).

1.5.1.2 Glucagon and intravenous dextrose

In any person whose conscious level is reduced, parenteral treatment is necessary. Glucagon, administered either intramuscularly or subcutaneously in a dose of 1mg (except children <5 yrs old who should receive 0.5 mg), or intravenous dextrose can be given. Family members, friends and colleagues can be taught to administer glucagon. This either restores consciousness or at least prevents progression to more profound coma, and may avoid the occurrence of a seizure or serious injury while waiting for emergency help to arrive. Glucagon can increase the blood glucose from between 3 to 12 mmol/l over the course of 60 minutes (Muhlhauser, 1985), and while usually effective, is slower to raise the blood glucose than intravenous dextrose (Collier, 1987[1]). It acts by mobilising glucose from the liver and by stimulating hepatic glycogenolysis. Treatment with glucagon can induce nausea and vomiting.

Occasionally, administration of glucagon is ineffective in patients who have protracted hypoglycaemia and have exhausted stores of hepatic glycogen. This may also occur in people with cachexia, anorexia nervosa, advanced malignancy or severe liver disease (Dewan, 2004). Intravenous glucose may be required to treat severe hypoglycaemic episodes that fail to respond to glucagon, or to treat severe iatrogenic hypoglycaemia, particularly when this has been caused by a long-acting sulfonylurea. Initially 25g of dextrose (50ml of 50% dextrose) should be administered, but in the case of prolonged hypoglycaemia a continuous infusion of glucose and frequent oral feeding will be required (Krentz, 2005). A 50% solution of dextrose is very irritant to veins and leakage can cause localised thrombosis

and extravascular tissue damage. As a consequence, 20% dextrose is now recommended in hospital practice. A study comparing intravenous glucagon with intravenous dextrose as the treatment of severe hypoglycaemia in a hospital emergency department showed that intravenous glucagon is a satisfactory alternative to dextrose, being easier to administer with very little risk of thrombosis or extravascular complications, although it takes longer to restore normoglycaemia (Collier, 1987[1]).

1.5.1.3 Management of severe hypoglycaemia/coma in hospital

If a person remains in a hypoglycaemic coma that has not responded to intravenous dextrose, hospital admission to an intensive care unit is essential. Neuroimaging must be performed to look for cerebral oedema, and to exclude other intracranial pathologies. Cerebral oedema is a dangerous complication of severe hypoglycaemia and is associated with a high mortality rate. Treatment includes intravenous mannitol and high dose corticosteroids, with other supportive measures such as high flow oxygen, sedation, and anticonvulsant therapy if indicated (MacCuish, 1993). People may not recover from hypoglycaemic coma until several days have passed, so it is difficult to gauge for how long treatment should be continued. If recovery occurs after prolonged unconsciousness, permanent neurological sequelae may be evident. Measurement of markers of acute neuronal damage such as neurone-specific endolase (NSE) and protein S100 have been advocated as a prognostic aid, as serum levels have been shown to rise acutely in fatal cases within 24-48 hours of the onset of hypoglycaemic coma (Strachan, 1999).

1.5.1.4 Sulfonylurea-induced hypoglycaemia

Severe hypoglycaemia secondary to long-acting sulfonylureas may be prolonged and is likely to be more dangerous in the elderly, particularly if they are frail. Severe hypoglycaemia in these circumstances may not respond to glucagon, and should be treated with intravenous dextrose followed by continuous glucose infusion, which may have to be

maintained for several hours or even days (Krentz, 2005). There is little evidence to support the suggestion that glucagon is contraindicated in the treatment of sulfonylurea-induced hypoglycaemia on the basis that it might encourage further secretion of endogenous insulin (Taylor, 1978). Those affected may require hospital admission to ensure that hypoglycaemia does not recur. Octreotide may be a useful adjunct in the treatment of refractory sulfonylurea-induced hypoglycaemia (Krentz, 1993).

1.5.1.5 Conclusion

Any guidelines proposed for the management of acute hypoglycaemia need to be flexible in order that treatment can be tailored to individual situations. With experience, many people learn how much carbohydrate they require to ingest to treat an isolated episode of mild hypoglycaemia, and this may be less or more than would be dictated if strict guidelines were to be followed. However, the suggested amount of carbohydrate can be used as a starting point, and as experience is gained, self-management can be modified by the individual to suit the severity and circumstances of an event, and their individual treatment regimen, therefore avoiding potential under- or over-treatment of the episode.

1.5.2 Treatment of chronic and recurrent hypoglycaemia

The treatment of an acute episode of hypoglycaemia must include an analysis of why the episode occurred and how it could be avoided in future. Prevention of hypoglycaemia is an important aspect of management because chronic fear of hypoglycaemia is a major limiting factor in the maintenance of optimal glycaemic control.

1.5.2.1 Dietary measures

Modification of diet in the longer term can influence the propensity to develop hypoglycaemia. It has been demonstrated that maintaining a high fibre, low glycaemic index

diet can both improve glycaemic control and reduce the frequency of hypoglycaemic events (Giacco, 2000).

1.5.2.2 Dealing with risk factors

The possible contribution of the previously described causes and risk factors should be considered for each individual patient to identify those at higher risk, and to assist with modification of those factors in order to avoid severe hypoglycaemia. Elderly people with diabetes who are receiving treatment with insulin or insulin secretagogues are at high risk of developing hypoglycaemia, and in these patients, less strict targets for glycaemic control may be appropriate (American Diabetes Association, 2007). This is especially important when considering the problems associated with hypoglycaemia in people who have multiple co-morbidities, including some which may also be risk factors for hypoglycaemia (such as renal impairment). In addition, many elderly people live alone, and they may not have family support at home to help treat a hypoglycaemic episode should early warning symptoms be missed. Patients should be asked routinely about exposure to hypoglycaemia, the symptoms they experience, and how easily these are identified and treated.

1.5.2.3 Impaired awareness of hypoglycaemia

A few studies have shown that in people with impaired awareness of hypoglycaemia, hypoglycaemic symptoms can be restored following a period of scrupulous avoidance of hypoglycaemia (Fanelli, 1993). Alternatively, by reducing insulin doses and relaxing glycaemic control, HbA1c can be allowed to rise (Liu, 1996). If hypoglycaemia awareness can be restored, it may be possible to re-establish better glycaemic control with a lower risk of severe hypoglycaemia subsequently. This restoration of symptoms is not guaranteed with these measures, however, particularly in those people who have chronic impaired awareness of hypoglycaemia, suggesting that they have suffered permanent dysfunction of blood glucose sensing within the brain. In this situation, acceptance of less strict glycaemic targets,

as with the elderly, is an appropriate strategy, and intensive insulin therapy should be avoided. Asymptomatic biochemical hypoglycaemia is more common in affected patients, so frequent home blood glucose monitoring is essential to detect an early fall in blood glucose to allow time for effective intervention. Re-education using structured education programmes can also be beneficial in this group, and these are discussed later in this chapter.

1.5.2.4 Sleep

To avoid the risk of developing nocturnal hypoglycaemia, patients should be advised to measure their blood glucose before they go to bed, as there is considerable risk of this occurring if the bedtime blood glucose is <6.0 mmol/l. A prophylactic bedtime snack can be taken if necessary, but is unlikely to last throughout the night (Raju, 2006, Kalergis, 2003). Inappropriate hyperinsulinaemia during the night is the underlying problem, so changes to the therapeutic regimen may be required to reduce the risk of developing nocturnal hypoglycaemia. Delaying administration of evening isophane insulin until bedtime can reduce the risk of nocturnal hypoglycaemia, and the use of a rapid-acting insulin analogue with the evening meal instead of using soluble insulin (Mohn, 1999) can lower the risk of hyperinsulinaemia in the early part of the night. Using a long-acting insulin analogue, injected in the morning, as the basal insulin may confer less risk of inducing nocturnal hypoglycaemia (Pieber, 2000), mainly because the duration of action is usually less than 24 hours. Continuous subcutaneous insulin infusion (CSII) also reduces nocturnal hypoglycaemia, as the nocturnal insulin requirement can be tailored using a variable rate of insulin infusion (Kanc, 1998). Pharmaceutical interventions could potentially reduce the risk of nocturnal hypoglycaemia, but remain in the experimental phase. Inhaled β-agonists such as terbutaline have been proposed as a method of increasing blood glucose overnight, and studies have shown that blood glucose rises for several hours following administration (Wiethrop, 1993). Unfortunately this may cause an elevated fasting blood glucose and its potential as a treatment option is unproven.

1.5.2.5 Exercise

Hypoglycaemia is a potential risk of strenuous exercise and is determined by the plasma insulin concentrations at the time of exercise. Hypoglycaemia occurs when plasma insulin concentrations are elevated; conversely hyperglycaemia may occur when plasma insulin is low. In addition, the nature of the exercise influences the propensity to develop hypoglycaemia, with an increased risk being more likely with exercise of long duration and high intensity. This is not always predictable, and can be influenced by other factors. The timing of exercise in relation to food intake and insulin doses has to be considered. Insulin doses may need to be reduced, or the timing altered, to avoid experiencing the peak of insulin action at the time of exercise. If prolonged exercise over several hours is planned, insulin dosage should be reduced and slowly absorbed carbohydrate should be consumed to reduce the likelihood of developing hypoglycaemia. Exercise of short duration and high intensity requires a different approach, with reduction of the preceding dose of rapid-acting insulin, and prophylactic consumption of some form of short-acting carbohydrate. Counterregulatory hormonal responses to hypoglycaemia are attenuated following antecedent exercise, and conversely antecedent hypoglycaemia reduces the counterregulatory response to subsequent exercise (Galassetti, 2001, Galassetti, 2003). This may potentiate the problem of exercise-related hypoglycaemia.

1.5.3 Education

Educating patients about all aspects of their diabetes care is of vital importance to their self-management of hypoglycaemia. This should include information regarding carbohydrate intake, both prophylactically to avoid hypoglycaemia, and for its immediate treatment, and also in relation to adjustment of insulin and dosage of oral medication, alcohol intake, exercise and driving. Review of this information should be routine practice in ongoing care. Lack of understanding of their therapeutic regimen and poor knowledge of the symptoms of

hypoglycaemia and how it should be treated may contribute to recurrent problems with hypoglycaemia (Sumner, 2000, Thomson, 1991, Mutch, 1985). Structured education programmes, such as DAFNE (Dose Adjustment for Normal Eating) or DESMOND (Diabetes Education and Self-Management for Ongoing and Newly Diagnosed), are now being adopted more widely to empower patients to self-adjust their treatment regimens in an effort to improve glycaemic control, and to promote flexible attitudes to Diabetes. DAFNE incorporates adjustment of insulin dose to match carbohydrate intake, and can help to minimise risk of hypoglycaemia although appears to be less effective in lowering HbA1c (DAFNE Study Group, 2002, Davies, 2008, Sämann, 2006). It follows a five day format for groups of patients, and has a curriculum covering education and training about dietary flexibility and insulin dose adjustment. DESMOND focuses on the diagnosis of Type 2 Diabetes, with initiation of the appropriate self-management skills. Structured education has been shown to decrease the risk of hypoglycaemia, but the resources required are significant, meaning it is not an option available to all, particularly in developing countries.

Family members, friends and colleagues also require education about hypoglycaemia and its treatment, particularly in an emergency situation. Knowledge of the symptoms of hypoglycaemia will help them to identify the onset of hypoglycaemia, and enable them to undertake emergency treatment, and they should be instructed how to administer glucagon to treat severe hypoglycaemia, which can be stored at home or in the workplace.

Specific training to improve the detection and avoidance of hypoglycaemia can improve overall glycaemic control, and reduce the risk of future hypoglycaemia. Blood Glucose Awareness Training (BGAT) is one such programme, developed in the USA to help people with impaired awareness of hypoglycaemia. The programme involves educating people about the identification and interpretation of physical symptoms, performance and mood changes as internal cues to blood glucose awareness. In addition, patients are advised about the influence of food, exercise, insulin doses and action profiles, time of day and previous

blood glucose values as external cues to help them determine the current level of blood glucose. It can be utilised when an intensified insulin regimen increases the frequency of hypoglycaemia and induces impaired hypoglycaemia awareness to improve the accuracy of estimating blood glucose, and BGAT has been shown to reduce the frequency of undetected biochemical hypoglycaemia (Cox, 1985, 1988, 1989, 1991). It also prevents further counterregulatory deficiencies and may modify the severity of hypoglycaemia associated with improved glycaemic control (Cox, 2001, Kinsley, 1999). However, a trial of a modified version of the BGAT programme in a European population suggested that the programme may not be so effective outside of the USA (Broers, 2002).

Another training programme called HyPOS has been developed in Germany to assist people who are suffering from recurrent severe hypoglycaemia. When compared with standard methods of education and optimisation of insulin regimens, HyPOS has been shown to improve hypoglycaemia awareness, promote the effective treatment of hypoglycaemia, and reduce the overall risk of hypoglycaemia (Hermanns, 2007). HyPOS focuses on avoiding low blood glucose values, on informing patients fully about the causes of impaired hypoglycaemia awareness, and attempts to modify health beliefs as these could be contributing to frequent hypoglycaemia by perpetuating the vicious cycle of recurrent episodes. People are taught how to detect falls in blood glucose more effectively, and how to treat them promptly. A further five day intensive in-patient education programme, devised in Germany, was shown to reduce the incidence of severe hypoglycaemia over a prolonged follow up period, while simultaneously improving HbA1c (Bott, 1997). Unfortunately, the extensive clinical resources that are required to operate these educational interventions are not available in many countries, as is the case for the general education strategies discussed previously.

1.5.4 Blood glucose monitoring

Measuring home blood glucose levels is necessary for effective management of insulin-treated diabetes, and is particularly valuable for the detection of asymptomatic biochemical hypoglycaemia. It allows both patients and healthcare professionals to review the results, recognise fluctuations and glycaemic patterns, and identify hypoglycaemia recurring at particular times of day. This assists with appropriate adjustment of therapeutic regimens. For people at higher risk of hypoglycaemia, such as those with impaired awareness of hypoglycaemia, frequent blood glucose monitoring may be the only way to identify asymptomatic biochemical hypoglycaemia and provide adequate warning to enable action to be taken. Monitoring blood glucose before, and during, demanding tasks such as driving and exercise can help to avoid hypoglycaemia at potentially dangerous times (Cox, 2000). However, this very much depends on how often blood glucose is measured, and testing at some times of day, such as during the night, may be impractical. Frequent home blood glucose monitoring may be considered unacceptable by the patient as it is invasive and can be painful, and it only provides single time point information, with no guidance to trends or direction and speed of change of glucose concentration (Gomis, 2004).

Continuous glucose monitoring systems (CGMS) measure glucose in interstitial fluid, and may be useful in people at increased risk of hypoglycaemia, as it can be employed to detect asymptomatic hypoglycaemia, and to observe fluctuations in glucose that may be missed by single point home testing. This is most valuable when the results are available in real time, which is possible with some systems. However, CGMS does have limitations. Nocturnal measurements may indicate a lower interstitial glucose value than the equivalent capillary blood glucose sample, thus giving the impression that hypoglycaemia is more prolonged than in reality (Wentholt, 2007, Kubiak, 2004). Continuous glucose monitoring systems have been shown to be useful in detecting problems with hypoglycaemia awareness, and in

reassessing these people following therapeutic interventions to try to restore their hypoglycaemic symptoms (Kubiak, 2004).

1.5.5 Modifying treatment strategies

Modification of a patient's insulin regimen may be necessary to reduce the frequency of hypoglycaemia. The time-action profile of particular insulins in different regimens has to be considered, and in some cases the use of an alternative insulin with a different time-action profile may be necessary. With a basal-bolus regimen, fasting hypoglycaemia implicates the effect of the long or intermediate-acting insulin preparation, administered the previous day. Daytime hypoglycaemia may be caused by either the rapid-acting or long-acting insulins, depending on the regimen used. If pre-prandial soluble (regular) human insulin is being used, changing to a rapid-acting analogue insulin may reduce daytime hypoglycaemia (Heller, 1999). The risk of nocturnal hypoglycaemia may be diminished by substituting the evening dose of intermediate-acting insulins such as isophane (NPH), or pre-mixed insulin formulations (30% rapid-acting/70% intermediate-acting or 50% rapid-acting/50% intermediate-acting for example), with a long-acting insulin analogue such as insulin glargine or insulin detemir (Home, 2004, Ashwell, 2006).

In people with type 2 diabetes, several new pharmacological therapies, including the incretin-mimetic class of agents, have a lower risk of hypoglycaemia in comparison with the sulfonylureas, especially when they are combined with other oral agents or insulin. Analogue insulins are also beneficial in lowering hypoglycaemia risk in type 2 diabetes. This can consequently reduce both the patient's and the clinician's fear of hypoglycaemia and make achievement of glycaemic targets more realistic (Boyle, 2008).

In cases of intractable recurrent hypoglycaemia, where the problem persists regardless of the insulin regimen being used, continuous subcutaneous insulin infusion (CSII) therapy may be beneficial, allowing greater flexibility with adjustment of basal insulin rates and prandial

bolus doses as necessary (Boland, 1999). A pilot study in people with type 1 diabetes who had impaired awareness of hypoglycaemia and recurrent severe hypoglycaemia compared the use of education alone in patients on conventional insulin regimens, with education plus multiple daily injections using analogue insulins, or education plus CSII using insulin pumps. Prevention of severe hypoglycaemia and restoration of hypoglycaemia awareness appeared to be possible while maintaining optimal glycaemic control, either with multiple injections of insulin analogues or with CSII (Thomas, 2007). A meta-analysis of trials assessing the impact of CSII on severe hypoglycaemia rates has shown that CSII significantly reduces the risk of severe hypoglycaemia when compared with multiple injection therapy (Pickup, 2008).

1.5.6 Future developments in treatment

Methods of improving recovery from hypoglycaemia have been explored using agents such as the amino acid, alanine, and the beta-2 agonist, terbutaline, and the effects of caffeine on enhancing symptom awareness. A suitable form of these pharmacological treatments may be useful to treat and prevent mild to moderate hypoglycaemia (Raju, 2006, Wiethrop, 1993, Kerr, 1993). Selective serotonin reuptake inhibitors (SSRIs) have also been investigated as a possible method of improving counterregulatory responses (Briscoe, 2008).

The use of continuous subcutaneous insulin delivery systems and real time CGMS are likely to increase. Hypoglycaemia detection should improve as a consequence of better glucose sensing technology, as users will receive more detailed and more frequent information about their blood glucose than can be provided by intermittent single-point testing. This will allow greater flexibility in the adjustment of basal rates and bolus doses of insulin, particularly as real-time glucose sensors become more readily available for routine clinical use (Klonoff, 2005). Real-time sensors will not only improve hypoglycaemia detection but will allow patients more freedom in their daily lives (Klonoff, 2005) and should help to reduce fear of

hypoglycaemia. The coupling of CSII with a real time glucose sensing system will allow the development of an effective closed loop system to deliver insulin. At present the limiting factor is the availability of an inexpensive and reliable glucose sensor. A cheap and effective glucose sensor with an alarm system would be of great help in detecting asymptomatic nocturnal hypoglycaemia.

Islet cell transplantation is a promising avenue in the treatment of recurrent severe hypoglycaemia unresponsive to analogue multiple injection therapy or CSII. Follow up data after five years has shown that even when a person is no longer insulin independent, the lower risk of severe hypoglycaemia is maintained (Shapiro, 2006). This, however, is an expensive treatment with very limited availability and the need for long term immunosuppression, and further investigation is needed prior to consideration of more widespread, routine use.

Despite these promising developments, hypoglycaemia remains a major limiting factor in the effective management of insulin-treated diabetes, with significant morbidity, so treatment and prevention are fundamental components of any therapeutic regimen.

1.6 Hypoglycaemia and cognitive function

1.6.1 General cognitive function

Hypoglycaemia has a detrimental effect on cognitive function, as the human brain relies solely on glucose as its source of energy. This decline in cognitive function may be perceived as symptoms by the person with diabetes, and may be their first warning to evolving hypoglycaemia if they have impaired hypoglycaemia awareness (Deary, 2007).

In order to characterise this effect in more detail, there have been many studies of experimental hypoglycaemia, assessing many different facets of cognition. Early studies

showed that at blood glucose concentrations of less than 3.0 mmol/l, there was a reduction in fine motor skill, mental speed, concentration and memory function (Deary, 2007). These early studies were performed using the insulin infusion technique to induce hypoglycaemia; this method results in variable insulin concentration, with a variable blood glucose nadir, making interpretation and comparison of results challenging. Since then, developments in the field of hypoglycaemia research, and attention to safety, mean that most experimental hypoglycaemia is induced by the hyperinsulinaemic clamp technique (DeFronzo, 1979). This doesn't accurately mimic the hypoglycaemia experienced in day-to-day life, in the way that the infusion method is more likely to, but it is a safe and effective method of inducing a decline in blood glucose which can be stabilised for a reasonable time period, allowing in-depth study of cognitive performance. Reproducible blood glucose target levels can also be achieved by this method, whereas variable levels will be achieved using alternative strategies such as the insulin infusion method.

Studies performed using the hyperinsulinaemic clamp technique have shown that at blood glucose concentrations between 3.1 and 3.4 mmol/l, there is a decrease in reaction times, slowing of mental arithmetic, impairment of verbal fluency and impairment of performance on parts of the Stroop test. There appears to be sparing of simple motor and sensory skills, and the speed of reading out loud (Deary, 2007). Even amongst studies using the same technique to induce hypoglycaemia, there remains heterogeneity between the studies, including the glycaemic target, the actual cognitive test battery used, whether there is a euglycaemic control arm to the study, and the baseline cognitive abilities of the subjects participating in the studies. There is no consensus on which cognitive tests are the best ones to identify a cognitive decline during experimental hypoglycaemia. Many of the tests used do not assess one pure domain of cognitive function, but encompass many different facets of cognition. In addition, the experimental setting of a clamp study is clearly an artificial one, which may affect a person's cognitive performance on the tests administered. The subjects

are stationary in a bed or chair, with infusions being administered through a cannula in one arm, and with blood sampling occurring regularly, which may discourage people from free movement and may affect their results on some tests.

A summary of studies assessing cognitive function in adults with type 1 diabetes is shown in table 1.5. There have also been many assessments of cognitive function in non-diabetic adults, which have shown similar findings in the majority of tests, although some aspects of psychomotor function were affected in the non-diabetic cohort but not the subjects with diabetes, suggesting a possible element of cerebral adaptation in those who have previous experience of hypoglycaemia (Geddes, 2008). Conversely, an early study by insulin infusion that had a mean glucose nadir of 2.0mmol/l revealed bigger decrements in cognitive function in those with diabetes than the non-diabetic subjects. It was postulated that this may have been due to the bigger drop in blood glucose experienced by those with diabetes (Wirsen, 1992).

Table 1.5: Summary of studies assessing cognitive function during hypoglycaemia in people with type 1 diabetes

Study	Method	Control Arm (euglycaemia)	Target Glucose Level (mmol/l)	Tests Affected	Tests Preserved
Holmes 1983	Infusion	No	3.3	Visual reaction time, simple addition	Reading, comprehension
Holmes 1984	Infusion	No	3.0	Naming and labelling skills	Word recognition
Holmes 1986	Infusion	No	3.0	Choice reaction time (CRT)	Simple motor and perceptual skills
Hoffman 1989	Infusion	No	2.8	Visual tracking, visuo-motor speed, concentration, planning ability	Visual reaction time
Widom 1990	Clamp	No	2.2	Visuo-spatial skills, visuo-motor skills, global cognition	
Wirsen 1992	Infusion	No	2.0	Reaction time, verbal fluency, short term memory	
Ewing 1998	Clamp	Yes	2.6	Visual information processing, contrast sensitivity	Visual acuity
McAulay 2001[2]	Clamp	Yes	2.6	Visual attention, auditory attention, attentional flexibility, speed of Information Processing	Sustained attention, intelligence scores
Sommerfield 2003[2]	Clamp	Yes	2.5	Immediate verbal memory, immediate visual memory, working memory, delayed memory	
Strachan 2003	Clamp	Yes	2.6	Simple auditory processing, auditory temporal processing, N240 potential amplitude	N100, P200, P300 event related potentials
Warren 2004	Clamp	Yes	2.6	Non verbal intelligence	
Warren 2007	Clamp	Yes	2.5	Prospective memory, immediate and delayed recall	Visual memory
Geddes 2008	Clamp	Yes	2.5	4CRT, pursuit rotor (fine motor, attention and co-ordination)	Hand grip, hand steadiness, total body co-ordination, line tracing time

It therefore appears that hypoglycaemia has a pronounced effect on complex cognitive tasks both in diabetic and non-diabetic humans, whereas simple mental tasks are less affected (Deary, 2007). On review it appears that this generally occurs at blood glucose levels below 3.0 mmol/l (Hoffman, 1989, Mitrakou, 1991, Widom, 1990, Wirsen, 1992, McAulay, 2001[2], Sommerfield, 2003[1], Sommerfield, 2003[2], Warren, 2004). This relative preservation of simple motor tasks may be explained by a functional MRI study which examined the effects of hypoglycaemia on brain function. There was a decrease in blood oxygen level-dependent (BOLD) activation in the motor area of the brain on MRI scanning when motor tasks were being performed (Rosenthal, 2001). Simple motor tasks such as finger tapping resulted in smaller areas of BOLD activation as compared with more complex tasks, such as the four choice reaction test (4CRT). This may reflect the increased cognitive load required with more complex tasks such as the four choice reaction test.

The recovery of different aspects of cognitive function varies from between 40 and 90 minutes following restoration of blood glucose to normal (Deary, 2007, Zammitt, 2008). This has particular relevance to the task of driving in daily life for people with diabetes. The Driver and Vehicle Licensing Authority (DVLA) in the UK now have comprehensive guidelines based on this evidence which suggest a delay of at least 45 minutes prior to continuation of driving following treatment of hypoglycaemia.

Of course, cognitive decline will not occur at a set blood glucose concentration for every individual. Other factors will influence this, and it is a dynamic process. The factors contributing to an increased likelihood of cognitive decline during hypoglycaemia include male sex, impaired hypoglycaemia awareness, type 1 diabetes and a high baseline IQ (Deary, 2007). Those patients with intensively controlled diabetes are more likely to experience hypoglycaemia, and are more at risk of developing impaired hypoglycaemia awareness; this can result in a reduction in the blood glucose threshold at which autonomic activation occurs, with the cognitive dysfunction beginning at higher blood glucose concentrations than the

sensation of autonomic warning symptoms. This can be a dangerous consequence of intensive glycaemic control, narrowing the window of opportunity to take appropriate action to avoid development of more severe hypoglycaemia.

In real life, most activities will actually involve a combination of multiple aspects of cognition, a good example of which is driving. This is a complex cognitive task requiring attention, memory, auditory and visual information processing, psychomotor function, reaction time and spatial perception, amongst others. Studies have been performed using a sophisticated driving simulator (Cox, 2000), which have demonstrated the decline in driving ability as blood glucose decreases. There is a slowing of reaction times, and an increase in accidents. These simulator studies make this cognitive dysfunction more relevant to real life activities.

1.6.2 Hypoglycaemia and spatial ability

Spatial ability can be defined as the ability to interpret the surrounding environment using visual cues. These visual cues must be generated, then structured and manipulated by the brain, in order to orientate and interpret the images appropriately. This translates into how a human being can deal with two- and three-dimensional objects, navigation and path-finding (Linn, 1985). Understandably, spatial ability is therefore a component of many cognitive function tests used experimentally, particularly the more complex or demanding tasks. It is a part of many daily tasks, with particular practical relevance to driving and map-reading, for example. No studies to date have specifically focused on the effects of hypoglycaemia on spatial abilities, although many tests that are impaired during experimental hypoglycaemia may include an element of spatial ability and spatial processing (Geddes, 2008).

1.6.3 Long-term effects of hypoglycaemia on the brain

1.6.3.1 Cognitive function

It is, as yet, unclear as to whether repeated episodes of hypoglycaemia may have progressive long term effects on mental abilities. The concept of diabetic encephalopathy has been described in the literature, and it is possible that exposure to repeated hypoglycaemia is one of the contributing factors to this phenomenon, along with the additional contribution by vascular disease and conventional cardiovascular risk factors (Dejgaard, 1991, Biessels, 1994, Makimattila, 2004). In essence, the brain can be considered to be an end-organ susceptible to the effects of diabetes in the same way that the retina, kidneys and nerves are, although the contribution of hypoglycaemia to this process remains uncertain.

Several studies in children with Type 1 diabetes have shown that repeated, severe hypoglycaemia, particularly before the age of 5, can reduce mental capabilities in adult life. This effect appeared more profound in those diagnosed at a younger age and with longer duration of diabetes (Ryan, 1984, Rovet, 1987, Golden, 1989, Hershey, 2005, Ryan, 2005). However, there have been some conflicting studies more recently suggesting this premise not to be true (Wysocki, 2003, Strudwick, 2005).

Adults with type 1 diabetes have been shown to have reduced mental abilities when compared with non-diabetic individuals (Ryan, 2006, Brands, 2005). The reasons for this have not been fully explained. It is believed that recurrent exposure to severe hypoglycaemia may result in a modest reduction in IQ, but the full explanation is likely to be multifactorial, and include vascular and blood flow related effects of diabetes (Brands, 2005). The DCCT and its follow up study, EDIC, showed that over the 10 years of follow up performed, there was no reduction in IQ or cognitive abilities associated with exposure to severe hypoglycaemia (DCCT Group, 1993, DCCT Group, 1996, DCCT/EDIC Group, 2007). It should again be noted that the DCCT was designed with very strict inclusion/exclusion

criteria, and that those included had low risk of hypoglycaemia, with resulting lower rates of severe hypoglycaemia than have been described in less selective studies. As a result, the subjects were not typical of routine clinical practice.

Retrospective cross-sectional studies have been undertaken to look at the impact of previous severe hypoglycaemia exposure on intellectual capacity in adults with type 1 diabetes. These studies have suggested that those with increased severe hypoglycaemia exposure in the past have lower intellectual functioning (Wredling, 1990, Langan, 1991, Lincoln, 1996, Deary, 1993[2]). Retrospective analysis of hypoglycaemia occurrence can be flawed, as previously discussed, however, and there are possible additional confounding variables in all of these analyses, including the contribution of the diagnosis of diabetes itself, and the premorbid IQ; participants experiencing more hypoglycaemia may have had lower IQ scores to begin with. These results therefore suggest an association between SH exposure and reduction in IQ, but do not confirm causality, and disagree with the findings of the prospective long term trials to date.

The evidence in type 2 diabetes of an association between cognitive decline and diabetes seems fairly robust (Cukierman, 2005). It has been reported, however, that because hypoglycaemia rates are relatively low in people with type 2 diabetes, that it is unlikely to play a part in the pathogenesis of this decline. There appears to be an accelerated rate of cognitive decline and an increased risk of dementia in type 2 diabetes, but the interplay of vascular risk factors and other risk factors is acknowledged (Stewart, 1999, Allen, 2004). The role of hypoglycaemia in this process remains to be established.

1.6.3.2 Structural and neurological changes

It is known that a severe, prolonged episode of hypoglycaemia can result in serious and permanent brain damage (Malouf, 1985, Yoneda, 2005). It should be noted that these

instances have often involved the consumption of alcohol which has compounded the effects of the episode of hypoglycaemia.

Hypoglycaemia can induce seizure activity. Neurophysiological changes are apparent during hypoglycaemia, including a decrease in alpha wave activity, increase in theta waves, and increased bursts of delta waves, particularly evident in the anterior brain. There is also increased latency and reduced amplitude of sensory-evoked potentials. These changes are more marked in those patients with impaired hypoglycaemia awareness, and can become permanent in those with recurrent severe hypoglycaemia (Pramming, 1988, Tallroth, 1990, Bjørgaas, 1998, Hyllienmark, 2005).

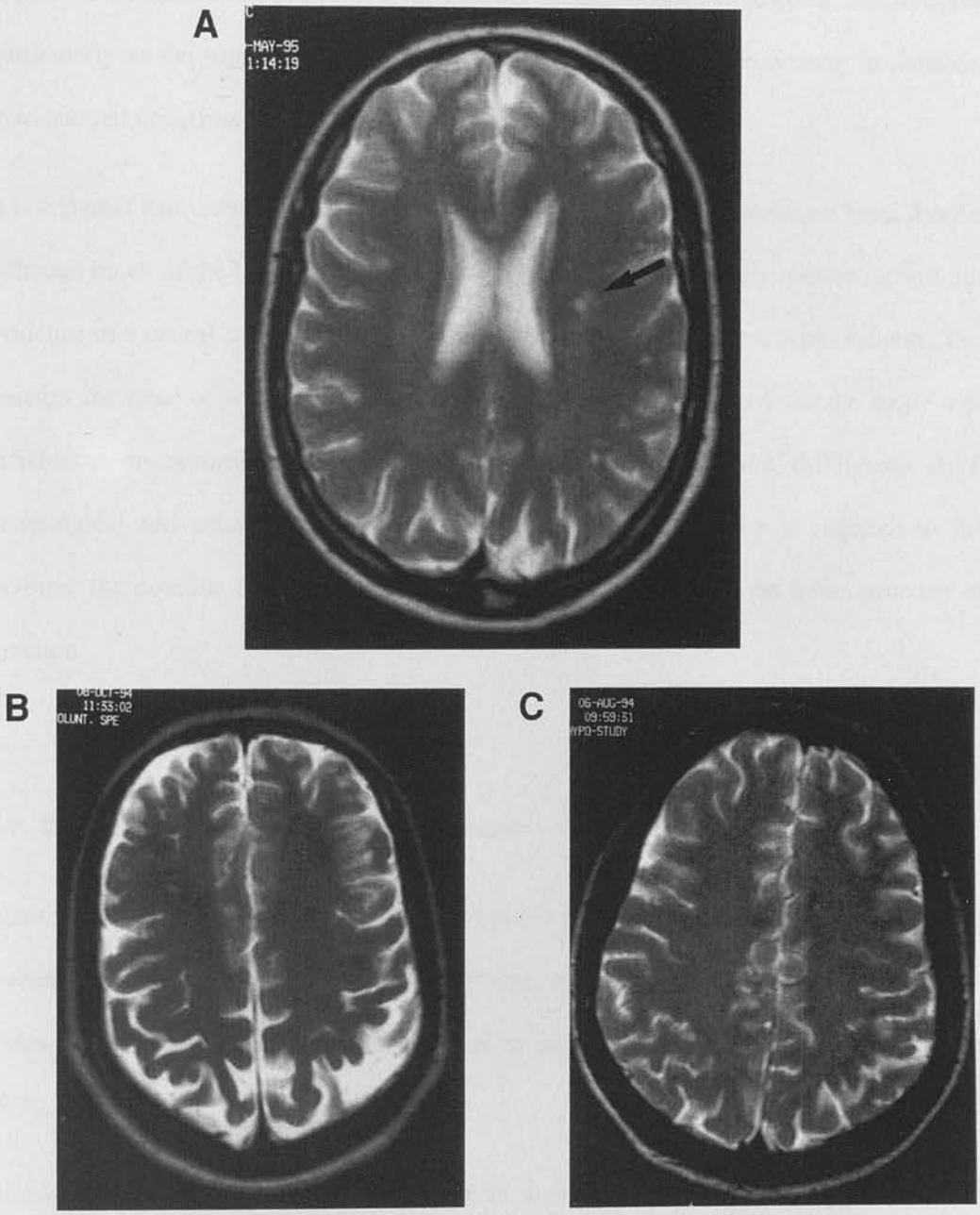
Regional blood flow changes occur, which are usually temporary and return to normal on recovery, but these may also become permanent in people with impaired hypoglycaemia awareness; an increase in blood flow to the frontal lobes is seen, which may be an adaptive phenomenon to protect a vulnerable region of the brain (MacLeod, 1996). These alterations will be discussed further in the section on cerebrovascular disease and hypoglycaemia later in this chapter. Hypoglycaemia can mimic the symptoms of acute stroke, causing, for example, hemiparesis. It has been shown that these clinical signs can correspond to transient abnormalities in cerebral imaging, suggesting that there has been focal reduction in blood flow to result in an area of ischaemia (Shirayama, 2004, Koppel, 1993, Gold, 1996, Cordonnier, 2005).

Furthermore, imaging studies have been undertaken in an attempt to clarify whether exposure to recurrent hypoglycaemia may impact on brain structure and function in the longer term. A study in 1997 demonstrated that people with type 1 diabetes exposed to recurrent severe hypoglycaemia had increased cerebral atrophy on MRI scanning as compared with a matched control group (Perros, 1997; figure 1.2).

Figure 1.2: Brain abnormalities seen in people with type 1 diabetes exposed to recurrent severe hypoglycaemia. Reproduced from Perros, 1997.

A. Small white lesions (leukoariosis); see arrow B. Cortical atrophy

C. Normal scan



These findings have not been replicated to date. It is evident that structural cerebral changes do occur in people with diabetes, as compared with non-diabetic control subjects, but there does not appear to be an association with exposure to recurrent severe hypoglycaemia. Instead, the main association is with the presence of microangiopathy, particularly retinopathy (Ferguson, 2003, Brands, 2005, Ryan, 2006). The implication that this removes the possible contribution of hypoglycaemia from the equation may, however, not be correct, particularly as the usual issues in hypoglycaemia studies with heterogeneity in definitions used and reporting methods are present in all of these studies.

It is apparent that there are potential long term sequelae of hypoglycaemia on brain function, although much of the longitudinal data thus far appears to be relatively reassuring with little evidence of a causal role in any abnormalities detected in those people with diabetes. There remains the issue of selection of subjects with low hypoglycaemia risk for the larger trials, differences in reporting hypoglycaemia and definitions used, and differences in the neurological and psychological assessments used. More information is required to fully examine the possible long-term effects of recurrent hypoglycaemia on brain structure and function.

1.7 Diabetes and Cardiovascular disease

Atherosclerosis is a major cause of cardiovascular morbidity and mortality in people with diabetes and is very common. It can develop prematurely, can be more diffuse and widespread and can be more aggressive than in people who do not have diabetes (Banga, 1994, Deckert, 1978, Kirpichnicov, 2001).

Several studies have now been undertaken to investigate the underlying pathogenesis of atherosclerosis and vascular complications in people with diabetes. These studies support the hypothesis that inflammation contributes to the pathogenesis of atherosclerosis and

cardiovascular disease, and it is likely that external influences on endothelial function and the blood components can have an effect on blood vessels and contribute to this. In the past, athero-thrombotic disease was believed to originate from collections of lipid in vessel walls, which form plaques, in combination with the effects of conventional cardiovascular risk factors such as hypertension, smoking and diabetes. Animal and human studies have now suggested that atherogenesis contains a significant inflammatory component, from the early stages of development, which contributes to its progression and the subsequent emergence of thrombotic complications (Libby, 2002). Research has therefore advanced from an examination of traditional cardiovascular risk factors to investigation of the processes that involve the vasculature at a cellular level, particularly examining the cells that are implicated in atherogenesis, including endothelial cells, macrophages, monocytes, platelets and smooth muscle cells.

1.7.1 Diabetes and vascular biology

There is a chronic systemic pro-inflammatory and pro-coagulant state in both type 1 and type 2 diabetes. The EURODIAB prospective complications study showed that inflammatory markers (C-reactive protein [CRP], interleukin-6 [IL-6] and tumour necrosis factor-α [TNF-α]) were found to be strongly, and independently, associated with vascular disease in people with type 1 diabetes (Schram, 2005). This has been supported by other studies that have shown an association between chronic inflammation, endothelial dysfunction and platelet hyperactivity, and the microvascular complications of retinopathy and nephropathy (Yngen, 2004, Vestra, 2005, van Hecke, 2005). Even in the absence of vascular complications, markers of endothelial dysfunction including CRP, von Willebrand factor (vWF) and vascular cell adhesion molecule (VCAM)-1 are elevated (Schalkwijk, 1999, Targher, 2005), while plasma concentrations of the anti-inflammatory cytokine interleukin (IL)-10 are lower in people with type 2 diabetes (van Exel, 2002).

Endothelin-1, a potent vasoconstrictor, has been demonstrated to be elevated in people with both type 1 and type 2 diabetes (Takahashi, 1990). Levels increase in the presence of microvascular disease, and it has therefore been postulated that endothelins may play a role in the development of these complications (Dhaun, 2006).

The evaluation of markers that may be associated with the presence of vascular disease, or signify increased future risk, has now gained momentum. C-reactive protein (CRP) has received much attention in this regard. CRP has been shown to be elevated in people with established vascular disease, and has been associated with a poorer prognosis in patients who present with unstable coronary heart disease (Mendall, 1996, Morrow, 1998).

Platelet-monocyte aggregation has been shown to be a sensitive indicator of platelet activation. Activated platelets bind to leucocytes, predominantly monocytes, to form aggregates. This induces secretion of interleukin-1β, interleukin-8, monocyte chemotactic protein, Mac-1 and tissue factor (Neumann, 1997, Celi, 1994). In apolipoprotein E-deficient mice, monocyte adhesion to the activated endothelium and atherogenesis per se are promoted by platelet-monocyte aggregation (Huo, 2003).

There is also increasing evidence to suggest that the CD40-CD40 ligand (CD40L) signalling pathway plays an important role in the pathogenesis of atherosclerosis and of plaque rupture (Schonbeck, 2000[1], Mach, 1998[1]). CD40 is a type I transmembrane protein and is a member of the tumour necrosis factor (TNF) receptor family. CD40L is a type II transmembrane protein (Schonbeck, 2000[2]). Their interaction regulates cell proliferation, differentiation and apoptosis, and is thought to induce a pro-inflammatory, prothrombotic response by causing a cascade of cytokine and chemokine release, and expression of adhesion molecules, matrix metalloproteinases and tissue factor (Schonbeck, 2000[2], Mach, 1997, Henn, 1998). A soluble, biologically active form also exists as soluble CD40 ligand (sCD40L). CD40 ligand and its receptor CD40 are present on the surface of platelets, monocytes, endothelial cells

and smooth muscle cells, all of which are associated with the formation of atheroma. Ligation of CD40-CD40L activates these atheroma-associated cells (Mach, 1997, Henn, 1998). Studies in hypercholesterolaemic mice show that interruption of the CD40/CD40L dyad slows the initiation and progression of atherosclerotic lesions (Mach 1998[2], Schonbeck, 2000[3]). In humans, clinical studies in patients with acute coronary syndromes have revealed the presence of an elevated level of sCD40L (Aukrust, 1999, Garlichs, 2001). Apparently healthy women who have increased plasma concentrations of sCD40L, have been shown to be at increased risk of acute vascular events (Schonbeck, 2001[2]). It has been postulated that this test could potentially be used for risk stratification.

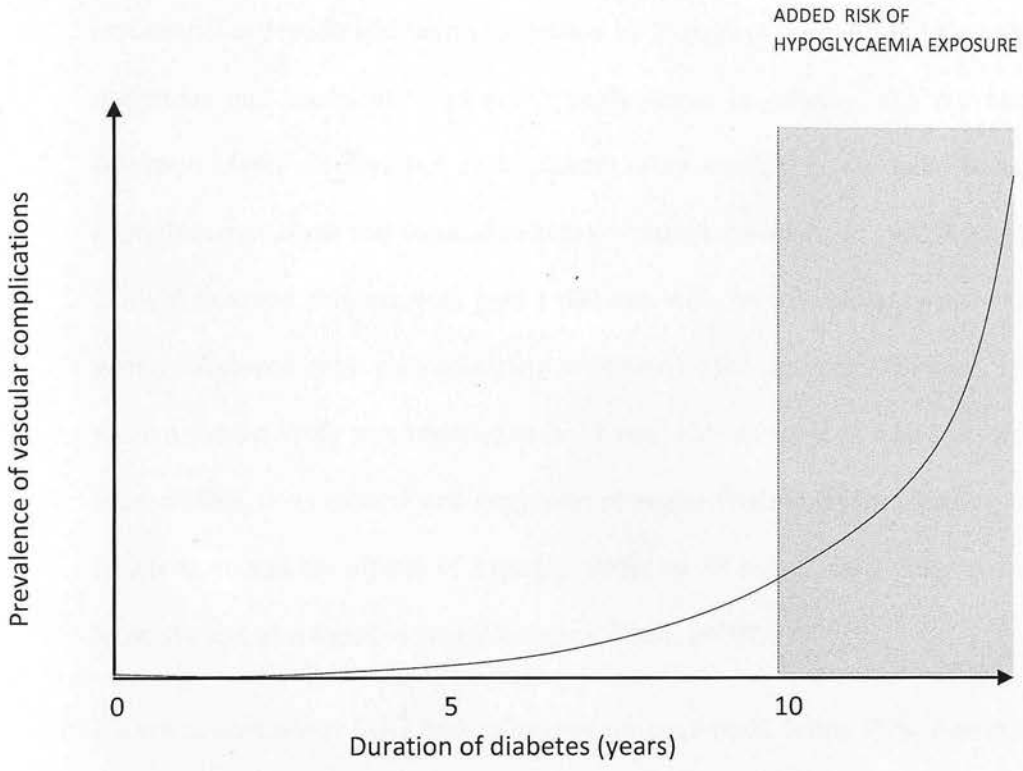
Recently it has been shown that diabetes is associated with increased CD40L expression and platelet-monocyte aggregation (Harding, 2004). Plasma levels of sCD40L are elevated, and correlate with HbA1c, suggesting that hyperglycaemia is responsible. An improvement in glycaemic control was associated with a reduction in plasma sCD40L by 37.5%, in 40% of patients with type 1 diabetes (Cipollone, 2005). Other studies, however, have suggested that improving metabolic control has no effect on endothelial dysfunction (Yudkin, 2000).

It remains controversial as to whether these vascular biomarkers are elevated in response to vascular disease rather than as promoters of vasculopathy. The fact that CRP and sCD40L are markers that predict the future risk of vascular disease is supportive of a causative role. In addition, it is known that systemic inflammation can precipitate vascular events. Such an example is viral infection with influenza, which provokes inflammatory and thrombotic effects on atherosclerotic plaques, and can increase the mortality from cardiovascular causes (Madjid, 2003).

1.8 Hypoglycaemia and vascular disease

It has been suggested that hypoglycaemia may aggravate the microvascular complications of diabetes (Frier, 1985). The profuse secretion of counterregulatory hormones, such as the catecholamines, and acute stimulation of the autonomic nervous system in response to hypoglycaemia, may contribute to these effects on haematological constituents, but to date investigation of this has been limited, partly due to a lack of sophisticated tests to assess this risk further. Figure 1.3 depicts a theoretical model of the increasing prevalence of diabetic complications associated with progressive duration of the disorder, and suggests the premise that recurrent exposure to hypoglycaemia may exert an increasingly adverse effect when the vasculature has already become compromised. Clinical evidence that hypoglycaemia may impact on the cardiovascular system has been accumulating for many years. Although much of the evidence is circumstantial, it does raise serious concerns, and indicates the need for more information on the potential hazards of hypoglycaemia.

Figure 1.3: Risks of hypoglycaemia on the vasculature: theoretical model of the increasing impact of hypoglycaemia once vascular complications have developed. Reproduced from Wright, 2008.



1.8.1 Myocardial ischaemia

It seems logical that the stress of acute hypoglycaemia, inflicted upon a diseased coronary arterial circulation may provoke myocardial ischaemia. But the evidence is limited and conflicting. A few anecdotal reports have been published indicating that angina or myocardial infarction had been precipitated by hypoglycaemia. It would now be considered dangerous and unethical to induce hypoglycaemia in patients who are known to have ischaemic heart disease, but such patients were studied in the past, particularly when hypoglycaemic coma was induced to treat psychiatric disorders. In 1932, hypoglycaemia was induced in seven subjects with type 1 diabetes with known cardiovascular disease, two of whom developed chest pain consistent with myocardial ischaemia (Strouse, 1932). In 1956 when a similar study was undertaken in 11 non-diabetic subjects who had known coronary heart disease, none experienced symptoms of angina (Judson, 1956). Another study in 1960, which examined the effects of hypoglycaemia on 38 non-diabetic patients with ischaemic heart disease, also failed to provoke angina (Egeli, 1960).

However, continuous ECG monitoring was not performed during these studies, and it is now recognised that people with type 1 diabetes, many of whom have autonomic dysfunction, often have 'silent' myocardial ischaemia and do not experience angina even when overt ischaemia is revealed by ECG changes. Ambulatory ECG studies of people with diabetes with known coronary heart disease have demonstrated ischaemia with ST segment depression in the absence of angina (Nesto, 1988). A case report of a patient who developed significant ST depression in the absence of angina during an episode of nocturnal hypoglycaemia has been described (Pladziewicz, 1989). So, although there are limited reports of hypoglycaemia-induced angina, this may reflect the relatively "silent" nature of ischaemic heart disease in people with diabetes.

Myocardial infarction or cardiovascular death have been associated with hypoglycaemia (Kinsey, 1941, Gilbert, 1946, Partaiman, 1965), and a direct association between hypoglycaemia and myocardial ischaemia has been described in a study of 21 patients who had co-existent insulin-treated type 2 diabetes and coronary heart disease, and were receiving treatment with basal-bolus insulin regimens (Desouza, 2003). Continuous blood glucose monitoring was combined with Holter monitoring to identify myocardial ischaemia. A significant association ($p<0.01$) was observed between hypoglycaemia and either symptoms or electrocardiographic changes of ischaemia, which was not observed with either hyperglycaemic or normoglycaemic states (Desouza, 2003). In addition, the Veterans Affairs Co-operative Study on Glycaemic Control and Complications (VACSDM) revealed that more cardiac events were recorded after the institution of strict glycaemic control (32% vs. 20%), when the intensively treated patients experienced 16.5 episodes/patient/year of mild hypoglycaemia vs. 1.5 in the conventional treatment group (Abraira, 1997). Studies of diabetic patients with coronary heart disease, which examined the influence of glycaemic control at the time of an acute coronary event on subsequent mortality have shown a positive association between both hypo- and hyperglycaemia and all-cause mortality risk over the course of two years (Svensson, 2005). In a large study from Israel that followed patients with coronary heart disease over an eight year period, hypoglycaemia at initial presentation emerged as a marker for increased all-cause mortality (Fisman, 2004).

1.8.2 Cardiac arrhythmia

Acute hypoglycaemia can precipitate T wave abnormalities and QT prolongation on the electrocardiogram. The evidence for any true pro-arrhythmic effect remains limited. Anecdotal reports have observed an increase in ectopic beats of atrial and ventricular origin, and of atrial fibrillation, during insulin-induced hypoglycaemia, although this occurred in patients who had established heart disease (Strouse, 1932). In diabetic subjects without any known cardiac disease, sporadic hypoglycaemia has provoked atrial fibrillation, which

reverted to sinus rhythm after correction of the low blood glucose (Collier, 1987[2]). These disturbances of cardiac rhythm are probably a consequence of the adrenergic response to hypoglycaemia, possibly mediated via a fall in serum potassium concentration, which may precipitate the cardiac arrhythmias (Burke, 1999, Thordarson, 1995, Marques, 1997, Harris, 1999). Alternatively, in those patients with pre-existing ischaemic heart disease, an arrhythmia may develop secondary to an ischaemic insult.

1.8.3 Cerebrovascular disease

Hypoglycaemia can induce localised neuroglycopenia and transient focal neurological signs, which resolve on correction of blood glucose. However, neuroimaging techniques have not demonstrated direct evidence that hypoglycaemia can cause cerebral ischaemia. It is not known whether the haemodynamic and haematological effects of hypoglycaemia on the vasculature can also contribute to the neurological deficit. In individual cases, when neuroimaging was performed after the development of a neurological deficit associated with severe hypoglycaemia, areas of reduced density that were consistent with ischaemia have been observed in the appropriate cerebral area; these changes have usually resolved following correction of hypoglycaemia (Shirayama, 2004, Koppel, 1993, Gold, 1996, Cordonnier, 2005). Permanent disability does occasionally result from severe hypoglycaemia, which may be mediated by cerebral damage secondary to glucose deprivation or by cerebral infarction. Transient ischaemic attacks and hemiplegia are recognised sequelae of hypoglycaemia, and the acute haemodynamic and haematological effects may provoke a stroke, presumably in the areas of the brain where the regional blood flow is compromised, but information is not available to determine the prevalence or mechanisms behind this (Malouf, 1985, Ben-Ami, 1999).

1.8.4 Microvascular disease

The DCCT confirmed that intensive glycaemic control limited the development and delayed the progression of long-term complications of diabetes over a mean follow up period of 6.5 years, but an initial deterioration in retinopathy was noted shortly after the intensive treatment regimen was instituted (DCCT Study Group, 1993, Agardh, 1992). In studies in the 1980s (Lauritzen, 1983, Dahl-Jorgensen, 1985), a similar phenomenon had been observed during the two to four months after rapid improvement of glycaemic control, although this gradually improved despite persistence of intensive therapy. Although the long-term progression of retinopathy is prevented by intensive control, established retinopathy is not reversed, and the temporary worsening of retinopathy can be clinically significant. A reduction in retinal blood flow is described as the most likely mechanism, but it is also noteworthy that hypoglycaemia frequency increases when tight control is instituted, as one might expect (Lauritzen, 1983, Dahl-Jorgensen, 1985). The haemodynamic and haematological effects of hypoglycaemia seem to be transient in an undamaged circulation, so are unlikely to contribute to the initial development of long term complications, but these effects may aggravate complications in an already compromised circulation (Frier, 1985). Acute vitreous haemorrhage has been reported following nocturnal hypoglycaemia (Tarman, 1979), which may be related to the rapid fall in intra-ocular pressure which occurs during hypoglycaemia (Frier, 1987), causing rupture of the fragile new capillary vessels that have developed as a consequence of retinal ischaemia.

At present the evidence for the possible effects of hypoglycaemia on microangiopathy remains circumstantial and the potential effects are hypothetical. It is possible, however, that hypoglycaemia may have adverse consequences on small blood vessels and thereby aggravate pre-existing microvascular disease.

1.8.5 Possible mechanisms of vascular damage

Hypoglycaemia may exacerbate vascular damage in a number of ways. These include direct effects on blood vessels through changes in vascular physiology, blood cell alterations and activation, vasoconstriction, and inflammation. Each of these will be examined in turn.

1.8.5.1 Physiological changes

In response to hypoglycaemia (see earlier section on the physiology of hypoglycaemia), epinephrine is released in large quantities through sympathetic neural activation and this hormone is known to have direct cardiovascular effects. Heart rate and systolic blood pressure rise, while a small decrement in diastolic blood pressure occurs (MacDonald, 2007). By studying patients with autonomic deficits, the increment in heart rate has been found to be mediated via sympathetic activity. This response is absent in tetraplegic patients with presynaptic sympathectomy caused by traumatic transection of the cervical cord (Corrall, 1979); it appears to be mediated via β_1 adrenoceptors (Kerr, 1990). The blood pressure changes are thought to be caused by epinephrine via α and β_2 adrenoceptors, and can be reproduced by infusing epinephrine intravenously (Kerr, 1990). Myocardial contractility and cardiac output both increase and peripheral vascular resistance declines (Hilsted, 1984). The left ventricular ejection fraction (LVEF) rises, as has been demonstrated using radionuclide ventriculography, and represents a surrogate measure for myocardial contractility. The change in LVEF can be abolished by β adrenoceptor blockade (Fisher, 1987).

Insulin-induced hypoglycaemia provokes cardiac electrophysiological changes including flattening or inversion of T waves in the ECG (Middleton, 1931), while QT prolongation (Parrish, 1952, Egeli, 1960) and ST depression (Parrish, 1952, Lloyd-Mosten, 1975) have been observed but are not readily reproducible. The T wave changes were shown to resolve during concomitant intravenous infusion of potassium during hypoglycaemia (Parrish, 1952, Egeli, 1960), and could be abolished by β blockade (Lloyd-Mosten, 1975). Administration of

selective β_1 blockade before the induction of acute hypoglycaemia prevented QT prolongation (Lee, 2005). This implicates a β adrenoceptor-mediated mechanism, probably acting via a fall in serum potassium and so precipitating the electrophysiological changes (Fisher, 1991[1]).

1.8.5.2 Regional blood flow

The sympathetic nervous system response to hypoglycaemia seems to play a major role in the regional changes in blood flow seen throughout the body. Total cerebral blood flow increases at blood glucose concentrations below 2.0 mmol/l and the regional distribution of blood flow within the brain alters to provide glucose to the areas that are most vulnerable to neuroglycopenia, such as the cerebral cortex and the basal ganglia (Bryan, 1990, MacLeod, 1994, Tallroth, 1992). Although this has been disputed by the results of one study (Segel, 2001), the hypoglycaemic nadir in that study was 3.0 mmol/l, which may not have been sufficient to stimulate a rise in cerebral blood flow.

Total splanchnic blood flow increases (Bearn, 1952, Braatvedt, 1993) and splenic blood flow decreases (Fisher, 1990[1]), while there is a relative increase in hepatic blood flow which should enhance hepatic glucose production. Blood flow to skeletal muscle increases (Allwood, 1959), while blood flow to the kidneys declines (Patrick, 1989). Blood flow to the skin is initially increased, causing vasodilatation and flushing, but it subsequently declines, causing pallor (Maggs, 1994). This redistribution of blood flow has two main hypothetical roles: (i) to protect vital organs such as the brain, and (ii) to maintain a supply of glucose by increasing the delivery of gluconeogenic precursors to the liver. It is possible that diversion of blood flow to more vital parts of the brain during hypoglycaemia may result in greater cerebral ischaemia in other areas.

1.8.5.3 Haemorrheological changes

1.8.5.3.1 Blood cells

Erythrocytes

Experimental studies of acute hypoglycaemia have demonstrated an increase in the number of circulating erythrocytes in relation to the peak of plasma epinephrine concentration in response to the hypoglycaemic stimulus (Frier, 1983, Hilsted, 1985). This apparent erythrocytosis is probably caused by haemoconcentration and a rise in packed cell volume, rather than the entry of new red blood cells into the circulation, as this response is preserved in patients post-splenectomy (Hilsted, 1990). When hypoglycaemia was induced in sympathectomized subjects, who do not generate an epinephrine response to hypoglycaemia, an increment in erythrocyte number was not observed, suggesting that this phenomenon is mediated by an adrenergic mechanism (Frier, 1983). The response can also be reproduced by the intravenous infusion of epinephrine (Hilsted, 1990), and while the increment can be abolished by α blockade with phentolamine (Fisher, 1991[2]), it is unaffected by selective or non-selective β blockade (Frier, 1983), implicating that the change is mediated by α adrenoceptors.

Leucocytes

The absolute number of leucocytes increases in response to acute insulin-induced hypoglycaemia (Collier, 1990). This leucocytosis consists of an initial increment in lymphocytes coinciding with the acute autonomic reaction, and this is followed within one hour by a rise in granulocytes (Frier, 1983, Fisher, 1989), and a fall in circulating T-lymphocytes (Fisher, 1989). The lymphocytosis that coincides with the autonomic reaction appears to be mediated predominantly through α adrenoceptors, as this response is absent in sympathectomized individuals and is abolished by α blockade with phentolamine, while being only mildly attenuated following unselective β blockade (Fisher, 1990[2]). That this

response is adrenergically-mediated has also been demonstrated by the administration of exogenous epinephrine, which provoked a lymphocytosis (Steel, 1971). In addition, acute experimental hypoglycaemia has been shown to induce a proliferation of β_2 adrenoceptors on the surface of mononuclear leucocytes (van Tits, 1990). By contrast, the later proliferation of granulocytes is mediated by cortisol, which was demonstrated by comparing the cortisol and granulocyte responses to hypoglycaemia in normal subjects with patients who had deficiencies of the hypothalamo-pituitary-adrenal axis (Fisher, 1989). Neutrophils are activated during hypoglycaemia, as demonstrated by the release of neutrophil elastase (Collier, 1990), a protease that has been implicated in the development of microangiopathic complications in diabetes.

Platelets

Platelet aggregation is promoted by hypoglycaemia (Hutton, 1979). Platelet factor 4 (Trovati, 1986) and plasma β thromboglobulin both increase, and are thought to represent the activation of platelets in vivo (Trovati, 1986, Monnier, 1984, Kishikawa, 1988, Takeda, 1988). In addition, the aggregation of platelets in response to ADP is promoted, and this probably develops secondary to the adrenergic stimulation that is associated with hypoglycaemia (Trovati, 1986). The mechanisms underlying platelet aggregation and activation have been investigated; both processes are inhibited by α_2 adrenoceptor blockade with either mianserin (Kishikawa, 1988) or midaglizole (Takeda, 1988).

1.8.5.3.2 Coagulation and fibrinolysis

In addition to the changes that involve blood cells, hypoglycaemia has substantial haemorrheological effects. Acute hypoglycaemia causes an increment in coagulation factor VIII activity of 96% (Corrall, 1980). This response was reproduced by the intravenous infusion of epinephrine, and was absent in sympathectomized subjects, while in non-diabetic subjects it was blocked by the administration of propranolol, but not metoprolol, indicating a

β_2 adrenoceptor-mediated process, a conclusion that has been supported by other studies (Ibbotson, 1995, Mikhailidis, 1985).

Von Willebrand factor rises in response to hypoglycaemia (Fisher, 1991[2]), and accelerated rates of thrombin generation have been documented (Ibbotson, 1995). Activation of the fibrinolytic system occurs, demonstrated by a rise in tissue plasminogen activator (tPA) and a fall in plasminogen activator inhibitor (PAI), suggesting the formation of complexes with tPA (Fisher, 1991[2]). Euglobulin lysis time (a test of plasminogen activator activity) is shortened and fibrin plate lysis increases, also indicating increased fibrinolysis (Fisher, 1991[2]). Fibrinolysis is altered by insulin infusion alone, while euglycaemia is maintained using a hyperinsulinaemic glucose clamp technique, with a simultaneous rise in tPA levels and fall in PAI levels (Landin, 1991). Changes in fibrinogen or fibrinogen degradation products (FDPs) have not been observed during hypoglycaemia (Fisher, 1991[2], Ibbotson, 1995).

1.8.5.3.3 Inflammation

The study of the inflammatory mechanisms underlying many disease states is currently receiving a lot of attention. It is known that acute hyperglycaemia, for example following an oral glucose load, provokes oxidative and inflammatory stress. This effect is also observed following induction of hyperglycaemia by intravenous glucose infusion (Dandona, 2007). Chronic hyperglycaemia has been shown to promote inflammatory changes and increase the generation of free radicals, which may be one of the mechanisms underlying the development of angiopathy in diabetes. It has also been reported that post-prandial glucose excursions can increase oxidative stress, thereby potentially increasing the risk of developing or aggravating vascular complications of diabetes (Monnier, 2006), suggesting that post-prandial hyperglycaemia should be targeted during treatment to achieve optimal glycaemic control. However, a recent study has disputed the importance of glucose fluctuations, being

unable to demonstrate a relationship with markers of oxidative stress (Wentholt, 2008). Conversely, insulin therapy has been shown to have potent anti-inflammatory properties when used to treat obese insulin-resistant individuals, and also demonstrates these effects when administered during a normoglycaemic clamp study (Dandona, 2007).

Investigation of the potential effect of hypoglycaemia on inflammatory factors has so far been limited, in part because it is only in recent years that access to sophisticated measures of inflammation and endothelial function has been widely available. Galloway et al (Galloway, 2000) have reported a significant increase in C-reactive protein at 24 hours after acute insulin-induced hypoglycaemia, both in subjects with type 1 diabetes (0.77mg/l to 2.31mg/l) and in healthy volunteers (0.32mg/l to 0.96mg/l). While it is unclear as to the role of CRP in the causation of atherogenesis, as discussed earlier, increasing evidence has implicated its involvement in acute vascular events, with data in vitro and in vivo demonstrating its possible pathogenetic qualities, and additionally CRP levels are predictive of outcome following cardiovascular events (Pepys, 2005, Jialal, 2004).

1.8.6 Conclusion

It is therefore imperative that further information is sought on the potential effects of hypoglycaemia on the vasculature. There is now a plethora of soluble marker assays and methods of assessing blood constituents available to examine the hypothesis that hypoglycaemia may aggravate micro- and macrovascular disease in people with diabetes.

Chapter 2: Methods

2 Methods

2.1 Subjects

Subjects recruited for this programme of research had to meet the following criteria for participation in any of the studies:

2.1.1 Non-diabetic subjects

Healthy adult volunteers recruited for the studies were only included if they met *all* of the following criteria:

1. Age range 18-45 years (either gender).
2. Normal body weight (Body Mass Index: 20-26kg/m^2).
3. On no medications (with the exception of the oral contraceptive pill).

2.1.2 Subjects with Type 1 Diabetes

Healthy adult patients with type 1 diabetes were recruited from the diabetes outpatient clinic at the Royal Infirmary of Edinburgh. They could be included in the studies if they met *all* of the following criteria:

1. Type 1 diabetes of at least 2 years duration.
2. HbA1c values between 7% and 9% for the preceding 6-12 months (non-diabetic range: 5.0-6.05%).
3. No microvascular or macrovascular complications.
4. On no medications other than insulin (with the exception of the oral contraceptive pill).
5. Age range 18-45 years (either gender).

Subjects were excluded from participation if they met *any* of the following criteria:

1. Impaired awareness of hypoglycaemia.
2. Co-existent systemic disease.
3. Past history of severe morbidity during or in response to hypoglycaemia (e.g. convulsions).
4. Past history of cerebral injury, seizure, alcoholism or psychiatric disorder.
5. Pregnancy.
6. Any evidence of microvascular complications.
7. Evidence of autonomic neuropathy using standard tests of cardiovascular reflexes (Ewing, 1985).
8. Documented clinical evidence of peripheral neuropathy.

All subjects gave written informed consent before participating in the studies, which had been approved by the Lothian Research Ethics Committee, in accordance with the Declaration of Helsinki.

2.2 Hyperinsulinaemic clamp technique (DeFronzo, 1979)

After an overnight fast, and confirmation of avoidance of biochemical hypoglycaemia in the preceding 48 hours in subjects with diabetes, a retrograde intravenous cannula is inserted into the non-dominant hand vein for regular sampling of blood glucose. This hand is placed in a heated blanket in order to arterialise the venous blood (Abumrad, 1981). A cannula is also inserted into a vein in the antecubital fossa of the same arm for intravenous infusion of 20% dextrose and soluble human insulin (Human Actrapid, Novonordisk Pharmaceuticals, Crawley, UK). Insulin is infused at a constant rate of 1.5mU/kg/min using a Gemini PCI pump (Alaris Medical Systems, San Diego, CA). Dextrose (20%) is infused at a rate that varies according to the arterialised blood glucose concentration, which is measured at 5

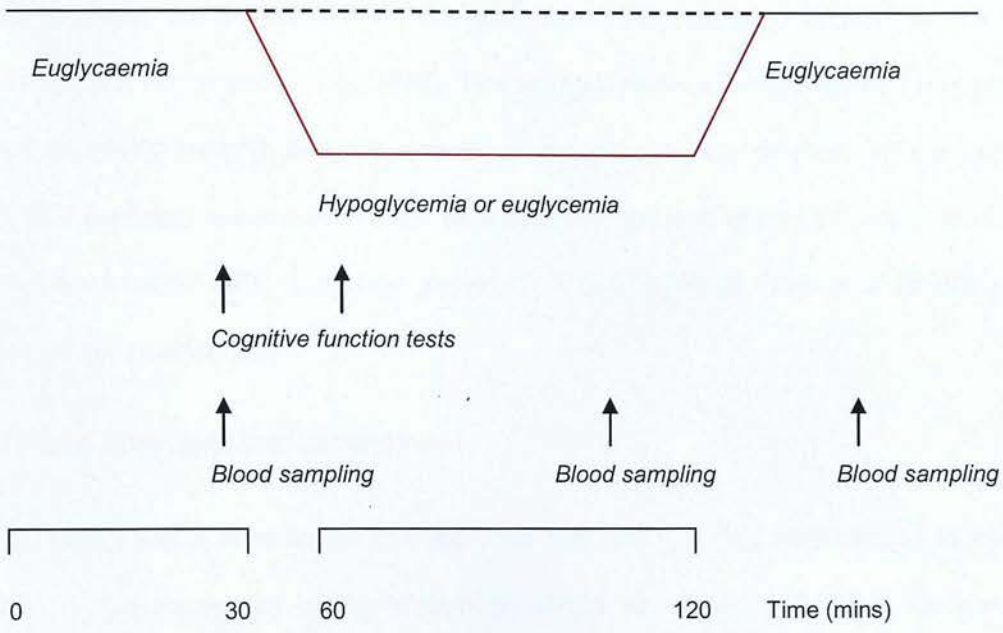
minute intervals using the glucose oxidase method (2300 Stat; Yellow Springs Instrument, Yellow Springs, OH). During a run-in period, arterialised blood glucose is maintained at 4.5 mmol/l for 30 minutes. Blood glucose is then stabilised and maintained at 4.5 mmol/l throughout (euglycaemia), or lowered over 20 minutes to 2.5 mmol/l (hypoglycaemia), and is maintained at this level for 1 hour, during which either cognitive testing or blood sampling can be undertaken. At the end of this experimental hour, euglycaemia (4.5 mmol/l) is restored and maintained (see figure 2.1). The glucose clamp is then discontinued and the subjects are given a standardised meal. Subjects with diabetes are then advised regarding doses and timing of insulin to be taken for the rest of the day.

The subjects are not informed as to which condition of the study is being performed. Hypoglycaemia and euglycaemia order is randomised and counterbalanced. Glucose for intravenous injection is available to reverse hypoglycaemia.

2.3 Insulin infusion method of hypoglycaemia induction

After an overnight fast, and confirmation of avoidance of hypoglycaemia in the preceding 48 hours, hypoglycaemia is induced by administering a continuous intravenous infusion of soluble insulin at a rate of 2.0mU/kg/min in 0.9% sodium chloride solution, through an indwelling venous cannula in the antecubital fossa. The insulin infusion is continued until the onset of symptoms of hypoglycaemia, which is usually coincidental with objective evidence of an acute autonomic reaction (R). This can be identified by a rapid increase in heart rate, a rise in systolic blood pressure, and the onset of sweating and finger tremor, as described previously (Hepburn, 1991). Euglycaemia is then restored by infusion of Dextrose (20%), and subjects are given a standardised meal. Subjects with diabetes are then given advice on insulin doses for the rest of the day. Glucose for intravenous injection is available throughout.

Figure 2.1: Protocol for hyperinsulinaemic clamp studies



2.4 Assessment of hypoglycaemia awareness

All subjects with and without diabetes underwent assessment of hypoglycaemia awareness and symptoms during their experimental procedures. The Edinburgh Hypoglycaemia Score was used for this purpose (Gold, 1994). This method utilises a Likert scale of 1 (not present) to 7 (intensely present) to assess a combination of autonomic, neuroglycopenic and non-specific (malaise) symptoms, and asks participants to rate how aware of hypoglycaemia they are on the same scale; 1 (always aware) to 7 (never aware). This is a validated self-assessment questionnaire.

2.5 Cognitive function assessment

The Digit Symbol Substitution test and Trail Making B test are administered in order to confirm the recognised effect of hypoglycaemia on cognitive function as described previously (McAulay, 2001[2], Sommerfield, 2003[1], Sommerfield, 2003[2], Warren, 2004). Tests of spatial ability were drawn from the French and Ekstrom Kit of Factor-Referenced (cognitive) Tests (Ekstrom, 1976, Ekstrom, 1979). As per the traditional design of studies assessing the effects of hypoglycaemia on cognition, the studies are randomised and counterbalanced, and different versions of each test, where available, are administered on each study day in order that no practice effects are seen.

2.5.1 Digit Symbol Substitution test

This test is from the Wechsler Adult Intelligence Scale-III (WAIS-III) and assesses the ability of the subject to perform coding as quickly as possible. It is particularly sensitive to cerebral insults, but is not sensitive to the location of the insult. The subject is given a key of numbers 1-9 which each have a corresponding symbol. They must then fill in as many symbols as possible for a list of numbers in 120 seconds.

2.5.2 Trail Making B

The Trail Making B test is a computerised version of the test, and similar in principle to the classic test, from the Halstead Reitan battery. It is used to assess complex visual processing, and also assesses motor function with regards to visual motor tracking. It is performed on a handheld computer. The subject is presented with a grid containing letters and numbers in a random order, and must connect the numbers and letters in numerical and alphabetical order, alternating number with the letter in the fashion '1-A-2-B-3-C…' etcetera.

2.5.3 Spatial Ability Tests (French and Ekstrom Kit of Factor Referenced (cognitive) Tests)

2.5.3.1 Hidden patterns test

The Hidden Patterns Test requires subjects to identify a figure that is hidden among other lines. The figure is the same throughout, with the same orientation, and subjects have 3 minutes to correctly identify as many of the patterns in which the figure is concealed as possible.

2.5.3.2 Card rotations test

The Card Rotations test requires the subject to look closely at a shape on the left hand side of a page, and then assess whether the 8 shapes on the right hand side are the same shape, rotated through a variable number of degrees, or whether the shapes are different, and have in fact been reversed or are a mirror image of the initial shape. Three minutes are allowed to complete as many items as possible.

2.5.3.3 Cube comparisons test

This test involves pairs of cubes, such as the wooden building blocks played with by children, with a letter or shape of each side of the cube. Subjects have 3 minutes to analyse

as many pairs of cubes as possible, and must determine whether the 2 cubes could be the same cube viewed from different sides, or whether they must be different cubes if the letters on the sides do not correspond with each other if the cube is turned over.

2.5.3.4 Paper folding test

The Paper Folding test involves showing participants a sequence of folds in a piece of paper, through which a set of holes is then punched. The participants must choose which one of a set of punched and unfolded papers corresponds to the one they have just seen.

2.5.3.5 Building memory test

This is a test of the subject's ability to remember the position of buildings on a street map. Four minutes are permitted to memorise the map, and then a further 4 minutes to place the buildings correctly on a blank version of the map.

2.5.3.6 Maze tracing test

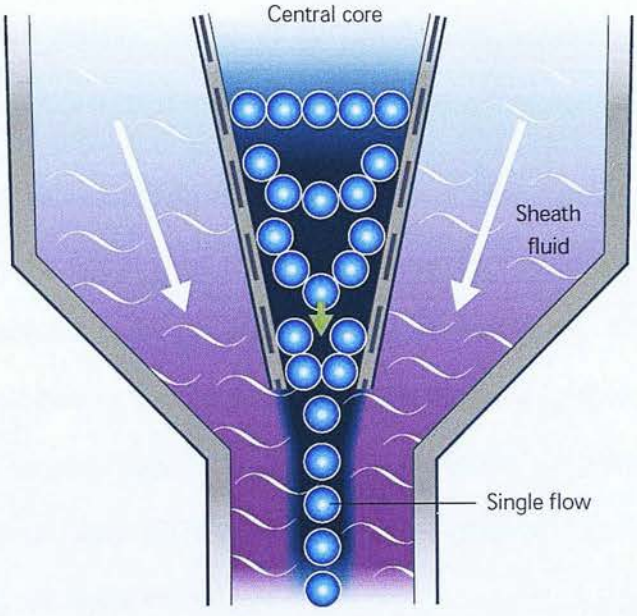
This is a test of the subject's ability to find a path through a maze quickly. A pencil line must be drawn through the maze without crossing any of the 'walls'. The maze is broken down into blocks, and the score is the number of blocks that are successfully navigated in 3 minutes.

2.6 Flow cytometry

2.6.1 Background

Flow cytometry allows the measurement of properties of individual particles. A sample solution can be injected into a flow cytometer, where the sample is ordered into a stream of individual particles which can be interrogated by the cytometer's detection system. Figure 2.2 illustrates the basic structure of a flow cytometer.

Figure 2.2: Flow cytometer structure – hydrodynamic focusing produces a single stream of particles (Reproduced from Rahman, 2006).

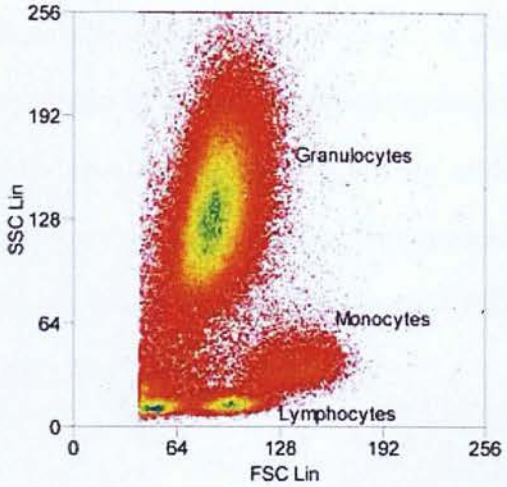


The system comprises a central channel through which the sample is injected. This is enclosed by an outer sheath that contains fluid which flows at a faster rate (sheath fluid). The drag effect of this fast-flowing outer fluid slows the central channel fluid down, so that a single file of particles is generated. The particles then pass through one or more beams of light, and light scattering can be used to determine the size and properties of the particle. Forward scatter of light roughly equates to the particle size, and the side scatter provides information about the granular content of the particle. Both aspects are unique for every particle, and can therefore be used to differentiate between cell types (figure 2.3).

Fluorochromes can be attached to the particles as well, and fluorescence emission will provide additional information about the particle. These can be used to identify cell surface receptors or intracellular molecules such as DNA and cytokines. The light emitted is detected and a small current is generated; this current is then amplified and can be plotted graphically as a histogram (figure 2.4). Typical fluorochromes include phycoerythrin (PE) and fluorescein isothiocyanate (FITC). The use of an isotype control allows the truly positive datasets to be identified as shown in figure 2.4.

Figure 2.3 a and b: Plot of forward scatter and side scatter demonstrating cell subtype differentiation in whole blood. Reproduced from Rahman, 2006.

a.



b.

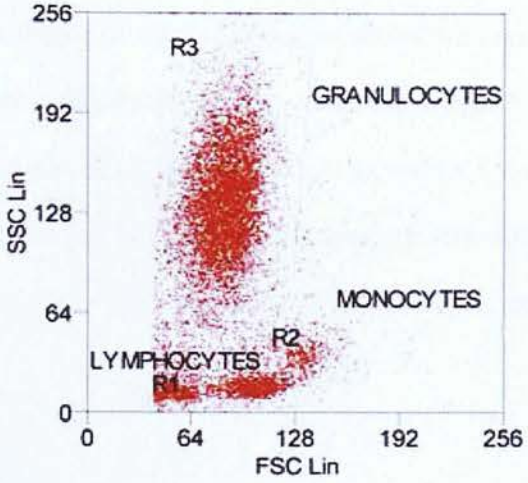
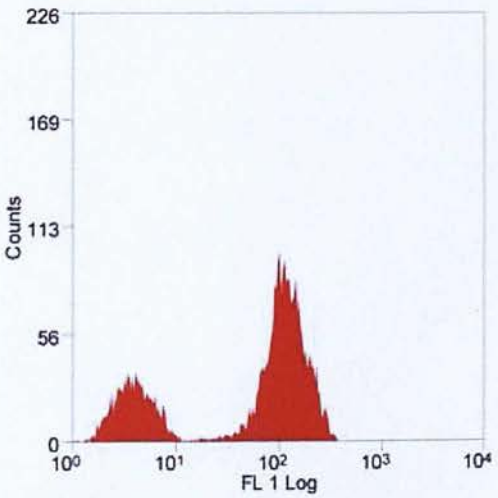


Figure 2.4 (Reproduced from Rahman, 2006):

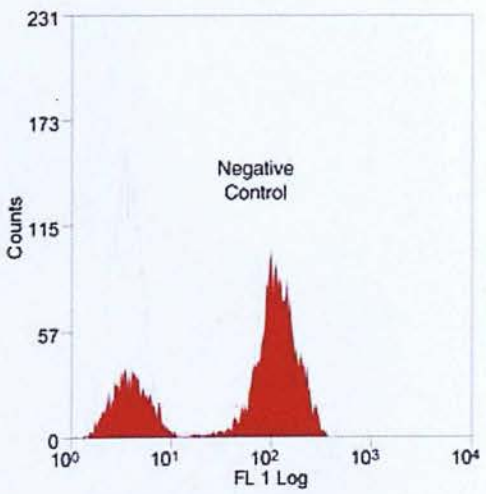
a. Histogram depicting 2 positive datasets identified using conjugated fluorochromes

b. Using an isotype control identifies the true positive dataset as the peak on the right

a.

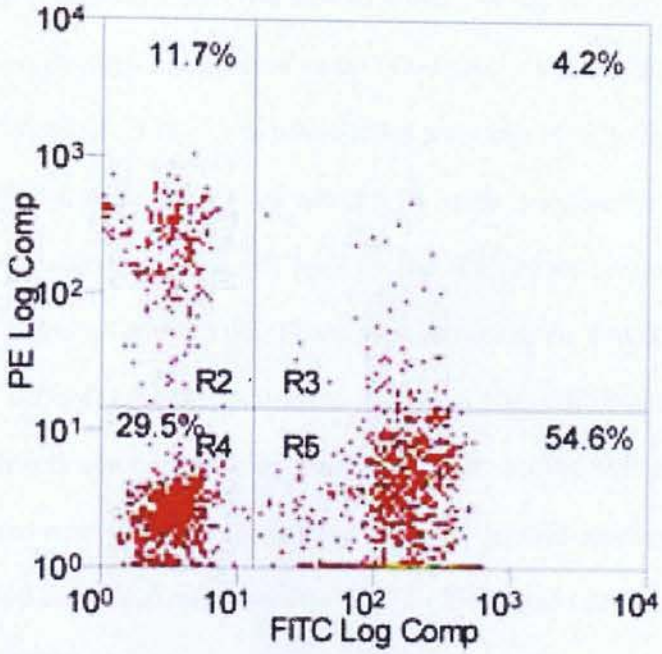


b.



By incubating blood cells with antibodies to cell surface markers with conjugated fluorochromes, it is possible to further clarify, by both size and wavelength of light emitted, which cell is being examined. And by incubating with more than one antibody of interest, information can be derived about aggregates of cells or suspected ligand-dyad pairings. Figure 2.5 shows the dot plot generated when a sample of whole blood is incubated with two antibodies of interest. The x-axis represents one antibody/fluorochrome, and the y-axis the other. Quadrants are then put in place on the dot plot (using the information gained from the histogram data) which allows the population of interest to be gated, and therefore identifies the molecules appearing in the right upper quadrant as being positive for both sets of antibodies/fluorochromes.

Figure 2.5: Dot plot of FITC-labelled molecules vs. PE-labelled molecules (Reproduced from Rahman, 2006).



2.6.2 Current method

Whole blood samples were collected using D-Phenylalanyl-L-prolyl-L-arginine chloromethyl ketone (PPACK), a selective thrombin inhibitor, as an anticoagulant, at pre-determined time points during the conduct of study 3 (baseline, test phase, during recovery, 6 hours and 24 hours). Samples (100 μL) of whole blood were immediately incubated with 10 μL of each monoclonal antibody for 30 minutes at room temperature (AbD Serotec, Kidlington, UK), with subsequent red cell lysis by the addition of 1 mL of FACS Lyse solution (Becton Dickinson, Oxford, UK). Flow cytometry using the FACS Calibur system (Becton Dickinson, Oxford, UK) was performed within 24 hours of obtaining the sample, preparation with antibody and red cell lysis. Isotype controls were performed in addition to both mono- and dual-stain for each parameter assessed: platelet-monocyte aggregation (CD14-CD42a), CD40 expression on monocytes (CD14-CD40), and CD40 ligand expression on platelets (CD154-CD42a).

2.6.2.1 CD14

Mouse anti-human CD14 monoclonal antibody conjugated to FITC was obtained from AbD Serotec, Kidlington, UK. CD14 is a cell surface receptor predominantly expressed by monocytes and macrophages, and in this programme of research has been use to select out the monocyte population.

2.6.2.2 CD42a

Mouse anti-human CD42a monoclonal antibody conjugated to RPE was obtained from AbD Serotec, Kidlington, UK. CD42a is a cell surface receptor expressed by platelets, and is the main platelet receptor for von Willebrand factor. It has been used to select out the platelet population.

2.6.2.3 CD40

Mouse anti-human CD40 monoclonal antibody conjugated to RPE was obtained from AbD Serotec, Kidlington, UK. CD40 is a tumour necrosis factor receptor family member.

2.6.2.4 CD154 (CD40 ligand)

Mouse anti-human CD154 monoclonal antibody conjugated to FITC was obtained from AbD Serotec, Kidlington, UK. CD154 (CD40L) is a member of the TNF family of molecules. It binds to CD40 on antigen presenting cells, and can be present on a wide variety of cells including T lymphocytes, platelets, mast cells, macrophages, B lymphocytes, NK cells in addition to endothelial cells.

2.6.2.5 Isotype controls

IgG1 and IgG2a isotype control antibodies were obtained from AbD serotec, Kidlington, UK.

2.6.3 Validity Experiments

During the design of the study protocol it became apparent that flow cytometry could not be done on the same day as the clamp study, particularly as it was planned to obtain a 24 hour sample from each study participant the day after the clamp itself. Therefore, we had concerns that the samples, having been incubated with the appropriate antibody combinations and red cells lysed, may not be robust enough to wait for later analysis. In order to ensure stability of the samples over time after preparation, validity experiments were performed.

2.6.3.1 Method

100 μL of whole blood collected in a PPACK tube was incubated with 10 μL of each monoclonal antibody combination for the required 30 minutes at room temperature. Red cell

lysis was subsequently performed using 1 ml of FACS lyse solution. The samples were then analysed using the flow cytometer at the following times:

0 hours (immediate testing)

24 hours

48 hours

The samples were refrigerated until the 24 hour and 48 hour time points as per manufacturer advice.

2.6.3.2 Results

Platelet-monocyte aggregation samples remained stable over the 48 hour period. CD40 expression on monocytes increased at 24 hours, and very dramatically again at 48 hours. CD40 ligand expression on platelets appeared to be stable over the 48 hours. Results are shown in table 2.1.

2.6.3.3 Conclusions

This validity study has shown that both platelet-monocyte aggregation and CD40 ligand expression on platelets remain stable over a 48 hour period. CD40 expression on monocytes dramatically increased at 48 hours, so any analysis beyond the 24 hour period of storage would be likely to endanger the accuracy of the results obtained. It was therefore ensured that all samples were analysed within the 24 hour window to prevent degradation of sample quality.

Table 2.1: Flow cytometry validity results

	0 hours	24 hours	48 hours
Platelet-monocyte aggregation	0.52%	0.47%	0.50%
CD40 expression	6.71%	11.52%	27.23%
CD40 ligand expression	2.72%	2.79%	2.20%

2.7 Endothelin-1 (ET-1)

Immunoreactive ET was measured by radioimmunoassay (ITS Production BV) after samples were collected at the pre-specified time points, cold-centrifuged and flash frozen at -70°C until analysis. The sensitivity of this assay is 2 pg/ml immunoreactive ET. Cross-reactivity of the assay with ET-1, ET-2, ET-3 and big ET-1 is 100%, 52%, 96% and 7% respectively. Plasma ET concentrations were measured at baseline, at the onset of the hypoglycaemic reaction (R), and at 15 and 60 minutes following R in study 2.

2.8 Soluble Blood Marker Analysis

Citrated plasma and serum samples were collected at the predetermined time points during hyperinsulinaemic clamp studies in study 3: baseline, test phase, during recovery, 6 hours and 24 hours. These samples were separated immediately and frozen at -80°C until analysis for the soluble markers, described below.

2.8.1 Von Willebrand Factor (In-house assay [Royal Infirmary of Edinburgh Haematology Laboratory]; ELISA; CV 7.3%).

2.8.2 Tissue Plasminogen Activator antigen (Hyphen Biomed Zymutest; intra-assay CV 3.5%, inter-assay CV 4.4%).

2.8.3 Soluble CD40 ligand (high sensitivity ELISA, Bender Medsystems; intra-assay CV 5.5%, inter-assay CV 7.2%).

2.8.4 Soluble P-selectin (ELISA, R&D Systems; intra-assay CV 5.1%, inter-assay CV 8.8%).

2.8.5 Interleukin-6 (High sensitivity ELISA, R&D Systems; intra-assay CV 5.9%, inter-assay CV 9.9%).

2.8.6 High sensitivity CRP (DRG Diagnostics, DRG Instruments GmbH Germany; intra-assay CV 4.2%, inter-assay CV 4.1%).

2.9 Counterregulatory hormone assays

Samples for counterregulatory hormone assessment were collected in EDTA tubes and immediately separated and frozen at -80°C until analysis. Epinephrine and norepinephrine were analysed by high performance liquid chromatography and electrochemical detection (Epinephrine: Intra-assay CV 8.5%, Inter-assay CV 9.9%; Norepinephrine: intra-assay CV 1.2%, inter-assay CV 3.9%). Samples were also assessed by radioimmunoassay for glucagon (interassay CV 12%) and pancreatic polypeptide (interassay CV 10%).

2.10 Statistical analysis

Results were analysed using SPSS version 15.0 for Windows (SPSS, Chicago, IL, USA), and Graphpad Prism 2.0 (GraphPad Software, San Diego, CA, USA). Specific details pertaining to each study are described in the appropriate chapter. In general, hyperinsulinaemic clamp studies require general linear modelling to compare hypoglycaemia with euglycaemia (repeated measures ANOVA), with order of session (euglycaemia-hypoglycaemia or hypoglycaemia-euglycaemia) as a between-subjects factor, and condition (euglycaemia or hypoglycaemia) as a within-subjects factor. A p-value <0.05 was considered to be significant. Where applicable, paired t-tests were utilised.

Chapter 3:
The effects of acute insulin-induced hypoglycaemia on spatial abilities in adults with type 1 diabetes

3 Study 1: The effects of acute insulin-induced hypoglycaemia on spatial abilities in adults with type 1 diabetes

3.1 Introduction

It is well documented that hypoglycaemia impairs many domains of cognitive function, but the effect of hypoglycaemia on spatial abilities has not previously been investigated in detail. However, spatial ability is undoubtedly a component of some of the tests used to assess other aspects of cognition in previous studies (Geddes, 2008). Spatial ability may be defined as the ability to generate, retain, retrieve, and transform or manipulate structured visual images in order to orientate and interpret the surrounding environment. Translating this into real life terms, spatial ability is concerned with how human beings deal with interpreting two- and three-dimensional shapes and objects, space, navigation, and pathfinding. On a day to day basis, this involves interpreting how shapes and objects will appear and function when they are rotated or viewed differently. This process is very important, with particular relevance for complex tasks such as driving and map reading. A large variety of mental tests are available for the assessment of spatial abilities. Largely, these tests can be separated into tests of *spatial perception*, which is the ability to determine spatial relations despite distracting information, *spatial visualisation*, which is the ability to manipulate complex, multi-step spatial information, and *mental rotation*, which is the ability to rotate two- or three-dimensional figures in the mind (Linn, 1985).

The aim of this study was to investigate the effects of acute insulin-induced hypoglycaemia on spatial abilities in adults with type 1 diabetes, utilising a well-characterised battery of spatial tests aimed at incorporating all of the afore-mentioned components of spatial cognition.

3.2 Methods

3.2.1 Subjects

Sixteen adults with type 1 diabetes (7 male, 9 female) participated in the study. Subjects were recruited from the diabetes clinic at the Royal Infirmary of Edinburgh. Baseline demographic characteristics are shown in table 3.1. HbA1c was measured by high performance liquid chromatography (non-diabetic reference range 5.0-6.05%; Bio-Rad Laboratories, Munich, Germany), and was DCCT-aligned. The subjects had no history of hypertension or macrovascular disease, and microvascular disease was excluded prior to recruitment. The presence of retinopathy was sought using digital retinal photography, neuropathy was assessed by clinical examination, and nephropathy was identified by the presence of microalbuminuria.

Subjects were excluded if they had a history of impaired awareness of hypoglycaemia, or a history of previous severe reaction to hypoglycaemia. None of the participants had a history of head injury, seizure, blackouts, alcohol or drug abuse or psychiatric illness. Subjects were not on any medications other than insulin or the contraceptive pill. All subjects gave written informed consent before participating in the study, which had been approved by the Lothian Research Ethics Committee.

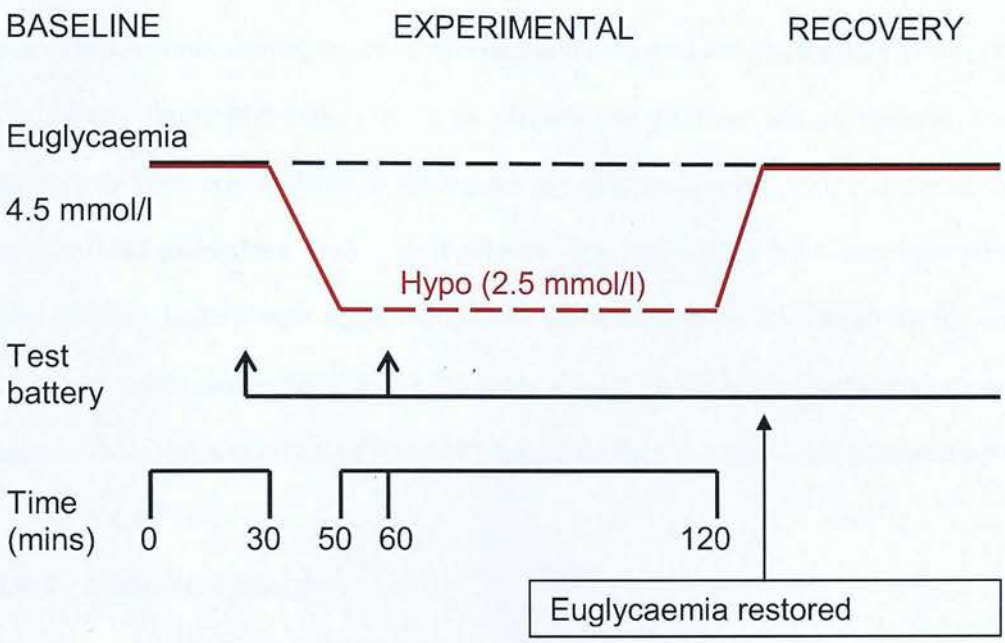
Table 3.1: Baseline demographic characteristics of study participants

Age (median [interquartile range]) (years)	28 (25-37.5)
Diabetes duration (median [IQR]) (years)	10 (4.2-19)
BMI (mean [SD]) (kg/m^2)	26.4 (4.01)
HbA1c (mean [SD]) (%)	7.91 (0.92)
National Adult Reading Test (NART) (mean [SD])	34.2 (5.1)

3.2.2 Study Design

The study was conducted at the Clinical Research Facility at the Royal Infirmary of Edinburgh. Each subject underwent two laboratory sessions, separated by at least two weeks. Subjects attended after an overnight fast, and subjects with diabetes had to confirm absence of biochemical hypoglycaemia in the preceding 48 hours prior to proceeding with the session. Intravenous cannulae were inserted into the non-dominant antecubital fossa for variable infusion of dextrose (20%) and soluble human insulin, and retrogradely into the non-dominant hand vein for regular blood glucose sampling at the bedside. The hand was heated to arterialise the venous blood (Ábumrad, 1981). Insulin was infused at a constant rate of 1.5mU/kg/min. A modified hyperinsulinaemic glucose clamp (DeFronzo, 1979) was used to maintain blood glucose at a predetermined level; euglycaemia at 4.5 mmol/l and hypoglycaemia at 2.5 mmol/l, as previously described in the methods section. Following the experimental period, blood glucose was returned to the euglycaemic target level, or maintained at that level if already euglycaemic. The clamp was then discontinued and subjects consumed a standardised meal. Subjects with diabetes were given advice on doses and timing of insulin for the rest of the day. Each subject underwent a euglycaemic study and a hypoglycaemic study in a randomised, counterbalanced fashion. The subjects were blinded to the experimental condition.

Figure 3.1: Study outline



3.2.3 Cognitive function testing

The previously described cognitive effects of hypoglycaemia were confirmed using the Trailmaking B and Digit Symbol Substitution tasks. These were performed during the run-in period and repeated during the experimental session once blood glucose targets were stable. The spatial ability test battery from the French and Ekstrom Kit of Factor-Referenced (cognitive) Tests was undertaken during the experimental period, with a different version being utilised during each session to avoid practice effects. These 2 different versions of the spatial ability battery were again randomised and counterbalanced. Details of the specific tasks undertaken are included in the Methods section. Subjects also completed a symptom questionnaire and assessment of hypoglycaemia awareness during the experimental period.

3.2.4 Statistical analysis

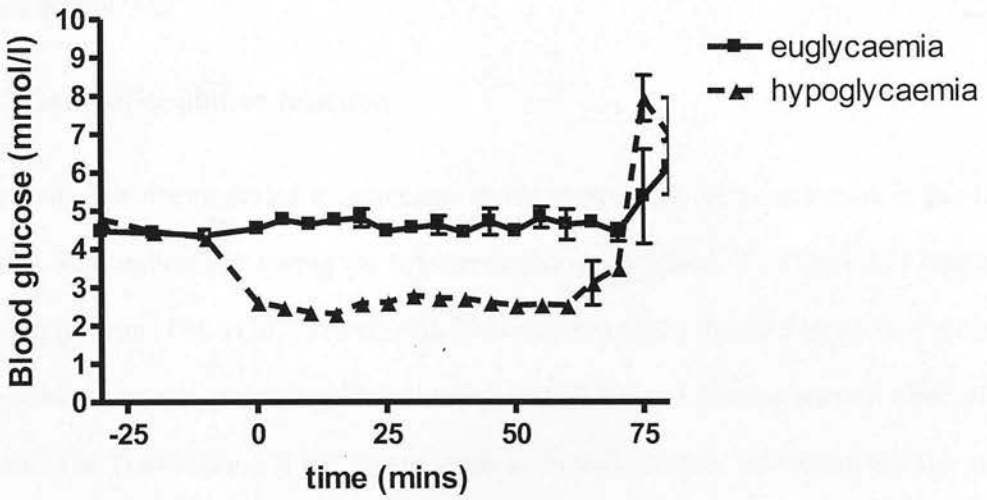
Results were analysed using SPSS version 15.0 for Windows (SPSS, Chicago, IL). A general linear model (repeated measures ANOVA) was used, with order of session (euglycaemia-hypoglycaemia or hypoglycaemia-euglycaemia) as a between-subjects factor, and condition (euglycaemia or hypoglycaemia) as a within-subjects factor. A p-value <0.05 was considered to be significant.

3.3 Results

3.3.1 Blood glucose

The target blood glucose levels were achieved for each experimental condition, as shown in figure 3.2. The mean (SD) blood glucose concentration achieved during the hypoglycaemia condition was 2.46 (0.22) mmol/l, and during the euglycaemia condition was 4.53 (0.24) mmol/l.

Figure 3.2 Blood glucose concentrations during hyperinsulinaemic euglycaemic and hypoglycaemic clamps



3.3.2 Symptom scores

Total autonomic ($p<0.001$), total neuroglycopenic ($p<0.001$), and non-specific (malaise) symptom scores ($p<0.001$) all increased significantly during hypoglycaemia. Results are shown in table 3.2.

3.3.3 General cognitive function

This study has demonstrated a decrement in the mean (SD) scores achieved in the Digit Symbol Substitution task during the hypoglycaemia study period (72.4 (20.2)) as compared with euglycaemia (84.6 (20.7)) ($p<0.001$). This confirms that a standard measure of speed of information processing was significantly impaired at a blood glucose concentration of 2.5 mmol/l. The Trail Making B test was affected by hypoglycaemia, but statistically this result did not achieve significance, though indicated a trend towards a decrease in score during hypoglycaemia ($p=0.07$). It took 50.45 (24.9) seconds to complete task during hypoglycaemia compared with 38.90 (11.6) seconds during euglycaemia (results are mean (SD)).

3.3.4 Spatial ability

The present study demonstrated that acute insulin-induced hypoglycaemia resulted in a significantly lower mean (SD) score on all but one of the spatial ability test battery. The score achieved during euglycaemia on the Hidden Patterns test was 94.5 (21.8) compared with 73.7 (21.0) during hypoglycaemia ($p<0.001$; figure 3.3). On the Card rotations test the euglycaemia score was 51.9 (15.5), compared with 40.4 (18.7) during hypoglycaemia ($p=0.001$). The Paper Folding test was again significantly affected with scores of 6.0 (1.9) vs. 4.7 (2.0) ($p=0.001$) as was the Maze Tracing test with scores of 11.1 (3.0) vs. 9.4 (2.5) ($p<0.001$). These tests require analysis of shapes and lines in addition to mental manipulation of shapes and figures. The Cube Comparisons test was also significantly affected with scores

of 11.7 (4.1) vs. 9.4 (5.7) (p=0.03). The only spatial ability test that was unaffected by hypoglycaemia as compared with euglycaemia was the Building Memory test (p=0.3).

Table 3.3 summarises the differences in performance on the tests of spatial ability during euglycaemia and hypoglycaemia (as mean (SD)). No significant effects were observed of order of exposure to glycaemic condition or test battery.

It is important to consider estimates of effect size in addition to conventional measures of statistical significance in order to establish how important an observation is. Table 3.3 shows, using Cohen's *d* values, that the impact of hypoglycaemia on these spatial abilities was of medium to large effect. In addition, the partial eta squared values in table 3.3 show that the hypoglycaemia condition accounted for a large proportion of the variance in the results.

Table 3.2: Symptom scores during experimental conditions (mean [SD]; *=p<0.05)

	Euglycaemia	Hypoglycaemia
Autonomic	1.36 (0.48)	2.76 (1.36)*
Neuroglycopenic	1.41 (0.80)	3.25(1.23)*
Non-specific	1.15 (0.28)	1.71 (0.80)*

Figure 3.3: Hidden patterns test scores in hypoglycaemia and euglycaemia conditions ($p<0.001$).

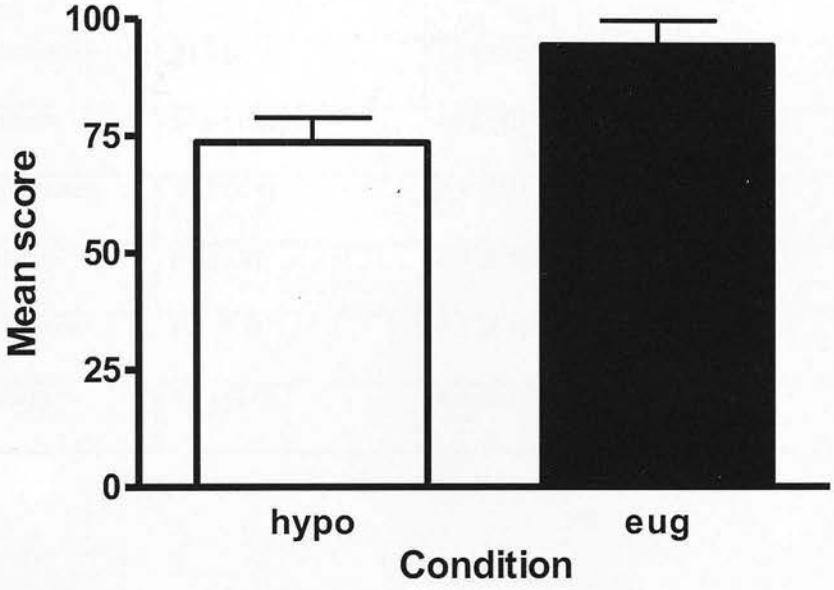


Table 3.3: Spatial ability test scores (results shown as mean [SD]; significance level p<0.05; effect sizes computed as Cohen's *d* and partial eta-squared).

Spatial test	Euglycaemia score	Hypoglycaemia score	P-value	Cohen's *d*	η_p^2
Hidden patterns	94.5 (21.8)	73.7 (21.0)	<0.001	0.97	0.627
Card rotations	51.9 (15.5)	40.4 (18.7)	0.001	0.67	0.580
Cube comparison	11.7 (4.1)	9.4 (5.7)	0.03	0.46	0.298
Paper folding	6.0 (1.9)	4.7 (2.0)	0.001	0.67	0.604
Building memory	8.6 (3.1)	7.8 (2.1)	0.3	0.30	0.081
Maze tracing	11.1 (3.0)	9.4 (2.5)	<0.001	0.62	0.621

3.4 Discussion

Acute insulin-induced hypoglycaemia causes significant decrements in most spatial cognitive abilities examined here in a group of 16 adults with uncomplicated type 1 diabetes. The impairment of this specific cognitive domain was accompanied, as anticipated, by a deterioration in the speed of mental processing as demonstrated by the decrease in score for the Digit Symbol Substitution task. The effect sizes obtained indicate the development of medium to large decrements in spatial abilities during hypoglycaemia in adults with type 1 diabetes.

One limitation of the present study was the lack of a control group of non-diabetic subjects. This would have allowed the opportunity to assess whether the diagnosis of diabetes *per se* has any impact on spatial ability at baseline, and also to examine whether the decrease in performance was as marked in the group of non-diabetic subjects, or if people with diabetes actually perform better having been exposed to hypoglycaemia previously. This observation was seen in a recent study of the effects of hypoglycaemia on psychomotor function, where some form of cerebral adaptation appeared to exist in the subjects with diabetes, who performed much better whilst hypoglycaemic (Geddes, 2008). Regardless of these issues, it is the everyday effect of hypoglycaemia on the people with diabetes that remains of clinical importance.

Other studies assessing the effects of hypoglycaemia on aspects of cognitive function have utilised tests that require a spatial ability component (Geddes, 2008), but no previous study has utilised a test battery specifically examining spatial abilities. Spatial cognition has a clear role in the safe conduct of tasks such as driving, which rely heavily upon the interpretation of the surrounding environment, and it was therefore felt that an examination of specific effects was necessary.

The Building Memory test exhibited little response to glycaemic condition, and did not achieve statistical significance. This test requires the subject to scrutinise a map which contains pictures of various buildings for a period of 4 minutes. The subjects are then given a blank map without the pictures of buildings, and they must mark on the map the positions where they remember the buildings were placed. This test therefore assesses both spatial ability and visual memory. This finding is consistent with previous studies that have examined memory function using visual memory tests from the Wechsler Adult Intelligence Scale, which have also shown that visual memory is preserved during acute hypoglycaemia (Warren, 2007). It is also notable that the Building Memory test, unlike the other tests used here, only requires participants to complete one task, rather than having the opportunity to try several tasks in the one test, and so its scores might be more idiosyncratic.

The right cerebral hemisphere, particularly the parietal lobe, is the area of the brain that dominates the conduct of spatial ability function. The frontal cortex, thalamus and to some extent, the cerebellum, are also involved in the co-ordination of spatial cognition (Harris, 2000, Vogel, 2003). Rosenthal et al showed, using neuroimaging during hypoglycaemia, that there was an attenuation of functional response (BOLD activation) in the pre-motor and supplementary motor cortex (Rosenthal, 2001). This is consistent with the recognised areas of importance in spatial functioning. In addition, it has been shown previously that general fluid intelligence is impaired during hypoglycaemia, and it is fluid intelligence, rather than crystallised intelligence, that is responsible for spatial cognition (Warren, 2004).

In conclusion, this study has shown that acute insulin-induced hypoglycaemia has an adverse effect on spatial abilities. This information improves our understanding of the domains of cognitive function that are affected during hypoglycaemia. Spatial abilities had not previously been examined, and now it is clear that they are significantly impacted upon by hypoglycaemia. Moreover, spatial abilities are highly relevant to the everyday activities of people with type 1 diabetes, and there are now data to show that part of the inability to

manage complex tasks during hypoglycaemia is the inability to effectively carry out spatial cognitive operations.

Chapter 4:

The effects of acute insulin-induced hypoglycaemia on Endothelin concentrations in adults with type 1 diabetes

4 Study 2: The effects of acute insulin-induced hypoglycaemia on Endothelin-1 concentrations in adults with type 1 diabetes

4.1 Introduction

Acute hypoglycaemia has been shown to cause a collection of haemodynamic, haemorrheological and haemostatic responses which are thought to be secondary to sympatho-adrenal activation and counterregulatory hormonal secretion, as demonstrated in previous experimental studies of acute insulin-induced hypoglycaemia (Fisher, 1993). The cardiovascular effects of these changes appear to be transient in healthy young adults with no pathophysiological consequences, but people with diabetes who have underlying vascular disease may be at risk of tissue ischaemia (Fisher, 1993). Myocardial and cerebral ischaemia, and ultimately acute vascular events, can be precipitated by hypoglycaemia (Desouza, 2003, Fisher, 2007). This has been reported anecdotally, as well as in a few small observational studies. Worsening of diabetic retinopathy may occur when strict glycaemic control is implemented (Frier, 1985, Hanssen, 1986), suggesting a further potential adverse effect on the microvasculature. The possible mechanisms by which hypoglycaemia might exacerbate vascular disease include haemorrheological changes, platelet and neutrophil activation, vasoconstriction (Fisher, 1993, Frier, 1985), and release of inflammatory mediators and cytokines (Galloway, 2000, Fisher, 1991[2], Collier, 1990, Corrall, 1980, Frier, 1983).

The endothelins are potent vasoconstrictors that have a central role in cardiovascular regulation. The endothelium-derived peptide, endothelin-1 (ET-1), is the principal isoform in humans and causes prolonged vasoconstriction, and promotes smooth muscle proliferation (Gossl, 2006). It is released predominantly from the vascular endothelium, with its main role being as a regulator of vascular tone, with resultant effects on systemic blood pressure. Once released from the vascular endothelium, ET-1 binds to endothelial receptors and initially mediates release of nitric oxide and consequent relaxation of vessel walls (Clozel, 1992);

there is then subsequent stimulation of two further subtypes of ET receptor, which induces slow and sustained vasoconstriction (Bobik, 1990). Endothelins have been implicated in the pathogenesis of several disorders including atherosclerosis, diabetes and hypertension (Hopfner, 1999, Luscher, 2000, Dhaun, 2006). Plasma endothelin production is stimulated by hypoxia, ischaemia, and epinephrine release amongst other factors (Gossl, 2006). Plasma levels are elevated in people with uncomplicated type 1 and type 2 diabetes (Takahashi, 1990), increase with presence of microalbuminuria and retinopathy (Collier, 1992), and may have a pathogenetic role in microangiopathy (Dhaun, 2006).

The aim of this study was therefore to examine changes in plasma endothelin-1 during acute insulin-induced hypoglycaemia in adults with type 1 diabetes, with the hypothesis that an increment in endothelin-1 concentration would be observed.

4.2 Methods

Twenty subjects with type 1 diabetes were studied. Subject characteristics are presented in table 4.1. All participants were receiving a combination of short and intermediate-acting insulins, either twice daily or as a basal bolus regimen. They were taking no medications other than insulin, and had no other medical disorders. Subjects were screened for diabetes complications prior to inclusion; retinopathy was assessed using ophthalmoscopy, peripheral neuropathy by clinical examination, and autonomic neuropathy using a standard battery of cardiovascular reflexes (Ewing, 1985). No participants had any clinical evidence of microangiopathy. The Lothian Medical Research Ethics Committee granted approval, and written informed consent was obtained from all subjects.

Table 4.1: Characteristics of participants with type 1 diabetes. Results are expressed as mean (SEM) unless stated otherwise. Non-diabetic range for HbA1c is 5.0-6.05% (measured by ion exchange high performance liquid chromatography).

Age (median {range}) (years)	25.5 {19-42}
Sex (male:female)	19:1
Duration of diabetes (median {range}) (years)	2 {1-26}
Body mass index (kg/m^2)	24.6 (2.8)
Insulin dose (units/kg)	0.6 (0.3)
Systolic blood pressure (mmHg)	124 (8)
Diastolic blood pressure (mmHg)	74 (18)
HbA1c (%)	7.8 (2.4)

After an overnight fast, and confirmation of the absence of hypoglycaemia in the preceding 48 hours, hypoglycaemia was induced with a continuous intravenous infusion of soluble insulin at a rate of 2.0mU/kg/min in 0.9% sodium chloride solution, through an indwelling venous cannula in the antecubital fossa. The insulin infusion was continued until the onset of symptoms of hypoglycaemia, which was usually coincidental with objective evidence of an acute autonomic reaction (R). This was identified by a rapid increase in heart rate, a rise in systolic blood pressure, and the onset of sweating and finger tremor, as described previously (Hepburn, 1991). This insulin infusion method produces a gradual, predictable and controlled decline in blood glucose to induce hypoglycaemia and simulates the development of hypoglycaemia in everyday life. It enables the glycaemic threshold for the acute autonomic activation to be identified objectively (Hepburn, 1993). Blood sampling was timed subsequently in relation to R, the onset of which differs between individuals (Hepburn, 1991).

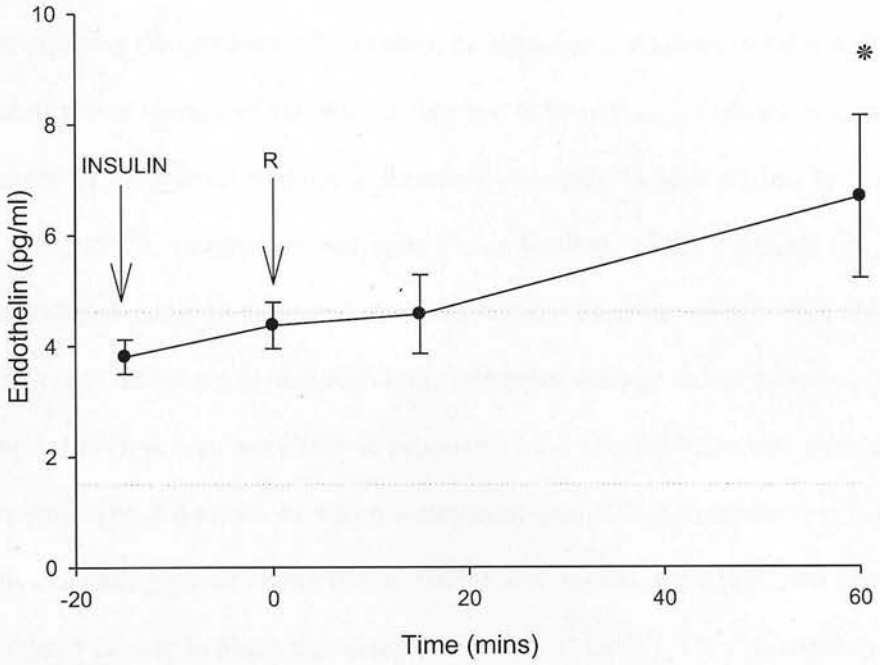
Arterialised venous blood was collected at 5 minute intervals for measurement of whole blood glucose concentration using a Yellow Springs Analyser (Yellow Springs, Ohio, USA). Blood samples were cold-centrifuged, the plasma separated and flash-frozen, and stored at -70°C for measurement of immunoreactive ET by radioimmunoassay (ITS Production BV). The sensitivity of this assay is 2 pg/ml immunoreactive ET. Cross-reactivity of the assay with ET-1, ET-2, ET-3 and big ET-1 is 100%, 52%, 96% and 7% respectively. Plasma ET concentrations were measured at baseline, at the onset of the hypoglycaemic reaction, and at 15 and 60 minutes following R.

Mean endothelin concentrations were compared by paired t test using SPSS version 15.0 for Windows.

4.3 Results

Plasma glucose concentration declined steadily from a mean (SEM) basal concentration of 5.2 (0.2) mmol/l, until the symptomatic hypoglycaemic reaction (R) occurred at the nadir of 1.9 (0.6) mmol/l. The mean (SEM) ET concentration rose from 3.80 (0.31) pg/ml at baseline to a peak of 6.72 (1.47) pg/ml at R + 60 minutes ($p<0.05$). These results are displayed graphically in figure 4.1. The different values at R and R + 15 minutes did not achieve statistical significance. The observed increase in ET concentration was wide, ranging from 0.11 pg/ml to 20.18 pg/ml.

Figure 4.1: Plasma endothelin concentrations in response to acute insulin-induced hypoglycaemia in people with type 1 diabetes. Results are shown as mean (SEM). R= autonomic reaction. Grey dotted lines = Normal range (1.5-4.5 pg/ml). * p<0.05.



4.4 Discussion

This study has shown that a significant rise in plasma endothelin-1 occurs in response to insulin-induced hypoglycaemia in adults with type 1 diabetes.

Although there is now a strong evidence base to support a role for ET in the pathogenesis of diseases affecting the cardiovascular system, its influence in diabetes remains undefined, and its reliability as a marker of endothelial function in people with diabetes is debatable. The significance of the current findings is therefore uncertain. In vitro studies have shown that insulin (Hu, 1993), vasopressin and epinephrine (Collier, 1992) stimulate ET production from endothelial cells. In animal studies, the urinary excretion of ET rises following the development of diabetes and may represent endothelial damage induced by the diabetic state (Morabito, 1994). A regulatory role of insulin on ET-1 concentration has been suggested in patients with type 2 diabetes in whom a rise in plasma ET concentrations occurs during a hyperinsulinaemic glucose clamp (Ferri, 1995[1]), and plasma ET and insulin concentrations are correlated closely in obese non-diabetic men (Ferri, 1995[2]). The diabetic state in general (Takahashi, 1990), and diabetic complications in particular (Morise, 1995), are associated with an elevated concentration of plasma endothelin. It is possible that the observed rise in plasma endothelin during hypoglycaemia could exacerbate ischaemia through its vasoconstrictive effects in a vasculature that is already compromised by micro- or macrovascular disease, and thereby enhance the risk of promoting an acute macrovascular event and possibly aggravate existing microvascular disease (Fisher, 1993, Frier, 1985). It is also plausible that endothelin-induced vasoconstriction may serve as a defense mechanism to protect vital organs such as the brain, and also to increase delivery of gluconeogenic precursors to the liver, through the regional blood flow alterations seen throughout the body in response to hypoglycaemia.

These results permit speculation as to the possible role of endothelin in the pathogenesis of hypoglycaemia-induced vascular injury, or in regional blood flow alterations, but do not provide definitive conclusions. One limitation of the current study was the absence of a control group of non-diabetic volunteers to allow the endothelin responses to be compared; the response in healthy controls may be similar to subjects with diabetes. The mechanism inducing the rise in plasma endothelin is not known. It may have been provoked by insulin *per se*, or it may have been secondary to the hormonal response to acute hypoglycaemia (Fisher, 1993) in the form of elevated epinephrine or vasopressin concentrations (Attinà, 2005). The increased sympathetic nervous system activity may have, through haemodynamic effects, increased shear stress on blood vessels and consequently triggered release of endothelin. Alternatively there may be a direct effect of neuroglycopenia causing ET release.

Another potential limitation of the present study design is the method of induction of hypoglycaemia, using an insulin infusion to reach the point of symptoms of hypoglycaemia. The insulin infusion method does not allow for controlled reduction in blood glucose, and cannot be precisely replicated from subject to subject, leading to more inter-individual variability, which may be why the degree of endothelin rise varied so much between participants. In addition, this method does not allow for a control arm of the study, and in current research practice it is rarely utilised so other studies in this field will not be directly comparable. However, this method could be seen as a strength in that it simulates the development of hypoglycaemia in real life more accurately than the hyperinsulinaemic clamp method does. Therefore the degree of rise in endothelin may be more true to that we would see in real life.

Despite these limitations, this preliminary study has demonstrated a significant rise in a peptide with potent vasoconstrictive properties, in a group of patients who are at high risk of developing vascular complications, caused by an incredibly common side-effect of their

treatment. This study adds fuel to the premise that hypoglycaemia may adversely impact on the vasculature.

Chapter 5:

The effects of acute insulin-induced hypoglycaemia on indices of inflammation

5 Study 3: The effects of acute insulin-induced hypoglycaemia on indices of inflammation

5.1 Introduction

Diabetes is associated with an increased risk of microvascular and macrovascular disease. Atherosclerosis develops prematurely and can be more aggressive and widespread in the diabetic population (Deckert, 1978, Banga, 1994, Kirpichnikov, 2001). In people with type 1 diabetes the rapid institution of strict glycemic control has been shown to aggravate microvascular complications, particularly retinopathy (Hanssen, 1986). Although this has been attributed to reduced capillary blood flow causing localised ischaemia (Hanssen, 1986), greater exposure to hypoglycaemia may have worsened microangiopathy through its potential effects on local vasculature (Frier, 1985). In addition, cardiovascular stress associated with hypoglycaemia may precipitate acute macrovascular events in a diseased circulation (Desouza, 2003, Fisher, 2007).

Experimental studies of hypoglycaemia in humans with and without diabetes have suggested several possible mechanisms by which hypoglycaemia may damage blood vessels. These include changes in regional blood flow, mobilisation and activation of leucocytes, particularly neutrophils, platelet activation, and enhanced coagulation and viscosity of the blood (Frier, 1983, Fisher, 1991[2], Trovati, 1986). In addition, more recently, plasma concentrations of C-reactive protein have been shown to increase during hypoglycaemia (Galloway, 2000), suggesting promotion of a proinflammatory state, which may aggravate vascular disease (Libby, 2002).

There has been increasing interest in the investigation of processes operating at a cellular level to cause atherosclerosis in recent years given the growing economic burden that the management of vascular disease occupies. Recent research has predominantly focused on the

potential influences of vascular inflammation, endothelial dysfunction, coagulation and platelet activation, with the intention that elucidating the underlying pathogenetic processes may help in the search for novel, early, therapeutic strategies to treat or even prevent the development of vascular disease.

The aims of the present study were to determine the effects of acute insulin-induced hypoglycaemia on inflammation, coagulation, and platelet and monocyte function in adults with and without type 1 diabetes. The battery of tests used was specifically selected to investigate cellular processes identified as important in the pathogenesis of acute and chronic vascular complications in type 1 diabetes.

5.2 Methods

5.2.1 Subjects

Sixteen non-diabetic adult volunteers and 16 healthy adults with type 1 diabetes participated in the study (Table 5.1). Subjects had no medical problems other than type 1 diabetes in the cohort with diabetes.

Table 5.1: Baseline demographic characteristics (shown as mean [SD] unless otherwise indicated)

	Non-diabetic subjects (n=16)	Subjects with diabetes (n=16)
Age (years) (median[IQR])	28 (26.7-35)	28 (25-37.5)
BMI (kg/m^2)	22.86 (2.4)	26.40 (4.0)
Male:Female	6:10	7:9
Duration of diabetes (years) (median[IQR])	N/A	10 (4.2-19)
HbA1c (%)	N/A	7.91 (0.9)

Subjects had no history of hypertension or macrovascular disease, and microvascular disease was excluded in the cohort with diabetes who underwent screening prior to inclusion in the study. Digital retinal photography was performed to exclude diabetic retinopathy, absence of neuropathy was confirmed by clinical examination, and nephropathy was excluded by the absence of microalbuminuria. Subjects with a history of impaired awareness of hypoglycaemia or a previous serious reaction to hypoglycaemia were excluded from the study. Participants had no history of head injury, seizure, blackouts, alcohol or drug abuse and psychiatric illness. They were on no medication other than insulin and the oral contraceptive pill. DCCT-aligned HbA1c was measured using high performance liquid chromatography (non-diabetic reference range 5.0-6.05%; Bio-Rad Laboratories, Munich, Germany); the mean (SD) of the participants with diabetes was 7.91 (0.92)%. All subjects gave written informed consent before participation and the study was approved by the Lothian Medical Research Ethics Committee.

5.2.2 Study Design

A modified hyperinsulinaemic glucose clamp (DeFronzo, 1979) was used to maintain blood glucose at a predetermined level; euglycaemia at 4.5 mmol/l and hypoglycaemia at 2.5 mmol/l. Each subject underwent two laboratory sessions, one hypoglycaemic study and one euglycaemic study, separated by at least two weeks (mean 7.2 weeks). Studies were assigned in a randomised, counterbalanced fashion.

5.2.3 Study Procedure

The participants with type 1 diabetes monitored blood glucose intensively during the 48 hours preceding each study, which was postponed if any blood glucose value was <3.5 mmol/l or if symptoms suggestive of hypoglycaemia were experienced. After fasting overnight, morning insulin was withheld. A retrograde intravenous cannula for blood glucose sampling was inserted into the non-dominant hand, which was heated to arterialise the

venous blood (Abumrad, 1981). A cannula in the non-dominant antecubital fossa was used to infuse 20% dextrose and soluble insulin (Human Actrapid, NovoNordisk, Crawley, UK) at a constant rate of 1.5mU/kg/min using a Gemini PCI pump (Alaris Medical Systems, San Diego, CA). Dextrose (20%) was infused at a variable rate depending on arterialised blood glucose concentrations, which were measured at 5 minute intervals using the glucose oxidase method (2300 Stat; Yellow Springs Instrument, Yellow Springs, OH). A third cannula in the other antecubital fossa was dedicated to blood sampling for inflammatory markers.

On each study day, the arterialised blood glucose was stabilised initially at 4.5 mmol/l for 30 minutes and either maintained at that level (euglycaemia) or lowered over 20 minutes to 2.5 mmol/l for 60 minutes (hypoglycaemia), after which blood glucose was restored to 4.5 mmol/l. Subjects consumed a standardised meal after each study. Blood sample time points were: baseline, during the experimental session (+45mins), during recovery (+105mins), at +6hours and at +24hours.

5.2.4 Flow cytometry

Whole blood samples were collected at the predetermined time points using D-Phenylalanyl-L-prolyl-L-arginine chloromethyl ketone (PPACK), a selective thrombin inhibitor, as an anticoagulant. 100 µl samples of whole blood were immediately incubated with 10 µl of each monoclonal antibody/antibody combination for 30 minutes at room temperature (AbD Serotec, Kidlington, UK). Subsequently red cell lysis was performed by the addition of 1 ml of FACS Lyse solution (Becton Dickinson, Oxford, UK). Flow cytometry using the FACS Calibur system (Becton Dickinson, Oxford, UK) was performed immediately following the experimental session to assess platelet-monocyte aggregation (CD14/CD42a), CD40 expression on monocytes (CD14/CD40), and CD40 ligand expression on platelets (CD154/CD42a). Isotype controls were performed in addition to both mono- and dual-stain for each parameter assessed at each time point.

5.2.5 Soluble marker assays

Citrated plasma and serum samples were collected at the predetermined time points. These were separated immediately and frozen at -80°C until analysis for the soluble markers:

Von Willebrand Factor (ELISA; CV 7.3%), tissue Plasminogen Activator antigen (Hyphen Biomed Zymutest; intra-assay CV 3.5%, inter-assay CV 4.4%), soluble CD40 ligand (high sensitivity ELISA, Bender Medsystems; intra-assay CV 5.5%, inter-assay CV 7.2%), soluble P-selectin (ELISA, R&D Systems; intra-assay CV 5.1%, inter-assay CV 8.8%), interleukin-6 (High sensitivity ELISA, R&D Systems; intra-assay CV 5.9%, inter-assay CV 9.9%), and high sensitivity CRP (DRG Diagnostics, DRG Instruments GmbH Germany; intra-assay CV 4.2%, inter-assay CV 4.1%).

5.2.6 Counterregulatory hormone assays

Samples for counterregulatory hormone quantification were collected in EDTA tubes and immediately separated and frozen at -80°C until analysis. Epinephrine and norepinephrine were assessed by high performance liquid chromatography and electrochemical detection. Glucagon and pancreatic polypeptide were determined by radioimmunoassay.

5.2.7 Hypoglycaemia symptom score

The Edinburgh Hypoglycaemia Scale (Gold, 1994) was used to assess the symptoms experienced during each experimental session.

5.2.8 Statistical analyses

Results were analysed using SPSS version 15.0 for Windows (SPSS, Chicago, IL). A general linear model (repeated measures ANOVA) was used, with order of session (euglycaemia-hypoglycaemia or hypoglycaemia-euglycaemia) as a between-subjects factor, and condition (euglycaemia or hypoglycaemia) as a within-subjects factor, to compare hypoglycaemia with

euglycaemia. Additional analysis using paired t-tests was performed to assess the change in any given parameter from baseline. A p-value <0.05 was considered to be significant. Results are reported as mean (SD) unless otherwise stated.

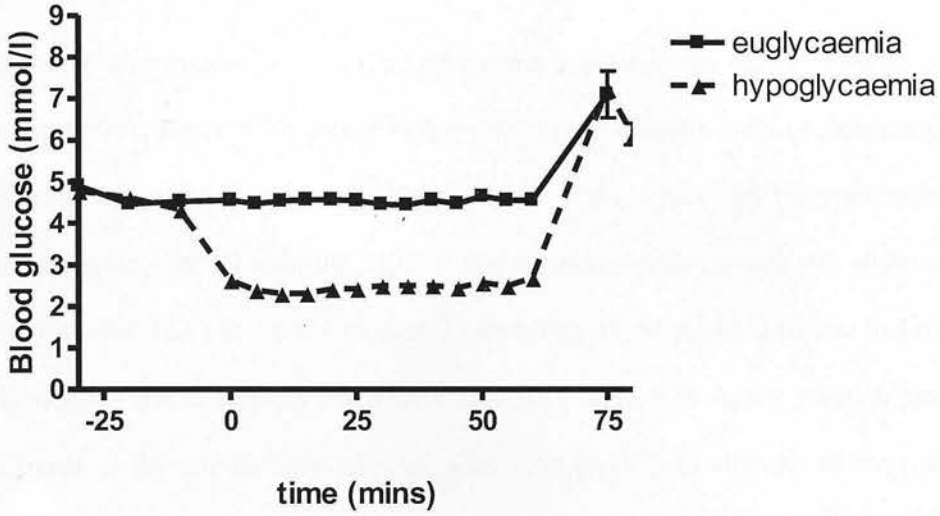
5.3 Results

5.3.1 Blood Glucose

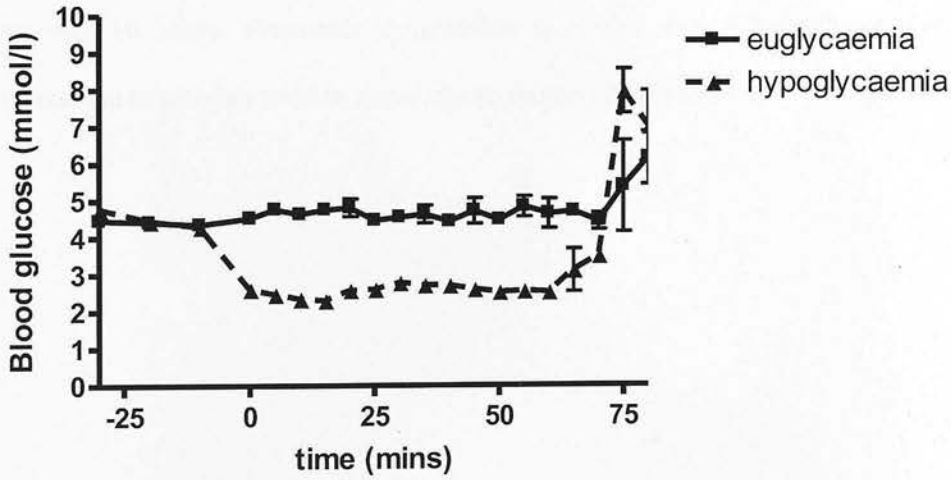
Target blood glucose concentrations were achieved as shown in Figure 5.1. In non-diabetic subjects, the mean (SD) blood glucose concentrations were 2.58 (0.2) and 4.42 (0.5) mmol/l during hypoglycaemia and euglycaemia respectively. In those with type 1 diabetes, blood glucose concentrations were 2.46 (0.22) and 4.53 (0.24) mmol/l respectively. The blood glucose nadir was similar in both groups.

Figure 5.1: Blood glucose concentrations during hyperinsulinaemic hypoglycaemic and euglycaemic clamp studies. i. Non-diabetic subjects; ii. Subjects with type 1 diabetes.

i.



ii.



5.3.2 Symptoms

Hypoglycaemia provoked a symptomatic response in all subjects with increased scores of autonomic ($p \leq 0.002$), neuroglycopenic ($p < 0.001$), and non-specific (malaise) ($p \leq 0.008$) symptoms compared to baseline. These scores are shown in table 5.2.

5.3.3 Counterregulatory response

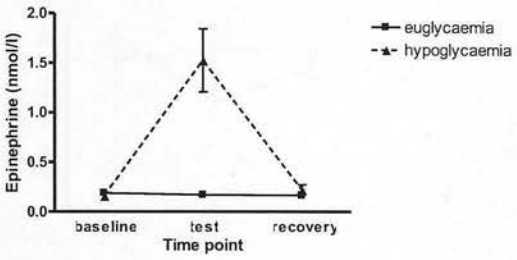
Plasma epinephrine increased during hypoglycaemia in participants with and without type 1 diabetes ($p \leq 0.001$; figure 5.2), with a higher peak in the subjects without diabetes (p=ns). The epinephrine response occurred only during hypoglycaemia and returned rapidly to baseline as anticipated (Thompson, 1993). Plasma norepinephrine did not show such a dramatic response, but was seen to increase more during hypoglycaemia studies in both non-diabetic subjects and in subjects with type 1 diabetes (figure 5.3). Again, a higher peak was demonstrated in the non-diabetic subjects. Glucagon levels rose slightly, or were at least preserved during hypoglycaemia in non-diabetic subjects (figure 5.4). Levels were suppressed in the euglycaemia studies, and were attenuated during all studies in subjects with type 1 diabetes. This is consistent with the well-established loss of glucagon response in people with type 1 diabetes of over 5 years duration; in this study the median duration of diabetes was 10 years. Pancreatic polypeptide increased during hypoglycaemia in all participants, but to a higher level in non-diabetic subjects (figure 5.5).

Table 5.2: Symptom data (reported as mean symptom score (SD); *= p<0.05)

	Non-diabetic subjects - Euglycaemia	Non-diabetic subjects - Hypoglycaemia	Subjects with type 1 diabetes - Euglycaemia	Subjects with type 1 diabetes - Hypoglycaemia
Autonomic	1.37 (0.37)	2.47 (1.11)*	1.36 (0.48)	2.76 (1.36)*
Neuroglycopenic	1.35 (0.64)	2.53 (0.83)*	1.41 (0.80)	3.25(1.23)*
Non-specific	1.18 (0.37)	1.82 (0.69)*	1.15 (0.28)	1.71 (0.80)*

Figure 5.2: Epinephrine responses to experimental procedures. i. Non-diabetic subjects; ii. Subjects with type 1 diabetes

i.



ii.

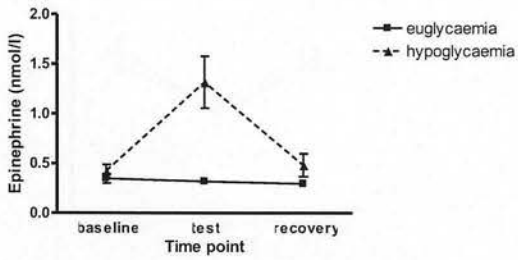
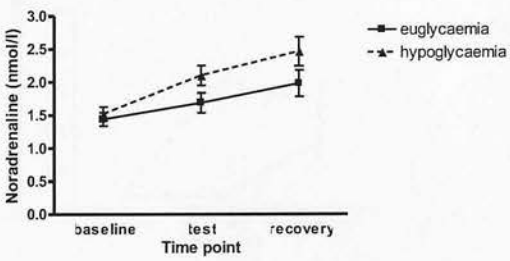


Figure 5.3: Norepinephrine responses. i. Non-diabetic subjects; ii. Subjects with type 1 diabetes

i.



ii.

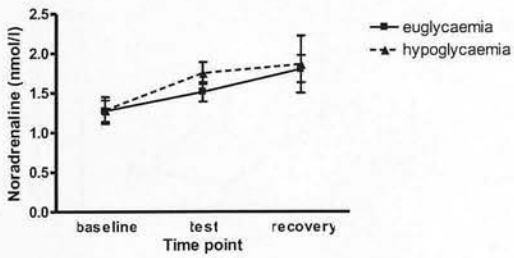
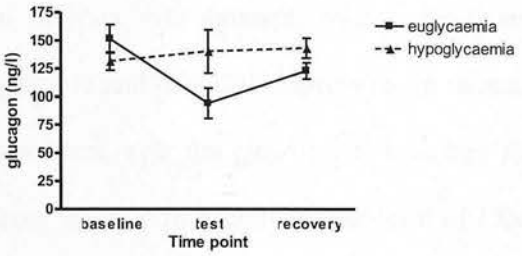


Figure 5.4: Glucagon response to hypoglycaemia. i. Non-diabetic subjects; ii. Subjects with type 1 diabetes.

i.



ii.

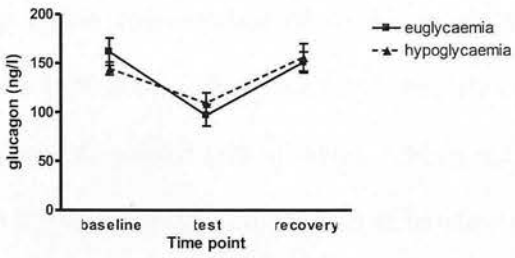
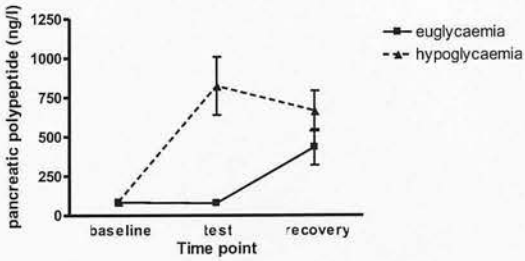
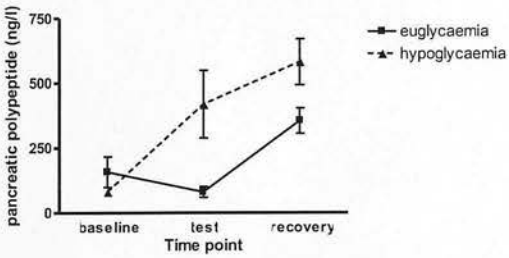


Figure 5.5: Pancreatic polypeptide responses. i. Non-diabetic subjects; ii. Subjects with type 1 diabetes

i.



ii.



5.3.4 Baseline levels of inflammation

Comparison of baseline levels of inflammatory, endothelial and platelet markers in non-diabetic subjects and subjects with type 1 diabetes showed that levels of all parameters except platelet-monocyte aggregation and CD40 ligand on platelets were elevated at baseline in subjects with diabetes, with a significantly higher concentration of soluble p-selectin ($p=0.01$) and of CD40 expression on monocytes ($p=0.006$) in those with diabetes. This is consistent with the chronic inflammatory response associated with diabetes. Surprisingly, there was a significantly higher level of CD40 ligand expression on platelets at baseline in non-diabetic subjects ($p=0.01$). These results are shown in table 5.3.

Table 5.3: Baseline levels of inflammation in non-diabetic subjects and subjects with type 1 diabetes. Data shown as mean (SD). P-value <0.05 denotes significance (*).

	Non-diabetic subjects	Subjects with type 1 diabetes	p-value
tPA	9.28 (10.2)	16.63 (29.7)	0.204
vWF	0.82 (0.3)	0.92 (0.2)	0.163
IL-6	0.79 (0.5)	0.99 (1.3)	0.427
sCD40L	2.78 (3.2)	3.15 (2.8)	0.629
P-selectin	32.50 (9.6)	39.48 (13.2)	*0.018
CD40 on monocytes	1.82 (1.9)	3.49 (2.7)	*0.006
PMA	0.65 (0.8)	0.43 (0.4)	0.200
CD40L on platelets	7.14 (4.9)	4.66 (2.8)	*0.017

5.3.5 Platelet activation

5.3.5.1 Platelet-monocyte aggregation

In non-diabetic subjects, mean (SD) platelet-monocyte aggregation appeared to rise, from a baseline level of 0.72 (0.8)% to 3.09 (8.1)% during hypoglycaemia, and this elevated level persisted for 24 hours, with a peak of 3.49 (10.4)% at 24h (figure 5.6). Platelet-monocyte aggregation remained unchanged throughout euglycaemia, with a small decline from a baseline of 0.70 (0.9)%, to 0.59 (0.4)% during the experimental period. The differences between conditions, and from baseline, did not achieve statistical significance.

In participants with diabetes, there was a late rise in platelet-monocyte aggregation following hypoglycaemia at 24h compared with baseline (p=0.03).

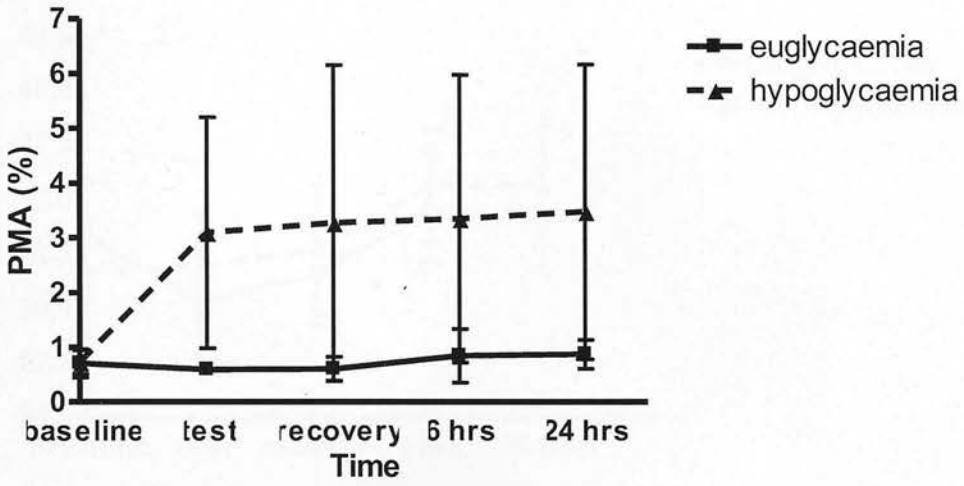
5.3.5.2 Soluble P-selectin

P-selectin increased significantly after hypoglycaemia in the non-diabetic group, exhibiting a late response at 6 hours (p=0.01) and 24 hours (p=0.02). There was a paradoxical decrease in P-selectin in those same subjects during euglycaemia (p=0.006).

P-selectin also decreased during euglycaemia in the subjects with diabetes (p=0.04), but no significant increment was identified during hypoglycaemia (figure 5.7).

Figure 5.6: Platelet-monocyte aggregation. i. Non-diabetic subjects; ii. Subjects with type 1 diabetes.

i.



ii.

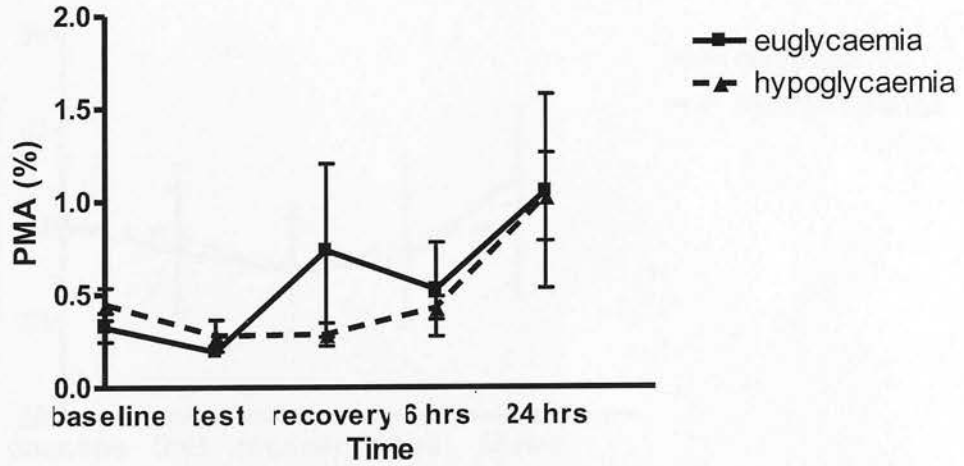
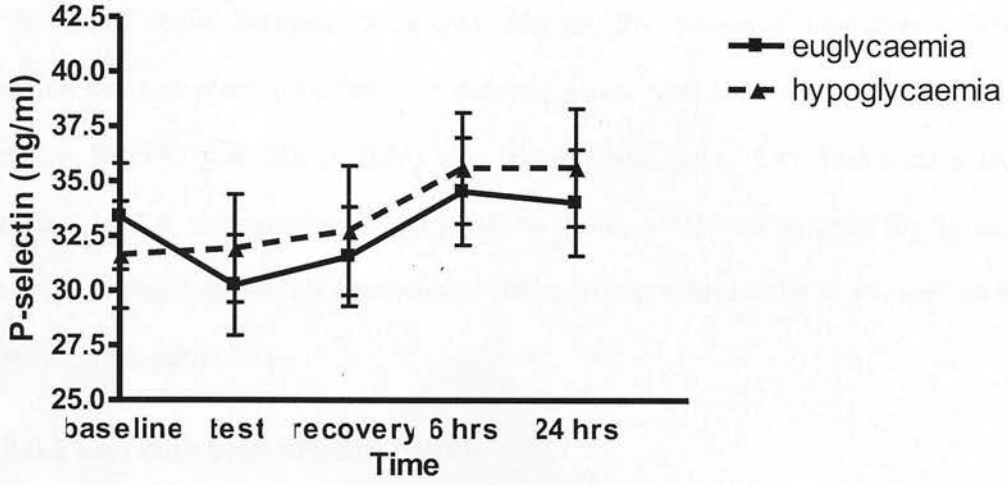
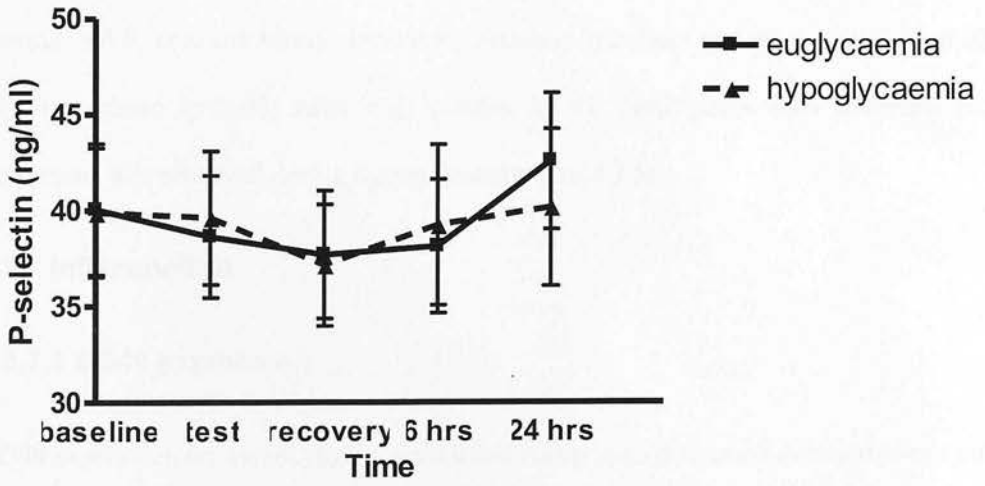


Figure 5.7: P-selectin. i. Non-diabetic subjects; ii. Subjects with type 1 diabetes.

i.



ii.



5.3.6 Endothelial markers

5.3.6.1 Tissue Plasminogen Activator (tPA)

In non-diabetic subjects, mean (SD) plasma tPA concentrations increased during hypoglycaemia, with a higher peak tPA concentration (12.55 (16.7) compared with 6.80 (7.9) ng/ml) (p=ns between conditions). Plasma tPA decreased significantly between baseline and test phase (p=0.004) and recovery phase (p=0.006), with a paradoxical rise between baseline and 24h (p=0.06) after euglycaemia (table 5.4). However, a diurnal variation in tPA concentration is recognised to occur, which may account for the decline observed during euglycaemia (Rydzewski, 1997). No significant differences occurred in the diabetic group (table 5.5).

5.3.6.2 Von Willebrand Factor (vWF)

A trend towards a difference in plasma vWF concentrations was observed between hypoglycaemia and euglycaemia at 6h in the non-diabetic subjects (p=0.07) (table 5.4).

Plasma vWF concentrations decreased between baseline and test phase (p=0.02) and recovery phase (p=0.03) after euglycaemia in the participants with diabetes. No such decrement was observed during hypoglycaemia (table 5.5).

5.3.7 Inflammation

5.3.7.1 CD40 expression

CD40 expression on monocytes increased following hypoglycaemia in non-diabetic subjects, from a baseline of 1.92 (2.2)% to a maximum of 3.13 (2.3)% at 24h (p=0.009) (results are mean (SD)). A significant difference between hypoglycaemia and euglycaemia conditions was present at 6h (p=0.05) and at 24h (p=0.04) (table 5.4).

In participants with type 1 diabetes, mean (SD) monocyte CD40 expression increased from 3.69 (3.4)% to 5.54 (4.4)% during hypoglycaemia (p=0.006), compared with no change during euglycaemia (3.64 (2.0)% to 3.65 (1.8)% respectively; p=ns). The increment during hypoglycaemia had dissipated by the time of the recovery phase and remained unchanged thereafter (table 5.5).

5.3.7.2 CD40 ligand expression

CD40 ligand (CD154) expression on platelets was found to have a very small rise during and after hypoglycaemia in non-diabetic volunteers (p=ns; table 5.4) but did not change in subjects with type 1 diabetes when compared with a euglycaemic control period (table 5.5).

5.3.7.3 Soluble CD40 ligand (sCD40L)

In non-diabetic subjects, plasma sCD40L concentrations were higher during hypoglycaemia than during euglycaemia (mean (SD) 2.80 (3.2) ng/ml vs. 2.41 (2.8) ng/ml), with a trend towards significance (p=0.09). A significant reduction in sCD40L concentration occurred during euglycaemia between baseline and recovery phase (p=0.03) (table 5.4).

In those with diabetes, a significant difference was observed between the mean (SD) baseline levels on each study day: 3.36 (2.9) ng/ml on the hypoglycaemia day compared with 2.86 (2.8) ng/ml on euglycaemia day (p=0.03), rendering subsequent measurements difficult to compare. A significant difference was again observed between the experimental condition levels, with a level of 3.41 (3.2) ng/ml during hypoglycaemia and 2.85 (2.8) ng/ml during euglycaemia (p=0.03) (table 5.5). Changes from baseline did not achieve significance.

5.3.7.4 Interleukin-6 (IL-6)

IL-6 levels rose in all experiments, maximally at 6h, irrespective of condition, with no clear differences identifiable in either group between the study conditions (table 5.4 and 5.5).

5.3.7.5 High Sensitivity C-reactive protein (hsCRP)

Test phase hsCRP was higher in all subjects during hypoglycaemia (1.81 (1.9) vs. 1.22 (1.9) ng/ml in non-diabetic participants (p=0.02); 2.72 (3.1) vs. 2.20 (2.9) ng/ml in subjects with diabetes (p=ns); results are mean (SD)) (table 5.4 and 5.5). A significant difference was observed in the baseline concentrations in the non-diabetic participants (p=0.01), frustrating interpretation of subsequent responses.

Table 5.4: Endothelial function and inflammation in non-diabetic subjects (Results shown as mean (SD); *=significant decrease from baseline p<0.05; †=significant increase from baseline p<0.05); ‡=significant difference between hypoglycaemia and euglycaemia p<0.05)

	Non-diabetic subjects									
	Euglycaemia					Hypoglycaemia				
Time	baseline	test	recovery	+6	+24	baseline	Test	recovery	+6	+24
tPA (ng/ml)	7.37 (8.1)	6.80 (7.9)*	6.44 (7.5)*	6.99 (9.3)	8.51 (7.8)†	10.96 (11.8)	12.55 (16.7)	9.10 (10.2)*	9.83 (12.0)	11.45 (11.6)
vWF (iu/ml)	0.81 (0.3)	0.76 (0.3)	0.78 (0.2)	0.80 (0.3)	0.85 (0.3)	0.82 (0.3)	0.81 (0.3)	0.89 (0.5)	0.90 (0.3)	0.89 (0.3)
CD40 (%)	1.51 (1.4)	2.23 (3.2)†	2.40 (3.2)†	0.84 (0.7)	2.06 (1.0)	1.92 (2.2)	1.47 (1.1)	1.55 (1.5)	1.98 (2.4)‡	3.13 (2.3)†‡
CD40L (%)	7.44 (3.7)	7.52 (4.0)	7.78 (4.7)	8.06 (4.5)	7.67 (4.1)	6.84 (5.9)	7.75 (5.8)	6.94 (5.8)	7.05 (5.5)	7.66 (5.4)
sCD40L (ng/ml)	2.68 (3.1)	2.41 (2.8)	2.40 (2.9)*	2.63 (2.9)	3.08 (3.3)	2.88 (3.3)	2.80 (3.2)	2.55 (3.2)	2.72 (3.3)	2.79 (3.2)
IL-6 (pg/ml)	0.86 (0.5)	1.06 (1.2)	1.05 (1.0)	5.98 (4.6)†	1.23 (0.9)	0.72 (0.4)	0.92 (0.5)	1.62 (1.2)†	4.37 (4.3)†	1.00 (0.9)
hsCRP (ng/ml)	1.04 (1.1)	1.22 (1.9)	1.18 (1.9)	1.24 (1.6)	1.31 (1.5)	1.83 (1.5)‡	1.81 (1.9)‡	1.56 (1.3)*	1.69 (1.2)	1.90 (1.6)

Table 5.5: Endothelial function and inflammation in subjects with diabetes (Results shown as mean (SD); *=significant decrease from baseline p<0.05; †=significant increase from baseline p<0.05); ‡=significant difference between hypoglycaemia and euglycaemia p<0.05)

	Subjects with type 1 diabetes									
	Euglycaemia					Hypoglycaemia				
Time	baseline	test	recovery	+6	+24	baseline	test	recovery	+6	+24
tPA (ng/ml)	15.25 (30.2)	17.70 (31.1)	15.99 (27.5)	22.13 (46.2)	20.86 (34.8)	18.12 (30.1)	20.55 (36.1)	17.69 (31.1)	18.37 (32.6)	22.98 (40.3)
vWF (iu/ml)	0.91 (0.2)	0.85 (0.2)*	0.91 (0.3)	0.85 (0.2)*	0.99 (0.2)	0.93 (0.2)	0.95 (0.2)	0.91 (0.2)	0.90 (0.2)	1.02 (0.2)
CD40 (%)	3.64 (2.0)	3.65 (1.8)	4.14 (2.5)	3.97 (2.3)	4.35 (2.0)	3.69 (3.4)	5.54 (4.4)†	3.36 (3.0)	4.88 (2.4)	4.70 (2.8)
CD40L (%)	4.62 (3.1)	5.10 (3.1)	4.79 (3.0)	5.09 (3.4)	4.28 (2.3)	4.89 (2.7)	4.63 (2.3)	4.61 (2.4)	4.48 (2.2)	4.91 (2.3)
sCD40L (ng/ml)	2.86 (2.8)	2.85 (2.8)	2.84 (2.8)	2.91 (2.9)	3.25 (3.2)†	3.36 (2.9)‡	3.41 (3.2)‡	3.10 (2.9)*	3.05 (2.8)*	3.44 (2.9)
IL-6 (pg/ml)	0.69 (0.58)	1.38 (1.9)	1.58 (1.8)	2.25 (2.8)†	1.19 (1.2)	1.21 (1.7)	1.15 (1.5)	1.76 (1.5)	3.10 (4.9)	1.96 (2.2)
hsCRP (ng/ml)	2.52 (3.1)	2.20 (2.9)	2.32 (2.8)	1.92 (1.8)	3.40 (3.6)	2.84 (3.2)	2.72 (3.1)	2.70 (3.2)	2.89 (3.3)	2.34 (2.8)

5.4 Discussion

Previous studies have demonstrated that hypercoagulability, platelet and neutrophil activation and C-reactive protein are upregulated following acute hypoglycaemia (Frier, 1983, Fisher, 1991[2], Trovati, 1986, Galloway, 2000), while a euglycaemic insulin infusion (for at least 2 hours) has been shown to reduce inflammatory markers, consistent with an anti-inflammatory effect of insulin (Dandona, 2009). The present study sought to replicate these effects, while investigating other novel underlying mechanisms of vascular disease.

This study has shown that hypoglycaemia promoted a response in a number of the vascular biomarkers examined, suggesting the premise that hypoglycaemia-induced metabolic stress may have adverse pathophysiological consequences. At the same time, the euglycaemic insulin infusion caused a potentially beneficial decrement in some parameters. However, the actual magnitude of most observed changes was small, and not all markers changed to a statistically significant degree.

This study has confirmed that platelet activation is promoted by hypoglycaemia, with increments both in platelet-monocyte aggregation and soluble p-selectin. Conversely, p-selectin decreased during euglycaemia. Endothelial function, using vWF and tPA Ag as surrogate markers, may have been disrupted, as shown by the increase in vWF after hypoglycaemia in non-diabetic volunteers, but this change was not replicated in those with diabetes. However, a reduction in vWF occurred after euglycaemia in diabetic participants, which should confer vascular benefit. tPA Ag also appeared to increase in non-diabetic subjects during hypoglycaemia, while declining during euglycaemia, whereas no significant changes occurred in the diabetic group. Soluble markers of inflammation, sCD40L and hsCRP, were higher during hypoglycaemia, with an elevation of hsCRP being observed in all subjects. Unfortunately, baseline differences in hsCRP in non-diabetic subjects, and in sCD40L in the diabetic subjects, made subsequent interpretation of responses more difficult.

sCD40L was apparently reduced during euglycaemia in non-diabetic participants. Surprisingly, IL-6 increased in all experiments regardless of glycaemic status, with a maximal response at 6h. Monocyte CD40 expression also increased, suggesting promotion of the interaction of the CD40-CD40 ligand dyad (from the tumour necrosis factor receptor family), thus affecting another process in the pathway leading to atherosclerotic plaque rupture (Schonbeck, 2001[1], Mach, 1998[1]). This change occurred much earlier in the diabetic than the non-diabetic subjects, in whom the response was delayed, prolonged, and still present at 24 hours. The persistence of these vascular changes for 24 hours after the hypoglycaemic stimulus, or their later appearance, suggests that the period of risk following hypoglycaemia may be present long after blood glucose has recovered to normal levels. This is a very concerning observation, as previously it had been believed that any stress induced by hypoglycaemia would be transient, and only likely to cause any adverse effects in those with significant underlying vascular abnormalities. It appears from these study results that many markers exhibit a delayed or prolonged response, which may pose longer term risks to the vasculature.

For some markers, a positive trend following hypoglycaemia was evident, without achieving statistical significance, or the only measurable difference between conditions was a beneficial decrement associated with euglycaemia. The sample size may have been insufficient to achieve significance, particularly as the magnitude of responses was small. It was not feasible in this study to examine a larger number of subjects due to the labour-intensive nature of running a hyperinsulinaemic clamp study alongside flow cytometric analysis of samples within 24 hours of each experimental session. The depth of hypoglycaemia or speed of onset may not have been sufficient to generate a significant response, and an insulin infusion may have been more likely to simulate real life induction of hypoglycaemia and precipitate the degree of change of markers that is most relevant to people with diabetes.

A further limitation of the present study was the need to examine the experimental conditions on two separate days in a counterbalanced fashion. Because the baseline levels of many inflammatory markers can differ on separate days, as was observed with sCD40L and hsCRP, this biological variability hinders the interpretation and comparison of subsequent results. However, the present study design was necessary to allow comparison of the euglycaemia and hypoglycaemia conditions in individual subjects, as both time and insulin infusion *per se* may exert effects on biomarker levels. This study design cannot control for other day-to-day factors that could influence baseline levels of inflammatory markers. However, the effects of hypoglycaemia could be evaluated, as each participant acted as their own control. This produces less variability than a comparison of results between individuals, as inter-individual variation in inflammatory marker levels creates more problems during analysis than intra-individual variation, which must be accepted. In addition, it was possible to analyse each study separately, by examining changes in parameters from baseline on that particular day, enabling the detection of significant effects exerted by hypoglycaemia compared to euglycaemia. Baseline levels of most markers (except CD40 ligand expression on platelets and platelet-monocyte aggregation) were higher in the diabetic group (significant for p-selectin and CD40 expression). This could affect the magnitude of response induced by the experimental procedures. However, an analysis of the percentage change from baseline was consistent with the trends identified in the absolute results (table 5.6).

Table 5.6: Indices of inflammation: Percentage change from baseline

	Non-diabetic subjects								Subjects with type 1 diabetes							
	Euglycaemia				Hypoglycaemia				Euglycaemia				Hypoglycaemia			
Time	test	recovery	+6	+24	test	recovery	+6	+24	test	recovery	+6	+24	test	recovery	+6	+24
tPA (ng/ml)	-7.7	-12.6	-5.1	+15.4	+14.5	-16.9	-10.3	+4.4	+16.0	+4.8	+45.1	+36.7	+13.4	-2.3	+1.3	+26.8
vWF (iu/ml)	-6.1	-3.7	-1.2	+4.9	-1.2	+8.5	+9.7	+8.5	-6.5	0	-6.5	+8.7	+2.1	-2.1	-3.2	+9.7
CD40 (%)	+47.6	+58.9	-44.3	+36.4	-23.4	-19.2	+3.1	+63.0	+0.2	+13.7	+9.0	+19.5	+50.1	-8.9	+32.2	+27.3
CD40L (%)	+1.0	+4.6	+8.3	+3.1	+13.3	+1.46	+3.0	+12.0	+10.4	+3.6	+10.1	-7.4	-5.3	-5.7	-8.3	+0.4
sCD40L (ng/ml)	-10.0	-10.4	-1.8	+14.9	-2.7	-11.4	-3.4	-3.1	-0.3	-0.7	+1.7	+13.6	+1.5	-7.7	-9.2	+2.3
IL-6 (pg/ml)	+23.2	+22.0	+595.3	+43.0	+38.5	+125.0	+506.9	+38.8	+100.0	+128.0	+226.0	+72.4	-4.9	+45.4	+156.1	+61.9
hsCRP (ng/ml)	+17.3	+13.4	+19.2	+25.9	-1.0	-14.7	-7.6	+3.8	-12.7	-7.9	-23.8	+34.9	-4.2	-4.9	+1.7	-17.6
P-selectin (ng/ml)	-9.3	-5.4	+3.6	+2.1	+0.9	+3.5	+12.6	+12.9	-3.5	-5.7	-4.7	+6.5	-0.5	-6.7	-1.5	+0.7
PMA (%)	-15.7	-14.2	+20.0	+24.2	+329.1	+354.1	+365.2	+384.7	-40.6	+128.1	+62.5	+231.2	-40.0	-37.7	-6.7	+126.6

As anticipated, epinephrine secretion was stimulated by hypoglycaemia in all subjects. It is likely that hormonal changes underlie the activation and upregulation of the vascular biomarkers, although other factors must be involved to generate the prolonged and delayed effects seen in some parameters, as epinephrine levels returned to baseline by the recovery phase. Catecholamines are known to promote platelet activation (Steel, 1971), while adrenoceptor blockade attenuates these effects (Fisher, 1990[2], Takeda, 1988). The participants with type 1 diabetes exhibited attenuated plasma epinephrine responses to hypoglycaemia compared with the non-diabetic subjects, who were naïve to such a hypoglycaemic stimulus. This is consistent with the recognised decline in the magnitude of counterregulatory hormonal responses with increasing duration of type 1 diabetes (Kerr, 2007). This attenuated epinephrine response may to some extent explain the lower responses of vascular biomarkers to hypoglycaemia.

In summary, the effects of hypoglycaemia on several vascular biomarkers that are implicated in the pathogenesis of vascular disease would support the premise that acute hypoglycaemia may be detrimental to an already diseased vasculature (Frier, 1985). Euglycaemia, or insulin itself, may have a protective, anti-inflammatory effect, consistent with previous reports (Dandona, 2009). In the present study the participants had no overt vascular disease and were unlikely to develop any demonstrable effects from a short period of exposure to hypoglycaemia. However, in people with diabetes of long duration, who are likely to have underlying vascular disease, these responses may not be benign. The release of potent vasoactive substances could potentially aggravate chronic vasculopathy, and contribute to the precipitation of acute macrovascular events. The prolonged increment in some indices suggests a longer period of risk following the hypoglycaemic insult than was previously believed. These changes may aggravate established diabetic micro- and macrovascular disease in those who are exposed to recurrent hypoglycaemia.

Chapter 6:
Discussion, Conclusions and Future Research

6 Discussion, Conclusions and Future Research

6.1 Spatial ability

6.1.1 Summary

Many facets of cognition have been shown to deteriorate during acute insulin-induced hypoglycaemia, but to date no study has focused purely on the effects on spatial abilities. The study described in chapter 3 has demonstrated that acute insulin-induced hypoglycaemia had an adverse impact on spatial abilities in 16 adults with uncomplicated type 1 diabetes. A large battery of tests specifically pertaining to spatial ability was utilised in order to investigate this. The decrements seen in all but one assessment were statistically significant, with large effect sizes.

A potential limitation of the present study was the absence of a control group of non-diabetic subjects with which to compare responses. It was felt, however, that the effects would be most relevant in the subjects with type 1 diabetes, who are actually exposed to hypoglycaemia on a regular basis, as opposed to non-diabetic subjects who are hypoglycaemia-naive, and who will not experience hypoglycaemia in real life. It would, however, be of interest to examine the responses in a group of non-diabetic individuals, as any differences between the groups can inform us further with regards to the phenomenon of cerebral adaptation to hypoglycaemia in those people who experience it regularly, as compared with the non-diabetic volunteers who would never have experienced it previously.

Many previous studies of hypoglycaemia and cognitive function have been limited by a small sample size, a limited selection of cognitive tests and inadequate assessment of both physical and psychosocial variables that may affect the results (Amiel, 1998). In this study avoidance of these pitfalls was ensured. In addition, there have been many methodological issues in hypoglycaemia studies in the past; these have included heterogeneity in the method of induction of hypoglycaemia, inadequate depth of hypoglycaemia to detect changes in

cognition, issues with blood sampling for glucose estimation and issues with opportunity for practice effects to arise during conduct of the cognitive test batteries. This study protocol attempted to address all of these potential issues with a robust study design, using a controlled induction of hypoglycaemia by hyperinsulinaemic clamp, comparing hypoglycaemia with euglycaemia in a randomised, counterbalanced manner. A depth of hypoglycaemia of 2.5mmol/l was achieved, and standard cognitive assessment with the Digit Symbol Substitution Test and Trail-Making B test was used to confirm that hypoglycaemia sufficient to impair cognitive function was reached. Two versions of the spatial ability test battery were available to avoid practice effects, again using a randomised, counterbalanced approach. Practice effects can mean that performance improves with repeated exposure to the tests, but can also mean that performance deteriorates through boredom or irritation with repeatedly performing the same task. The drawback of only having 2 versions of each cognitive test, though, is that there was not the opportunity for the subject to familiarise themselves with the test beforehand, which can mean that the effects of hypoglycaemia are underestimated. As a large effect size was demonstrated in the present study, it can be assumed that any familiarisation with the tests would have potentially increased the effect size demonstrated. Arterialised venous blood collection was performed (Abumrad, 1981) in order to provide accurate estimation of blood glucose concentration at the bedside throughout the experimental procedures.

Previous studies of the effects of hypoglycaemia on cognition have used batteries of tests that are likely to include a spatial ability component, but the aims of this study were to concentrate on spatial ability alone, as it is a task that is so relevant to daily life. Driving, in particular, relies heavily on spatial ability, so it is therefore of considerable importance to know how hypoglycaemia will impact on it. The French and Ekstrom Kit of Factor Referenced (cognitive) tests was used (Ekstrom, 1976). This kit consists of 72 different psychological tests representing 23 cognitive factors, and a selection of those pertaining to

spatial ability factors was used for our spatial test battery. The selected tests needed to examine each factor associated with spatial ability but be manageable within the experimental study timeframe. It would have been unacceptable to subject each participant to a more prolonged episode of hypoglycaemia in order to administer a larger test battery; this would also introduce fatigue as a potential confounding variable as this may impair cognitive performance. It was felt that the chosen battery adequately assessed each facet of spatial functioning to give an overall assessment of spatial ability during the experimental period, and the battery was administered comfortably during the 1 hour experimental window, including the need for explanation of each test. The sub-divisions assessed were: Flexibility of Closure (Hidden Patterns), Spatial Orientation (Card Rotations and Cube Comparisons), Visualisation (Paper Folding), Visual Memory (Building Memory), and Spatial Scanning (Maze Tracing) (Ekstrom 1979). Each of these will now be discussed in turn (Ekstrom, 1976, Ekstrom, 1979).

Flexibility of Closure: this is defined as

'the ability to hold a given visual percept or configuration in mind so as to disembed it from other well-defined perceptual material'.

Tests of this factor require the subject to search a distracting visual field in order to find a given model/pattern.

Visual Memory:

'the ability to remember the configuration, location and orientation of figural material'.

It is felt that visual memory encompasses processes beyond other memory functions, with the existence of iconic memory used to store visual impressions.

Spatial Orientation:

'the ability to perceive spatial patterns or to maintain orientation with respect to objects in space'.

This is a complex sub-division of spatial ability in that it is difficult to completely extract this factor from others associated with spatial ability. It is intricately linked to Visualisation. It is thought that the figure is perceived as a whole in Spatial Orientation, but that it requires mental restructuring and manipulation in Visualisation (Carroll, 1974). Spatial Orientation may be a more difficult or less practiced aspect of perceptual speed, which is assessed by a different selection of tests not utilised in our present test battery. Both aspects require visual memory, but serial operations are required in Visualisation.

Spatial Scanning:

'speed in exploring visually a wide or complicated spatial field'.

The tests of this factor require the ability to scan the field quickly, follow paths with the eye and reject false leads. There is an element of planning involved, but it is felt to be limited in these maze-type tasks which simply require the desire to scan for the right path before wasting time drawing a line on the paper. These tasks are certainly at risk of practice effects, but we eliminated this possibility by only performing the task during experimental periods, and we had 2 versions of the test available for each experimental session.

Visualisation:

'the ability to manipulate or transform the image of spatial patterns into other arrangements'.

This requires restructuring and mental manipulation of the images seen. Many researchers feel that this is not a primary factor in itself, but comprises multiple factors including figural adaptive flexibility, speed of closure and flexibility of closure. Some subjects may utilise an analytical strategy to perform these tests, looking for symmetry and planes of reflection as clues to the solution (Cattell, 1971). Alternative suggestions are that there are primary and

higher order levels of visualisation (Royce, 1973). This is not a major issue in the present study as a collection of tests was used to represent spatial ability together.

Only one aspect of the spatial test battery was not significantly affected by hypoglycaemia, and that was the Building Memory test. This test requires the subject to memorise the position of various buildings on a map, and then try to re-site those buildings on a blank map thereafter. It therefore encompasses both a spatial ability function and visual memory function, and in the Kit of Factor-Referenced Cognitive Tests comes under the 'Visual Memory' factor. It is noteworthy that visual memory has been investigated previously, and that it was shown not to be affected by hypoglycaemia (Warren, 2007). In that study, the visual reproduction (immediate and delayed) test was utilised from the Wechsler Memory Scales (revised) battery. A 'ceiling' effect was demonstrated, where almost all candidates achieved a near perfect score, suggesting that this was not a very discriminatory test for the study of the effects of hypoglycaemia. This ceiling effect did not seem to be present in analysis of the scores for the Building Memory test, but there were fewer items in this test than in others used, and this seems more likely to have contributed to the lower discriminatory value of the test.

6.1.2 Mechanisms of cognitive dysfunction

6.1.2.1 Regional blood flow changes

It has been shown that hypoglycaemia causes an overall increase in total cerebral blood flow at blood glucose concentrations below 2.0 mmol/l. The regional distribution of blood flow within the brain is altered from baseline, with an increase to the frontal cortex and the thalamus, perhaps to provide glucose to the areas that are most vulnerable to neuroglycopenia (Bryan, 1990, MacLeod, 1994, Tallroth, 1992). Consequently there are also areas that experience a reduction in flow, for example the right posterior cingulate gyrus and putamen (MacLeod, 1996). These areas will experience a reduction in fuel delivery, and

these areas may have a role in co-ordinating the affected components of cognitive function. The putamen is certainly recognised to be of importance in regulating movement and learning. Neuroimaging methods have become very sophisticated in recent years, and are able to assist in our understanding of these regional blood flow alterations, to help translate them into the functional changes that we see in real life.

6.1.2.2 Data from functional imaging studies

In vivo neuroimaging studies are helping to shed light on the effects of hypoglycaemia on cognitive function. Changes in regional blood flow can be assessed directly using PET (Positron Emission Tomography), or indirectly using functional MRI (magnetic resonance imaging). Imaging studies are good at assessing how structure and function are affected by hypoglycaemia, but cannot tell us the mechanisms by which these changes are induced. Since the brain is dependent on glucose for metabolism and function, acute neurological consequences of hypoglycaemia are expected when a hypoglycaemic state is created. This has led to the use of imaging techniques to attempt to clarify which regions of the brain are most affected during hypoglycaemia. Areas controlling appetite show increased neuronal activity, whereas the lateral orbitofrontal cortex sees a downregulation of activity. Global blood flow decreases by 6-8% overall at a blood glucose concentration of 3.0 mmol/l, particularly evident at the hippocampi. Conversely, there is a relative increase in activity seen in the medial and orbital prefrontal cortex, thalamus, globus pallidus and periaqueductal grey (Teves, 2004).

Spatial ability processes predominantly involve the right cerebral hemisphere, particularly the parietal lobe, and spatial cognition is co-ordinated through the frontal cortex, thalamus and to some extent the cerebellum (Harris, 2007, Vogel, 2003). It has been demonstrated that BOLD activation (blood oxygenation level-dependent activation) is attenuated during

hypoglycaemia in the pre-motor and supplementary motor cortex, consistent with some of the areas known to be involved in spatial ability processes (Rosenthal, 2001).

Of considerable interest is the role of the brain in detecting and responding to evolving hypoglycaemia. Functional neuroimaging provides a method to assess which areas of the brain switch on or off during hypoglycaemia, and as well as being relevant to cognitive function, this is also helping to determine how the brain senses hypoglycaemia. Following on from this, the pathogenesis of impaired hypoglycaemia awareness or cerebral adaptation to hypoglycaemia in those who are prone to recurrent episodes is an area of intense research at the present time.

6.1.3 Clinical Relevance

The study described in chapter 3 has provided further evidence of the adverse impact that hypoglycaemia has on cognition. Spatial ability is one of the most important components involved in the task of driving, along with information processing, psychomotor function and attention. Across Europe, people with type 1 diabetes are offered a period-restricted driving licence which allows the opportunity for reassessment of clinical status on a regular basis to ensure no issues pertaining to vision, proprioception or hypoglycaemia have developed, that may hamper a person's ability to drive safely. The Driver and Vehicle Licencing Authority (DVLA) in the UK issues 3 year period-restricted licences for driving cars and motorcycles, and has recently, in 2011, introduced new guidance to allow annual licences to be issued to drivers of buses and lorries, where this was previously banned. Drivers of these larger vehicles have to comply with very strict criteria including an annual assessment to ensure that they: have had no SH in the preceding 12 months, have full hypoglycaemic awareness, prove adequate control through regular blood glucose measurement using a meter with a memory function, demonstrate an understanding of the risks of hypoglycaemia and have no other debarring complications of diabetes. An additional change to the UK guidelines has

been to impose a ban on driving if more than one episode of SH has occurred in the preceding year, or if impaired hypoglycaemia awareness is present (defined in this case as the *total* loss of warning symptoms). Previously a one year restricted licence was issued in the case of impaired awareness, so the new guidelines are stricter, showing that attention to safety is becoming ever more important.

Studies examining the effects of hypoglycaemia on driving using a driving simulator have been conducted in the USA. Using the hyperinsulinaemic clamp technique, as in our spatial ability study, the direct consequences of hypoglycaemia on driving have been assessed. At a blood glucose concentration of 3.8mmol/l or less, driving errors become commonplace. These errors include speeding, inappropriate braking, crossing the midline of the road, or even driving off the road, and also an increased frequency of crashes. All of these tasks involve malfunction of spatial and orientational ability. Only 30% of participants in these simulator studies requested treatment to correct their hypoglycaemia, and this only occurred at a blood glucose concentration below 2.8mmol/l. Less than 25% of subjects felt they had to stop driving once their blood glucose was low (Cox, 1993, Cox, 2000). Although a driving simulator is still not equivalent to driving a real vehicle, it is fairly close, and these studies demonstrate the relevance to real life that impairments in cognitive function have.

A further aspect of real life that may be affected by this cognitive impairment during hypoglycaemia is employment. Certain vocations are not compatible with insulin therapy and risk of hypoglycaemia due to the effect this will have on cognitive function, or even on conscious level if left untreated. These include vocations such as working as an airline pilot or train driver, and jobs involving working alone in isolated or dangerous areas, or at height. Serving in the armed forces is also banned, and work with emergency teams and in the offshore oil industry is not usually permitted due to the isolation that occurs, and the unpredictable nature of the jobs involved. Therefore people who experience regular hypoglycaemia, or have impaired hypoglycaemia awareness, can be limited in the

professions that they can follow, and may have restrictions imposed on them at work. It may also be necessary for an individual to take time off work following hypoglycaemic events in order to recover and restabilise blood glucose, which may again lead to conflict with an employer.

The present study findings are of considerable concern given the importance of spatial ability in normal day-to-day functioning. The effects of hypoglycaemia on the brain must not be underestimated.

6.2 Vascular biology

6.2.1 Summary

6.2.1.1 Endothelin-1

Chapter 4 describes a preliminary study performed to evaluate the effects of acute hypoglycaemia on levels of plasma endothelin-1, a potent vasoconstrictor. This study demonstrated, in 20 subjects with type 1 diabetes, that endothelin-1 concentrations increased following hypoglycaemia, with a maximum increase seen at 60 minutes following the autonomic reaction.

The practical aspects of this study were performed prior to the general acceptance that the hyperinsulinaemic glucose clamp technique was a safer and more controlled method of inducing experimental hypoglycaemia. In this study, hypoglycaemia was induced by the insulin infusion method, with the rate of infusion calculated according to body weight. The steady infusion is continued until the autonomic reaction is generated, which can occur after a variable amount of time in different individuals, and can also occur at different blood glucose concentrations. This will be particularly true in this group of patients with type 1 diabetes, who will have differing levels of prevailing glucose control, and different history of

exposure to previous hypoglycaemia. As a result, the exposure to insulin during the experiment will also have differed between individuals. There were, therefore, several confounding variables that may have influenced the results seen. Another drawback to using this method is that it does not allow for a control arm to the study, and therefore there is no euglycaemic control experiment to show that insulin infusion, *per se*, does not affect endothelin-1 levels. Additionally, these results will not be directly comparable with other studies in the field which have utilised the more commonly used clamp method. Despite this, there is no doubt that the levels did rise, but a large inter-individual variation in response was seen, which may have been contributed to by the confounders described above. However, in favour of this method of inducing hypoglycaemia is the fact that it is a better way of mimicking the day to day occurrence of hypoglycaemia in people with type 1 diabetes, and therefore perhaps these results are a more true to life representation of how individuals would respond.

The next questions to address are: i) the role of endothelin-1 in diabetes, and ii) the potential role and effects it may produce following hypoglycaemia. Patients with diabetes, and many other conditions associated with cardiovascular risk including hypertension and renal disease, have elevated baseline levels of endothelin-1 (Schneider, 2002, Dhaun, 2006) when compared with healthy controls. It is thought possible that elevation in ET-1 concentration may precede the development of vascular complications in disease states associated with cardiovascular risk (Schneider, 2002). There is evidence to suggest that the presence of microvascular complications may lead to increases in ET-1, and that ET-1 levels are correlated with presence of microangiopathy, hypertension, increased disease duration and family history of diabetes in people with type 2 diabetes (Collier, 1992, Ak, 2001, Dhaun, 2006). There has been no association found to date with metabolic control, treatment modality, age, sex, lipid control, obesity or smoking (Ak, 2001). It is believed that the role of ET-1 in health is to maintain vascular tone, so that in disease states increasing the risk of

vascular disease, there may be an insensitivity to ET-1, resulting in a compensatory increase in concentrations. This may reflect abnormal vascular reactivity in the at risk groups (Ang, 2001).

It is also postulated that ET-1 rises in a dose-response relationship with insulin, either endogenous or exogenous (Wollesen, 1999). It is therefore plausible that any increment in ET-1 concentration seen in these patients may have been induced by insulin infusion rather than hypoglycaemia itself. Without a euglycaemic control arm to the experiment this fact will be difficult to dispute or confirm. The fact remains that there was an identifiable and statistically significant rise in ET-1 concentration following induction of hypoglycaemia, and since it is known that ET-1 exerts a vasoconstrictive effect on the vasculature, it must be assumed that the potential for reduction in blood flow and ischaemia is a possible adverse consequence. The effects of the endothelin rise may also serve to alter the regional blood flow around the body, preserving nutrient delivery to important areas, and diverting it from those areas deemed less vital.

6.2.1.2 Platelet function, endothelial markers and inflammation

Chapter 5 describes a large study undertaken in 16 non-diabetic subjects and 16 subjects with type 1 diabetes. This study was designed to further investigate the hypothesis that hypoglycaemia may have adverse effects on vascular function, on a larger scale. All subjects underwent a euglycaemic and hypoglycaemic study period on two separate occasions, and had an extensive battery of soluble markers of platelet function, coagulation and inflammation examined, in addition to having platelet and inflammation studies conducted by flow cytometry. The battery of tests was selected to address three of the main pathophysiological mechanisms recognised to influence the development and progression of vascular disease, namely platelet activation, endothelial function and inflammation.

This study has demonstrated that moderate hypoglycaemia resulted in platelet activation, as shown by an increment in platelet-monocyte aggregation and soluble p-selectin, with suppression of p-selectin during the euglycaemic clamps. Endothelial markers, in this study using tPA and vWF, were variably affected; in non-diabetic subjects there was an increase in tPA during hypoglycaemia, and a suppression during euglycaemia, but these findings were not replicated in the group with diabetes. Again, vWF appeared to increase at a later stage post-hypoglycaemia in the non-diabetic subjects, with little change in the subjects with diabetes, although the euglycaemic clamp did result in suppression of vWF, which may confer a beneficial effect of insulin on endothelial function in people with diabetes. Lastly, inflammation was assessed using CD40 expression, soluble CD40 ligand, IL-6 and hsCRP as markers. CD40 expression increased during hypoglycaemia in subjects with diabetes, and also in non-diabetic subjects though this occurred after a delay at 6 and 24 hours following hypoglycaemia. Soluble CD40 ligand increased significantly during hypoglycaemia and decreased significantly during euglycaemia in non-diabetic subjects. There was also a significant difference between the peak response during the experimental phase in subjects with diabetes, although baseline levels differed on each study day and this may have influenced the outcomes. IL-6 rose regardless of experimental condition in all subjects, so this was not indicative of a response to experimental condition, and hsCRP was significantly higher in all subjects during hypoglycaemia. Although, again, there was a baseline difference in non-diabetic subjects between study days which may have influenced the results. Interestingly, some of the increments seen occurred after a delay, and some were even present 24 hours after the hypoglycaemic insult. This is a concerning finding, particularly if hypoglycaemia is a regular occurrence, as potentially damaging vascular substances may be elevated for some time following hypoglycaemia, and with repeated episodes these levels may increase further, posing additional risk. This contradicts the previous belief that only transient alterations in vascular biology occur following a hypoglycaemic insult, and certainly merits careful consideration.

Overall these results are suggestive of a pro-inflammatory, pro-atherogenic response to hypoglycaemia, although some issues in interpretation of the results are present. The baseline differences in sCD40L and hsCRP between study days have frustrated interpretation of these parameters. Unfortunately there is day to day intra-individual variation in inflammatory parameters, as so many external and internal influences can affect levels. This would, of course, be true in real-life too. It is hoped that by assessing a large battery of tests, however, that the overall picture becomes clearer. In addition, in some parameters there was only a trend towards significance indicated on statistical analysis, or a non-significant result was obtained despite a difference being evident on the absolute results. In order to assess the trends identified further, percentage change from baseline was also assessed, and this confirmed the trends seen in the absolute numbers. The only way to overcome this hurdle and improve the statistical power would have been to use a larger sample size, particularly as the magnitude of the changes seen was very small. Unfortunately due to time constraints and the complicated nature of running a clamp study alongside flow cytometric analysis of study samples with only one investigator, this was not practically possible during this programme of research. It is anticipated, however, that many other studies in this field will be conducted with a similar study design, and that cumulatively these studies together will add strength to these results, and lend support to the hypothesis.

6.2.2 Recent studies

Since the design and conduct of this study, several further studies in this field have been reported. Razavi Nematollahi et al investigated the effects of acute hypoglycaemia induced by the insulin tolerance test method, injecting an insulin bolus of 0.1units/kg into 13 male volunteers without diabetes. This resulted in a nadir blood glucose of 2.2mmol/l, as compared with 2.5mmol/l in the clamp study described in chapter 5, and the rate of fall of blood glucose was obviously much more rapid. They demonstrated a significant rise in TNF-α, IL-1β, IL-6 and IL-8, and confirmed a rise in counterregulatory hormones, which they

hypothesised were the cause of the increments in inflammatory markers. This study did not include a control arm, and was conducted in a small group of non-diabetic individuals, with the expected limitations that this would entail (Razavi Nematollahi, 2009).

Dotson et al showed that IL-6 increases during a hypoglycaemic hyperinsulinaemic glucose clamp, with no change seen in the euglycaemic control study. This contradicts the findings of our study that both experimental conditions resulted in a rise in IL-6, maximal at 6 hours. This study only reported results during the clamp period, however, up until 135 minutes, and did not comment on recovery levels or on delayed responses (Dotson, 2008). The duration of the experimental phase in this study was 120minutes approximately, which is double the length of time our subjects were exposed to hypoglycaemia.

More recently a study in the USA has been conducted with a very similar design to the one described in chapter 5. This group used hyperinsulinaemic clamps to compare euglycaemia and hypoglycaemia in both diabetic and non-diabetic subjects, and demonstrated a significant pro-inflammatory, antifibrinolytic response following hypoglycaemia. The depth of hypoglycaemia achieved in this study was a mean of 2.9mmol/l, but the experimental period again lasted for 120minutes compared with 60minutes in our study. Not surprisingly, they therefore saw a more impressive response to hypoglycaemia, with increments in intercellular adhesion molecule (ICAM), vascular cell adhesion molecule (VCAM), p-selectin, e-selectin, plasminogen activator inhibitor-1 (PAI-1), tumour necrosis factor-α (TNF-α), interleukin-6 (IL-6) and vascular endothelial growth factor (VEGF). They also demonstrated an anti-inflammatory effect of the euglycaemic-hyperinsulinaemic clamp, although their results were again more dramatic due to the increased duration of exposure to insulin (Gogitidze Joy, 2010). This group have also presented findings looking at similar parameters in people with type 2 diabetes following hypoglycaemia, and have compared hyperglycaemia with hypoglycaemia in non-diabetic volunteers. Their results indicate that there is, again, an increment in inflammatory markers, platelet activation and anti-

fibrinolytic mechanisms, and additionally that flow mediated dilation is impaired in subjects with type 2 diabetes after hypoglycaemia. Despite some methodological flaws in the study design, these findings are highly clinically relevant (Gogitidze Joy, 2011[1]). They also demonstrated that acute hypoglycaemia resulted in greater disruption of these parameters than acute hyperglycaemia in non-diabetic volunteers (Gogitidze Joy, 2011[2]). These studies add further evidence to our rapidly evolving knowledge of the detrimental effects of hypoglycaemia.

It has also been demonstrated that central arterial pressure falls during acute hypoglycaemia induced by the insulin infusion method (as used in chapter 4) (Sommerfield, 2007). This finding permits speculation that a resultant reduction in coronary blood flow may ensue, which may lead to precipitation of acute vascular events in a diseased circulation. The depth of hypoglycaemia achieved in this study ranged from 2.6-2.9 mmol/l, but measurements were timed in relation to the autonomic reaction, which would suggest that counterregulatory hormone secretion had been activated even at fairly modest degrees of hypoglycaemia.

Imaging studies have now been performed to assess the effects of hypoglycaemia on the cardiovascular system. One study examined, in non-diabetic subjects and subjects with type 1 diabetes, whether hypoglycaemia would have an impact on myocardial blood flow using myocardial contrast echocardiography. They confirmed that myocardial blood flow decreases during hypoglycaemia as compared with a euglycaemic control period. This group also looked at endothelin-1 concentrations, which rose during hypoglycaemia. The depth of hypoglycaemia achieved in this study was 2.8mmol/l (Rana, 2011). This confirms the effect of hypoglycaemia on ET-1 production that was demonstrated in our initial vascular study, and has also determined that the effect is independent of insulin infusion itself.

In the longer term, there are concerns that repeated exposure to hypoglycaemia, for example in those people with impaired hypoglycaemia awareness, will lead to chronic increased

vascular risk. A study reported in 2011 showed that this premise is of concern, with an increase in inflammatory markers, reduction in flow-mediated vasodilatation, and an increase in carotid intima-media thickness (CIMT) and femoral intima-media thickness (FIMT) in subjects with type 1 diabetes and impaired awareness with increased frequency of hypoglycaemia, when compared with subjects with type 1 diabetes and normal awareness (Gimenez, 2011). This team also conducted a short clamp study lasting 30 minutes to assess the effects of acute hypoglycaemia on the same individuals but did not see any significant increments in the parameters assessed. Furthermore, a longitudinal study of the effect of cardiovascular risk factors on evolution of CIMT in children with type 1 diabetes has shown that there is an inverse relationship between glycaemic control, as measured by HbA1c and CIMT, i.e. the lower the HbA1c, the higher the CIMT measurement (Dalla Pozza, 2011). These study findings are of considerable concern as for a long time the drive to lower HbA1c has been the clinical priority, at the expense of an increased frequency of hypoglycaemia, and mounting evidence would now suggest that this is at odds with our desire to reduce vascular risk.

6.2.3 Clinical relevance

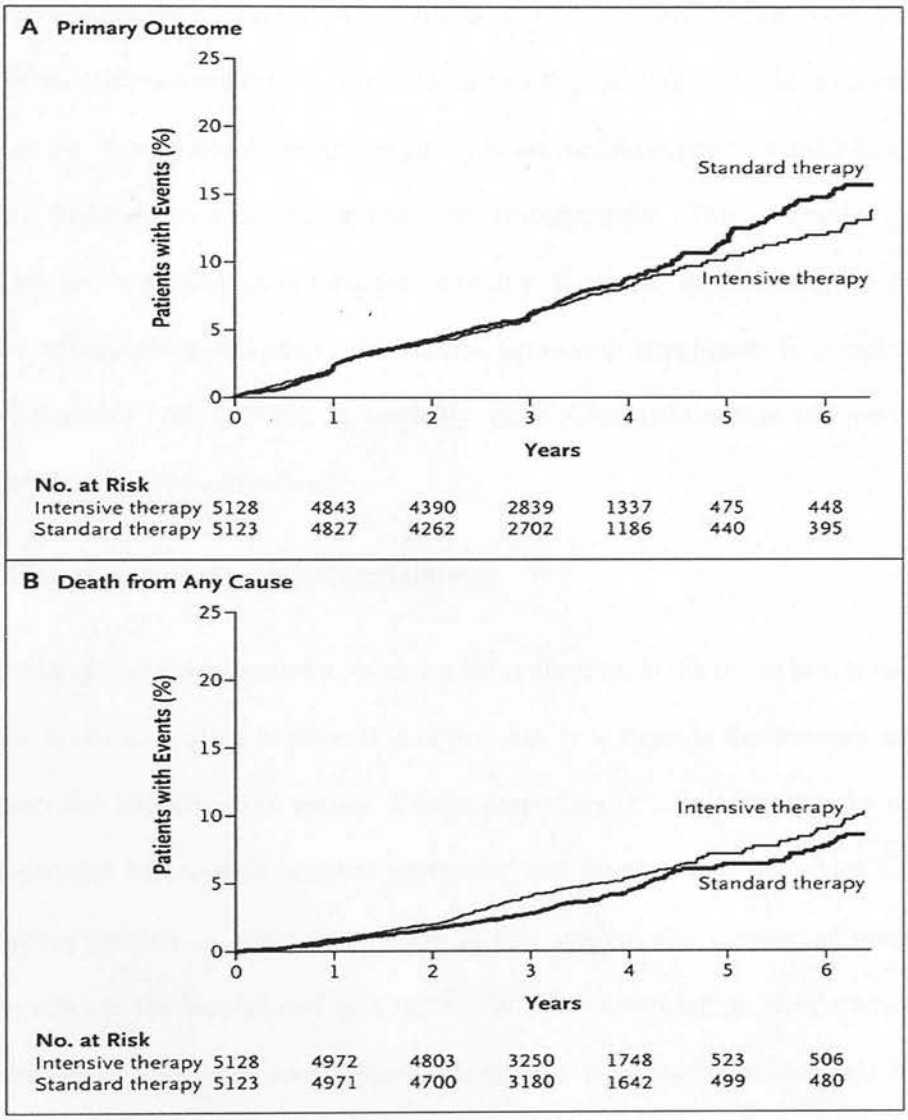
6.2.3.1 Clinical trial evidence

A plethora of clinical trial evidence has now been reported to indicate that striving for tight glycaemic control may confer no benefit to the patient, and may in fact have the potential to cause harm. Two trials examining intensive glucose control in people with type 2 diabetes using insulin give us cause for concern. The Action to Control Cardiovascular Risk in Diabetes (ACCORD) trial and the Veterans Affairs Diabetes Trial (VADT) showed no cardiovascular risk benefit in the groups receiving intensive insulin therapy (Duckworth, 2009, ACCORD Study Group, 2008). In fact, the ACCORD trial had to be terminated prematurely after 3.5 years due to increased all-cause (22% increase) and cardiovascular

(35% increase) mortality in the intensively treated arm, although rates of acute myocardial infarction were reduced (figure 6.1).

The glycaemic target in ACCORD was an HbA1c of 6.0% in the intensively treated arm, and 7.0-7.9% in the conventional therapy arm. The achieved levels were 6.4% and 7.5% respectively. In the VADT trial, levels of 6.9% and 8.4% were achieved. The rate of severe hypoglycaemia was significantly increased in both trials in the tight control groups (10.5% vs 3.5% in ACCORD – severe hypoglycaemia requiring medical assistance; 12 episodes per 100 patient-years vs. 4 episodes per 100 patient-years in VADT – episodes with some degree of impairment of conscious level). There was a threefold increase in sudden deaths in the intensively treated arm of the VADT trial. Subsequently, detailed post-hoc analysis of the ACCORD study data has suggested that the increased mortality seen was not attributable to symptomatic, severe hypoglycaemia. Both intensive and conventional study groups were shown to have increased mortality if they had experienced severe hypoglycaemia, but causality cannot be proven, and based on those results the mode of treatment (intensive vs conventional) cannot be held responsible. These findings do, however, remain concerning given the well-established fact that hypoglycaemia begets hypoglycaemia, and that much of it may be asymptomatic if impaired awareness becomes a factor. This means that the rates of hypoglycaemia reported may not be accurate. In addition, definitions of hypoglycaemia, and methods of reporting episodes vary greatly in these large scale clinical trials making comparisons and conclusions very challenging.

Figure 6.1 Kaplan-Meier Curves for the primary outcome (non-fatal MI, non-fatal stroke or death from cardiovascular causes) and death from any cause in the ACCORD trial. (Reproduced from ACCORD Study Group, 2008)



Moreover, if the mechanisms underlying the relationship between increased mortality and intensive control are considered in light of the findings reported in study 3, i.e. the prolonged and delayed effects following the hypoglycaemic insult, then this may suggest why absolute frequencies of severe hypoglycaemia did not explain the findings of ACCORD or VADT. If late and prolonged pro-atherogenic effects occur after each hypoglycaemic event, the overall burden of vascular disease may be gradually increasing, leading to increased mortality. In addition, it has been demonstrated that exposure to antecedent hypoglycaemia blunts cardiac autonomic function on repeated exposure to hypoglycaemia. This is another plausible mechanism for increasing cardiovascular mortality in people experiencing an increased frequency of hypoglycaemia, as cardiovascular autonomic impairment is associated with increased mortality (Adler, 2009). Interestingly, those abnormalities were still present at 16 hours after the hypoglycaemic insult.

6.2.3.2 Glycaemic control in critical illness

Another area of glycaemic control to receive a lot of attention in the recent past is the control of glucose levels in hospital in-patients, and particularly in those in the coronary care, high dependency and intensive care setting. A large proportion of hospital in-patients have pre-existing diabetes before their hospital admission, and coupled with the added burden of stress hyperglycaemia in patients who are acutely unwell, the number of people with hyperglycaemia in the hospital setting is substantial. We know that high blood glucose levels can predispose to infection, poor wound healing and increased morbidity and mortality (Capes, 2000). This has provoked the conduct of several large scale trials designed to assess the impact of careful glucose control on hospital outcomes.

Van den Berghe and colleagues examined intensive glucose control in surgical and medical intensive care patients and found it to impressively benefit mortality (Van den Berghe, 2001, Van den Berghe, 2006). The patients in these trials were highly selected, and the team

managing insulin infusion in this single intensive care unit was highly trained. DIGAMI assessed the impact of glucose control in acute myocardial infarction using an intensive intravenous insulin regimen followed by subcutaneous insulin for 3 months. This study was only conducted in people with pre-existing diabetes. This showed a survival benefit in those intensively controlled (Malmberg, 1997). Subsequently, DIGAMI 2 did not confirm the results of DIGAMI 1 from the point of view of intensive insulin therapy, but did reaffirm the importance of good glycaemic control around the time of the acute cardiac event (Malmberg, 2005). A larger scale multicentre trial followed to assess intensive treatment further; NICE-SUGAR (Normoglycaemia in Intensive Care Evaluation – Survival Using Glucose Algorithm Regulation) showed that intensive glucose control in the ICU led to increased mortality (NICE-SUGAR Study Investigators, 2009). Furthermore, the Volume and Insulin Therapy in Severe Sepsis and Septic Shock study (VISEP) showed increased hypoglycaemia in the intensively treated group, who had higher mortality rates (Brunkhorst, 2008). In all of these trials there was an approximately sixfold increase in hypoglycaemia in the intensively treated arms.

Therefore, the potential adverse impact of intensive glucose control must be recognised, whilst acknowledging that some improvement in glucose control in those who are hyperglycaemic will be beneficial. The anti-inflammatory effect of insulin infusion, *per se*, must also be recognised, and inducing hypoglycaemia and its potentially adverse consequences may reverse any possible benefit. This would suggest that a careful balance needs to be struck. Aiming for target ranges of 4.4-6.1mmol/l, as in Van den Berghe's studies, should not be recommended, and a more sensible range of 6-10mmol/l may be more practical, and safer, whilst still benefitting the patient.

6.2.3.3 Primary Care Quality and Outcomes Framework

The Quality and Outcomes Framework (QOF) is an annual reward and incentive programme for all general practice surgeries in England. It was originally designed to recognise important clinical targets, as specified by The National Institute for Clinical Excellence (NICE). The targets identify many specific healthcare domains and reward good practice, i.e. the achievement of the predefined targets. The incentives are financial, and the financial health of GP surgeries depends on this revenue stream. Until 2011, the diabetes target according to the QOF programme was to achieve an HbA1c of less than 7% in 50% of their patients. This year, in 2012, the guideline has been raised to 7.5%, in response to the recent influx of evidence relating to the lack of benefit gained from intensive glycaemic control. Thus, for several years, there was a financial incentive to tightly control HbA1c in primary care, with no scope for individualisation of targets. The move to adopt a higher HbA1c threshold in accordance with the current evidence is a sensible one, and should hopefully allow optimal glycaemic control to be encouraged without the adverse outcomes associated with strict control. Furthermore, with the exploding epidemic of type 2 diabetes necessitating that the majority of people with type 2 diabetes are cared for in the community and not by hospital specialists, there are a growing number of people who will be looked after in this treat to target manner. Even people with stable type 1 diabetes are being cared for by primary care in some areas of the United Kingdom, and if the target-based approach is adopted for all, there is considerable cause for concern even with these slightly higher HbA1c targets.

6.2.4 Potential mechanisms of vascular damage and cardiovascular morbidity and mortality

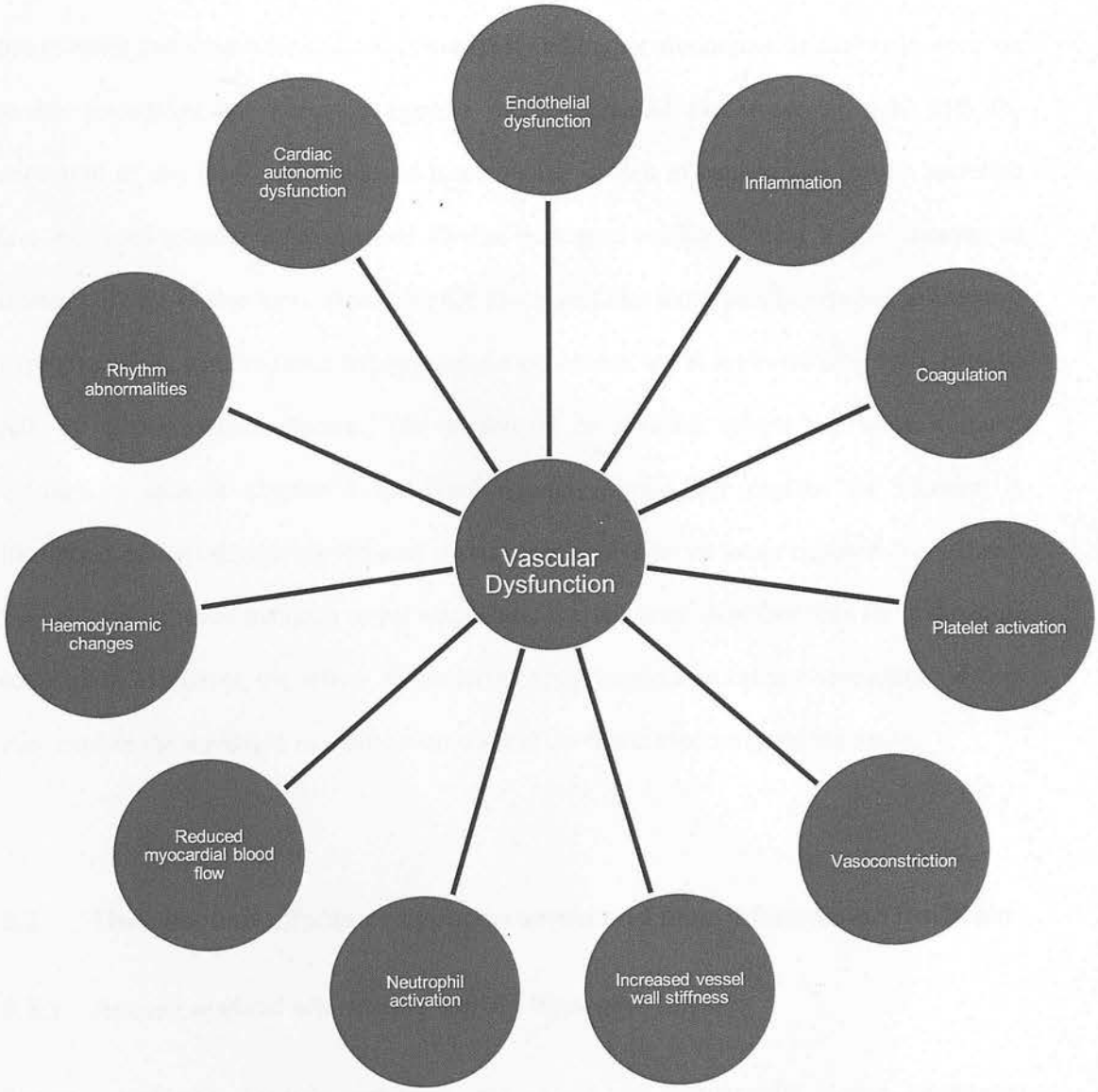
Although much of the clinical evidence behind the hypothesis of this body of research remains anecdotal, there is now mounting support from clinical trials in type 2 diabetes and in-patient glycaemic control, and from clinical research, in favour of this detrimental effect

of hypoglycaemia on vascular disease. Figure 6.2 summarises the plausible mechanisms behind this adverse effect. The profuse secretion of counterregulatory hormones in response to hypoglycaemia, particularly the catecholamines, is postulated as one way in which these changes may be stimulated. The epinephrine response has been shown to trigger many of the haematological changes observed in hypoglycaemia, and the magnitude of these responses can be attenuated by adrenoceptor- blockade.

The increase in coagulation and viscosity may restrict blood flow to distal tissues, while the activation of leucocytes and platelets may encourage thrombosis or release local factors that may damage the endothelium. Advances in vascular research are focusing increasingly on the inflammatory processes underlying the development of many disease states. The stress response of acute hypoglycaemia is associated with a rise in inflammatory markers, which may potentially cause endothelial damage and contribute to the development of vascular disease and thrombosis.

A further consequence of hypoglycaemia-induced epinephrine release is localised vasoconstriction, and promotion of the secretion of other factors such as endothelin-1, which can affect localised blood flow through vasoconstriction. This may precipitate capillary closure, leading to ischaemic damage in distal tissues. Vessel wall stiffness increases, more evident in those with longer duration of diabetes, and myocardial blood flow decreases. Whilst most of these alterations are likely to be transient in a normal circulation, in people with diabetes who have a diseased circulation, they may precipitate a catastrophic event.

Figure 6.2: Potential Mechanisms of Vascular Damage and Cardiovascular Mortality



The results of the ACCORD trial certainly suggested that there were a subgroup of patients who were more at risk of this link between severe hypoglycaemia and cardiovascular events, given that the association was not clear cut. Those individuals with longer duration of diabetes, older age, higher HbA1c at baseline and higher albumin:creatinine ratio were identified as at risk, and they were likely to have more pre-existing vascular dysfunction. The VADT showed that those with diabetes of longer duration, insulin treatment at baseline, pre-existing cardiovascular disease, lower BMI and higher albumin:creatinine ratio were the at-risk population; all factors suggestive of an increased cardiovascular risk, with the exception of low BMI which may be more related to lack of endogenous insulin secretion and increased reliance on exogenous insulin, perhaps a marker of their longer duration of diabetes. There is also now some evidence to suggest that those people exposed to frequent hypoglycaemia, with impaired hypoglycaemia awareness, are at increased long term, chronic risk of cardiovascular disease. The prolonged or delayed effects on some vascular biomarkers seen in chapter 5 following hypoglycaemia may explain the increase in likelihood of hypoglycaemia-induced macrovascular events in those exposed to recurrent hypoglycaemia, even though a direct association has not been identified thus far in the large scale trials. Moreover, the effects of recurrent hypoglycaemia on cardiac autonomic reflexes may explain the increased mortality seen without the direct association being made.

6.3 The vascular effects of hypoglycaemia and their influence on the brain

6.3.1 Acute cerebral alterations during hypoglycaemia

Severe, protracted hypoglycaemia is recognised to occasionally cause permanent neurological dysfunction, but usually, even after hypoglycaemic coma, the patient will make a full neurological recovery once blood glucose is restored to normal. Large blood vessels could be subject to the pro-atherogenic, pro-coagulant changes seen during hypoglycaemia,

leading potentially to an increased risk of stroke. There is unfortunately no strong trial evidence to confirm this, as recent focus has been on cardiac events, although several anecdotal case reports would agree with this possibility (Cordonnier, 2005, Ben-Ami, 1999). Small vessel effects, e.g. vasoconstriction, may be responsible for the diversion of blood flow to important areas during hypoglycaemia in a protective sense, with resultant areas of relative ischaemia where blood flow is deemed less important during the acute episode. The regional cerebral blood flow changes seen during hypoglycaemia appear to be temporary and reversible, however (MacLeod, 1994).

6.3.2 Chronic cerebral changes and association with hypoglycaemia

The chronic effects of hypoglycaemia on the brain are not yet well understood, but it is possible that the vascular effects seen in this and other research programmes may contribute to the development of cerebrovascular disease, if the brain is considered to be an end-organ susceptible to conventional risk factors. It is plausible that repeated exposure to hypoglycaemia may result in gradual development of small vessel disease. Some evidence suggests that recurrent exposure to severe hypoglycaemia may lead to the development of dementia (Whitmer, 2009). This longitudinal study in a large cohort of patients with type 2 diabetes examined associations of hypoglycaemia with dementia and found that there was an increased risk in those exposed to recurrent hypoglycaemia. Of course, the converse is also true, that people with developing cognitive impairment will manage their diabetes less well and may therefore be more prone to hypoglycaemia. This was demonstrated in the ADVANCE trial, with an increased risk of hypoglycaemia (HR 2.1) in those with known cognitive impairment (de Galan, 2009). Recently, the Edinburgh Type 2 Diabetes study has examined, in detail, the possible contributors to cognitive decline in type 2 diabetes. Severe hypoglycaemia occurrence has been shown to be associated with cognitive decline, even when adjusted for premorbid intelligence, duration of diabetes, vascular factors and other potential confounding variables (Aung, 2012). This study has also demonstrated an

association between raised inflammatory markers (IL-6, TNF-α and CRP) (Marioni, 2010[1]) and plasma viscosity (Marioni, 2010[2]) and poorer late life cognition in type 2 diabetes. The DCCT examined the effects of intensive control on cognitive decline and found no association (DCCT Group, 1996, DCCT/EDIC Study Group, 2007). The evidence for a causal link between hypoglycaemia and development of dementia therefore remains controversial, at least in type 1 diabetes.

The brain is also susceptible to exposure to inflammation, for example during infection, as cytokines may cross the blood-brain barrier. These substances can activate microglia, which are the cerebral version of the macrophage, which can then activate a cascade of events leading to cell and neuronal damage (van Gool, 2010). Whether hypoglycaemia may exert these same effects is as yet unknown, and requires further consideration.

Cerebral atrophy has been shown to be associated with increased exposure to severe hypoglycaemia using MRI (Perros, 1997), which may support an association between hypoglycaemia and microangiopathy. As neuroimaging techniques become more sensitive, and the population of people with diabetes become more concerned over possible long term effects on their brains, more studies have been conducted in this area. Evidence has been conflicting, however, with several studies indicating no strong link between previous hypoglycaemia exposure and cognition or brain structure (Ferguson, 2003, Brands, 2006, Kodl, 2008). It does appear, though, that exposure to hypoglycaemia at a young age, in childhood, may predispose to structural brain changes and cognitive decline (Hershey, 2010, Northam, 2009, Ferguson, 2005). One of the main issues in interpreting these imaging studies with respect to previous hypoglycaemia is that there is no long term measure that can be assessed to tell how much hypoglycaemia a subject has been exposed to in the past. Retrospective recall of hypoglycaemia history by subjects themselves is not always accurate either, particularly if asked to recall events over a lifetime, but this remains the best method of determining previous exposure. As with any study examining previous hypoglycaemia

experience, there is a skewed distribution with few subjects experiencing a lot of episodes, and the majority experiencing few, if any, episodes. Definitions of previous exposure also vary between studies.

In addition, cross-sectional studies have shown that intellectual decrements can occur in people diagnosed with type 1 diabetes in adulthood who are exposed to recurrent severe hypoglycaemia (Langan, 1991, Wredling, 1990, Lincoln, 1996). The patients in these studies were older than in the DCCT, so again we see that the individuals most susceptible to damage are the older ones, with longer diabetes duration, and higher likelihood of pre-existing abnormalities.

6.4 Future research

More evidence is needed from both clinical trials and experimental studies to determine the extent of the risk that hypoglycaemia poses for the vasculature in people with diabetes, both in the acute setting and in the longer term. It remains to be established how the depth of hypoglycaemia, duration of hypoglycaemia and speed of onset influence the changes in vascular biology that have been demonstrated, as these factors appear to influence the results in the studies performed to date. This would also allow us to better extrapolate these results into the real life setting, where these factors are significantly different from the experimental hyperinsulinaemic clamp setting. More information is required on the duration of alterations in blood constituents following a hypoglycaemic insult, as this may be a factor in increasing longer term risk. Further research utilising cardiac imaging modalities would also be useful to better inform us with regard to the longer term risks of recurrent hypoglycaemia on development of vascular disease. Investigation into cardiac autonomic dysfunction and its association with recurrent hypoglycaemia and possible increased mortality would be beneficial. Furthermore, there is a great need for more clinical trial evidence to establish

whether there is a causal role of hypoglycaemia in the increased mortality demonstrated by the ACCORD study. At the present time there is a clear association, but post-hoc analysis has been unable to shed more light on the subject. Large trials need to be designed with this specific question in mind. Additional research is also needed in the in-patient setting, as opinions remain divided on how best to target glycaemic control in the critically ill. It has also been demonstrated that spontaneous hypoglycaemia and iatrogenic hypoglycaemia are two distinct phenomena, with spontaneous hypoglycaemia more likely to cause harm in patients following acute myocardial infarction; this is not seen when the hypoglycaemia has been induced by treatment (Goyal, 2009, Kosiborod, 2009). This requires further consideration. In addition, the trial evidence suggesting an association between intensive glycaemic control and mortality has been gathered in people with type 2 diabetes. It remains to be established whether the same can occur in type 1 diabetes, particularly those patients with longer duration who may have pre-existing vascular abnormalities. More evidence of the mechanisms involved is needed in people with type 2 diabetes as well, as the majority of experimental studies to date have been in people with type 1 diabetes and in healthy volunteers.

With respect to the brain, we need more information with regards to the impact of recurrent hypoglycaemia on long term brain function and cognition. Studies until now have yielded conflicting results, and there has been much heterogeneity particularly in the hypoglycaemia definitions utilised, and in the methods of reporting hypoglycaemia. Imaging modalities are becoming ever more sophisticated and are likely to provide further information on the processes affecting the brain. At the present time, it is unclear as to how hypoglycaemia affects brain volume. In order to interpret such imaging studies accurately, longitudinal, prospective studies are required to determine whether hypoglycaemia issues precede brain volume changes, or whether the brain volume changes can occur before the hypoglycaemia. In addition, neuroimaging has the capacity to help inform our understanding of the brain's

response to hypoglycaemia, to identify the most vulnerable regions, to explain why these areas are vulnerable, and to elucidate their role in the development of recurrent hypoglycaemia and hypoglycaemia-associated autonomic failure or cerebral adaptation to hypoglycaemia.

6.5 Conclusions

There is no doubt that achieving good glycaemic control prevents the development and progression of microangiopathy, as evidenced by the landmark DCCT/EDIC and UKPDS studies. Additionally the legacy effect that ensues from early optimal control will follow an individual throughout their diabetes journey, conferring a protective effect against the development of future complications. However, achieving strict glycaemic control inevitably introduces the side effect of hypoglycaemia, as exogenous insulin therapy cannot be adjusted real-time in the way that endogenous secretion is physiologically altered. It is therefore imperative that we fully understand the impact that hypoglycaemia can have – on both the brain, which has been well recognised for some time, but also on the vasculature, an area of evolving understanding. Spatial cognition is crucial for every day functioning so exposure to hypoglycaemia understandably poses a potentially dangerous hazard to people with insulin-treated diabetes, particularly relevant to driving. People with diabetes are at greater risk of developing vascular disease, and with recent clinical trial evidence showing an association between strict glycaemic control and increased mortality, hypoglycaemia is of course postulated as the culprit in susceptible individuals. Causality has not been established, however, and this must be the focus for the next stream of research studies.

References

Abraira C, Colwell J A, Nuttall F Q, et al. Veterans Affairs Cooperative Study on glycemic control and complications in type II diabetes (VACSDM): results of the feasibility trial. *Diabetes Care* 1995; **18**: 1113-1123.

Abraira C, Colwell J, Nuttall F, Sawin C T, Henderson W, Comstock J P, Emanuele N V, Levin S R, Pacold I, Lee H S. Cardiovascular events and correlates in the Veterans Affairs Diabetes Feasibility Trial. Veterans Affairs Cooperative Study on Glycemic Control and Complications in Type II Diabetes. *Arch Int Med* 1997; **157**: 181-188.

Abumrad NN, Rabin D, Diamond MP, Lacy WW. Use of a heated superficial hand vein as an alternative site for the measurement of amino acid concentrations and for the study of glucose and alanine kinetics in man. *Metabolism* 1981; **30**: 936–940.

ACCORD Study Group, Gerstein H C, Miller M E, Byington R P, Goff D C Jr, Bigger J T, Buse J B, Cushman W C, Genuth S, Ismail-Beigi F, Grimm R H Jr, Probstfield JL, Simons-Morton DG, Friedewald WT. Effects of intensive glucose lowering in type 2 diabetes. *N Engl J Med* 2008; **358**: 2545–2559.

Adler G K, Bonyhay I, Failing H, Waring E, Dotson S, Freeman R. Antecedent hypoglycemia impairs autonomic cardiovascular function. Implications for rigorous glycemic control. *Diabetes* 2009; **58**: 360-366.

Agardh C D, Eckert B, Agardh E. Irreversible progression of severe retinopathy in young type I insulin-dependent diabetes mellitus patients after improved metabolic control. *J Diab Complications* 1992; **6**: 96-100.

Ak G, Buyukberber S, Sevinc A, Turk H M, Ates M, Sari R, Savli H, Cigli A. The relation between plasma endothelin-1 levels and metabolic control, risk factors, treatment modalities, and diabetic microangiopathy in patients with type 2 diabetes mellitus. *J Diabetes Complications* 2001; **15**: 150-157.

Allen K V, Frier B M, Strachan M W J. The relationship between type 2 diabetes and cognitive dysfunction: longitudinal studies and their methodological limitations. *Eur J Pharmacol* 2004; **490**: 169-175.

Allwood M J, Hensel H, Papenberg J. Muscle and skin blood flow in the human forearm during insulin hypoglycaemia. *J Physiol* 1959; **147**: 269-273.

American Diabetes Association Workgroup on Hypoglycemia. Defining and Reporting Hypoglycemia in Diabetes. *Diabetes Care* 2005; **28**: 1245-49.

American Diabetes Association Position Statement. Standards of Medical Care in Diabetes-2007. *Diabetes Care* 2007; **30** (Suppl 1): S4-S41.

Amiel S A, Dixon T, Mann R, Jameson K. Hypoglycaemia in Type 2 diabetes. *Diabet Med* 2008; **25**: 245-254.

Amiel S A. Cognitive function testing in studies of acute hypoglycaemia: rights and wrongs? *Diabetologia* 1998; **41**: 713-719.

Ang C, Hillier C, Cameron A D, Greer I A, Lumsden M A. The effect of type 1 diabetes on vascular responses to endothelin-1 in pregnant women. *J Clin Endocrinol Metab* 2001; **86**: 4939-4942.

Ashwell S G, Amiel S A, Bilous R W, Dashora U, Heller S R, Hepburn D A, Shutler S D, Stephens J W, Home P D. Improved glycaemic control with insulin glargine plus insulin lispro: a multicentre, randomized, cross-over trial in people with Type 1 diabetes. *Diabet Med* 2006; **23**: 285-292.

Attinà T, Camidge R, Newby DE, Webb DJ. Endothelin antagonism in pulmonary hypertension, heart failure, and beyond. *Heart* 2005; **91**: 825–831.

Aukrust P, Muller F, Ueland T et al. Enhanced levels of soluble and membrane bound CD40 ligand in patients with unstable angina. Possible reflection of T lymphocyte and platelet involvement in the pathogenesis of acute coronary syndromes. *Circulation* 1999; **100**: 614-20.

Aung P P, Strachan M W, Frier B M, Butcher I, Deary I J, Price J F. Severe hypoglycaemia and late-life cognitive ability in older people with type 2 diabetes: the Edinburgh Type 2 Diabetes Study. *Diabet Med* 2012 (in press).

Banga J D. Lower extremity arterial disease in diabetes mellitus. *Diab Rev Int* 1994; **4**: 7-11.

Bearn A G, Billing B H, Sherlock S. The response of the liver to insulin in normal subjects and in diabetes mellitus: hepatic vein catheterisation studies. *Clin Sci* 1952; **11**: 151-164.

Ben-Ami H, Nagachandran P, Mendelson A, Edoute Y. Drug-induced hypoglycemic coma in 102 diabetic patients. *Arch Intern Med* 1999; **159**: 281-284.

Biessels G J, Kappelle A C, Bravenboer B, Erkelens D W, Gispen W H. Cerebral function in diabetes mellitus. *Diabetologia* 1994; **37**: 643-650.

Bjørgaas M, Sand T, Vik T, Jorde R. Quantitative EEG during controlled hypoglycaemia in diabetic and non-diabetic children. *Diabet Med* 1998; **15**: 30-37.

Bobik A, Grooms A, Millar J A, Mitchell A, Grinpukel S. Growth factor activity of endothelin on vascular smooth muscle. *Am J Physiol* 1990; **258**: C408-C415.

Boland E A, Grey M, Oesterle A, Fredrickson L, Tamborlane W V. Continuous subcutaneous insulin infusion. A new way to lower risk of severe hypoglycemia, improve metabolic control, and enhance coping in adolescents with type 1 diabetes. *Diabetes Care* 1999; **22**: 1779-1784.

Bolli G, de Feo P, Compagnucci P, Cartechini M G, Angeletti G, Santeusanio F, Brunetti P, Gerich J E. Abnormal glucose counterregulation in insulin-dependent diabetes mellitus. Interaction of anti-insulin antibodies and impaired glucagon and epinephrine secretion. *Diabetes* 1983; **32**: 134-141.

Bolli G, Tsalikian E, Haymond M, Cryer P, Gerich J E: Defective glucose counterregulation after subcutaneous insulin in noninsulin dependent diabetes mellitus: paradoxical lack of compensatory increase in glucose production, roles of insulin resistance, abnormal neuroendocrine responses and islet paracrine interactions. *J Clin Invest* 1984; **73**: 1532–1541.

Bott S, Bott U, Berger M, Muhlhauser I. Intensified insulin therapy and the risk of severe hypoglycaemia. *Diabetologia* 1997; **40**: 926-932.

Boyle P J, Zrebiec J. Impact of therapeutic advances on hypoglycaemia in type 2 diabetes. *Diabetes Metab Res Rev* 2008; **24**: 257-285.

Braatvedt G D, Flynn M D, Stanners A, Halliwell M, Corrall R J M. Splanchnic blood flow in man: evidence for mediation via a beta-adrenergic mechanism. *Clin Sci* 1993; **84**: 201-207.

Brands A M, Biessels G J, de Haan E H, Kappelle L J, Kessels R P. The effects of type 1 diabetes on cognitive performance: a meta-analysis. *Diabetes Care* 2005; **28**: 726-735.

Brands A M, Kessels R P, Hoogma R P, Henselmans J M, van der Beek Boter J W, Kappelle L J, de Haan E H, Biessels G J. Cognitive performance, psychological well-being, and brain magnetic resonance imaging in older patients with type 1 diabetes. *Diabetes* 2006; **55**: 1800–1806.

Briscoe V J, Ertl A C, Tate D B, Davis S N. Effects of the selective serotonin reuptake inhibitor fluoxetine on counterregulatory responses to hypoglycemia in individuals with type 1 diabetes. *Diabetes* 2008; **57**: 3315-3322.

Brodows R G, Williams C, Amatruda J M. Treatment of insulin reactions in diabetics. *J Am Med Assoc* 1984; **252**: 3378-3381.

Broers S, le Cessie S, van Vliet K P, Spinhoven Ph, van der Ven N C W, Radder J K. Blood glucose awareness training in Dutch type 1 diabetes patients. Short term evaluation of individual and group training. *Diabet Med* 2002; **19**: 157-161.

Brunkhorst F M, Engel C, Bloos F, Meier- Hellmann A, Ragaller M, Weiler N, et al, for the German Competence Network Sepsis (SepNet). Intensive insulin therapy and pentastarch resuscitation in severe sepsis. *N Engl J Med* 2008; **358**: 125–139.

Bryan R M. Cerebral blood flow and energy metabolism during stress. *Am J Physiol* 1990; **259**: H269-H280.

Burke B J Kearney T K. Hypoglycaemia and cardiac arrest. *Pract Diab Internat* 1999; **16**:189 – 190.

Capes S E, Hunt D, Malmberg K, Gerstein H C. Stress hyperglycaemia and increased risk of death after myocardial infarction in patients with and without diabetes: a systematic overview. *Lancet* 2000; **355**: 773–778.

Carroll J B. Psychometric tests as cognitive tasks: a new "structure of intellect". Educational Testing Service, Research Bulletin 1974; 14-16.

Cattell R B. Abilities: Their structure, growth and action. Boughton Mifflin, Boston, 1971.

Celi A, Pellegrini G, Lorenzet R et al. P-selectin induces the expression of tissue factor on monocytes. *Proc Natl Acad Sci* USA 1994; **91**: 8767-8771.

Cipollone F, Chiarelli F, Davi G et al. Enhanced soluble CD40 ligand contributes to endothelial cell dysfunction in vitro and monocyte activation in patients with diabetes mellitus: effect of improved metabolic control. *Diabetologia* 2005; **48**: 1216-1224.

Clozel M, Gray G A, Breu V, Loffler B-M, Osterwalder R. The endothelin ET_B receptor mediates both vasodilatation and vasoconstriction in vivo. *Biochem Biophys Res Commun* 1992; **183**: 566-571.

Collier A, Steedman D J, Patrick A W, Nimmo GR, Matthews DM, MacIntyre CC, Little K, Clarke BF. Comparison of intravenous glucagon and dextrose in treatment of severe hypoglycemia in an accident and emergency department. *Diabetes Care* 1987[1]; **10**: 712-715.

Collier A, Matthews D M, Young R J, Clarke B F. Transient atrial fibrillation precipitated by hypoglycaemia: two case reports. *Postgrad Med J* 1987[2]; **63**: 895-897.

Collier A, Patrick A W, Hepburn D A, et al. Leucocyte mobilisation and release of neutrophil elastase following acute insulin-induced hypoglycaemia in normal humans. *Diabet Med* 1990; **7**: 506-509.

Collier A, Leach JP, McLellan A, Jardine A, Morton JJ, Small M. Plasma endothelin-like immunoreactivity levels in IDDM patients with microalbuminuria. *Diabetes Care* 1992; **15**: 1038–1040.

Cordonnier C, Oppenhein C, Lamy C, Meder J-F, Mas J-L. Serial diffusion and perfusion weighted MR in transient hypoglycemia. *Neurology* 2005; **65**: 175.

Corrall R J M, Frier B M, McClemont E J W, Taylor S J, Christie N E. Recovery mechanisms from acute hypoglycaemia in complete tetraplegia. *Paraplegia* 1979; **17**: 314-318.

Corrall R J M, Webber R G, Frier B M. Increase in coagulation factor VIII activity in man following acute hypoglycaemia: mediation via an adrenergic mechanism. *Br J Haematol* 1980; **44**: 301-305.

Cox D J, Gonder-Frederick L, Clarke W. Driving decrements in type 1 diabetes during moderate hypoglycemia. *Diabetes* 1993; **42**: 239-243.

Cox D J, Gonder-Frederick L A, Kovatchev B P, Julian D M, Clarke W L. Progressive hypoglycemia's impact on driving simulation performance: occurrence, awareness, and correction. *Diabetes Care* 2000; **23**: 163-170.

Cox D J, Clarke W L, Gonder-Frederick L, Pohl S, Hoover C, Snyder A, Zimbelman L, Carter W R, Bobbitt S, Pennebaker J. Accuracy of perceiving blood glucose in IDDM. *Diabetes Care* 1985; **8**: 529-536.

Cox D, Carter W R, Gonder-Frederick L, Clarke W L, Pohl S. Blood glucose awareness training in IDDM patients. *Biofeedback Self Regul* 1988; **13**: 201-217.

Cox D J, Gonder-Frederick L, Lee J H, Julian D, Carter W R, Clarke W L. Blood glucose awareness training among patients with IDDM: effects and correlates. *Diabetes Care* 1989; **12**: 313-318.

Cox D, Gonder-Frederick L, Julian D, Cryer P. Intensive versus standard blood glucose awareness training (BGAT) with insulin-dependent diabetes: mechanisms and ancillary effects. *Psychosom Med* 1991; **53**: 453-462.

Cox D J, Gonder-Frederick L, Polonsky W, Schlundt D, Kovatchev B, Clarke W. Blood glucose awareness training (BGAT-2). Long term benefits. *Diabetes Care* 2001; **24**: 637-642.

Cryer P E. Hypoglycaemia: the limiting factor in the glycaemic management of type 1 and type 2 diabetes. *Diabetologia* 2002; **45**: 937-948.

Cukierman T, Gerstein HAC, Williamson JD. Cognitive decline and dementia in diabetes-systematic overview of prospective observational studies. *Diabetologia* 2005; **48**: 2460–2469.

DAFNE Study Group. Training in flexible, intensive insulin management to enable dietary freedom in people with type 1 diabetes: dose adjustment for normal eating (DAFNE) randomised controlled trial. *Br Med J* 2002; **325**: 746.

Dahl-Jorgensen K, Brinchmann-Hanssen O, Hansen K F, Sandvik L, Aagenaes O, Aker Diabetes Group. Rapid tightening of blood glucose control leads to transient deterioration of retinopathy in insulin dependent diabetes mellitus: the Oslo study. *Br Med J* 1985; **290**: 811-815.

Dalla Pozza R, Beyerlein A, Thilmany C, Weissenbacher C, Netz, H, Schmidt H, Bechtold S. The effect of cardiovascular risk factors on the longitudinal evolution of the carotid intima medial thickness in children with type 1 diabetes mellitus. *Cardiovascular Diabetology* 2011; **10**: 53.

Dandona P, Chauduri A, Ghanim H, Mohanty P. Proinflammatory effects of glucose and anti-inflammatory effect of insulin: relevance to cardiovascular disease. *Am J Card* 2007; **99** (suppl): 15B-26B.

Dandona P, Chauduri A, Ghanim H, Mohanty P. Insulin as an anti-inflammatory and antiatherogenic modulator. *J Am Coll Cardiol* 2009; **53** (Suppl. 5): S14–S20.

Davies M J, Heller S, Skinner T C, et al. Effectiveness of the diabetes education and self management for ongoing and newly diagnosed (DESMOND) programme for people with newly diagnosed type 2 diabetes: cluster randomised controlled trial. *Br Med J* 2008; **336**: 491-495.

Davis E A, Keating B, Byrne G C, Russell M, Jones T W. Hypoglycemia: incidence and clinical predictors in a large population-based sample of children and adolescents with IDDM. *Diabetes Care* 1997; **20**: 22-25.

De Fronzo R, Tobin J D, Andres R. Glucose clamp technique: a method for quantifying insulin secretion and resistance. *Am J Physiol* 1979; **273**: E214-E223.

De Galan B E, Hoekstra J B L. Glucose counterregulation in type 2 diabetes mellitus. *Diabet Med* 2001; **18**: 519-527.

De Galan B E, Zoungas S, Chalmers J, Anderson C, Dufouil C, Pillai A, et al, for the ADVANCE Collaborative Group. Cognitive function and risks of cardiovascular disease and hypoglycaemia in patients with type 2 diabetes: the Action in Diabetes and Vascular Disease: Preterax and Diamicron Modified Release Controlled Evaluation (ADVANCE) trial. *Diabetologia* 2009; **52**: 2328-2336.

Deary I J. Effects of hypoglycaemia on cognitive function. In: *Hypoglycaemia and Diabetes: Clinical and Physiological Aspects*. Frier B M and Fisher B M, eds. Edward Arnold, London, 1993[1]: 80-92.

Deary I J, Crawford J R, Hepburn D A, Langan S J, Blackmore L M, Frier B M. Severe hypoglycemia and intelligence in adult patients with insulin-treated diabetes. *Diabetes* 1993[2]; **42**: 341-344.

Deary I J. Symptoms of hypoglycaemia and effects on mental performance and emotions. In: *Hypoglycaemia in Clinical Diabetes*. 2nd edition. Frier B M and Fisher M, Eds. John Wiley and Sons, Chichester, 2007: 25-48.

Deckert T, Poulsen J E, Larsen M. Prognosis of diabetics with diabetes onset before age 31. *Diabetologia* 1978; **14**:463-77.

Dejgaard A, Gade A, Larsson H, Balle V, Parving A, Parving H-H. Evidence for diabetic encephalopathy. *Diabet Med* 1991; **8**: 162-167.

Desouza C, Salazar H, Cheong B, Murgo J, Fonseca V. Association of hypoglycemia and cardiac ischemia. *Diabetes Care* 2003; **26**: 1485–1489.

Dewan S, Gillett A, Mugarza J A, Dovey T M, Halford J C G, Wilding J P H. Effects of insulin-induced hypoglycaemia on energy intake and food choice at a subsequent test meal. *Diabetes Metab Res Revs* 2004; **20**: 405-410.

Dhaun N, Goddard J, Webb DJ. The endothelin system and its antagonism in chronic kidney disease. *J Am Soc Nephrol* 2006; **17**: 943–955.

The Diabetes Control and Complications Trial Research Group. Diabetes Control and Complications Trial (DCCT): results of feasibility study. *Diabetes Care* 1987; **10**: 1-19.

The Diabetes Control and Complications Trial Research Group. The effect of intensive insulin treatment of diabetes on the development and progression of long term complications in insulin-dependent diabetes mellitus. *N Engl J Med* 1993; **329**: 977-986.

The Diabetes Control and Complications Trial Study Group. Effects of intensive diabetes therapy on neuropsychological function in adults in the Diabetes Control and Complications Trial. *Ann Intern Med* 1996; **124**: 379-388.

The Diabetes Control and Complications Trial/Epidemiology of Diabetes Interventions and Complications (DCCT/EDIC) Study Research Group. Intensive diabetes treatment and cardiovascular disease in patients with type 1 diabetes. *N Engl J Med* 2005; **353**: 2643-2653.

The DCCT/EDIC Study Research Group. Long-term effect of diabetes and its treatment on cognitive function. *N Engl J Med* 2007; **356**: 1842-1852.

Donnelly L A, Morris A D, Frier B M, Ellis J D, Donnan P T, Durrant R, Band M M, Reekie G, Leese G P (for the DARTS/MEMO Collaboration). Frequency and Predictors of hypoglycaemia in type 1 and insulin-treated type 2 diabetes: a population-based study. *Diabet Med* 2005; **22**: 449-455.

Dotson S, Freeman R, Failing H J, Adler G K. Hypoglycemia increases serum interleukin-6 levels in healthy men and women. *Diabetes Care* 2008; **31**: 1222–1223.

Duckworth W, Abraira C, Moritz T, Reda D, Emanuele N, Reaven P D, et al, for the VADT Investigators. Glucose control and vascular complications in veterans with type 2 diabetes. *N Engl J Med* 2009; **360**: 129–139.

Egeli E S, Berkmen R. Action of hypoglycemia on coronary insufficiency and mechanism of ECG alteration. *Am Heart J* 1960; **59**: 527-540.

Ekstrom R B, French J W, Harman H H, Dermen D. Kit of Factor-Referenced Tests. Princeton, NJ: Educational Testing Service, 1976.

Ekstrom R B, French J W, Harman H H. Cognitive factors: Their identification and replication. *Multivariate Behavioral Research Monographs* 1979; **79**: 3-84.

The EURODIAB IDDM Complications Study Group. Microvascular and acute complications in IDDM patients: the EURODIAB IDDM Complications Study. *Diabetologia* 1994; **37**: 278-285.

Ewing D J, Martyn C N, Young R J, Clarke B F. The value of cardiovascular autonomic function tests: 10 years experience in diabetes. *Diabetes Care* 1985; **8**: 491–498.

Ewing F E, Deary I J, McCrimmon R J, Strachan M W J, Frier B M. Effect of acute hypoglycaemia on visual information processing in adults with type 1 diabetes mellitus. *Physiol & Behav* 1998; **64**: 653-660.

Fanelli C G, Epifano L, Rambotti A M, Pampanelli S, Di Vincenzo A, Modarelli F, Lepore M, Annibale B, Ciofetta M, Bottini P. Meticulous prevention of hypoglycemia normalizes the glycemic thresholds and magnitude of most of neuroendocrine responses to, symptoms of, and cognitive function during hypoglycemia in intensively treated patients with short-term IDDM. *Diabetes* 1993; **42**: 1683-1689.

Ferguson S C, Blane A, Perros P, McCrimmon R J, Best J K, Wardlaw J, Deary I J, Frier B M. Cognitive ability and brain structure in type 1 diabetes. Relation to microangiopathy and preceding severe hypoglycemia. *Diabetes* 2003; **52**: 149-156.

Ferguson S C, Blane A, Wardlaw J, Frier B M, Perros P, McCrimmon R J, Deary I J. Influence of an early-onset age of type 1 diabetes on cerebral structure and cognitive function. *Diabetes Care* 2005; **28**: 1431-1437.

Ferri C, Carlomagno A, Coassin S, Baldoncini R, Cassone Faldetta MR, Laurenti O, Properzi G, Santucci A, De Mattia G. Circulating endothelin-1 levels increase during euglycemic hyperinsulinemic clamp in lean NIDDM men. *Diabetes Care* 1995[1]; **18**: 226–233.

Ferri C, Bellini C, Desideri G, Di Francesco L, Baldoncini R, Santucci A, De Mattia G. Plasma endothelin-1 levels in obese hypertensive and normotensive men. *Diabetes* 1995[2]; **44**: 431–436.

Fisher B M, Gillen G, Dargie H J, Inglis G C, Frier B M. The effects of insulin-induced hypoglycaemia on cardiovascular function in normal man: studies using radionuclide ventriculography. *Diabetologia* 1987; **30**: 841-845.

Fisher B M, Thomson I, Hepburn D A, Frier B M. Effects of adrenergic blockade on serum potassium changes in response to acute insulin-induced hypoglycemia in nondiabetic humans. *Diabetes Care* 1991[1]; **14**: 548-552.

Fisher B M, Gillen G, Hepburn D A, Dargie H J, Barnett E, Frier B M. Splenic responses to acute insulin-induced hypoglycaemia in humans. *Clin Sci* 1990[1]; **78**: 469-474.

Fisher B M, Quin J D, Rumley A, et al. Effects of acute insulin-induced hypoglycaemia on haemostasis, fibrinolysis, and haemorrheology in insulin-dependent diabetic patients and control subjects. *Clin Sci* 1991[2]; **80**: 525-531.

Fisher B M, McCruden D C, Smith J G, et al. The role of cortisol in the peripheral granulocyte response to insulin-induced hypoglycaemia in man. *Horm Metabol Res* 1989; **21**: 253-257.

Fisher B M, Hepburn D A, Smith J G, Frier B M. The effect of α-adrenergic blockade on responses of peripheral blood cells to acute insulin-induced hypoglycaemia in humans. *Eur J Clin Inv* 1990[2]; **20**: 51-55.

Fisher B M, Frier B M. Effect on vascular disease. In: *Hypoglycaemia and Diabetes: Clinical and Physiological Aspects*. Frier B M and Fisher B M, eds. Edward Arnold, London, 1993: 355–361.

Fisher M, Heller S. Mortality, cardiovascular morbidity and possible effects of hypoglycaemia on diabetic complications. In: *Hypoglycaemia in Clinical Diabetes*. Frier B M and Fisher M, eds. 2nd edn. John Wiley and Sons, Chichester, 2007: 265–284.

Fisman E Z, Motro M, Tenenbaum A, Leor J, Boyko V, Mandelzweig L, Sherer Y, Adler Y, Behar S. Is hypoglycaemia a marker for increased long-term mortality risk in patients with coronary artery disease? An 8-year follow-up. *Eur J Cardiovasc Prev Rehabil* 2004; **11**: 135-143.

Frier B M. Hypoglycaemia in the diabetic adult. In: *Hypoglycaemia*. Gregory J W and Aynsley-Green A Eds. *Baillere's Clin Endocrinol and Metab* 1993; **7**: 757-777.

Frier B M, Hilsted J. Does hypoglycaemia aggravate the complications of diabetes? *Lancet* 1985; **326**: 1175-1177.

Frier B M, Hepburn D A, Fisher B M, Barrie T. Fall in intraocular pressure during acute hypoglycaemia in patients with insulin dependent diabetes. *Br Med J* 1987; **294**: 610-611.

Frier B M, Corrall R J M, Davidson N McD, et al. Peripheral blood cell changes in response to acute hypoglycaemia in man. *Eur J Clin Inv* 1983; **13**: 33-39.

Frier B M. Impaired awareness of hypoglycaemia. In: *Hypoglycaemia in Clinical Diabetes*. Frier B M, Fisher M, eds. 2nd edn. John Wiley and Sons, Chichester, 2007: 141-170.

Frier B M. Defining hypoglycaemia: what level has clinical relevance? *Diabetologia* 2009; **52**: 31-34.

Galassetti P, Mann S, Tate D, Neill R A, Costa F, Wasserman D H, Davis S N. Effects of antecedent prolonged exercise on subsequent counterregulatory responses to hypoglycemia. *Am J Physiol Endocrinol Metab* 2001; **280**: E908-E917.

Galassetti P, Tate D, Neill R A, Morrey S, Wasserman D H, Davis S N. Effect of antecedent hypoglycemia on counterregulatory responses to subsequent euglycemic exercise in type 1 diabetes. *Diabetes* 2003; **52**: 1761-1769.

Galloway P J, Thomson G A, Fisher B M, Semple C G. Insulin-Induced Hypoglycemia Induces a Rise in C-Reactive Protein. (Letter) *Diabetes Care* 2000; **23**: 861.

Garlichs C D, Eskafi S, Raaz D et al. Patients with acute coronary syndromes express enhanced CD40 ligand/CD154 on platelets. *Heart* 2001; **86**: 649-655.

Geddes J, Deary I J, Frier B M. Effects of acute insulin-induced hypoglycaemia on psychomotor function: people with type 1 diabetes are less affected than non-diabetic adults. *Diabetologia* 2008; **51**: 1814-1821.

Gerich J E, Langlois M, Noacco C, Karam J H, Forsham P H. Lack of glucagon response to hypoglycemia in diabetes: evidence for an intrinsic pancreatic alpha cell defect. *Science* 1973; **182**: 171-173.

Giacco R, Parillo M, Rivellese A A, Lasorella G, Giacco A, D'Episcopo L, Riccardi G. Long-term dietary treatment with increased amounts of fiber-rich low-glycemic index natural foods improves

blood glucose control and reduces the number of hypoglycemic events in type 1 diabetic patients. *Diabetes Care* 2000; **23**: 1461-1466.

Gilbert R A, Goldzieher J W. The mechanism and prevention of cardiovascular changes due to insulin. *Ann Intern Med* 1946; **25**: 928-940.

Gimenez M, Gilabert R, Monteagudo J, Alonso A, Casamitjana R, Pare C, Conget I. Repeated episodes of hypoglycemia as a potential aggravating factor for preclinical atherosclerosis in subjects with type 1 diabetes. *Diabetes Care* 2011; **34**: 198-203.

Gogitidze Joy N, Hedrington MS, Briscoe VJ, Tate DB, Ertl AC, Davis SN. Effects of acute hypoglycemia on inflammatory and pro-atherothrombotic biomarkers in individuals with type 1 diabetes and healthy individuals. *Diabetes Care* 2010; **33**: 1529–1535.

Gogitidze Joy N, Mikeladze M, Hedrington M, Younk L, Pulliam L, Davis I, Tate D B, Davis S N. Effects of hypoglycemia on endothelial function and atherothrombotic balance in type 2 diabetes (T2DM)(Abstract). *Diabetes* 2011[1]; **60** (Suppl 1): 481P.

Gogitidze Joy N, Perkins J, Richardson A, Hedrington M, Younk L, Davis I, Tate D, Davis S N. Acute effects of hypoglycemia and hyperglycemia on pro-atherothrombotic risk in non-diabetic humans (Abstract). *Diabetes* 2011[2]; **60** (Suppl 1): 480P.

Gold A E, MacLeod K M, Frier B M. Frequency of severe hypoglycemia in patients with type 1 diabetes with impaired awareness of hypoglycemia. *Diabetes Care* 1994; **17**: 697–703.

Gold A E, Marshall S M. Cortical blindness and cerebral infarction associated with severe hypoglycemia. *Diabetes Care* 1996; **19**: 1001-1003.

Golden M P, Ingersol G M, Brack C J, Russell B A, Wright J C, Huberty T J. Longitudinal relationship of asymptomatic hypoglycemia to cognitive function in IDDM. *Diabetes Care* 1989; **12**: 89-93.

Gomis R, Esmatjes E. Asymptomatic hypoglycaemia: identification and impact. *Diabetes Metab Res Revs* 2004; **20** (Suppl 2): 547-549.

Gossl M, Lerman A. Endothelin: beyond a vasoconstrictor. *Circulation* 2006; **113**: 1156–1158.

Goyal A, Mehta S R, Díaz R, Gerstein H C, Afzal R, Xavier D, Liu L, Pais P, Yusuf S. Differential clinical outcomes associated with hypoglycemia and hyperglycemia in acute myocardial infarction. *Circulation* 2009; **120**: 2429–2437.

Gray R O, Butler P C, Beers T R, Kryshak E J, Rizza R A. Comparison of the ability of bread versus bread plus meat to treat and prevent subsequent hypoglycemia in patients with insulin-dependent diabetes mellitus. *J Clin Endocrinol Metab* 1996; **81**: 1508-1511.

Hanssen KF, Dahl-Jorgensen K, Lauritzen T, Feldt-Rasmussen B, Brinchmann-Hansen O, Deckert T. Diabetic control and microvascular complications. The near-normoglycaemic experience. *Diabetologia* 1986; **29**: 677–684.

Harding S A, Sommerfield A J, Sarma J et al. Increased CD40 ligand and platelet-monocyte aggregates in patients with type 1 diabetes mellitus. *Atherosclerosis* 2004; **176**: 321-325.

Harris N D, Heller S R. Sudden death in young patients with type 1 diabetes: a consequence of disease, treatment or both? *Diabet Med* 1999; **16**: 1-3.

Harris I M, Egan G F, Sonkkila C, Tochon-Danguy H J, Paxinos G, Watson J D G. Selective right parietal lobe activation during mental rotation. *Brain* 2000; **123**: 65-73.

Heller S R, Macdonald I A, Tattersall R B: Counterregulation in type II (non-insulin-dependent) diabetes mellitus: normal endocrine and glycaemic responses up to 10 years after diagnosis. *Diabetologia* 1987[1]; **30**: 924 –929.

Heller S R, Herbert M, Macdonald I A, Tattersall R B. Influence of sympathetic nervous system on hypoglycaemia warning symptoms. *The Lancet* 1987[2]; **330**: 359-363.

Heller S R, Cryer P E. Reduced neuroendocrine and symptomatic responses to subsequent hypoglycemia after 1 episode of hypoglycemia in nondiabetic humans. *Diabetes* 1991; **40**: 223-226.

Heller S R, Amiel S A, Mansell P. Effect of the fast-acting insulin analog lispro on the risk of nocturnal hypoglycemia during intensified insulin therapy. *Diabetes Care* 1999; **22**: 1607-1611.

Henderson J N, Allen K V, Deary I J, Frier B M. Hypoglycaemia in insulin-treated Type 2 diabetes: frequency, symptoms and impaired awareness. *Diabet Med* 2003; **20**: 1016-1021.

Henn V, Slupsky J R, Gräfe M, Anagnostopoulos I, Förster R, Müller-Berghaus G, Kroczek R A. CD40 ligand on actvated platelets triggers an inflammatory reaction of endothelial cells. *Nature* 1998; **391**: 591-4.

Hepburn D A, Patrick A W, Eadington D W, Ewing D J, Frier B M. Unawareness of hypoglycaemia in insulin-treated diabetic patients: prevalence and relationship to autonomic neuropathy. *Diabet Med* 1990; **7**: 711-717.

Hepburn DA, Patrick AW, Brash HM, Thomson I, Frier BM. Hypoglycaemia unawareness in Type 1 diabetes: a lower plasma glucose is required to stimulate sympathoadrenal activation. *Diabet Med* 1991; **8**: 934–945.

Hepburn DA, MacLeod KM, Frier BM. Physiological, symptomatic and hormonal responses to acute hypoglycaemia in Type 1 diabetic patients with autonomic neuropathy. *Diabet Med* 1993; **10**: 940–949.

Hermanns N, Kulzer B, Kubiak T, Krichbaum M, Haak T. The effect of an education programme (HyPOS) to treat hypoglycaemia problems in patients with type 1 diabetes. *Diabetes Metab Res Revs* 2007; **23**: 528-538.

Hershey T, Perantie D C, Warren S L, Zimmerman E C, Sadler M, White N H. Frequency and timing of severe hypoglycemia affects spatial memory in children with type 1 diabetes. *Diabetes Care* 2005; **28**: 2372-2377.

Hershey T, Perantie D C, Wu J, Weaver P M, Black K J, White N H. Hippocampal volumes in youth with type 1 diabetes. *Diabetes* 2010; **59**: 236–241.

Hilsted J, Bonde-Petersen F, Norgaard M-B, Greniman M, Christensen N J, Parving H-H, Suzuki M. Haemodynamic changes in insulin-induced hypoglycaemia in normal man. *Diabetologia* 1984; **26**: 328-332.

Hilsted J, Bonde-Peterson F, Madsbad S, et al. Changes in plasma volume, in transcapillary escape rate of albumin and in subcutaneous blood flow during hypoglycaemia in man. *Clin Sci* 1985; **69**: 273-277.

Hilsted J, Bendtsen F, Christensen N J, Henriksen J H. Plasma volume changes during hypoglycaemia: the effect of arterial blood sampling. *Scand J Clin Lab Inv* 1990; **50**: 797-800.

Hirsch I B, Heller S R, Cryer P E. Increased symptoms of hypoglycemia in the standing position in insulin-dependent diabetes mellitus. *Clin Sci* 1991; **80**: 583-586.

Hoffman R G, Speelman D J, Hinnen D A, Conley K L, Guthrie R A, Knapp R K. Changes in cortical functioning with acute hypoglycemia and hyperglycemia in type 1 diabetes. *Diabetes Care* 1989 ; **12**: 193-197.

Holmes C S, Hayford J T, Gonzalez J L, Weydert J A. A survey of cognitive functioning at different glucose levels in diabetic persons. *Diabetes Care* 1983; **6**: 180-185.

Holmes C S, Koepke K M, Thompson R G, Gyves P W, Weydert J A. Verbal fluency and naming performance in type 1 diabetes at different blood glucose concentrations. *Diabetes Care* 1984; **7**: 454-459.

Holmes C S, Koepke K M, Thompson R G. Simple versus complex performance impairments at three blood glucose levels. *Psychoneuroendocrinology* 1986; **11**: 353-357.

Home P D, Bartley P, Russell-Jones D, Hanaire-Broutin H, Heeg J E, Abrams P, Landin-Olsson M, Hylleberg B, Lang H, Draeger E. Insulin detemir offers improved glycemic control compared with NPH insulin in people with Type 1 diabetes. *Diabetes Care* 2004; **27**: 1081-1087.

Hopfner RL, Gopalakrishnan V. Endothelin: emerging role in diabetic vascular complications. *Diabetologia* 1999; **42**: 1383–1394.

Hu R, Levin ER, Pedram A, Frank HJL. Insulin stimulates production and secretion of endothelin from bovine endothelial cells. *Diabetes* 1993; **42**: 351–358.

Huo Y, Schober A, Forlow S B et al. Circulating activated platelets exacerbate atherosclerosis in mice deficient in apolipoprotein E. *Nat Med* 2003; **9**: 61-67.

Hutton R A, Mikhailidis D, Dormandy K M, Ginsburg J. Platelet aggregation studies during transient hypoglycaemia. *J Clin Pathol* 1979; **32**: 434-438.

Hyllienmark L, Maltez J, Dandenell A, Ludvigsson J, Brismar T. EEG abnormalities with and without relation to severe hypoglycaemia in adolescents with type 1 diabetes. *Diabetologia* 2005; **48**: 412-419.

Ibbotson S H, Catto A, Davies J A, Grant P J. The effect of insulin-induced hypoglycaemia on Factor VIII:C concentrations and thrombin activity in subjects with type 1 (insulin-dependent) diabetes. *Thromb Haemost* 1995; **73**: 243-246.

Jaap A J, Jones G C, McCrimmon R J, Deary I J, Frier B M. Perceived symptoms of hypoglycaemia in elderly type 2 diabetic patients treated with insulin. *Diabet Med* 1998; **15**: 398-401.

Jialal I, Devaraj S, Venogopal S K. C-reactive protein: risk marker or mediator in atherothrombosis. *Hypertension* 2004; **44**: 6-11.

Jones T W, Porter P, Sherwin R S, et al. Decreased epinephrine responses to hypoglycemia during sleep. *N Engl J Med* 1998; **338**: 1657-1662.

Judson W E, Hollander W. The effects of insulin-induced hypoglycemia in patients with angina pectoris. Before and after intravenous hexamethonium. *Am Heart J* 1956; **52**: 198-209.

Kalergis M, Schiffrin A, Gougeon R, Jones P J, Yale J F. Impact of bedtime snack composition on prevention of nocturnal hypoglycaemia in adults with type 1 diabetes undergoing intensive insulin management using lispro insulin before meals: a randomized, placebo-controlled, crossover trial. *Diabetes Care* 2003; **26**: 9-15.

Kanc K, Janssen M M J, Keulen E T P, Jacobs M A J M, Popp-Snijders C, Snoek F J, Heine R J. Substitution of night-time continuous subcutaneous insulin infusion therapy for bedtime NPH insulin in a multiple injection regimen improves counterregulatory hormonal responses and warning symptoms of hypoglycaemia in IDDM. *Diabetologia* 1998; **41**: 322-329.

Kerr D, Macdonald I A, Heller S R, Tattersall R B. β-adrenoceptor blockade and hypoglycaemia. A randomised, double-blind, placebo controlled comparison of metoprolol CR, atenolol and propranolol LA in normal subjects. *Br J Clin Pharmacol* 1990; **29**: 685-693.

Kerr D, Sherwin R S, Pavalkis F, Fayad P B, Sikorski L, Rife F, Tamborlane W V, During M J. Effect of caffeine on the recognition of and responses to hypoglycemia in humans. *Ann Int Med* 1993; **119**: 798-804.

Kerr D, Richardson T. Counterregulatory deficiencies in diabetes. In: *Hypoglycaemia in Clinical Diabetes.* Frier B M and Fisher M, eds. 2nd edn. John Wiley and Sons, Chichester, 2007: 121–140.

Kinsey J L. Incidence and cause of death in shock therapy. *Archives of Neurology and Psychiatry* 1941; **46**: 55-58.

Kinsley B T, Widom B, Simonson D C. Differential regulation of counterregulatory hormone secretion and symptoms during hypoglycemia in IDDM. Effect of glycemic control. *Diabetes Care* 1995; **18**: 17-26.

Kinsley B T, Weinger K, Bajaj M, Levy C J, Simonson D C, Quigley M, Cox D J, Jacobson A M. Blood glucose awareness training and epinephrine responses to hypoglycemia during intensive treatment in type 1 diabetes. *Diabetes Care* 1999; **22**: 1022-1028.

Kirpichnikov D, Sowers J. Diabetes Mellitus and diabetes-associated vascular disease. *Trends Endocrinol Metab*. 2001; **12**: 225-30.

Kishikawa H, Takeda H, Kiyota S, Sakakida M, Fukushima H, Ichinose K, Matsuda H, Nakamura N, Uzawa H. Role of α2-adrenergic receptor in platelet actvation during insulin-induced hypoglycemia in normal subjects. *Diabetes* 1988; **36**: 407-412.

Klonoff D C. Continuous glucose monitoring. Roadmap for 21st century diabetes therapy. *Diabetes Care* 2005; **28**: 1231-1239.

Kodl C T, Franc D T, Rao J P, Anderson F S, Thomas W, Mueller B A, Lim K O, Seaquist E R. Diffusion tensor imaging identifies deficits in white matter microstructure in subjects with type 1 diabetes that correlate with reduced neurocognitive function. *Diabetes* 2008; **57**: 3083–3089.

Koppel B S, Daras M. Transient hypodensity on CT scan during hypoglycemia. *Eur Neurol* 1993; **33**: 80-82.

Kosiborod M, Inzucchi S E, Goyal A, Krumholz H M, Masoudi F A, Xiao L, Spertus J A. Relationship between spontaneous and iatrogenic hypoglycemia and mortality in patients hospitalized with acute myocardial infarction. *JAMA* 2009; **301**: 1556–1564.

Krentz A J, Bailey C J. Oral antidiabetic agents: current role in type 2 diabetes mellitus. *Drugs* 2005; **65**: 385-411.

Krentz A J, Boyle P J, Justice K M, Wright A D, Schade D S. Successful treatment of severe refractory sulfonylurea-induced hypoglycemia with octreotide. *Diabetes Care* 1993; **16**: 184-186.

Kubiak T, Hermanns N, Schreckling H J, Kulser B, Haak T. Assessment of hypoglycaemia awareness using continuous glucose monitoring. *Diabet Med* 2004; **21**: 487-490.

Landin K, Tengborn L, Chmielewska J, von Schenck H, Smith U. The acute effect of insulin on tissue plasminogen activator and plasminogen activator inhibitor in man. *Thromb Haemost* 1991; **65**: 130-133.

Langan SJ, Deary IJ, Hepburn DA, Frier BM: Cumulative cognitive impairment following recurrent severe hypoglycaemia in adult patients with insulin-treated diabetes mellitus. *Diabetologia* 1991; **34**: 337–344.

Lauritzen T, Frost-Larsen K, Larsen H W, Deckert T and the Steno Study Group. Effect of 1 year of near-normal blood glucose levels on retinopathy in insulin-dependent diabetics. *Lancet* 1983; **i**: 200-208.

Lee S P, Harris N D, Robinson R T et al. Effect of atenolol on QTc interval lengthening during hypoglycaemia in type 1 diabetes. *Diabetologia* 2005; **48**: 1269-1272.

Leese G P, Wang J, Broomhall J, et al (for the DARTS/MEMO Collaboration). Frequency of severe hypoglycemia requiring emergency treatment in type 1 and type 2 diabetes: a population-based study of health service resource use. *Diabetes Care* 2003; **26**; 1176-1180.

Levy C J, Kinsley B T, Bajaj M, Simonson D C: Effect of glycemic control on glucose counterregulation during hypoglycaemia in NIDDM. *Diabetes Care* 1998; **21**: 1330–1338.

Libby P, Ridker P M, Maseri A. Inflammation and atherosclerosis. *Circulation* 2002; **105**: 1135.

Lincoln N B, Faleiro R M, Kelly C, Kirk B A, Jeffcoate W J: Effect of long-term glycemic control on cognitive function. *Diabetes Care* 1996; **19**: 656–658.

Linn, M C, Petersen A C. Emergence and characterisation of gender differences in spatial abilities: A meta-analysis. *Child Development* 1985; **56**: 1479-1498.

Liu D, McManus R M, Ryan E A. Improved counter-regulatory hormonal and symptomatic responses to hypoglycaemia in patients with insulin-dependent diabetes mellitus after 3 months of less strict glycemic control. *Clin Inv Med* 1996; **19**: 71-82.

Lloyd-Mosten R H, Oram S. Modification by propranolol of cardiovascular effects of induced hypoglycaemia. *Lancet* 1975; **i**: 1213-1215.

Luscher T F, Barton M. Endothelins and endothelin receptor antagonists. *Circulation* 2000; **102**: 2434–2440.

MacCuish A C. Treatment of Hypoglycaemia. In: *Hypoglycaemia and Diabetes: Clinical and Physiological Aspects*. Frier B M and Fisher B M, eds. Edward Arnold, London, 1993: 212-221.

Macdonald I A, King P. Normal glucose metabolism and responses to hypoglycaemia. In: *Hypoglycaemia in Clinical Diabetes*. Frier B M and Fisher M, Eds. 2nd edition. John Wiley and Sons, Chichester, 2007: 1-24.

Mach F, Schonbeck U, Libby P. CD40 signalling in vascular cells: a key role in atherosclerosis? *Atherosclerosis* 1998[1]; **137** Suppl: S89-95.

Mach F, Schonbeck U, Sukhova G K, Bourcier T, Bonnefoy J Y, Pober J S, Libby P. Functional CD40 ligand is expressed on human vascular endothelial cells, smooth muscle cells, and macrophages: Implications for CD40-CD40 ligand signalling in atherosclerosis. *Proc Natl Acad Sci USA* 1997; **94**: 1931-1936.

Mach F, Schonbeck U, Sukhova G K, et al. Reduction of atherosclerosis in mice by inhibition of CD40 signalling. *Nature* 1998[2]; **394**: 200-3.

MacLeod K M, Hepburn D A, Frier B M. Frequency and morbidity of severe hypoglycaemia in insulin-treated diabetic patients. *Diabet Med* 1993; **10**: 238-245.

MacLeod K M, Hepburn D A, Deary I J D, Goodwin G M, Dougall N, Ebmeier K P, Frier B M. Regional cerebral blood flow in IDDM patients: effects of diabetes and of recurrent severe hypoglycaemia. *Diabetologia* 1994; **37**: 257-263.

MacLeod K M, Gold A E, Ebmeier K P, Hepburn D A, Deary I J, Goodwin G M, Frier B M. The effects of acute hypoglycemia on relative cerebral blood flow distribution in patients with type 1 (insulin-dependent) diabetes and impaired hypoglycemia awareness. *Metabolism* 1996; **45**: 974-980.

Madjid M, Naghavi M, Litovsky S, Casscells S W. Influenza and cardiovascular disease. *Circulation* 2003; **108**: 2730-2736.

Maggs D G, Scott A R, Macdonald I A. Thermoregulatory responses to hyperinsulinemic hypoglycemia and euglycemia in humans. *Am J Physiol* 1994; **267**: R1266-R1272.

Makimattila S, Malmberg-Ceder K, Hakkinen A M, Vuori K, Salonen O, Summanen P et al. Brain metabolic alterations in patients with type 1 diabetes-hyperglycaemia-induced injury. *J Cereb Blood Flow Metab* 2004; **24**: 1393-1399.

Malmberg K. Prospective randomised study of intensive insulin treatment on long term survival after acute myocardial infarction in patients with diabetes mellitus. DIGAMI (Diabetes Mellitus, Insulin Glucose Infusion in Acute Myocardial Infarction) Study Group. *Br Med J* 1997; **314**: 1512–1515.

Malmberg K, Ryden L, Wedel H, Birkeland K, Bootsma A, Dickstein K, Efendic S, Fisher M, Hamsten A, Herlitz J, Hildebrandt P, MacLeod K, Laakso M, Torp-Pedersen C, Waldenstrom A, DIGAMI 2 Investigators. Intense metabolic control by means of insulin in patients with diabetes mellitus and acute myocardial infarction (DIGAMI 2): effects on mortality and morbidity. *Eur Heart J* 2005; **26**: 650–661.

Malouf R, Brust J C M. Hypoglycemia: causes, neurological manifestations, and outcome. *Ann Neurol* 1985; **17**: 421-430.

Marioni R E, Starchan M W J, Reynolds R M, Lowe G D O, Mitchell R J, Fowkes G R, Frier B M, Lee A J, Butcher I, Rumley A, Murray G D, Deary I J, Price J F. Association between raised inflammatory markers and cognitive decline in elderly people with type 2 diabetes: The Edinburgh Type 2 Diabetes Study. *Diabetes* 2010[1]; **59**: 710-713.

Marioni R E, Deary I J, Strachan M W, Lowe G D, Rumley A, Murray G D, Price J F. Blood rheology and cognition in the Edinburgh Type 2 Diabetes Study. *Age Ageing* 2010[2]; **39**: 354-359.

Marques J L, George E, Pearcey S R, Harris N D, MacDonald I A, Cochrane T, Heller S R. Altered ventricular repolarisation during hypoglycaemia in patients with diabetes. *Diabet Med* 1997; **14**: 648-654.

McAulay V, Deary I J, Frier B M. Symptoms of hypoglycaemia in people with diabetes. *Diabet Med* 2001[1]; **18**: 690-705.

McAulay V, Ferguson S C, Deary I J, Frier B M. Acute hypoglycemia in humans causes attentional dysfunction while non-verbal intelligence is preserved. *Diabetes Care* 2001[2]; **24**: 1745-1750.

Mendall M A, Patel P, Ballam L, Strachan D, Northfield T C. C reactive protein and its relation to cardiovascular risk factors: a population based cross sectional study. *Br Med J* 1996; **312**: 1061-1065.

Middleton W S, Oatway W H. Insulin shock and the myocardium. *Am J Med Sci* 1931; **181**: 39-52.

Mikhailidis D P, Barradas M A, Hutton R A, Jeremy J Y, Sabur M, Dandona P. The effect of non-specific β-blockade on metabolic and haemostatic variables during hypoglycaemia. *Diabetes Res* 1985; **2**: 127-134.

Mitrakou A, Ryan C, Veneman T et al. Hierarchy of glycemic thresholds for counterregulatory hormone secretion, symptoms, and cerebral dysfunction. *Am J Physiol* 1991; **260**: E67-E74.

Mohn A, Matyka K A, Harris D A, Ross K M, Edge J A, Dunger D B. Lispro or regular insulin for multiple injection therapy in adolescence. Differences in free insulin and glucose levels overnight. *Diabetes Care* 1999; **22**: 27-32.

Monnier L H, Lachkar H, Richard J L, Colette C, Borgel D, Orsetti A, Mirouze J. Plasma β-thromboglobulin response to insulin-induced hypoglycemia in type 1 diabetic patients. *Diabetes* 1984; **33**: 907-909.

Monnier L, Mas E, Ginet C, Michel F, Villon L, Cristol J, Colette C. Activation of oxidative stress by acute glucose fluctuations compared with sustained chronic hyperglycaemia in patients with type 2 diabetes. *JAMA* 2006; **295**: 1681-1687.

Morabito E, Corsico N, Martelli EA. Endothelins urinary excretion is increased in spontaneously diabetic rats BB/BB. *Life Sci* 1994; **56**: 13–18.

Morise T, Takeuchi Y, Kawano M, Koni I, Takeda R. Increased plasma levels of immunoreactive endothelin and von Willebrand factor in NIDDM patients. *Diabetes Care* 1995; **18**: 87–89.

Morrow D A, Rifai N, Antman E M, Weiner D L, McCabe C H, Cannon C P, Braunwald E. C-reactive protein is a potent predictor of mortality independently of and in combination with troponin T in acute coronary syndromes: a TIMI 11A substudy. Thrombolysis in myocardial infarction. *J Am Coll Cardiol* 1998; **31**: 1460-1465.

Muhlhauser I, Toth G, Sawicki P T, Berger M. Severe hypoglycemia in type 1 diabetic patients with impaired kidney function. *Diabetes Care* 1991[1]; **14**: 344-346.

Muhlhauser I, Heinemann L, Fritsche E, von Lennek K, Berger M. Hypoglycemic symptoms and frequency of severe hypoglycemia in patients treated with human and animal insulin preparations. *Diabetes Care* 1991[2]; **14**: 745-749.

Muhlhauser I, Koch J, Berger M. Pharmacokinetics and bioavailability of injected glucagon: differences between intramuscular, subcutaneous and intravenous administration. *Diabetes Care* 1985; **8**: 39-42.

Mutch W J, Dingwall-Fordyce I. Is it a hypo? Knowledge of the symptoms of hypoglycaemia in elderly diabetic patients. *Diabet Med* 1985; **2**: 54-56.

Nesto R W, Phillips R T, Kett K G, Hill T, Perper E, Young E, Leland O S. Angina and exertional myocardial ischemia in diabetic and non-diabetic patients: assessment by exercise thallium scintigraphy. *Ann Intern Med* 1988; **108**: 170-175.

Neumann F-J, Marx N, Gawaz M et al. Induction of cytokine expression in leucocytes by binding of thrombin-stimulated platelets. *Circulation* 1997; **95**: 2387-2394.

NICE-SUGAR Study Investigators, Finfer S, Chittock D R, Su S Y, Blair D, Foster D, Dhingra V, et al. Intensive versus conventional glucose control in critically ill patients. *N Engl J Med* 2009; **360**: 1283–1297.

Northam E A, Rankins D, Lin A, Wellard R M, Pell G S, Finch S J, Werther G A, Cameron F J. Central nervous system function in youth with type 1 diabetes 12 years after disease onset. *Diabetes Care* 2009; **32**: 445–450.

Parrish A E, Sugar S J N, Fazekas J F. A relationship between electrocardiographic changes and hypokalemia in insulin-induced hypoglycemia. *Am Heart J* 1952; **43**: 815-820.

Partaiman J O, Bradley R F. Acute myocardial infarction in 258 cases of diabetes. Immediate mortality and five-year survival. *N Engl J Med* 1965; **273**: 455-461

Patrick A W, Hepburn D A, Craig K J, Thompson I, Swainson C D, Frier B M. The effects of acute insulin-induced hypoglycaemia on renal function in normal human subjects. *Diabet Med* 1989; **6**: 703-708.

Pederson-Bjergaard U, Pramming S, Thorsteinsson B. Recall of severe hypoglycaemia and self-estimated state of awareness in type 1 diabetes. *Diabetes Metab Res Revs* 2003; **19**: 232-240.

Pedersen-Bjergaard U, Pramming S, Heller S R, Wallace T M, Rasmussen A K, Jorgensen H V, Matthews D R, Hougaard P, Thorsteinsson B. Severe hypoglycaemia in 1076 adult patients with type 1 diabetes: influence of risk markers and selection. *Diabetes Metab Res Revs* 2004; **20**:479-486.

Pepys M B. CRP or not CRP? That is the question. *Arterioscler Thromb Vasc Biol* 2005; **25**: 1091-1094.

Perros P, Deary I J, Sellar R J, Best J J, Frier B M: Brain abnormalities demonstrated by magnetic resonance imaging in adult IDDM patients with and without a history of recurrent severe hypoglycemia. *Diabetes Care* 1997; **20**: 1013–1018.

Pieber T R, Eugene-Jolchine I, Derobert E. Efficacy and safety of HOE 901 versus NPH insulin in patients with type 1 diabetes. The European Study Group of HOE 901 in type 1 diabetes. *Diabetes Care* 2000; **23**: 157-162.

Pickup J C, Sutton A J. Severe hypoglycaemia and glycaemic control in type 1 diabetes: meta-analysis of multiple daily insulin injections compared with continuous subcutaneous insulin infusion. *Diabet Med* 2008; **25**: 765-774.

Pladziewicz D S, Nesto R W. Hypoglycemia-induced silent myocardial ischemia. *Am J Cardiol* 1989; **63:** 1531-1532.

Pramming S, Thorsteinsson B, Stigsby B, Binder C. Glycaemic threshold for changes in electroencephalograms during hypoglycaemia in patients with insulin-dependent diabetes. *Br Med J* 1988; **296**: 665-667.

Pramming S, Thorsteinsson B, Bendtson I, Binder C. Symptomatic hypoglycaemia in 411 type 1 diabetic patients. *Diabet Med* 1991; **8**: 217-222.

Rabkin R, Ryan M P, Duckworth W C. The renal metabolism of insulin. *Diabetologia* 1984; **27**: 351-357.

Rahman M. Introduction to Flow Cytometry. AbD Serotec Information Pack, 2006.

Raju B, Arbalaez A M, Breckenridge S M, Cryer P E. Nocturnal hypoglycemia in type 1 diabetes: an assessment of preventive bedtime treatments. *J Clin Endocrinol Metab* 2006; **91**: 2087-2092.

Rana O, Byrne C D, Kerr D, Coppini D V, Zouwail S, Senior R, Begley J, Walker J J, Greaves K. Acute hypoglycemia decreases myocardial blood flow reserve in patients with type 1 diabetes and in healthy humans. *Circulation* 2011; **124**: 1548-1556.

Razavi Nematollahi L, Kitabchi AE, Kitabchi AE, Stentz FB, Wan JY, Larijani BA, Tehrani MM, Gozashti MH, Omidfar K, Taheri E. Proinflammatory cytokines in response to insulin-induced hypoglycaemic stress in healthy subjects. *Metabolism* 2009; **58**: 443–448.

Rosenthal J M, Amiel S A, Yaquez L, Bullmore E, Hopkins D, Evans M, Pernet A, Reid H, Giampietro V, Andrew C M, Suckling J, Simmons A, Williams, S C. The effect of acute hypoglycemia on brain function and activation: a functional magnetic resonance imaging study. *Diabetes* 2001; **50**: 1618-1626.

Rovet J F, Ehrlich R M, Hoppe M. Intellectual deficits associated with early onset of insulin-dependent diabetes in children. *Diabetes Care* 1987; **10**: 510-515.

Royce J R. The conceptual framework for a multi-factor theory of individuality. In: *Multivariate Analysis and Psychological Theory*. Royce J R, ed. Academic Press, 1973: 305-407.

Ryan C, Vega A, Longstreet C, Drash A. Neuropsychological changes in adolescents with insulin-dependent diabetes. *J Consult Clin Psych* 1984; **52**: 335-342.

Ryan C M, Gurtunca N, Becker D. Hypoglycemia: a complication of diabetes therapy in children. *Pediatr Clin North Am* 2005; **52**: 1705-1733.

Ryan C M. Diabetes and brain damage: more (or less) than meets the eye? *Diabetologia* 2006; **49**: 2229-2233.

Rydzewski A, Urano T, Nagai N, Takada Y, Katoh-Oishi Y, Taminato T, Yoshimi T, Takada A. Diurnal variation in serum remnant-like lipoproteins, platelet aggregation and fibrinolysis in healthy volunteers. *Haemostasis* 1997; **27**: 305–314.

Sämann A, Mühlhauser I, Bender R, Hunger-Dathe W, Kloos C, Müller U A. Flexible intensive insulin therapy in adults with Type 1 diabetes and high risk for severe hypoglycemia and diabetic ketoacidosis. *Diabetes Care* 2006; **29**: 2196-2199.

Schalkwijk C G, Poland D C W, van Dijk W, Kok A, Emeis J J, Drager A M, Doni A, van Hinsbergh V W, Stehouwer C D. Plasma concentration of C-reactive protein is increased in Type 1 diabetic patients without clinical macroangiopathy and correlates with markers of endothelial dysfunction: evidence for chronic inflammation. *Diabetologia* 1999; **42**: 351-357.

Schneider J G, Tilly N, Hierl T, Sommer U, Hamann A, Dugi K, Leidig-Bruckner G, Kasperk C. Elevated plasma endothelin-1 levels in diabetes mellitus. *Am J Hypertens* 2002; **15**: 967-972.

Schonbeck U, Mach F, Libby P. CD154 (CD40L). *Int J Biochem Cell Biol* 2000[1]; **32**: 687-93.

Schonbeck U, Mach F, Sukhova G K, Herman M, Graber P, Kehry M R, Libby P. CD40 Ligation Induces Tissue Factor Expression in Human Vascular Smooth Muscle Cells. *Am J Path* 2000[2]; **156**: 7-14.

Schonbeck U, Sukhova G K, Shimizu K, Mach F, Libby P. Inhibition of CD40 signalling limits evolution of established atherosclerosis in mice. *Proc Natl Acad Sci USA* 2000[3]; **97**: 7458-7463.

Schonbeck U, Libby P. CD40 signalling and plaque instability. *Circulation Research* 2001[1]; **89**: 1092.

Schonbeck U, Varo N, Libby P, Buring J, Ridker P M. Soluble CD40L and Cardiovascular Risk in Women. *Circulation* 2001[2]; **104**: 2266-2268.

Schopman J E, Geddes J, Frier B M. Prevalence of impaired awareness of hypoglycaemia and frequency of hypoglycaemia in insulin-treated type 2 diabetes. *Diabetes Research and Clinical Practice* 2010; **87**: 64-68.

Schram M T, Chaturvedi N, Schalkwijk C G, Fuller J H, Stehouwer C D A. Markers of inflammation are cross-sectionally associated with microvascular complications and cardiovascular disease in type 1 diabetes – the EURODIAB Prospective Complications Study. *Diabetologia* 2005; **48**: 370-378.

Segel S A, Paramore D A, Cryer P E: Hypoglycemia-associated autonomic failure in advanced type 2 diabetes. *Diabetes* 2002; **51**: 724–733.

Segel S A, Fanelli C G, Dence C S, Markham J, Videen T O, Paramore D S, Powers W J, Cryer P E. Blood-to-brain glucose transport, cerebral glucose metabolism and cerebral blood flow are not increased after hypoglycemia. *Diabetes* 2001; **50**: 1911-1917.

Shapiro A M J, Ricordi C, Hering B J, et al. International trial of the Edmonton protocol for islet transplantation. *N Engl J Med* 2006; **355**: 1318-1330.

Shirayama H, Ohshiro Y, Kinjo Y, Taira S, Teruya I, Nakachi K, Tawata M, Takasu N. Acute brain injury in hypoglycaemia-induced hemiplegia. *Diabet Med* 2004; **21**: 623-624.

Slama G, Traynard P Y, Desplanque N, Pudar H, Dhunputh I, Letanoux M, Bornet F R, Tchobroutsky G. The search for an optimised treatment of hypoglycemia. Carbohydrates in tablets, solution, or gel for the correction of insulin reactions. *Arch Intern Med* 1990; **150**: 589-593.

Sommerfield A J, Deary I J, McAulay V, Frier B M. Moderate hypoglycemia impairs multiple memory functions in healthy adults. *Neuropsychology* 2003[1]; **17**: 125-132.

Sommerfield A J, Deary I J, McAulay V, Frier B M. Short-term, delayed, and working memory are impaired during hypoglycemia in individuals with type 1 diabetes. *Diabetes Care* 2003[2]; **26**: 390-396.

Sommerfield A J, Wilkinson I B, Webb D J, Frier B M. Vessel wall stiffness in type 1 diabetes and the central hemodynamic effects of acute hypoglycemia. *Am J Physiol Endocrinol Metab* 2007; **293**: E1274-1279.

Steel C M, French E B, Aitchison W R C. Studies on adrenaline-induced leucocytosis in normal man. *Br J Haematol* 1971; **21**: 413-421.

Stewart R, Liolitsa D. Type 2 diabetes mellitus, cognitive impairment and dementia. *Diabet Med* 1999; **16**: 93-112.

Strachan M W J. Frequency, causes and risk factors for hypoglycaemia in type 1 diabetes. In: *Hypoglycaemia in Clinical Diabetes*. Frier B M and Fisher M, eds. 2nd edn. John Wiley and Sons, Chichester, 2007: 49-82.

Strachan M W J, Abraha H D, Sherwood R A, Lammie G A, Deary I J, Ewing F M, Perros P, Frier B M. Evaluation of serum markers of neuronal damage following severe hypoglycaemia in adults with insulin-treated diabetes mellitus. *Diab Metab Res Revs* 1999; **15**: 5-12.

Strachan M, Ewing F, Frier B, McCrimmon R, Deary I J. Effects of acute hypoglycaemia on auditory information processing in adults with type 1 diabetes. *Diabetologia* 2003; **46**: 97-105.

Strouse S, Soskin S, Katz L N, Rubinfield S H. Treatment of older diabetic patients with cardiovascular disease. *JAMA* 1932; **98**: 1703-1706.

Strudwick S K, Carne C, Gardner J, Foster J K, Davis E A, Jones T W. Cognitive functioning in children with early onset type 1 diabetes and severe hypoglycemia. *J Pediatr* 2005; **147**: 680-685.

Sumner J, Baber C, Williams V. What do patients with type 1 diabetes know about hypoglycaemia? *Pract Diabetes Int* 2000; **17**; 187-190.

Svensson A, McGuire D K, Abrahamsson P, Dellborg M. Association between hyper- and hypoglycaemia and 2 year all-cause mortality risk in diabetic patients with acute coronary events. *Eur Heart J* 2005; **26**: 1255-1261.

Takahashi K, Ghatei MA, Lam HC, O'Halloran J, Bloom SR. Elevated plasma endothelin in patients with diabetes mellitus. *Diabetologia* 1990; **33**: 306–310.

Takeda H, Kishikawa H, Shinohara M, Miyata T, Suzaki K, Fukushima H, Ichinose K, Shichiri M. Effect of α2-adrenoceptor antagonist on platelet actvation during insulin-induced hypoglycaemia in type 2 (non-insulin-dependent) diabetes mellitus. *Diabetologia* 1988; **31**: 657-663.

Tallroth G, Ryding E, Agardh C D. Regional cerebral blood flow in normal man during insulin-induced hypoglycemia and in the recovery period following glucose infusion. *Metabolism* 1992; **41**: 717-721.

Tallroth G, Lindgren M, Stenberg G, Rosen I, Agardh C-D. Neurophysiological changes during insulin-induced hypoglycaemia and in the recovery period following glucose infusion in type 1 (insulin-dependent) diabetes mellitus and in normal man. *Diabetologia* 1990; **33**: 319-323.

Targher G, Bertolini L, Zoppini G et al. Increased plasma markers of inflammation and endothelial dysfunction and their association with microvascular complications in Type 1 diabetic patients without clinically manifest macroangiopathy. *Diabet Med* 2005; **22**: 999-1004.

Tarman W. Diabetic vitreous hemorrhage and its relationship to hypoglycaemia. *Mod Probl Ophthal* 1979; **20**: 413-414.

Taylor J R, Sheratt H S, Davies D M. Intramuscular or intravenous glucagon for sulphonylurea hypoglycaemia? Eur *J Clin Pharmacol* 1978; **14**: 125-127.

ter Braak E W M T, Appelman A M M F, Van de Laak M F, Stolk R P, Van Haeften T W, Erkelens D W. Clinical characteristics of type 1 diabetic patients with and without severe hypoglycemia. *Diabetes Care* 2000; **23**:1467-1471.

Teves D, Videen T O, Cryer P E, Powers W J. Activation of human medial prefrontal cortex during autonomic responses to hypoglycaemia. *Proc Natl Acad Sci USA* 2004; **101**: 6217-6221.

Thomas R M, Aldibbiat A, Griffin W, Cox M A A, Leech N J, Shaw J A M. A randomized pilot study in Type 1 diabetes complicated by severe hypoglycaemia, comparing rigorous hypoglycaemia avoidance with insulin analogue therapy, CSII or education alone. *Diabet Med* 2007; **24**: 778-783.

Thompson C J, Baylis P H. Endocrine changes during insulin-induced hypoglycaemia. In: *Hypoglycaemia and Diabetes: Clinical and Physiological Aspects.* Frier B M and Fisher B M, eds. Edward Arnold, London, 1993: 116-131.

Thomson F J, Masson E A, Leeming J T, Boulton A J M. Lack of knowledge of symptoms of hypoglycaemia by elderly diabetic patients. *Age and Ageing* 1991; **20**: 404-406.

Thordarson H, Sovik O. Dead in bed syndrome in young diabetic patients in Norway. *Diabet Med* 1995; **12:** 782-787.

Trovati M, Anfossi G, Cavalot F, Vitali S, Massucco P, Mularoni E, Schinco P, Tamponi G, Emanuelli G. Studies on mechanisms involved in hypoglycemia-induced platelet activation. *Diabetes* 1986; **35**: 818-825.

UK Hypoglycaemia Study Group. Risk of hypoglycaemia in types 1 and type 2 diabetes: effects of treatment modalities and their duration. *Diabetologia* 2007; **50**: 1140-1147.

United Kingdom Prospective Diabetes Study Group: Intensive blood glucose control with sulphonylureas or insulin compared with conventional treatment and risk of complications in patients with type 2 diabetes (UKPDS 33). *Lancet* 1998; **352**: 837-852.

Van den Berghe G, Wouters P, Weekers F, Verwaest C, Bruyninckx F, Schetz M, Vlasselaars D, Ferdinande P, Lauwers P, Bouillon R. Intensive insulin therapy in critically ill patients. *N Engl J Med* 2001; **345**: 1359-1367.

Van den Berghe G, Wilmer A, Hermans G, Meersseman W, Wouters P J, Milants I, Van Wijngaerden E, Bobbaers H, Bouillon R. Intensive insulin therapy in the medical ICU. *N Engl J Med* 2006; **354**: 449–461.

van Exel E, Gussekloo J, de Craen A JU M et al. Low Production Capacity of Interleukin-10 Associates With the Metabolic Syndrome and Type 2 Diabetes. *Diabetes* 2002; **51**: 1088-1092.

van Gool W A, van de Beek D, Eikelenboom P. Systemic infection and delirium: when cytokines and acetylcholine collide. *Lancet* 2010; **375**: 773–775

Van Hecke M V, Dekker J M, Nijpels G, et al. Inflammation and endothelial dysfunction are associated with retinopathy: the Hoorn Study. *Diabetologia* 2005; **48**: 1300-1306.

van Tits L J H, Daul A, Bauch H J, et al. Effects of insulin-induced hypoglycaemia on β2-adrenoceptor density and proliferative responses of human lymphocytes. *J Clin Endocr Metab* 1990; **71**: 187-192.

Veneman T, Mitrakou A, Mokan M, Cryer P, Gerich J. Induction of hypoglycemia unawareness by asymptomatic nocturnal hypoglycemia. *Diabetes* 1993; **42**: 1233-1237.

Vestra M D, Mussap M, Gallina P, et al. Acute-Phase Markers of Inflammation and Glomerular Structure in Patients with Type 2 Diabetes. *J Am Soc Nephrol* 2005; **16**: 78-82.

Vogel J J, Bowers C A, Vogel D S. Cerebral lateralization of spatial abilities: a meta-analysis. *Brain Cogn* 2003; **52**: 197-204.

Warren R E, Allen K A, Sommerfield A J, Deary I J, Frier B M. Acute hypoglycemia impairs non-verbal intelligence. *Diabetes Care* 2004; **27**: 1447-1448.

Warren R E, Zammitt N N, Deary I J, Frier B M. The effects of acute hypoglycaemia on memory acquisition and recall and prospective memory in type 1 diabetes. *Diabetologia* 2007; **50**: 178-185.

Wentholt I M E, Kulik W, Michels R P J, Hoekstra J B L, De Vries J H. Glucose fluctuations and activation of oxidative stress in patients with type 1 diabetes. *Diabetologia* 2008; **51**: 183-190.

Wentholt I M E, Maran A, Masurel N, Heine R J, Hoekstra J B L, DeVries J H. Nocturnal hypoglycaemia in type 1 diabetic patients, assessed with continuous glucose monitoring: frequency, duration and associations. *Diabet Med* 2007; **24**: 527-532.

Whitmer R A, Karter A J, Yaffe K, Quesenberry C P Jr, Selby J V. Hypoglycemic episodes and risk of dementia in older patients with type 2 diabetes mellitus. *JAMA* 2009; **301**: 1565–1572.

Widom B, Simonson D J. Glycemic control and neuropsychologic function during hypoglycemia in patients with insulin-dependent diabetes. *Ann Intern Med* 1990; **112**: 904-912.

Wiethrop B V, Cryer P E. Alanine and terbutaline in treatment of hypoglycemia in IDDM. *Diabetes Care* 1993; **16**: 1131-1136.

Wirsen A, Tallroth G, Lindgren M, Agardh C. Neuropsychological performance differs between type 1 diabetic and normal men during insulin-induced hypoglycaemia. *Diabet Med* 1992; **9**: 156-165.

Wollesen F, Berglund L, Berne C. Plasma endothelin-1 and total insulin exposure in diabetes mellitus. *Clin Sci* 1999; **97**: 149-156.

Wredling R, Levander S, Adamson U, Lins P E. Permanent neuropsychological impairment after recurrent episodes of severe hypoglycaemia in man. *Diabetologia* 1990; **33**: 152–157.

Wright R J, Frier B M. Vascular disease and diabetes: is hypoglycaemia an aggravating factor? *Diabetes Metab Res Revs* 2008; **24**: 353–363.

Wysocki T, Taylor A, Harris M A, Jackson S C, Mauras N, White N H, Fox L. Absence of adverse effects of severe hypoglycaemia on cognitive function in school-aged children with diabetes over 18 months. *Diabetes Care* 2003; **26**: 1100-1105.

Yoneda Y, Yamamoto S. Cerebral cortical laminar necrosis on diffusion-weighted MRI in hypoglycaemic encephalopathy. *Diabet Med* 2005; **22**: 1098-1100.

Yngen M, Ostenson C-G, Hu H, et al. Enhanced P-selectin expression and increased soluble CD40 Ligand in patients with Type 1 diabetes mellitus and microangiopathy: evidence for platelet hyperactivity and chronic inflammation. *Diabetologia* 2004; **47**: 537-540.

Yudkin J S, Panahloo C, Stehouwer J, et al. The influence of improved glycaemic control with insulin and sulphonylureas on acute phase and endothelial markers in Type II Diabetic subjects. *Diabetologia* 2000; **43**: 1099-1106.

Zammitt N N, Frier B M. Hypoglycemia in type 2 diabetes: pathophysiology, frequency, and effects of different treatment modalities. *Diabetes Care* 2005; **28**: 2948-2961.

Zammitt N N, Warren R E, Deary I J, Frier B M. Delayed recovery of cognitive function following hypoglycemia in adults with type 1 diabetes: effect of impaired awareness of hypoglycemia. *Diabetes* 2008; **57**: 732-736.

Appendix: Published Papers

With kind permission of the publishers

Effects of Acute Insulin-Induced Hypoglycemia on Spatial Abilities in Adults With Type 1 Diabetes

ROHANA J. WRIGHT, MRCP[1]
BRIAN M. FRIER, MD[1,2]
IAN J. DEARY, PHD[2,3]

OBJECTIVE — To examine the effects of acute insulin-induced hypoglycemia on spatial cognitive abilities in adult humans with type 1 diabetes.

RESEARCH DESIGN AND METHODS — Sixteen adults with type 1 diabetes underwent two counterbalanced experimental sessions: euglycemia (blood glucose 4.5 mmol/l [81 mg/dl]) and hypoglycemia (2.5 mmol/l [45 mg/dl]). Arterialized blood glucose levels were maintained using a hyperinsulinemic glucose clamp technique. During each session, subjects underwent detailed assessment of spatial abilities from the Kit of Factor-Referenced Cognitive Tests and two tests of general cognitive function.

RESULTS — Spatial ability performance deteriorated significantly during hypoglycemia. Results for the Hidden Patterns, Card Rotations, Paper Folding, and Maze Tracing tests were all impaired significantly ($P \leq 0.001$) during hypoglycemia, as were results for the Cube Comparisons Test ($P = 0.03$). The Map Memory Test was not significantly affected by hypoglycemia.

CONCLUSIONS — Hypoglycemia is a common side effect of insulin therapy in individuals with type 1 diabetes, and spatial abilities are of critical importance in day-to-day functioning. The deterioration in spatial abilities observed during modest experimental hypoglycemia provides novel information on the cerebral hazards of hypoglycemia that has potential relevance to everyday activities.

Diabetes Care 32:1503–1506, 2009

Hypoglycemia is a common side effect of insulin treatment of diabetes. Strict glycemic control limits the development and severity of vascular complications of diabetes, but hypoglycemia is a frequent consequence. Strict glycemic control can increase the incidence of severe hypoglycemia by threefold (1). Hypoglycemia has an adverse effect on cognitive functions, as the human brain relies solely on glucose as its source of energy (2). It has a pronounced effect on complex cognitive tasks both in diabetic and nondiabetic individuals, whereas simple mental tasks are relatively unaffected (2). Cognitive function deteriorates when arterialized blood glucose concentrations decline to <3.0 mmol/l (3–6). Simple and choice reaction times, speed of mathematical calculation, verbal fluency, attention, memory, and psychomotor function have all been demonstrated to be affected during hypoglycemia (7–10). The recovery of different aspects of cognitive function may vary from between 40 and 90 min after restoration of blood glucose to normal (2,11).

Whereas hypoglycemia impairs many domains of cognitive function, the effect of hypoglycemia on spatial cognitive abilities has not been investigated in detail, although spatial ability is undoubtedly a component of some of the tests used to assess other aspects of cognition (12). Spatial abilities may be defined as the ability to generate, retain, retrieve, and transform or manipulate structured visual images to orientate and interpret the surrounding environment. In real-life terms, spatial ability is concerned with how human beings deal with issues concerning two- and three-dimensional objects, space, navigation, and pathfinding. Practical daily cognition often involves inferring how shapes and objects will appear and function when they are rotated or otherwise oriented or viewed differently. In everyday interactions with the environment, this process is very important, with particular relevance for complex tasks such as driving and map reading. A large variety of mental tests are available for the assessment of spatial abilities. Largely, these tests can be separated into tests of spatial perception, namely the ability to determine spatial relations despite distracting information; spatial visualization, which is the ability to manipulate complex, multistep spatial information; and mental rotation, which is the ability to rotate two- or three-dimensional figures in one's mind (13). The present study was designed to investigate the effects of acute insulin-induced hypoglycemia on spatial abilities in adults with type 1 diabetes, using a well-characterized battery of spatial tests that incorporate all of these components of spatial cognition.

RESEARCH DESIGN AND METHODS

— Sixteen adults with type 1 diabetes (seven male and nine female) participated in the study. Subjects were recruited from the diabetes clinic at the Royal Infirmary of Edinburgh. Baseline demographic characteristics were a median age of 28 years (interquartile range 25–37.5 years), median duration of diabetes 10 years (4.2–19 years), BMI (means ± SD) 26.4 ± 4.01 kg/m^2, and A1C 7.91 ± 0.92%. A1C was measured by high-performance liquid chromatography (nondiabetic reference range 5.0–6.05%; Bio-Rad Laboratories, Munich, Germany) and was Diabetes Control and Complications Trial–aligned. The subjects had no history of hypertension or macrovascular disease, and microvascu-

From the [1]Department of Diabetes, Royal Infirmary of Edinburgh, Edinburgh, U.K.; the [2]Centre for Cognitive Ageing and Cognitive Epidemiology, University of Edinburgh, Edinburgh, U.K.; and the [3]Department of Psychology, University of Edinburgh, Edinburgh, U.K.
Corresponding author: Ian J. Deary, i.j.deary@ed.ac.uk.
Received 4 February 2009 and accepted 30 April 2009.
Published ahead of print at http://care.diabetesjournals.org on 1 June 2009. DOI: 10.2337/dc09-0212.

lar disease was excluded before recruitment. The presence of retinopathy was sought using digital retinal photography, neuropathy was assessed by clinical examination, and nephropathy was identified by the presence of microalbuminuria. Subjects were excluded if they had a history of impaired awareness of hypoglycemia or a history of a previous severe reaction to hypoglycemia. None of the participants had a history of head injury, seizure, blackouts, alcohol or drug abuse, or psychiatric illness. Subjects were not taking any medications other than insulin or the oral contraceptive pill. All subjects gave written informed consent before participating in the study, which had been approved by the local research ethics committee.

Each subject underwent two laboratory sessions, separated by at least 2 weeks. The study was conducted at the Clinical Research Facility at the Royal Infirmary of Edinburgh. A modified hyperinsulinemic glucose clamp (14) was used to maintain blood glucose at a predetermined level: euglycemia at 4.5 mmol/l (81 mg/dl) and hypoglycemia at 2.5 mmol/l (45 mg/dl). Each subject underwent a euglycemia study and a hypoglycemia study in a randomized, counterbalanced fashion. The subjects were blinded to the experimental condition.

Study procedure

The experimental session began at 0830 h. All subjects monitored their blood glucose with care for the preceding 48 h, including bedtime testing, and the study was postponed if they had any blood glucose value <3.5 mmol/l or any symptoms suggestive of hypoglycemia. After an overnight fast the subjects omitted their morning insulin dose. A retrograde intravenous cannula for regular blood glucose sampling was inserted into the nondominant hand and was placed in a heated blanket to arterialize the venous blood (15). A further cannula in the nondominant antecubital fossa was used to infuse soluble insulin (Human Actrapid; Novo Nordisk Pharmaceuticals, Crawley, U.K.) and 20% dextrose. Insulin was infused at a constant rate of 1.5 mU · kg^{-1} · min^{-1} using a Gemini PCI pump (Alaris Medical Systems, San Diego, CA). Dextrose (20%) was infused at a rate that varied according to the arterialized blood glucose concentration, which was measured at 5-min intervals using the glucose oxidase method (2300 Stat; YSI, Yellow Springs, OH).

On each study day, the arterialized blood glucose was initially stabilized at 4.5 mmol/l for a period of 30 min. It was then either maintained at that level throughout the study (euglycemia condition) or it was lowered over 20 min to 2.5 mmol/l and maintained at that level for the duration of the study (hypoglycemia condition). The experimental period lasted for 60 min, after which time the blood glucose concentration was restored to 4.5 mmol/l. Subjects were given a meal after completion of each study.

Cognitive function tests

Tests of spatial ability were drawn from the French and Ekstrom Kit of Factor-Referenced Cognitive Tests (16,17). In addition, the Digit Symbol Substitution Test and Trail Making B Test were administered to confirm the recognized effect of hypoglycemia on cognitive function, as described previously (7–10).

Spatial ability tests

Hidden patterns test. The Hidden Patterns Test requires subjects to identify a figure that is hidden among other lines. The figure is the same throughout, with the same orientation, and subjects have 3 min to correctly identify as many of the patterns in which the figure is concealed as possible.

Card rotations test. The Card Rotations Test requires the subject to look closely at a shape on the left-hand side of a page and then assess whether the eight shapes on the right-hand side are the same shape rotated through a variable number of degrees or whether the shapes are different and have in fact been reversed or are a mirror image of the initial shape. Three minutes are allowed to complete as many items as possible.

Cube comparisons test. This test involves pairs of cubes, such as the wooden building blocks played with by children, with a letter or shape on each facet of the cube. Subjects have 3 min to analyze as many pairs of cubes as possible and must determine whether the two cubes could be the same cube viewed from different sides or whether they must be different cubes if the letters on the sides did not correspond with each other had the cube been turned over.

Paper folding test. The Paper Folding Test involves showing participants a sequence of folds in a piece of paper, through which a set of holes is then punched. The participants must choose which of a set of punched and unfolded papers corresponds to the one they have just seen.

Map memory test. This is a test of the subject's ability to remember the position of buildings on a street map. Four minutes are permitted to memorize the map and then a further 4 min to place the buildings correctly on a blank version of the map.

Maze tracing test. This is a test of the subject's ability to find a path through a maze quickly. A pencil line must be drawn through the maze without crossing any of the "walls." The maze is broken down into blocks, and the score is the number of blocks that are successfully navigated in 3 min.

Other cognitive function tests

Digit symbol substitution test. This test is from the Wechsler Adult Intelligence Scale-III and assesses the ability of the subject to perform coding as quickly as possible. The subject is given a key of numbers 1–9, which each have a corresponding symbol. They must then fill in as many symbols as possible for a list of numbers in 120 s.

Trail making B test. The Trail Making B Test is a computerized version of the test and similar in principle to the classic test from the Halstead Reitan battery. It is used to assess complex visual processing and also assesses motor function with regard to visual motor tracking. It is performed on a handheld computer. The subject is presented with a grid containing letters and numbers in a random order and must connect the numbers and letters in numerical and alphabetical order, alternating the number with the letter in the fashion "1-A-2-B-3-C . . ." etc.

Hypoglycemia symptom score. The Edinburgh Hypoglycemia Scale was used to assess the symptoms experienced by subjects during each experimental session. It is a validated self-rating questionnaire comprising a list of common symptoms of hypoglycemia that can be classified into autonomic, neuroglycopenic, and nonspecific symptoms. Each symptom is scored on a Likert scale from 1 (not present) to 7 (intensely present) (18).

Statistical analysis

Results were analyzed using SPSS (version 15.0 for Windows; SPSS, Chicago, IL). A general linear model (repeated-measures ANOVA) was used, with order of session (euglycemia-hypoglycemia or hypoglycemia-euglycemia) as a between-subjects factor and condition (euglycemia

Table 1—*Spatial ability test scores*

Spatial test	Euglycemia score	Hypoglycemia score	*P*	Cohen's *d*	η_p^2
Hidden Patterns	94.5 ± 21.8	73.7 ± 21.0	<0.001	0.97	0.627
Card Rotations	51.9 ± 15.5	40.4 ± 18.7	0.001	0.67	0.580
Cube Comparison	11.7 ± 4.1	9.4 ± 5.7	0.03	0.46	0.298
Paper Folding	6.0 ± 1.9	4.7 ± 2.0	0.001	0.67	0.604
Map Memory	8.6 ± 3.1	7.8 ± 2.1	0.3	0.30	0.081
Maze Tracing	11.1 ± 3.0	9.4 ± 2.5	<0.001	0.62	0.621

Data are means ± SD. Significance level was $P < 0.05$; effect sizes were computed as Cohen's *d* and η_p^2.

or hypoglycemia) as a within-subjects factor. $P < 0.05$ was considered to be significant. Effect sizes were calculated using η_p^2 to assess the degree to which hypoglycemia accounts for the variance in results, and Cohen's *d* was used to establish the extent of any effects of hypoglycemia on spatial abilities. Results are expressed as means ± SD unless stated otherwise.

RESULTS

Blood glucose

The target blood glucose levels were achieved for each experimental condition. The blood glucose concentration achieved during the hypoglycemia condition was 2.46 ± 0.22 mmol/l and during the euglycemia condition was 4.53 ± 0.24 mmol/l.

Symptom scores

Significant increments occurred in total autonomic ($P < 0.001$), total neuroglycopenic ($P < 0.001$), and malaise symptom scores (<0.001) during hypoglycemia.

General cognitive function

In the present study, scores achieved for the Digit Symbol Substitution Test were significantly lower during the hypoglycemia study period (72.4 ± 20.2) compared with those during euglycemia (84.6 ± 20.7) ($P < 0.001$), confirming that a standard measure of speed of information processing was significantly impaired at blood glucose concentrations of 2.5 mmol/l. Performance on the Trail Making B Test was statistically not impaired by hypoglycemia, with a score of 50.4 ± 20.9 s during hypoglycemia and a score of 38.9 ± 11.5 s during euglycemia ($P = 0.07$).

Spatial ability

Hypoglycemia resulted in a significantly lower score on all of the spatial ability tests except the Map Memory Test (Table 1). Cohen's *d* results have shown that the impact of hypoglycemia on these spatial abilities was medium to large. Moreover, the η_p^2 values indicate that the hypoglycemia condition accounted for a large proportion of the variance in the results (Table 1). No significant effects were observed of order of exposure to glycemic condition or test battery.

CONCLUSIONS— Acute, insulin-induced hypoglycemia causes significant decrements in most spatial cognitive abilities examined here in a group of adults with uncomplicated type 1 diabetes. This impairment of function was accompanied by a deterioration in speed of mental processing as demonstrated by the decrement in score for the Digit Symbol Substitution Test. The effect sizes obtained indicate the development of medium to large decrements in spatial abilities during hypoglycemia in adults with type 1 diabetes.

The present study examined a group of subjects with type 1 diabetes and did not include a control group of nondiabetic subjects. Although this is a limitation of the present study, in reality it is the everyday effect of hypoglycemia on this group of individuals that is of clinical importance.

Other studies assessing the effects of hypoglycemia on aspects of cognitive function have used tests that require a spatial ability component (12), but to our knowledge no previous study has used a test battery specifically examining spatial abilities, although it has clear importance in the safe conduct of tasks such as driving, which rely heavily upon the interpretation of the surrounding environment.

The Map Memory Test was not affected significantly by the glycemic condition. This test assesses both spatial ability and visual memory. This finding is consistent with previous studies that examined memory function using visual memory tests from the Wechsler Adult Intelligence Scale, which also showed that visual memory is preserved during acute hypoglycemia (19). It is also notable that the Map Memory Test, unlike the other tests used here, does not have multiple items, and so its scores may be more idiosyncratic.

Spatial ability relies on cerebral pathways that predominantly involve the right cerebral hemisphere, particularly the parietal lobe. The frontal cortex, thalamus, and, to some extent, the cerebellum are also involved in the coordination of spatial cognition (20,21). Neuroimaging studies during hypoglycemia have shown attenuation of functional response, e.g., blood oxygenation level–dependent activation, in the premotor and supplementary motor cortex, consistent with recognized areas of importance in spatial functioning (22). In addition, it has been shown previously that general fluid intelligence is impaired during hypoglycemia, and it is fluid intelligence rather than crystallized intelligence that is responsible for spatial cognition (10).

In summary, the present study has shown that acute hypoglycemia has an adverse effect on spatial abilities. These novel data are important for two reasons. First, with regard to our understanding of the domains of cognitive function that experience decrements during hypoglycemia, spatial abilities were a lacuna that has now been partly filled. Second, spatial abilities are relevant to the everyday activities of individuals with type 1 diabetes, and there are now data to show that part of the inability to manage complex tasks during hypoglycemia is the inability to efficiently carry out spatial cognitive operations.

Acknowledgments— This work was supported by the Biotechnology and Biological Sciences Research Council, the Engineering and Physical Sciences Research Council, the Economic and Social Research Council, and the Medical Research Council. B.M.F. and I.J.D. are members of The University of Edinburgh Centre for Cognitive Ageing and Cognitive Epidemiology, part of the cross-council Lifelong Health and Wellbeing Initiative.

No potential conflicts of interest relevant to this article were reported.

References

1. The Diabetes Control and Complications Trial Research Group. The effect of intensive insulin treatment of diabetes on the

development and progression of long term complications. N Engl J Med 1993; 329:977–986
2. Deary IJ. Symptoms of hypoglycaemia and effects on mental performance and emotions. In *Hypoglycaemia in Clinical Diabetes.* 2nd ed. Frier BM, Fisher M, Eds. Chichester, U.K., John Wiley & Sons, 2007, p. 25–48
3. Hoffman RG, Speelman DJ, Hinnen DA, Conley KL, Guthrie RA, Knapp RK. Changes in cortical functioning with acute hypoglycemia and hyperglycemia in type 1 diabetes. Diabetes Care 1989;12: 193–197
4. Mitrakou A, Ryan C, Veneman T, Mokan M, Jenssen T, Kiss I, Durrant J, Cryer P, Gerich J. Hierarchy of glycemic thresholds for counterregulatory hormone secretion, symptoms, and cerebral dysfunction. Am J Physiol 1991;260:E67–E74
5. Widom B, Simonson DJ. Glycemic control and neuropsychologic function during hypoglycemia in patients with insulin-dependent diabetes. Ann Intern Med 1990;112:904–912
6. Wirsen A, Tallroth G, Lindgren M, Agardh C. Neuropsychological performance differs between type 1 diabetic and normal men during insulin-induced hypoglycaemia. Diabet Med 1992;9:156–165
7. McAulay V, Ferguson SC, Deary IJ, Frier BM. Acute hypoglycemia in humans causes attentional dysfunction while nonverbal intelligence is preserved. Diabetes Care 2001;24:1745–1750
8. Sommerfield AJ, Deary IJ, McAulay V, Frier BM. Moderate hypoglycemia impairs multiple memory functions in healthy adults. Neuropsychology 2003; 17:125–132
9. Sommerfield AJ, Deary IJ, McAulay V, Frier BM. Short-term, delayed, and working memory are impaired during hypoglycemia in individuals with type 1 diabetes. Diabetes Care 2003;26:390–396
10. Warren RE, Allen KA, Sommerfield AJ, Deary IJ, Frier BM. Acute hypoglycemia impairs non-verbal intelligence. Diabetes Care 2004;27:1447–1448
11. Zammitt NN, Warren RE, Deary IJ, Frier BM. Delayed recovery of cognitive function following hypoglycemia in adults with type 1 diabetes: effect of impaired awareness of hypoglycemia. Diabetes 2008;57:732–736
12. Geddes J, Deary IJ, Frier BM. Effects of acute insulin-induced hypoglycaemia on psychomotor function: people with type 1 diabetes are less affected than non-diabetic adults. Diabetologia 2008;51: 1814–1821
13. Linn MC, Petersen AC. Emergence and characterisation of gender differences in spatial abilities: a meta-analysis. Child Dev 1985;56:1479–1498
14. De Fronzo R, Tobin JD, Andres R. Glucose clamp technique: a method for quantifying insulin secretion and resistance. Am J Physiol 1979;273:E214–E223
15. Abumrad NN, Rabin D, Diamond MP, Lacy WW. Use of a heated superficial hand vein as an alternative site for the measurement of amino acid concentrations and for the study of glucose and alanine kinetics in man. Metabolism 1981; 30:936–940
16. Ekstrom RB, French JW, Harman HH, Dermen D. *Kit of Factor-Referenced Cognitive Tests.* Princeton, NJ: Educational Testing Service, 1976
17. Ekstrom RB, French JW, Harman HH. Cognitive factors: their identification and replication. Multivariate Behav Res Monogr 1979;79:3–84.
18. Gold AE, MacLeod KM, Frier BM. Frequency of severe hypoglycemia in patients with type 1 diabetes with impaired awareness of hypoglycemia. Diabetes Care 1994;17:697–703
19. Warren RE, Zammitt NN, Deary IJ, Frier BM. The effects of acute hypoglycaemia on memory acquisition and recall and prospective memory in type 1 diabetes. Diabetologia 2007;50:178–185
20. Harris IM, Egan GF, Sonkkila C, Tochon-Danguy HJ, Paxinos G, Watson JDG. Selective right parietal lobe activation during mental rotation. Brain 2000;123: 65–73
21. Vogel JJ, Bowers CA, Vogel DS. Cerebral lateralization of spatial abilities: a meta-analysis. Brain Cogn 2003;52:197-204
22. Rosenthal JM, Amiel SA, Yaguez L, Bullmore E, Hopkins D, Evans M, Pernet A, Reid H, Giampetro V, Andrew CM, Suckling J, Simmons A, Williams SCR. The effect of acute hypoglycemia on brain function and activation: a functional magnetic resonance imaging study. Diabetes 2001;50:1618–1626

DIABETICMedicine

DOI: 10.1111/j.1464-5491.2007.02199.x

Short Report

Plasma endothelin response to acute hypoglycaemia in adults with Type 1 diabetes

R. J. Wright, K. M. Macleod, P. Perros, N. Johnston*, D. J. Webb* and B. M. Frier

Department of Diabetes, Royal Infirmary of Edinburgh and *Clinical Pharmacology Unit, Queen's Medical Research Institute, Royal Infirmary of Edinburgh, UK

Accepted 1 March 2007

Abstract

Aims To determine whether acute insulin-induced hypoglycaemia provokes a detectable alteration in peripheral plasma endothelin (ET) concentrations in humans with Type 1 diabetes.

Methods Serial plasma concentrations of ET were measured in 20 patients with Type 1 diabetes during controlled hypoglycaemia induced by intravenous infusion of soluble insulin.

Results A significant increase was observed in plasma ET concentrations, from 3.80 ± 0.31 pg/ml at baseline to 6.72 ± 1.47 pg/ml at 60 min after the onset of the hypoglycaemic reaction ($P < 0.05$).

Conclusions Acute insulin-induced hypoglycaemia induces a rise in plasma endothelin concentrations in people with Type 1 diabetes. This finding is consistent with a putative role for ET in the mediation of hypoglycaemia-induced vasoconstriction, and the possible precipitation of macrovascular or microvascular events.

Diabet. Med. 24, 1039–1042 (2007)

Keywords adrenaline, endothelin, hypoglycaemia, insulin, Type 1 diabetes mellitus

Abbreviations ET, endothelin; R, autonomic reaction

Introduction

Acute hypoglycaemia induces haemodynamic, haemorrheological and haemostatic responses, secondary to sympatho-adrenal activation and counter-regulatory hormonal secretion [1]. The cardiovascular effects are transient in healthy young adults with no pathophysiological consequences, but people with diabetes who have vascular disease may be at risk of tissue ischaemia [1]. Myocardial and cerebral ischaemia, and acute vascular events, can be precipitated by hypoglycaemia [2,3], and worsening of diabetic retinopathy may occur when strict glycaemic control is implemented [4,5]. The possible mechanisms by which hypoglycaemia might exacerbate vascular disease include haemorrheological changes, platelet and neutrophil activation, vasoconstriction [1,4], and release of inflammatory mediators and cytokines [6–11].

The endothelins are potent vasoconstrictors that have a central role in cardiovascular regulation. The endothelium-derived peptide, endothelin-1 (ET-1), is the principal isoform in humans, causes prolonged vasoconstriction, and promotes smooth muscle proliferation [12]. Endothelins have been implicated in the pathogenesis of several disorders, including atherosclerosis, diabetes and hypertension [13–15]. Plasma endothelin production is stimulated by hypoxia, ischaemia and adrenaline release, amongst other factors [12,15]. Plasma levels are elevated in people with uncomplicated Type 1 and Type 2 diabetes [16], increase with the presence of microalbuminuria and retinopathy [17], and may have a pathogenetic role in microangiopathy [15]. Changes in plasma endothelin during acute insulin-induced hypoglycaemia were examined in adults with Type 1 diabetes, with the hypothesis that an increment in endothelin concentration would be observed.

Patients and methods

Twenty subjects with Type 1 diabetes were studied. Subject characteristics are presented in Table 1. All were receiving short- and intermediate-acting insulins in combination, or as multiple-injection therapy. They were taking no medications other than insulin, and had no other medical disorders. Subjects were screened for retinopathy using ophthalmoscopy, peripheral

Correspondence to: Professor B. M. Frier, Department of Diabetes, Royal Infirmary of Edinburgh, 51 Little France Crescent, Edinburgh EH16 4SA, UK. E-mail: brian.frier@luht.scot.nhs.uk

Table 1 Characteristics of participants with Type 1 diabetes

Age [median (range)] (years)	25.5 (19–42)
Sex (male : female)	19 : 1
Duration of diabetes [median (range)] (years)	2 (1–26)
Body mass index (kg/m^2)	24.6 ± 2.8
Insulin dose (units/kg)	0.6 ± 0.3
Systolic blood pressure (mmHg)	124 ± 8
Diastolic blood pressure (mmHg)	74 ± 18
HbA_{1c} (%)	7.8 ± 2.4

Results are expressed as mean ± SEM unless stated otherwise. Non-diabetic range for glycated haemoglobin (HbA_{1c}) is 5.0–6.5% (measured by ion exchange high-performance liquid chromatography).

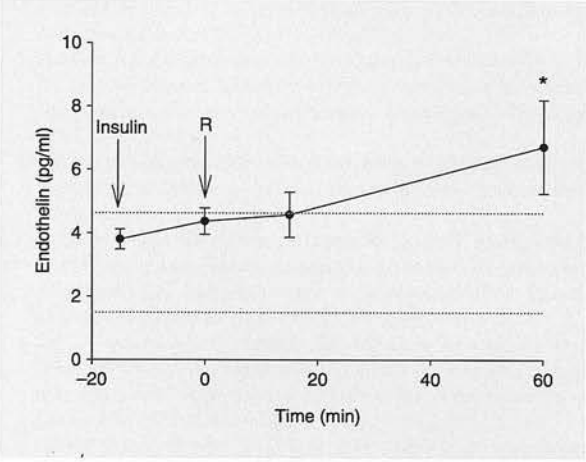


FIGURE 1 Plasma endothelin concentrations in response to acute insulin-induced hypoglycaemia in people with Type 1 diabetes. Results are shown as mean ± SEM. Grey dotted lines indicate normal range (1.5–4.5 pg/ml). R, autonomic reaction. *$P < 0.05$.

neuropathy by clinical examination, and autonomic neuropathy using a standard battery of cardiovascular reflexes [18]. None had any clinical evidence of microangiopathy. The local medical research ethics committee granted approval, and written informed consent was obtained from all subjects.

After an overnight fast, hypoglycaemia was induced with a continuous intravenous infusion of soluble insulin at a rate of 2.0 mU/kg/min in 0.15 mmol/l saline, through an indwelling venous cannula in the antecubital fossa. The insulin infusion was continued until the onset of symptoms of hypoglycaemia, which was usually coincidental with objective evidence of an acute autonomic reaction (R). This was identified by a rapid increase in heart rate, a rise in systolic blood pressure, and the onset of sweating and finger tremor, as described previously [19]. This insulin infusion method produces a gradual, predictable and controlled decline in blood glucose to induce hypoglycaemia and simulates the development of hypoglycaemia in everyday life. It enables the glycaemic threshold for the acute autonomic activation to be identified objectively [20]. Blood sampling was timed subsequently in relation to R, the onset of which differs between individuals [19].

Arterialized venous blood was collected at 5-min intervals for measurement of whole blood glucose concentration using a Yellow Springs Analyser (Yellow Springs, OH, USA). Blood samples were cold centrifuged, the plasma separated and flash-frozen, and stored at –70°C for measurement of immunoreactive ET by radioimmunoassay (ITS Production BV). The sensitivity of this assay is 2 pg/ml immunoreactive ET. Cross-reactivity of the assay with ET-1, ET-2, ET-3 and big ET-1 is 100, 52, 96 and 7%, respectively. Plasma ET concentrations were measured at baseline, at the onset of the hypoglycaemic reaction, and at 15 and 60 min following R.

Mean endothelin concentrations were compared by paired *t*-test using SPSS version 12.0 for Windows (SAS Institute, Cary, NC, USA).

Results

Plasma glucose concentration declined steadily from a mean basal concentration of 5.2 ± 0.2 mmol/l (mean ± SEM), until the symptomatic hypoglycaemic reaction (R) occurred at the nadir of 1.9 ± 0.6 mmol/l. The mean (± SEM) ET concentration rose from 3.80 ± 0.31 pg/ml at baseline to a peak of 6.72 ± 1.47 pg/ml at R + 60 min ($P < 0.05$; Fig. 1). The different values at R and R + 15 min did not achieve significance. The observed increase was wide, ranging from 0.11 pg/ml to 20.18 pg/ml.

The temporal pattern of the rise in plasma endothelin response coincided with the rise in plasma adrenaline, cortisol and growth hormone (results not shown), but did not correlate significantly with any single variable measured. Haemodynamic changes (blood pressure and heart rate) returned to normal by R + 15 min (not shown).

Discussion

The present study has shown that a significant rise in plasma endothelin occurs in response to insulin-induced hypoglycaemia in adults with Type 1 diabetes.

Although there is strong evidence to support a role for ET in the pathogenesis of diseases affecting the cardiovascular system, its influence in diabetes remains undefined, and its reliability as a marker of endothelial function in people with diabetes is debatable. The significance of the current findings is therefore uncertain. *In vitro* studies have shown that insulin [21], vasopressin and adrenaline [17] stimulate ET production from endothelial cells. In animal studies, the urinary excretion of ET rises following the development of diabetes and may represent endothelial damage induced by the diabetic state [22]. A regulatory role of insulin on ET-1 concentration has been suggested in patients with Type 2 diabetes in whom a rise in plasma ET concentrations occurs during a hyperinsulinaemic glucose clamp [23], and plasma ET and insulin concentrations are correlated closely in obese non-diabetic men [24]. The diabetic state in general [16], and diabetic complications in particular [16,25], are associated with an elevated concentration

of plasma endothelin. It is possible that the observed rise in plasma endothelin during hypoglycaemia could exacerbate ischaemia through its vasoconstrictive effects in a vasculature that is already compromised by micro- or macrovascular disease, and thereby enhance the risk of promoting an acute macrovascular event and possibly aggravate existing microvascular disease [1,4]. It is also plausible that endothelin-induced vasoconstriction may serve as a defence mechanism to protect vital organs such as the brain, and also to increase delivery of gluconeogenic precursors to the liver. Total cerebral blood flow increases at blood glucose concentrations below 2.0 mmol/l and the regional distribution of blood flow within the brain alters to provide glucose to the areas most vulnerable to neuroglycopenia, such as the cortex and the basal ganglia [26,27]. Total splanchnic blood flow is increased [28] (while splenic blood flow is reduced [29]), with a relative rise in hepatic blood flow, as mechanisms which should promote the production of glucose in the liver.

These results permit speculation as to the possible role of endothelin in the pathogenesis of hypoglycaemia-induced vascular injury, or regional blood flow alterations, but do not provide definitive conclusions. One limitation of the current study is the absence of a control group of non-diabetic volunteers to allow the endothelin responses to be compared, and examine whether the response in healthy control subjects is similar to that observed in people with diabetes. The mechanism inducing the rise in plasma endothelin is not known. It may have been provoked by insulin per se, or it may have been secondary to the hormonal response to acute hypoglycaemia [1], in the form of elevated adrenaline or vasopressin concentrations [30]. The increased sympathetic nervous system activity, through haemodynamic effects, may increase shear stress on blood vessels and consequently trigger the release of endothelin. Alternatively, endothelin release may be a direct consequence of neuroglycopenia.

Despite the limitations of this study, in response to hypoglycaemia a significant rise has been demonstrated of a peptide with potent vasoconstrictive properties, in a group of patients who are at high risk of developing vascular complications. The role of endothelin in this situation has yet to be explored.

Competing interests

None to declare.

References

1 Fisher BM, Frier BM. Effect on vascular disease. In: Frier, BM, Fisher, BM, eds. *Hypoglycaemia and Diabetes: Clinical and Physiological Aspects*. London: Edward Arnold, 1993: 355–361.

2 Desouza C, Salazar H, Cheong B, Murgo J, Fonseca V. Association of hypoglycemia and cardiac ischemia. *Diabetes Care* 2003; **26**: 1485–1489.

3 Fisher BM, Heller S. Mortality, cardiovascular morbidity and possible effects of hypoglycaemia on diabetic complications. In: Frier, B M, Fisher, B M, eds. *Hypoglycaemia in Clinical Diabetes*, 1st edn. Chichester: John Wiley and Sons, 1999: 167–186.

4 Frier BM, Hilsted J. Does hypoglycaemia aggravate the complications of diabetes? *Lancet* 1985; **326**: 1175–1177.

5 Hanssen KF, Dahl-Jorgensen K, Lauritzen T, Feldt-Rasmussen B, Brinchmann-Hansen O, Deckert T. Diabetic control and microvascular complications. The near-normoglycaemic experience. *Diabetologia* 1986; **29**: 677–684.

6 Galloway PJ, Thomson GA, Fisher BM, Semple CG. Insulin-induced hypoglycemia induces a rise in C-reactive protein. *Diabetes Care* 2000; **23**: 861.

7 Fisher BM, Quin JD, Rumley A, Lennie SE, Small M, MacCuish AC *et al.* Effects of acute insulin-induced hypoglycaemia on haemostasis, fibrinolysis and haemorrheology in insulin-dependent diabetic patients and control subjects. *Clin Sci* 1991; **80**: 525–531.

8 Collier A, Patrick AW, Hepburn DA, Bell D, Jackson M, Dawes J, Frier BM. Leucocyte mobilization and release of neutrophil elastase following acute insulin-induced hypoglycaemia in normal humans. *Diabet Med* 1990; **7**: 506–509.

9 Corrall RJM, Webber RG, Frier BM. Increase in coagulation factor VIII activity in man following acute hypoglycaemia: mediation via an adrenergic mechanism. *Br J Haematol* 1980; **44**: 301–305.

10 Wieczorek I, Pell AC, McIver B, MacGregor IR, Ludlam CA, Frier BM. Coagulation and fibrinolytic systems in Type 1 diabetes: effects of venous occlusion and insulin-induced hypoglycaemia. *Clin Sci* 1993; **84**: 79–86.

11 Frier BM, Corrall RJM, Davidson NM, Webber RG, Dewar A, French EB. Peripheral blood cell changes in response to acute hypoglycaemia in man. *Eur J Clin Invest* 1983; **13**: 33–39.

12 Gossl M, Lerman A. Endothelin: beyond a vasoconstrictor. *Circulation* 2006; **113**: 1156–1158.

13 Hopfner RL, Gopalakrishnan V. Endothelin: emerging role in diabetic vascular complications. *Diabetologia* 1999; **42**: 1383–1394.

14 Luscher TF, Barton M. Endothelins and endothelin receptor antagonists. *Circulation* 2000; **102**: 2434–2440.

15 Dhaun N, Goddard J, Webb DJ. The endothelin system and its antagonism in chronic kidney disease. *J Am Soc Nephrol* 2006; **17**: 943–955.

16 Takahashi K, Ghatei MA, Lam HC, O'Halloran J, Bloom SR. Elevated plasma endothelin in patients with diabetes mellitus. *Diabetologia* 1990; **33**: 306–310.

17 Collier A, Leach JP, McLellan A, Jardine A, Morton JJ, Small M. Plasma endothelin-like immunoreactivity levels in IDDM patients with microalbuminuria. *Diabetes Care* 1992; **15**: 1038–1040.

18 Ewing DJ, Martyn CN, Young RJ, Clarke BF. The value of cardiovascular autonomic function tests: 10 years experience in diabetes. *Diabetes Care* 1985; **8**: 491–498.

19 Hepburn DA, Patrick AW, Brash HM, Thomson I, Frier BM. Hypoglycaemia unawareness in Type 1 diabetes: a lower plasma glucose is required to stimulate sympathoadrenal activation. *Diabet Med* 1991; **8**: 934–945.

20 Hepburn DA, MacLeod KM, Frier BM. Physiological, symptomatic and hormonal responses to acute hypoglycaemia in Type 1 diabetic patients with autonomic neuropathy. *Diabet Med* 1993; **10**: 940–949.

21 Hu R, Levin ER, Pedram A, Frank HJL. Insulin stimulates production and secretion of endothelin from bovine endothelial cells. *Diabetes* 1993; **42**: 351–358.

22 Morabito E, Corsico N, Martelli EA. Endothelins urinary excretion is increased in spontaneously diabetic rats BB/BB. *Life Sci* 1994; **56**: 13–18.

23 Ferri C, Carlomagno A, Coassin S, Baldoncini R, Cassone Faldetta MR, Laurenti O *et al.* Circulating endothelin-1 levels increase during euglycemic hyperinsulinemic clamp in lean NIDDM men. *Diabetes Care* 1995; **18**: 226–233.

24 Ferri C, Bellini C, Desideri G, Di Francesco L, Baldoncini R, Santucci A *et al.* Plasma endothelin-1 levels in obese hypertensive and normotensive men. *Diabetes* 1995; **44**: 431–436.

25 Morise T, Takeuchi Y, Kawano M, Koni I, Takeda R. Increased plasma levels of immunoreactive endothelin and von Willebrand factor in NIDDM patients. *Diabetes Care* 1995; **18**: 87–89.
26 Bryan RM. Cerebral blood flow and energy metabolism during stress. *Am J Physiol* 1990; **259**: H269–H280.
27 MacLeod KM, Hepburn DA, Deary IJ, Goodwin GM, Dougall N, Ebmeier KP *et al.* Regional cerebral blood flow in IDDM patients: effects of diabetes and of recurrent severe hypoglycaemia. *Diabetologia* 1994; **37**: 257–263.
28 Bearn AG, Billing BH, Sherlock S. The response of the liver to insulin in normal subjects and in diabetes mellitus: hepatic vein catheterisation studies. *Clin Sci* 1952; **11**: 151–164.
29 Braatvedt GD, Flynn MD, Stanners A, Halliwell M, Corrall RJM. Splanchnic blood flow in man: evidence for mediation via a beta-adrenergic mechanism. *Clin Sci* 1993; **84**: 201–207.
30 Attinà T, Camidge R, Newby DE, Webb DJ. Endothelin antagonism in pulmonary hypertension, heart failure, and beyond. *Heart* 2005; **91**: 825–831.

Effects of Acute Insulin-Induced Hypoglycemia on Indices of Inflammation

Putative mechanism for aggravating vascular disease in diabetes

ROHANA J. WRIGHT, MRCP[1]
DAVID E. NEWBY, PHD[2]
DAVID STIRLING, PHD[3]
CHRISTOPHER A. LUDLAM, PHD[3]
IAN A. MACDONALD, PHD[4]
BRIAN M. FRIER, MD[1]

OBJECTIVE — To examine the effects of acute insulin-induced hypoglycemia on inflammation, endothelial dysfunction, and platelet activation in adults with and without type 1 diabetes.

RESEARCH DESIGN AND METHODS — We studied 16 nondiabetic adults and 16 subjects with type 1 diabetes during euglycemia (blood glucose 4.5 mmol/l) and hypoglycemia (blood glucose 2.5 mmol/l). Markers of inflammation, thrombosis, and endothelial dysfunction (soluble P-selectin, interleukin-6, von Willebrand factor [vWF], tissue plasminogen activator [tPA], high-sensitivity C-reactive protein [hsCRP], and soluble CD40 ligand [sCD40L]) were measured; platelet-monocyte aggregation and CD40 expression on monocytes were determined using flow cytometry.

RESULTS — In nondiabetic participants, platelet activation occurred after hypoglycemia, with increments in platelet-monocyte aggregation and P-selectin ($P \leq 0.02$). Inflammation was triggered with CD40 expression increasing maximally at 24 h (3.13 ± 2.3% vs. 2.06 ± 1.0%) after hypoglycemia ($P = 0.009$). Both sCD40L and hsCRP ($P = 0.02$) increased with a nonsignificant rise in vWF and tPA, indicating a possible endothelial effect. A reduction in sCD40L, tPA, and P-selectin occurred during euglycemia ($P = 0.03$, $P \leq 0.006$, and $P = 0.006$, respectively). In type 1 diabetes, both CD40 expression (5.54 ± 4.4% vs. 3.65 ± 1.8%; $P = 0.006$) and plasma sCD40L concentrations increased during hypoglycemia (peak 3.41 ± 3.2 vs. 2.85 ± 2.8 ng/ml; $P = 0.03$). Platelet-monocyte aggregation also increased significantly at 24 h after hypoglycemia ($P = 0.03$). A decline in vWF and P-selectin occurred during euglycemia ($P \leq 0.04$).

CONCLUSIONS — Acute hypoglycemia may provoke upregulation and release of vasoactive substances in adults with and without type 1 diabetes. This may be a putative mechanism for hypoglycemia-induced vascular injury.

Diabetes Care 33:1591–1597, 2010

In people with type 1 diabetes the rapid institution of strict glycemic control aggravates microvascular complications, particularly retinopathy (1). Although attributed to reduced capillary blood flow causing localized ischemia (1), greater exposure to hypoglycemia may have worsened microangiopathy through its putative effects on local vasculature (2). In addition, cardiovascular stress associated with hypoglycemia may precipitate acute macrovascular events in a diseased circulation. While supported by anecdotal reports (3), the increase in cardiovascular mortality in people with type 2 diabetes in the Action to Control Cardiovascular Risk in Diabetes (ACCORD) trial (4) (and possibly in the Veterans Affairs Diabetes Trial [5]), in which intensive treatment had tripled the frequency of severe hypoglycemia, has caused concern.

Possible mechanisms by which hypoglycemia may damage blood vessels include changes in regional blood flow, mobilization and activation of neutrophils, platelet activation, and enhanced coagulation and viscosity of the blood (3,6–8). Plasma concentrations of C-reactive protein, interleukin-6 (IL-6), and endothelin-1 increase during hypoglycemia (9–11) and may promote vascular disease (12).

Investigation of processes operating at a cellular level to cause atherosclerosis has focused on the potential influences of vascular inflammation, endothelial dysfunction, coagulation, and platelet activation. The present study sought to determine the effects of acute insulin-induced hypoglycemia on inflammation, coagulation, and platelet and monocyte function in adults with and without type 1 diabetes.

RESEARCH DESIGN AND METHODS

— Participants in the study included 16 nondiabetic adult volunteers with no medical history and 16 healthy adults with type 1 diabetes (Table 1). Those with diabetes had no history of hypertension or macrovascular disease, and microvascular disease was excluded. Screening for retinopathy used digital retinal photography, absence of neuropathy was confirmed by clinical examination, and nephropathy was excluded by the absence of microalbuminuria. Subjects with a history of impaired awareness of hypoglycemia or a previous serious reaction to hypoglycemia were excluded. None had a history of head injury, seizure, blackouts, alcohol or drug abuse and psychiatric illness, and their only other medication was the contraceptive pill. Diabetes Control and Complications Trial–aligned A1C was measured using high performance liquid chromatography (nondiabetic reference range 5.0–6.05%; Bio-Rad Laboratories, Munich, Germany); the mean ± SD of the participants with diabetes was 7.91 ± 0.92%. All gave written informed consent before participation, and the study was approved by the Local Medical Research Ethics Committee.

From the [1]Department of Diabetes, Royal Infirmary of Edinburgh, Edinburgh, U.K.; the [2]Centre for Cardiovascular Science, University of Edinburgh, Edinburgh, U.K.; the [3]Department of Haematology, Royal Infirmary of Edinburgh, Edinburgh, U.K.; and the [4]School of Biomedical Sciences, University of Nottingham, Nottingham, U.K.
Corresponding author: Brian M. Frier, brian.frier@luht.scot.nhs.uk.
Received 4 January 2010 and accepted 22 March 2010. DOI: 10.2337/dc10-0013.

See accompanying original article, p. 1529, and editorial, p. 1686.

Table 1—*Baseline demographic characteristics*

	Nondiabetic subjects	Subjects with diabetes
n	16	16
Age (years)	28 (26.7–35)	28 (25–37.5)
BMI (kg/m^2)	22.86 ± 2.4	26.40 ± 4.0
Male/female	6/10	7/9
Duration of diabetes (years)	N/A	10 (4.2–19)
A1C (%)	N/A	7.91 ± 0.9

Data are median (interquartile range) and means ± SD unless otherwise indicated.

A modified hyperinsulinemic glucose clamp (13) was used to maintain blood glucose at a predetermined level: euglycemia at 4.5 mmol/l and hypoglycemia at 2.5 mmol/l. Each subject underwent two laboratory sessions, separated by at least 2 weeks (mean 7.2 weeks), of a euglycemic study and a hypoglycemic study in a randomized, counterbalanced fashion.

The participants with type 1 diabetes monitored blood glucose intensively during the 48 h preceding each study, which was postponed if any blood glucose value was <3.5 mmol/l or if symptoms suggestive of hypoglycemia were experienced. After fasting overnight, morning insulin was withheld. A retrograde-intravenous cannula for blood-glucose sampling was inserted into the nondominant hand, which was heated to arterialize the venous blood (14). A cannula in the nondominant antecubital fossa was used to infuse 20% dextrose and soluble insulin (Human Actrapid; Novo Nordisk, Crawley, U.K.) at a constant rate of 1.5 mU/kg/min using a Gemini PCI pump (Alaris Medical Systems, San Diego, CA). The dextrose was infused at a variable rate depending on arterialized blood glucose concentrations, which were measured at 5 min intervals using the glucose oxidase method (2300 Stat; Yellow Springs Instruments, Yellow Springs, OH). A third cannula in the other antecubital fossa was dedicated to blood sampling for inflammatory markers.

On each study day, the arterialized blood glucose was stabilized initially at 4.5 mmol/l for 30 min and either maintained at that level (euglycemia) or lowered over 20 min to 2.5 mmol/l for 60 min (hypoglycemia), after which blood glucose was restored to 4.5 mmol/l. Subjects consumed a standardized meal after each study. Blood sample time points were: baseline, during the experimental session (+45 min), during recovery (+105 min), at +6 h, and at +24 h.

Flow cytometry

Whole blood samples were collected at the predetermined time points using D-Phenylalanyl-L-prolyl-L-arginine chloromethyl ketone, a selective thrombin inhibitor, as an anticoagulant. Samples (100 μl) of whole blood were immediately incubated with 10 μl of each monoclonal antibody (AbD Serotec, Kidlington, U.K.) for 30 min at room temperature, with subsequent red cell lysis by the addition of 1 ml of fluorescent-activated cell sorter (FACS) Lyse solution (Becton Dickinson, Oxford, U.K.). Flow cytometry using the FACS Calibur system (Becton Dickinson, Oxford, U.K.) was performed immediately after the experimental session to assess platelet-monocyte aggregation (CD14/CD42a) and CD40 expression on monocytes (CD14/CD40). Isotype controls were performed in addition to both mono- and dual-stain for each parameter assessed at each time point.

Soluble marker assays

Citrated plasma and serum samples were collected at the predetermined time points. These were separated immediately and frozen at −80°C until analysis for the soluble markers:

Von Willebrand factor (vWF) (enzyme-linked immunosorbent assay [ELISA]; coefficient of variation [CV] 7.3%), tissue plasminogen activator (tPA) antigen (Hyphen Biomed Zymutest; intra-assay CV 3.5%, inter-assay CV 4.4%), soluble CD40 ligand (sCD40L) (high sensitivity ELISA, Bender Medsystems; intra-assay CV 5.5%, inter-assay CV 7.2%), soluble P-selectin (ELISA, R&D Systems; intra-assay CV 5.1%, inter-assay CV 8.8%), IL-6 (High sensitivity ELISA, R&D Systems; intra-assay CV 5.9%, inter-assay CV 9.9%), and high sensitivity CRP (DRG Diagnostics; DRG Instruments, Marburg, Germany; intra-assay CV 4.2%, inter-assay CV 4.1%).

Catecholamine assays

Samples for epinephrine quantification were collected in EDTA tubes and immediately separated and frozen at −80°C until analysis by high-performance liquid chromatography and electrochemical detection (intra-assay CV 1.2%, inter-assay CV 3.9%).

Hypoglycemia symptom score

The Edinburgh Hypoglycemia Scale (15) was used to assess the symptoms experienced during each experimental session.

Statistical analyses

Results were analyzed using SPSS version 15.0 for Windows (SPSS, Chicago, IL). A general linear model (repeated-measures ANOVA) was used, with order of session (euglycemia-hypoglycemia or hypoglycemia-euglycemia) as a between-subjects factor, and condition (euglycemia or hypoglycemia) as a within-subjects factor, to compare hypoglycemia with euglycemia. Additional analysis using paired *t* tests was performed to assess the change in any given parameter from baseline. A *P* value <0.05 was considered to be significant. Results are reported as mean ± SD unless otherwise stated.

RESULTS — Hypoglycemia provoked a symptomatic response in all subjects with increased scores of autonomic ($P \leq 0.002$), neuroglycopenic ($P < 0.001$), and malaise ($P \leq 0.008$) symptoms compared with baseline. Comparison of baseline levels of inflammatory, endothelial and platelet markers in nondiabetic subjects and subjects with type 1 diabetes showed a significantly higher concentration of soluble P-selectin ($P = 0.01$) and of CD40 expression on monocytes ($P = 0.006$) in those with diabetes, demonstrating the chronic inflammatory response associated with diabetes.

Blood glucose

Target blood glucose concentrations were achieved (Fig. 1). In nondiabetic subjects, blood glucose concentrations were 2.58 ± 0.2 and 4.42 ± 0.5 mmol/l during hypoglycemia and euglycemia, respectively. In those with type 1 diabetes, blood glucose concentrations were 2.46 ± 0.22 and 4.53 ± 0.24 mmol/l, respectively. The blood glucose nadir was similar in both groups.

Counterregulatory response

Plasma epinephrine increased during hypoglycemia in participants with and with-

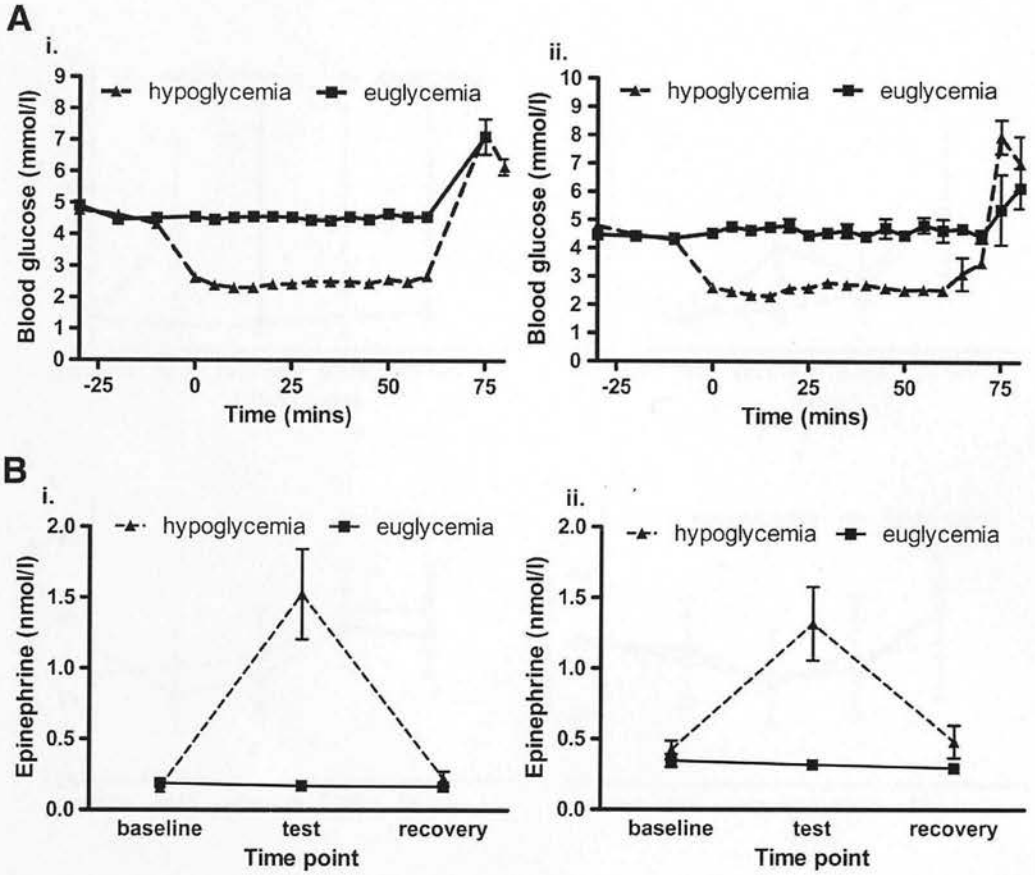


Figure 1—A: *Blood glucose concentrations during hyperinsulinemic hypoglycemic and euglycemic clamp studies.* B: *Epinephrine responses to experimental procedures.* i. *nondiabetic subjects;* ii. *subjects with type 1 diabetes.*

out type 1 diabetes ($P \leq 0.001$; Fig. 1). The epinephrine response occurred only during hypoglycemia and returned rapidly to baseline as anticipated (16).

Platelet activation

Platelet-monocyte aggregation. In nondiabetic subjects, platelet-monocyte aggregation appeared to rise, from a baseline level of 0.72 ± 0.8% to 3.09 ± 8.1% during hypoglycemia, with a peak of 3.49 ± 10.4% at 24 h (Fig. 2). Platelet-monocyte aggregation remained unchanged throughout euglycemia. The difference between conditions, and from baseline, did not achieve statistical significance.

In participants with diabetes, there was a late rise in platelet-monocyte aggregation after hypoglycemia at 24 h compared with baseline ($P = 0.03$).

Soluble P-selectin. Soluble plasma P-selectin concentrations increased after hypoglycemia in nondiabetic subjects, exhibiting a late response at 6 h ($P = 0.01$) and 24 h ($P = 0.02$; Fig. 2) but decreasing during euglycemia ($P = 0.006$).

P-selectin also decreased during euglycemia in the diabetic group ($P = 0.04$), but did not change during hypoglycemia.

Endothelial markers

tPA. In nondiabetic subjects, plasma tPA concentrations increased during hypoglycemia, with a higher peak tPA concentration (12.55 ± 16.7 compared with 6.80 ± 7.9 ng/ml) (NS between conditions). Plasma tPA decreased significantly between baseline and test phase ($P = 0.004$) and recovery phase ($P = 0.006$), with a paradoxical rise between baseline and 24 h ($P = 0.06$) after euglycemia (Table 2). However, a diurnal variation in tPA concentration is recognized to occur, which may account for the decline observed during euglycemia (17). No significant differences occurred in the diabetic group (Table 2).

vWF. A trend toward a difference in plasma vWF concentrations was observed between hypoglycemia and euglycemia at 6 h in the nondiabetic subjects ($P = 0.07$) (Table 2).

Plasma vWF concentrations decreased between baseline and test phase ($P = 0.02$) and recovery phase ($P = 0.03$) after euglycemia in the participants with diabetes. No such decrement was observed during hypoglycemia (Table 2).

Inflammation

CD40 expression. CD40 expression on monocytes increased after hypoglycemia in nondiabetic subjects, from a baseline of 1.92 ± 2.2% to a maximum of 3.13 ± 2.3% at 24 h ($P = 0.009$). A significant difference between hypoglycemia and euglycemia conditions was present at 6 h ($P = 0.05$) and at 24 h ($P = 0.04$) (Table 2).

In participants with type 1 diabetes, monocyte CD40 expression increased from 3.69 ± 3.4% to 5.54 ± 4.4% during hypoglycemia ($P = 0.006$), compared with no change during euglycemia (3.64 ± 2.0% to 3.65 ± 1.8%, respectively; P = NS). The increment during

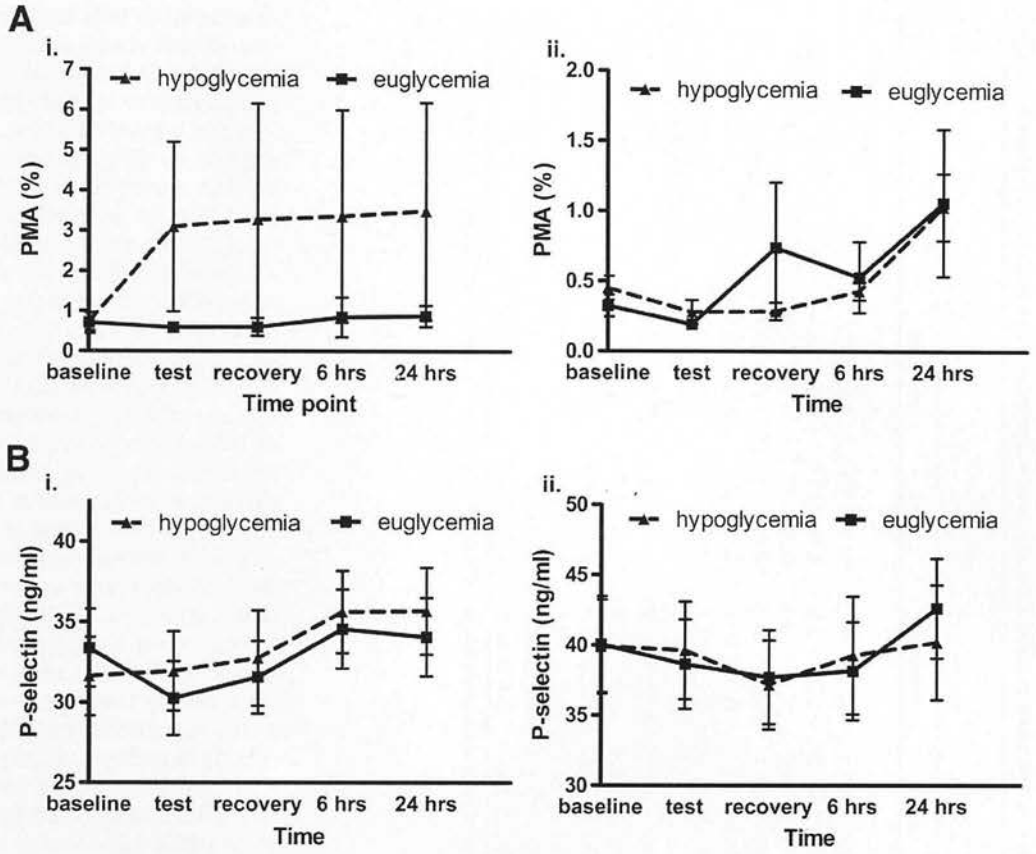


Figure 2—*Platelet activation in response to experimental hypoglycemia and euglycemia.* A: *Platelet-monocyte aggregation.* B: *Soluble P-selectin,* i. *Nondiabetic subjects,* ii. *Subjects with type 1 diabetes.*

hypoglycemia had dissipated by the time of the recovery phase and remained unchanged thereafter (Table 2).

sCD40L. In nondiabetic subjects, plasma sCD40L concentrations were higher during hypoglycemia than during euglycemia (2.80 ± 3.2 vs. 2.41 ± 2.8 ng/ml), with a trend toward significance ($P = 0.09$). A significant reduction in sCD40L concentration occurred during euglycemia between baseline and recovery phase ($P = 0.03$) (Table 2).

In those with diabetes, a significant difference was observed between the baseline levels on each study day: 3.36 ± 2.9 ng/ml on the hypoglycemia day compared with 2.86 ± 2.8 ng/ml on euglycemia day ($P = 0.03$), rendering subsequent measurements difficult to compare. A significant difference was again observed between the experimental condition levels, with a level of 3.41 ± 3.2 ng/ml during hypoglycemia and 2.85 ± 2.8 ng/ml during euglycemia ($P = 0.03$) (Table 2). Changes from baseline did not achieve significance.

IL-6. IL-6 levels rose in all experiments, maximally at 6 h, irrespective of condition, with no clear differences identifiable in either group between the study conditions (Table 2).

hsCRP. Test phase hsCRP was higher in all subjects during hypoglycemia (1.81 ± 1.9 vs. 1.22 ± 1.9 ng/ml in nondiabetic participants [$P = 0.02$]; 2.72 ± 3.1 vs. 2.20 ± 2.9 ng/ml in subjects with diabetes [P = ns]) (Table 2). A significant difference was observed in the baseline concentrations in the nondiabetic participants ($P = 0.01$), frustrating interpretation of subsequent responses.

CONCLUSIONS — Previous studies have demonstrated that hypercoagulability, platelet and neutrophil activation, C-reactive protein, IL-6, and Endothelin-1 are upregulated after acute hypoglycemia (3,6–11), while a euglycemic insulin infusion (for at least 2 h) was shown to reduce inflammatory markers, consistent with an anti-inflammatory effect of insulin (18). The present study sought to replicate these effects, while investigating other underlying mechanisms of vascular disease, and tests were selected to investigate the effect of acute hypoglycemia on important cellular processes (platelet activation, endothelial dysfunction and inflammation) underlying the development of acute and chronic vascular complications in type 1 diabetes.

The present study showed that hypoglycemia generated a response in some of these markers, suggesting that hypoglycemia-induced metabolic stress may have adverse pathophysiological consequences while the euglycemic insulin infusion caused a potentially beneficial decrement in some parameters. However, the magnitude of most observed changes was small, and not all markers changed significantly.

The present study confirmed that platelet activation is promoted by hypoglycemia (8), with increments both in platelet-monocyte aggregation and soluble P-selectin. Conversely, P-selectin decreased during euglycemia. Endothelial function, using vWF and tPA Ag as surrogate markers, may have been disrupted, as shown by the increase in vWF after hypoglycemia in nondiabetic volunteers, but this change was not replicated in those with diabetes. However, a reduc-

Table 2—*Endothelial function and inflammation in nondiabetic subjects and subjects with type 1 diabetes*

	Euglycemia					Hypoglycemia				
	Baseline	Test	Recovery	+6 h	+24 h	Baseline	Test	Recovery	+6 h	+24 h
Nondiabetic subjects										
tPA (ng/ml)	7.37 ± 8.1	6.80 ± 7.9*	6.44 ± 7.5*	6.99 ± 9.3	8.51 ± 7.8†	10.96 ± 11.8	12.55 ± 16.7	9.10 ± 10.2*	9.83 ± 12.0	11.45 ± 11.6
vWF (IU/ml)	0.81 ± 0.3	0.76 ± 0.3	0.78 ± 0.2	0.80 ± 0.3	0.85 ± 0.3	0.82 ± 0.3	0.81 ± 0.3	0.89 ± 0.5	0.90 ± 0.3	0.89 ± 0.3
CD40 (%)	1.51 ± 1.4	2.23 ± 3.2†	2.40 ± 3.2†	0.84 ± 0.7	2.06 ± 1.0	1.92 ± 2.2	1.47 ± 1.1	1.55 ± 1.5	1.98 ± 2.4‡	3.13 ± 2.3†‡
sCD40L (ng/ml)	2.68 ± 3.1	2.41 ± 2.8	2.40 ± 2.9*	2.63 ± 2.9	3.08 ± 3.3	2.88 ± 3.3	2.80 ± 3.2	2.55 ± 3.2	2.72 ± 3.3	2.79 ± 3.2
IL-6 (pg/ml)	0.86 ± 0.5	1.06 ± 1.2	1.05 ± 1.0	5.98 ± 4.6†	1.23 ± 0.9	0.72 ± 0.4	0.92 ± 0.5	1.62 ± 1.2†	4.37 ± 4.3†	1.00 ± 0.9
hsCRP (ng/ml)	1.04 ± 1.1	1.22 ± 1.9	1.18 ± 1.9	1.24 ± 1.6	1.31 ± 1.5	1.83 ± 1.5‡	1.81 ± 1.9‡	1.56 ± 1.3*	1.69 ± 1.2	1.90 ± 1.6
Subjects with type 1 diabetes										
tPA (ng/ml)	15.25 ± 30.2	17.70 ± 31.1	15.99 ± 27.5	22.13 ± 46.2	20.86 ± 34.8	18.12 ± 30.1	20.55 ± 36.1	17.69 ± 31.1	18.37 ± 32.6	22.98 ± 40.3
vWF (IU/ml)	0.91 ± 0.2	0.85 ± 0.2*	0.91 ± 0.3	0.85 ± 0.2*	0.99 ± 0.2	0.93 ± 0.2	0.95 ± 0.2	0.91 ± 0.2	0.90 ± 0.2	1.02 ± 0.2
CD40 (%)	3.64 ± 2.0	3.65 ± 1.8	4.14 ± 2.5	3.97 ± 2.3	4.35 ± 2.0	3.69 ± 3.4	5.54 ± 4.4†	3.36 ± 3.0	4.88 ± 2.4	4.70 ± 2.8
sCD40L (ng/ml)	2.86 ± 2.8	2.85 ± 2.8	2.84 ± 2.8	2.91 ± 2.9	3.25 ± 3.2†	3.36 ± 2.9‡	3.41 ± 3.2‡	3.10 ± 2.9*	3.05 ± 2.8*	3.44 ± 2.9
IL-6 (pg/ml)	0.69 ± 0.5	1.38 ± 1.9	1.58 ± 1.8	2.25 ± 2.8†	1.19 ± 1.2	1.21 ± 1.7	1.15 ± 1.5)	1.76 ± 1.5	3.10 ± 4.9	1.96 ± 2.2
hsCRP (ng/ml)	2.52 ± 3.1	2.20 ± 2.9	2.32 ± 2.8	1.92 ± 1.8	3.40 ± 3.6	2.84 ± 3.2	2.72 ± 3.1	2.70 ± 3.2	2.89 ± 3.3	2.34 ± 2.8

Data are means ± SD. *Significant decrease from baseline ($P < 0.05$); †significant increase from baseline ($P < 0.05$); ‡significant difference between hypoglycemia and euglycemia ($P < 0.05$).

tion in vWF occurred after euglycemia in diabetic participants, which should confer vascular benefit. tPA Ag also appeared to increase in nondiabetic subjects during hypoglycemia, while declining during euglycemia, whereas no significant changes occurred in the diabetic group. Soluble markers of inflammation, sCD40L and hsCRP, were higher during hypoglycemia, with an elevation of hsCRP being observed in all subjects. Unfortunately, baseline differences in hsCRP in nondiabetic subjects, and in sCD40L in the diabetic subjects, frustrated interpretation of subsequent responses. sCD40L was apparently reduced during euglycemia in nondiabetic participants. Surprisingly, IL-6 increased in all experiments regardless of glycemic status, with a maximal response at 6 h. This response is inexplicable, and contrasts with a previous report (10). Monocyte CD40 expression also increased, suggesting promotion of the interaction of the CD40-CD40 ligand dyad (from the tumor necrosis factor receptor family), thus affecting another process in the pathway leading to atherosclerotic plaque rupture (19,20). This change occurred much earlier in the diabetic than the nondiabetic subjects, in whom the response was delayed, prolonged, and still present at 24 h. The persistence of these vascular changes for 24 h after the hypoglycemic stimulus, or their later emergence, suggests that the period of risk after hypoglycemia may be present long after blood glucose recovery.

For some markers, a positive trend after hypoglycemia was evident, without achieving statistical significance, or the only measurable difference between conditions was a beneficial effect associated with euglycemia. The sample size may have been insufficient to achieve significance, particularly as the magnitude of responses was small. It was not feasible to study a larger number of subjects using a procedure that is labor-intensive and costly. In a previous study, larger increments in inflammatory markers were observed during an insulin tolerance test, where hypoglycemia of <39 mg/dl (<2.2 mmol/l) was induced (21). The more rapid reduction to a lower blood glucose causing a greater hypoglycemic stimulus may have heightened the magnitude of the responses, compared with the more modest changes that occurred during a controlled glucose clamp (blood glucose 2.5 mmol/l [45 mg/dl]), as observed in the present study. A further limitation of the present study was the need to ex-

amine the experimental conditions on two separate days in a counterbalanced fashion. Because the baseline levels of many inflammatory markers can differ on separate days, as was observed with sCD40L and hsCRP, this biological variability hinders the interpretation and comparison of subsequent results. However, the present study design was necessary to allow comparison of the euglycemia and hypoglycemia conditions in individual subjects, as both time and insulin infusion per se may exert effects on biomarker levels. This study design cannot control for other day-to-day factors that could influence baseline levels of inflammatory markers. However, the effects of hypoglycemia could be evaluated, as each participant acted as their own control. This produces less variability than a comparison of results among individuals, as more inter-individual variation in inflammatory marker levels is present than intra-individual variation. In addition, it was possible to analyze each study separately, by examining changes in parameters from baseline on that particular day, enabling the detection of significant effects exerted by hypoglycemia compared with euglycemia. Baseline levels of all markers (except IL-6) were higher in the diabetic group (significant for P-selectin and CD40 expression). This could affect the magnitude of response induced by the experimental procedures. However, an analysis of the percentage change from baseline was consistent with the trends identified in the absolute results (shown as in the online appendix available at http://care.diabetesjournals.org/cgi/content/full/dc10-0013/DC1).

As anticipated, epinephrine secretion was stimulated by hypoglycemia. It is likely that hormonal changes underlie the activation and upregulation of the vascular biomarkers. Catecholamines promote platelet activation (22), while adrenoceptor blockade attenuates these effects (23,24). The participants with type 1 diabetes exhibited attenuated plasma epinephrine responses to hypoglycemia compared with the nondiabetic subjects, who were naïve to such a hypoglycemic stimulus, this being consistent with the recognized decline in the magnitude of counterregulatory hormonal responses with increasing duration of type 1 diabetes (25). This attenuated epinephrine response may explain the lower responses of vascular biomarkers to hypoglycemia.

In summary, the effects of hypoglycemia on several vascular biomarkers that are implicated in the pathogenesis of vascular disease, would support the premise that acute hypoglycemia may be detrimental to an already diseased vasculature (2). Euglycemia may have a protective, anti-inflammatory effect. In the present study, the participants had no overt vascular disease and were unlikely to develop any demonstrable effects from a short period of exposure to hypoglycemia. However, in people with diabetes of long duration, who are likely to have underlying vascular disease, these responses may not be benign. The release of potent vasoactive substances could potentially aggravate chronic vasculopathy, and contribute to the precipitation of acute macrovascular events. This may aggravate established diabetic micro- and macrovascular disease in those who are exposed to recurrent hypoglycemia.

Acknowledgments— The cost of assays was supported by research grants from the Scottish Society of Physicians and the Edinburgh branch of Diabetes UK.

No potential conflicts of interest relevant to this article were reported.

References

1. Hanssen KF, Dahl-Jørgensen K, Lauritzen T, Feldt-Rasmussen B, Brinchmann-Hansen O, Deckert T. Diabetic control and microvascular complications: the near-normoglycaemic experience. Diabetologia 1986;29:677–684
2. Frier BM, Hilsted J. Does hypoglycaemia aggravate the complications of diabetes? Lancet 1985;2:1175–1177
3. Wright RJ, Frier BM. Vascular disease and diabetes: is hypoglycaemia an aggravating factor? Diabetes Metab Res Rev 2008;24:353–363
4. Action to Control Cardiovascular Risk in Diabetes Study Group, Gerstein HC, Miller ME, Byington RP, Goff DC Jr, Bigger JT, Buse JB, Cushman WC, Genuth S, Ismail-Beigi F, Grimm RH Jr, Probstfield JL, Simons-Morton DG, Friedewald WT Effects of intensive glucose lowering in type 2 diabetes. N Engl J Med 2008;358:2545–2559
5. Duckworth W, Abraira C, Moritz T, Reda D, Emanuele N, Reaven PD, Zieve FJ, Marks J, Davis SN, Hayward R, Warren SR, Goldman S, McCarren M, Vitek ME, Henderson WG, Huang GD, for the VADT Investigators. Glucose control and vascular complications in veterans with type 2 diabetes. N Engl J Med 2009;360:129–139
6. Frier BM, Corrall RJ, Davidson NM, Webber RG, Dewar A, French EB. Peripheral blood cell changes in response to acute hypoglycaemia in man. Eur J Clin Inv 1983;13:33–39
7. Fisher BM, Quin JD, Rumley A, Lennie SE, Small M, MacCuish AC, Lowe GD. Effects of acute insulin-induced hypoglycaemia on haemostasis, fibrinolysis and haemorheology in insulin-dependent diabetic patients and control subjects. Clin Sci 1991;80:525–531
8. Trovati M, Anfossi G, Cavalot F, Vitali S, Massucco P, Mularoni E, Schinco P, Tamponi G, Emanuelli G. Studies on mechanisms involved in hypoglycemia-induced platelet activation. Diabetes 1986;35:818–825
9. Galloway PJ, Thomson GA, Fisher BM, Semple CG. Insulin-induced hypoglycemia induces a rise in C-reactive protein (Letter). Diabetes Care 2000;23:861
10. Dotson S, Freeman R, Failing HJ, Adler GK. Hypoglycemia increases serum interleukin-6 levels in healthy men and women. Diabetes Care 2008;31:1222–1223
11. Wright RJ, Macleod KM, Perros P, Johnston N, Webb DJ, Frier BM. Plasma endothelin response to acute hypoglycaemia in adults with type 1 diabetes. Diabet Med 2007;24:1039–1042
12. Libby P, Ridker PM, Maseri A. Inflammation and atherosclerosis. Circulation 2002;105:1135–1143
13. De Fronzo R, Tobin JD, Andres R. Glucose clamp technique: a method for quantifying insulin secretion and resistance. Am J Physiol 1979;273:E214–E223
14. Abumrad NN, Rabin D, Diamond MP, Lacy WW. Use of a heated superficial hand vein as an alternative site for the measurement of amino acid concentrations and for the study of glucose and alanine kinetics in man. Metabolism 1981;30:936–940
15. Gold AE, MacLeod KM, Frier BM. Frequency of severe hypoglycemia in patients with type 1 diabetes with impaired awareness of hypoglycemia. Diabetes Care 1994;17:697–703
16. Thompson CJ, Baylis PH. Endocrine changes during insulin-induced hypoglycaemia. In *Hypoglycaemia and Diabetes: Clinical and Physiological Aspects*. Frier BM, Fisher BM, Eds. Edward Arnold, London, U.K., 1993, p.116–131
17. Rydzewski A, Urano T, Nagai N, Takada Y, Katoh-Oishi Y, Taminato T, Yoshimi T, Takada A. Diurnal variation in serum remnant-like lipoproteins, platelet aggregation and fibrinolysis in healthy volunteers. Haemostasis 1997;27:305–314
18. Dandona P, Chauduri A, Ghanim H, Mohanty P. Insulin as an anti-inflammatory and antiatherogenic modulator. J Am Coll Cardiol 2009;53 (Suppl. 5):S14–S20
19. Schönbeck U, Libby P. CD40 signaling and plaque instability. Circ Res 2001;89:1092–1103

20. Mach F, Schönbeck U, Libby P. CD40 signaling in vascular cells: a key role in atherosclerosis? Atherosclerosis 1998; 137(Suppl.):S89–S95
21. Razavi Nematollahi L, Kitabchi AE, Kitabchi AE, Stentz FB, Wan JY, Larijani BA, Tehrani MM, Gozashti MH, Omidfar K, Taheri E. Proinflammatory cytokines in response to insulin-induced hypoglycemic stress in healthy subjects. Metabolism 2009;58:443–448
22. Steel CM, French EB, Aitchison WR. Studies on adrenaline-induced leucocytosis in normal man. I. The role of the spleen and of the thoracic duct. Br J Haematol 1971;21:413–421
23. Fisher BM, Hepburn DA, Smith JG, Frier BM. The effect of alpha-adrenergic blockade on responses of peripheral blood cells to acute insulin-induced hypoglycaemia in humans. Eur J Clin Invest 1990; 20:51–55
24. Takeda H, Kishikawa H, Shinohara M, Miyata T, Suzaki K, Fukushima H, Ichinose K, Shichiri M. Effect of alpha 2-adrenoceptor antagonist on platelet activation during insulin-induced hypoglycaemia in type 2 (noninsulin-dependent) diabetes mellitus. Diabetologia 1988;31:657–663
25. Kerr D, Richardson T. Counterregulatory deficiencies in diabetes. In *Hypoglycaemia in Clinical Diabetes*. 2nd edition. Frier BM, Fisher M, Eds. John Wiley and Sons, Chichester, U.K., 2007, p. 121–140