# Insulin sensitivity: measurements in the whole body and in isolated tissues

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by

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## Summary

As the incidence of type 2 diabetes rises exponentially in the early part of the 21st century, the global healthcare and financial burdens associated with this chronic condition rise in tandem. As such, it becomes increasingly important to characterise this disease further, and unravel the molecular mechanisms which lead to hyperglycaemia and, ultimately, premature vascular disease. Insulin resistance, while pathognomonic of type 2 diabetes, is not confined to this condition. It has become clear that insulin resistance lies at the centre of a constellation of cardiovascular risk factors, which include hypertension, obesity and dyslipidaemia. To date there are no large prospective studies that have examined the role of insulin sensitivity in the development of vascular disease, however epidemiological evidence suggests that there is a relationship. Thus, studies exploring the factors that determine insulin sensitivity are of considerable clinical importance.

In this thesis, a series of studies is described which were designed to measure insulin action in the whole body and in isolated tissues. Insulin sensitivity was measured in the whole body using the hyperinsulinaemic euglycaemic clamp, though also in adipose tissue by measuring insulin-mediated suppression of lipolysis and in human resistance vessels by measuring insulin-mediated vasodilation. In association with this, the effect of activation of endogenous hormonal axes that may influence insulin sensitivity was investigated, not only in the whole body but also in individual tissues.

As an overview of the experimental chapters:

**i) Insulin-mediated suppression of lipolysis; development and application of an assay of insulin sensitivity**

In chapter 3, the development of an assay designed to measure insulin sensitivity in isolated adipocytes is described. Initial experiments are described that establish the fundamentals of this assay, which is based on insulin-mediated suppression of isoproterenol-induced lipolysis. There follows investigation of the hypothesis that insulin sensitivity in isolated adipocytes reflects the phenotype of the patient from whom they are obtained; phenotype characterisation included fasting insulin, blood pressure, fasting lipids and measurement of basal metabolic index. In addition to demonstrating a reduction in insulin sensitivity in obese individuals, a continuous relationship was observed between insulin sensitivity and BMI. Thus, there was evidence of a relationship between clinical phenotype and the response to insulin at a cellular level.

**ii) Dietary sodium restriction and insulin sensitivity**

The hypothesis that modest dietary sodium restriction, by activation of the renin-angiotensin system, reduces systemic insulin sensitivity is investigated. This study followed previous observations that severe dietary sodium restriction reduces insulin sensitivity, and that, in keeping with this, use of the angiotensin converting enzyme inhibitors may improve insulin sensitivity. Using a double-blind, randomised, placebo-controlled crossover trial, an 11% reduction in systemic insulin sensitivity was demonstrated in association with modest dietary sodium restriction and significant activation of the circulating renin angiotensin system. Potential explanations for the

heterogeneity of previous study findings, as well as the clinical implications of the observed fall in insulin sensitivity, are discussed.

**iii) Angiotensin II and insulin sensitivity in human adipocytes**

In chapter 5, in an attempt to explain the findings in the previous chapter, the hypothesis that angiotensin II may have a direct cellular effect on insulin action is explored. This may be explained by intracellular crosstalk between second messengers of the insulin and angiotensin II signalling cascades. Having confirmed the presence of angiotensin II binding to isolated adipocytes, no effect of angiotensin II on insulin-stimulated glucose transport, insulin-mediated suppression of lipolysis or glucose transport in the absence of insulin was demonstrated. The conclusion was reached that the findings in chapter 4 might be explained by angiotensin II action in alternative tissue, or action of an alternative hormone such as aldosterone. There was no evidence to support the suggestion that angiotensin II contributes to insulin resistance in human adipose tissue.

**iv) Glucocorticoids and insulin sensitivity: dissociation of insulin's metabolic and vascular actions**

In chapter 6, the effect of a short period of exposure to glucocorticoid on insulin sensitivity is investigated, again using a double-blind, randomised, placebo-controlled design. In this study, insulin sensitivity was measured both systemically and again in isolated tissue, though on this occasion in small resistance vessels dissected from subjects at the end of both dexamethasone and placebo phases. As anticipated, systemic insulin sensitivity was reduced significantly by glucocorticoids (mean reduction of 29%), however there was no difference in insulin action in isolated vessels between

phases. While confirming the effect of glucocorticoids on insulin sensitivity, this observation is also important in light of the debate regarding the coupling of insulin action in different tissues. Possible explanations for the dissociation of the vascular and metabolic effects of insulin in this model are discussed.

## Conclusions

From the above experimental chapters, several conclusions were made

i) Insulin sensitivity in isolated human adipocytes is reduced in obese, though otherwise healthy, females with normal fasting plasma glucose.

ii) Dietary sodium restriction is associated with a reduction in systemic insulin sensitivity.

iii) Intracellular crosstalk between angiotensin II and insulin does not result in insulin resistance in human adipocytes.

iv) Dexamethasone treatment is associated with a significant reduction in systemic insulin sensitivity, though no change in insulin action in the vasculature.

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## Abbreviations used in this thesis

| | |
|---|---|
| ADP | Adenosine diphosphate |
| AGT | Angiotensinogen |
| AII | Angiotensin II |
| ACE | Angiotensin converting enzyme |
| ATP | Adenosine triphosphate |
| AT1 | Angiotensin II type 1 receptor |
| AT2 | Angiotensin II type 2 receptor |
| AUC | Area under the curve |
| BMI | Basal metabolic index |
| CAP | c-Cbl-associating protein |
| cAMP | cyclic adenosine monophosphate |
| cGMP | cyclic guanosine monophosphate |
| CHD | Coronary heart disease |
| CI | Confidence interval |
| CRC | Concentration response curve |
| CRP | C-reactive protein |
| CVD | Cardiovascular disease |
| DAG | Diacyl glycerol |
| DNA | Deoxyribonucleic acid |
| ECG | Electrocardiogram |
| EDHF | Endothelium dependent hyperpolarizing factor |
| EGIR | European group on insulin resistance |
| FAS | Fatty acid synthase |
| FAT | Fatty acid translocase |
| FIRI | Fasting insulin resistance index |
| GDP | Guanosine diphosphate |
| GPDH | Glycerol-3-phosphate dehydrogenase |
| GTP | Guanosine triphosphate |
| HDL | High density lipoprotein |
| HOMA | Homeostatic modelling assessment |
| HOPE | Heart outcomes prospective evaluation |
| HSD | Hydroxysteroid dehydrogenase |
| HSL | Hormone sensitive lipase |
| HUVEC | Human umbilical vein endothelial cell |
| IGF-1 | Insulin-like growth factor |
| IGT | Impaired glucose tolerance |
| IHD | Ischaemic heart disease |
| IL-6 | Interleukin-6 |
| $IP_3$ | inositol 1,4,5-triphosphate |
| IRAS | Insulin resistance and atherosclerosis study |
| IRS | Insulin receptor substrate |
| ISBAT | Interscapular brown adipose tissue |
| JAK | Janus kinase |
| LDL | Low density lipoprotein |
| LDM | Low density microsomes |
| LPL | lipoprotein lipase |
| L-NMMA | L-$N^{\omega}$-monomethylarginine |
| MAP | Mean arterial pressure |
| MAPK | Mitogen associated protein kinase |
| MKP-1 | Mitogen activated protein kinase phosphatase-1 |
| MI | Myocardial infarction |

| | |
|---|---|
| NADH | Nicotinamide-adenine dinucleotide |
| NADPH | Nicotinamide-adenine dinucleotide phosphate |
| NE | Norepinephrine |
| NEFA | Non-esterified fatty acid |
| NO | Nitric oxide |
| NOS | Nitric oxide synthase |
| NSF | N-ethylmaleimide-sensitive fusion protein |
| OGTT | Oral glucose tolerance test |
| OR | Odds ratio |
| PAI-1 | Plasminogen activation inhibitor-1 |
| PDGF | Platelet derived growth factor |
| PDK-1 | Phosphoinositide-dependent protein kinase -1 |
| PCR | Polymerase chain reaction |
| PEPCK | Phosphoenolpyruvate carboxykinase |
| PG | Prostaglandin |
| PI3`-kinase | Phosphatidyl inositol 3` kinase |
| $PIP_3$ | Phosphatidyl inositol 3,4,5, -triphosphate |
| PKA | Protein kinase A |
| PKB | Protein kinase B |
| PKC | Protein kinase C |
| PLC | Phospholipase C |
| PPAR | Peroxisome proliferator activated receptor |
| PRA | Plasma renin activity |
| RAS | Renin angiotensin system |
| RIA | Radioimmunoassay |
| RNA | Ribonucleic acid |
| RT | Reverse transcriptase |
| SD | Standard deviation |
| SEM | Standard error of the mean |
| SH2 domain | Src homology domain 2 |
| SHR | Spontaneously hypertensive rat |
| SH-PTP2 | Src homology protein tyrosine phosphatase 2 |
| SNARE | Soluble NSF attachment protein receptors |
| SR | Salt resistant |
| SS | Salt sensitive |
| SSPG | Steady state plasma glucose |
| STAT | Signal transduction and activation of transcription |
| TG | Triglycerides |
| TNF-$\alpha$ | Tumour necrosis factor alpha |
| tPA | Tissue plasminogen activator |
| TZD | Thiazolidinedione |
| UKPDS | United Kingdom Prospective Diabetes Study |
| VAMP | Vesicle associated membrane peptide |
| VSMC | Vascular smooth muscle cells |
| WHO | World health organisation |
| WHR | Waist hip ratio |

**Presentations of work undertaken in this thesis to learned societies**

Several aspects of the work undertaken in this thesis have been presented to learned societies:

*Dissociation of the metabolic and vascular action of insulin*
Presented to the Hypertension in Diabetes Study Group of the European Association for the study of Diabetes, April 2001

*Corticosteroids and insulin sensitivity*
Presented to the Scottish Society for Experimental Medicine, November 2000

*Differing effects of glucocorticoids on metabolic and vascular insulin sensitivity*
Presented in poster form to the Society for Endocrinology, November 2000

*Intracellular crosstalk does not mediate the insulin resistance associated with sodium restriction*
Presented to the British Hypertension Society, September 2000

*Obesity is characterised by insulin resistance in human adipose tissue*
Presented in poster form to the European Association for the Study of Diabetes, September 2000

*Angiotensin II has a metabolically neutral effect on insulin sensitivity in human adipose tissue*
Presented in poster form to the International Society for Hypertension, August 2000

*The renin angiotensin system and insulin sensitivity in human adipose tissue*
Presented to the Hypertension in Diabetes Study Group of the European Association for the Study of Diabetes, May 2000

*Angiotensin II and insulin sensitivity in human adipocytes*
Presented to the Scottish Society for Experimental Medicine, November 1999

*The renin angiotensin system and insulin sensitivity in human adipose tissue*
Presented in poster form to the Society for Endocrinology, November 1999

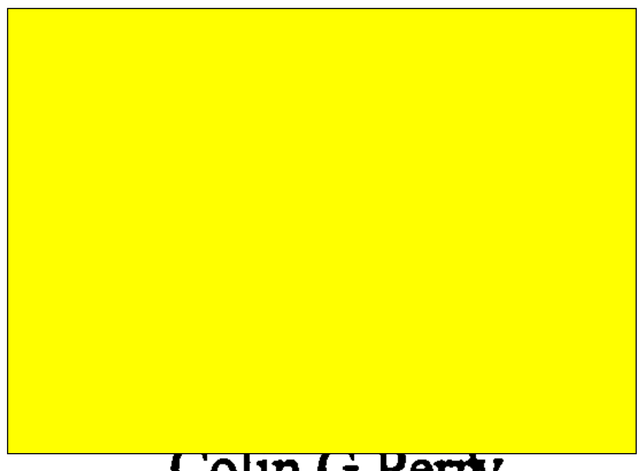
*The development of an adipocyte glucose transport assay*
Presented to the British Hypertension Research Group, May 1998

*Studies of insulin signalling in human adipocytes*
Presented to British Hypertension Research Group, October 1997

## Declaration

I declare that this thesis has been composed by myself and that, other than the myography studies, it is a record of work performed by myself. It has not been submitted previously for a higher degree.

The work described in this thesis was carried out under the supervision of Professor JMC Connell, Department of Medicine and Therapeutics, University of Glasgow, and Professor GW Gould, Institute of Biomedical and Life Sciences, University of Glasgow.



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# Chapter 1: Introduction

## 1.1 Overview

Over a century has passed since the concept of treating diabetes with pancreatic material was proposed by Minkowski and Von Merings in the University of Strasbourg in 1889 (1). In 1923, the Nobel Prize in physiology and medicine was awarded to Banting and McLeod, who shared the award with Best and Collip respectively, for the discovery of insulin. Very soon afterwards, insulin was widely available as treatment for diabetes, a condition with a hitherto appalling prognosis. Indeed, Joslin described the first patients receiving insulin therapy as "near resurrections".

In 1936, Himsworth made the observation (2) that there was differential sensitivity to insulin in patients with diabetes, leading to the realisation that although insulin acted as an efficient blood glucose-lowering agent, there was a group of patients with diabetes who seemed less sensitive to insulin action. Soon after insulin assays were available, it became clear that this less insulin sensitive group of patients did not in fact lack circulating insulin; indeed there was an abundance (3). As such, the concept of insulin resistance was developed. The high cardiovascular mortality associated with type 2 diabetes and hyperinsulinaemia (and therefore insulin resistance), in conjunction with the observation that in the non-diabetic population there were associations between hyperinsulinaemia, hypertension (4) and coronary heart disease (5) led several groups to speculate that insulin resistance was an important metabolic variable that conferred a poor cardiovascular prognosis.

The series of studies contained in this thesis describe several techniques used to measure insulin sensitivity in both the whole body and in isolated tissues, and the relationship between these measurements and the cardiovascular phenotype of subjects. In addition, I have studied the influence of activity of other endocrine systems, such as the renin angiotensin aldosterone system and the hypothalamic pituitary adrenal axis, on insulin sensitivity. Lastly, I have considered the relationship between the metabolic sensitivity to insulin and the role of insulin in the vasculature, in an attempt to develop a direct link between insulin resistance and vascular disease.

## 1.2 Insulin: basic biology and physiology

### 1.2.1 Synthesis and secretion of insulin

The insulin molecule is made up of two polypeptide chains (A and B), which are linked by disulphide bonds. Circulating insulin is the result of the proteolytic cleavage of preproinsulin to proinsulin, and then to insulin and C-peptide, which are released from $\beta$ cells in the Islets of Langerhan's in equimolar amounts. Small amounts of the structurally very similar proinsulin are also secreted in this process, giving rise to a potential source of interference with non-specific insulin assays. Insulin is secreted in response to various physiological stimuli, including glucose, arginine, lysine, ketone bodies and free fatty acids (reviewed in (6)).

### 1.2.2 Physiological actions of insulin

Insulin binds to its transmembrane tyrosine kinase coupled receptor (see section 1.7) and, through a series of second messenger events, stimulates its cellular actions (reviewed in (6)), the exact nature of which depend on the target tissue and organ.

- *Glucose Uptake.* Insulin stimulates glucose uptake into muscle and fat, in a process involving the translocation of specific insulin responsive glucose transporters to the cell surface.
- *Glycogen Synthesis*. Insulin stimulates the storage of glucose as glycogen in skeletal muscle, a process that involves the activation of glycogen synthase by glycogen synthase kinase.

- *Lipogenesis / suppression of lipolysis*. Much of the glucose taken up into adipose tissue is stored as triglyceride. Insulin activates acetyl coenzyme A (CoA) carboxylase, which favours the diversion of acetyl coenzyme A away from oxidation and toward lipid synthesis. This may be mediated by activity of the adenosine 3`, 5`-cyclic monophosphate (cAMP) dependent protein kinase.

- *Hepatic glucose output.* Amino acids derived from muscle and glycerol released from triglyceride lipolysis may be converted into substrates for gluconeogenesis. While glucagon facilitates this process, insulin inhibits the hepatic production of glucose. The breakdown of hepatic glycogen, an additional hepatic source of glucose, is again regulated by the balance of glucagon`s agonist and insulin's antagonist action.

- *Vasodilation*. In addition to the metabolic actions of insulin, it has become clear that insulin acts in the vasculature as a vasodilator, involving the endothelial-dependent release of vasoactive substances such as nitric oxide. This is discussed later in this thesis.

Certain common conditions, such as type 2 diabetes and essential hypertension, are characterised by a reduction in the ability of insulin to transport glucose into cells – insulin resistance (reviewed in (7-9) . In addition, other insulin-regulated processes, such as suppression of lipolysis and possibly insulin-mediated vasodilation may also be attenuated. This thesis will focus on the mechanism and nature of insulin resistance measured in human tissue and in the whole body. In order to do so, it is necessary to define, more specifically, what insulin resistance implies and the risks that it may carry.

## 1.3 Insulin resistance

### 1.3.1 Definition

Insulin resistance, like other syndromes of hormone resistance, may be defined conceptually as "a subnormal biological response to insulin at physiological concentrations". This simple definition hides the complexity of the biological parameter that it attempts to summarise, a situation that becomes clearer when attempts are made to measure this variable.

### 1.3.2 Measurement of whole body insulin sensitivity

A variety of methods employed in the *in vivo* measurement of insulin sensitivity have been described, and the field is characterised by a lack of standardisation. These methods are of varying complexity, ranging from simple fasting plasma insulin and insulin resistance index, to the time consuming, though more direct, euglycaemic hyperinsulinaemic clamp (10). The methods of measuring insulin sensitivity are detailed below (reviewed in (11)), and summarised in table 1.1.

- *Euglycaemic hyperinsulinaemic clamp(10)*: A primed, then constant, exogenous insulin infusion suppresses endogenous pancreatic $\beta$ cell insulin secretion and hepatic glucose production, while glucose is infused at a variable rate in order to maintain blood glucose at a predetermined level. At the steady state the glucose infusion rate reflects directly the rate of glucose uptake into tissues, i.e. insulin sensitivity. Initial clamp studies used arterial blood samples for analysis of serum glucose levels, but hand warming is now widely used to "arterialise" venous blood (12). Reproducibility is improved when a three hour rather than two hour clamp is performed (13)

- *Insulin suppression test:* Glucose and insulin are co-infused, with rates held constant. Steady state is achieved once plasma glucose reaches a plateau. Endogenous insulin release is suppressed using somatostatin. Steady state plasma glucose is used as an index of insulin sensitivity.

- *Short insulin tolerance test:* This is based on the rate at which plasma glucose disappears during the first twenty minutes following an intravenous bolus of insulin.

- *Serum insulin:* Serum insulin has been used as a surrogate measure of insulin resistance, based on the assumption that compensatory hyperinsulinaemia occurs under normal circumstances in proportion to the degree of insulin resistance (this may not be the case in the context of pancreatic failure). Both fasting and post-prandial measurements have been used in prospective studies of insulin resistance and cardiovascular disease (14) (15). The main difficulties in interpreting these values are variations in the performance of assay kits across centres and differences in the specificity of insulin vs. its precursor molecules(16).

- *Homeostatic Modelling Assessment (HOMA) and Fasting Insulin Resistance Index (FIRI):* These simple indices are derived from fasting insulin and glucose levels (17,18) In non-diabetic populations, positive correlation with fasting insulin is almost unity. However, in datasets from which diabetic subjects have not formally been excluded, these may provide a better surrogate measurement of insulin sensitivity than fasting insulin(16).

- *Frequently sampled iv glucose tolerance test with minimal model analysis:* This technique, in which up to 22 glucose samples are obtained for the mathematical calculation of insulin sensitivity, has been reported as having a high

degree of correlation with values of insulin sensitivity generated using the euglycaemic hyperinsulinaemic clamp in healthy volunteers (19). In subjects with type 2 diabetes, however, the correlation is less strong, particularly when the number of glucose samples is reduced to 12 (as in the Insulin Resistance and Atherosclerosis Study(20)). As such, this technique is best employed in studies of populations with normal glucose tolerance.

## Table 1.1 The methods used to measure whole body insulin sensitivity

| Method | r-value to clamp | Parameter | Published coefficient of variation | Advantages | Disadvantages |
|---|---|---|---|---|---|
| **Euglycaemic Hyperinsulinaemic Clamp** | N/A | M | 6% [21] | Direct<br>No assumptions | Labour intensive and requires on-line glucose monitoring.<br>Theoretical risk of hypoglycaemia<br>Bias if operator not blinded. |
| **Insulin Suppression Test** | 0.93 [22] | SSPG | 10% [22] | No assumptions<br>Relatively cheap and easy to perform. | Requires specialist supervision and octreotide infusion.<br>Difficulty in achieving steady state glucose concentrations.<br>Inaccurate at high and low values. |
| **Short Insulin Tolerance Test** | Not known | $K_{ITT}$ | Not known | Easily performed.<br>Limited data suggest values correlate with clamp. | Risk of hypoglycaemia.<br>Limited validation.<br>Assumptions regarding glucose disappearance *in vivo*. |
| **Plasma Insulin (fasting/2-hour)** | -0.82 (area under insulin curve for OGTT)[23] | ρmol/l | Assay dependent | Simple and cheap | Depends on compliance with fast. |
| **HOMA/FIRI** | FIRI -0.67[23]<br>HOMA -0.83[17] | R | 30% [17] | Simple and cheap | Physiologically limited, though better than plasma insulin in diabetic subjects. |
| **Frequently sampled iv GTT (Bergman's Minimal Model)** | 0.62 [20] | $S_i$ | 20-28% [24] | Gives additional information about insulin secretion.<br>Cheap, easily performed | Assumes a single compartment model of glucose distribution.<br>Requires an insulin response.<br>Negative results in severe insulin resistance.<br>Complicated analysis, and large number of glucose samples. |

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## 1.4 Insulin resistance, the metabolic syndrome and cardiovascular disease

Scientific and clinical awareness of the link between insulin resistance and vascular disease has increased exponentially over the last 20 years. A relationship between atherosclerosis and plasma insulin was hypothesised more than 30 years ago (5), based on the presence of hyperinsulinaemia in acute myocardial infarction. More recent cross sectional studies, suggesting that plasma insulin is associated with other cardiovascular risk factors (25), provided firmer evidence on which to base larger prospective projects. Reaven's description of Syndrome X, or the metabolic syndrome, in 1988 (26), was a synthesis of his and others' data that offered a definition of the metabolic derangement accompanying insulin resistance. His hypothesis states that insulin resistance lies at the centre of a constellation of cardiovascular risk factors, with each individual component associated with vascular disease, however these factors coexist in certain individuals more than could be explained by chance. Despite insulin resistance *per se* being a prerequisite of the syndrome, diabetic levels of hyperglycaemia were not an absolute requirement; more important was resistance to the metabolic effects of insulin, with secondary hyperinsulinaemia. Insulin resistance is a continuous biological variable with wide variability throughout the non-diabetic and diabetic populations; up to a ten fold variation has been reported in subjects with normal glucose tolerance (27), (28).

The baseline characteristics of recruits to cohorts such as the Atherosclerosis Risk in Communities study (29) and the San Antonio Heart Study (30) demonstrate clearly statistical clustering of hypertriglyceridaemia, low concentrations of high density lipoprotein (HDL)-cholesterol, hypertension, diabetes and hyperuricaemia with fasting insulin, basal metabolic index (BMI) and waist hip ratio (WHR). Detailed metabolic characterisation of a population of nearly 3000 subjects clearly illustrates the co-clustering

of these individual risk factors (table 1.2) (31). The prevalence of the individual components of the syndrome in isolation was far less than in combination, while even in the isolated forms, associations with hyperinsulinaemia, implying insulin resistance, were noted. This led the authors of this analysis to hypothesise that insulin resistance lies at the centre of the metabolic syndrome.

**Table 1.2: The metabolic syndrome: prevalence of individual components in a Western population**

| Component | Definition | Prevalence in population (%) | Prevalence alone (%) |
|---|---|---|---|
| **Obesity** | BMI>27kg/m$^2$ | 54.3 | 29 |
| **Type 2 diabetes** | Fasting glucose ≥7.8mmol/l or 2h glucose ≥11.1mmol/l, or on treatment | 9.3 | 1.3 |
| **Impaired glucose tolerance** | Fasting glucose < 7.8mmol/l and 2h glucose 7.8-11.1mmol/l | 11.1 | 1.8 |
| **Hypertension** | Diastolic blood pressure ≥ 95mmHg, or on treatment | 9.8 | 1.5 |
| **Hypertriglyceridaemia** | Serum triglyceride >2.9mmol/l | 10.3 | 1 |
| **Hypercholesterolaemia** | Serum total cholesterol >6.5mmol/l | 9.2 | 1.7 |

### 1.4.1 Diagnostic criteria for the metabolic syndrome

Clearly if there are to be prospective studies of the metabolic syndrome and its attendant cardiovascular risk, strict criteria are required for its definition. There have been two main attempts to define these diagnostic criteria (table 1.3). Both definitions are hampered by the lack of standardisation in the measurement of the individual components of the syndrome across centres, in particular the specificity of insulin assays and the precise protocol for the euglycaemic hyperinsulinaemic clamp technique are highly variable

.

Though the constituents are similar for both sets of guidelines, there are notable differences that may have important effects on projected population prevalence. The World Health Organisation (WHO) criteria (32) require either proven glucose intolerance or the performance of a euglycaemic clamp. In the European Group on Insulin Resistance (EGIR) guidelines (33), the blood pressure threshold is lower, the triglyceride threshold is higher, the diagnosis of central obesity does not require a measurement of hip circumference and microalbuminuria is not included. When the WHO set of criteria is applied to the EGIR database of 1500 non-diabetic, normotensive European adults (34), a prevalence of 15.6% (WHO) (35) is derived. Subsequent application of diagnostic guidelines to other datasets has yielded prevalence of between 7% and 30% using WHO criteria and between 1% and 22% using EGIR criteria (33).

**Table 1.3: Diagnostic criteria for the metabolic syndrome**

| | WHO 1998[32] | EGIR 1999[33] |
|---|---|---|
| **Name** | "metabolic syndrome" | "insulin resistance syndrome" |
| **Population Eligible** | All | Non-diabetic |
| **Criteria** | Diabetes mellitus or impaired glucose tolerance [a] and/or insulin resistance (euglycaemic hyperinsulinaemic clamp) [b]<br><br>*Plus*<br>two of<br>▪ BP ≥160/90mmHg<br>▪ Triglycerides ≥1.7mmol/l [c]<br>▪ WHR >0.9 in males/ >0.85 in females [d]<br>▪ Microalbuminuria [e] | Insulin resistance (euglycaemic hyperinsulinaemic clamp) or fasting hyperinsulinaemia [f]<br><br>*Plus*<br>two of<br>▪ BP ≥140/90 mmHg (or treated)<br>▪ Triglycerides >2.0mmol/l[g]<br>▪ Waist circumference ≥94cm in males/≥80cm in females<br>▪ Fasting glucose ≥6.1mmol/l but <7.0mmol/l [h] |

a) according to WHO criteria

b) lowest quartile of glucose disposal for background population under study

c) and /or HDL <0.9mmol/l in males, <1.0mmol/l in females

d) and /or BMI>30kg/m$^2$

e) Albumin excretion rate ≥ 20μg/min or albumin : creatinine ratio ≥20mg/g

f) Lowest quartile of glucose disposal rate or highest quartile of insulin level

g) Or HDL < 1.0mmol/l or on therapy

h) i.e. non-diabetic fasting hyperglycaemia

### 1.4.2 The metabolic syndrome, insulin resistance and lipid profile

The metabolic syndrome, insulin resistance and type 2 diabetes are associated with an atherogenic lipid profile, traditionally consisting of raised total cholesterol, low HDL cholesterol and elevated triglycerides. Additional, qualitative, aspects of the dyslipidaemia prevalent in insulin resistance have become apparent in recent years. In males with type 2 diabetes and hypertriglyceridaemia, there is a preponderance of small dense low-density lipoprotein (LDL) particles and an associated reduction in larger, buoyant particles (36). This relationship is also applicable to subjects without diabetes; analysis of the San Antonio Heart Study cohort revealed that small dense LDL was associated with elevated triglycerides, decreased HDL, hypertension and impaired glucose tolerance. There was also an association between increasing numbers of these individual metabolic disorders and decreasing LDL size (37). The smaller LDL subtype is in addition more easily oxidised that its larger counterparts (38), and again there is evidence demonstrating an association between LDL "oxidizability" and insulin resistance (39). That this small, dense, partially oxidised LDL is related to atherosclerosis is demonstrated in a cross-sectional study of middle-aged males, in whom there was a negative relationship between LDL particle size and intimal media thickness of the internal carotid artery (40).

## 1.5 Cardiovascular risk associated with insulin resistance: are insulin resistance and hyperinsulinaemia cardiovascular risk factors?

From the preceding discussion it may be extrapolated that, given the traditional cardiovascular risk factors encompassed in the metabolic syndrome, as a syndrome there will be an association with cardiovascular disease. What is less clear is whether insulin resistance *per se* is an independent risk factor once the other associated factors are accounted for. As the absolute risk of cardiovascular events in apparently healthy middle-

aged subjects remains small, large well-designed prospective studies with detailed metabolic investigation at baseline are required to answer this fundamentally important question. All but a very few studies to date have used plasma insulin as a surrogate marker of insulin sensitivity.

### 1.5.1 Prospective studies of insulin as a cardiovascular risk factor

Several large studies have examined the relationship between plasma insulin, either fasting or post-load, and while a detailed review of this literature is beyond the scope of this thesis, I have considered certain important aspects of these studies; while some have suggested that insulin is an important cardiovascular risk marker, even after adjustment for other risk factors, others have found no relationship whatsoever. Several factors may help explain these discrepancies.

- *Age:* The age of the population may be important. For instance, in the Busselton study, 60-69 year old males with high (values in the upper 20$^{th}$ percentile) one-hour post load insulin levels had a risk ratio of 2.0 for the six-year incidence of coronary heart disease (14) and a risk ratio of 2.3 for twelve-year coronary heart disease mortality (41) after adjustment for other risk factors. No such relationship was observed in the younger or older groups studied.

  In younger populations, it may be that the risk of experiencing a cardiovascular event during follow-up is too low to demonstrate statistical significance, while in older populations relevant subjects may be excluded on the basis of already having clinically apparent cardiovascular disease. In addition, studies in older subjects may be focussing on "survivors" that are not representative of the general population.

- *Duration of Follow Up:* Several prospective studies have analysed the relationship between serum insulin and cardiovascular disease at more than one time-point. This has demonstrated inconsistencies in insulin's strength as a risk factor. In the first follow up of the Paris Prospective Study (mean follow up 63 months, mean age 49) (42), fasting serum insulin was a significant risk factor after multivariate analysis for the development of coronary heart disease (CHD) (non-fatal MI and coronary related deaths). However, in the 15-year follow up data from the same cohort, no relationship was found between fasting insulin and overall CHD mortality. Two hour post load insulin levels were identified as an independent risk factor, though only when considered as a categorical variable (below or above 452 ρmol/l i.e. the lower limit of the fifth quintile of the distribution) (15). Similarly, in Helsinki policemen (n=982), 9.5 year follow up (43) revealed that elevated plasma insulin one and two hours following a glucose load was an independent risk factor for CHD (CHD death or non-fatal MI), while in the longer term follow up of the same cohort, (44) only an association between one-hour insulin (highest area under the curve (AUC) insulin quintile vs. lower four quintiles) and CHD events persisted, and with less strength than that seen initially ( 5-years hazard ratio 2.36 (1.00-5.57 95%CI), 10-year hazard ratio 2.29 (1.31-4.02 95%CI), 15-year hazard ratio 1.76 (1.09-2.82 95%CI) and 22-year hazard ratio 1.32 (0.89-1.97 95%CI) after adjustment for other factors).

- *Assay specificity:* It has been argued that differential cross-reactivity of insulin radioimmunoassays with insulin precursor molecules, intact insulin and 32,33 split insulin may be a further source of heterogeneity between studies. It is interesting to note that both studies which have used specific insulin assays (the

British Regional Heart Study (45) and a nested-case control study of Canadian males (46)) have found serum insulin to be a risk factor for CHD after multivariate analysis.

- *Ethnicity:* While most studies have focused on Caucasian populations, the evidence for serum insulin as an independent cardiovascular risk factor in other populations is less compelling. Studies of the insulin resistant, obese Pima Indians have demonstrated a low prevalence of cardiovascular disease (47), while fasting plasma insulin was not a predictor of ischaemic ECG abnormalities in either diabetic or non-diabetic members of this ethnic group (48). Similarly, the relationship between plasma insulin and blood pressure that is seen in some Caucasian populations is less strong in other populations (49).

- *Cause specific outcomes:* One factor that may not be initially obvious, though which may explain apparent discrepancies amongst studies, is the cardiovascular endpoint. For example, some investigators have focused exclusively on fatal or non-fatal CHD, while others have used composite endpoints of cardiovascular disease (CVD) in general. Few details of classification of sudden death (i.e. CHD or otherwise) are given by some investigators, which may have a considerable bearing on risk ratios.

- *Adjustment for other risk factors:* Perhaps one of the most important points in this debate is how best to analyse the results. In univariate analysis, insulin may be associated with development of disease. However, after adjustment for related

risk factors (HDL-cholesterol, obesity, blood pressure) statistical significance is often diminished or lost. The mathematics of multiple regression mean that the variable that is most accurately and reproducibly measured will be favoured over related variables that are more difficult to quantify. Standard statistical approaches will not identify a phenotype as an "independent" risk marker if it lies on a chain of definite biological causation and its upstream or downstream regulator has been included in the model. In practice, those risk markers that are fixed (age/sex) or at least partially reversible (obesity, dyslipidaemia, blood pressure, glycaemia) will be entered into models in preference to others.

Factor analysis is a more recent approach which allows for biological interactions amongst variables. Analysis of a cohort of 1069 Finnish subjects revealed that the "insulin resistance" factor (reflecting BMI, WHR, triglycerides, fasting glucose and insulin) was associated with a hazard ratio of 1.33 (95% CI 1.08-1.65) for the development of coronary heart disease death or non-fatal MI at seven year follow-up in men, though carried no increased risk in women (50). Pyorala *et al* ((51) applied the same technique to the Helsinki Policeman Study. After 22-year follow-up, the insulin resistance factor (BMI, subscapular skinfold, AUC insulin during oral glucose tolerance test (OGTT), mean blood pressure and triglycerides) was associated with an age-adjusted hazard ratio of 1.28 (1.10-1.50 95% CI) for CHD risk, and 1.64 (1.29-2.08 95% CI) for stroke risk.

### 1.5.2 Insulin as a cardiovascular risk factor: a meta-analysis

Given the heterogeneous methodology and the apparently contradictory nature of the studies of insulin as a cardiovascular risk marker, a meta-analysis published by Ruige *et al*

(52) was a timely addition to the literature. The analysis was limited to prospective population-based and nested case-control studies which included data on fasting or non-fasting insulin and considered cardiovascular events as outcomes (fatal/non fatal MI, new ECG abnormalities). Twelve datasets were included in the final analysis. Heterogeneity between studies was noted, mainly with regard to insulin assays, ethnic composition of study groups and endpoints. However, an overall relative risk of 1.18 (1.01-1.29 95% CI) for an increase of 50ρmol/l in fasting insulin concentration was derived. Thus it was concluded that hyperinsulinaemia is indeed associated with cardiovascular risk, although the magnitude of the risk was small in comparison with "established" risk factors, especially in non-whites. As in all meta-analyses, there was a potential for publication bias.

From these prospective studies of insulin (fasting or otherwise), it can be deduced either that insulin resistance is not a strong risk factor for cardiovascular disease, or that plasma insulin is not a particularly sensitive or specific marker of insulin resistance. To date, there is only one comparatively small prospective study in which insulin resistance was measured at baseline (53). In this study, cardiovascular end-points occurred in the most insulin resistant tertile of 147 subjects during the 4.7-year follow-up; however, these end-points included peripheral vascular disease and hypertension and the nature (if any) of the risk associated with insulin resistance remains unclear.

### 1.5.3 Cross-sectional studies of insulin sensitivity and cardiovascular disease

Although prospective studies provide better information regarding causation in the relationship between a risk factor and disease, data from cross-sectional studies are also of considerable value, and may in the future provide prospective data. Furthermore, some

studies have measured insulin sensitivity rather than serum insulin concentrations, and have characterised larger numbers of subjects than the prospective trials described above.

In a cross sectional study of 1,373 non-diabetic Finnish subjects (54), increasing quintiles (I+II vs. III+IV vs. V) of fasting plasma insulin in males were associated with an increase in the age-adjusted prevalence of CHD (diagnosed by symptoms or ECG changes). No independent relationship was, however, present between insulin and CHD after adjustment for other cardiovascular risk factors.

The Insulin Resistance and Atherosclerosis Study (IRAS) investigators (55) chose to use a more direct measurement of insulin sensitivity - the frequently sampled intravenous glucose tolerance test with minimal model analysis - rather than potentially misleading surrogate markers. A tri-ethnic population was studied in an attempt to clarify the role of ethnicity in the relationship between insulin sensitivity and atherosclerosis. In all, 542 white non-Hispanic, 457 Hispanic and 398 black subjects underwent measurement of insulin sensitivity and carotid artery intimal media thickness (IMT), the prognostic significance of which has been shown in a healthy population; increasing IMT was associated with increased incidence of myocardial infarction and stroke at six-year follow-up (56). In the IRAS study, the average IMT in Hispanics and non-Hispanic whites was 17$\mu$m thinner for every one-unit increase in insulin sensitivity, once corrected for other cardiovascular risk factors and glucose tolerance. The important finding in this study was that asymptomatic atherosclerosis was associated with insulin resistance in Hispanic and non-Hispanic whites (though not in black subjects); this was only partly explained by adjustment for traditional cardiovascular risk factors, glucose tolerance, fasting insulin and adiposity.

## 1.6 Is insulin atherogenic?

The association observed between plasma insulin levels and ischaemic heart disease has led some investigators to suggest that insulin itself might be atherogenic (57). Proponents of this theory suggest that insulin, as a growth factor, stimulates vascular smooth muscle cell (vsmc) proliferation (58) and lipid synthesis (59). Data from the United Kingdom Prospective Diabetes Study, in which over 3000 subjects with type 2 diabetes were randomised to receive intensive blood glucose lowering treatment modalities, including insulin therapy in approximately 30%, may have refuted this hypothesis (60) in that the insulin treated group, who had iatrogenic hyperinsulinaemia, did not suffer excess cardiovascular morbidity. Reassuring data of a mechanistic nature are also provided from a study of patients with poorly controlled type 2 diabetes who underwent *in vivo* tests of endothelial function prior to, and following, six months of insulin therapy. Insulin therapy, in association with a fall in HbA1c, triglycerides and free fatty acids, resulted in an improvement in both endothelium–dependent and –independent vasodilation (61).

To summarise the role of insulin and insulin resistance as cardiovascular risk factors, the present lack of prospective studies of insulin sensitivity and cardiovascular endpoints means that it is difficult to determine the magnitude of associated cardiovascular risk. While there are data examining serum insulin as a predictor of cardiovascular outcome, the shortfalls of using insulin as a surrogate marker of insulin sensitivity and the heterogeneity between studies limits the applicability of these datasets to this question. Useful data does come from factor analysis studies, where the relationship between clusters of cardiovascular risk factors and endpoints may be studied, thus overcoming some of the limitations of multivariate analysis. The IRAS study is to be commended in its use of a

measure of insulin sensitivity (the insulin suppression test), and has shown that insulin sensitivity is associated with carotid artery atherosclerosis in a cross sectional study. To address the temporal nature of this relationship, the EGIR investigators have co-ordinated the pooling of clamp data from 20 European research centres to create a database that is large enough to consider important epidemiological issues. The group proposes a prospective study of insulin sensitivity and atherosclerotic vascular disease; 1,500 subjects will have detailed metabolic and cardiovascular profile at baseline, then undergo follow up assessments to examine the development of clinical and laboratory markers of atherosclerosis. Clearly this will enable conclusions to be drawn regarding the nature of the relationship between insulin resistance and cardiovascular disease.

## 1.7 Insulin-signalling in health and disease

The above studies have focussed on whole body insulin sensitivity as a variable, and considered its role as a cardiovascular risk factor. As discussed earlier, the generation of such a value must take into consideration a complex series of physiological interactions. As such, only limited interpretations can be made on the action of insulin at the tissue and cellular level. Furthermore, these whole body insulin sensitivity values may be influenced by the effect of changes in blood flow and therefore substrate delivery to metabolically active tissue beds. The study of insulin action in individual tissue, be it vascular or metabolic in nature, generally requires the isolation of that tissue. By examining individual tissues, both in tissue culture and in primary isolates, advances have been made in the characterisation of the intracellular signalling pathways activated in response to insulin stimulation, illustrated in figure 1.1. These events are outlined below, and the potential defects in the pathway are considered as candidates for the underlying abnormality in insulin resistance. It must be emphasised that, despite these considerable scientific advances in characterising the intracellular action of insulin, no single defect has been identified in population studies as being a strong candidate to underlie the majority of cases of insulin resistance and type 2 diabetes seen so commonly in the clinical setting.

**Figure 1.1: Insulin signalling in metabolic tissues**




**Key:** PI 3 kinase, phosphatidyl inositol 3` kinase; PI (4,5)$P_2$, phosphatidyl inositol 4,5 bisphosphate; PI (3,4,5) $P_3$, phosphatidyl inositol (3,4,5) triphosphate.

### 1.7.1 The insulin receptor

The insulin receptor is present in nearly all tissues, though the local concentration varies greatly between tissues. The insulin-like growth factor –1 (IGF-1) and insulin receptor-related receptors are members of the same family, with considerable homology in the intracellular kinase domain, though little similarity in the extracellular binding domain (62). The main insulin responsive tissues, namely the liver, adipose tissue and muscle, have the greatest number of insulin receptors.

The insulin receptor gene is found on the short arm of chromosome 19 (63). The receptor itself consists of two extracellular $\alpha$-subunits and two intracellular $\beta$-subunits that are linked by disulphide bonds.

Activation of the insulin receptor, and the subsequent intracellular second messenger events, are regulated by the phosphorylation of tyrosine residues. Insulin binding to the external $\alpha$-subunit stimulates the intrinsic tyrosine kinase activity of the insulin receptor, and results in the autophosphorylation of key regulatory tyrosine residues. When the insulin receptor is vacant, there is tonic inhibition of the intrinsic kinase activity. Phosphorylation of serine/threonine residues may inhibit insulin receptor activation, thus providing a mechanism for fine control of the insulin receptor involving ligand binding, and the balance of serine and tyrosine phosphorylation.

The insulin receptor also has a role in the internalisation of bound insulin. The insulin-receptor complex is internalised by endocytosis, and while insulin is then degraded, the

receptor recycles to the plasma membrane. Prolonged insulin stimulation may also lead to degradation, and so down regulation, of the receptor (64).

### 1.7.1.1 Defects in the insulin receptor

Insulin receptor gene defects underlie the severe insulin resistance syndromes, such as leprechaunism, the Rabson-Mendenhall syndrome and Type A insulin resistance (reviewed in (65), (66)). These are characterised by acanthosis nigricans, glucose intolerance and abnormalities of growth and development. These receptor abnormalities lie mainly in the absolute number of receptors, the binding of insulin ($\alpha$-subunit) or in the activation of tyrosine kinase activity ($\beta$-subunit). Since the description of the first insulin receptor mutation in 1988, over 50 further mutations have been described (reviewed in (67)). However, though small reductions in receptor number and binding have been observed, as well as defects in receptor and substrate phosphorylation, these seem unlikely to be the primary cause of the insulin resistance seen in type 2 diabetes.

The application of gene knockout technology to the area of insulin action has offered new insights into the role of the insulin receptor and signalling intermediates in insulin action. Homozygous insulin receptor knockout mice ($IR^{-/-}$) have a phenotype characterised by severe insulin resistance, with diabetes, ketoacidosis, hypertriglyceridaemia and growth retardation, followed by death within one week. Mice heterozygous for the deletion, however, have no dramatic increase in the incidence of type 2 diabetes. From a mechanistic standpoint, data from specific tissue knockouts, where the insulin receptor may only be absent in one tissue, are more intriguing. Muscle specific insulin receptor knockout mice (MIRKO) show reduced insulin-mediated glucose uptake into skeletal muscle(68). However, these animals are not diabetic and have normal serum insulin concentrations.

They do display an increase in fat mass and triglycerides. These data demonstrate that near total insulin resistance in skeletal muscle is not sufficient to cause a reduction in glucose tolerance, and suggest that glucose uptake into other metabolically active tissue, such as adipose tissue, must be considerable and may even compensate. In addition, exercise stimulated glucose uptake into skeletal muscle is unaffected in MIRKO mice, implying a distinct pathway for this process.

The insulin receptor may also have a sensing role in the pancreas, as pancreatic insulin receptor knockout mice lose pancreatic insulin secretion in response to a glucose load(69). Lastly liver specific insulin receptor knockout mice are severely insulin resistant, though do not display fasting hyperglycaemia once four months old(70)

### 1.7.2. Insulin receptor substrates

The main substrates of the insulin receptor are the insulin receptor substrates 1-4 (IRS1-4), named in order of discovery. Most is known about IRS-1 and IRS-2, however the chromosomal locations of all the IRS genes have been determined – IRS-1 on chromosome 1, IRS-2 on chromosome 8, IRS-3 on chromosome 5 and IRS-4 on the X chromosome.

#### 1.7.2.1 IRS-1/-2

IRS-1 was cloned in 1991 (71), and is a 180kDa protein that resides in the cytosol. IRS-1 contains many potential sites for tyrosine phosphorylation by the activated insulin receptor. Once phosphorylated, these residues dock proteins that contain src homology 2 (SH2) domains. These proteins include SH-PTP2, Grb2, Nck and the regulatory subunit of the lipid kinase PI3'-kinase.

IRS-2 was purified and cloned in 1995 (72). In comparison with IRS-1, IRS-2 exhibits a highly conserved amino terminus containing a pleckstrin homology (PH) and phosphotyrosine-binding (PTB) domain, though there is less homology of the carboxy terminus. IRS-2 is also tyrosine phosphorylated by the insulin receptor, and binds the regulatory subunit of phosphatidylinositol 3' kinase (PI3'-kinase).

### 1.7.2.2 IRS-3/-4

Initially referred to as pp60, IRS-3 was cloned from rat adipocytes by Lienhard's group (73) in 1997. A 60kDa protein was identified that had been reported to be tyrosine phosphorylated and activate PI3'-kinase in response to insulin. IRS-3 shares 50% structural homology with IRS-1 and –2, in that it has a PH domain, a PTB domain and tyrosine phosphorylation sites. The structurally similar IRS-4 is a 160kDa protein that was cloned from HEK 293 cells by Lienhard's group in 1997 (74), and later from the mouse (75). IRS-3 and -4 share 40-50% homology of the PH and PTB domains with IRS-1 and -2. Like the other IRS proteins, IRS-4 is activated in response to insulin and binds PI3'-kinase. Despite the observation made in over-expression models (76) that both IRS-3 and -4 translocate GLUT4 to the cell surface in adipocytes, Fantin *et al* (75) found no IRS-4 protein in adipose tissue, liver or skeletal muscle, raising questions about its absolute importance.

Unlike IRS-1 and –2, which reside in the low-density microsomal (LDM) fraction of the cell, IRS-3 and 4 are found in the plasma membrane fraction (77) and so contribute to PI3'-kinase activation in this cellular locale. The subcellular distribution of these proteins may be important in the response to insulin, as it appears that activation of PI3'-kinase in the LDM fraction may be more important in mediating insulin action (78). As such, the observations that in IRS-3 null mice (79) there is no defect in growth or glucose transport,

and that in 32D myeloid progenitor cells, transfection of IRS-4 only weakly activates PI3'-kinase (though does stimulate protein kinase B) are all the more interesting (80). In addition, in healthy Danes, (81) IRS-4 polymorphisms were not associated with insulin resistance or type 2 diabetes, while IRS-4 null mice show only mild glucose homeostatic defects with no hyperinsulinaemia or hyperglycaemia (82). There are suggestions that IRS-3 may play a more important role in liver glucose homeostasis (83).

### 1.7.2.3 Defects in the insulin receptor substrates

As the IRS proteins play a central role in insulin-signalling, any deficiencies in their function would be potential candidates in the pathogenesis of insulin resistance.

- ***IRS-1***

It is unlikely that abnormalities of IRS-1 are responsible for type 2 diabetes. Although polymorphisms in IRS-1 have been identified in insulin resistant populations, such as Met 614 Val in severe insulin resistance (84) and Gly 972 Arg in type 2 diabetes (85), the effect of these on glucose tolerance is not clear. The latter polymorphism has a prevalence of 5.8% in normal populations and 10.7% in patients with type 2 diabetes. This defect may lead to a reduction in insulin-stimulated IRS-1 activation of PI3'-kinase through the p85 subunit. Furthermore, mice homozygous for disruption of the IRS-1 gene (86), while displaying a hypertensive, dyslipidaemic (hypertriglyceridaemia with low HDL-cholesterol) phenotype with reduced endothelial function, are not diabetic, and have normal levels of free fatty acids (87). Thus, features of the human insulin resistant phenotype are present in these animals, though they have normal levels of blood glucose. Normoglycaemia is maintained by increased pancreatic insulin secretion, although a heightened intracellular role for IRS-2 is also proposed. In (IRS-$1^{-/-}$) knockout models, reduced insulin stimulated PI3'-kinase activity is seen in skeletal muscle, though not in

liver. Thus, the increase in the amount of IRS-2-associated PI3'-kinase activity is greater in liver than in skeletal muscle. In keeping with these observations, adipocytes from diabetic humans have a reduced total amount of IRS-1. However, in diabetic adipocytes PI3'-kinase is nearly entirely associated with IRS-2 (88), suggesting that while IRS-1 may be the main substrate for insulin stimulation and activation of PI3 kinase, this role may be assumed by IRS-2 or perhaps IRS-3 in insulin resistant states to maintain normal cellular insulin responsiveness.

- ***IRS-2***

In contrast to IRS-1 knockouts, IRS-2 deficient mice display abnormalities of glucose tolerance as early as 3 weeks of age, and by 10 weeks are diabetic (89). In comparison with the (IRS-$1^{-/-}$) mice, far less severe growth retardation is observed, suggesting that IGF-1 may preferentially require the presence of IRS-1 for signal transduction. In neonates, there is no difference in fasting insulin between the deleted and wild type animals, although their blood glucose is elevated, implying insulin resistance. By six weeks of age, however, plasma insulin is greater than in the wild type animals, and the response to the hypoglycaemic action of insulin is reduced (89). The use of clamp studies with measurement of hepatic glucose output demonstrates that hepatic and muscle insulin resistance is present. In addition, and contrary to the (IRS-$1^{-/-}$) mice, the IRS-2 deficient animals show a lack of the $\beta$-cell hyperplasia that would normally accompany peripheral insulin resistance. While this lack of insulin secretory capacity is not sufficient to cause abnormalities of glucose tolerance at birth, over the next 10 weeks, perhaps in association with glucose toxicity, the capacity of the pancreas to overcome peripheral insulin resistance falls. Thus, IRS-2, normally present in the pancreatic ductal epithelium, may also have a role in pancreatic growth. While these observations would favour a major role for IRS-2 disruption in the pathogenesis of insulin resistance, disruption of the IRS-2 gene was

investigated as a candidate mechanism for insulin resistance in the Ashkenazi Jews, and no major role for this gene in determining susceptibility to type 2 diabetes was identified (90).

- ***IRS-3***

IRS-3$^{-/-}$ (79) mice display normal growth, blood glucose, serum insulin and adipocyte glucose transport, though clearly this maintenance of homeostasis may be the result of IRS-1/2 compensation.

- ***IRS-4***

Again, defects in this gene seem unlikely to underlie type 2 diabetes, as despite there being common genetic polymorphisms, no association with type 2 diabetes or insulin resistance was identified in a Caucasian population (81).

### 1.7.3 Phosphatidylinositol 3` kinase

PI3'-kinase is a lipid kinase with two functionally defined subunits-the p85 regulatory subunit, which the IRS proteins bind to via a SH2 domain interaction, and the catalytic (p110) subunit, which phosphorylates the D-3 position of the inositol ring in PI(3)P, PI-(3,4)$P_2$, and PI(3,4,5)$P_3$. Eight polymorphisms of the regulatory subunit have been identified, including p85$\alpha$, p55$\alpha$, p50$\alpha$, p85$\beta$ and p55 PI3'-kinase. These isoforms are differentially expressed in different tissues, and this may be important in determining individual tissues responses to insulin. Activation of PI3'-kinase is necessary (but not sufficient) for the translocation of GLUT4 to the cell surface, and thereby glucose uptake, as inhibition with wortmannin or LY294002 prevents this process (91). In addition to glucose transport, insulin-stimulated activation of PI3'-kinase is also necessary for the suppression of lipolysis, activation of glycogen synthesis, phosphorylation of protein kinase B and stimulation of protein synthesis.

### 1.7.3.1 Abnormalities of PI3`- kinase

Disruption of the gene encoding the p85α subunit is associated, counter-intuitively, with increased insulin sensitivity in mice (92). This may be due to the increased expression of alternative isoforms of this subunit, such as p50α. A polymorphism of the p85 subunit in which methionine is replaced by isoleucine has been identified, however the prevalence of this polymorphism is not greater in diabetic than non-diabetic populations (93).

### 1.7.4 Phosphoinositide-dependent protein kinase -1

PI (3,4,5) $P_3$ binds to and activates the phosphoinositide-dependent protein kinase (PDK-1), which then activates other intermediates such as PKB and various isoforms of PKC (α and β (94) and ξ (95)). In addition, PDK-1 regulates activity of the p70S6 kinase, a regulator of cell proliferation, through PKC ξ activation (96). Emphasising the potential importance of PDK-1, overexpression of PDK-1 in rat adipocytes results in an increase in activation of PKC ξ and an increase in the translocation of epitope tagged GLUT 4 to the cell surface (97). Expression of inactive PDK-1 inhibits both of these processes.

### 1.7.5 Protein kinase B

PKB (also known as akt) is activated in turn by PI3`-kinase-induced stimulation of PDK-1, and is involved not only in glucose transport, but also in the inhibition of glycogen synthase kinase-3, thereby activating glycogen synthase. The processes downstream of PKB activation that stimulate the translocation of GLUT4 from storage vesicles to the cell surface are, as yet, unclear.

### 1.7.5.1 Defects in protein kinase B

As PKB lies downstream of PI3'-kinase, any defect in its action may have considerable importance. In a comparison of six healthy volunteers and six patients with type 2 diabetes (98), the diabetic patients showed reduced insulin sensitivity, measured by euglycaemic hyperinsulinaemic clamp, reduced insulin stimulated glucose uptake into isolated skeletal muscle strips and reduced insulin-stimulated activation of PKB, despite no reduction in expression of the protein. Studies of insulin-signalling in rat skeletal muscle (99) have revealed that activation of PKB is diminished by 60% in media containing 25mmol/l glucose, compared with that of muscles preincubated in a glucose-free medium. Importantly, activation of PI3'-kinase and mitogen-activated protein (MAP) kinase is unaffected. Similar results are obtained from hyperglycaemic Zucker rats (100), in which liver PKB activity is reduced despite normal or increased PI3'-kinase activity. These data imply that, certainly in hyperglycaemia induced insulin resistance, reduced activation of PKB may be of primary importance. Against this, however, are data from Kim *et al* (101), who compared vastus lateralis biopsies from lean, obese non-diabetic, and obese type 2 diabetic patients. They found that insulin activation of protein kinase B isoforms is normal in muscle of obese non-diabetic and obese diabetic subjects, despite decreases of 50% and 39% in IRS-1- and IRS-2-associated PI3'-kinase activity, respectively, in obese diabetic subjects. Thus they concluded that PKB did not have an important role in the genesis of insulin resistance in human skeletal muscle. A later study from this group (102) also showed that in obese Zucker rats, the reduction in insulin-stimulated activation of PI3'-kinase is greater than the reduction in PKB activity, reinforcing the previous study.

### 1.7.6 Events downstream of PI3 kinase/ PDK-1/PKB

While events downstream of PKB activation are not fully characterised, it is clear that PI3'-kinase activation alone is not sufficient to stimulate glucose transport, as evidenced by platelet derived growth factor induced stimulation of PI3 kinase. An additional signal, or accessory pathway, may need to be activated in addition to the generation by insulin of $PIP_3$ (reviewed in (65))

### 1.7.7c-CBL-associating protein; non-PI3`-kinase dependent insulin signalling

Observations suggest that while PI3'-kinase activation is necessary for insulin action, it is not, on its own, sufficient. This has led to the concept of alternative, or parallel, insulin-signalling pathways. One such pathway involves the insulin-stimulated translocation of phospho-c-Cbl to lipid rafts, via its association with c-Cbl-associating protein (CAP). Blockade of this pathway, by overexpression of a dominant negative CAP mutant, blocks insulin-stimulated glucose transport in 3T3-L1 adipocytes (103). Further characterisation of this novel pathway in human tissue is required.

### 1.7.8 Contraction-induced glucose uptake

While it is appreciated that exercise improves insulin sensitivity, the precise mechanism underlying this remains unclear. Early observations in rat hindlimbs demonstrated that contraction-induced glucose uptake may not involve tyrosine phosphorylation of the insulin receptor, suggesting an alternative pathway (104). The observation that obese Zucker rats display resistance to insulin-stimulated glucose transport, though no defect in contraction-induced glucose transport, supported the notion that these pathways may involve different signalling intermediates(105). One intermediate in the contraction-induced pathway may be nitric oxide (the neuronal form of nitric oxide synthase is expressed in skeletal muscle

(106)), with downstream activation of cyclic GMP (107). Activation of this NO/cGMP pathway occurs during skeletal muscle contraction, as demonstrated in rat soleus muscle using the NO donor sodium nitroprusside (108).

Young *et al* ((109)) used soleus muscle from lean and obese Zucker rats to investigate this pathway in insulin sensitive and resistant models. Incubation with a cGMP phosphodiesterase inhibitor increased cGMP and glucose uptake in lean though not obese rats. Maximal NOS activity was also reduced in the obese animals. Lastly, NO donors (sodium nitroprusside) resulted in less increase in cGMP in muscle from obese than lean animals. These data demonstrate a reduction in NO/cGMP activity in the insulin resistant animals.

In primary vascular smooth muscle cell cultures (110), insulin increases expression of mitogen-activated protein kinase phosphatase (MKP-1) by a pathway sensitive to iNOS and cGMP inhibition, and also wortmannin. That this is PI3'-kinase dependent does seem to contradict the data from skeletal muscle. In vascular smooth muscle cells from insulin resistant spontaneously hypertensive rats (SHR), insulin-stimulated induction of MKP-1 expression is reduced due to a decrease in insulin stimulated PI3'-kinase activity and so iNOS expression. Thus a pathway of iNOS-cGMP-MKP-1, downstream of insulin-stimulated activation of PI3'-kinase was proposed.

These data suggest that there may be at least one non-insulin mediated signalling pathway that stimulates glucose uptake, and may in the future provide additional therapeutic targets for insulin-sensitising agents.

### 1.7.9 GLUT4: translocation and trafficking

Insulin stimulates glucose transport by the translocation of a transport protein to the cell surface. In 1989 the protein was identified as GLUT 4, one a family of seven glucose transporters. GLUT4 is the dominant transporter in adipose tissue and skeletal muscle, particularly in red muscle fibres. GLUT 1, the constitutively active glucose transporter, is also present in both of these tissue types (reviewed in (111)).

While there is a reduction in the number of GLUT4 transporters in adipose tissue from diabetic patients, there is no equivalent defect in skeletal muscle, and as such this defect alone is not likely to account for the resistance to insulin stimulated glucose uptake. There may, however, be abnormal translocation of GLUT4 to the cell surface in association with insulin resistance, as demonstrated in muscle biopsies from patients with type 2 diabetes and normoglycaemic insulin resistant subjects (112). Here, in both the basal and insulin-stimulated state, GLUT4 resided in a more dense membrane fraction than in insulin sensitive subjects. The tracking of GLUT4 in its storage vesicles has been aided by the identification of protein markers that co-localise with these vesicles, such as the insulin-regulated membrane aminopeptidases (IRAP), the translocation of which is insulin-responsive and wortmannin-sensitive (113,114).

#### 1.7.9.1 The SNARE hypothesis

One potential defect that may explain mistargeting of GLUT4 in response to insulin lies at the level of the SNARE proteins (soluble NSF (N-ethylmaleimide-sensitive fusion protein) attachment protein receptors). It is proposed in the SNARE hypothesis that the targeting of specific vesicles to specific sites involves the formation of a complex between the membrane SNARE (t-SNARE) and the vesicle (v-SNARE). This has led to the elucidation

of several such membrane (syntaxin-3) and vesicle (vesicle associated membrane peptide-2, -3/cellulobrevin) SNAREs, and the suggestion that defects in the function or expression of these may result in resistance to insulin-stimulated glucose uptake (reviewed in (115)).

### 1.7.9.2 GLUT 4 knockout models

*GLUT 4* $^{-/-}$ mice are hyperinsulinaemic, and so by implication insulin resistant, however they are not diabetic(116). These mice also demonstrate a reduction in adiposity and in circulating non-esterified fatty acids (NEFA). Once these mice have skeletal muscle GLUT 4 restored, glucose metabolism returns to normal, but the defect in adipose tissue metabolism remains. Heterozygous mice (*GLUT 4* $^{+/-}$) (117) develop hyperinsulinaemia, hypertension and hyperglycaemia, though β cell function remains unaffected. Peripheral, though not hepatic, insulin resistance is demonstrable using the euglycaemic hyperinsulinaemic clamp (118). The restoration of skeletal muscle GLUT 4 expression in these animals returns whole body insulin sensitivity to normal (119), and prevents the development of diabetes. In parallel with this, specific skeletal muscle GLUT 4 knockout mice develop hyperinsulinaemia and diabetes. While these studies demonstrate the importance of glucose uptake into the skeletal muscle bed, it is important to note that mice with adipose-selective reduction in GLUT 4 develop liver and skeletal muscle insulin resistance. Thus, while glucose uptake into skeletal muscle has clearly an important role in determining systemic insulin sensitivity, insulin resistance in adipose tissue is also involved in the regulation of phenotype and may play a part in the development of systemic insulin resistance and type 2 diabetes (120)

### 1.7.10 Genetic knockout models: the "two hit" hypothesis

As the diabetic phenotype is characterised by a reduction in pancreatic secretory reserve in association with peripheral insulin resistance, Terauchi *et al* created a model to mimic this by crossing *IRS-1* $^{-/-}$ animals with mice displaying a β cell specific glucokinase (GK) deficiency (resulting in reduced glucose-stimulated pancreatic insulin secretion) (121). These *GK/IRS-1* mice were indeed diabetic, as despite the pancreatic hyperplasia, the GK deficiency evidently prevented sufficient insulin production to overcome the severe peripheral insulin resistance.

### 1.7.11 Obesity, adipose tissue and insulin resistance

In obesity, there are reports of reduced insulin receptor number and tyrosine kinase activity in skeletal muscle and adipocytes (122,123). While weight loss corrects both of these defects in the non-diabetic population, in patients with obesity and type 2 diabetes, weight loss does not restore insulin sensitivity to normal, suggesting an additional defect in type 2 diabetes. It does seem that there is a reduction in IRS-1 associated PI3'-kinase activity in obesity and type 2 diabetes(124); IRS-2 may however be able to compensate (88) and the relevance of this reduction in IRS-1 associated PI3'-kinase is not clear.

80% of patients with type 2 diabetes are obese; the strength of the relationship between obesity and insulin resistance suggests that there may be a direct link between the two, perhaps mediated by factors that are released by adipocytes. Several candidates are proposed, and are discussed below.

### 1.7.11.1 Adipose tissue cytokines

Tumour necrosis factor- α (TNF-α) and interleukin 6 (IL-6) are cytokines with important roles in inflammation, though with more recently appreciated additional metabolic effects. Both are expressed in human adipose tissue, with circulating levels increased in obesity (125). The physiological importance of this elevation in circulating cytokines in unclear, however *in vitro* studies of TNF- α in skeletal muscle show reduced insulin-stimulated IRS-1 tyrosine phosphorylation, and increased serine/threonine phosphorylation, either by an increase in serine kinase or a reduction in serine phosphatase activity (126). Reduced expression of IRS-1 and GLUT4 has also been demonstrated in association with TNF- α (127). Elevated IL-6 and TNF- α may also reduce insulin action by increasing basal intracellular calcium, which has a negative effect on GLUT 4-mediated glucose uptake (128). Interleukin-6 regulates the release of hepatic C-reactive protein, and while the main source of IL-6 remains the activated leucocyte, cross-sectional data that show relationships between CRP, IL-6 and TNF- α as well as between CRP, insulin resistance and obesity (129) support the proposal that there may be a link between low level inflammation and insulin resistance / atherogenesis, with adipose tissue cytokine secretion playing a central role.

Recently two additional adipocytokines have been reported. Low levels of adiponectin (130), an adipocytokine which is reduced in obesity, are associated with insulin resistance in mice. In addition, infusion of this hormone improves insulin sensitivity in lipoatrophic mice. Mechanistically, it has been suggested that expression of this hormone may be partially regulated by a peroxisome proliferator activated receptor (PPAP)-γ dependent pathway. Contrasting findings are reported for resistin, the gene for which is expressed during adipocyte differentiation, though is downregulated in adipocytes exposed to PPAP-γ

agonists. Circulating resistin is increased in obese mice, while administration of resistin to mice impairs insulin action and glucose uptake. Future studies in humans are keenly awaited to clarify the role of these hormones in man

### 1.7.11.2 Non-esterified (free) fatty acids and insulin resistance

Over 30 years ago, Randle (131) hypothesised that high circulating levels of non-esterified fatty acids (NEFA) may induce insulin resistance, possibly by competing with glucose in intracellular metabolism. There is recent evidence to suggest that NEFA induce cellular insulin resistance; using the model of intralipid infusion in rats, Magnan *et al* (132) demonstrated impaired insulin-induced glucose disposal during a clamp. In an elegant series of studies in humans, Dresner *et al* (133) investigated the mechanism underlying NEFA induced insulin resistance in human skeletal muscle. Following heparin and lipid infusion, glucose oxidation and glycogen synthesis fell by 50-60%. Insulin-stimulated IRS-1 associated PI3'-kinase activity was significantly reduced by lipid infusion, implying that NEFA were acting at the level of intracellular second messengers. Further animal studies demonstrated similar findings, but in addition showed an increase in protein kinase ξ activity in response to lipid infusion, which the authors suggested may explain the defects in the insulin-signalling cascade by the process of serine phosphorylation (134).

### 1.7.11.3 Cd 36 and insulin resistance

In 1999, Aitman and colleagues (135) identified a single gene that was defective in the spontaneously hypertensive rat (SHR) and whose protein product was undetectable in the adipocyte plasma membrane of these insulin resistant animals. This protein was Cd36, also known as fatty acid translocase (FAT) and has been shown to play a role in transmembrane long chain fatty acid transport as well as being a receptor for oxidised low density

lipoprotein. Humans with genetic Cd36 deficiency are characterised as having high blood pressure, insulin resistance and hypertriglyceridaemia (136). The prevalence of this genetic defect in the wider population, and the mechanism through which it is related to cellular insulin resistance, remains unknown.

### 1.7.11.4 Peroxisome proliferator activated receptor (PPAR) γ

The recent discovery of a new class of antidiabetic drugs – the thiazolidinediones (TZDs) – has provided a new insight into potential intracellular mechanisms that may underlie obesity and insulin resistance. These agents act on PPAR γ receptors, which are abundant in adipocytes, and increase insulin sensitivity; indeed it appears that the presence of white adipose tissue is necessary for TZDs glucose lowering action(137), while a preferential effect in subcutaneous depots may explain the modest weight gain associated with their use(138). Patients heterozygous for a dominant-negative PPAR γ allele are insulin resistant (139). The mechanism linking PPAR γ with insulin sensitivity is unclear; while they increase expression of Cd36(140), it has also recently been proposed that the hormones resistin (141) and adiponectin may play a role (130).

## 1.7.12 Hyperinsulinaemia and insulin resistance

As hyperinsulinaemia accompanies insulin resistance, elevation in serum insulin may be proposed as playing a part in the development of cellular insulin resistance by reducing receptor expression. Though reversal of hyperinsulinaemia by streptozotocin administration may return insulin receptor expression to normal (142), insulin secretion increases in response to insulin resistance, and so it seems more likely that hyperinsulinaemia is a result rather than a cause of insulin resistance.

### 1.7.13 Counter-regulatory hormones and insulin resistance

The role of hormones such as cortisol, norepinephrine and glucagon in the development of the metabolic syndrome and type 2 diabetes is not clear, however, insulin resistance associated with glucocorticoid action is discussed below and explored in chapter 6.

### 1.7.14 Glucose and insulin resistance

Elevation in blood glucose is known to impair pancreatic insulin secretion, but glucose may also act in peripheral tissues to reduce insulin sensitivity. Glucose, by increasing intracellular diacyl glycerol (DAG), stimulates intracellular PKC, which in turn phosphorylates serine residues on the insulin receptor and reduces activation(143). This process of serine phosphorylation may be a more generic mechanism through which an insulin signal may be "dampened" by reducing the association of the insulin receptor with the insulin receptor substrates (144). Indeed, PI3'-kinase may serine phosphorylate IRS-1, providing a mechanism for fine control and self-limitation of the insulin signal (145). Certainly in hyperglycaemic animal models, reduction in blood glucose has been shown to be associated with an improvement in insulin-stimulated activation of signalling intermediates, such as PKB (146).

## 1.8 Insulin and suppression of lipolysis

In addition to its effects on glucose transport, insulin is also involved in determining the rate of lipolysis in adipocytes. Lipolysis is the breakdown of cellular triacylglycerol (triglycerides) into its constituent parts-one glycerol and three long chain free fatty acids per molecule of triglyceride (reviewed in(147,148)). The formation and breakdown of

triglyceride in the cell is generally the result of a balance between lipolytic (e.g. sympathetic nervous system) and anti-lipolytic stimuli, such as insulin.

As is the case for glucose uptake, insulin stimulates the insulin receptor and recruits the IRS proteins, leading to the activation of PI3`-kinase; this step is necessary for the suppression of lipolysis, as in rat adipocytes (149), inhibition of PI3`-kinase by wortmannin abolishes both insulin-stimulated glucose and the normal suppression by insulin of isoproterenol-induced lipolysis and glycerol release.

Hormone sensitive lipase (HSL), the rate-limiting step in triglyceride lipolysis (150), is activated following protein kinase A (PKA) mediated phosphorylation. While β-adrenergic stimuli increase the activity PKA, insulin inhibits it. Again, as is the case for glucose transport, the insulin pathway downstream of PI3`-kinase is not well characterised, although stimulation of phosphodiesterase 3B may be involved.

### 1.9 Cellular insulin sensitivity: variation with the insulin resistant phenotype

As discussed in section 1.4, the insulin resistance or metabolic syndrome has a classical phenotype of hypertension, obesity and dyslipidaemia, with resistance to insulin stimulated glucose uptake at its core. In the WHO and EGIR criteria for diagnosis of the syndrome, whole body insulin sensitivity, measured using the euglycaemic hyperinsulinaemic clamp, must be in the lowest quartile for the group under study. While it is appreciated that whole body insulin sensitivity varies greatly between subjects (27), it has proven more difficult to study the sensitivity of individual tissues to insulin; it would be anticipated that insulin response at the tissue level would reflect the phenotype, however there are few studies that have examined this in the healthy population.

Kashiwagi *et al* demonstrated a reduction in maximum insulin stimulated glucose uptake, glucose utilisation and basal glucose transport rates in adipocytes isolated from patients with type 2 diabetes compared with normal subjects (151). In a subsequent publication (152), comparison of lean, moderately obese and obese Pima Indians revealed increasing adipocyte insulin resistance across the three groups, and while abnormalities of insulin binding were noted, this was not thought to be the sole factor in the pathogenesis of cellular insulin resistance. Foley *et al* (153) demonstrated that in obese Pima Indians, insulin stimulated glucose transport and antilipolysis were both reduced in the most insulin resistant subjects; in 74 Southwest American (Pima) Indians with normal glucose tolerance, the $ED_{50}$ of insulin for glucose transport and antilipolysis correlated with fasting insulin, two hour insulin and BMI. Again the suggestion was that the cellular defect was located downstream of insulin binding to it's receptor, opposing the findings of Ciaraldi (154), who demonstrated reduced insulin binding in subjects with impaired glucose tolerance (IGT) and type 2 diabetes. Foley used data from Pima Indians to test the reproducibility of these techniques for measuring insulin sensitivity in subcutaneous fat. This type of study is difficult as the nature of the procedure means that two fat samples are rarely obtained from the same subject, though here the needle biopsy technique seemed sufficiently well tolerated for the procedure to be repeated. The coefficients of variation for the $ED_{50}$ of insulin for glucose transport and antilipolysis were 33% and 31% respectively. Resistance to insulin stimulated glucose uptake and antilipolysis was greater in obese diabetic and obese non-diabetic female Pima Indians than in non-obese controls from the same ethnic background. No difference in the $ED_{50}$ for antilipolysis was, however, seen between the obese diabetic and obese non-diabetic groups, suggesting that elevated blood glucose plays

only a minor role in determining insulin sensitivity in adipocytes (155). In this study, there was no difference in insulin binding between diabetic and non-diabetic obese subjects.

While these studies in Pima Indians illustrate cellular insulin resistance, it must be borne in mind that this population is severely obese (mean BMI in (155) =35kg/m$^2$) and insulin resistant. The applicability of these results to an insulin sensitive Caucasian population has been questioned, and though in a comparison of 10 obese Pima Indians and 10 age and weight matched Caucasians, insulin sensitivity in adipocytes was similar (156), in the context of cardiovascular risk, the metabolic syndrome in Caucasians and Pima Indians seem quite different(48). Furthermore, in a comparison of age and weight matched Pima and Caucasian children (157), Pima children had a higher fasting plasma glucose and insulin in association with larger abdominal adipocytes than the Caucasian children.

In two groups of healthy individuals matched for age and weight (158), one group having a family history of type 2 diabetes in two first-degree relatives, subcutaneous adipose tissue lipolysis was measured using microdialysis. Clamp studies demonstrated that the group with a family history of diabetes had a lower insulin sensitivity index than those without. During insulin infusion, subcutaneous adipose tissue glycerol release was greater in the group with a family history of diabetes, in keeping with reduced insulin sensitivity. However, fat cells obtained by needle biopsy prior to the commencement of the clamp showed no difference between groups in basal or insulin stimulated glucose uptake or suppression of lipolysis. Thus the literature remains unclear with regard to the presence of cellular insulin resistance in insulin-resistant, though euglycaemic, subjects.

As well as an association between increases in BMI and insulin resistance, the distribution of fat may also be important. A high waist: hip ratio, representing preferential abdominal deposition of fat, seems more closely associated with insulin resistance in obesity than gluteofemoral adiposity. This may be explained by the observation that adipocytes from visceral and abdominal fat deposits are more prone to lipolysis (159), and therefore the release of NEFA that may contribute to insulin resistance. In a study of 930 obese patients, higher triglycerides, glucose and insulin were observed in subjects with abdominal obesity (highest waist/hip ratio) vs. a more peripheral distribution (160). Landin *et al* (161) demonstrated that in obese postmenopausal women, adipocytes obtained from those with a high WHR were larger and more sensitive to isoproterenol induced lipolysis than cells from obese women with a lower WHR. In this study, there was no difference in the antilipolytic effect of high dose insulin between obese and lean women.

Most lipolytic stimuli to adipose tissue act via the cell surface adrenoceptors. Catecholamines stimulate lipolysis via the $\beta$1, $\beta$2 and $\beta$3 and inhibit lipolysis via the $\alpha$2 adrenoceptors (162). Lonnqvist *et al* (163) compared omental adipose tissue from obese and lean women, and found that norepinephrine-induced lipolysis was twofold greater in the obese women. These women also had greater $\beta$3 adrenoceptor sensitivity, which has its main effect in visceral adipocytes (164), and reduced $\alpha$2 adrenoceptor sensitivity. Thus the increase in NEFA release in obesity may not only be due to reduced insulin suppression of lipolysis, but also involve increased sensitivity to lipolysis in visceral fat that may increase portal NEFA.

Free fatty acid turnover is not only important in adipose tissue. Bolinder *et al* (165) used the technique of microdialysis to examine glycerol concentrations in skeletal muscle,

adipose tissue and venous plasma in lean and obese volunteers. Fasting glycerol in all three compartments was 50% higher in the obese group, however the important observation was that significant amounts of glycerol were released from skeletal muscle as well as adipose tissue.

## 1.10 Insulin as a vascular hormone

The previous section focused on the relationship between the metabolic effects of insulin at a cellular and whole body level. However, the realisation that insulin is also active in the vasculature has raised questions about the relationship between metabolic and vascular insulin responsiveness. It has been suggested that a defect in the action of insulin in the vasculature may provide a more direct link between insulin resistance, hypertension and vascular disease.

Though insulin increases fluid retention and facilitates sympathetic neurotransmission, two models of hyperinsulinaemia do not support a direct relationship with hypertension; in dogs infused with high physiological doses of insulin for 28 days, no elevation in blood pressure was observed (166), while in patients with insulinoma, there is no association with hypertension (167). As such, insulin may have a direct vasodilatory effect, preventing increases in blood pressure by lowering systemic vascular resistance. Laakso *et al* (168) demonstrated that insulin caused a dose-dependent increase in limb blood flow, and went on to show that in obese subjects with reduced insulin-mediated glucose uptake there was an associated reduction in insulin-mediated increments in limb blood flow. Thus the vascular and metabolic actions seemed related. Subsequent studies confirmed that, as well as in obesity, vascular insulin resistance was also present in non-insulin dependent diabetes (169) and hypertension (170).

### 1.10.1 Insulin as a vasodilator: the role of the endothelium

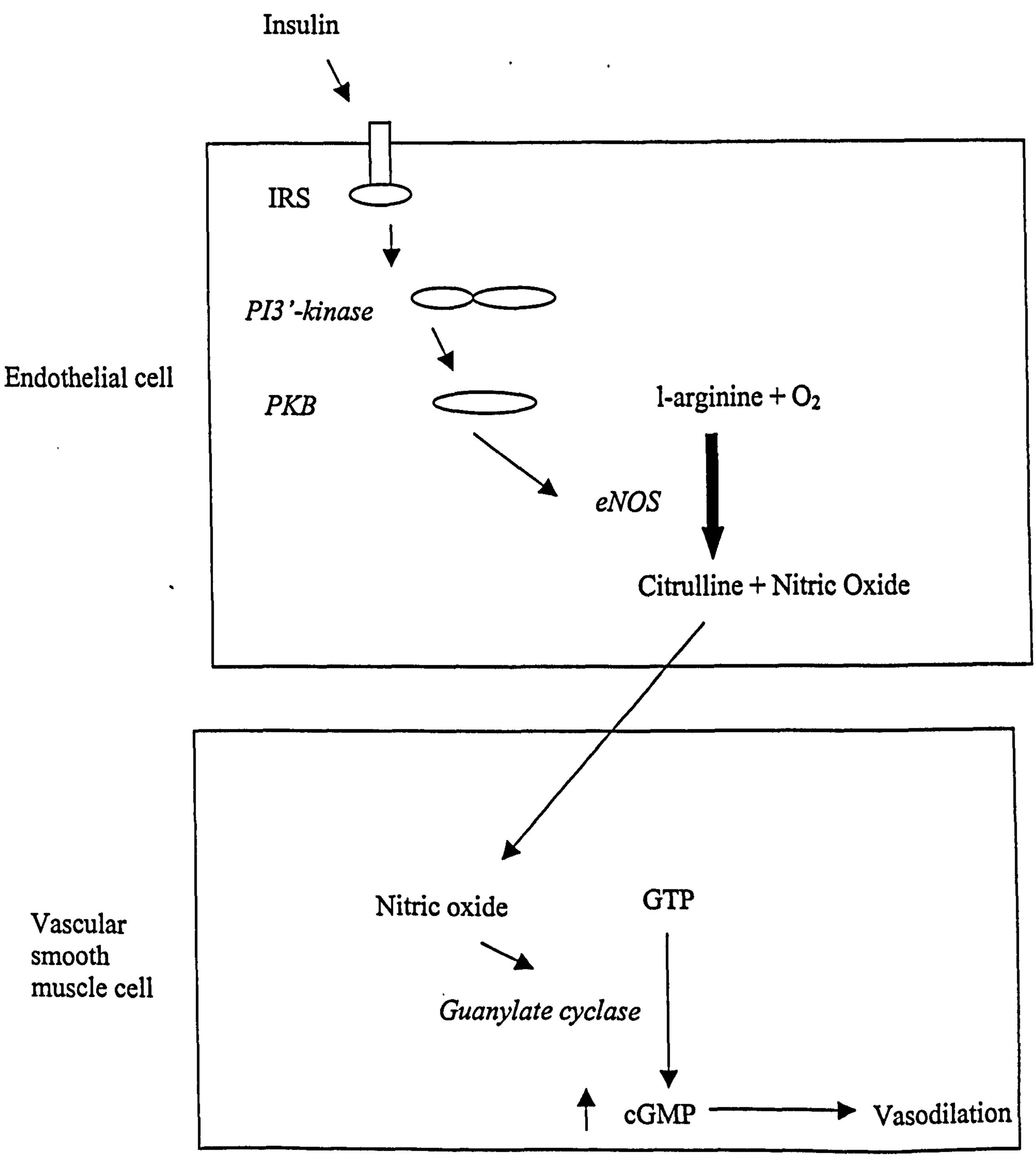
The above early studies demonstrated that insulin is indeed a vasodilator, however they provided no mechanistic information. Insulin may vasodilate by modulating vascular

responsiveness to the $\alpha$ and $\beta$ adrenoceptors (171), and by direct effects on vascular smooth muscle membrane ion channels involved with vasorelaxation.

Most interest has however focussed on insulin's action as a vasodilator involving the endothelium, a cellular monolayer lining the vasculature. Far from being an inert barrier, the endothelium responds to a wide range of stimuli by secreting biologically active agents involved in the maintenance of vascular tone, platelet aggregation, vascular thrombosis and inflammation.

One such agent was identified as the endothelium-dependent vasodilator- nitric oxide (NO). NO is synthesised in the endothelium by nitric oxide synthase (NOS) using L-arginine as a substrate, a process that is inhibited by the addition of L-NMMA, a stereospecific inhibitor of NOS that competes with L-arginine.

**Figure 1.2: Insulin-mediated vasodilation, the endothelium and vascular smooth muscle.** IRS, insulin receptor substrate; PI3'-kinase, phosphatidylinositol 3' kinase; PKB, protein kinase B; eNOS, endothelial nitric oxide synthase; GTP, guanosine triphosphate; cGMP, cyclic guanosine monophosphate.



Acetylcholine induced vascular relaxation is NO mediated (172), and requires the presence of an intact endothelium (173), implying that NO is endothelium derived. Insulin induced vasodilation is similarly endothelium dependent and at least partly NO mediated. Steinberg (174) found that in normotensive, non-diabetic volunteers, the infusion of L-NMMA into the femoral artery resulted in a 20% fall in leg blood flow at baseline, and a 40% fall during euglycaemic hyperinsulinaemia, essentially returning flow to baseline levels. These percentages were assumed to be the proportion of flow that was NO dependent in basal conditions and during hyperinsulinaemia respectively. Importantly, hyperinsulinaemia had no effect on sodium nitroprusside (endothelium independent) vasodilation. Reinforcing insulin's role as an endothelium dependent vasodilator, in rat cremaster muscle arterioles (175), insulin-mediated vasodilation is completely abrogated by the removal of the endothelium.

In disease states such as hypertension, type 2 diabetes and obesity, there is clearly resistance to the metabolic effects of insulin, and this appears also to be the case for endothelial insulin sensitivity. Laakso's early study demonstrated that insulin-induced vasodilation was reduced in obesity, though did not prove conclusively that this was secondary to an endothelial defect. However, increases in leg blood flow in response to methacholine were 40% lower in obesity and 55% lower in type 2 diabetes compared with controls(176). The methacholine response was augmented during euglycaemic hyperinsulinaemia only in the lean controls. In contrast, the sodium nitroprusside vasodilatory response was comparable in all three groups. Further evidence relating metabolic insulin sensitivity and endothelial function came from the observation that there was a correlation between metabolic insulin sensitivity and basal nitric oxide production

(measured by the reduction in forearm blood flow following intra-arterial infusion of L-NMMA) (177).

### 1.10.2 Endothelial function and the metabolic syndrome

Many studies have examined the association of endothelial function with features of the insulin resistant phenotype

- *Obesity and type 2 diabetes:* In obesity and type 2 diabetes, basal methacholine induced leg blood flow is reduced compared with controls. Additionally, during euglycaemic hyperinsulinaemia, these groups have no augmentation of methacholine induced leg blood flow, while sodium nitroprusside induced vasodilation is comparable between groups. Thus, obesity and type 2 diabetes are associated with reduced endothelial dependent vasodilation, and normal endothelium independent responses (176).

Not all studies have found a reduction in endothelial function in association with insulin resistance and type 2 diabetes (178) however, some groups demonstrating no variation from normal volunteers in the vascular effects of acetylcholine.

- *Dyslipidaemia:* The relationship between dyslipidaemia and the endothelium has been studied at some length. Mechanistically, a dysfunctional endothelium may be related to dyslipidaemia via the local expression of LDL receptors and the presence of lipoprotein lipase. In the insulin resistance or metabolic syndrome, there is an excess of easily oxidised, small, dense LDL, which reduces NOS expression in platelets. HDL cholesterol may offset the negative effect of oxidised LDL on the

endothelium, however relative amounts of this lipoprotein are reduced in the metabolic syndrome as discussed earlier. An excess of free fatty acids in the systemic circulation, as is seen in insulin resistance, as well as those released locally by the action of lipoprotein lipase, may have a deleterious effect on endothelial function (179).

- *Hypertension:* Endothelium mediated relaxation is reduced in hypertension, as demonstrated in the reduction in forearm vasodilation seen in response to intra-arterial acetylcholine in hypertensive patients (180). L-NMMA infusion into hypertensive volunteers, either alone or in combination with acetylcholine, has less effect on basal forearm vascular flow than in normotensives. These data suggest that both basally and in response to endothelium dependent vasodilators, there is an abnormality in nitric oxide availability in hypertension (181). The observation that there is also reduced endothelial response to bradykinin, another endothelium derived vasodilator, in hypertension, implies that the underlying abnormality is not confined to a particular intracellular pathway, rather that the endothelium has a general defect that is involved in mediating the response to many extracellular stimuli (182). Furthermore, there does not seem to be a defect in the availability of nitric oxide substrate; in hypertensive patients, the defect in endothelium-dependent vasodilation is not reversed by L-arginine infusion (183) .Not all studies concur with these findings however; some studies have found no difference in forearm vascular response to intra-arterial acetylcholine between hypertensive and normotensive volunteers (184).

Thus, metabolic insulin resistance and endothelial function seem to be linked in the majority of studies. It is more difficult to uncover the temporal relationship between these features, which would allow speculation regarding causality. In a recent study (185) comparing three groups– healthy normotensives, non diabetic hypertensives and type 2 diabetics- well matched for age and lipid profile, no difference was demonstrated between the three groups with respect to insulin sensitivity or endothelial function. There was however a correlation between metabolic insulin sensitivity and endothelial function on combining the data from all three groups. This suggests that there may be an as yet unidentified, and perhaps unifying, factor(s) that affects both metabolic insulin sensitivity and endothelial function similarly, irrespective of glycaemic or blood pressure status.

### 1.10.3 Insulin-signalling in the vasculature; the cellular interface between metabolic and vascular insulin resistance

The culture of human endothelial cells has led to a greater understanding of insulin action in vascular cells, and has highlighted similarities and differences between vascular and metabolic insulin action. Zeng et al (186) demonstrated a dose dependent increase in NO release from cultured human umbilical vein endothelial cells (HUVECs) treated with insulin, and found that this action shared similar properties with the better-characterised insulin signal in muscle and adipose cells. They showed that inhibitors of tyrosine kinase, NOS and PI3'-kinase reduced insulin-stimulated release of NO in these cells. In bovine aortic endothelial cells (187), physiological insulin concentrations increased endothelial NOS mRNA, protein and activity over a 2-8 hour time period, an effect that was again sensitive to PI3'-kinase inhibitors, and also to PKC$\beta$ overexpression. Reinforcing the importance of these signalling intermediates in vascular insulin responsiveness, overexpression of insulin receptors into HUVECs (188) resulted in a 3-fold increase in

insulin stimulated NO production. In cells overexpressing a tyrosine kinase-deficient mutant insulin receptor, this effect was not seen. Similarly overexpression of PI3'-kinase or PKB mutants inhibited insulin stimulated NO production.

Extending these observations to models of insulin resistance, in vascular tissue from obese (insulin resistant) and lean (insulin sensitive) Zucker rats (189), insulin stimulated tyrosine phosphorylation of the insulin receptor, IRS-1 and –2 was reduced in obese compared with lean animals. In aortic tissue from obese animals, association of p85 with IRS-1, IRS-1 associated PI3'-kinase activity and insulin stimulated serine phosphorylation of PKB were all reduced, however there was no reduction in insulin-stimulated activation of MAP kinase, implying selective insulin resistance. The insulin-stimulated activation of PKB in HUVECs has also been suggested as having an important role in the apoptotic suppressive effect of insulin (190).

The physiological role of insulin as a vasodilator, and the influence of insulin-mediated vasodilation in determining glucose uptake, remains unclear. Scientific debate continues surrounding this important topic. Some groups suggest that insulin action in various tissues may be influenced by similar defects given the commonality of the insulin-signalling cascade in vascular, muscle and adipose tissues. Data from endothelial cell culture is discussed below that seem to support this notion. Other groups have proposed that a reduction in insulin-mediated glucose uptake may be at least partly the result of a reduction in the sensitivity of vascular tissue to insulin's vasodilatory stimulus – the concept of substrate delivery (reviewed in (191)), though the strength of this observation may be influenced by the methodology employed (192).

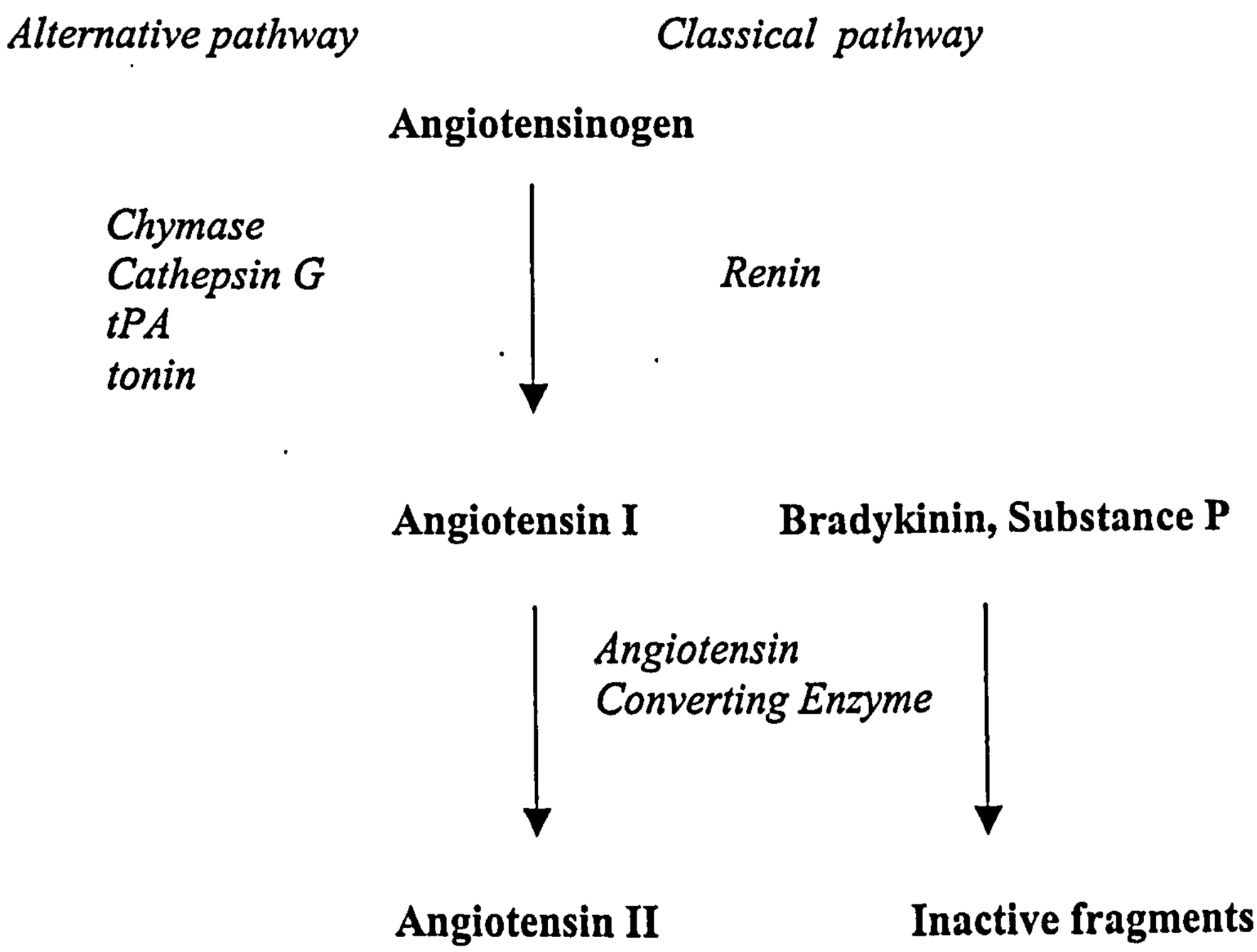
Having discussed the definition, associations and risk associated with insulin resistance, and considered the normal and deranged processes of insulin action, I will go on to examine the role that two endocrine systems – the renin angiotensin system (RAS) and the hypothalamo pituitary adrenal (HPA) axis – may play in determining endogenous insulin sensitivity.

## 1.11 The renin angiotensin system and insulin sensitivity

### 1.11.1 Overview

Circulating angiotensinogen (AGT) is synthesised predominantly in the liver, though in recent years it has become apparent that an increasing number of alternative tissues also have the capacity to secrete this initial substrate in the formation of angiotensin II (AII). The protease renin, which is secreted by the juxtaglomerular apparatus in the kidney, converts angiotensinogen to angiotensin I. Renin is released in response to hypovolaemia or impaired renal blood flow, beta adrenergic stimulation and sodium depletion. Angiotensin I is then converted to angiotensin II by either the action of Angiotensin Converting Enzyme (ACE), a metalloprotease, or through the "non-ACE" pathway, involving the chymostatin-sensitive AII generating enzyme, chymase, cathepsin G, tissue plasminogen activator (tPA) and tonin (193,194) (figure 1.3). The relative contribution of each pathway varies between tissues and pathological states, however it has been suggested that the chymase pathway may convert up to 60% of angiotensin I (195). That the two pathways are distinct is confirmed in studies demonstrating an additive effect of chymase and ACE inhibitors. In addition, the relatively non-specific ACE also deactivates bradykinin, providing an important difference between the action of ACE inhibitors and angiotensin II type 1 (AT1) receptor blockers.

**Figure 1.3: The classical and alternative pathways for conversion of angiotensinogen to angiotensin II in humans.**



### 1.11.2 Local renin angiotensin system

In the last 15 years it has become clear that certain hormone systems, in addition to their systemic control of secretion and feedback loops, have sufficient levels of production in local tissues to support the notion that local, self regulating, systems exist. These may have distinct stimuli and inhibitors of synthesis, and may behave independently of the systemic system. One such hormonal system appears to be the renin angiotensin system; indeed, to date, 12 extrahepatic tissues have been identified in the rat that have detectable angiotensinogen and renin mRNA, including the kidney, heart, adrenal glands, adipose tissue and the gonads (reviewed in (196,197).

Locally generated AII may act in several ways: it may have a classical endocrine effect at distant receptors, it may have a more local paracrine or even autocrine effect, and it may act intracellularly. It is unlikely that the circulating levels of AII are influenced to a great degree by these local systems, however they allow tissues to create very high, or low concentrations of ambient AII, which may be important in determining local blood flow and metabolism.

I will now focus on the evidence for the presence of a local RAS in adipose tissue, the role that the systemic and local RAS may have in adipocytes, and the potential for crosstalk between the AII and insulin-signalling cascade that may exist in these and other cells.

### 1.11.3 The renin angiotensin system in human adipose tissue

#### 1.11.3.1 Constituents of the pathway

The first groups to describe adipose tissue as a site expressing elements of the RAS did so while studying the local expression of the AGT gene in the vasculature and its supporting tissues. AGT mRNA was detected in rat periatrial and periaortic brown adipose tissue using *in situ* hybridisation and Northern blot analysis(198). The local expression of AGT was found to be under the influence of systemic factors, as nephrectomy increased levels in interscapular and periaortic adipose tissue, while ACE inhibition increased only the levels in interscapular tissue.

More sensitive molecular biological techniques, such as polymerase chain reaction (PCR) amplification of mRNA, have provided evidence of widespread expression of components of the RAS. PCR analysis of DNA from rat interscapular brown adipose tissue confirmed AGT gene expression, however it initially proved more difficult to confirm the presence of the renin gene, despite Western blotting of adipose tissue demonstrating the presence of two isoforms of renin. It was postulated that perhaps the sensitivity of the PCR was not sufficient to detect the expression of the renin gene(199), and to date only one group has identified the presence of renin mRNA in adipose tissue. In a study examining adipose tissue from obese subjects, a Swedish group demonstrated the presence of AGT in isolated adipocytes, along with mRNA for both renin and ACE. Transcripts for cathepsin D and G, components of the non-RAS, were also present (200) In a larger study (201), expression of AGT, ACE and the AT1 receptor genes was again confirmed in human subcutaneous and omental adipose tissue samples from a non-obese population. Though no mRNA was found for the renin gene, chymase and renin binding protein genes were expressed.

### 1.11.3.2 Angiotensin II receptors

Although adipose tissue may be able to generate AII locally, any autocrine or paracrine effect of this hormone on adipose tissue metabolism requires the presence of specific receptors that bind AII. Crandall *et al* (202) confirmed AII binding in rodent epididymal adipocytes, and went on to show that this was displaced by a specific AT1 receptor antagonist, suggesting that the observed binding was to the AT1 receptor (203). Using similar binding techniques in human adipocytes, the presence of high affinity AII binding, displaced by type 1 though not type 2 blockers, was subsequently confirmed (204).

## 1.11.4 The physiological role of the RAS in adipose tissue

### 1.11.4.1 Relationship to body weight and obesity

In an elegant series of carefully controlled experiments, Cassis *et al*(205) found that, in rats matched for caloric intake, low dose AII infusion over a 14 day period resulted in an increase in systolic blood pressure and lack of weight gain. This lack of weight gain appeared to be independent of AII's effect on blood pressure. Explantation of organs from AII infused rats suggested that these animals had a reduction in the relative mass of retroperitoneal white fat and diaphragmatic muscle. Other groups have however suggested that AII's effect on body weight may be partially anorectic. Rats studied after 14 days of AII infusion at pressor doses lost 18% of their body weight after seven days and maintained this weight at the end of the second week, whereas sham infused rats continued to gain weight over the study period. This weight loss was prevented by co-administration of losartan, though not hydralazine, suggesting an AT1 mediated effect that was not pressor dependent. Analysis of eating patterns in the studied animals showed that AII infused rats' food intake was 25% of those receiving a sham infusion. After matching for

food intake, 74% of the observed weight loss was accounted for by differences in intake, while the remainder seemed secondary to an alternative property of AII (206).

These observations suggest that the RAS has a potentially important role in the regulation of body weight and fat mass. This may be partially mediated by a central, anorectic effect via the AT1 receptor. There may also be effects in adipose tissue, possibly by local potentiation of the sympathetic nervous system.

The adipose tissue RAS seems also to be influenced by nutritional status. In mice subjected to two days of fasting, local expression of AGT mRNA falls, while on refeeding this is restored then rises to double the levels seen in control fed animals. Epididymal fat from ob/ob and db/db mice secretes twice as much AGT per cell as age matched controls. Taken together, these findings demonstrate that the regulation of AGT synthesis in adipose tissue is to some extent dependent on nutritional status, and suggest that in genetically obese animals, this situation may be exaggerated (207). It may be that local AGT secretion has a regulatory role in adipose tissue perfusion, modifying the release of fatty acids into the systemic circulation depending on nutritional status.

### 1.11.4.2 Angiotensin II and prostaglandin synthesis

Prostaglandin $PGI_2$, and to a lesser extent $PGE_2$ and $PGF_{2\alpha}$, are produced by isolated adipocytes in response to noradrenaline-induced lipolysis (208). This effect is inhibited by insulin (209). Arachidonic acid, the substrate for prostaglandin synthesis in adipocytes, may be derived from two sources – cellular phospholipids and triglycerides. In isolated rat (210) and human (211) adipocytes, AII, bradykinin and vasopressin all stimulate the production of $PGI_2$ from adipocytes, with no associated rise in $PGE_2$ or glycerol (210). As

$PGI_2$ is a potent vasodilator, release of this prostaglandin in response to AII may modify the local perfusion of adipose tissue. Similarly, the AII regulated release of $PGI_2$ from perivascular and visceral fat in other tissues or organs may regulate flow to various vascular beds (212). These *in vitro* findings have been reproduced *in vivo* using *in situ* microdialysis of rat peri-epididymal fat. Through an indomethacin sensitive mechanism, AII increased local production of 6 keto-$PGF_{1\alpha}$, a stable degradation product of $PGI_2$, with no associated effect on $PGE_2$ or glycerol(213). This suggests that, in contrast to isoproterenol or forskolin, which have a cAMP mediated lipolytic effect and result in glycerol accumulation, AII mediates its effect on $PGI_2$ production through an alternative route, perhaps via phospholipase $A_2$, releasing arachidonic acid from cellular lipids. There is some evidence that this is indeed the case; in isolated rat adipocytes, isoproterenol induced prostaglandin production is sensitive to insulin, presumably by an antagonistic effect on cAMP production, however the AII induced production of $PGI_2$ is not inhibited by the addition of insulin (214).

### 1.11.4.3 Adipocyte differentiation

In co-culture of preadipose and adipose cells, the addition of AII resulted in an increase in the differentiation of the preadipose cells. Addition of AII to undifferentiated cells alone had no effect, as had the addition of AII to the co-culture experiments in the presence of an AT2 blocker, implying that this effect required stimulation of differentiated cells at the AT2 receptor. Activity of the enzyme glycerol-3-phosphate dehydrogenase (GPDH), the rate limiting step in triglyceride synthesis (reviewed in (215)) and an indicator of differentiation, rose fourfold in the preadipose cells. This process was again sensitive to cyclooxygenase inhibition and to the addition of antibodies directed against the stable analogue of $PGI_2$. Therefore, AII stimulates the mature adipocyte, promoting release of

$PGI_2$, which then stimulates preadipocytes at the AT2 receptor and promotes differentiation.

Further investigations, using the murine 3T3 L1 adipocyte cell line, which express the AT2 receptor, reinforce AII's role as an adipogenic hormone, and provide some insight into its intracellular action(216). 48 hours of physiological levels of AII treatment resulted in increased activity of both GPDH, and fatty acid synthase (FAS), the rate-limiting step in long chain saturated fatty acid synthesis, and led to an increase in intracellular triglyceride stores. This process was abolished by the addition of the AT2 receptor blocker P-186. Parallel experiments in human adipocytes also demonstrated that 72 hours of exposure to AII results in elevation of FAS, and to a lesser extent GPDH activity.

In summary therefore, treatment with AII, acting at the AT2 receptor in differentiated adipocytes, and perhaps at the AT1 receptor in preadipocytes, results in the maturation and differentiation of cells into adipocytes.

In addition to AII's effect on adipocyte differentiation via production of $PGI_2$, the degree of differentiation of adipose tissue is an important determinant of the expression of the RAS in adipose tissue. Cultured adipocytes express AGT mRNA depending on the method and extent of cellular differentiation; the amount of AGT mRNA in 3T3-F442A adipocytes increases with the degree of differentiation into adipocytes(217). Glucocorticoids also influence expression of these components; in cultured Ob1771 cells exposed to dexamethasone(218), levels of AGT mRNA and protein synthesis increased 12 fold. Similar results were observed in explanted rat adipocytes incubated in the presence or absence of dexamethasone. Fatty acids, which differentiate adipocytes via the PPARs,

may have a similar role, as treatment of preadipocytes with fatty acids resulted in a 17.6 fold increase in cellular AGT mRNA after 48 hours (219).

Thus a situation exists in which a stimulus to adipocyte AII production, such as feeding or obesity, may increase the differentiation of adipose tissue. Once fully differentiated, adipocytes are then more able to produce AII locally by the increased expression of AGT mRNA, which may then increase differentiation of preadipocytes, and the formation of triglyceride stores. Once able to produce AII, these adipocytes may also regulate local blood flow and nutrient flux, both by the direct effect of AII on the vasculature, and perhaps also by secondary production of other vasoactive hormones such as the prostaglandins.

### 1.11.4.4 Thermogenesis

Mammals have a highly developed adaptive response to cold exposure, central to which is the sympathetic nervous system, involving an increase in metabolic rate, circulating catecholamines, and sensitivity of beta adrenoceptors. In addition to an increase in blood pressure, changes also occur at the tissue level, and in particular an increase in the thermogenic interscapular brown adipose tissue (ISBAT), is seen. These adaptive response are prevented by captopril therapy (220). In cold exposed male Sprague Dawley rats, plasma AII rises acutely, perhaps secondary to an increase in sympathetic activity, then falls to baseline within 24 hours before rising again after seven days (221). AT1 receptor density in ISBAT also increases, along with ISBAT tissue mass, while in some other tissues, such as lung, no changes in AT1 receptor density occur, implying tissue specificity The elevation of plasma AII, though not AT1 receptors, depends on the associated increase in food intake following cold exposure.

These data demonstrate that the adaptive response to cold in rodents involve changes in the systemic and adipose tissue RAS. These changes are particularly important in ISBAT, where local AII may enhance the thermogenic response to sympathetic stimulation. Furthermore, in models of defective thermogenesis, such as the Zucker rats, this process is disrupted, and may explain in part the development of obesity by defective energy expenditure. The relevance of this homeostatic system to human physiology is questionable, as humans have no brown adipose tissue, however it does provide an example of the physiological regulation and role of the adipose tissue RAS.

### 1.11.5 The renin angiotensin system and the endothelium

Cardiovascular risk factors such as hypertension, type 2 diabetes and hypercholesterolaemia are associated with endothelial dysfunction, and as ACE inhibitors may have an effect on cardiovascular mortality in high risk patients (222), possibly outwith their antihypertensive action, the possibility arises that they may act by improving the function of the endothelium.

In a hypertensive rat model of endothelial dysfunction, Clozel *et al* (223) showed that treatment with ACE inhibition improved this defect in isolated aortic rings. Aortic vasodilation in response to acetylcholine and bradykinin (224) was greater in ramipril treated rats than those receiving hydralazine or placebo. The ramipril effect was negated by the co administration of a bradykinin antagonist. Thus it was suggested that ACE inhibition improves endothelial function, most likely by a reduction in bradykinin breakdown. Certainly, bradykinin is a potent vasodilator and stimulates the production of NO, $PGI_2$ and possibly the endothelium dependent hyperpolarizing factor (EDHF), all of which are vasodilators (reviewed in (225)).

Creager *et al* ((226)) found no improvement in endothelial function in association with ACE inhibition, measured by recording the changes in forearm blood flow in response to local arterial methacholine infusion, despite a significant effect of ACE inhibition on systemic blood pressure. In a randomised cross over study of patients with CHD (227), comparing eight weeks of quinapril, enalapril, losartan and amlodipine, only quinapril (an ACE inhibitor with high affinity) was associated with an improvement flow mediated vasodilation. In hypercholesterolaemic patients, six months of lisinopril therapy was associated with an improvement in both endothelium dependent and independent

vasodilation, when compared with placebo, though of course this was in association with a fall in systemic blood pressure (228).

Most of the actions of AII in the endothelium seem to be mediated by the AT1 receptor. Certainly in cultured human umbilical vein endothelial cells, the AT1 receptor is expressed and binds AII (229), though this may be lost at higher passages (230). Using reverse transcriptase (RT) PCR, however, Zhang *et al* ((231)) were able to identify the presence of both the AT1 and 2 receptors in HUVECs.

AII may have a direct deleterious effect on the endothelium, by increasing the production of superoxide anions, which in turn reduce levels of nitric oxide and $PGI_2$ (232), (233). The main source of vascular superoxide is from reduced nicotinamide-adenine dinucleotide (NADH)-dependent oxidase. In HUVECs, AII exposure for one hour induced a dose-dependent increase in superoxide generation that was inhibited by the addition of an AT1 receptor blocker, and to a lesser extent by AT2 blockade (231).

AII may also have additional effects on vascular homeostasis, by acting as a stimulant to the production of local athero- and thrombogenic factors, such as PAI-1 (234), PDGF (235), Endothelin –1 (236) and E-selectin (237) from the endothelium.

An additional mechanism through which AII's effect on the endothelium may be harmful has been proposed in relation to the atherogenic, oxidised form of LDL-cholesterol, receptors for which are present in the endothelium (238). Incubation of human coronary artery endothelial cells with AII increased the expression of these receptors, and so the cellular uptake of LDL, and was associated with cell injury, measured by lactate

dehydrogenase (LDH) release. This effect was blocked by AT1 receptor antagonism *in vitro*, while in internal mammary artery biopsies from patients treated with ACE inhibitors prior to surgery, there was a reduction in the mRNA for these receptors (239).

While the focus of these papers has been on the effects of AII on endothelial function, the recently reported randomised aldactone evaluation study, which identified a reduction in mortality in patients with chronic heart failure treated with spironolactone in addition to their conventional therapy (240), has prompted investigation into the role of aldosterone antagonism. In a randomised, double blind, placebo-controlled crossover study in patients with chronic heart failure (241), one month of spironolactone therapy improved endothelium dependent vasodilation in the forearm, possibly by increasing NO bioavailability.

Thus, there is increasing evidence to support the notion that AII, and perhaps aldosterone may have deleterious effects on the vasculature, possibly mediated at the level of the endothelium.

### 1.11.6 Dietary sodium restriction

#### 1.11.6.1 The metabolic effects of dietary sodium restriction; an overview

There is now a wide literature concerning the metabolic effects of a low sodium diet. Despite the recommendations by guidance bodies, there is little or no prospective evidence to support the notion that a low salt diet prolongs life (242). This is despite the well-documented reduction in blood pressure that accompanies dietary sodium restriction (243), which would be anticipated to be associated with a reduction in cardiovascular mortality. As such, interest has focused on the metabolic effects of dietary sodium restriction that may be deleterious; possibilities include a reduction in insulin sensitivity, adverse effects on lipid profile and stimulation of counter regulatory hormones. Of particular relevance here is the effect on insulin sensitivity, as this may be a direct consequence of activation of the RAS, and as such these data are reviewed below.

The gold standard investigation of insulin sensitivity, as outlined earlier, is the euglycaemic hyperinsulinaemic clamp. Similarly, the ideal experimental design is a double blinded, randomised, placebo controlled trial of crossover design that compares low and high salt phases. Not all the trials have used either of these techniques or designs, and so the relative merits of each study must be considered individually.

#### 1.11.6.2 Low sodium diet and lipids

Early studies were designed to clarify the relationship between dietary sodium reduction and plasma lipid profile. To observe an effect on insulin sensitivity was not the primary goal, and as such simple serum insulin was used as a marker of insulin sensitivity. In a large study of 19-78 year old non-obese normotensive subjects (244) entered into a single blind crossover study comparing seven days of 20mmol/day and 300mmol/day sodium

intake, total and LDL cholesterol were greater in the low sodium phase, as was serum insulin (245).

Fliser *et al* (246) undertook a parallel comparison of two groups, randomised to placebo or the alpha 1 receptor blocker doxazosin, with both groups undergoing two seven day periods of low sodium diet (20mmol/day) supplemented with placebo or slow sodium (200mmol/day). No washout period was incorporated into the design. Low sodium diet was associated with an increase in total cholesterol and LDL cholesterol after two and seven days of diet, though more marked after two days. Insulin and C-peptide were greater on low sodium diet after two days, as was circulating norepinephrine. Doxazosin therapy was associated with blunting of the lipid, insulin and C-peptide changes, though no changes in the activation the sympathetic nervous system. Goodfriend *et al* (247) included a measurement of insulin sensitivity (ITT) in addition to an oral glucose tolerance test after seven days of low (20mEq/day) then high (200mEq/day) sodium intake in 30 subjects. A negative correlation was observed between aldosterone and HDL during both dietary phases. A weaker relationship was also seen between insulin and aldosterone, and between the aldosterone–renin ratio and insulin during the high sodium phase.

### 1.11.6.3 Low sodium diet and insulin sensitivity

As early studies examining the metabolic effects of sodium restriction had suggested metabolic derangement, further studies were designed to examine specifically the relationship between insulin sensitivity and sodium intake. Different subject groups have been studied:

- *Normotensive Subjects*: Fliser *et al* (248) investigated the hypothesis that the differing results seen in response to sodium restriction may in part be explained by variation in the duration of diet. In healthy males, insulin sensitivity, assessed by the clamp technique, was measured in seven males after one week of randomly assigned high (200mmol/day) and low (20mmol/day) sodium, and in seven males after three days of the same dietary protocol. While no difference in M value was seen after seven days, clamps after three days showed a reduction in insulin sensitivity associated with the low sodium phase (M value 7.4 ± 2 vs. 8.6 ± 1.1 mg/kg/min)

In 1996, Grey *et al* (249) compared more modest sodium restriction in 34 healthy normotensive males, aiming for two seven day periods of sodium restriction to less than 80 mmol/day, with one week supplemented with placebo and the other by 120mmol/day of slow sodium tablets. The study had a randomised double blind crossover design, though notably there was no washout period between phases. Insulin sensitivity was measured using fasting plasma insulin (RIA) and CIGMA. Mean urinary sodium after the low sodium phase was 52 ± 25 mmol/day compared with 185 ± 46 mmol/day after high sodium. No change in blood pressure, insulin sensitivity or lipids other than an increase in $HDL_3$ on low sodium was observed.

Seven subjects were salt sensitive (fall in MAP ≥3 mmHg on low sodium), though this subgroup did not behave differently to that in the main analysis

Not all studies have shown a fall in insulin sensitivity associated with dietary sodium restriction. Facchini *et al* (250) found no difference in steady state plasma glucose (SSPG) during insulin suppression test in 19 healthy volunteers undergoing five day dietary periods of 25 and 200mmol/day sodium intake, despite excellent compliance (12 ± 3mmol/24h vs. 174 ± 14 mmol/24h).

- *Hypertensive Subjects:* Iwaoko *et al* (251) demonstrated that in 31 hypertensive subjects, seven days of low sodium intake vs. seven days of high sodium intake was associated with no change in fasting plasma insulin or glucose after correction for haematocrit. However, during an OGTT, the AUC for insulin and glucose was less in the high sodium phase, suggesting that there was a reduction in insulin sensitivity associated with dietary sodium restriction. The fall in AUC glucose on a low sodium diet correlated with the absolute value of AUC glucose, suggesting that the most glucose intolerant subjects were most susceptible to the effects of dietary sodium restriction. The group in fact contained seven diabetics and 18 subjects with IGT after the OGTT, suggesting that the group was heterogeneous. There was no mention of blood pressure therapy. Compliance was documented by the urinary sodium of 32mmol/24 hours and 298mmol/24 hours on low and high sodium phases respectively, and despite the fact that MAP rose from 100 to 108 on high sodium, no correlation was observed between salt sensitivity and insulin sensitivity.

After 12 weeks of modest sodium restriction in 34 hypertensive patients (20 male, mean age 53), (252) C-peptide rose by 40% and glucose rose by 6% (both $p<0.05$) compared with baseline. There was no associated evidence of sympathetic activation. The design, and lack of a measure of insulin sensitivity limits the value of this study, though the duration of diet is of interest. The same authors published data from a double blind cross over study (253) of 16 mild to moderate hypertensive subjects (mean age 50, 13 males) randomly assigned to a period of modest sodium restriction for two periods of eight weeks, one supplemented with placebo and the other with 50mmol/day of sodium. No significant difference in blood pressure, glucose or insulin was found between phases.

- *Type 2 diabetes:* Petrie *et al* (254) undertook studies of dietary sodium restriction in eight diet controlled type 2 diabetics, with a double blind randomised placebo controlled crossover design, measuring M values after four days of sodium replete (160mmol/day) and deplete (40mmol/day) diet. Insulin sensitivity was 12 % lower ($p=0.04$) on low sodium intake.

- *Other cardiovascular high risk groups:* To determine if the response to dietary sodium restriction was related to the cardiovascular risk profile, Egan *et al* (255) tested the effect of seven days of dietary sodium restriction (<20mmol) and seven days of normal sodium intake (200mmol) in a group of 29 subjects and analysed the responses having subdivided the cohort into groups with increasing cardiovascular risk factors. In those with the highest number of risk factors, the response of the RAS to a low sodium diet, measured by plasma renin activity and aldosterone, was greatest. In the low score group, low sodium was associated with a higher insulin

AUC, fasting total cholesterol and LDL cholesterol. In the medium score group, similar lipid and insulin changes were observed, and in the high score group, elevations in TG, total cholesterol and insulin (both fasting and AUC) were seen. Only in the high risk group were NEFA greater during sodium restriction. Thus in those with a metabolic profile more prone to cardiovascular disease, dietary sodium restriction was associated with the greatest activation of the RAS, and the firmest evidence of a reduction in insulin sensitivity.

### 1.11.6.4 Dietary sodium restriction and NEFA

Circulating free fatty acids may bind directly to the AII receptor, thus reducing AII induced increases in plasma aldosterone (256). Thus studies were undertaken to determine the effect of dietary sodium on plasma NEFA. This interaction could affect not only the RAS, but would also have repercussions on insulin sensitivity and the function of the endothelium, both of which are reduced by plasma NEFA. Goodfriend *et al* (257) demonstrated that saline infusion increased plasma NEFA. High sodium diet was associated with borderline significant increases in plasma NEFA when compared with low sodium intake, though these data were the sum of three different experimental protocols. Further studies revealed that saline infusion was accompanied by a fall in plasma insulin, and that high sodium diet was associated with a reduction in plasma insulin. No correlation was observed between the rises in NEFA and the falls in plasma insulin, suggesting a lack of direct causality. In subjects on low, though not high, sodium intake, a correlation was seen between norepinephrine and NEFA. These interactions may provide some explanation for the observed increase in the aldosteronogenic effect of AII during insulin infusion; insulin reduces NEFA, which may block the AII receptor.

### 1.11.6.5 Dietary sodium restriction, insulin sensitivity and salt sensitivity

Rocchini first observed a relationship between the metabolic and pressor response to dietary sodium restriction (258). In comparison of obese and lean volunteers undergoing two weeks of high sodium (>250mmol/day) then low sodium (<30mmol/day) diet, the fall in blood pressure in the obese group was 12 ± 1 mmHg compared with an increase of 1 ± 2mmHg in the lean group. The magnitude of this sensitivity (salt sensitivity) was predicted by fasting insulin, though also by the percentage of body weight made up by fat, which may be related. In keeping with these observations, the obese subjects were then entered into a weight loss programme, and those who lost more than 1kg of weight were then less salt sensitive. Those who lost no weight remained salt sensitive as in the initial phase.

In a study of 23 healthy young males (259) in a single blind randomised crossover consisting of six days of 20mmol/day and six days of 260 mmol/day sodium intake, comparisons were made between salt sensitive (SS) and resistant (SR) subjects. Response to dietary sodium differed, in that the SS group showed higher plasma glucose during OGTT than the SR group during the high, but not low sodium phase. In the SS group, glucose tolerance improved on low sodium, though in the SR group the opposite occurred on low sodium. Thus on high sodium intake, those at risk of later developing hypertension (SS) were more insulin resistant, and this was negated on low sodium. These results must be interpreted with caution, however, as a surrogate measure of insulin sensitivity was employed, and it is unclear whether dietary sodium intake may affect the absorption of glucose during OGTT. To answer some of these criticisms the same authors (260) measured insulin sensitivity by insulin suppression test in 18 healthy male volunteers after two weeks of 20mmol/day sodium intake, one week of which was randomly supplemented with 220mmol sodium daily. In the seven SS subjects, plasma glucose was higher than in

the SR subjects, in keeping with insulin resistance, though no difference in plasma insulin was observed.

In another study of 28 subjects with untreated hypertension (261) who underwent one week of sodium intake of 220mmol/day, then one week of 30mmol/day, MAP was measured at the end of both phases, and salt sensitivity defined as a fall in MAP >10%. Blood pressures were the mean of 25 readings taken every five minutes after a 30-minute rest period. Ten subjects were salt sensitive, eight salt resistant (<3% fall in blood pressure) and ten intermediate. OGTT with plasma insulin was performed at the end of the high sodium period. The baseline data revealed that those in the salt sensitive group were, on retrospect, more obese and had higher fasting plasma insulin than the salt resistant group. Furthermore, plasma insulin was greater during the OGTT in the salt sensitive group. A relationship just below statistical significance was seen between the reduction in MAP on the low sodium phase and the integration of the area of insulin and glucose curves during OGTT when all subjects were included. Bigazzi *et al* (262) demonstrated that in hypertensive patients, comparison of the 29 SS and 23 SR subjects (defined after one week of 20mmol/day sodium then one week of 250mmol/day sodium) revealed no difference in baseline plasma glucose and insulin, however after OGTT AUC glucose and insulin was greater in the SR group. More recently, a study of 16 non-obese patients with mild hypertension (263) and on no treatment for 4 weeks, examined insulin sensitivity as measured by the insulin suppression test after one week of low sodium (40-60mEq/day) then one week of high sodium intake (300mEq/day). Salt sensitivity was defined as an increase in MAP >10% in response to high sodium. No difference in fasting insulin or glucose was observed between the groups, however, the insulin suppression test revealed

that glucose disposal was less and steady state glucose was greater in SS than SR hypertensives. This difference was observed during both low and high sodium phases.

These data suggested that in normotensive individuals, salt sensitivity is associated with insulin resistance. Not all groups have found this however, as in a study of 27 normotensive males(264), 18 of whom were salt resistant and nine of whom were salt sensitive, there was no difference in the mean M value between the groups. In addition, there was no effect of dietary sodium restriction (60mmol vs. 260mmol/day for seven days) on insulin sensitivity in either group. In fact, in untreated hypertensive patients, greater salt sensitivity was associated with greater insulin sensitivity (265), a finding similar to that observed in 21 SS males (266) whose salt sensitivity correlated significantly and positively with insulin sensitivity.

An additional component of the relationship between insulin resistance and salt sensitivity may be a reduction in the normal nocturnal fall in blood pressure (267). In hypertensive subjects, sodium induced increase in blood pressure between phases was associated with the SSPG during an insulin sensitivity test while on the high sodium phase. SSPG was negatively correlated with the fall in nocturnal blood on high sodium intake, while sodium induced increase in blood pressure was negatively correlated with nocturnal fall in MAP, suggesting that the lesser the nocturnal blood pressure fall the greater the salt sensitivity, the greater the insulin resistance.

Thus there seems to be a relationship between salt sensitivity and insulin resistance, certainly at baseline, though the metabolic response to sodium loading and restriction seems less clear, and may differ between normotensives and hypertensives. Similarly, some

of the variation in findings may be accounted for by variability in the methods used to measure insulin sensitivity.

If salt sensitivity precedes hypertension, then it may be expected to be common in populations with a high prevalence of hypertension, such as American blacks (268). There is some evidence to suggest that this might be the case (269), however, dietary potassium plays an important role in determining the sensitivity of MAP to sodium restriction. After sodium loading for four weeks, American blacks receiving potassium supplementation suppressed the frequency and severity of salt sensitivity to levels seen in similarly treated whites (270). However, when potassium intake was limited to 30mmol/day, the frequency of SS was greater in blacks than whites

### 1.11.6.6 Dietary sodium, insulin sensitivity and the vasculature

In addition to the potentially deleterious effects that sodium restriction may have on lipids and metabolic insulin sensitivity, activation of the RAS by this means may also affect vascular function. This may be mediated by AII, aldosterone or even catecholamines that are produced in response to a low sodium diet. Alternatively, if dietary sodium is associated with a fall in insulin responsiveness in muscle and fat, then there may be an associated reduction in endothelial insulin sensitivity.

In 18 obese subjects, no difference was seen in plasma insulin or glucose between SS and SR groups, however during the high sodium phase insulin increased calf blood flow only in the SR subjects(271). 13 subjects, eight of whom had borderline hypertension, were entered into a double blind randomised crossover trial (272) consisting of one week on low sodium intake (20mEg/day) and one week of normal intake (240mEq/day), with vascular

sensitivity to insulin measured in dorsal hand veins at the end of each phase (using the linear variable differential transformer technique). On normal sodium, vascular insulin sensitivity was lower in hypertensives than in normotensives. On low sodium, vascular insulin sensitivity decreased in both hypertensives and normotensives, paralleled by an increase in norepinephrine. Limb blood flow in 18 healthy male volunteers was compared after six days of 220mmol/day and 40mmol/day sodium intake (273). No change in metabolic insulin sensitivity during two stage clamps was observed between phases, however leg blood flow in response to high dose insulin was significantly greater during high than low sodium. These vascular changes were dissociated from the metabolic changes; no relationship was seen between the metabolic and vascular responses.

In WKY rats, no difference in insulin sensitivity (M) was demonstrated after three weeks of low, normal and high sodium diet, however insulin enhanced $\alpha$-2 adrenergic vasorelaxation in aortic rings was present only after high and normal sodium intake, again suggesting that low sodium diet may be associated with endothelial dysfunction (274).

### 1.11.7 ACE inhibitors and insulin sensitivity

If dietary sodium restriction reduces insulin sensitivity, possibly through a direct effect of angiotensin II (see section 1.11.10), then it would be anticipated that inhibition of the generation of angiotensin II by ACE inhibition would have an insulin sensitising action. The evidence for this is reviewed below.

The first study to demonstrate an improvement in insulin sensitivity in association with ACE inhibition was reported by Pollare *et al* (275) in 1989. They found that in hypertensive patients there was an 11% increase in insulin sensitivity (clamp measured) after four weeks of captopril therapy compared with placebo. Many studies followed this,

in healthy volunteers (276), patients with type 2 diabetes (277), (278)) and patients with hypertension (276). These have varied in design (crossover, parallel group and before and after), duration (1-12 weeks), the use of a placebo group and the technique employed to measure insulin sensitivity (clamp, ivGTT). As such the literature is heterogeneous, making it difficult to reach a consensus. Of the studies demonstrating a positive effect, Paolisso 1992 (279) found that two weeks of lisinopril increased insulin sensitivity (ivGTT) by 18% in a double blind placebo controlled crossover study in elderly hypertensive patients. The same authors (280) later found that in a similar patient group, 8 weeks of lisinopril increased insulin sensitivity by 33% (M value), though here there was only single blinding. De Mattia (278) reported a 50% increase in insulin sensitivity (clamp generated) in patients with type 2 diabetes treated for ten days with captopril though notably changes in insulin sensitivity were not a primary end-point of the study. If changes of this magnitude are genuinely the case in diabetes, then this is difficult to reconcile with data from the UKPDS treatment groups in which there was no reduction in HbA1c in the captopril vs. the $\beta$ blocker group (281). In a recent study, using double-blind, placebo controlled crossover methodology to compare four weeks of trandolapril therapy with placebo in hypertensive patients with type 2 diabetes(282), no effect on M value was seen. The authors took this opportunity to review the published data in this area, and in particular consider the methodologies employed. The only other trial identified as using gold standard methodology was undertaken by Wiggam *et al* (283), and reported no effect of eight weeks of captopril treatment on insulin sensitivity in a hypertensive cohort.

Less scientifically robust, though nonetheless intriguing, population-based data on ACE inhibition and hypoglycaemia have been reported by two groups. Herings *et al* (284) found, using a retrospective nested case control design, that hypoglycaemia requiring

hospitalisation occurred more frequently in diabetic patients using ACE inhibitors (OR 2.8[95% CI 1.4-5.7]), irrespective of hypoglycaemic therapy. Using similar methodology, Morris *et al* (285) analysed data obtained from over 6, 000 diabetic patients in Tayside, and again identified an OR of 4.3 [95% CI 1.2-16.0] for the risk of hypoglycaemia in subjects treated with ACE inhibitors, after adjustment was made for other factors. No other antihypertensive agent was identified as having a similar effect.

Two major studies have reported a reduction in the incidence of type 2 diabetes in association with ACE inhibition. In the Heart Outcomes Prevention Evaluation (HOPE) study(222), a 33% reduction in new cases of type 2 diabetes was reported in the ACE inhibitor group, however the diagnosis of diabetes was based only on patients reporting the diagnosis; it may also be argued that as the patients in the placebo group experienced more events requiring hospitalisation, they would most likely have undergone more testing of random and fasting blood glucose. In the Captopril Prevention Project (286), a reduction in new cases of diabetes was reported in hypertensive patients treated with captopril compared with those treated with conventional antihypertensive medication (predominantly diuretics and $\beta$ blockers). Importantly, there was no placebo group. In addition, this study has been subsequently criticised on the basis of the randomisation process, raising questions about the validity of the findings.

It must be borne in mind that ACE inhibition not only reduces circulating AII, so reducing AII stimulation of the AT1 and 2 receptors, but also increases bradykinin by its effect on kininase. In contrast, AT1 receptor blockade increases AII, and so increases the AII available to the AT2 receptor. There is no associated effect on kinins. Thus the effect of

blockade must be examined separately, and in assessing the particular role of the AT1 receptor in modifying insulin sensitivity, these agents may provide a "purer" model.

Analysis of this literature reveals similar methodological and design heterogeneities to those encountered when considering the effects of ACE inhibition. In a randomised, double blind study (287) comparing losartan and metoprolol in 20 hypertensive patients selected also because they were hyperinsulinaemic, there was no change in insulin sensitivity (M value) after 12 weeks of therapy in either group. In keeping with this finding, in a further study of hypertensive patients treated with losartan for four weeks (288), though on this occasion with a crossover design and placebo control group, there was no effect on clamp measured insulin sensitivity. In 25 hypertensives, comparing losartan to lisinopril, no effect of losartan on insulin sensitivity was observed. There was an improvement in insulin sensitivity associated with lisinopril therapy, however the absence of a placebo limb to the study makes interpretation of this finding difficult (289). In a placebo controlled crossover study of normotensive offspring of hypertensive parents, losartan had no effect on insulin sensitivity after 14 days of therapy, though insulin sensitivity was measured using the minimal model method of Bergman (290).

Some studies have suggested a positive effect of AT1 receptor blockade; Paolisso *et al* (291) demonstrated an improvement in insulin sensitivity following four weeks of losartan therapy vs placebo, though there was no crossover design to the study, while two weeks of candesartan therapy (292) was reported as improving insulin sensitivity in an uncontrolled "before and after"study of eight patients.

In summary, there is no real consensus as to the effects of AT1 receptor blockade and ACE inhibition on insulin sensitivity, as the heterogeneity of publications makes it very difficult to compare studies. There is no firm evidence to suggest that either class of drug has a positive effect in this regard.

### 1.11.8 Infusion of AII and insulin sensitivity

The results of the above studies, which suggest a fall in insulin sensitivity in response to dietary sodium restriction and a potential improvement in association with blockade of the RAS, may be interpreted as inferring that AII has a direct effect to reduce insulin sensitivity. As such, several studies have tested the effect of AII infusion on insulin sensitivity.

A series of studies in normotensive males suggested that AII infusion during a euglycaemic hyperinsulinaemic clamp improved insulin sensitivity. The doses and timing of AII administration during the clamp varied between groups, as did the magnitude of improvement in insulin sensitivity. Fliser *et al* (293) demonstrated a significant elevation in M value during the last hour of a clamp associated with an infusion of AII at 1.5ng/kg/min compared with sham, while there was no increase in blood pressure. In a study of 39 healthy males, Widgren at al (294) found that a 30-minute infusion of AII (0.1ng/kg/min) was associated with increased insulin sensitivity compared with the preceding period of the clamp. Using a higher dose of AII (15ng/kg/min) Townsend *et al* (295) showed a 15% improvement in insulin sensitivity compared with placebo.

These studies are limited by the lack of formal blinding and randomisation. Morris and colleagues undertook a double-blind, randomised, placebo-controlled crossover study in 12

healthy volunteers (296), and found that infusion of 1ng/kg/min (subpressor) and 5ng/kg/min (pressor) AII throughout the clamp had no effect on insulin sensitivity.

Clearly higher doses of AII may have confounding pressor effects that may influence insulin sensitivity. In the Fliser study, there was no increase in blood pressure, however AII infusion in the Townsend and Widgren studies was accompanied by an elevation in blood pressure. Morris documented an elevation in blood pressure in response to 5ng/kg/min, though not 1ng/kg/min.

In attempt to address the association of the metabolic and pressor effects of AII, Buchanan (297) devised an elegant series of studies in healthy males. In the absence of insulin during a sham clamp, AII had no effect on whole body glucose turnover or leg glucose extraction, implying no direct effect of AII to stimulate glucose uptake. A clamp performed at physiological insulinaemia demonstrated that AII infusion increased blood pressure, whole body glucose clearance and plasma insulin. In clamps performed at insulin levels sufficiently high to remove insulin as a rate-limiting step in glucose uptake (maximal insulin), AII increased whole body glucose clearance with no effect on glucose extraction across the leg. In combination with these studies, it was shown that AII infusion redistributed flow from the insulin insensitive splanchnic circulation to insulin sensitive skeletal muscle beds. These observations suggested that the effect of AII to increase insulin sensitivity was mediated not by a direct stimulation of glucose uptake or an increase in glucose extraction, but by a redirecting of flow to insulin sensitive vascular beds. This explained why AII only augmented insulin sensitivity in the presence of insulin. In keeping with this, Jamerson *et al* (298) infused AII into the brachial artery of healthy volunteers,

and found no direct effect on insulin stimulated glucose uptake into forearm skeletal muscle.

In subjects with type 2 diabetes, Morris *et al* (299) demonstrated that at subpressor and weakly pressor doses of AII, insulin sensitivity increased. The AII doses were calculated as too low to have an effect of renal plasma flow, and so an additional action of AII was suggested. Both of these sets of observations may be explained by Buchanan`s postulate (297) that AII may increase capillary recruitment in insulin sensitive microcirculations, thus increasing the delivery of substrate. The vascular responsiveness of the microcirculation may therefore be important in determining insulin sensitivity, and as such may be abnormal in insulin resistant states. Certainly in type 1 diabetes, the vascular responsiveness to AII is abnormal in streptozotocin-induced diabetes animal models (300) and humans with type 1 diabetes (301). In a double blind randomised placebo controlled study of 15 volunteers with recent onset type 2 diabetes (302), AII infusion of 2ng/kg/min induced no increase in insulin sensitivity during a clamp, whereas in a matched group of healthy volunteers, insulin sensitivity increased significantly. AII increased MAP by similar absolute values in both groups, however the AII induced increase in leg blood flow was less in the diabetic group. Interestingly, the change in insulin sensitivity and leg blood flow were correlated in both groups, suggesting that the metabolic effect of AII was related to its effect on redistribution of flow to insulin sensitive, though not insensitive, local circulations.

### 1.11.9 Effects of AII on the pancreas

AII may also modify the secretion of insulin, providing a potential confounder in some of the previously discussed studies. Fliser *et al* (303) demonstrated that, in a double-blind,

randomised, placebo-controlled study of healthy volunteers, pressor doses of AII (5ng/kg/min) reduced basal and glucose stimulated insulin secretion. A more modest effect was seen with subpressor doses. The pattern and spontaneous pulsatility of insulin secretion was unaffected. As in many other tissues, the pancreas may have a local RAS. In dogs, (304) AT2 receptors were identified in the endocrine and exocrine pancreas. Thus AII may influence pancreatic insulin secretion, and therefore glucose tolerance, by effects either on the pancreatic circulation or the directly on the islet.

### 1.11.10 Intracellular crosstalk as a potential cause of AII induced reduction in insulin sensitivity

#### 1.11.10.1 Angiotensin II signalling

Human vascular smooth muscle cells have two well-characterised angiotensin II receptors, the AT1 and AT2 receptors, which were cloned in 1991 and 1992 respectively. They are members of the G protein coupled, seven transmembrane domain receptor superfamily. While two subtypes of the type 1 receptor are present in rodent tissue, this does not seem to be the case in equivalent human cell lines. These receptors are expressed in various tissues, including the traditional AII target tissues such as vascular smooth muscle, cardiac muscle and the adrenal gland, but more recently significant numbers of receptors have been shown to be present in tissues such as adipose tissue, neural tissue and skeletal muscle (see section 1.11.3). It has been suggested that the effect of angiotensin II in different tissues may be explained by the relative amounts of the type 1 and 2 receptor.

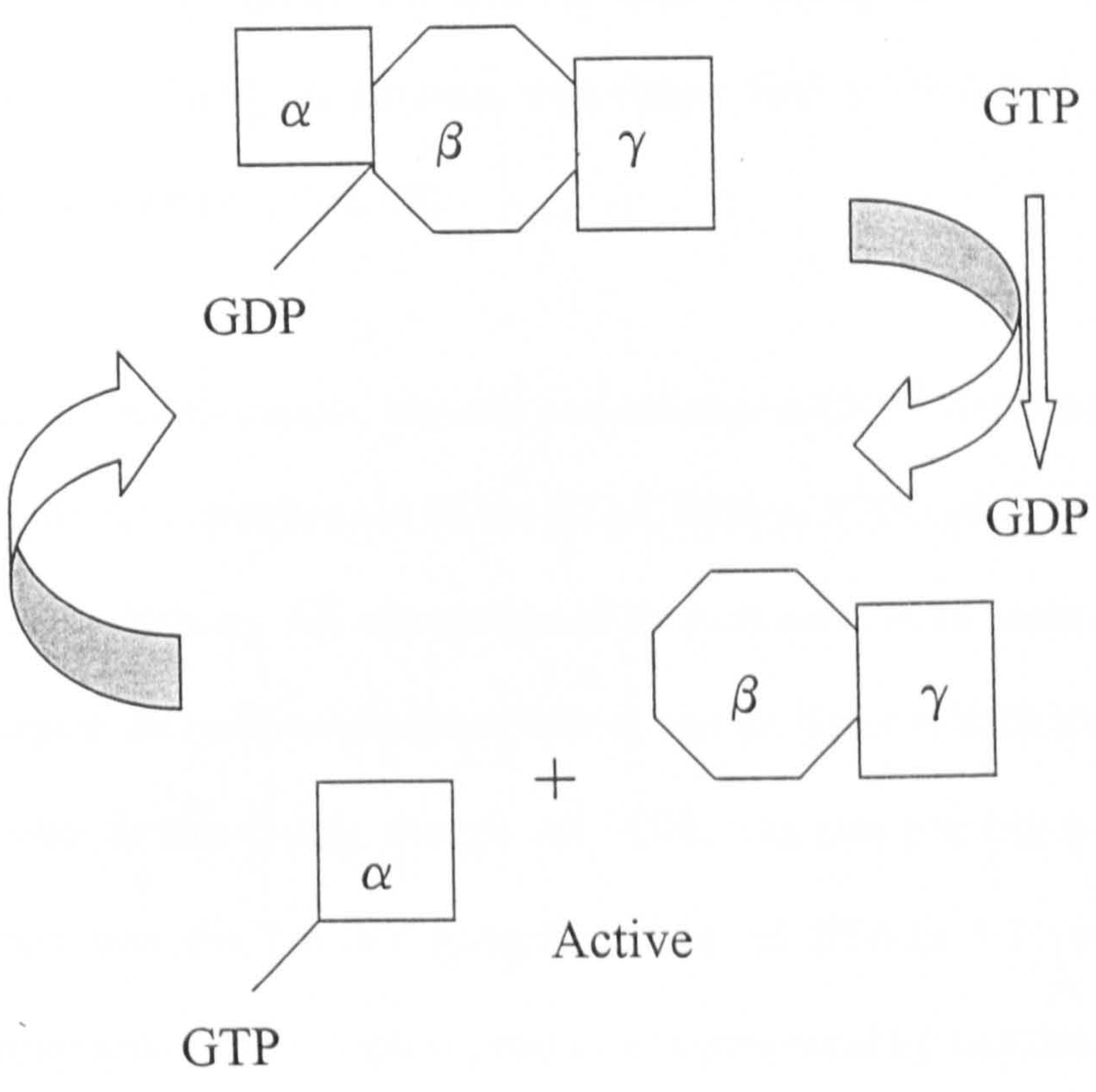
Much of our understanding of the intracellular events that follow angiotensin II binding is based on studies in cultured vascular smooth muscle cells, with assumptions made that similar events occur in other tissues. The initial post receptor events of the AT1 receptor are typical of all G protein coupled receptors (Figure 1.4). While inactive, the proteins exist in their guanosine diphosphate (GDP) bound form, as a heterotrimer composed of an $\alpha$, $\beta$, and $\gamma$ subunit associated with the receptor. Upon ligand binding, conformational changes occur in the $\alpha$ subunit, allowing GDP to be replaced by guanosine triphosphate (GTP). The $\alpha$ and $\beta\gamma$ subunits then become dissociated from the receptor (reviewed in (305).

The G protein coupled receptors are classified according the properties of the $\alpha$ subunit, namely $G\alpha_s$, $G\alpha_i$, and $G\alpha_q$. The AT1 receptor is an example of a $G\alpha_q$ receptor. The released

α, β, and γ subunits are free to initiate further downstream signalling events. In response to AII, the $G\alpha_q$ GTP activates phospholipase C (PLC) -β, which in turn catalyses the conversion of phosphatidylinositol-4,5 phosphate into inositol-1,4,5- triphosphate ($IP_3$) and diacylglycerol (DAG). Vascular smooth muscle cells have very little PLC-β, though stimulation of the AT1 receptor still results in $IP_3$ formation. This raises the possibility that PLC- γ may be involved with this process. In addition to PLC, phospholipases $A_2$ and D are also activated, resulting in the formation of the prostaglandin precursor arachidonic acid (306).

The result of DAG accumulation in the cell is activation of the serine threonine kinase PKC. It is not clear which isoform is activated in response to AII, though it is suggested that this may be cell type specific; in cultured vascular smooth muscle cells it may be PKC-ξ that is the main target.

**Figure 1.4: G protein activation and subunit dissociation**




Abbreviations: GTP, guanosine triphosphate; GDP, guanosine diphosphate

Again in vsmc, AT1 receptor activation has also been shown to activate components of the Janus Kinase (JAK) / signal transduction and activation of transcription (STAT) pathway. This is similar in nature to the response seen in cytokine receptors. Activation of the AT1 receptor by angiotensin II certainly results in the recruitment of JAK2 and so gives the activated receptor the capacity for tyrosine residue phosphorylation. One target for this kinase is the STAT family of proteins, which then bind to DNA in the nucleus following dimerization (reviewed in (307,308).

Using rat aortic smooth muscle, Marrero and colleagues (309) investigated the role that AII activation of the AT1 receptor has on the STAT family. These proteins are activated by the JAK intracellular kinases. AII stimulation of the cell resulted in association of JAK2 with the AT1 receptor, its tyrosine phosphorylation, and an increase in its kinase activity. Tyk 2, another member of this family, though not JAK1, was also phosphorylated. The result of this activation was the tyrosine phosphorylation of STATs 1-3, and their subsequent nuclear translocation. This complete process was prevented by addition of the AT1 receptor blocker, losartan. Thus AII, acting via its G-protein coupled receptor, stimulates intracellular kinase activity in smooth muscle cells. This has relevance to the later discussion of intracellular crosstalk between signalling systems.

While it is established that stimulation of the AT1 receptor leads to vasoconstriction, aldosterone secretion and sympathetic augmentation, the AT2 receptor remains less well characterised; a role in the regulation of apoptosis (310) and stimulation of vasodilation (311) has been suggested. The AT2 receptor is coupled to $G\alpha_i$, (312) and inactivates the extracellular signal regulated kinase (ERK).

### 1.11.10.2 Intracellular crosstalk between insulin and angiotensin II

In the last five years, a novel cellular process has emerged that suggests a mechanism through which AII may reduce insulin responsiveness at the level of individual cells. In the investigation of it's role as a growth factor, AII, acting via the AT1 receptor, was found to induce tyrosine phosphorylation of IRS-1 in rat cardiac muscle (313), suggesting intracellular crosstalk between the second messengers activated in response to AII and insulin. Velloso *et al* (314) developed this theory further by studying the tip of rats' left ventricle after AII and insulin had been injected into the inferior vena cava. Both insulin and AII stimulated the phosphorylation of tyrosine residues on IRS-1 and 2, which in turn resulted in the association of these substrates with the p85 subunit of PI3'-kinase. Immunoprecipitation experiments confirmed that, in response to AII, JAK2 was associated with IRS-1 and 2, as well as the AT1 receptor. Despite the activation of these early signalling responses, AII induced a reduction in both basal and insulin stimulated PI3'-kinase activity, which was blocked by co-administration of a specific AT1 receptor blocker. This was not secondary to any effect on the insulin receptor, IRS-1/2 association with p85 or the association of p85 with the catalytic p110 subunit of PI3'-kinase. Further studies were undertaken in rat aortic smooth muscle (315), with similar, though subtly different findings. Importantly, AII pre-treatment again reduced insulin stimulated, IRS-1 associated PI3'-kinase activity by 60%. However, in these cells, AII reduced insulin stimulated tyrosine phosphorylation of IRS-1, and its subsequent association with PI3'-kinase by 30-50%. The non-specific AII receptor blocker saralasin blocked these effects, though specific AT1 or 2 blockers had no effect, raising the possibility that a third, uncharacterised AII receptor is important in this process.

A mechanism for the inhibition of PI3'-kinase was also suggested; AII activates protein kinase C, a serine threonine kinase, and as phosphorylation of serine residues on the beta subunit of the insulin receptor or IRS-1 inhibits the insulin-signalling pathway, this was investigated as the site of crosstalk. In keeping with this, phosphoserine content of the insulin receptor and IRS-1 was increased by 70% and 75% respectively in response to AII. The observed reduction in the association of IRS-1 with the activated insulin receptor also favoured this hypothesis.

Thus, these two related papers provided a novel mechanism through which AII may cause insulin resistance in the vasculature. Importantly, neither study measured any cellular endpoint of PI3'-kinase, such as glucose transport. If this process was inhibited by AII, then an effect of AII to inhibit insulin signalling in skeletal muscle or adipose tissue may translate to insulin resistance associated with activation of the RAS in dietary sodium restriction, and any insulin sensitising effect of ACE inhibitors. This hypothesis is investigated in this thesis.

While AII may crosstalk with insulin and modify its post receptor signalling, it is important to consider a potential role for bradykinin in any influence that activity of the RAS may have on insulin sensitivity. This is especially relevant in the context of ACE inhibition, as in addition to inhibiting the ACE, these drugs also inhibit the kininase enzyme, and as such increase local and circulating kinin concentrations.

### 1.11.10.3 Bradykinin and insulin stimulated glucose transport

Henriksen *et al* (316) demonstrated that insulin stimulated glucose uptake into the epitrochlearis muscle of obese Zucker rats was greater after short term captopril treatment,

and that this effect was abolished by a bradykinin antagonist. In canine adipocytes (317), bradykinin, in the presence of insulin, increased insulin stimulated 2-deoxyglucose uptake. Mechanistically, bradykinin increased the translocation of GLUT 4 to the cell surface in response to insulin, possibly by increasing insulin-stimulated receptor and IRS-1 phosphorylation. 20-month old rats treated acutely with captopril (318) showed increased insulin-stimulated receptor and IRS-1 phosphorylation in liver and muscle. IRS-1 association with PI3'-kinase also rose. Unlike captopril, losartan had no effect on these early events in the insulin-signalling cascade, implying that the captopril effect was not one of a reduction in AII. In fact, administration of bradykinin increased insulin receptor and IRS-1 tyrosine phosphorylation, and so the effect of ACE inhibition was more likely secondary to associated inhibition of kininase.

Bradykinin treated obese Zucker rats (319) had lower fasting insulin and free fatty acids, as well as lower insulin and glucose response to an OGTT, than saline treated controls. Insulin stimulated glucose uptake into isolated epitrochlearis muscle was 52% greater in the bradykinin treated animals. Interestingly, contraction-stimulated glucose uptake was also improved, suggesting that the bradykinin effect may not be exclusive to insulin-signalling pathways. In L6 myotubes (320) bradykinin-induced translocation of GLUT4 to the plasma membrane was unaffected by pre-treatment with wortmannin, as is the case in exercise-induced glucose transport. A potential link between the vasodilatory and metabolic properties of bradykinin was proposed after Henriksen (321) demonstrated that the bradykinin-associated increases in insulin sensitivity in skeletal muscle of the obese Zucker rat were inhibited not only by blockade of the B2 receptor, but also by inhibition of NOS.

Though these animal and cell culture studies present intriguing mechanisms through which the RAS may modify insulin sensitivity in humans, no studies have examined the effect of AII or bradykinin on glucose transport in human tissue. Baba *et al* (322), found no effect of AII or bradykinin on insulin-stimulated activation of the insulin receptor tyrosine kinase in human adipocytes, though no measurement of glucose transport was included in the communication.

## 1.12 Glucocorticoids and insulin sensitivity

Glucocorticoid excess, seen clinically in Cushing's syndrome, is associated with obesity, hypertension and glucose tolerance. This observation has led some to hypothesise that glucocorticoid levels may underlie the development of the metabolic syndrome, especially given data showing that rats treated with glucocorticoids *in utero* are born with a low birthweight, while later developing insulin resistance and hypertension (323). In addition, higher 0900 plasma cortisol concentrations are found in men born with a low birthweight, and are associated with the development of hypertension, hypertriglyceridaemia and glucose intolerance (324).

### 1.12.1 Biology of cortisol

Adrenocorticotrophic hormone (ACTH) stimulates the secretion of cortisol into the circulation from the adrenal cortex. Cortisol circulates either unbound (5-10%) and therefore active, or bound to cortisol binding globulin (70-75%) or albumin (20%). At high physiological levels of cortisol, these binding mechanisms are saturated, and so large excursions may occur in free cortisol.

Cortisol activates either GR (corticosteroid type 2) or MR (corticosteroid type 1) receptors. GR are high capacity, low affinity receptors that are widely distributed, in contrast to the low capacity high affinity MR receptors that bind cortisol depending on its local availability (determined by the 11$\beta$ hydroxysteroid dehydrogenase (11$\beta$ HSD) enzymes).

11$\beta$ HSD-1 increases availability of cortisol, and is found in liver, adipose tissue and skeletal muscle. Carbenoxolone, which inhibits hepatic 11$\beta$ HSD-1, increases whole body insulin sensitivity, with no effect on peripheral insulin sensitivity, suggesting that a

lowering of intra-hepatic cortisol may be the mechanism underlying a potential improvement in hepatic insulin responsiveness.

### 1.12.2 Mechanism of glucocorticoid induced insulin resistance

That glucocorticoids reduce insulin sensitivity is not a novel observation, though the underlying mechanism remains unclear. Early observations were that cortisol excess reduced hepatic and peripheral insulin sensitivity, probably by a post receptor mechanism (325). Rooney and colleagues (326) investigated the effect of a 28 hour cortisol infusion on insulin sensitivity and found an increase in fasting insulin, a reduction in peripheral insulin sensitivity (by nearly 50%) and a reduction in the effect of infused insulin to reduce hepatic glucose output. It was suggested that the increase in hepatic glucose output was secondary to an effect on phosphoenolpyruvate carboxykinase (PEPCK), the rate-limiting step in gluconeogenesis. In addition free fatty acids increased, which may contribute to insulin resistance and hyperglycaemia by substrate competition in the Randle cycle. A physiological rise in glucocorticoid concentration may also increase hepatic glucose output by reducing pancreatic insulin secretion (seen in cultured mouse islet cells by Lambillotte *et al* (327) 1997) and increasing glucagon secretion (328). It was suggested that cortisol reduced peripheral glucose uptake, as demonstrated by a reduction in muscle glucose uptake in the presence rather the absence of a physiological nocturnal cortisol rise. In keeping with this, Paquot *et al* (329), observed a 51.5% reduction in glucose uptake in healthy volunteers during a hyperinsulinaemic euglycaemic clamp, and a 54% reduction in glucose oxidation after 2 days of dexamethasone 1mg bd.

Several authors have examined the nature of the glucocorticoid-insulin interaction at the cellular level. Montiel et (330) used rat glial cells to demonstrate a reduction in insulin

binding at the plasma membrane in association with dexamethasone exposure, an effect that was sensitive to inhibition of protein synthesis by cycloheximide. Similarly, Yasuda *et al* (331) found that equivalent doses of hydrocortisone and dexamethasone, though not prednisolone, reduced insulin binding in erythrocytes. It seems unlikely that this is the sole explanation for corticosteroid induced insulin resistance, as all three agents reduce insulin sensitivity. In addition, these findings are at odds with the observations of other authors, who found that in patients with Cushing's syndrome there was an increase in insulin binding to erythrocytes (332) and monocytes (333). Lastly, Watanabe *et al* (334) incubated isolated rat adipocytes with hydrocortisone for up to eight hours and found no effect on insulin binding site number or affinity, however there was a rightward shift in the uptake of 3-O-methylglucose in response to insulin, favouring a post receptor mechanism underlying insulin resistance. Giorgino *et al* (335) investigated the theory that the defect may lie at the level of the insulin receptor and IRS-proteins tyrosine phosphorylation, and found a reduction in tyrosine phosphorylated insulin receptors and in skeletal muscle IRS-1. Interestingly, these changes were seen when the animals were pre-treated with streptozotocin, suggesting that hyperinsulinaemia may be important in mediating signalling disturbances.

Additional studies in rodents suggested that a defect may also lie at the level of GLUT 4's subcellular distribution; in rat adipocyte membranes, dexamethasone resulted in a relative redistribution of GLUT4 away from the plasma membrane and toward the LDM fraction in response to insulin (336), while in isolated rat soleus muscle from animals treated with dexamethasone for 5 days, reduced insulin-stimulated glucose transport, glucose phosphorylation, glucose oxidation and glycogen synthesis was observed, in association

with a reduction in the translocation of GLUT4 to the plasma membrane in response to insulin (337).

These abnormalities of GLUT4 trafficking may display tissue specificity; while Coderre *et al* (338) observed a decrease in adipocyte GLUT4 expression following dexamethasone treatment, an increase in expression was seen in skeletal muscle. Again, however, GLUT4 translocation in response to insulin was reduced.

Thus, glucocorticoids induce insulin resistance in the liver, possibly by an effect on PEPCK, and in skeletal muscle and fat, though the mechanism in these tissues is not entirely clear.

## 1.13 Aims of this thesis

1. To develop a methods of determining endogenous insulin sensitivity in human tissue

2. To examine the relationship with the insulin resistant phenotype and insulin response in isolated tissue

3. To determine the effect of dietary sodium restriction on insulin sensitivity

4. To explore the hypothesis that angiotensin II may have a direct effect on insulin action

5. To examine the effect of glucocorticoid exposure on insulin sensitivity in metabolic and vascular tissue, and consider the coupling of these effects.

# Chapter 2: Materials and Methods

## 2.1 Materials

All materials used in the course of this thesis were of good quality and were obtained from the following sources:

### 2.1.1 General reagents

**Alpha Laboratories, Eastleigh, Hampshire, UK**

NEFA-C assay, Alpha laboratories, Eastleigh, Hampshire, UK

**Amersham International Plc, Aylesbury, Buckinghamshire, UK**

ECL Western Blotting Detection Kit

**Astra, King`s Langley, Herts., U.K.**

1% lignocaine hydrochloride

**Bio-Rad Laboratories Ltd, Hemel Mempstead, Hertfordshire, UK**

Bio-Rad protein assay

*N,N,N',N'*, -tetramethylenediamine (TEMED)

**Boehringer Mannheim GmbH, Germany**

Complete ™ Protease Inhibitor Cocktail Tablets

**Fisons, Loughborough, Leicestershire, UK**

Acrylamide

Ammonium persulphate

Diaminoethanetetra-acetic acid, disodium salt (EDTA)

Glucose

HEPES

Magnesium sulphate heptahydrate

*N,N* methylene-bis-acrylamide

Potassium chloride

Sodium dodecyl sulphate

Sodium chloride

Sodium dihydrogen phosphate

Sodium hydrogen carbonate

**Gibco BRL, Paisley, UK**

Tris base

**Kodak Ltd, Hemel Mempstead, Hertfordshire, UK**

X-Omat S film

**Lorne Laboratories, Twyford, Essex, UK**

Collagenase, Worthington Type 1

**Merck Ltd (BDH), Lutterworth, Leicestershire, UK**

Calcium chloride hexahydrate

Dow Corning silicone oil

Magnesium chloride heptahydrate

Potassium dihydrogen phosphate

**New England Biolabs, Hitchin, Hertfordshire, UK**

Broad range pre-stained protein standards (6-175kDa)

**NovoNordisk A/S, DK 2880, Bagsvaerd, Denmark**

Actrapid human insulin

**Packard Instruments, Groningen, The Netherlands**

Scintillation fluid

**Premier Brands UK, Knighton Adbaston, Staffordshire, UK**

Marvel powdered milk

**Schleider & Scheull, Dassel, Germany**

Nitrocellulose membrane (0.45$\mu$m)

**Sigma Chemical Company Ltd, Poole, Dorset, U.K.**

Acetylcholine

Adenosine

Bovine Serum Albumin

Bromophenol blue

DL-dithiothreitol

Human angiotensin II

Isoproterenol

L-$N^{\omega}$-monomethylarginine (L-NMMA)

Norepinephrine

Sucrose

Triton X-100

**Seton Healthcare Group, Tubiton House, Oldham, U.K.**

Iodine

**2.1.2 Radioactive materials**

**NEN Dupont (UK) Ltd, Stevenage, Hertfordshire, UK**

2-deoxy-D- [$^{3}$H] glucose

**NEN Life Science Products, Boston, MA**

$^{125}$I labelled [Sar$^{1}$Ile$^{8}$] Angiotensin II

### 2.1.3 Physiological buffers

**KRH buffer**

Fresh KRH buffer (NaCl 118mM, $NaHCO_3$ 5mM, KCl 4.7mM, $KH_2$ $PO_4$ 1.2mM, $MgSO_4.7H_20$ 1.2mM, HEPES 25mM, pH adjusted to 7.4) was prepared for each assay. Adenosine 100nM and $CaCl_2$ 2.5mM were added for preparation of collection, digestion and wash buffer.

**Wash buffer**

To 400mls of KRH buffer, 4g of Bovine Serum Albumin (BSA) were added, and the pH readjusted to 7.4, constituting the wash buffer.

**Collection buffer**

To 100mls of wash buffer, 0.054g of glucose was added (final glucose concentration 3mM) to make the collection buffer.

**Digestion buffer**

For digestion, 4ml collection buffer with 2mg/ml collagenase was added to each gram of fat.

**HES buffer**

HEPES 20mM, EDTA 1mM, Sucrose 255mM, pH 7.4

**Radioligand binding buffer**

$MgCL_2$10mM, EDTA 1mM, Tris-HCl 50mM, pH 8.26 at 4°C

**Phosphate buffered saline (PBS)**

NaCl 150mM, $NaH_2PO_4.2H_2O$, pH 7.4

**Physiological salt solution (PSS)**

NaCl 118.4mM, KCl 4.7mM, $MgSO_4.7H_2O$ 1.2mM, $NaHCO_3$ 24.9mM, $CaCl_2$ 2.5mM, glucose 11.1mM, EDTA 0.023mM

### 2.1.4 SDS-PAGE buffers

**Electrode buffer**

Tris base 25mM, glycine 192mM, 0.1% (w/v) SDS

**Sample buffer**

Tris. HCl 93mM; pH 6.8, dithiothreitol 20mM (added immediately before use), EDTA 1mM, 10% (w/v) glycerol, 2% (w/v) SDS, 0.002% (w/v) bromophenol blue.

### 2.1.5 Western blot buffers

**Blotting buffer**

$NaH_2PO4.2H_2O$ 25mM; pH 6.5

**TBST-1**

2.42g Tris.HCl, 8.77g NaCl, 0.2ml Tween-20; pH 7.4

## 2.2 Methods

### 2.2.1 Laboratory methods

#### 2.2.1.1 Collection of subcutaneous adipose tissue

All patients were approached the day prior to theatre and informed consent obtained. On the day of theatre, a piece of adipose tissue was removed at the start of the surgical procedure by the operator; tissue weight varied between 1.5g and 5g. The adipose tissue was placed immediately in collection buffer (see 2.1.3), prewarmed to 37°C, and transported to the laboratory.

#### 2.2.1.2 Digestion of adipose tissue

Once in the laboratory, any attached skin, subcutaneous connective tissue or visible blood vessels were dissected from the adipose sample and discarded. The tissue was then weighed, and washed by repeated collection buffer changes (section 2.1.3), while ensuring the temperature of the tissue's immediate environment did not change. For tissue digestion, 4ml of a 2mg/ml solution of collagenase (section 2.1.3) (374) was added for each gram of tissue to a 50ml centrifuge tube containing the adipose tissue sample. The tissue was then chopped into smaller pieces using scissors and agitated in a shaking water bath for 30 minutes. At the end of this period, the digestate was passed through a filter to leave a layer of adipocytes floating on the surface above buffer. This buffer layer was aspirated and replaced with fresh wash buffer (section 2.1.3) five times.

Finally the floating adipocytes were suspended at 90% cytocrit by aspirating the appropriate volume of buffer from below the adipocyte layer. Aliquots were taken and

added to wash buffer to achieve a final adipocyte concentration of 10%, 100µl of cells in 900µl of buffer, in preparation for further assay.

### 2.2.1.3 Glucose transport assay

Adipocytes were resuspended at 10% cytocrit as above. The cells were then incubated with agonist / antagonist. All insulin stimulations were for 30 minutes, and with five minutes of stimulation remaining, a cocktail of $^{3}$[H] 2-deoxy D-glucose, unlabelled 2-deoxy D-glucose and KRH buffer was added, achieving a final concentration of 10µM 2-deoxyglucose / 2.5µCi /ml (described in (351)). Angiotensin II was added with, or 15 minutes prior to, insulin. Each assay condition was matched with an equivalent preparation containing cytochalasin B (10µM). Cytochalasin B, a fungal metabolite, is an effective inhibitor of insulin-stimulated glucose transport. Following the completion of the incubation times, 50µl aliquots of cells were removed from each assay condition and then centrifuged at 3500g through an oil layer. The overlying cells were then aspirated, solubilised in triton-X 100, and $^{3}$[H] counts determined in triplicate using a Beckman scintillation counter. The cytochalasin B counts were then subtracted from each assay result in order to account for non-specific glucose transport. Results were expressed as fold increase in uptake relative to the basal counts.

### 2.2.1.4 Assay of non-esterified (free) fatty acids

Adipocytes were prepared as described in section 2.2.1.1. Into 900µl of wash buffer, 100µl of adipocytes (from suspension at 90% cytocrit) were added, and left to quiesce for 15 minutes. Agents were added at time (t) = 0 minutes. For the basic analysis of insulin-mediated suppression of isoproterenol-induced lipolysis, insulin (0.01-100nM) and isoproterenol (200nM) were added to adipocyte suspensions. In any given experiment, one

assay vial contained adipocytes in buffer alone for analysis of basal NEFA release, one vial contained adipocytes and isoproterenol (200nM), and the other five vials contained isoproterenol (200nM) and increasing concentrations of insulin (0.01-100nM). For the assessment of the effect of angiotensin II on insulin action, angiotensin II was added either with, or 15 minutes prior to, insulin and isoproterenol. For wortmannin assays, wortmannin was added 30 minutes prior to insulin. Once agonist/antagonists were added, the adipocyte suspension was left for 45 minutes shaking at $37^0$C in a water bath. After 45 minutes, 50µl aliquots of buffer were removed from each individual reaction condition and placed in plastic tubes, still at $37^0$C. The amount of liberated fatty acid in the reaction was then assayed using a commercially available kit (NEFA C, Alpha Laboratories). To each 50µl aliquot, 1ml of reagent A, then after ten minutes, 2ml of reagent B were added. Five minutes later 1ml of the resulting mixture was decanted into a cuvette for spectrophotometric analysis at a wavelength of 550nm. Each assay was conducted with a standard at 1mmol/l of free fatty acid (supplied with the assay kit), allowing standardisation, and the absorbance of the media alone taken as the zero value. The basis of the NEFA assay is a two-step enzymatic reaction, involving the activity of acyl CoA synthetase, acyl CoA oxidase and peroxidase.

The recovery of the different free fatty acids (stated in the accompanying data sheet), as a reflection of the assay accuracy, is detailed in table 2.1.

**Table 2.1: Recovery of non-esterified fatty acids, using the NEFA-C WAKO assay**

| Free fatty acid | Recovery (%) | Accuracy (%) |
|---|---|---|
| **Acetic Acid** | 0 | 0 |
| **Propionic Acid** | 0 | 0 |
| **Butyric Acid** | 0 | 0 |
| **Valeric Acid** | 14 | 9 |
| **Caproic Acid** | 98 | 99 |
| **Caprylic Acid** | 97 | 94 |
| **Capric Acid** | 89 | 86 |
| **Lauric Acid** | 98 | 97 |
| **Myristic Acid** | 103 | 93 |
| **Palmitic Acid** | 97 | 94 |
| **Stearic Acid** | 90 | - |
| **Arachidic Acid** | 94 | - |
| **Palmitoleic Acid** | 100 | 103 |
| **Oleic Acid** | 100 | 102 |
| **Linoleic Acid** | 102 | 100 |
| **Arachidonic Acid** | 89 | 85 |
| **Citric Acid** | 0 | 0 |
| **Oxalic Acid** | 0 | 0 |

### 2.2.1.5 Preparation of human adipocyte membrane fractions

Adipocytes were digested as above, and re-suspended at 35% cytocrit. After two washes in ice-cold phosphate buffered saline the cells were resuspended at 66% cytocrit in HES buffer containing protease inhibitors. The remaining steps were carried out at 4°C. The cells were homogenised by 20 up and down strokes of a Dounce homogeniser. They were then centrifuged at 3000g at 4°C for 5 minutes. The resulting fat cake and pellet were discarded, while the supernatant was centrifuged at 100,000g at 4°C for 60 minutes. The membrane pellet was washed then resuspended in binding buffer with protease inhibitor cocktail tablets and protein concentration assayed as described in section 2.2.1.7.

### 2.2.1.6 Radioligand binding studies

Membranes were prepared from either adipocytes or vascular smooth muscle cells (positive control, provided by Dr Jill Wakefield, Institute of Biomedical and Life Sciences, University of Glasgow) and resuspended in radioligand binding buffer. Duplicate 150μl aliquots of the resuspended membranes were then added to glass tubes containing 50μL $^{125}$I labelled [Sar$^{1}$Ile$^{8}$] Angiotensin II and either 50μL of $H_2O$ or losartan (provided by Dr J Brosnan, University of Glasgow) (10μM final concentration) to define non-specific binding. Equilibrium binding was achieved by incubation at 37°C for 45 minutes, and reaction terminated by vacuum filtration over 0.3% (v/v) polyethylenimine-soaked GF/B filters and washing with ice-cold radioligand binding buffer supplemented with 0.01% (w/v) CHAPS using a Brandel cell harvester. Receptor bound ligand was quantified using a gamma counter.

### 2.2.1.7 Determination of sample protein concentration

This method was used to determine the protein concentration in membrane preparations obtained from human adipocytes. A 10$\mu$l aliquot of each sample was added to 200$\mu$l of Bio-Rad protein assay reagent and 790 $\mu$l of water. After mixing and equilibration, the absorbance was read in a spectrophotometer at a wavelength of 595nm. The concentration of the sample was determined from a standard curve constructed from bovine serum albumin standards (0.125mg/ml-2mg/ml) treated in the same manner.

### 2.2.1.8 SDS/Polyacrylamide gel electrophoresis

Sodium dodecyl sulphate (SDS)/ Polyacrylamide gel electrophoresis (PAGE) was undertaken using the Bio-Rad mini-Protean II gel apparatus. 4cm of resolving gel (10% acrylamide / 0.28% bisacrylamide in 0.383mM Tris.HCl, pH 8.8; 0.1% SDS, polymerised with 0.1% ammonium persulphate and 0.019% *N,N,N',N'*, -tetramethylenediamine (TEMED)) underlay 2cm of stacking gel (5% acrylamide / 0.136% bisacrylamide in 125mM Tris.HCl, pH 6.8; 0.1% SDS, polymerised with 0.1% ammonium persulphate and 0.05% TEMED).

After separation of the proteins, the gels were removed and equilibrated in blotting buffer (see 2.1.5) for 30 minutes. Each gel was then placed on nitrocellulose paper and sandwiched between two layers of filter paper. This was placed in a cassette and the proteins transferred onto the nitrocellulose in a transfer tank. After 3 hours, the nitrocellulose was removed and placed in blocking buffer (TBST-1 with 5% (w/v) non-fat milk) overnight. The membrane was then placed in 1% (w/v) non-fat milk/TBST-1 containing primary antibody and shaken at 37°C for one hour. Following five washes with TBST-1 over the next hour, the membrane was incubated for one hour at room temperature

in 1% non-fat milk/TBST-1 containing species-specific HRP-linked anti-IgG. Following this, the nitrocellulose membrane was washed then submerged in solution one and two (Amersham ECL Western Blotting detection kit) for one minute. The nitrocellulose was then placed in a X-ray film cassette and exposed to Kodak X-Omat S film prior to developing.

### 2.2.2 Clinical methods

#### 2.2.2.1 Recruitment of patients and volunteers

The Ethics Committee of the West Glasgow Hospitals University NHS Trust approved all clinical and experimental protocols. After full explanation of experimental procedures, informed consent was obtained. Each volunteer was issued with details of the study as well as investigators` contact telephone numbers.

Healthy volunteers for clinical studies were recruited in the Clinical Investigation and Research Unit (CIRU), Department of Medicine and Therapeutics, Western Infirmary, Glasgow. Medical and nursing students were excluded. All volunteers were aged 18-40 years.

Volunteers for clinical studies were screened by health questionnaire and physical examination. Venous blood was obtained for haematology, biochemistry and virology to assess suitability for studies prior to recruitment. Volunteers with elevated fasting plasma glucose, hypertension and intercurrent acute or chronic illness were excluded. In addition, volunteers underwent routine electrocardiography and were excluded if a resting ECG was abnormal. Female volunteers were not recruited into clinical studies. Volunteers were instructed to fast overnight prior to clinical procedures and assessments. Taxis were available to transfer volunteers to and from the CIRU.

For laboratory studies requiring human tissue, gynaecology patients suitable for donation of subcutaneous adipose tissue during routine laparotomy were approached 24 hours prior to theatre, and their consent obtained for tissue extraction as well as peripheral venous

blood sampling. All patients were undergoing surgery for benign disease. Patients with non-gynaecological illness, including hypertension and diabetes, were not approached. Any abnormalities of peripheral blood subsequently found were relayed to the appropriate General Practitioner.

#### 2.2.2.2 Clinical and morphometric measurements

##### 2.2.2.2.1 Body mass index

Body weight and height were measured with subjects in light clothes without shoes to the nearest 0.5 kg of weight and to the nearest 0.5cm height. The weighing scales were calibrated regularly (CIRU and ward G9 scales, Western Infirmary, Glasgow).

Body mass index (BMI, $kg/m^2$) was calculated from the formula

Body Mass Index = Body weight (kg) / (height (m)) $^2$

##### 2.2.2.2.2 Blood pressure and heart rate

All blood pressure and pulse measurements were recorded using a Dinamap Critikon (Johnson and Johnson Professional Products Ltd UK). At volunteer screening, and in the assessment of gynaecological patients, readings were taken supine after ten minutes rest. Blood pressure readings prior to and during the euglycaemic hyperinsulinaemic clamp were taken with the volunteer lying supine.

#### 2.2.2.3 Clinical procedures

##### 2.2.2.3.1 Hyperinsulinaemic euglycaemic clamp

Insulin sensitivity was assessed using a modification (180 rather than 120 minutes) of the hyperinsulinaemic euglycaemic clamp (10). Volunteers were asked to attend the CIRU having fasted from midnight the preceding evening. With the volunteer lying at 45° on a

bed, two 18-gauge intravenous cannulae were inserted; the first was placed anterogradely into the left antecubital fossa for administration of glucose and insulin infusions, and the second retrogradely into the right hand for the sampling of venous blood. The right hand was then placed in a heated box for the remainder of the morning, with the temperature inside the box kept at 55°C to arterialise sampled venous blood.

Intravenous soluble insulin was infused in 0.9% saline with a 10% v/v dilution of the volunteer's own venous blood to minimise adsorption of insulin to the plastic syringe or infusion device. This was infused into the left antecubital fossa cannula using a Braun Perfusor pump at a constant rate for 180 minutes after a brief initial period of priming. The aim was to achieve steady state circulating insulin at approximately 120μU/ml above the basal fasting level.

The insulin infusion regime was as follows

| | |
|---|---|
| 0-4 minutes | 4.5mU/kg/min |
| 4-7 minutes | 3.0mU/kg/min |
| 7-180 minutes | 1.5mU/kg/min |

Prior to the commencement of the infusions, the volunteer lay rested for 30 minutes after insertion of the cannulae to allow for acclimatisation. During this period, basal blood samples were taken. Blood pressure and heart rate were recorded at baseline, and every 15 minutes thereafter during the clamp. 20% glucose (Baxter Healthcare, Norfolk, U.K.) was infused into the left antecubital cannula at a variable rate via an IMED infusion device (IMED, Abingdon, U.K.).

Insulin was commenced at time (t) = zero minutes and adjusted according to the above regimen, while glucose infusion commenced at t = two minutes and was altered accordingly thereafter. While the insulin infusion was held at a constant rate following the priming period, the glucose infusion rate was adjusted throughout the clamp to maintain serum glucose at 5.2mmol/l. Arterialised venous blood was drawn from the warmed hand cannula every five minutes, centrifuged for ten seconds at 1000 revolutions per minute (rpm), and serum glucose determined by the glucose oxidase method using a Beckman 2 glucose analyser (Beckman Instruments, Fullerton, CA, USA). The glucose infusion was altered at increments of 1mg glucose/kg body weight/min (mg/kg/min). The retrograde cannula in the warmed hand was kept patent by the infusion of 0.9% saline (<100ml total volume infused per clamp) and frequent saline bolus flushes.

**2.2.2.3.2 Calculation of insulin sensitivity**

During the euglycaemic hyperinsulinaemic clamp, steady state was achieved once the serum glucose was stable at around 5.2mmol/l at a constant glucose infusion rate. Once reached, this was maintained until the last 40 minutes of the clamp, when the glucose infusion was held constant and the serum glucose documented every five minutes. Central to the calculation of insulin sensitivity (M) is the assumption that at this dose of insulin infusion, there is total suppression of hepatic glucose output, and so the glucose infusion rate equates to the disappearance of glucose into insulin sensitive tissues i.e. insulin sensitivity.

In the calculation of M, corrections are made for urinary glucose loss (UC) and for any difference between the desired serum glucose of 5.2mmol/l and the actual average serum glucose value during steady state.

Thus, to calculate insulin sensitivity (M)

**M =** **I – UC + SC**

I = average glucose infusion rate during steady state

UC = correction for urinary loss (negligible during hyperinsulinaemic euglycaemic clamp)

SC = space correction (mg/kg/min)

**SC =** **(5.2-G) x 17.86 x 0.095**

G = mean glucose during steady state

17.86 = unit conversion factor (mmol/l to mg/dl)

0.095= glucose space constant

Morris *et al* calculated the between day intra individual coefficient of variation for insulin sensitivity to be 6% using this technique (21).

**2.2.2.3.3 Buttock biopsy**

Subjects were invited to attend the CIRU at 0830, having fasted from midnight. With the subject lying prone, one buttock was exposed and the area sterilised with 10% Iodine. 1% lignocaine hydrochloride was then instilled subcutaneously to cover an area of 6cm x 4cm. Once the area was anaesthetised, an elliptical incision no greater than 3cm x 0.75 cm was made and a diamond shaped segment of adipose tissue was removed. Four skin stitches were then inserted to close the wound. The volunteer was then given a light meal and asked to refrain from exertion for five days while keeping the wound clean. Ten days later the stitches were removed.

### 2.2.2.4 Assay methodology for analysis of venous blood

#### 2.2.2.4.1 Analysis of routine blood samples in Gartnavel General Hospital

Samples for urea, creatinine, electrolytes, total cholesterol, triglycerides, HDL-cholesterol, LDL-cholesterol, glucose, cortisol and growth hormone were collected and placed immediately into the appropriate containers. They were then analysed as routine samples in the Biochemistry Department, Gartnavel General Hospital.

#### 2.2.2.4.2 Assay of non-routine blood samples

Samples for measurement of renin, angiotensin II, aldosterone and catecholamines were undertaken in the laboratory of Dr JJ Morton, Blood Pressure Unit, Western Infirmary, Glasgow. Total plasma renin concentration was measured using a micro assay based on antibody trapping as described by JA Millar *et al* (339). Plasma AII was measured using a kit from Diagnostic Products Ltd, Glyn Rhonwy, Gwynedd, UK. Plasma aldosterone was measured using a commercially available radioimmunoassay (RIA) kit (Biodata, Milan), using the method of Mackenzie and Clements (340). Catecholamines were measured by high pressure liquid chromatography (HPLC) with electrochemical detection (341). Samples for insulin and C-peptide were analysed by commercially available radioimmunoassay (DiaSorin, UK). The fasting insulin resistance index was calculated as described by Duncan (18);

FIRI = (fasting glucose x fasting insulin) / 25

Plasma levels of tissue plasminogen activator were measured with a commercially available enzyme linked immunosorbent assay (ELISA) from Biopool AB, Umea, Sweden.

Plasma von Willebrand factor (vWF) antigen levels were measured using an in-house (Professor GDO Lowe, Dr A Rumley, Glasgow Royal Infirmary) ELISA, employing rabbit anti-human polyclonal antibodies obtained from DAKO plc, High Wycombe, UK.

#### 2.2.2.4.3 Assay of urinary biochemistry

Urine was collected in plain containers over a 24-hour period for assay of urinary sodium and cortisol. Samples were assayed in the Biochemistry Department, Gartnavel General Hospital.

### 2.2.2.5 Myography protocol

#### 2.2.2.5.1 Preparation of arteries

Resistance arteries were dissected as described in section 2.2.2.3.3. Where possible, four segments of artery (approx. 200-400μm average diameter and 2mm in length) were mounted as ring preparations on two 40μm stainless steel wires in a four-channel small vessel myograph (Danish MyoTechnology, Aarhus, Denmark), as initially described by Mulvany and Halpern (342).

One wire was attached to an isometric force transducer and the other to a movable micrometer. The vessels were bathed in a physiological salt solution (PSS). The temperature was maintained at 37 $^{0}$C, and pH maintained at 7.4, with a gas mixture of 5%$CO_2$ and 95%$O_2$ being bubbled throughout the experiment.

#### 2.2.2.5.2 Normalisation of vessels

Following a rest period of 30 minutes, each artery was stretched at one-minute intervals to determine the resting tension-internal circumference (L) relationship. The LaPlace

equation, P=T/r (P is the effective pressure, T is the wall tension and r is the internal radius), was used to determine $L_{100}$. This is the calculated internal diameter the vessel would have *in vivo* when relaxed and subjected to a transmural pressure of 100mmHg (13.3Kpa). To achieve optimal contraction, each vessel was then set to the normalised internal diameter $L_1 = 0.9L_{100}$

**2.2.2.5.3 Myography protocol**

A trained technician, blinded to study randomisation phases, undertook all myography studies. Following the normalisation procedure described above, the vessels were maintained in physiological salt solution (PSS) at $37^0$C for a further 60 minutes. The vessels were then exposed twice to KPSS (PSS solution with KCl substituted for NaCl on an equimolar basis). Vessels were then incubated for 30 minutes in PSS before a cumulative concentration response curve (CRC) to norepinephrine (NE) from 1nM to 30$\mu$M. After a further 30-minute incubation, a plateau contraction was obtained with 10$\mu$M NE before a concentration response curve to acetylcholine (ACh) from1nM to 30$\mu$M was performed. If vessels were either unable to contract to KPSS or NE, or showed no relaxation to ACh, they were excluded from the study.

Vessels were then pre-incubated for 30 minutes in PSS alone (control) or insulin (1nM), insulin (100pM), or insulin (10pM) before a NE concentration response curve. Finally, vessels were pre-incubated with insulin as before with the addition of L-NG-monomethyl-arginine (L-NMMA) (1$\mu$M) to all vessels. A final concentration response curve to NE was then obtained.

#### 2.2.2.6 Statistical techniques employed in this thesis

All statistical analysis was undertaken using the Minitab statistical package (Minitab Inc., Pennsylvania, USA). The level of statistical significance was taken to be a p value of <0.05. Data are expressed as mean and standard errors unless stated otherwise. The Anderson-Darling test was used to assess the distribution of a dataset, and if not normally distributed, data were log transformed.

##### 2.2.2.6.1 Metabolic studies

For the studies comparing metabolic variables during active and placebo phases, statistical analysis was undertaken using the paired Student's t-test. In the comparison of unpaired groups with normally distributed variables, an unpaired t-test was used.

##### 2.2.2.6.2 Studies in human adipocytes

For comparison of the effect of angiotensin II on insulin action in isolated adipocytes, the unpaired t-tèst was used. Where appropriate, area under the curve was calculated as a summary measure of serial observations (343). Simple correlations were tested using Pearson's correlation, after logarithmic transformation where appropriate.

##### 2.2.2.6.3 Myography studies

In this thesis, two parameters were used to compare myography data between phases. Response was first expressed as the $pD_2$, which is the negative log of the concentration of agonist (norepinephrine) required to produce 50% of the maximal contractile response (1$\mu$M norepinephrine). This is referred to as the sensitivity. In addition, using the Law of LaPlace, the maximum active effective pressure ($mN/mm^2$) was calculated from wall tension and internal radius, and expressed as an absolute number. This second parameter

allowed comparison of the maximum effect of agonist, in the presence and absence of insulin.

# Chapter 3: Insulin-mediated suppression of lipolysis; development and application of an assay of insulin sensitivity

## 3.1 Introduction

Insulin sensitivity is considered by convention to be sensitivity to the glucose lowering action of insulin, measured systemically using the techniques described in chapter 1. It remains difficult, however, to measure insulin action in isolated human tissue. Furthermore, it cannot be assumed that whole body and cellular insulin sensitivity are closely correlated in an individual; whole body insulin sensitivity (M) values generated from the euglycaemic hyperinsulinaemic clamp are the result of integrated insulin responses in various tissues, and may also be influenced by properties of the vascular beds supplying these tissues. In this chapter the development of an assay of adipocyte insulin sensitivity is described, however rather than examining the glucose lowering effects of insulin, the action of insulin to attenuate the process of lipolysis and release of non-esterified fatty acids (NEFA) is measured. Having developed this technique, associations of insulin action in isolated adipocytes with the phenotypic characteristics of the insulin resistance syndrome are described. While intuition would suggest that insulin resistance in isolated tissue is associated with type 2 diabetes, hypertension, dyslipidaemia and obesity, the literature that supports this is limited largely to the study of populations with severe syndromes of insulin resistance, such as pathological obesity (153) and type 2 diabetes (151).

Two hypotheses are investigated:

- That insulin will attenuate the process of isoproterenol-induced lipolysis in isolated human adipocytes.
- That the application of this assay to healthy females will show a relationship between insulin action in isolated tissues and characteristics of the insulin resistant phenotype.

## 3.2 Methods

### 3.2.1 Identification and recruitment of subjects

Women were recruited from the gynaecology wards of the Western Infirmary, Glasgow. All subjects were undergoing laparotomy for non-malignant gynaecological disease.

Exclusion criteria were as follows

- Treated or untreated hypertension (BP > 160/90 mmHg)
- Type 1 or 2 diabetes
- Family history of diabetes in first degree relative
- Intercurrent drug therapy
- Malignant disease
- Post menopausal or <6 periods in last twelve months

The protocol was approved by the Ethics Committee of the Western Infirmary, and all subjects gave informed consent. Peri-umbilical subcutaneous adipose tissue was obtained and prepared as described in Chapter 2.2.1.1

### 3.2.2 Preliminary studies of NEFA release

Preliminary experiments were undertaken to develop the assay of insulin-mediated attenuation of isoproterenol-induced lipolysis. In all experiments, adipocytes were obtained as described in section 2.2.1.1 and assays undertaken to determine;

1. The time course of isoproterenol-induced lipolysis ± adenosine. Cells were incubated with isoproterenol 200nM for 20, 40 and 60 minutes, having been digested and suspended in the presence or absence of adenosine (100nM).
2. The concentration-response relationship of isoproterenol-induced lipolysis ± adenosine. Cells were incubated with increasing concentrations of isoproterenol, from 2nM to 5$\mu$M, for 45 minutes and NEFA release assayed. Cells were digested either in the presence or absence of adenosine 100nM.
3. The time course of insulin-mediated suppression of isoproterenol-induced lipolysis. The temporal nature of insulin's suppression of isoproterenol-induced lipolysis was examined using two insulin concentrations (0.1nM, 10nM) incubated for 15, 30, 45 and 60 minutes with adipocytes treated for 45 minutes with isoproterenol 200nM. Adenosine 100nM was present in the buffer.
4. The effect of wortmannin on basal and insulin-attenuated lipolysis. Adipocytes were incubated in the presence and absence of wortmannin 100nM for 30 minutes, then the release of NEFA in either the unstimulated state or the insulin-mediated suppression of isoproterenol-induced lipolysis measured. Assays were undertaken in the presence of adenosine 100nM.

### 3.2.3 Studies of insulin action and subject phenotype

Adipose tissue was obtained at laparotomy from 20 females. Results were analysed in two ways; the subjects were divided into two discrete groups defined on the basis of BMI, providing a lean (BMI $\leq$25kg/m$^2$) and obese (BMI$\geq$30kg/m$^2$) group (as defined by the WHO (344)), and also by examining data from all 20 volunteers and testing for a continuous relationship between insulin action and characteristics of the insulin resistant phenotype. The index of insulin sensitivity was expressed as the area under the curve (AUC) of insulin-mediated attenuation of isoproterenol-induced lipolysis between concentrations of 0.01nM and 10nM insulin – the greater the area under the curve, the lesser the sensitivity to insulin. As the response to insulin at 1nM-10nM was maximal, and falling at 100nM concentrations, analysis of the AUC was not extended to include that seen at 100nM insulin.

### 3.2.4 Measurement of BMI and blood pressure

BMI and blood pressure were measured as described in Chapter 2.

### 3.2.5 Serum insulin and fasting insulin resistance index (FIRI)

Samples for fasting insulin and glucose were obtained on the day of theatre when possible, and assayed as described in Chapter 2. FIRI was calculated as described by Duncan *et al* (18).

### 3.2.6 Serum lipid analysis

Samples for fasting lipid profile were obtained on the day of theatre when possible and processed as described in materials and methods.

### 3.2.7 Statistical analysis

In all assays, each expressed value was determined from the mean of two values assayed in exactly the same manner. Comparison of groups was undertaken using an unpaired t-test. For the correlation analyses, variables were tested for normality using the Anderson-Darling normality test and logarithmically transformed as appropriate. In all cases, $p<0.05$ was taken as signifying conventional levels of statistical significance.

## 3.3 Results

### 3.3.1 Preliminary assays of NEFA release and insulin suppression

Preliminary assays were undertaken to demonstrate and confirm several basic tenets of the assay technique.

#### 3.3.1.1 Time course of isoproterenol-induced lipolysis

A time course of isoproterenol incubation and NEFA release was undertaken in isolated human adipocytes (figure 3.1), demonstrating increasing release of NEFA in response to a single concentration of isoproterenol (200nM) for up to one hour.

#### 3.3.1.2 Concentration-response of isoproterenol-induced lipolysis

Figure 3.2 demonstrates a concentration response curve of isoproterenol-induced lipolysis in isolated human adipocytes. Notably, incubation with 200nM isoproterenol (subsequently used as the standard concentration in the final assay) induced a sub-maximal release of NEFA into the media. In addition, figure 3.2 shows the anticipated anti-lipolytic action of pre-incubation with adenosine (100nM) on isoproterenol-induced lipolysis.

#### 3.3.1.3 Time course of insulin action

Insulin's attenuation of lipolysis was confirmed in this preliminary experiment and its action was shown to be time-dependent across the limited range of incubation periods studied (figure 3.3).

#### 3.3.1.4 Wortmannin and insulin-mediated attenuation of lipolysis

Isolated cells were incubated with 100nM wortmannin for 30 minutes, then the basal release of NEFA assayed and this value compared with cells untreated with wortmannin

(Figure 3.4.1). A significant increase in the basal release of NEFA was demonstrated in the presence of wortmannin ($p=0.026$ comparing wortmannin present and absent, $n=4$). No effect of wortmannin *per se* was seen on the assay standard with a predetermined NEFA concentration of 1mmol/l, confirming that there was no confounding effect of wortmannin on the readings obtained from light spectrophotometry.

Cells were preincubated with 100nM wortmannin for 30 minutes and insulin-mediated attenuation of isoproterenol-induced lipolysis assayed (figure 3.4.2). Wortmannin abrogated the effect of insulin to suppress NEFA release ($p<0.01$ comparing AUC ± wortmannin 100nM, $n=4$). These assays confirmed the dependence on PI3 kinase of insulin's attenuation of lipolysis, and in addition suggested a role for PI3 kinase in the determination of the basal rate of lipolysis.

### 3.3.2 The relationship between adipocytes *ex vivo* and the clinical phenotype

#### 3.3.2.1 Baseline characteristics

Table 3.1 details the mean baseline characteristics of all 20 subjects recruited, while in table 3.2 the characteristics of the lean ($n=9$) and obese ($n=9$) groups are shown. Importantly, while BMI, fasting insulin and LDL-cholesterol were lower in the lean subjects, there was no difference in blood pressure, age or fasting glucose.

#### 3.3.2.2 Basal release of NEFA and phenotypic characteristics

The relationship between the basal release of NEFA (i.e. the amount of NEFA in media after 45 minutes incubation of adipocytes in buffer alone) and phenotypic characteristics was examined. Given the non-parametric distribution of the values of the basal NEFA release, data were logarithmically transformed. Significant relationships were observed

between BMI (figure 3.5.1), serum total/HDL-cholesterol ratio (figure 3.5.2), and age (figure 3.5.4).

### 3.3.2.3 Insulin-mediated suppression of isoproterenol-induced lipolysis and phenotypic characteristics

Insulin-mediated suppression of isoproterenol-induced lipolysis was significantly less in obese subjects compared with their lean counterparts (expressed as the AUC of the insulin (10pM-10nM) concentration response curve, figure 3.6, $p<0.01$ comparing lean and obese groups). Indeed, in correlation analyses, the AUC of insulin concentration response curve was shown to correlate significantly with BMI and total/HDL-cholesterol ratio, though not FIRI or age (figure 3.7.1-4). There was no correlation between the release of NEFA in response to isoproterenol alone and BMI (figure 3.8).

**Table 3.1: Baseline characteristics of all 20 subjects studied**

| | Mean ± S.D. |
|---|---|
| **Number** | 20 |
| **Age (years)** | 38.7 ± 7.3 |
| **Mean Arterial Pressure** | 91.8 ± 9.1 |
| **BMI ($kg/m^2$)** | 29.1 ±5.5 |
| **Fasting Insulin Resistance Index** | 3.1 ± 1.3 |
| **Total cholesterol (mmol/l)** | 4.0± 0.7 |
| **Total:HDL cholesterol** | 3.6 ± 1.4 |
| **Triglycerides (mmol/l)** | 1.14 ± 0.7 |
| **Smokers** | 6 |

**Table 3.2: Characteristics of the lean and obese subgroups**

| | Lean (mean±SD) | Obese (mean±SD) | t-test (unpaired) |
|---|---|---|---|
| **Number** | 9 | 9 | |
| **Age (years)** | 40 ± 5.1 | 37.66 ± 11 | 0.79 |
| **Mean Arterial Pressure (mmHg)** | 87.66 ± 7.2 | 95.2 ± 9.9 | 0.09 |
| **BMI ($kg/m^2$)** | 24.2 ± 1.4 | 34.22 ± 3.2 | <0.001 |
| **Fasting Insulin Resistance Index** | 2.4 ± 0.6 | 3.9 ± 1.5 | 0.014 |
| **Total cholesterol (mmol/l)** | 3.64± 0.5 | 4.31± 0.8 | 0.06 |
| **Total: HDL cholesterol** | 3.0 ± 0.5 | 3.8 ± 1.5 | 0.13 |
| **LDL cholesterol (mmol/l)** | 2.0 ± 0.4 | 2.5 ± 0.5 | 0.034 |
| **Triglycerides (mmol/l)** | 0.9 ± 0.23 | 1.3 ± 0.9 | 0.25 |
| **Smokers** | 3 | 3 | |

**Figure 3.1: Time course of isoproterenol-induced lipolysis**

Cells were incubated with isoproterenol 200nM for 20, 40 and 60 minutes, both in the presence and absence of adenosine (100nM).

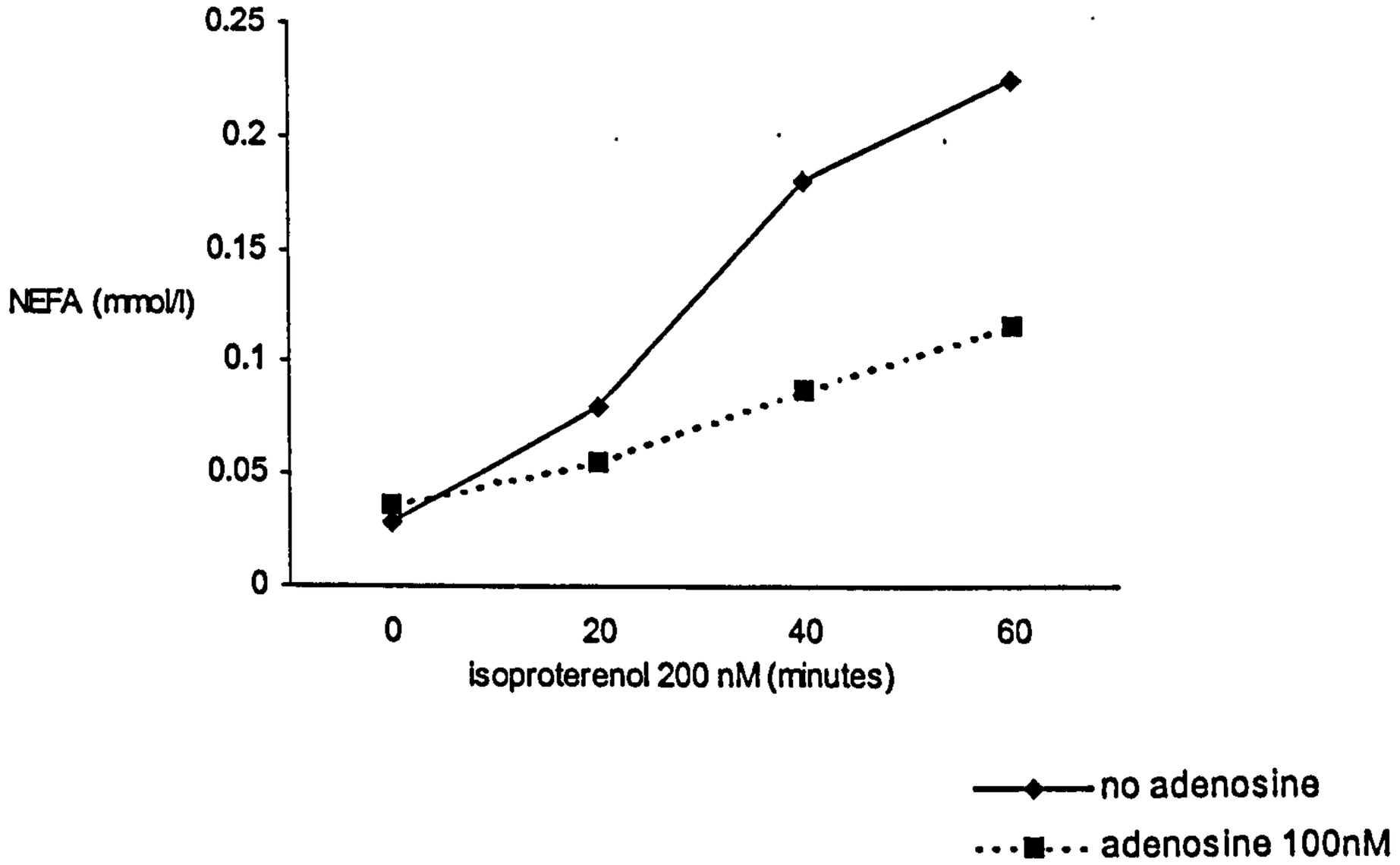


**Figure 3.2: Concentration-response relationship of isoproterenol-induced lipolysis**

Cells were incubated with increasing concentrations of isoproterenol, from 2nM to 2$\mu$M, for 45 minutes and NEFA release assayed. This was performed in the presence of adenosine 100nM.

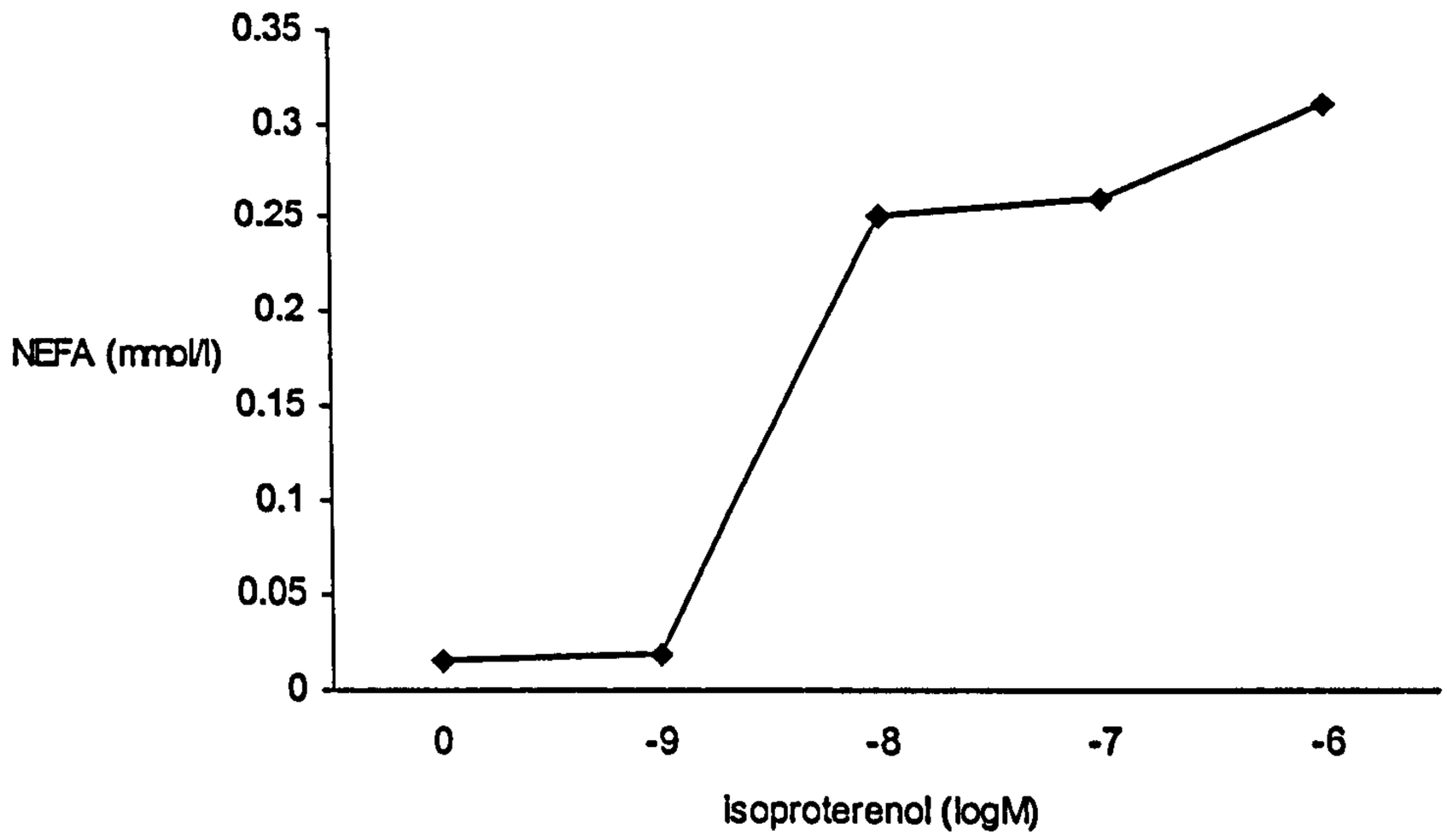


**Figure 3.3: Time course of insulin-mediated suppression of isoproterenol-induced lipolysis.**

The temporal nature of insulin's suppression of isoproterenol-induced lipolysis was examined using two insulin concentrations (100pM, 10nM) incubated for 15, 30, 45 and 60 minutes with adipocytes treated for 45 minutes with isoproterenol 200nM. Adenosine 100nM was present in the buffer.

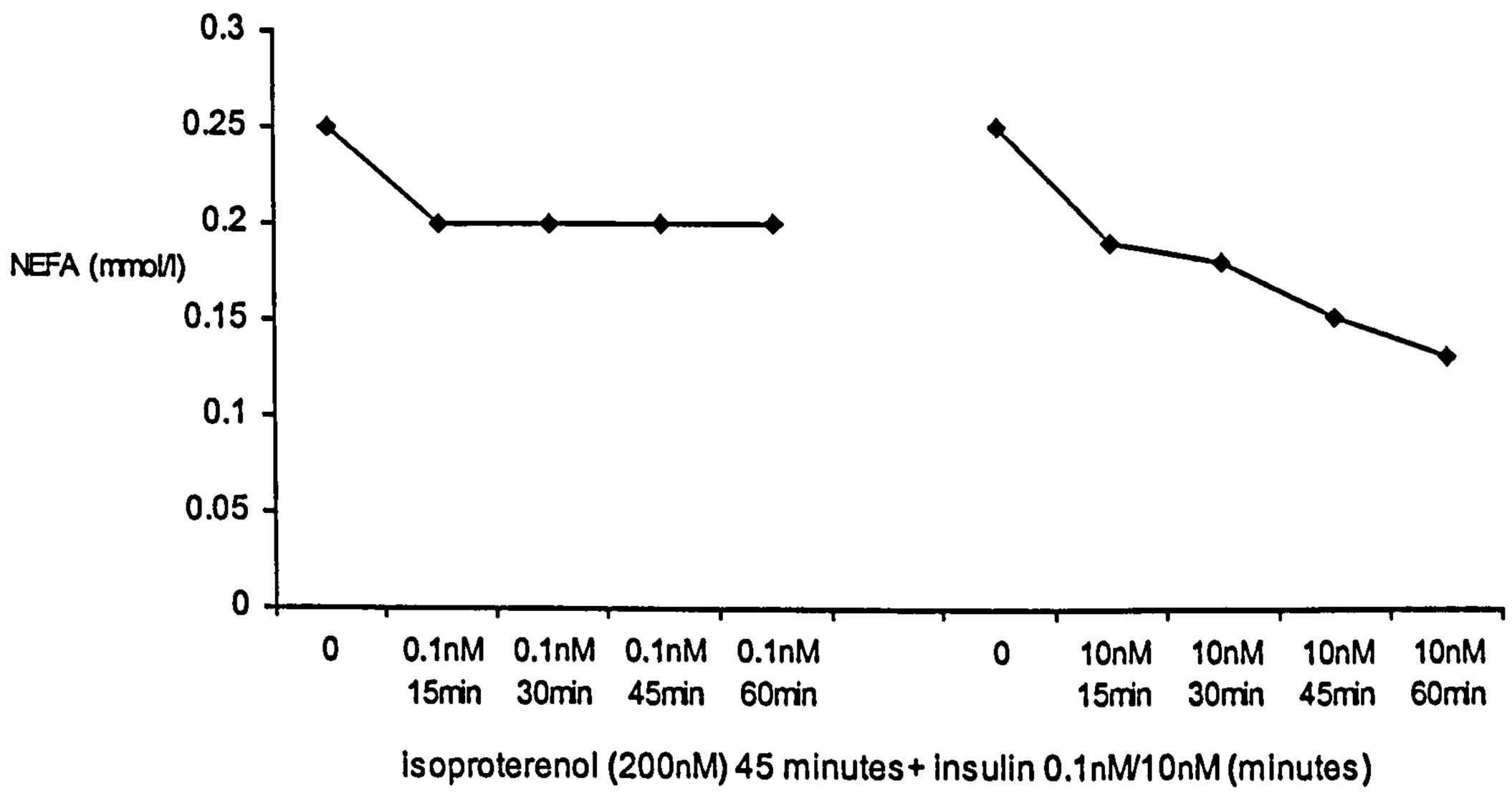


**Figure 3.4: Effect of wortmannin on basal and insulin-attenuated lipolysis.**

Adipocytes were incubated in the presence and absence of wortmannin 100nM for 30 minutes, then either the release of NEFA in the unstimulated state (Figure 3.4.1 ($p<0.05$ for comparison of basal NEFA release ± wortmannin) or the insulin-mediated suppression of isoproterenol-induced lipolysis (Figure 3.4.2) ($p<0.05$ for comparison of AUC of insulin-mediated attenuation of isoproterenol-induced lipolysis ± wortmannin) measured. Assays were undertaken in the presence of adenosine 100nM.Error bars denote standard errors.

**Figure 3.4.1**

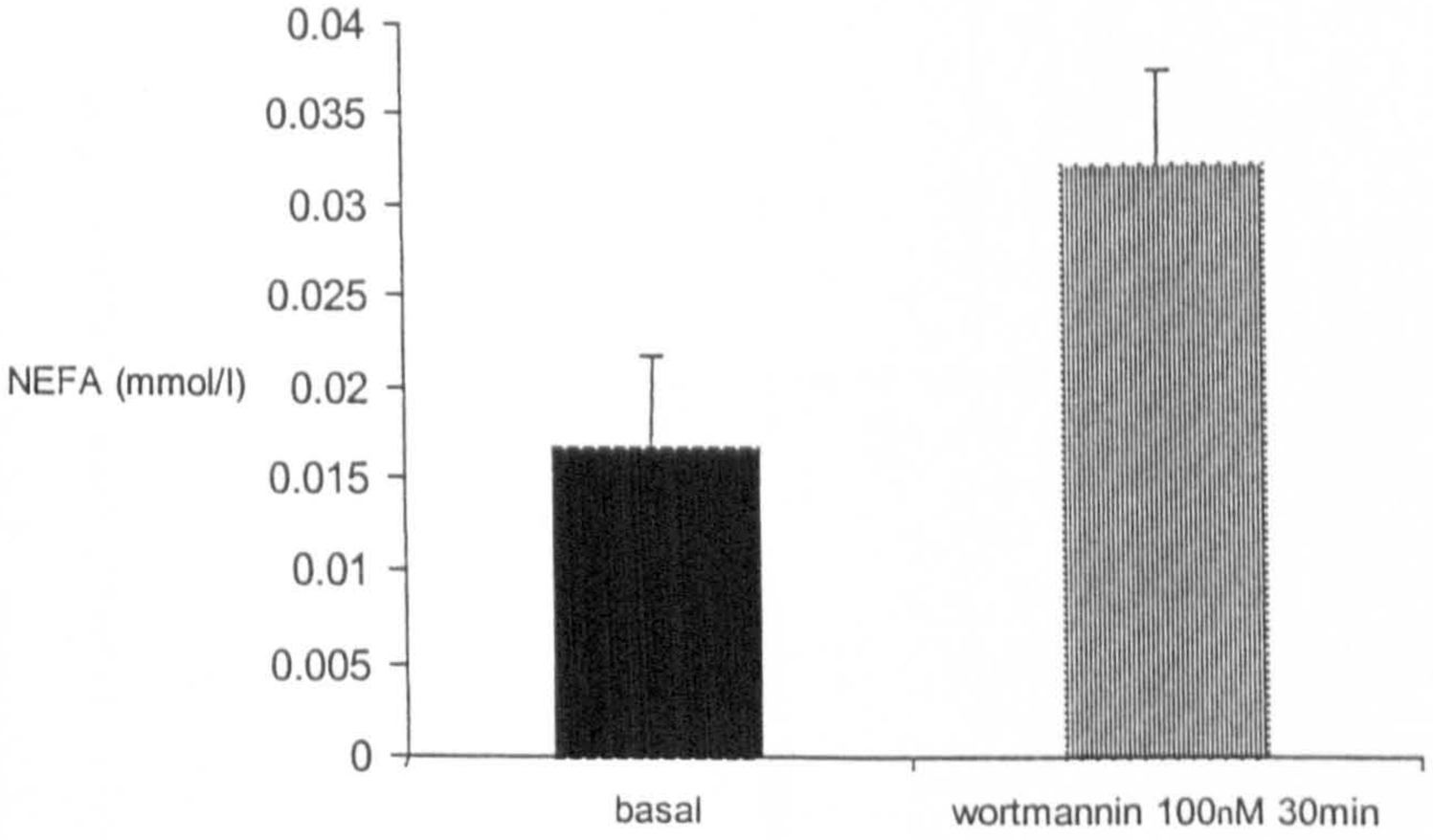



**Figure 3.4.2**

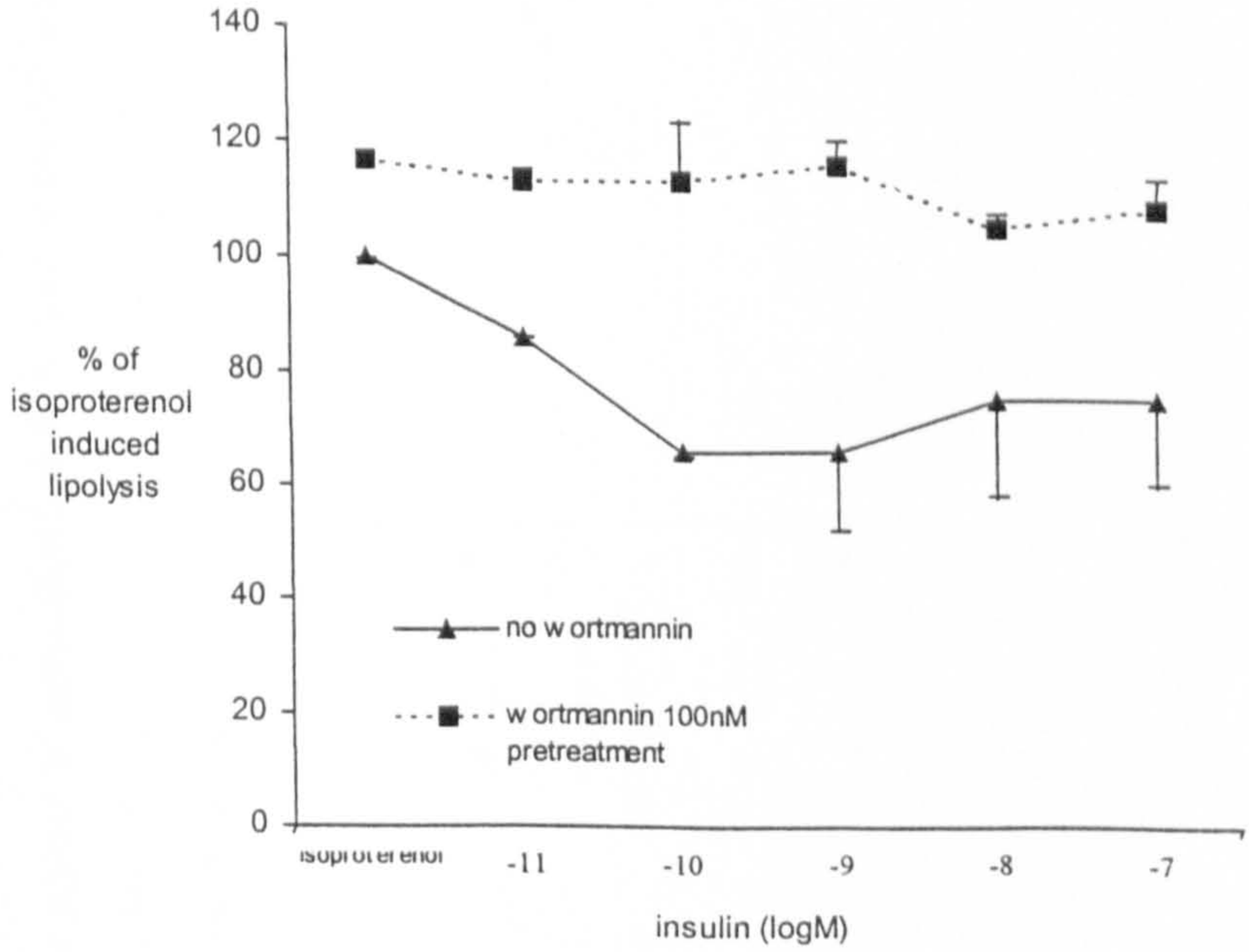
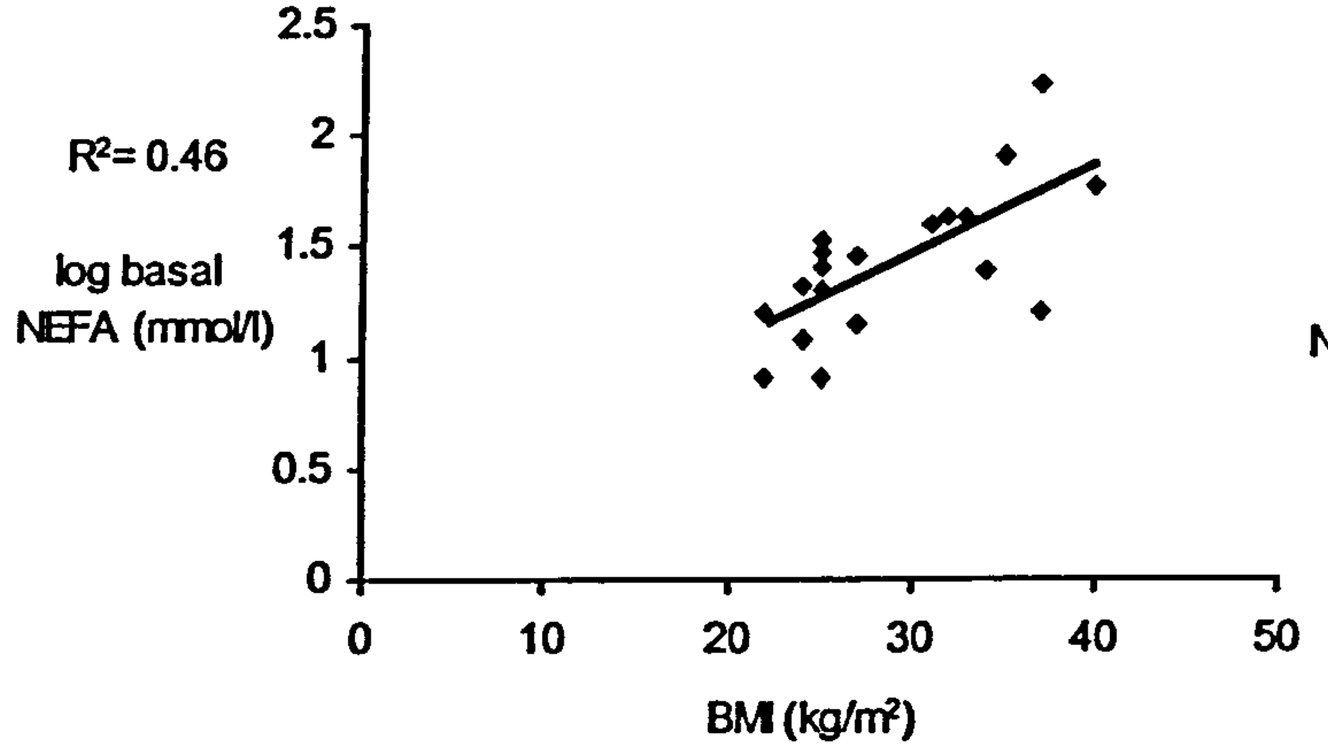
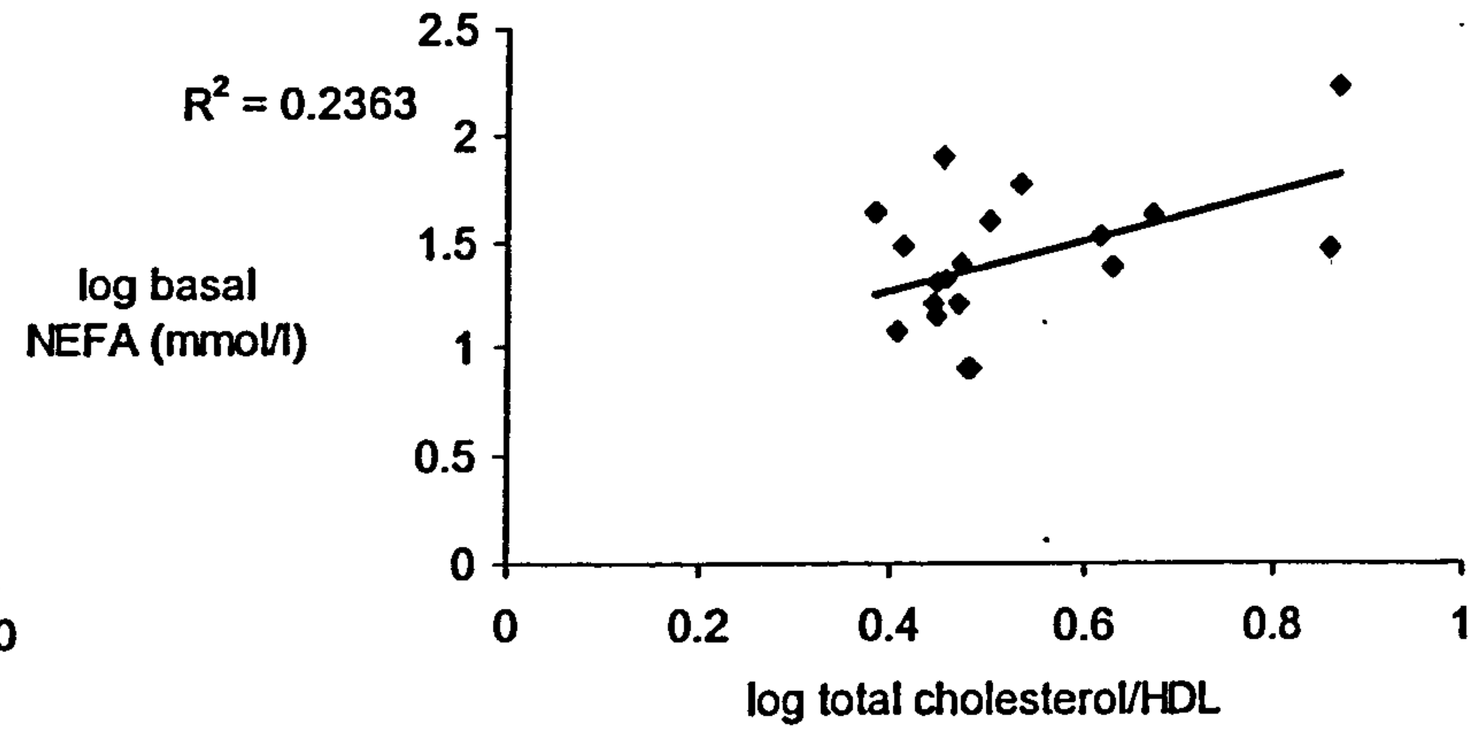
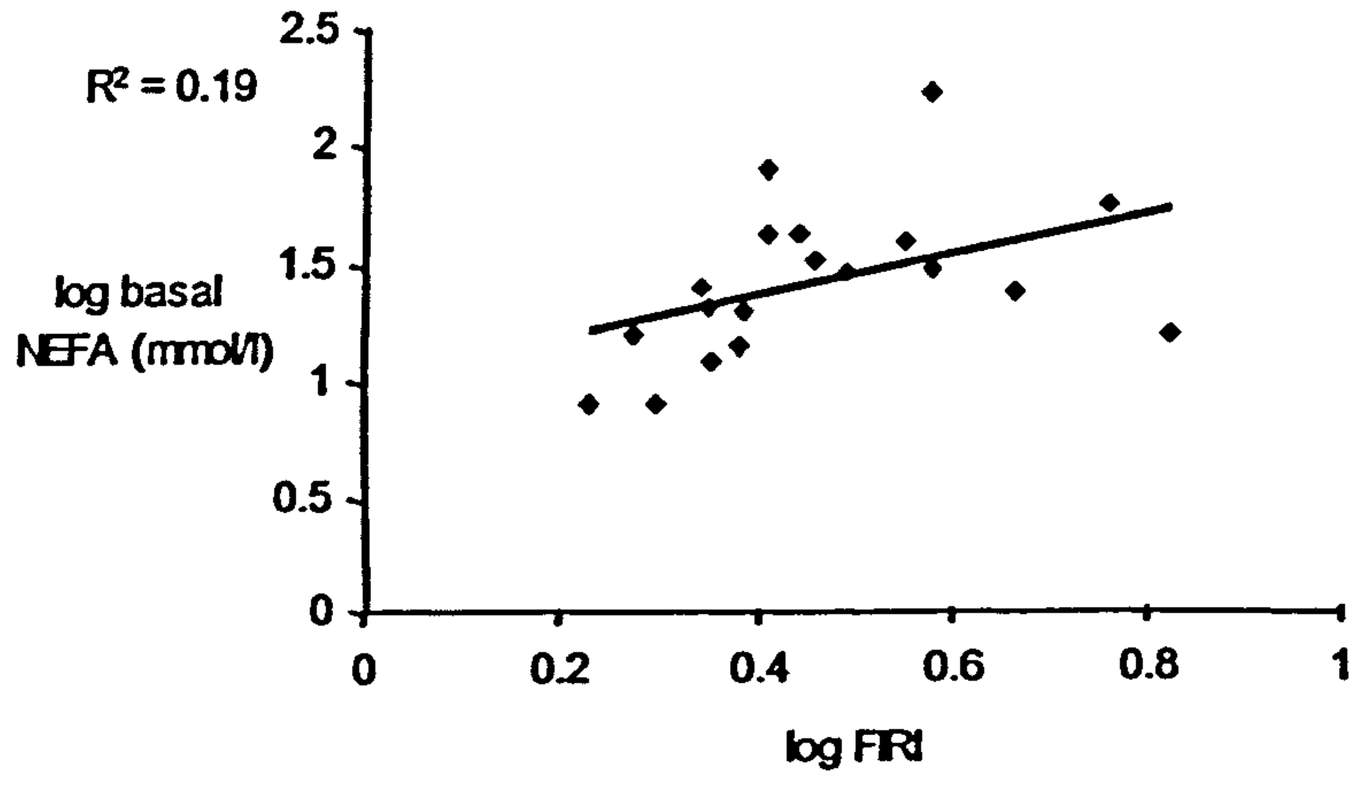


**Figure 3.5: Basal adipocyte non-esterified fatty acid release and phenotype characteristics.**

The basal release of non-esterified fatty acid from subcutaneous adipocytes obtained from 20 healthy females undergoing laparotomy was examined in relation to BMI ($p<0.01$) Figure 3.5.1, total/HDL-cholesterol ratio ($p<0.01$) Figure 3.5.2, FIRI ($p>0.05$) Figure 3.5.3 and age ($p<0.05$) Figure 3.5.4. Error bars represent standard errors.



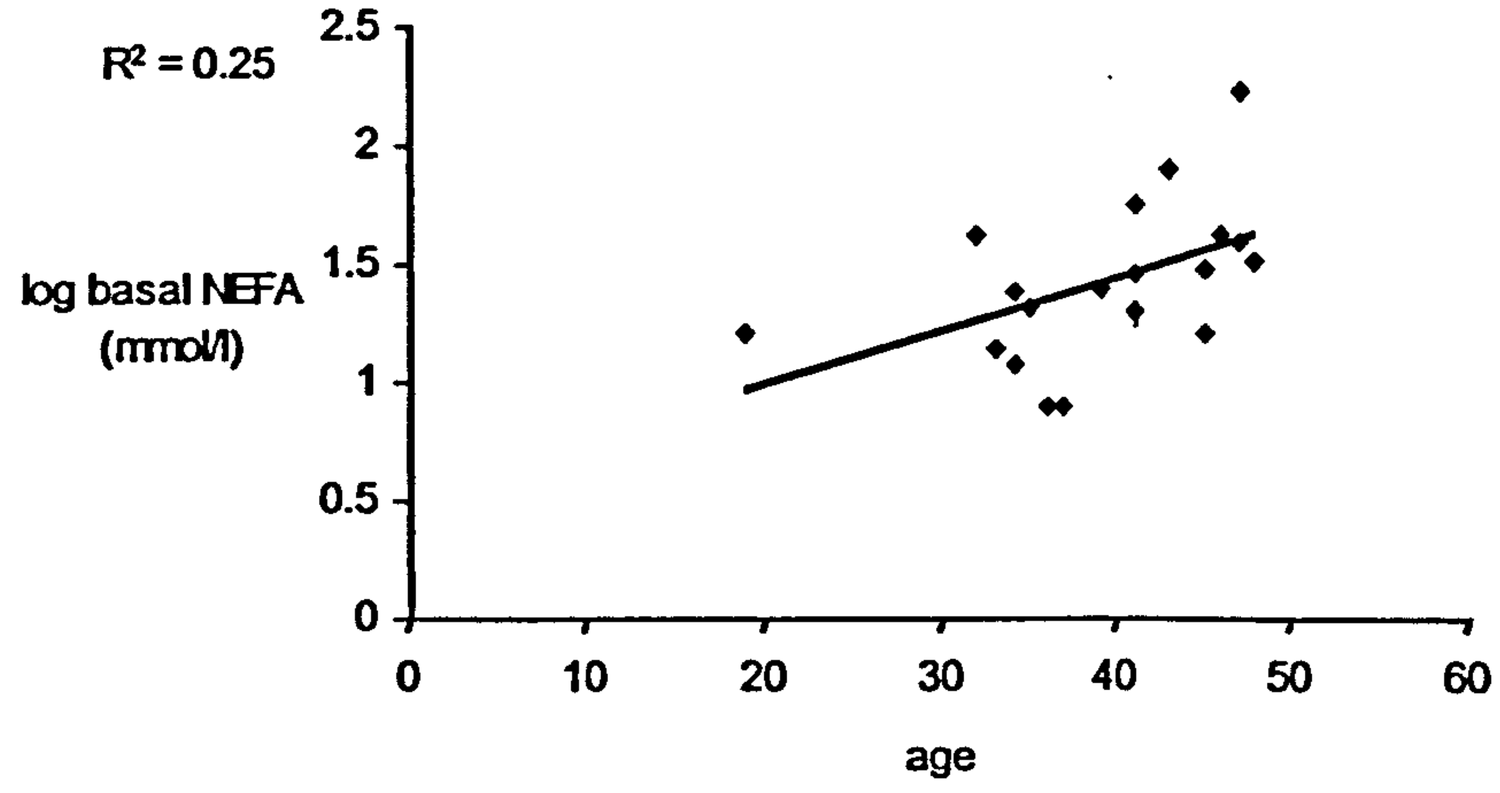
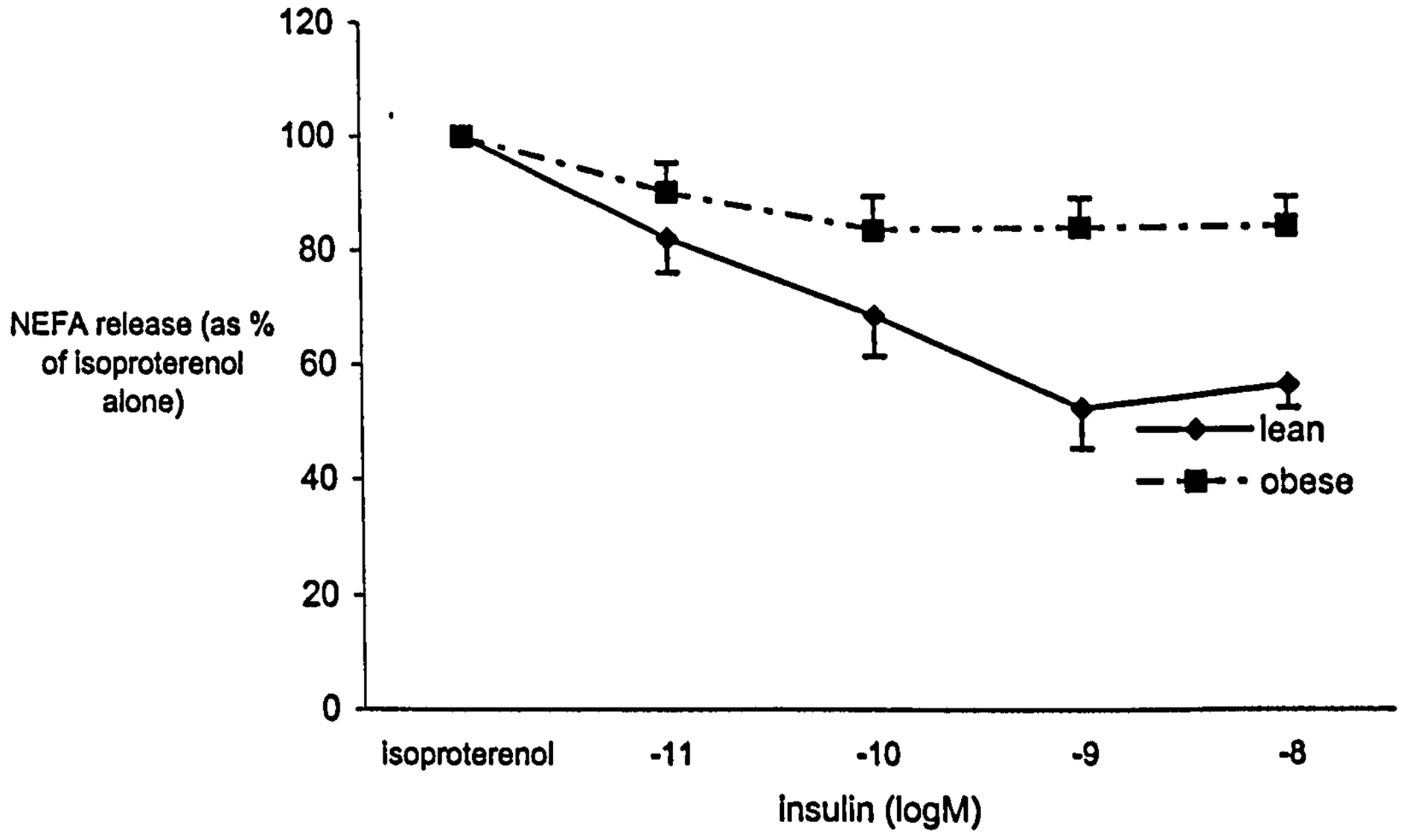



**Figure 3.6: Insulin sensitivity in adipocytes from lean and obese subjects**

Insulin-mediated attenuation (10pM-10nM) of isoproterenol-induced (200nM) lipolysis in subcutaneous adipocytes obtained from nine lean (BMI≤25 $kg/m^2$) and nine obese (BMI≥30$kg/m^2$) females (p=0.001 for comparison of AUC between lean and obese groups). Error bars denote standard errors.



## Figure 3.7: Insulin sensitivity in isolated adipocytes and phenotype characteristics.

The relationship between insulin resistance (the area under the curve of (10pM-10nM) insulin-mediated attenuation of isoproterenol-induced lipolysis in subcutaneous adipocytes obtained from 20 healthy females undergoing laparotomy, expressed in arbitrary units) and phenotype characteristics associated with insulin resistance was examined (Figure 3.7.1 BMI ($kg/m^2$) ($p<0.01$), Figure 3.7.2 total cholesterol: HDL –cholesterol ratio ($p<0.05$), Figure 3.7.3 fasting insulin resistance index ($p>0.05$), Figure 3.7.4 age ($p>0.05$)).

**Figure 3.7.1**

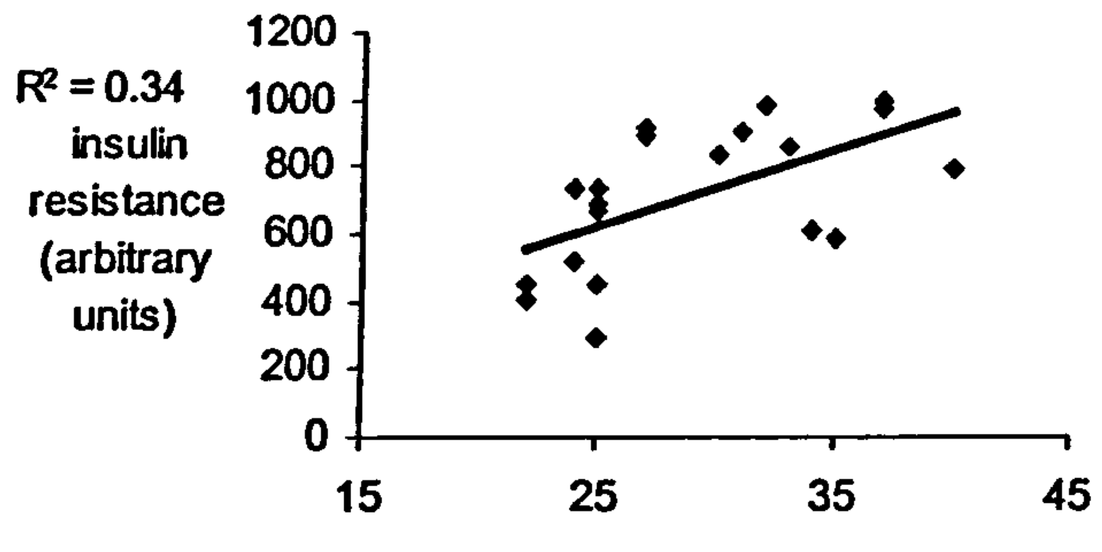



**Figure 3.7.2**

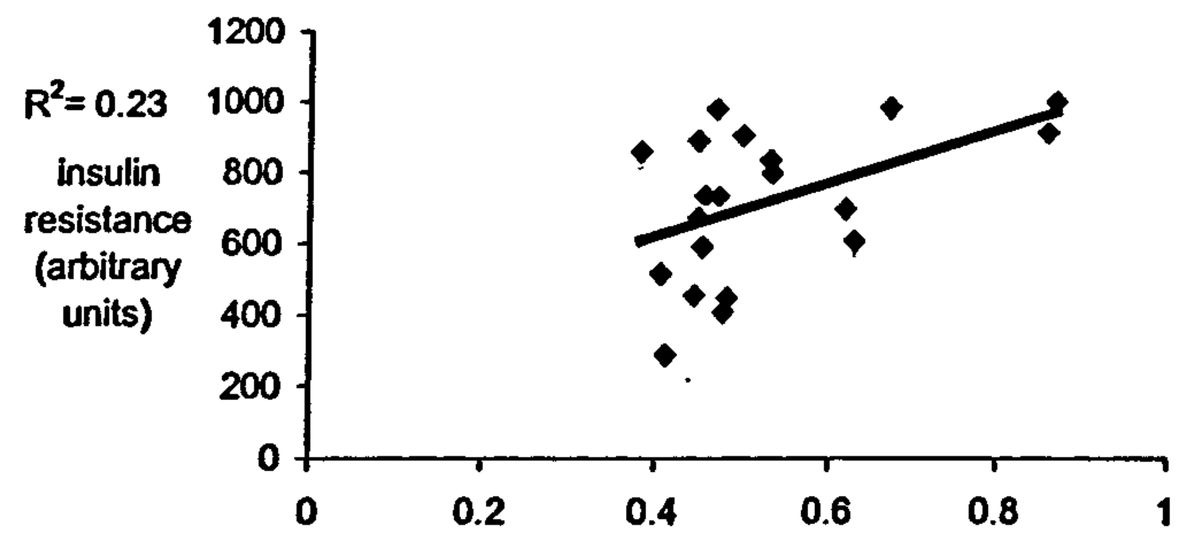



**Figure 3.7.3**

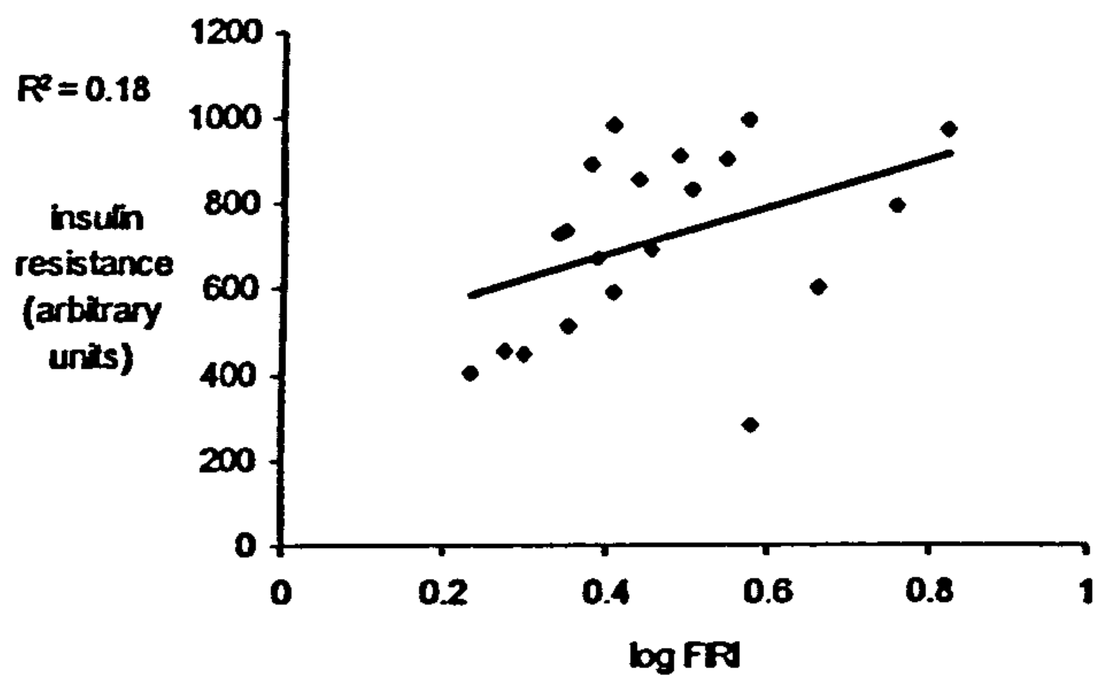



**Figure 3.7.4**

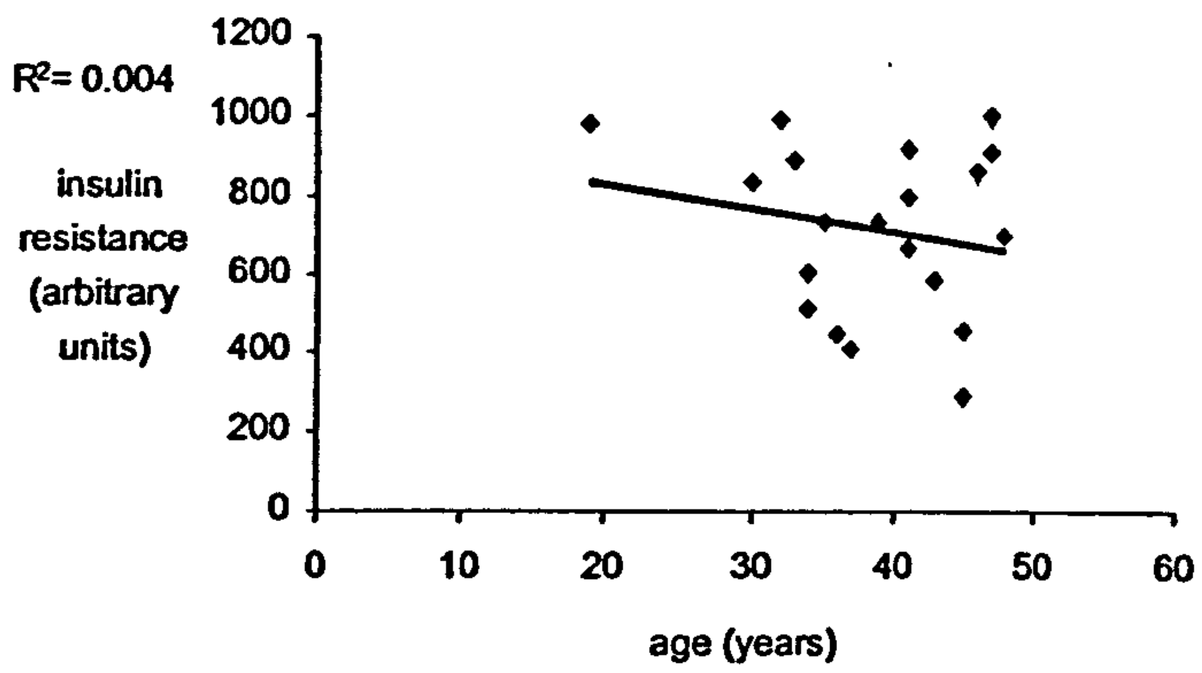
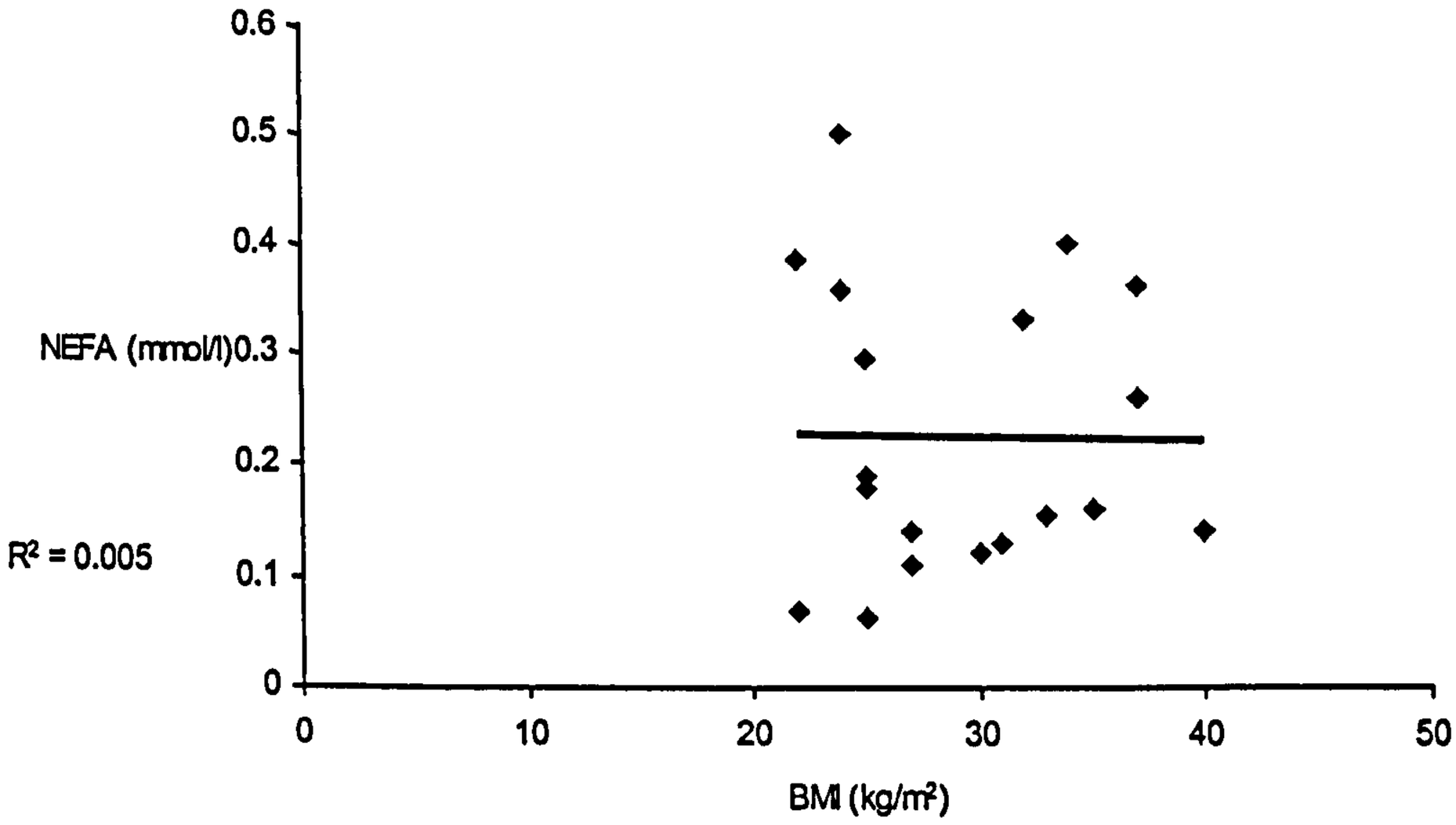


**Figure 3.8: Isoproterenol-induced non-esterified fatty acid release and BMI.**

Media concentration of non-esterified fatty acid (mmol/l) released in response to incubation of subcutaneous adipocytes with 200nM isoproterenol vs BMI ($p>0.05$) in 20 healthy women undergoing laparotomy



## 3.4 Discussion

In this chapter, the development of an assay of insulin responsiveness in human adipose tissue is described, based on the insulin-mediated suppression of isoproterenol-induced lipolysis. This assay was then used to measure insulin sensitivity in adipose tissue from well-characterised, otherwise healthy females undergoing laparotomy, and showed a relationship between the clinical phenotype and insulin action in isolated tissue.

The central tenet of this assay is the inhibitory effect of insulin on cellular release of NEFAs. Isoproterenol, acting via its cell surface $\beta$ adrenoceptors, stimulates adenylate cyclase activity, generating cAMP. This then stimulates activation of the cAMP dependent protein kinase A (PKA), which phosphorylates and activates hormone sensitive lipase; this enzyme is the rate-limiting step in the hydrolysis of stored triglyceride to glycerol and NEFA (reviewed in (345)). Released NEFAs then activate the colorimetric reaction used in the assay – NEFAs in the presence of added acyl-CoA synthetase form acyl CoA. This is then oxidised by added acyl-CoA oxidase to produce hydrogen peroxide which, in the presence of added peroxidase, allows the oxidative condensation of 3-methyl-N-ethyl-N-($\beta$-hydroxy-ethyl)-aniline and forms a purple adduct with an absorption maximum of 550nm.

The precise mechanism through which insulin attenuates lipolysis is as yet uncharacterised, though it appears that insulin acts via the tyrosine phosphorylation of the insulin receptor and IRS proteins leading to the activation of PI3'-kinase. That this step is necessary for the suppression of lipolysis is demonstrated by my results that show pre-treatment with the PI3'-kinase inhibitor wortmannin blocking this effect (figure 3.4). It is suggested that, through further second messenger events, insulin also reduces activation of

PKA, perhaps involving phosphodiesterase E3 (346), thus reducing activation of hormone sensitive lipase.

Important preliminary assays were undertaken to characterise the cellular response to isoproterenol. Isoproterenol is a non-specific agonist of the adipocyte $\beta$-adrenoceptor. Alternative stimuli used by other groups in similar systems include forskolin (stimulates adenylate cyclase), 8-bromo cyclic AMP (cyclic AMP analogue) and individual $\beta$-adrenoceptor agonists (dobutamine and terbutaline). It is proposed that the sensitivity to catecholamine-induced lipolysis is reduced in obesity, possibly mediated by impaired expression of hormone sensitive lipase (347).

In the present assay, neither the time courses employed (figure 3.1,figure 3.3), nor the concentration of isoproterenol (figure 3.2) saw isoproterenol's or insulin's maximal lipolytic stimulus. In addition, there was no suggestion that obese subjects released more NEFA in response to isoproterenol (figure 3.8). These observations in combination suggest that the release of NEFA observed, both basally and after insulin suppression, was not simply a function of cell size and triglyceride capacity. This is of particular importance as the cell size was not measured, and so the results are not expressed with a correction for cell size. Despite this, however, and again to avoid generating results that were more due to sensitivity to lipolysis than insulin sensitivity, results were expressed in terms of insulin action relative to the effect of isoproterenol alone, rather than as absolute numbers.

While these studies were not designed to define formally the role of adenosine in the regulation of lipolysis, adenosine does have anti-lipolytic activity, mediated via the A1 receptor, which modifies the activity of adenylate cyclase (348). The net anti-lipolytic

activity of adenosine is likely to be at least partly dependent on the ratio of A1:A2 receptors in a given tissue. The content of adenosine is higher and the response to it less in omental than subcutaneous adipocytes (349). It has been demonstrated that in adipocytes from obese animal models, there is increased sensitivity to adenosine-mediated inhibition of lipolysis (350). Given the confounding effects of adenosine, assays were undertaken at a "clamped" supraphysiological, though not maximally effective, concentration of adenosine (100nM). Other groups have addressed this problem in a similar manner, though some have added adenosine deaminase to the buffer to reduce endogenous adenosine levels, and then added exogenous adenosine (351).

Having developed this assay, the technique was employed to measure cellular insulin sensitivity in humans and to consider this in the setting of the clinical phenotype. Phenotype characterisation consisted of measurement of BMI, blood pressure, fasting glucose, total, LDL- and HDL- cholesterol and triglycerides. In addition, age, family history, smoking and menopausal status were recorded. Insulin sensitivity in human adipose tissue has been assessed previously, mainly using measurements of glucose uptake. Here, the suppression of lipolysis was used, mainly because personal experience of glucose transport assays in human tissue had shown that use of this parameter was confounded by the effects of the basal rate of glucose transport, and limited by the larger volume of tissue required to construct a dose response curve of insulin-stimulated glucose uptake. The additional advantage of the present assay is that there is no need for the use of radioisotopes. Other groups have used microdialysis techniques to analyse *in vivo* adipose tissue lipolysis, though it cannot be assumed that this reflects only the action of insulin at the cell; these measurements may be confounded by the effects of local blood flow. In keeping with this, in a study of family members of patients with obesity or type 2 diabetes,

insulin-mediated attenuation of lipolysis was reduced using *in vivo* microdialysis measurements, though not in an *in vitro* assay undertaken in parallel (158).

While there is a literature that demonstrates a reduction in insulin action in isolated tissue from subjects with type 2 diabetes, there are fewer published data exploring insulin sensitivity in isolated adipose tissue from patients without diabetes. Bogardus *et al* ((352) found that there was no correlation between obesity and maximal glucose uptake in isolated adipocytes from non-diabetic Pima Indians, and concluded that obesity had only a minor role in determining *in vivo* and *in vitro* glucose uptake. Kashiwagi *et al* (152), however, did find that the $EC_{50}$ of insulin's antilipolytic action (measured by glycerol release) was greater in mildly obese Pima Indians (mean BMI 33 $kg/m^2$) than lean volunteers (mean BMI 22 $kg/m^2$).

In the present study, a close relationship was observed between the basal release of NEFA and BMI, independent of blood pressure, FIRI and lipid profile. This may represent an effect of total cell volume or number in the obese subjects; the effect of wortmannin to increase this value (figure 3.4.1) also suggests however that the basal NEFA value is to some extent influenced by PI3'-kinase activity, and so insulin. Obese patients, who have an increased risk of developing further characteristics of the insulin resistance syndrome, were shown to be insulin resistant even when normoglycaemic and normotensive, implying that cellular insulin resistance is an early feature. The data were analysed not only by comparing lean and obese groups (figure 3.6), but also by examining the cohort as a whole (figure 3.7) to demonstrate a continuous relationship between cellular insulin resistance and obesity, independent of age and blood pressure. It must be borne in mind that these relationships between isolated tissue and the whole body phenotype are evident after the adipocytes have

been extracted, digested and maintained *ex vivo* for at least 90-minutes prior to analysis, reinforcing the strength of the observations. This is of fundamental importance; while there is little doubt that there is a circulating influence of agents such as glucose and TNF-α *in vivo,* it seems unlikely that the findings in isolated tissue reflect these influences after digestion etc. It is possible that the cells continue to secrete agents, such as resistin (141) that may have a paracrine effect once *in vitro*, however it is most likely that the observation of a relationship between clinical and cellular features of insulin resistance reflects an intrinsic cellular defect.

The role of adipose tissue in the development of insulin resistance, type 2 diabetes and vascular disease is an area of great current scientific interest. More than thirty years ago, however, Randle hypothesised that the release of NEFA from adipose tissue, secondary to resistance to insulin, may, by substrate competition, result in hyperglycaemia. While there have been subsequent advances in this particular field, mainly at the molecular level, the central tenet behind Randle's hypothesis still holds true, while further work has also suggested there may be a role for NEFA in mediating the endothelial dysfunction that is associated with insulin resistance. Recent publications propose that adipose tissue releases other factors, such as TNF-α, IL-6, resistin and angiotensin II that may all have a role in modifying insulin action in a variety of target organs, and may even couple the metabolic and vascular manifestations of insulin resistance. Intriguingly, endothelial NOS message and protein have recently been isolated in human adipocytes (353). Given these observations, it may become more appropriate to consider adipose tissue as an endocrine organ.

In summary, I have developed an assay of insulin action in isolated human adipose tissue, using insulin's well-described role in the suppression of lipolysis. I have also shown that the insulin sensitivity of isolated tissue reflects the host phenotype. I have gone on to use this assay in chapter 5 to investigate the effect of angiotensin II on insulin action in adipose tissue.

# Chapter 4: Dietary sodium restriction and insulin sensitivity

## 4.1 Introduction

The relationship between the RAS and insulin sensitivity remains unclear. While angiotensin II infusion has been reported to increase insulin sensitivity(293,299), activation of the RAS by dietary sodium restriction may have the opposite effect (248,254) . Using the euglycaemic hyperinsulinaemic clamp, some, though not all, groups have found that low sodium diet is associated with a reduction in insulin sensitivity (reviewed in Chapter 1). As moderate dietary sodium restriction is now a major part of the non-pharmacological management of hypertension, potentially adverse metabolic consequences need to be thoroughly explored.

Analysis of the current literature gives no clear message; differences in the degree and duration of sodium restriction, the populations under study, and the method of measuring insulin sensitivity make it difficult to reach a consensus. Therefore, a double-blind, randomised, placebo-controlled crossover study was designed to test the effect of modest dietary sodium restriction on insulin sensitivity. Only modest sodium restriction was used in an attempt to maintain clinical relevance to the result; the use of a simple diet sheet and the avoidance of pre-prepared meals mean that similar results may be anticipated were these conditions applied to the general population. Additionally, the euglycaemic hyperinsulinaemic clamp was used as the gold standard method for the assessment of insulin sensitivity. The metabolic effects of sodium restriction may be linked to its pressor effects (354), and as such detailed blood pressure measurements were obtained at the end of each dietary phase.

There is also variability in published studies with regard to the assessment of neurohumoral activation in response to sodium restriction. In addition to measuring renin, aldosterone and angiotensin II (AII), serum cortisol, noradrenaline and growth hormone were also measured. Lastly, as the endothelium expresses AT1 receptors, and may have a role in determining insulin sensitivity, markers of endothelial function during both normal and restricted sodium intake were also measured.

## 4.2 Methods

The study was designed as double-blind, randomised, placebo-controlled crossover trial comparing low and normal sodium intake for five days, with a washout period of seven days between phases.

### 4.2.1 Volunteers

15 healthy males (mean age 26.5 ± 3.66 (SD) years, mean BMI 23.33 ± 2.4 (SD) kg/m$^2$), were recruited to the study by advertisement. Subjects with any intercurrent medical condition, including hypertension and diabetes mellitus, were excluded. Volunteers underwent a standard physical assessment prior to enrolment into the study, and all gave written informed consent. All aspects of the study protocol were approved by the Ethics Committee of the West Glasgow Hospitals University NHS Trust.

### 4.2.2 Low sodium diet

Volunteers were asked to limit oral sodium intake for two periods of five days, separated by at least one week. During these periods, ten tablets were taken daily, either Slow Sodium (100mmol/day) (Ciba-Geigy, Horsham, UK) or matched placebo, in random order.

A diet sheet was given to each volunteer at the screening visit (figure 4.1), detailing low sodium foodstuffs and a sample daily diet. Dietary advice was reinforced by the study nurse and investigator. Volunteers were asked not to smoke, or drink alcohol during the study.

### 4.2.3 Urinary electrolytes

Urine was collected into preservative for the 24 hours preceding the euglycaemic clamp.

On the fifth morning of each phase, volunteers attended the Clinical Investigation and

Research Unit, having fasted overnight. To measure salt sensitivity, blood pressure was recorded lying recumbent in a quiet room every two minutes for one hour, using a semi-automated sphygmomanometer (Dinamap Critikon, Johnson and Johnson Professional Products Ltd., U.K.).

### 4.2.4 The euglycaemic hyperinsulinaemic clamp

The euglycaemic hyperinsulinaemic clamp protocol was derived from that described by DeFronzo *et al* 1979 (10) and is detailed in Chapter 2.

### 4.2.5 Analysis of serum biochemistry

At baseline, 60, 120, 150 and 180 minutes, blood was taken for insulin and electrolytes. Samples for C-peptide, plasma renin, aldosterone, angiotensin II and cortisol were collected at the start and end of the clamp. Baseline samples were also obtained for total cholesterol and triglycerides, markers of endothelial function, noradrenaline, growth hormone and glucose. The methods for all of these assays are detailed in Chapter 2.

### 4.2.6 Statistical analysis

Analysis was performed using the Minitab statistical package. All means are expressed ± the standard error of the mean, except where stated otherwise. Equivalent values from each study phase were compared using paired t-tests. For studies of correlation, non-normally distributed variables were first expressed as their natural logarithm.

## 4.3 Results

All 15 recruits completed both phases of the protocol, and experienced no adverse events.

### 4.3.1 Body weight

There was no significant difference in body weight between phases (table 4.1).

### 4.3.2 Urinary sodium excretion

Urinary excretion of sodium for the 24 hours prior to the clamp was measured to estimate compliance with diet and medication. Mean urinary sodium during the 24 hours prior to the clamp after the placebo phase was 70.6 ± 45.1 mmol/24 hours, compared with 175 ± 72.1 mmol/24 hours ($p<0.01$) (table 4.1) after sodium supplementation.

### 4.3.3 Serum sodium

Serum sodium at the commencement of the clamps was lower after the period of sodium depletion in comparison with the sodium replete phase (table 4.1).

### 4.3.4 Activation of other counter-regulatory endocrine systems

There were no differences in baseline or post clamp noradrenaline, cortisol or growth hormone between phases (table 4.1)

### 4.3.5 Lipids

There were no significant differences in total cholesterol or triglycerides between phases (table 4.1).

### 4.3.6 Blood pressure

No significant changes in systolic or diastolic blood pressure were observed between phases (table 4.1) when group means were compared. However, eight of the fifteen volunteers were salt sensitive (SS), defined as a fall in mean arterial pressure (MAP) of at least 3mmHg in response to sodium restriction. When salt sensitivity was considered as a continuous variable, no statistically significant correlation was seen between the degree of salt sensitivity and the change in insulin sensitivity (figure 4.4).

### 4.3.7 Activation of the renin angiotensin system

Before each clamp, plasma renin activity, aldosterone and angiotensin II were measured after 20 minutes supine rest to document the degree of activation of the RAS (table 4.2). All three parameters were significantly greater at the start and end of the euglycaemic clamp during sodium restriction.

### 4.3.8 Insulin sensitivity

Fasting insulin, C-peptide and glucose were not significantly different between phases (table 4.3). Despite this, insulin sensitivity, measured as the M value derived from a euglycaemic hyperinsulinaemic clamp, was reduced during sodium depletion compared with the sodium-replete phase (M=11.0±2.4 (SD) mg/kg/min vs 12.4±3.5 (SD) mg/kg/min) (table 4.3 and figure 4.3). There was no difference in the mean insulin during the 180-minute clamp, or during the final 40 minutes of the clamp, between phases (table 4.3 and figure 4.2). However, even after correcting for potential confounding effects of differences in serum insulin during steady state, there was a significant difference in the insulin sensitivity index between phases (M/I during sodium deplete phase 0.08 ± 0.03 (SD) vs 0.1 ± 0.05 (SD) during sodium replete phase p=0.043). The mean effect was a fall in insulin

sensitivity of 1.4 mg/kg/min (95% CI 0.2-2.6), which equates to a mean 11% reduction (95%CI 2%-21%).

While data from all volunteers who underwent randomisation were included in the main analysis, a subgroup of subjects with arbitrarily defined "good" compliance with dietary sodium restriction (table 4.4) (urinary sodium on low sodium diet <80mmol/24 hours) was analysed. In this group (n=11), dietary sodium restriction was similarly associated with a fall in insulin sensitivity (M value during sodium deplete phase 11.45 ± 2.7 (SD) mg/kg/min vs 13.1 ± 3.8 (SD) mg/kg/min during sodium replete phase p=0.045).

### 4.3.9 Parameters of RAS activation and insulin sensitivity

After logarithmic transformation, baseline values of renin, angiotensin II and aldosterone following sodium restriction were expressed as a percentage of the sodium replete equivalent values, and plotted against the change in insulin sensitivity (the M value during sodium restriction as a percentage of the M value when sodium replete). No statistically significant correlation was observed between any of these variables and the percentage change in insulin sensitivity (figure 4.5).

### 4.3.10 Markers of endothelial function

Markers of endothelial function (von Willebrand factor, tissue plasminogen activator) were measured at the end of each study phase to determine whether sodium restriction has an effect on the endothelium. There was no statistically significant difference in any of these markers between phases (table 4.1).

**Figure 4.1: Sample diet sheet, issued to volunteers at commencement of sodium restricted diet.**

| Food Group | Salty Foods to Avoid | Suitable Alternatives |
|---|---|---|
| **Breakfast Cereals** | All others | Weetabix, Shredded Wheat |
| **Bread, Cakes and Biscuits** | All others | Bread (Wholemeal/white max 4 slices/day), sweet cakes or sweet biscuits only. |
| **Starchy Carbohydrates** | All others | Potatoes, rice, pasta. |
| **Eggs** | Scotch Eggs | Boiled, poached, scrambled or fried |
| **Milk** | Salted Lassi | ½ pint semi-skimmed milk daily |
| **Cheese** | | Cottage/cream cheese, maximum of 50g daily |
| **Meats and Poultry** | Bacon, ham, gammon. All smoked meats. Salami, sausages, beefburgers. Tinned meats, e.g. corned beef, luncheon meat. Meat pies and manufactured meat dishes. Dehydrated packed meals. Meat paste and pate. | All fresh and frozen meats e.g. beef, lamb, pork, turkey, chicken etc. |
| **Fish** | All smoked, cured, salted, tinned or pickled fish. Fish paste and pate, taramasalata. Shellfish e.g. prawns, shrimps. Manufactured fish dishes. | All fresh or frozen fish. Fish in batter or breadcrumbs. |
| **Soups** | Packet and tinned. | Home made soup with no added salt or stock |
| **Drinks** | | Fruit juice, reduced sugar fruit squash, diet fizzy drinks, mineral water, tea, coffee, tonic water. |
| **Fruit** | Olives, salted nuts. | Fresh, frozen or tinned in natural juice. Unsalted nuts. |
| **Vegetables** | Tinned unless labelled "no added salt". Baked beans. | Fresh, frozen or tinned in natural juice. |
| **Spreads** | All others. | Polyunsaturated margarine. |
| **Miscellaneous** | Meat or yeast extract e.g. Bovril, Marmite, Oxo. Gravy granules, stock cubes, garlic salt. Pickles, chutneys, tomato ketchup, Worcester sauce, tomato juice, chilli sauce. | Use black pepper, herbs and spices to flavour food. Use unsalted snacks or fresh or dried fruit as snacks |

**Table 4.1: Comparison of body weight, blood pressure and serum biochemistry after five days of sodium restricted and supplemented diet.**

All values are quoted as mean ± one standard deviation

| Parameter | Sodium deplete | Sodium replete | p value |
|---|---|---|---|
| **Weight (kg)** | 73 ± 9.1 | 74 ± 7.8 | 0.19 |
| **Urinary sodium (mmol/24 hours)** | 70 ± 45 | 175 ± 72.1 | <0.001 |
| **Baseline serum sodium (mmol/l)** | 139.4 ± 1.72 | 141.2 ± 1.82 | 0.015 |
| **Systolic blood pressure (mmHg)** | 115 ± 12 | 115 ± 9.6 | 0.97 |
| **Diastolic blood pressure (mmHg)** | 58 ± 10.7 | 60 ± 8.2 | 0.33 |
| **Fasting total cholesterol (mmol/l)** | 4.0 ± 0.83 | 3.9 ± 0.81 | 0.14 |
| **Fasting triglycerides (mmol/l)** | 0.8 ± 0.34 | 0.7 ± 0.45 | 0.8 |
| **Growth hormone (mU/l 0900 hrs))** | 1.5 ± 3.12 | 0.51± 0.69 | 0.3 |
| **Noradrenaline (nmol/l)** | 1.7 ± 0.86 | 1.7 ± 0.8 | 0.25 |
| **Cortisol (nmol/l 0900 hrs)** | 342 ± 135.0 | 286 ± 91.7 | 0.14 |
| **von Willebrand factor (%)** | 90 ± 0.4 | 85 ± 10.2 | 0.4 |
| **Tissue plasminogen activator (ng/ml)** | 6.3 ± 0.61 | 5.3 ± 0.52 | 0.12 |

**Table 4.2: Comparison of the components of the circulating renin angiotensin system after sodium deplete and supplemented diet.**

All values are quoted as mean ± one standard deviation

| Parameter | Sodium deplete | Sodium replete | p value |
|---|---|---|---|
| **Plasma renin activity (μU/ml)** | | | |
| **Time 0** | 25.0 ± 10.43 | 13.4 ± 7.28 | <0.001 |
| **Time 180** | 24.9 ± 11.16 | 12.3 ± 6.58 | <0.001 |
| **Plasma aldosterone (ng/100ml)** | | | |
| **Time 0** | 15.8 ± 9.91 | 8.4 ± 5.77 | <0.05 |
| **Time 180** | 11.6 ± 9.18 | 6.1 ± 3.89 | <0.05 |
| **Plasma angiotensin II (pg/ml)** | | | |
| **Time 0** | 28.5 ± 17.27 | 11.0 ± 6.89 | <0.001 |
| **Time 180** | 28.3 ± 22.03 | 12.3 ± 6.85 | <0.005 |

**Table 4.3: Comparison of euglycaemic hyperinsulinaemic clamp results on day five after sodium deplete and supplemented diet.**

All values are quoted as mean ± one standard deviation

| Parameter | Sodium deplete | Sodium replete | p value |
|---|---|---|---|
| Fasting insulin (μU/ml) | 11.6 ± 3.24 | 11.4 ± 3.02 | 0.83 |
| Fasting C-peptide (ng/ml) | 1.7 ± 0.51 | 1.6 ± 0.53 | 0.21 |
| Fasting glucose (mmol/l) | 4.5 ± 0.33 | 4.5 ± 0.45 | 0.43 |
| M (mg/kg/min) | 11.0 ± 2.4 | 12.4 ± 3.5 | 0.027 |
| Insulin sensitivity index (M/I) | 0.08 ± 0.03 | 0.1 ± 0.05 | 0.043 |
| Mean plasma insulin | | | |
| 180 minutes | 142.1 ± 60.34 | 136.9 ± 70.24 | 0.37 |
| final 40 minutes | 147.2 ± 68.16 | 138.1± 67.52 | 0.1 |

**Table 4.4: Dietary sodium restriction and insulin sensitivity; subgroup analysis of "good"compliers (n=11)**

All values are quoted as mean ± one standard deviation

| Parameter | Sodium deplete | Sodium replete | p value |
|---|---|---|---|
| **M (mg/kg/min)** | 11.4± 2.7 | 13.1± 3.8 | <0.001 |
| **Urinary sodium (mmol/24 hours)** | 47± 23 | 152± 62 | 0.045 |

**Figure 4.2: Serum insulin during euglycaemic hyperinsulinaemic clamp**

Serum insulin was measured throughout the euglycaemic hyperinsulinaemic clamps during both phases. At no individual time point, nor as a mean value throughout the procedure, was there any difference in serum insulin between phases. Error bars denote the standard error of the mean.

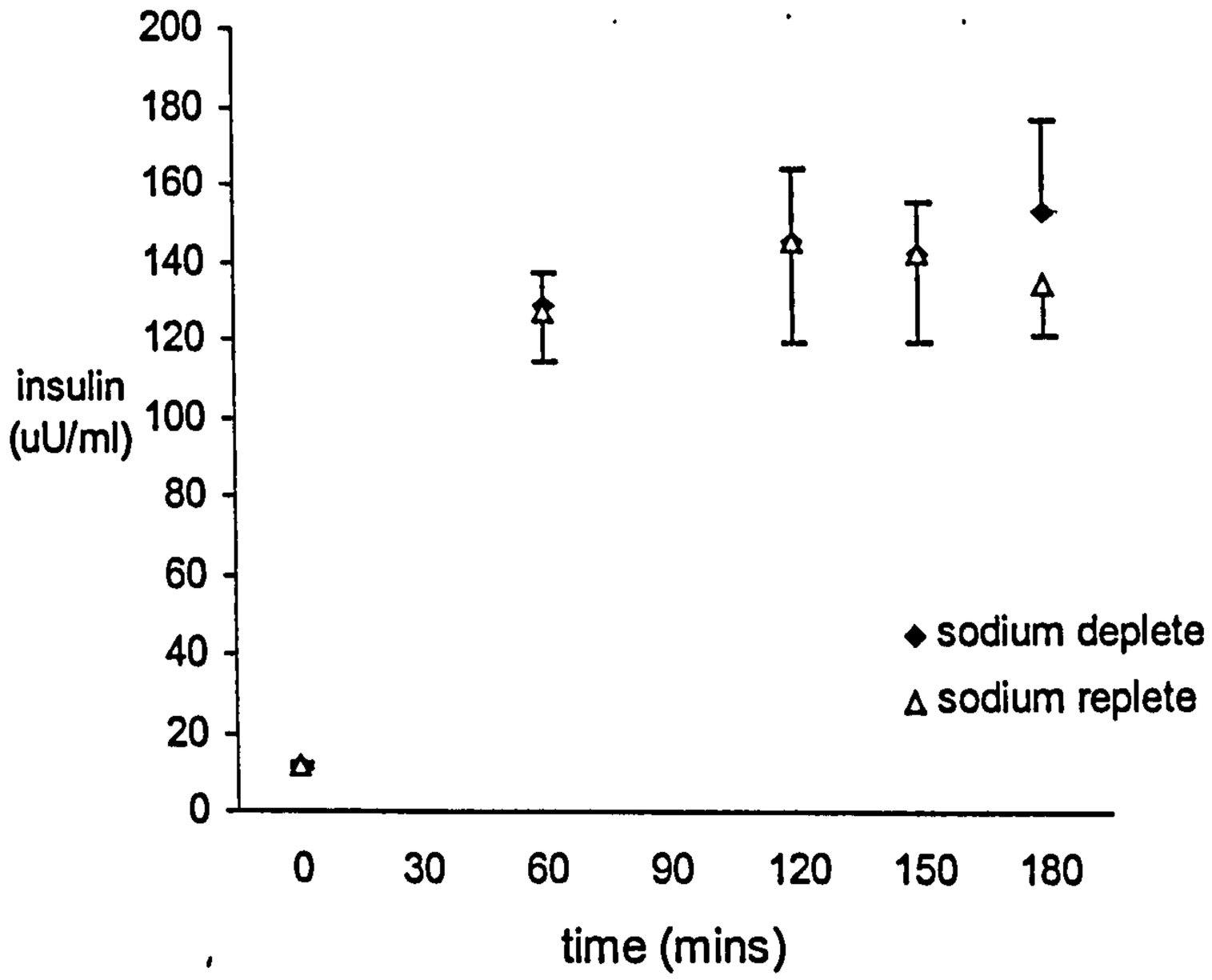


**Figure 4.3: Insulin sensitivity during sodium deplete and replete phases.**

Insulin sensitivity, measured by the euglycaemic hyperinsulinaemic clamp, was measured after the sodium deplete and replete phases. Insulin sensitivity was significantly reduced during dietary sodium depletion (M value 12.4 ± 3.5 (SD) mg/kg/min sodium replete vs.11.0 ± 2.4 (SD) mg/kg/min sodium deplete, p=0.027). Values shown illustrate the mean (——) and 95% confidence intervals(━)for each phase.

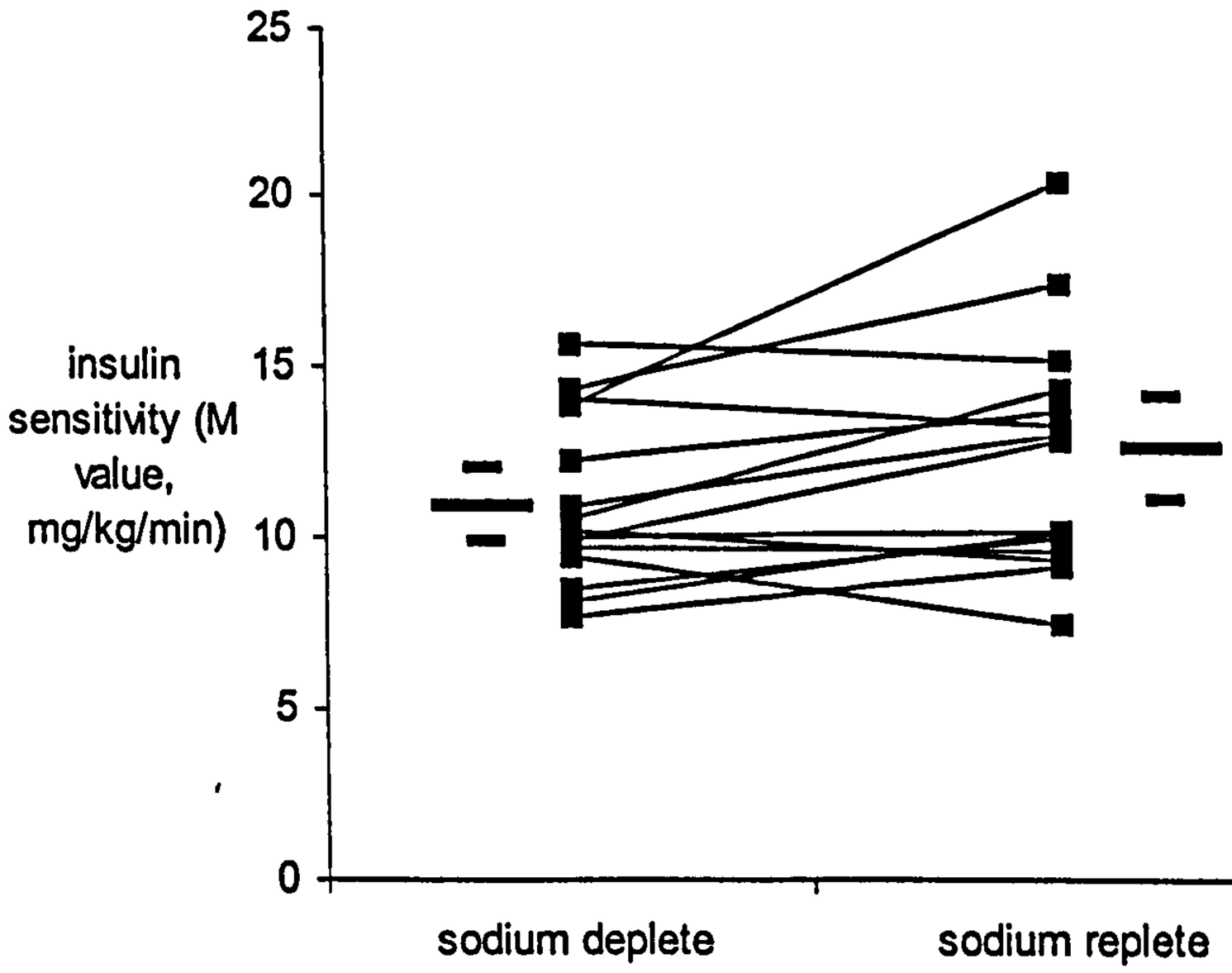
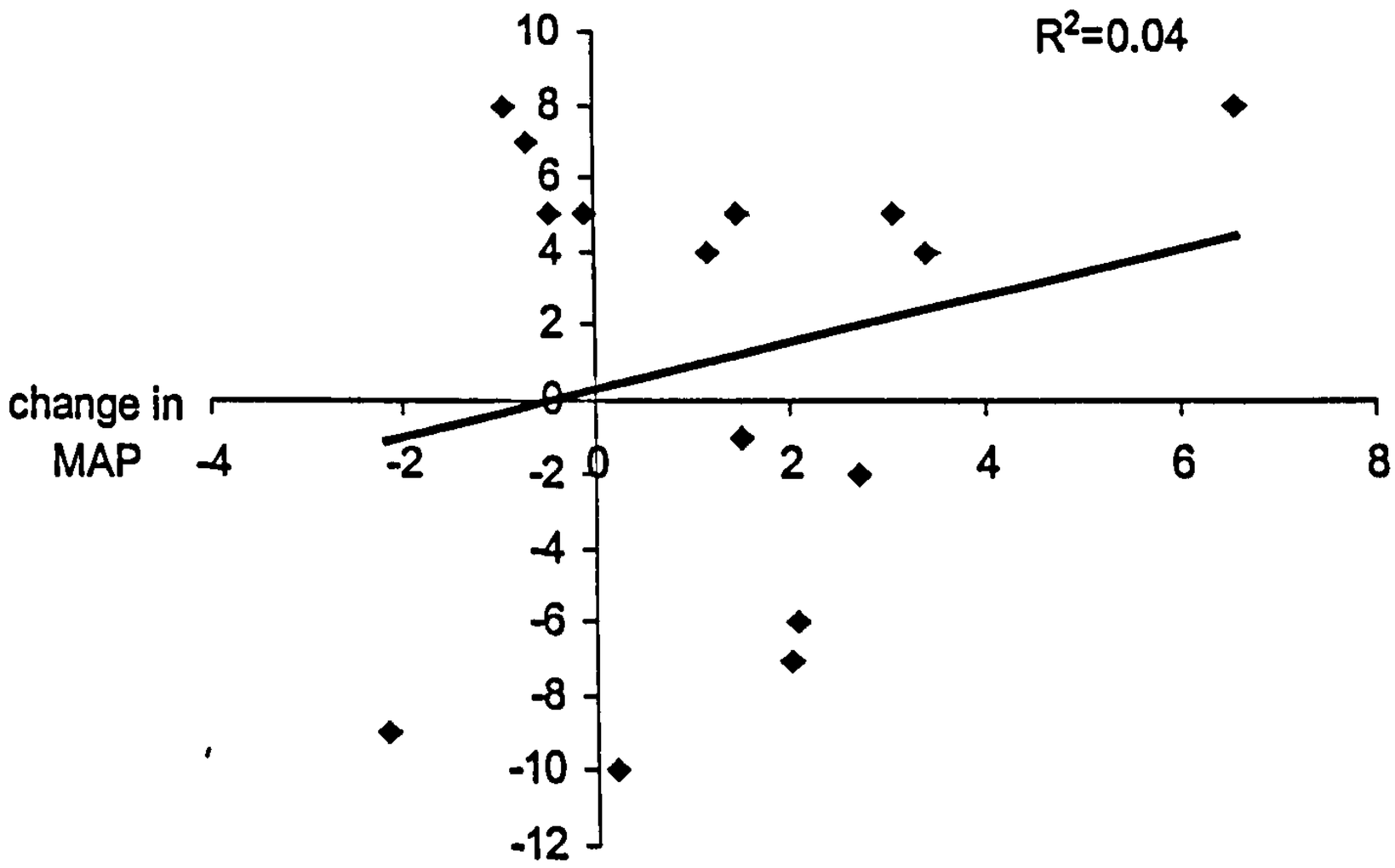


**Figure 4.4: Salt sensitivity and the change in insulin sensitivity between sodium replete and deplete phases.**

Salt sensitivity (MAP during sodium replete minus MAP during sodium deplete) was plotted against the absolute change in insulin sensitivity (M during sodium replete minus M during sodium deplete). A trend was observed though no statistically significant correlation was observed ($p>0.05$ for correlation).



## Figure 4.5: The relationship between the degree of activation of the renin angiotensin system and the change in insulin sensitivity.

To determine whether there was a relationship between the degree of change (delta) in an individual component of the renin angiotensin and the change in insulin sensitivity between phases, these values were plotted, after log transformation of the values for percentage change (delta) in renin / angiotensin II /aldosterone, in figures 4.5.1 (delta renin vs delta M, $p>0.05$) figure 4.5.2 delta angiotensin II vs delta M, $p>0.05$) and figure 4.5.3 (delta aldosterone vs delta M $p>0.05$).

**Figure 4.5.1**

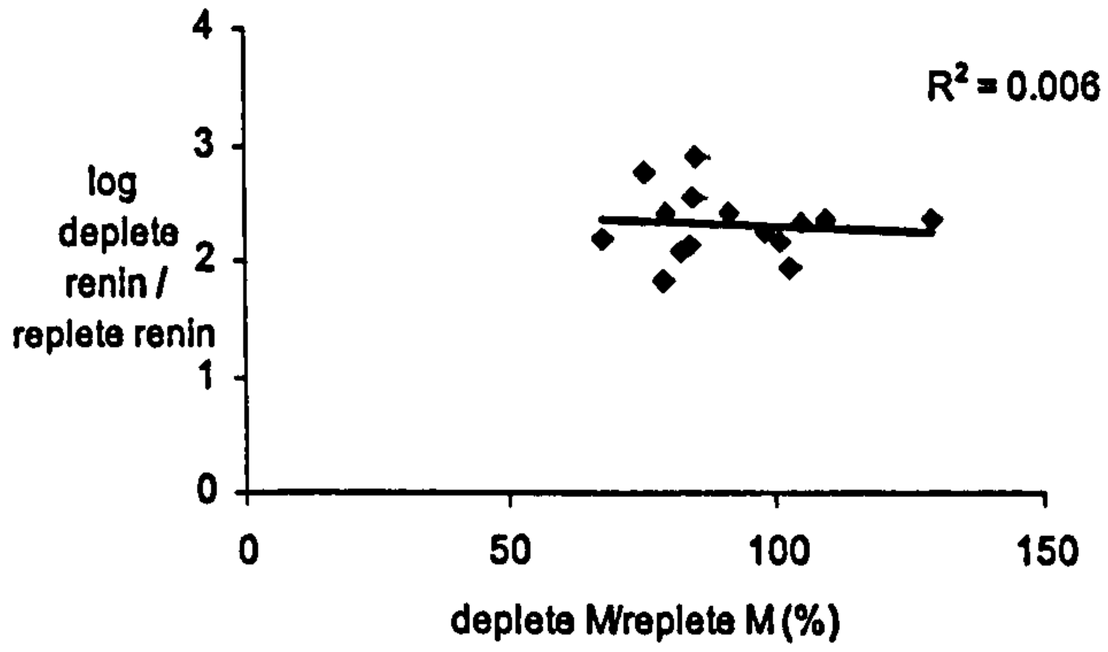



**Figure 4.5.2**

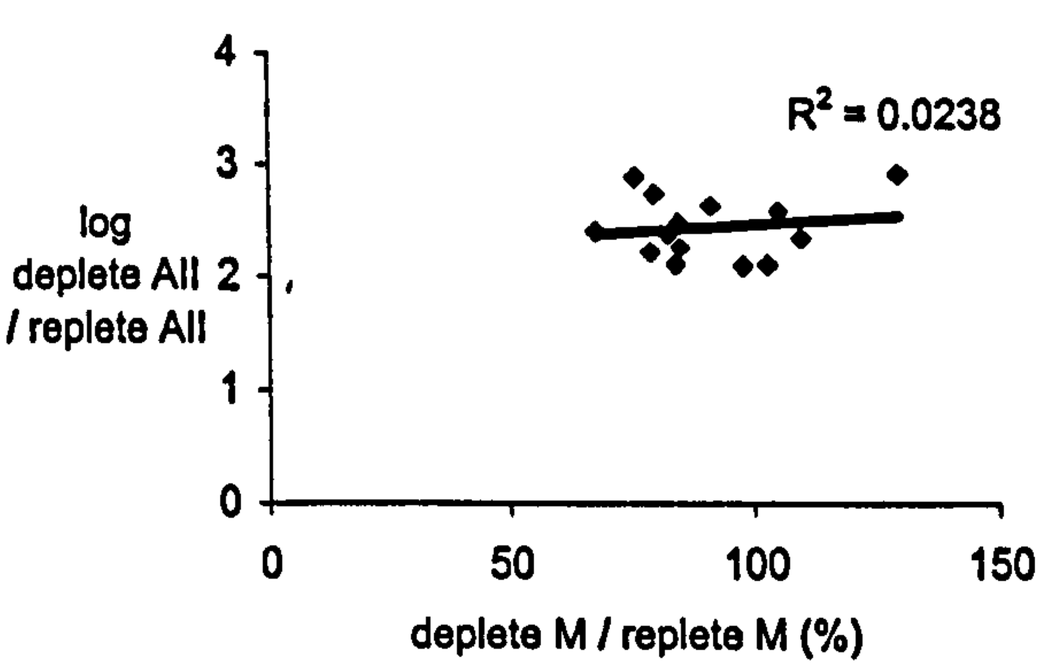
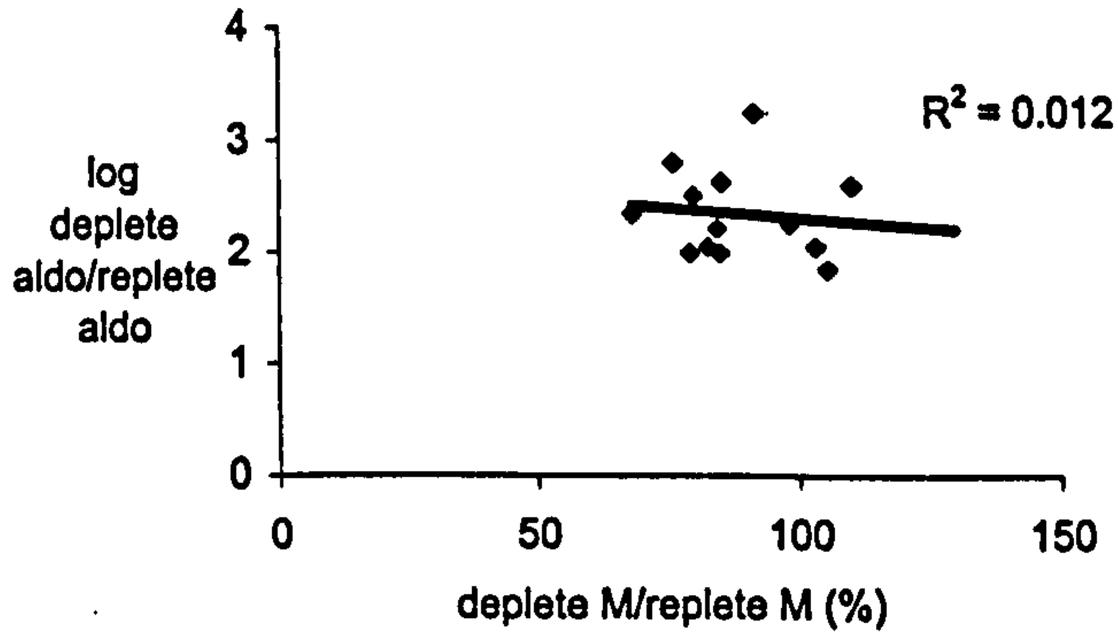



**Figure 4.5.3**



## 4.4 Discussion

This is the first study to show that a modest reduction in dietary sodium is associated with a fall in insulin sensitivity measured by the euglycaemic hyperinsulinaemic clamp in normal volunteers.

Although other groups have demonstrated that sodium restriction may be associated with insulin resistance, most have used severe dietary restriction. In contrast, the present study aimed to examine the effect of modest sodium restriction to between 60 and 80 mmol/day. Rather than supplying volunteers with prepared food, they were simply asked to follow a diet sheet during the study. Thus, the measures used to reduce dietary sodium were simple, and as such the sodium restriction in this study is similar to the strategies recommended to the general population in advice on low sodium diet. It is therefore possible that a similar metabolic outcome may be anticipated, were dietary sodium restriction to be applied to a larger population.

Despite the relatively large number of studies that have investigated the effect of sodium restriction on insulin sensitivity, there is no clear consensus of opinion. Several potential explanations for the heterogeneity of results are proposed. Different methods used to measure insulin sensitivity may explain some of these findings; studies have measured fasting and post-load insulin (259), the insulin tolerance test(255), the CIGMA (249), the insulin suppression test (260) and the euglycaemic hyperinsulinaemic clamp(248). The clamp technique is the accepted gold standard in the measurement of insulin sensitivity and has a high level of reproducibility (see table 1.1). As such, the change in insulin sensitivity demonstrated in this study is likely to reflect a definite biological change, though the magnitude of the difference may have been beyond the resolution of detection using

alternative techniques. The populations studied also vary between studies, as in addition to normal volunteers, some groups have considered effects on hypertensives ((251) (252) and subjects with type 2 diabetes (254). Not all studies have been of a double-blind crossover design with randomised phases of dietary intervention, incorporating a washout period. Lastly, and perhaps most importantly, the degree of sodium restriction varies from a comparison of <20mmol/day with >200mmol/day, to the more modest range used in this study.

In a randomised, placebo-controlled crossover trial in 34 normotensive males, Grey *et al* (249) compared insulin sensitivity, measured by continuous infusion of glucose and modelling assessment (CIGMA), after seven days of dietary sodium intake of <80mmol/day plus placebo with one week of the same diet though supplemented with 120mmol oral sodium daily. Thus, the trial design was similar the present study but insulin sensitivity was measured using CIGMA, which makes certain physiological assumptions (table 1.1). Urinary sodium results were similar to those described in the present study (low sodium 52±25mmol/day, high sodium 186±46 mmol/day), though there was no change in insulin sensitivity between phases. It is unclear why I have found a difference in insulin sensitivity using a similar protocol. As discussed, the use of the euglycaemic clamp technique may have enabled me to detect more subtle changes in insulin sensitivity. It must also be noted that the Grey study (249) had no washout period between phases. Lastly, the duration of diet may be important. In type 2 diabetes, Petrie *et al* (254) reported a reduction in insulin sensitivity associated with dietary sodium restriction for five days, while Fliser *et al* (248) demonstrated that three days of severe sodium restriction was associated with a reduction in insulin sensitivity, however seven days of the same diet had no effect.

By measuring renin, aldosterone and angiotensin II after both phases of the protocol, I have characterised the activity of the RAS in response to our dietary intervention. This has shown that even modest sodium restriction is associated with significant elevations of all three of these components, and therefore overall activation of the system. In a meta-analysis of trials involving low sodium diets, analysis of 20 populations with sodium excretion reduced to between 40 and 100mmol sodium/24 hours demonstrated that this degree of dietary sodium restriction resulted in a twofold increase in renin and aldosterone (355). My own data are in keeping with these figures; in this study a mean sodium excretion of 70.6 mmol/24 hours was associated with a doubling of renin and aldosterone. To put these in some context, more severe sodium restriction, resulting in levels of urinary sodium excretion less than 20mmol/24 hours, was associated with a five- to six fold increase in renin and aldosterone (355). Thus, studies using more severe sodium restriction than my own have resulted in more dramatic activation of the RAS. However, using a less severe sodium restriction I was still able to elevate renin, aldosterone and AII. As the increase in the activity of the system in my study was almost exactly to the level seen in similar studies of sodium restriction, it seems unlikely that the reason for demonstrating an effect on insulin sensitivity was due to unusually high activity in response to the diet.

Overall, there was no change in systolic or diastolic blood pressure between phases. While severe sodium restriction is associated with a reduction in blood pressure (354) more modest reductions have previously been reported as having no effect (249). Salt sensitivity, considered as a continuous variable or defined as a fall in MAP $\geq$3mmHg, may be involved in the subsequent development of hypertension (356) and may also be related to the metabolic response to sodium restriction. Melander *et al* (354) demonstrated recently that in males with hypertensive first-degree relatives, the improvement in insulin sensitivity

associated with high (240mmol/24 hours) vs low (10mmol/24 hours) sodium intake correlated with salt sensitivity. In the present study, a similar, though weaker, relationship was observed (figure 4.4). I did not observe a relationship between the degree of salt sensitivity and the activation of the RAS. This may reflect the more modest sodium restriction and more physiological, though still biologically significant, activation of the RAS than seen in other studies.

The analyses in the present study were performed on all 15 patients recruited. Four volunteers failed to reduce urinary sodium excretion to less than 100mmol sodium/24 hours during sodium restriction, however it was felt inappropriate to exclude volunteers on this basis. Three of these volunteers demonstrated a 100% increase in two of renin, aldosterone and AII between high and low sodium, while the remaining volunteer increased aldosterone by >100% between phases. Nonetheless, after exclusion of these four volunteers from the analysis, insulin sensitivity continued to remain lower during the low sodium phase (table 4.4).

No difference in total cholesterol or triglycerides was seen between phases. Fliser *et al* (246) demonstrated that total cholesterol rose in association with severe sodium restriction, however using a less severe regime, Grey *et al* (249) found no effect on total cholesterol, though noted a rise in $HDL_3$ during sodium restriction. In addition, sodium restriction was not associated with any change in the concentrations of circulating markers of endothelial function. It has been demonstrated that 3 weeks of severe sodium restriction in WKY rats (274) has a deleterious effect on the endothelium; these results would not support that observation, however this was not the primary question that the present study was designed to answer, and the strength of the relationship between circulating markers and more direct

functional measurements (the WKY studies were undertaken in aortic ring preparations) of endothelial integrity is not clear.

The mechanism through which sodium restriction has reduced insulin sensitivity in this study is unclear. The lack of a significant difference in noradrenaline, cortisol and growth hormone between phases suggests that these hormones are not responsible, though it is acknowledged that, for example, measurement of plasma noradrenaline is not an ideal marker of activity of the sympathetic nervous system. In addition, there are other hormones that may be involved which were not measured, such as antidiuretic hormone and atrial natriuretic hormone. The most obvious candidate hormones that may underlie a direct effect are the activated components of the RAS, in particular aldosterone and angiotensin II. In two recent studies, aldosterone was found to correlate positively with insulin resistance (357) and negatively with insulin sensitivity (358). A potential molecular explanation for this has been proposed from work in cultured human promonocytes, where aldosterone treatment reduced insulin receptor mRNA in a dose and time dependent manner (359). Interestingly, aldosterone has also been shown to correlate negatively with plasma levels of HDL-cholesterol, another component of the metabolic syndrome (247). In 50 untreated hypertensive patients, however, Lind *et al* (360) observed no correlation between aldosterone and insulin sensitivity, though did find a relationship between insulin resistance and plasma renin activity. In my study, there was no strong relationship between these two variables and insulin sensitivity.

It has also been demonstrated in rodent cardiac and aortic smooth muscle that AII may have an effect of cellular insulin responsiveness by intracellular crosstalk between signalling

intermediates (314). This potential mechanism through which activation of the RAS may reduce insulin sensitivity is explored further in chapter 5.

The lack of prospective studies of insulin sensitivity and cardiovascular end-points makes it difficult to quantify the risk associated with an 11% fall (as seen here in response to sodium restriction) in insulin sensitivity. Cross sectional data from the IRAS study (361) demonstrate a continuous relationship between insulin sensitivity (measured using the frequently sampled intravenous glucose tolerance test) and the intimal media thickness of the internal carotid artery. Furthermore, longitudinal follow up of cohorts of healthy males from Paris and Helsinki suggest that fasting hyperinsulinaemia, as a surrogate for insulin resistance, is associated with an adverse cardiovascular outcome. To put this reduction in insulin sensitivity in some context, several authors have considered a negative effect on insulin sensitivity as a potential mechanism to explain the disappointing effects on cardiovascular mortality of beta-blockers in the treatment of hypertension. Atenolol was shown to reduce insulin sensitivity (measured by the euglycaemic hyperinsulinaemic clamp) by 12% after 4-6 weeks of treatment (362), leading the authors to speculate that this metabolically adverse action may reduce the overall gain otherwise associated with a reduction in blood pressure.

In summary, I have demonstrated that modest sodium restriction is associated with a reduction in insulin sensitivity. While this has been demonstrated previously using strict dietary regimes that are difficult to adhere to over prolonged periods, the present study used a more modest, and therefore palatable, diet. Despite this, activation of the RAS was achieved in all volunteers, with no significant increase in the activity of other neurohumoral systems. I suggest that this modest reduction in insulin sensitivity may be of clinical

importance, perhaps contributing to the disappointing effects of sodium restriction on mortality in the general population (242).

# Chapter 5: Angiotensin II and insulin sensitivity in human adipocytes

## 5.1 Introduction

In the previous chapter modest dietary sodium restriction was shown to be associated with a significant reduction in whole body insulin sensitivity. The underlying mechanism is unclear, however it has been suggested that this effect may secondary to activation of the RAS, possibly mediated by a direct effect of angiotensin II on insulin action. In support of this, some groups have found an improvement in insulin sensitivity in association with ACE inhibition (279) (278), while others have observed increased frequency of hypoglycaemia in patients with type 1 diabetes treated with ACE inhibition (285,363) (see section 1.11.7).

Data from rodent cardiac myocytes and aortic smooth muscle cells have highlighted a potential mechanism through which angiotensin II may reduce insulin sensitivity at the cellular level. Angiotensin II was found to reduce the insulin stimulated activation of Insulin Receptor Substrate 1 (IRS-1) - associated phosphatidylinositol 3' kinase (PI3'-kinase) in both of these cell lines, possibly via AT1 receptor mediated phosphorylation of serine/threonine residues on the regulatory subunit of this key signalling intermediate (314,315). Downstream effects of insulin stimulation are mediated predominantly by IRS-1 and –2 associated PI3'-kinase. This step plays a pivotal role in the translocation of the insulin responsive glucose transporter GLUT4 to the cell surface and in the regulation of hormone sensitive lipase; as such, any reduction in the activity of this enzyme may be anticipated to reduce insulin-stimulated glucose transport and insulin-mediated suppression

of lipolysis. These important metabolic end-points were not measured in the initial reports of intracellular crosstalk between insulin and angiotensin II signalling.

This chapter tests the hypothesis that angiotensin II will reduce insulin-stimulated glucose uptake and insulin-mediated suppression of lipolysis in human adipose tissue. While this intracellular crosstalk has thus far only been demonstrated in rodent muscle cells, I have studied this relationship in human adipocytes. As well as being more accessible than human muscle cells, adipocytes are a well-characterised insulin responsive tissue. In addition, over the last ten years it has become evident that adipose tissue is one of the many sites that possess a local RAS (200,201), and as such any interaction between AII and insulin may be of physiological importance in determining local and systemic insulin sensitivity.

## 5.2 Methods

### 5.2.1 Recruitment of subjects

Subjects were recruited as outlined in materials and methods. All subjects were female in-patients undergoing gynaecological surgery for non-malignant disease, and were excluded if they were hypertensive, aged over 60 years, had a BMI > 33 kg/m$^2$ or were using intercurrent drug therapy. Between one and five grams of peri-umbilical subcutaneous adipose tissue was dissected from each subject.

### 5.2.2 Laboratory methods

A detailed description of the methods of adipocyte isolation, preparation of membrane fractions, radioligand binding, Western Blotting, glucose transport and NEFA assays is found in Chapter 2.

#### 5.2.2.1 Radioligand binding studies

Adipocyte membranes were incubated in the presence of 1nM $^{125}$ I-labelled (Sar$^1$,Ile$^8$) angiotensin II with or without 10$\mu$M losartan (a specific AT1 receptor antagonist). The losartan displaced binding was taken to be the amount of AII bound to the AT1 receptor, as is fully described in Chapter 2.2.1.6.

#### 5.2.2.2 Western blotting of human adipocyte membranes for the AT1 receptor

Human adipocyte membranes were prepared as described, and electrophoresed on a 10% polyacrylamide gel. Protein was then transferred onto nitrocellulose, and probed with anti-angiotensin II type 1 receptor antibody (Autogen Bioclear UK Ltd), then anti-rabbit HRP

labelled secondary antibody. Membranes prepared from rodent vascular smooth muscle cells were included as a positive control.

### 5.2.2.3 Glucose transport assays

A full description of the glucose transport assay is included in the materials and methods section. Cells were incubated with angiotensin II for 30 minutes to examine whether AII has an effect on glucose transport in the absence of insulin. To assess the effect of angiotensin II on insulin-stimulated glucose transport, increasing concentrations of AII were added to a single concentration of insulin, and increasing concentrations of insulin were added to a single concentration of AII.

### 5.2.2.4 Angiotensin II and lipolysis

Equivalent experiments were undertaken for the analysis of the effect of AII on lipolysis, both in the presence and absence of insulin, using the previously described methods.

## 5.2.3 Statistical analysis

For binding assays, mean values of specific AII binding were compared with non-specific binding using a paired t-test. The area under the curve (AUC) for glucose uptake and suppression of lipolysis was calculated for each assay using the method described by Matthews for the analysis of serial measurements (343). Comparisons of these parameters with and without AII were made using a paired t-test. Glucose uptake and lipolysis were compared for each concentration of AII with basal levels using paired t-tests. Data are expressed as mean and standard error.

## 5.3 Results

### 5.3.1 The angiotensin II receptor and human adipose tissue

Immunoblotting unstimulated adipose tissue membranes with a specific antibody (figure 5.1) confirmed the presence of the Angiotensin II Type 1 (AT1) receptor, as evidenced by a cross-reacting species at the expected molecular weight and at a similar position to the AT1 receptor expressed in vascular smooth muscle cells.

Radioligand binding, using vascular smooth muscle cells as a positive control and losartan to displace specific binding at the AT1 receptor, demonstrated the presence of binding to the AT1 receptor in human adipocyte membranes (figure 5.2) with a mean value of 22 ± 3 fmol AII bound per mg membrane protein (n=4). As anticipated, binding to the AT1 receptor in vascular smooth muscle cell membranes was of far greater magnitude (706 ± 147 fmol AII bound per mg membrane protein, n=4) (figure 5.3).

### 5.3.2 Angiotensin II and glucose transport

#### 5.3.2.1 Angiotensin II alone

Human adipocytes were incubated with increasing doses of AII and glucose uptake assayed as described in materials and methods. As shown in figure 5.4, no effect of physiological or pharmacological concentrations of AII was observed on adipocyte glucose transport in the absence of insulin (p=ns for comparison of glucose transport between any single AII concentration and basal glucose uptake).

### 5.3.2.2 Angiotensin II and insulin

Co-incubation of increasing doses of angiotensin II with 10nM insulin (figure 5.5) had no effect on insulin-stimulated glucose uptake into adipocytes (p=ns for comparison of glucose transport at 10nM insulin ± AII at all concentrations).

Preliminary experiments, limited by tissue availability, showed no effect of 100nM AII (figure 5.6.1) or 1$\mu$M AII (figure 5.6.2) on glucose transport (p=ns for comparison of insulin-stimulated glucose transport ±AII). More complete concentration response curves comparing insulin-stimulated glucose transport with and without AII incubation again demonstrated no effect of AII (figure 5.7,5.8) (p=ns for comparison of AUC of insulin-stimulated glucose transport ±AII).

## 5.3.3 Angiotensin II and insulin-mediated suppression of lipolysis

AII concentration response curves showed no significant effect on NEFA release over the concentrations tested (p=ns for NEFA release at any concentration of AII, compared with basal NEFA release (figure 5.9.1). As adenosine has an anti-lipolytic action, I considered the possibility that a weak lipolytic action of angiotensin II may be overwhelmed by adenosine; however, in adenosine-free buffer, again no effect of increasing concentrations of AII was observed on adipocyte release of NEFA (figure 5.9.2).

As shown in chapter 3, preliminary studies using the PI3'-kinase inhibitor wortmannin confirmed that insulin-mediated suppression of isoproterenol-induced lipolysis is indeed PI3'-kinase dependent. As such, any negative effect of angiotensin II on insulin – mediated activation of PI3'-kinase would be anticipated to translate to a reduction in insulin-mediated suppression of lipolysis; however, co-incubation of angiotensin II with insulin

and isoproterenol had no effect on the liberation of NEFA from adipocytes (p=ns for comparison of AUC of insulin-mediated attenuation of isoproterenol-induced lipolysis ± AII) (figure 5.10).

**Figure 5.1: Western blot of human adipocyte membrane fraction for angiotensin II type I receptor.**

Human adipocyte membrane fraction (20μg) (far right lane), with vascular smooth muscle membranes (50μg) (far left lane) as a positive control, probed with anti-angiotensin II type 1 receptor antibody. The middle three lanes represent subcellular fractionation of murine 3T3 L1 adipocytes. (HDM= high-density microsomes, LDM= low-density microsomes, PM= plasma membrane) and, for the purposes of this thesis, should be disregarded. The result shown is representative of three similar experiments.

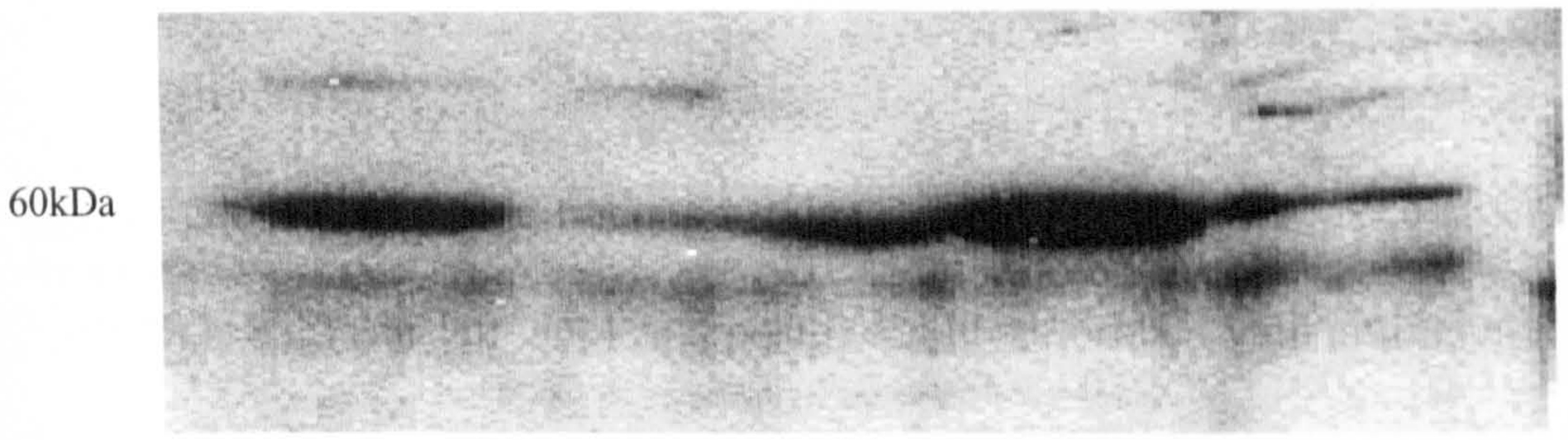


**Figure 5.2: Angiotensin II binding to human adipocyte membranes.**

The results shown represent the mean (± sem) of four experiments, each of which was analysed in duplicate. A significant difference was demonstrated between the total binding of radiolabelled angiotensin II and non-specific binding (binding of radiolabelled angiotensin II in the presence of the AT1 receptor blocker losartan) (p=0.008) which represented specific binding to the AT1 receptor. Mean binding to the AT1 receptor in human adipocyte membranes was 22±3 fmol AII bound per mg membrane protein.

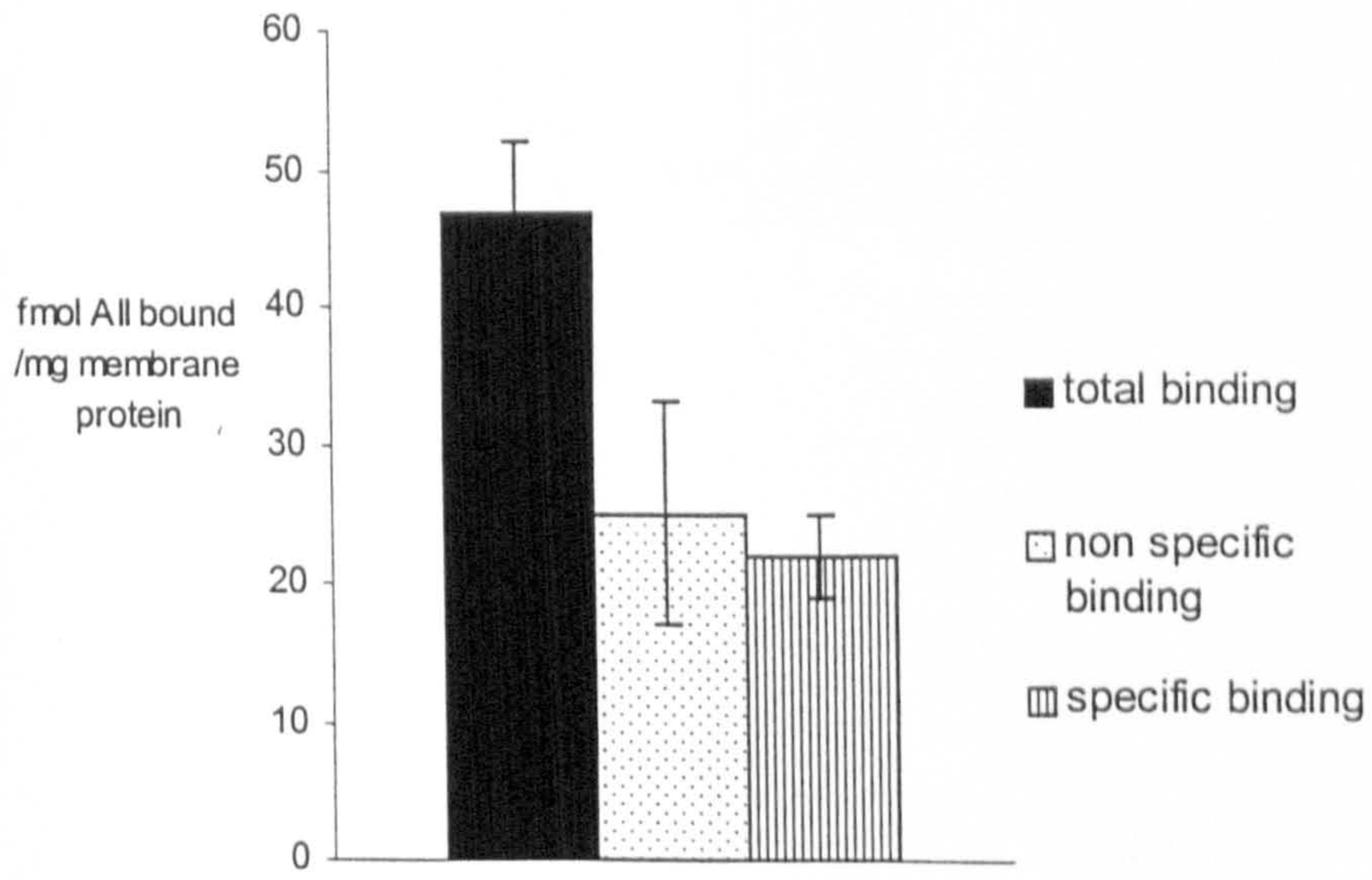


**Figure 5.3: Angiotensin II binding to human vascular smooth muscle membranes.**

The results shown represent the mean (± sem) of four experiments, each of which was analysed in duplicate. A significant difference was demonstrated between total binding and non-specific binding, representing specific binding to the AT1 receptor ($p<0.001$).

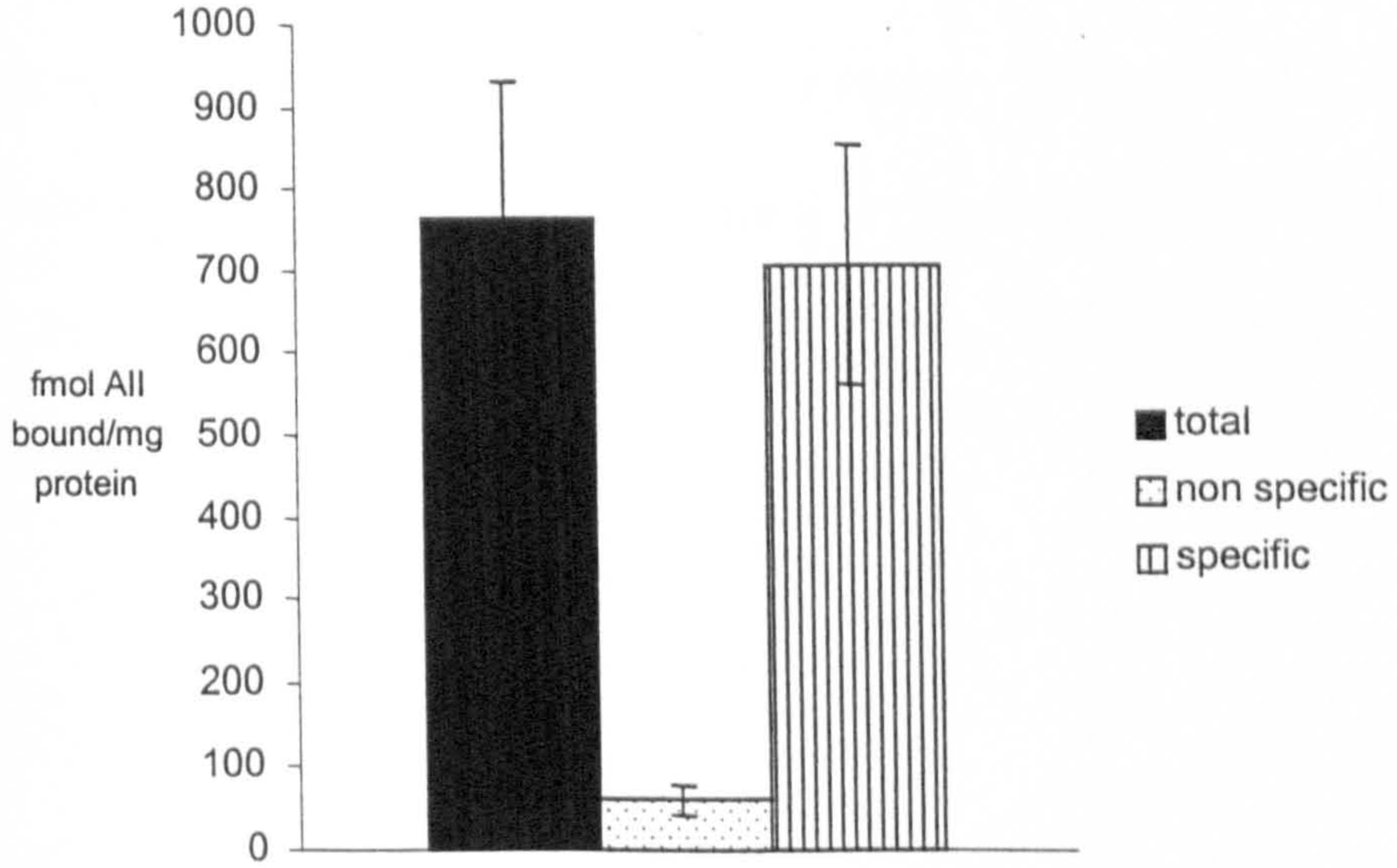


**Figure 5.4: Angiotensin II and glucose uptake in human adipocytes**.

Adipocytes were incubated with increasing concentrations of angiotensin II (1nM-1$\mu$M) for 30 minutes and glucose uptake assayed. Adipocyte glucose uptake was considered the amount of $^{3}$H 2-deoxyglucose associated with adipocytes after incubation with angiotensin II for 30 minutes and expressed as a fold change relative to the amount of $^{3}$H 2-deoxyglucose taken up into adipocytes in the basal state. No difference in glucose uptake was evident at the concentrations of angiotensin II tested (p=ns for comparison of glucose uptake at all AII concentrations vs. glucose uptake basally). The results shown represent the mean (± sem) of five experiments, in which each individual sample was analysed in triplicate.

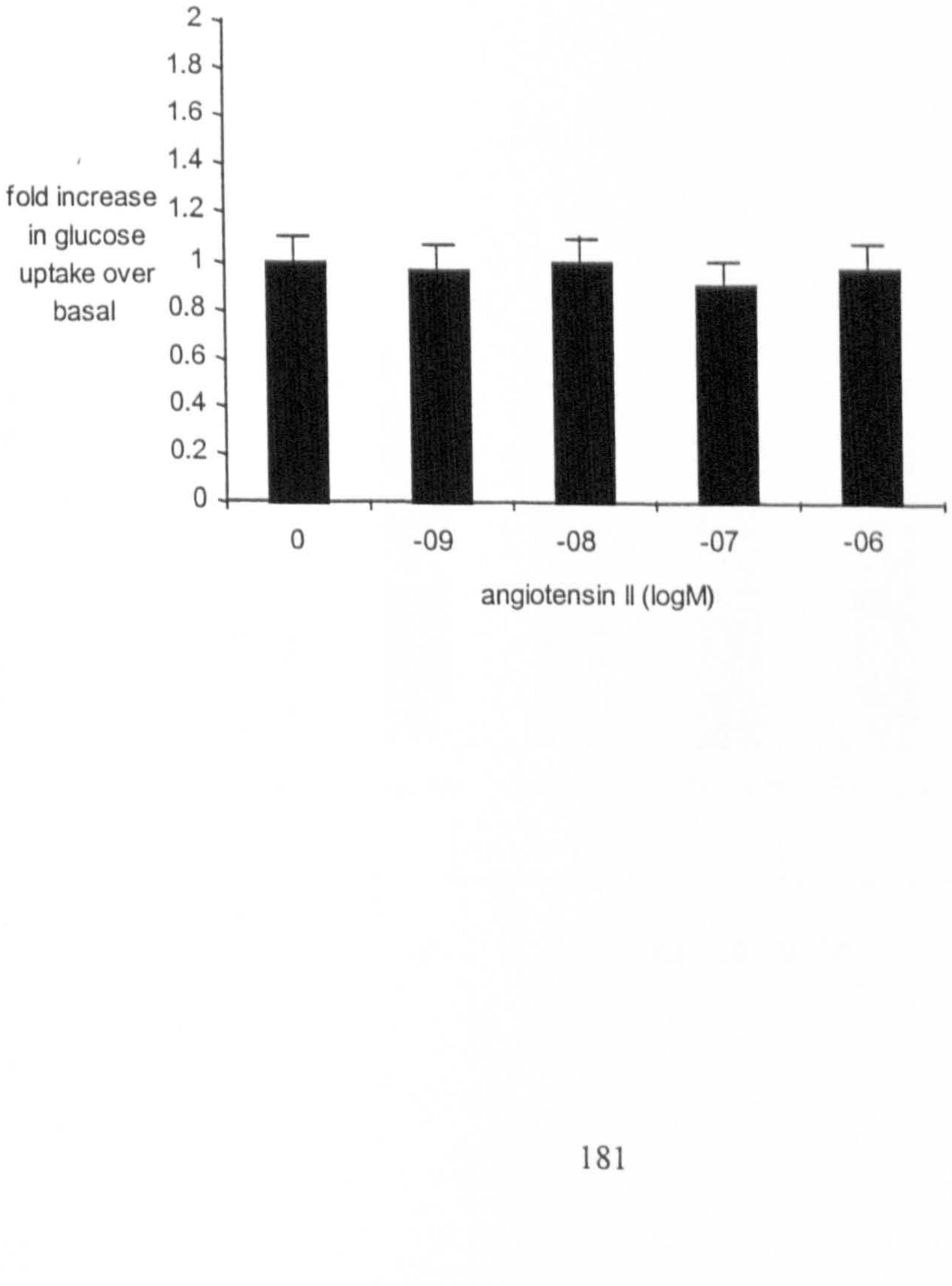


**Figure 5.5: Insulin-stimulated glucose uptake and coincubation with increasing concentrations of angiotensin II**

Insulin-stimulated glucose transport in human adipose tissue in response to insulin10nM alone was compared with coincubation with increasing doses of AII + insulin 10nM. Adipocyte glucose uptake was considered the amount of $^{3}H$ 2-deoxyglucose associated with adipocytes after incubation with insulin (doses shown) ± angiotensin II (doses shown) for 30 minutes and expressed as a fold change relative to the amount of $^{3}H$ 2-deoxyglucose taken up into adipocytes in the basal state (having excluded the $^{3}H$ 2-deoxyglucose uptake in the presence of cytochalasin B, considered non-facilitative). The values are generated from the means of four identical experiments, ± sem. All individual samples were measured in triplicate. There was no significant effect of angiotensin II on insulin-stimulated glucose uptake ($p>0.05$ for all comparisons).

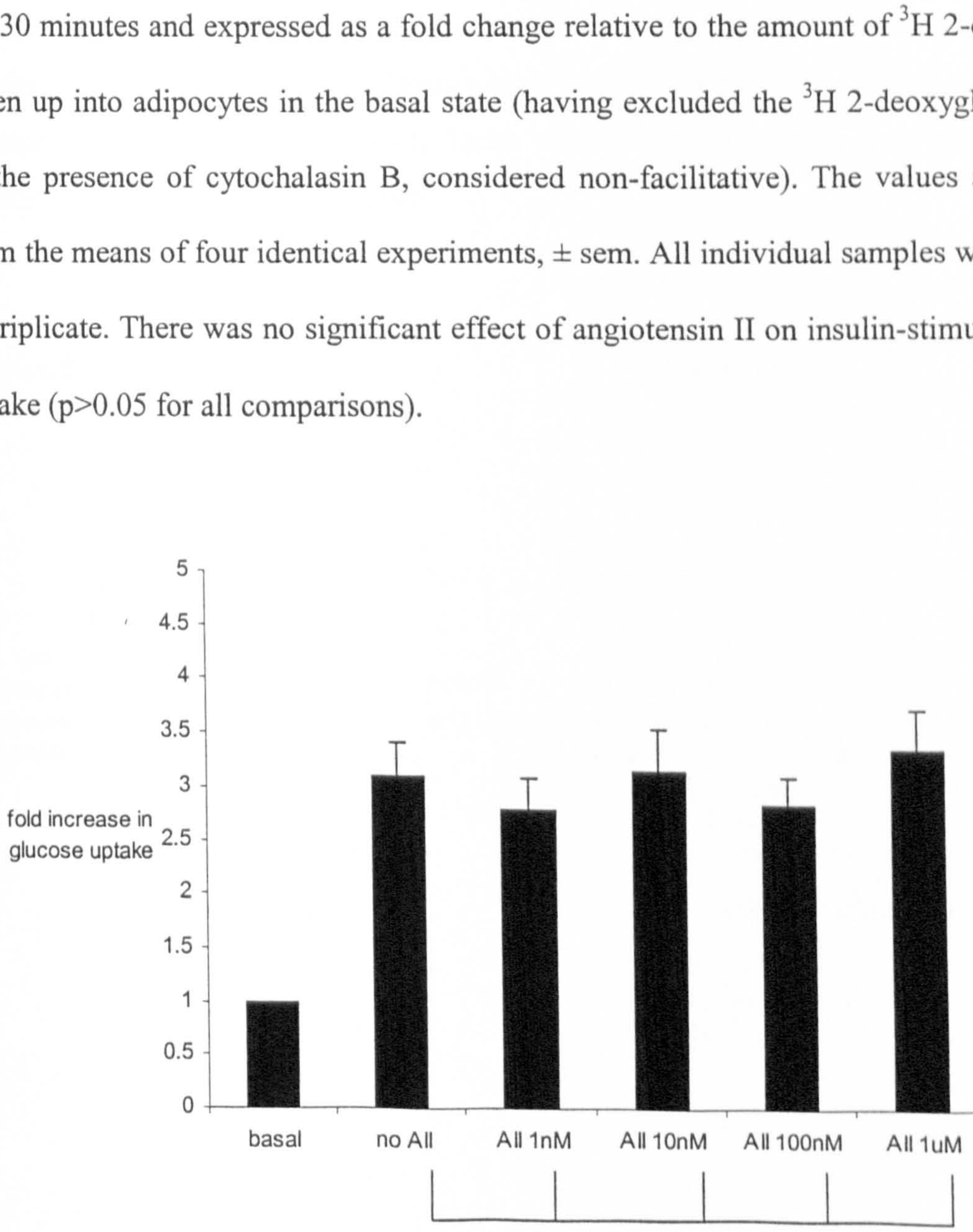


**Figure 5.6: Angiotensin II and insulin-stimulated glucose uptake in human adipocytes.** The effect of 15 minutes pre-incubation with AII 100nM (figure 5.6.1) and AII 1$\mu$M (figure 5.6.2) on insulin-stimulated glucose uptake in human adipose tissue was examined. The values shown represent the means ± s.e.m. of three sets of experiments for both datasets, with all individual samples analysed in triplicate. Adipocyte glucose uptake was considered to be the amount of $^{3}$H 2-deoxyglucose associated with adipocytes after incubation with insulin (doses shown) ± angiotensin II (doses shown) for 30 minutes and expressed as a fold change relative to the amount of $^{3}$H 2-deoxyglucose taken up into adipocytes in the basal state (having excluded the $^{3}$H 2-deoxyglucose uptake in the presence of cytochalasin B, considered non-facilitative). These assays were limited by tissue availability, hence the limited concentrations of AII used. There was no significant effect of angiotensin II on insulin-stimulated glucose transport.

**Figure 5.6.1**

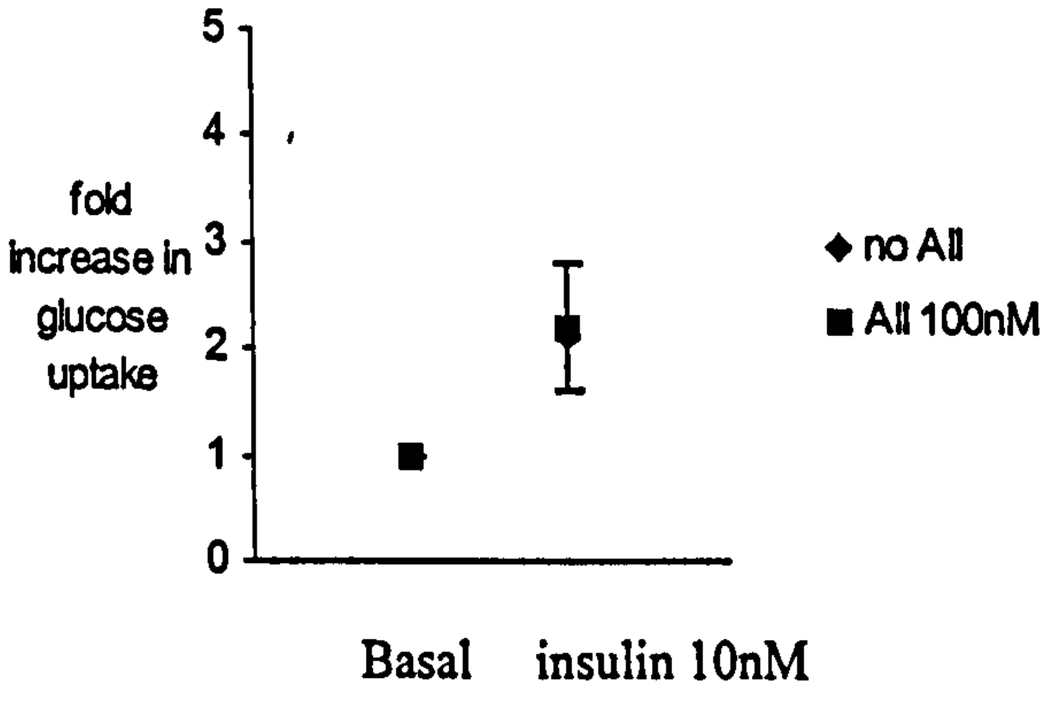



**Figure 5.6.2**

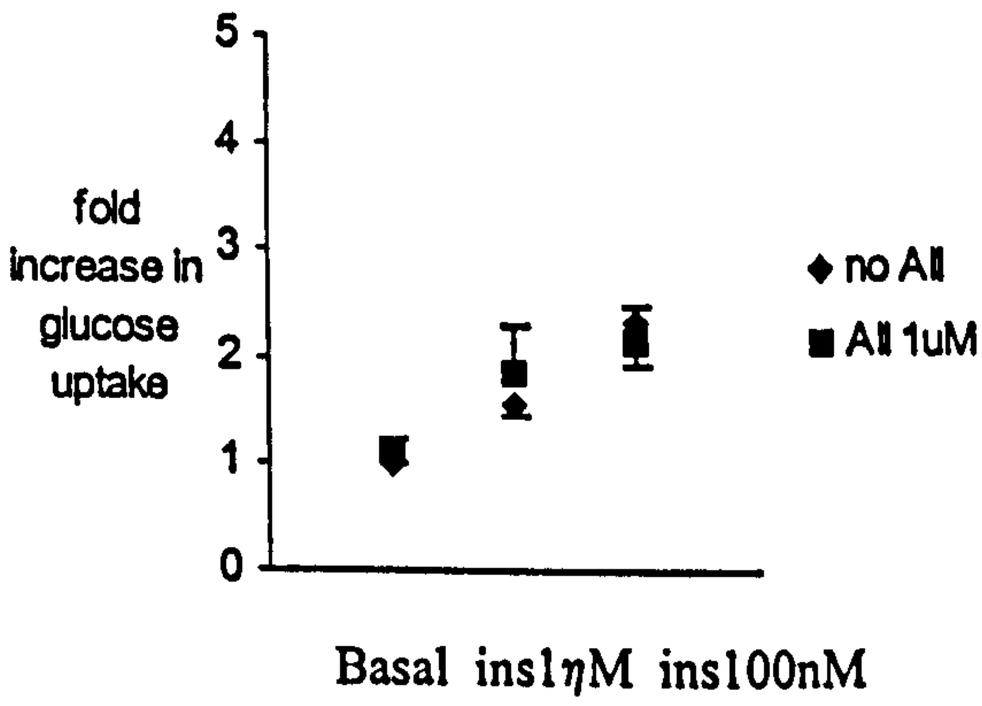


**Figure 5.7: Angiotensin II pre-incubation and insulin-stimulated glucose uptake in human adipose tissue.**

The effect of angiotensin II (100nM) pre-treatment (15 minutes) on insulin-stimulated glucose transport in human adipose tissue was examined. Adipocyte glucose uptake was considered the amount of $^{3}H$ 2-deoxyglucose associated with adipocytes after incubation with insulin (doses shown) ± angiotensin II (doses shown) for 30 minutes and expressed as a fold change relative to the amount of $^{3}H$ 2-deoxyglucose taken up into adipocytes in the basal state (having excluded the $^{3}H$ 2-deoxyglucose uptake in the presence of cytochalasin B, considered non-facilitative). The results shown represent the mean (±sem) of four experiments, each sample of which was analysed in triplicate. There was no effect of angiotensin II on insulin stimulated glucose uptake ($p>0.05$ for comparison of AUC insulin alone vs angiotensin II + insulin).

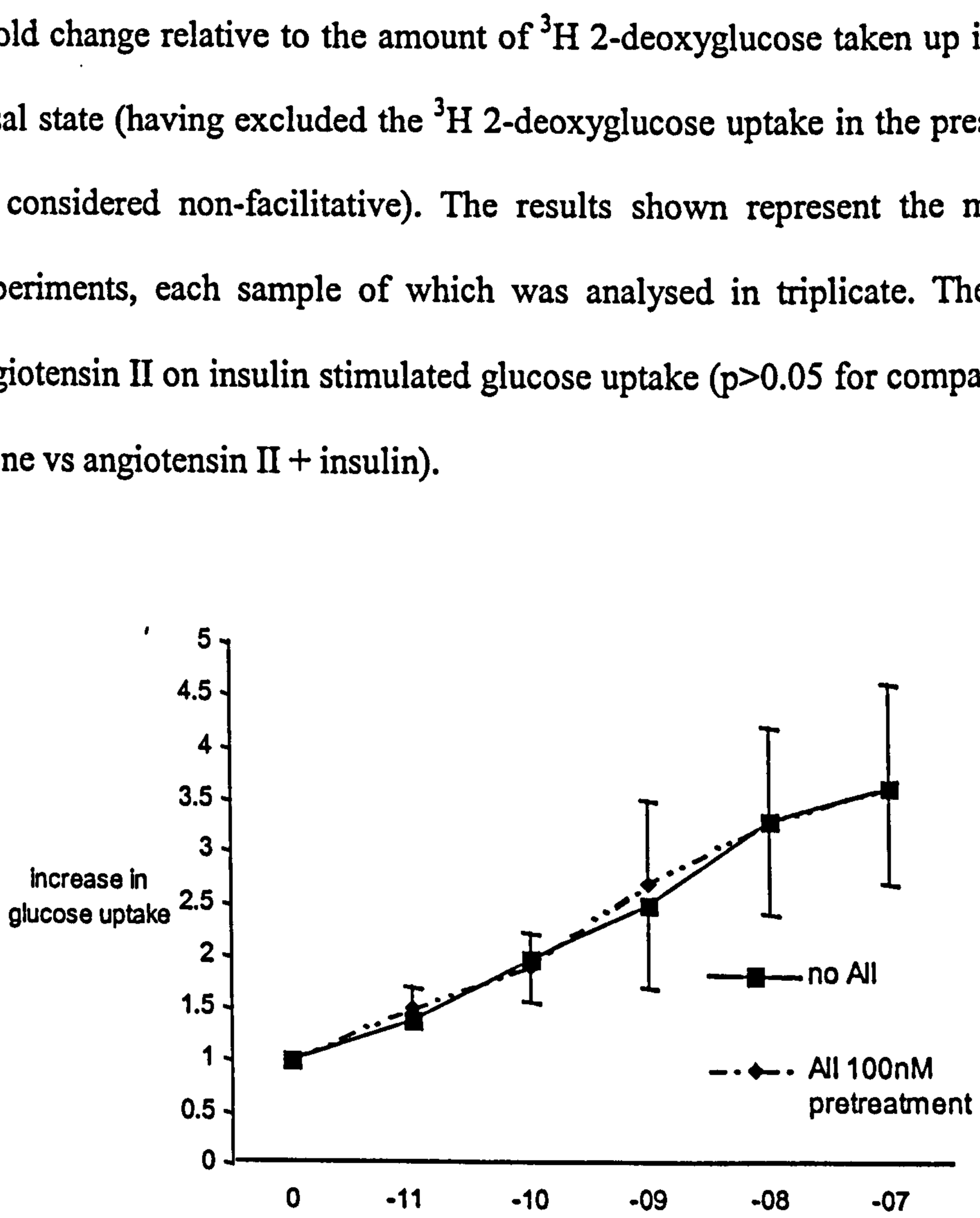


**Figure 5.8:Insulin-stimulated glucose uptake and coincubation with angiotensin II.**

The effect of angiotensin II (100nM) coincubation on insulin-stimulated glucose transport was examined in human adipose tissue. Adipocyte glucose uptake was considered the amount of $^{3}H$ 2-deoxyglucose associated with adipocytes after incubation with insulin (doses shown) ± angiotensin II 100nM for 30 minutes and expressed as a fold change relative to the amount of $^{3}H$ 2-deoxyglucose taken up into adipocytes in the basal state (having excluded the $^{3}H$ 2-deoxyglucose uptake in the presence of cytochalasin B, considered non-facilitative). The results shown represent the mean (±sem) of four experiments, each sample of which was analysed in triplicate. There was no effect of angiotensin II on insulin stimulated glucose uptake ($p>0.05$ for comparison of AUC insulin alone vs angiotensin II + insulin).

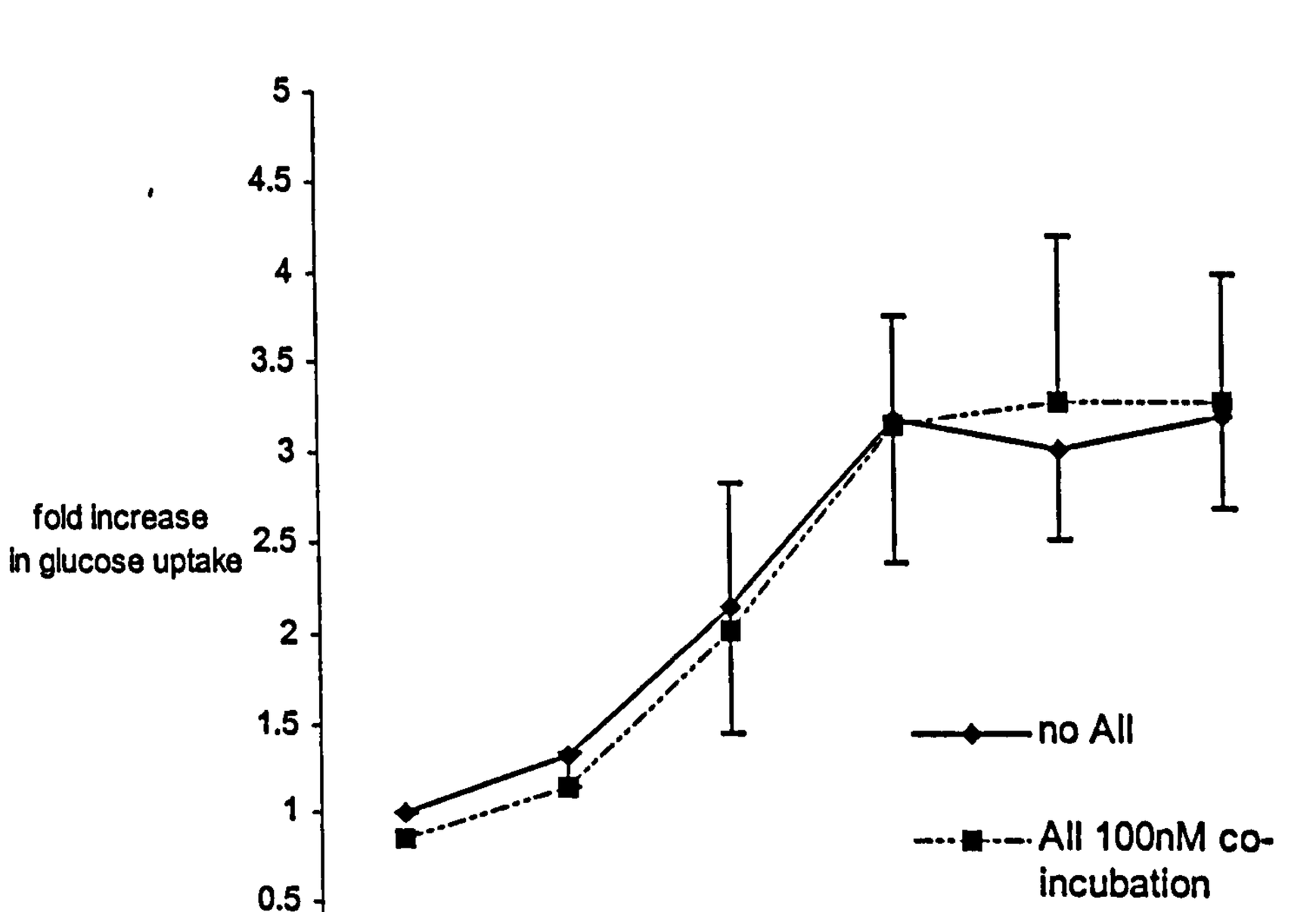


**Figure 5.9: Angiotensin II and release of NEFA from adipocytes.**

The effect of 45 minutes of coincubation with angiotensin II on NEFA release from isolated human adipocytes in the presence (figure 5.9.1) and absence (figure 5.9.2) of adenosine (100nM) and isoproterenol (200nM). The NEFA release in response to isoproterenol alone is taken as the 100% value in 5.9.1, while the basal value in 5.9.2 is the NEFA release from unstimulated adipocytes, with all other values expressed as a percentage of this. For both graphs, the results shown represent the means (±sem) of four experiments, with the individual samples analysed in duplicate. There was no effect of angiotensin II on NEFA release, either in the presence or absence of adenosine and isoprenaline ($p>0.05$ comparing angiotensin II NEFA release with no angiotensin II).

**Figure 5.9.1**

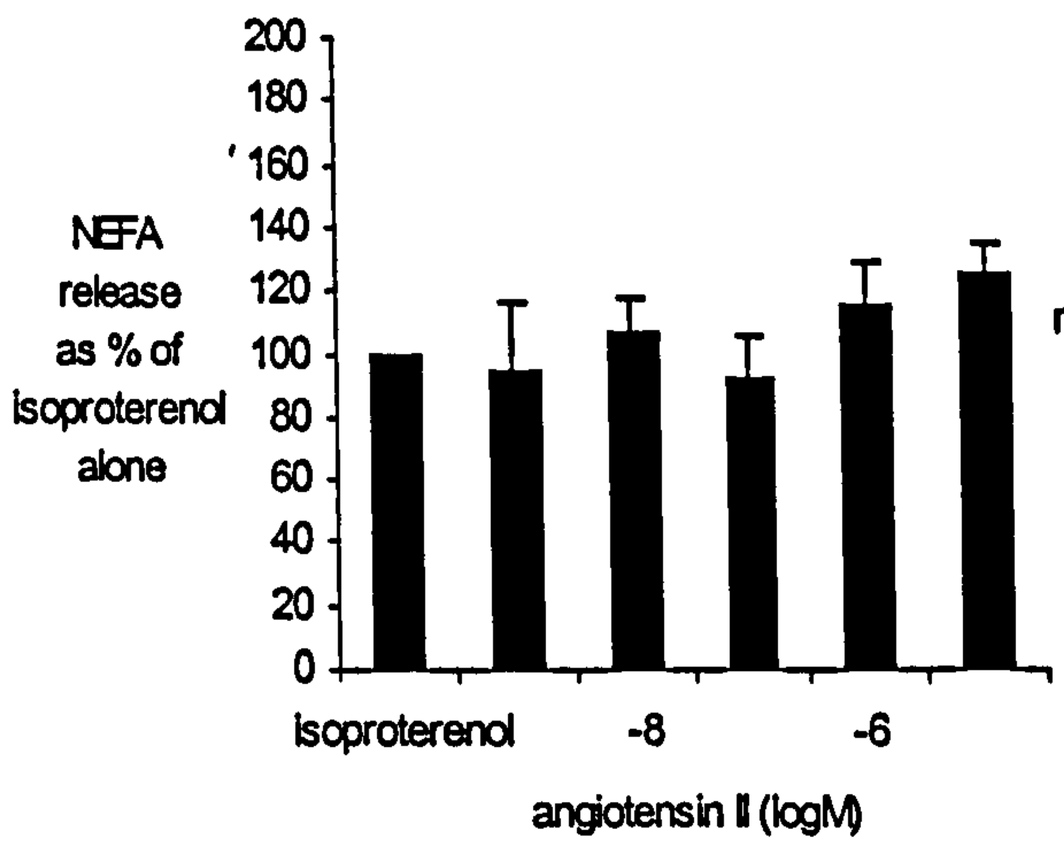



**Figure 5.9.2**

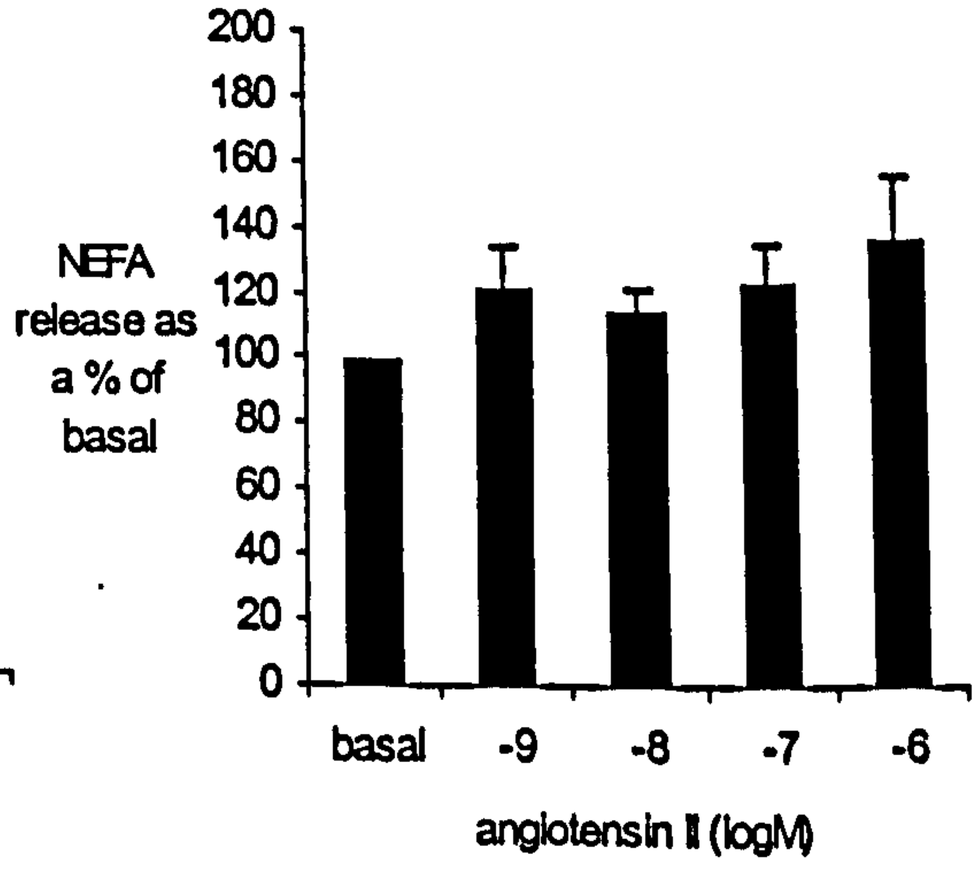
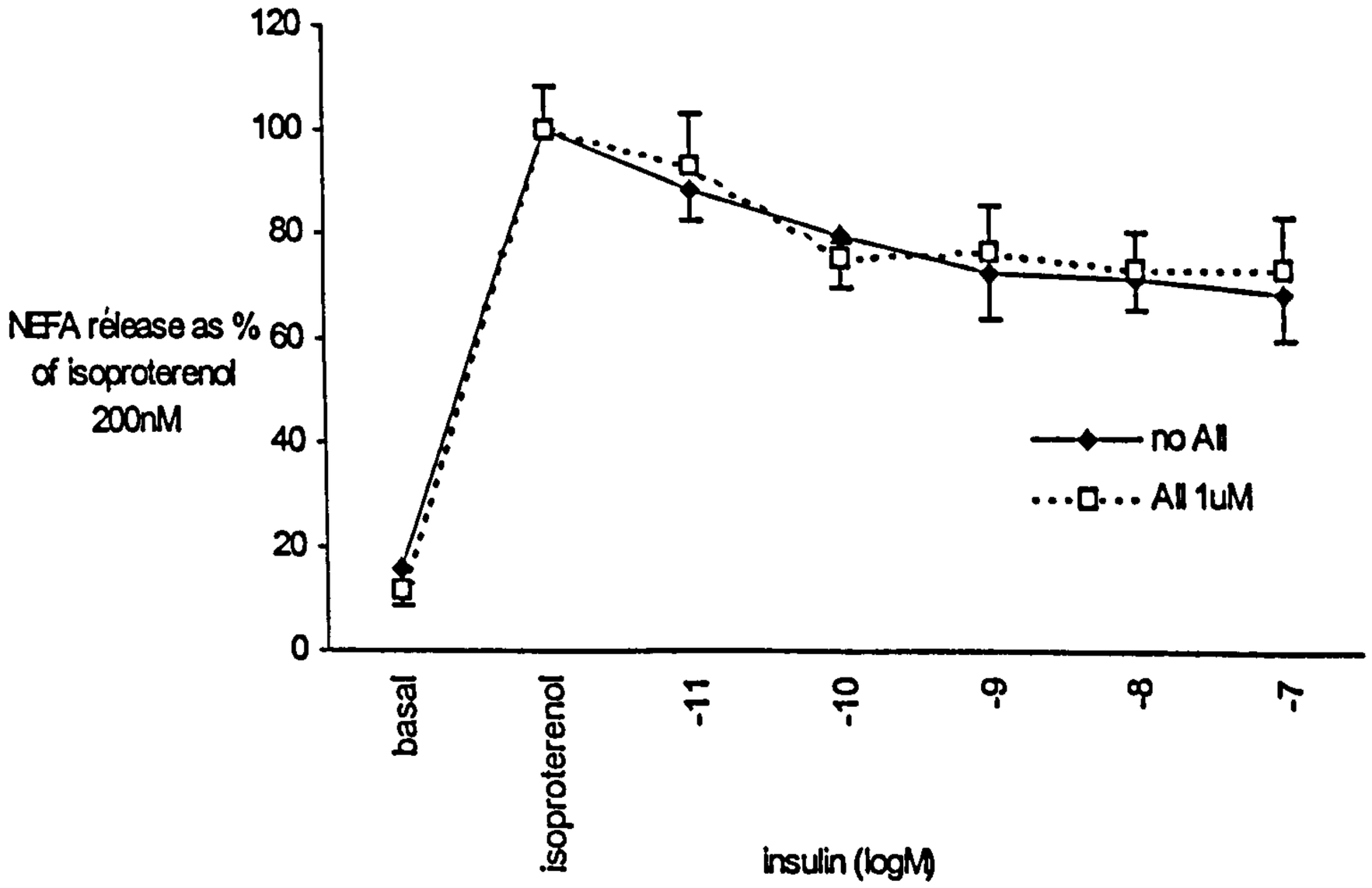


**Figure 5.10: Angiotensin II and insulin-mediated suppression of lipolysis**

The effect of angiotensin II (1$\mu$M) co-incubation on insulin-mediated suppression of lipolysis was examined in human adipocytes. The NEFA release in response to isoproterenol alone is taken as the 100% value, with all other values expressed as a percentage of this. The results shown represent the mean (±sem) of four experiments, each sample of which was analysed in duplicate. There was no effect of angiotensin II on insulin-mediated suppression NEFA release ($p>0.05$ comparing AUC insulin alone with insulin +angiotensin II)



## 5.4 Discussion

I have demonstrated that human adipose tissue expresses the AT1 receptor, and that AII binds to this receptor after digestion and isolation of cells according to my protocol. However, AII treatment had no effect on insulin-stimulated glucose uptake or suppression of lipolysis.

These experiments were undertaken to test the hypothesis that AII may reduce insulin responsiveness at the cellular level, and explain why activation of the RAS in response to dietary sodium restriction is associated with a reduction in whole body insulin sensitivity. The main sites of peripheral glucose uptake *in vivo* are skeletal muscle and adipose tissue, with recent evidence proposing that the contribution of adipose tissue insulin sensitivity to the phenotype is considerably more important than hitherto appreciated (119), (120). Skeletal muscle and adipose tissue have similar insulin-signalling cascades, suggesting that any effect seen in adipose tissue may be extrapolated to muscle. From a more practical standpoint, it is very difficult to obtain the volume of human skeletal muscle necessary to undertake the assays described above.

Two techniques were employed to confirm the presence of the AT1 receptor in adipocytes prepared using my digestion and isolation protocol. Immunoblotting of adipocyte membranes prepared from fresh adipocytes demonstrated that the protein was expressed in these cells. This did not however confirm that the receptor binds AII under the conditions used in the laboratory, and so competitive radioligand binding studies were undertaken. These confirmed that adipocytes do indeed bind AII, and that this is displaced by losartan, implying specific binding to the AT1 receptor.

The finding that human adipose tissue expresses constituents of the RAS is not novel, as there is now considerable evidence to suggest that a locally regulated RAS is present in adipose tissue(364). In particular, Crandall *et al* (204) undertook detailed binding studies in human subcutaneous adipocytes, using similar methods to those employed in this thesis, and demonstrated AT1 receptor binding with a mean maximum binding capacity of 27.3 ± 9.3 fmol AII/mg protein, and an $IC_{50}$ for losartan of $1.61 \pm 0.45 \times 10^{-8}$. Competitive binding studies using the AT2 receptor blocker PD 123 319 suggested no binding at this receptor. This binding capacity is similar to the findings described in the present chapter. The combination of my own studies with data from other groups (204) provides firm evidence of the presence of the AT1 receptor in human adipose tissue, and confirms that adipose tissue is a suitable tissue in which to investigate my hypothesis.

The notion that AII may have a direct effect on cellular insulin sensitivity, mediated at the level of intracellular second messengers, was proposed in two papers. The first of these found that in cardiac myocytes from animals injected with AII 2 minutes prior to excision, insulin stimulated activation of PI3'-kinase was reduced by 40% (314). This effect of AII was mediated via the AT1 receptor, and involved the recruitment of the intracellular Janus Kinase-2 enzyme. The second paper studied the nature of the intracellular crosstalk in rodent aortic vascular smooth muscle cells. Again, AII reduced insulin stimulated IRS-1 associated PI3'-kinase activity by 60% (315), though in these cells blocking the AT1 receptor did not diminish this action. This effect was mediated by crosstalk between intracellular AII and insulin-signalling, involving the phosphorylation of serine/threonine residues on the regulatory subunit of PI3'-kinase.

There are no published data investigating the effects of AII on insulin stimulated glucose transport in human tissue. Baba *et al* (322) did examine the effect of AII on insulin stimulated tyrosine kinase activity in isolated human adipocytes, and found no effect. Patiag *et al* (365) investigated the effect of AII, and AII receptor blockade, in cultured myoblasts and again showed that AII co-incubation did not alter insulin sensitivity at the cellular level.

Thus, this work is the first to consider the potentially important effect of AII on insulin regulated cellular processes in human tissue. I have found no effect of AII on insulin-stimulated glucose transport or suppression of lipolysis. In the crosstalk studies, the concentration of AII that inhibited PI3'-kinase activation was 100nM. I have used AII doses of between 1nM and 1$\mu$M and found no effect. It is possible that I have missed an effect of AII at very high or low doses, though this seems unlikely given the range of doses that I have tested. It may be that, despite demonstrating that AT1 receptors are present in adipocytes isolated using my protocol, other important components of the signalling machinery necessary to generate this crosstalk, such as JAK2, are expressed at insufficient levels in adipocytes compared with myocytes. Were this the case, however, an effect would be anticipated in myoblasts, and this is not the case (365).

It seems more likely that this crosstalk in response to AII and insulin does occur in metabolically active cell lines, however the reduction in IRS-1 associated PI3'-kinase activity is not translated into effects on glucose transport or lipolysis. This raises questions about the absolute requirement for IRS-1 associated PI3'-kinase activity in cellular insulin action, and has support from some of the published literature. In insulin resistant adipocytes, IRS-1 associated PI3'-kinase activity is reduced, however IRS-2 associated

PI3'-kinase activity shows a compensatory increase (88). Furthermore, in IRS-1 knockout mice, though the animals are growth retarded and hypertriglyceridaemic, they are not diabetic. In contrast, mice lacking IRS-2 do indeed develop diabetes at an early age (89). Thus there are situations, manufactured and natural, in which a reduction in IRS-1 associated PI3'-kinase is not associated with a reduction in glucose transport, and so perhaps the effect of AII on IRS-1 associated PI3'-kinase is compensated similarly by alternative metabolic pathways.

No effect of AII was seen on glucose transport in the absence of insulin, though incubation times were of a relatively short duration (though long enough to demonstrate binding). It addition, a relatively small effect on glucose transport cannot be excluded as this may be beyond the sensitivity of the assay system.

I was not able to demonstrate a statistically significant effect of AII on the release of NEFA from isolated adipocytes, however it is suggested in the concentration response curves, both with and without isoproterenol and adenosine (figure 5.9.1 and 5.9.2) that there may be a modest effect to increase release of NEFA. AII has been reported to stimulate the release of prostaglandins from isolated rat adipocytes (210), using arachidonic acid as a substrate derived from cellular phospholipid breakdown, though not triacylglycerol lipolysis. The reagents in the NEFA assay detect the presence of arachidonic acid, and so this modest increase in NEFA at high AII doses may represent the liberation of arachidonic acid rather than triglyceride lipolysis. Angiotensin II may, however, play a relatively minor role in the regulation of lipolysis, as suggested in a recent publication which found no effect of infusing subpressor or pressor concentrations of angiotensin II on whole body lipolysis in healthy volunteers (366). My own data would largely support this.

The finding that intracellular crosstalk between insulin and AII activated second messengers does not result in a reduction in insulin regulated cellular processes is of relevance to the local adipose tissue microcirculation as well as the systemic circulation. Adipose tissue has a highly developed local RAS and is capable of producing AII, independent of the activity of the circulating RAS. The local production of AII seems to be important mainly in cellular differentiation and growth (213,216). The observation made in Chapter 4, that dietary sodium restriction activates the renin-angiotensin system and reduces insulin sensitivity, in combination with the demonstration of intracellular crosstalk between second messengers of the angiotensin II and insulin-signalling cascades, raises the possibility that the local RAS may in addition modify insulin sensitivity. The results presented herein, however, would refute this.

In summary, I have found no inhibition of insulin sensitivity by AII in isolated human adipose tissue, despite confirming binding at the AT1 receptor. Any reduction in insulin sensitivity seen in response to AII may be mediated in alternative tissues, or through alternative mechanisms.

# Chapter 6: Glucocorticoids and insulin sensitivity: dissociation of insulin's metabolic and vascular actions

## 6.1 Introduction

In chapter 4, I demonstrated that dietary sodium restriction is associated with a reduction in insulin sensitivity. This reduction in metabolic insulin sensitivity was not associated with any difference in the circulating levels of markers of endothelial function, suggesting that despite the association of metabolic insulin resistance and endothelial dysfunction, manipulation of one variable did not necessarily alter the other. In the present chapter, I have investigated this relationship further, using systemic exposure to glucocorticoids as a model of reduced metabolic insulin sensitivity. There is reasonable scientific evidence, on a background of considerable clinical evidence, to suggest that glucocorticoids reduce metabolic insulin sensitivity; it remains unclear if this metabolic defect is an isolated response or alternatively, whether there may a reduction in sensitivity to insulin action in the vasculature. While there are some data suggesting that metabolic and vascular insulin sensitivity are coupled (i.e. the most metabolically insulin responsive subjects exhibit the greatest vasodilation in response to insulin) (177) (367,368), there are few data demonstrating alteration of both in response to one stimulus. To show this *in vivo* would suggest there being a defect common to both metabolic and vascular tissues. One such defect may be at the level of the intracellular signalling intermediates that are shared between both tissue types; the classical metabolic pathway of insulin stimulating the insulin receptor and insulin receptor substrate proteins through a series of tyrosine phosphorylation events culminating in the activation of PI3'-kinase seems also to be the case in endothelial

cells. It is of importance, therefore, to bear in mind that the predominant mechanisms through which glucocorticoids induce insulin resistance in peripheral tissues appears to be mediated at the level of GLUT4 trafficking and subcellular distribution(336,338). As such, this model of insulin resistance is one in which the impairment in insulin-stimulated glucose uptake is most likely due to a defect beyond the level of the insulin-signalling cascade.

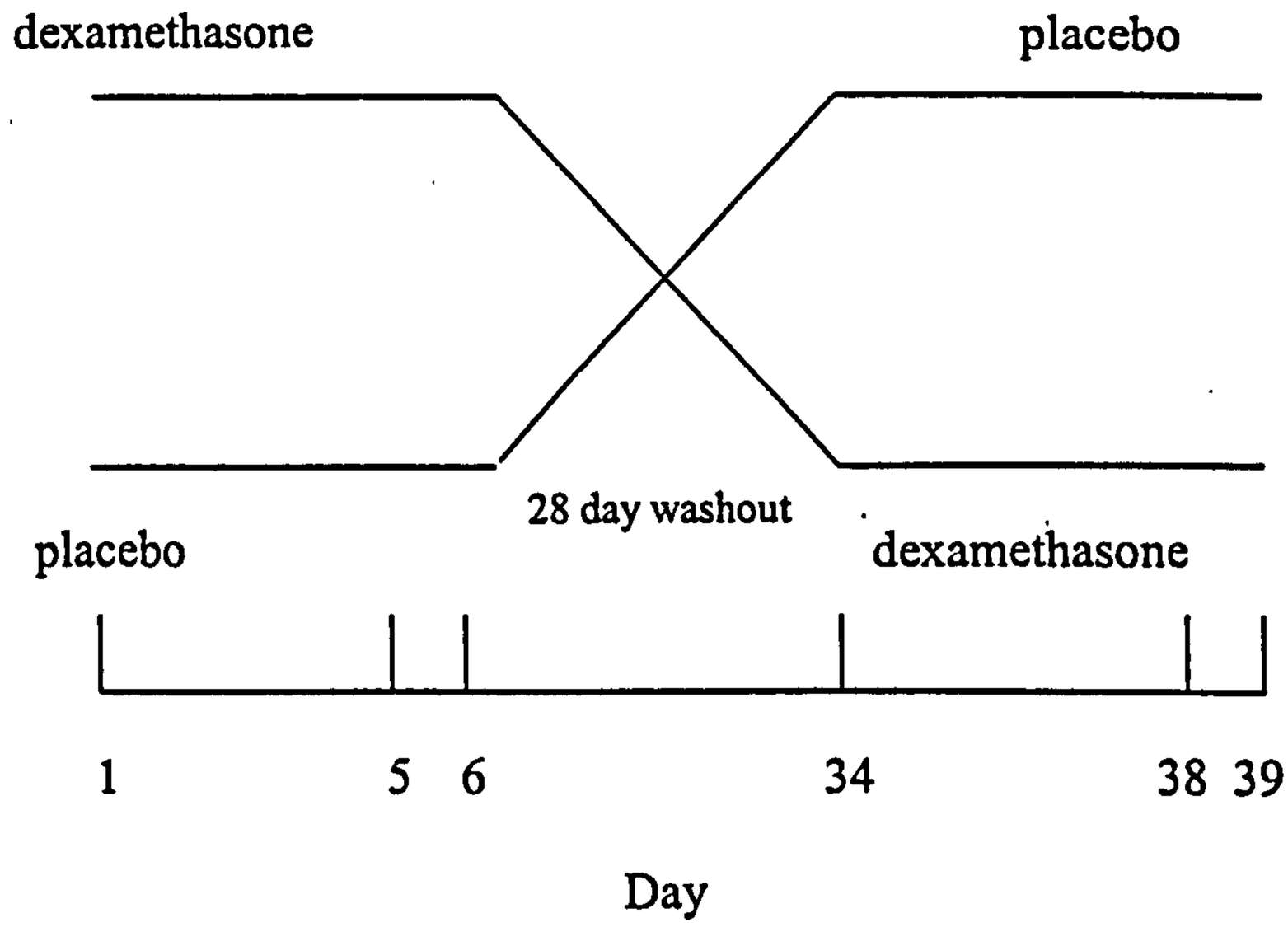
In addition, it has been suggested (369) that excessive tissue exposure to glucocorticoid action as a result of dysregulation of the 11$\beta$ hydroxysteroid dehydrogenase enzymes (types I and II), may play a part in the aetiology of the insulin resistance or metabolic syndrome. If this is indeed the case, then the metabolic and vascular features of the metabolic syndrome might be expected to develop during dexamethasone treatment.

## 6.2 Methods

### 6.2.1 Study Protocol

A double-blind, randomised, placebo-controlled crossover trial with a four week washout period was utilised to compare the effect of dexamethasone 1mg twice daily for six days with matching placebo. The Ethics Committee of the West Glasgow Hospitals University NHS Trust approved the protocol. Twenty volunteers were recruited as described in Chapter 2. Exclusion criteria were BMI $\geq 27 kg/m^2$, blood pressure $\geq 150/85$ mmHg, fasting glucose $\geq 6.0$ mmol/l and any history of intercurrent illness or drug therapy. Volunteers were asked not to smoke or drink alcohol during the two study phases. On the fifth day of dexamethasone or placebo, volunteers attended fasted for a gluteal biopsy (Chapter 2.2.2.3.3). A technician, blinded to the study phase and the result of metabolic investigations, dissected small resistance vessels from the biopsy for vascular studies using the Mulvany wire myograph method (Chapter 2.2.2.5). For the next 24 hours, the volunteers collected urine for measurement of urinary free cortisol. The following morning volunteers underwent fasting serum lipid measurements followed by a euglycaemic hyperinsulinaemic clamp.

**Figure 6.1: Study protocol**




Day 1and 34 – start placebo/dexamethasone

Day 5 and 38 – gluteal biopsy and wire myography

Day 6 and 39 – euglycaemic hyperinsulinaemic clamp

### 6.2.2 Clinical procedures

#### 6.2.2.1 Euglycaemic hyperinsulinaemic clamp

Insulin sensitivity was assessed using the hyperinsulinaemic euglycaemic clamp (10) (Chapter 2.2.2.3.1). At time 0 minutes of the clamp protocol, serum samples were withdrawn from the left antecubital fossa for lipid profile. The methods for all peripheral blood analysis are described in Chapter 2.

#### 6.2.2.2. Vascular studies

i) Gluteal Biopsy

Subjects were invited to attend the Clinical Investigation and Research Unit at 0830, having fasted from midnight. A gluteal biopsy was obtained as described in Chapter 2.2.2.3.3

ii) Myography studies

Resistance arteries were dissected from the biopsy; these underwent myography studies as described in full in Chapter 2. In brief, four vessels were obtained where possible from each volunteer during both study phases. After the initial normalisation protocol, vessels underwent a cumulative concentration response curve to norepinephrine (NE), then pre-constriction with norepinephrine followed by an acetylcholine relaxation phase, then insulin (1nM, 100pM, 10pM) pre-incubation followed by a further norepinephrine concentration-response curve. Where sufficient vessels remained viable after the insulin + NE concentration response curve, 30 minute incubations were undertaken with L-NMMA (1$\mu$M) prior to repeating the NE concentration response curve in the presence of insulin 10pM-1nM.

Wherever possible one vessel was used as a time control. This vessel underwent an identical protocol to the others, except no insulin incubation was undertaken, allowing analysis of the effect of time alone on contractile response in vessels.

### 6.2.3 Statistical analysis

i) Comparisons of insulin sensitivity (M-value), lipids and circulating markers of endothelial function between phases were made using a paired t-test.

ii) For myography studies, two parameters were used for comparison of either concentration response curves with baseline curves, or between phase comparisons. First, the $pD_2$, which is the negative log of the NE concentration at which the half maximal response is seen ($EC_{50}$), was calculated. This has also been referred to as the sensitivity value. In addition, the maximum active effective pressure ($mN/mm^2$, calculated from the radius and the wall tension using the Law of LaPlace P=T/r, where P is the active effective pressure, T is the wall tension and r is the internal radius) was calculated, allowing comparison of the maximal effect of agonist. Baseline NE concentration response curve and NE concentration response curve following insulin were compared between placebo and dexamethasone phases using these parameters. Where subjects had undergone two biopsies, a paired t-test was used, whereas, when comparing data that included subjects with only one successful myography result, an unpaired t-test was used. In assessing the effect of L-NMMA on insulin-mediated vasodilation, a paired t-test was used to compare $pD_2$ between the insulin pre-treated NE concentration response curve and the equivalent insulin concentration + L-NMMA.

## 6.3 Results

Twenty volunteers were recruited to the study, with one failing to attend for the second study phase due to work commitments. The study protocol was well tolerated by all of the subjects, the only complication being four minor infections at the biopsy site. Sixteen volunteers had at least one successful biopsy, with twelve having full myography data. An absence of vessels of suitable diameter was the reason for failure to obtain vessels from the biopsies with no myography data. The baseline characteristics of the subjects are detailed in Table 6.1.

### 6.3.1 Blood pressure, body weight and insulin sensitivity

No significant change in blood pressure or weight was observed between phases (Table 6.2). Insulin sensitivity, measured by the euglycaemic hyperinsulinaemic clamp, was significantly reduced following six days of dexamethasone (mean fall of 29% (95%CI 8-50%)) (Table 6.1, Figure 6.2). In addition, fasting serum insulin was higher during this phase (Table 6.2, Figure 6.3). There was no between phase difference in serum insulin at any time point during the clamps (Figure 6.3), and in particular, there was no difference in the mean serum insulin during steady state (115± 36 $\mu$U/ml (SD) during placebo vs. 123± 36$\mu$U/ml (SD) during dexamethasone p=0.33).

**Table 6.1: Baseline characteristics of recruits**

All values are expressed as mean and one standard deviation

| | **Mean (± S.D.)** |
|---|---|
| **Number** | 20 |
| **Age (years)** | 24.3± 4.1 |
| **Weight (kg)** | 76.1± 9.2 |
| **BMI ($kg/m^2$)** | 23.9± 3.1 |
| **Blood Pressure (mm/Hg)** | 129±10.4/66±8.6 |
| **Total Cholesterol (mmol/l)** | 4.3±0.98 |
| **Triglycerides (mmol/l)** | 1.49±0.84 |

### 6.3.2 Vascular studies

#### 6.3.2.1 Analysis of the effect of time on control vessels

Comparison of the norepinephrine-induced vasoconstriction between vessels after the first and third concentration response curves to norepinephrine demonstrated no change in $pD_2$ ($pD_2$ for first NE concentration response curve 7.14±0.1 vs 7.13±0.13 for third NE concentration response curve, p=0.91) when all 17 available results were examined (Figure 6.4). When these were examined according to study phase, there was no difference in $pD_2$ between these curves during placebo (n=6, p=0.84) or dexamethasone phases (n=11, p=0.88). Having demonstrated that there was no effect of time on vessel response, further comparisons of the effect of insulin or acetylcholine on NE induced vasoconstriction were made for each vessel versus the first curve in response to NE alone, on the same vessel.

#### 6.3.2.2 Dexamethasone and norepinephrine induced vasoconstriction

For this analysis, data were obtained from 12 volunteers, accounting for 32 arteries studied in each phase. All volunteers contributed between two and four arteries to the final analysis and each volunteer contributed the same number of arteries in each phase. In volunteers where, for example, two arteries were available from one phase and three from the other, the vessel used to subsequently study insulin 10nM was discarded arbitrarily. To achieve greater statistical accuracy, only subjects with data from both phases were studied, and the $pD_2$ of NE induced vasoconstriction compared using a paired t-test. The results are shown in Figure 6.5. There was no statistically significant change in the $pD_2$ of NE induced vasoconstriction between phases ($pD_2$ during placebo 6.86±0.08 vs $pD_2$ during dexamethasone 7.0±0.05, p=0.1).

### 6.3.2.3 Acetylcholine-mediated vasodilation

Data were available from eleven subjects during the placebo phase and thirteen subjects during the dexamethasone phase. There was no difference in the $pD_2$ of acetylcholine-mediated vasodilation between phases, as shown in figure 6.6 ($pD_2$ for acetylcholine mediated vasodilation during placebo 6.87±0.09 vs. 7.04±0.11 during dexamethasone, p=0.5). In addition, on comparing the ten volunteers with data from both phases, again no difference in acetylcholine-mediated vasodilation was observed.

### 6.3.2.4 Insulin-mediated attenuation of NE induced vasoconstriction

i) placebo phase

Figures 6.7.1-3 and table 6.3 demonstrate the effect of pre-incubation of vessels with insulin on NE-mediated vasoconstriction during the placebo phase. Vessels were pre-incubated with insulin 1nM (Figure 6.7.1), 100pM (Figure 6.7.2) and 10pM (Figure 6.7.3). A significant rightward shift in the NE concentration response curve (reduction in $pD_2$) was seen at insulin concentrations of 100pM and 10pM. No significant change in $pD_2$ was seen in association with insulin 1nM. In response to insulin 1nM and 100pM, a significant reduction in the calculated maximum active effective pressure was observed. No change in maximum active effective pressure was observed in response to insulin 10pM.

ii) dexamethasone phase

Figures 6.8.1-3 and table 6.3 demonstrate the effect of pre-incubation of vessels with insulin on NE-mediated vasoconstriction during the dexamethasone treatment phase. Vessels were pre-incubated with insulin 1nM (Figure 6.8.1), 100pM (Figure 6.8.2) and 10pM (Figure 6.8.3). A significant rightward shift in the NE concentration response curve was seen at insulin concentrations of 1nM and 100pM. In addition, a significant reduction in calculated maximum active effective pressure was observed at insulin concentrations of

1nM and 100pM. There was no significant effect on the $pD_2$ or maximum active effective pressure in response to insulin 10pM.

### 6.3.2.5 Comparison of insulin action during dexamethasone and placebo phases

Figures 6.9.1-2 and table 6.4 illustrate the results of NE concentration response curve and corresponding insulin pre-treated NE concentration response curve during placebo and dexamethasone phases for 1nM (n=11) and 100pM (n=10) insulin. In both of these graphs, only data from volunteers with two sets of myography results (i.e. on placebo and dexamethasone treatment phases) were included. Importantly, there was no difference in the $pD_2$ or maximum active effective pressure for the baseline NE concentration response curves when placebo and dexamethasone phases were compared. Similarly, no between phase difference in $pD_2$ or calculated maximum active effective pressure was seen at either insulin dose. These data are also represented in figure 6.10.1-4, comparing the $pD_2$ and calculated maximum active effective pressure between phases after incubation with insulin 1nM (n=11) and 100pM (n=10). The data from 10pM insulin were not analysed due to low numbers of matched results. To absolutely exclude a confounding effect of dexamethasone on the NE concentration response curve between phases, after correcting for the corresponding NE concentration response curve by expressing $pD_2$ ins/ $pD_2$ baseline and maximum active effective pressure ins/maximum active effective pressure baseline for each individual, still no effect of dexamethasone was observed.

### 6.3.2.6 Insulin-mediated vasodilation-the effect of nitric oxide synthase inhibition

Figures 6.11.1-3 and 6.12.1-2, and table 6.5, demonstrate, during placebo and dexamethasone phases respectively, the effect of L-NMMA pre-incubation on insulin-mediated attenuation of NE induced vasoconstriction. Numbers are lower due to some

protocols being abandoned at a stage where vessels were clearly non-viable, limiting conclusions that can be drawn from these data. Despite this, the impression is that inhibition of the endothelial isoform of nitric oxide synthase abrogates most of the insulin effect during both phases. During both placebo and dexamethasone phases, L-NMMA had a significant effect on insulin action (figures 6.11.1-3 and 6.12.1-2, table 6.5)

**Figure 6.2: Insulin sensitivity after placebo and dexamethasone phases.**

Insulin sensitivity after placebo and dexamethasone study phases was measured and compared using the euglycaemic hyperinsulinaemic clamp. There was a significant reduction in insulin sensitivity after six days of dexamethasone exposure compared with placebo ($p<0.001$). Values shown illustrate the mean for each phase.

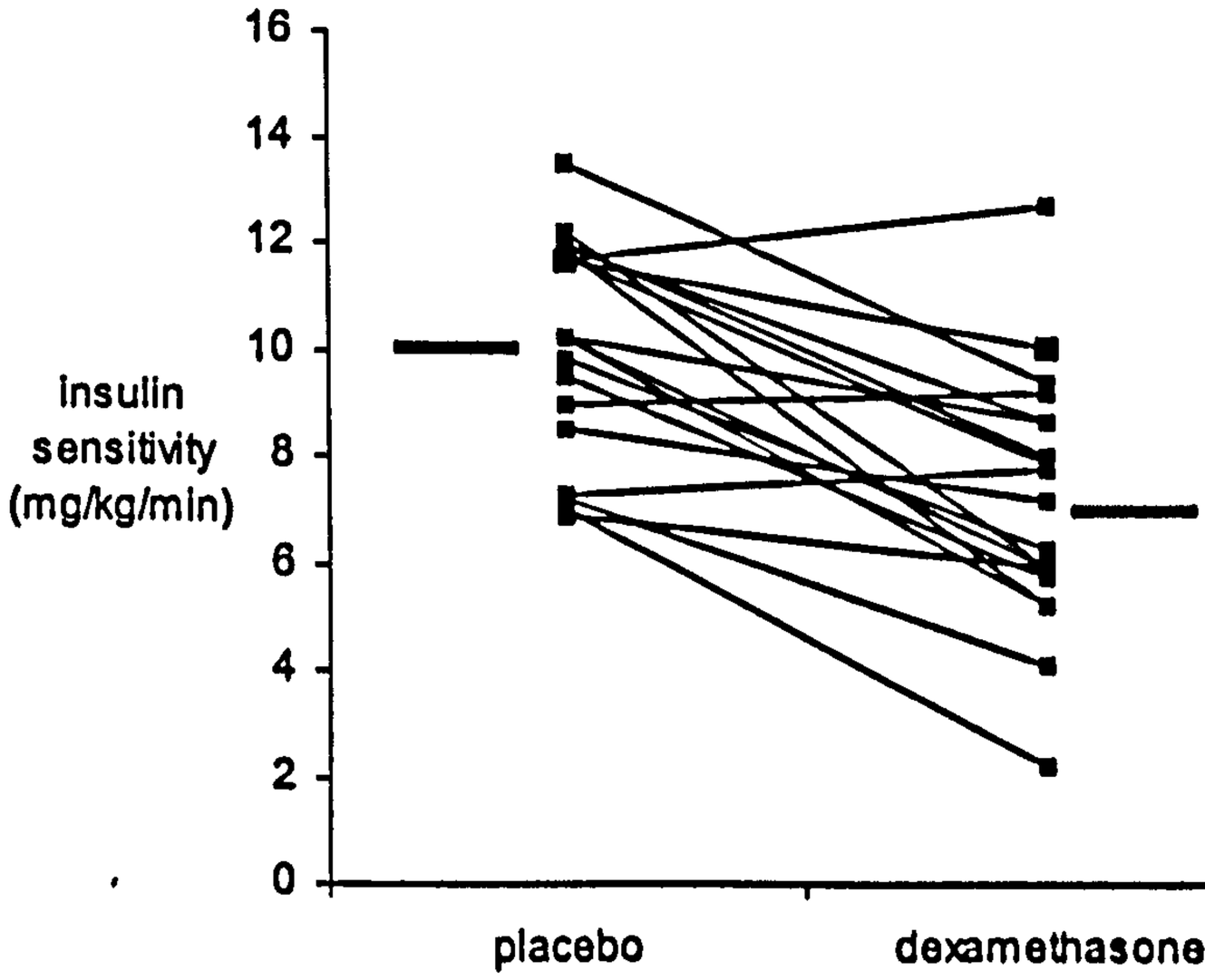


**Figure 6.3: Serum insulin during the euglycaemic hyperinsulinaemic clamp**

Fasting and steady-state serum insulin concentrations during euglycaemic hyperinsulinaemic clamp were compared. Fasting insulin was significantly greater during the dexamethasone study phase ($p<0.05$), however serum insulin was no different at any point during the clamp, and in particular during the final 40 minutes. Error bars represent standard errors.

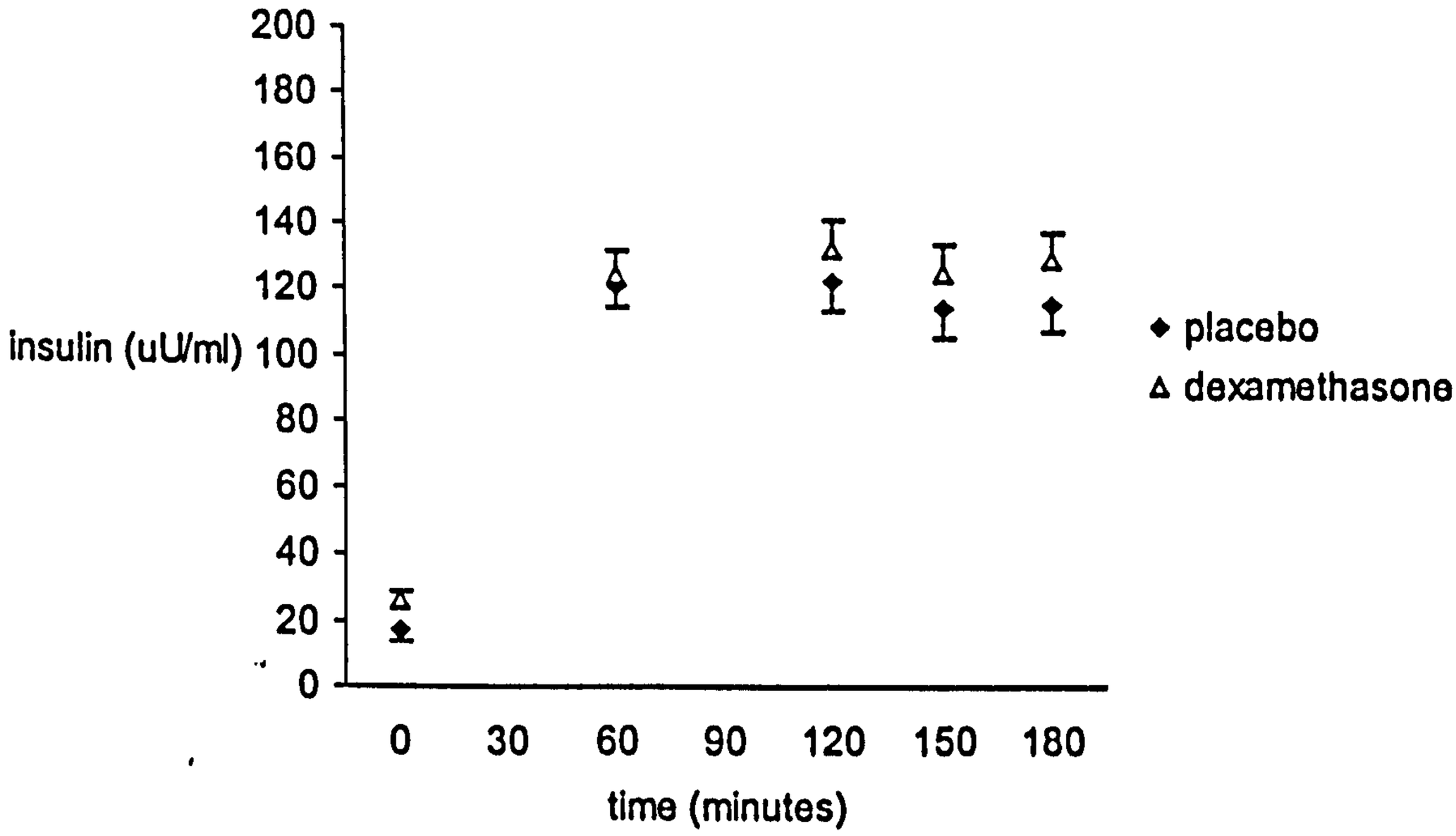


**Figure 6.4: The effect of time and norepinephrine-induced vasoconstriction.**

The effect of time on NE induced vasoconstriction in human resistance vessels was compared during placebo and dexamethasone phases ($p=0.91$ for comparison of $pD_2$, n=17 vessels). Error bars represent standard errors.

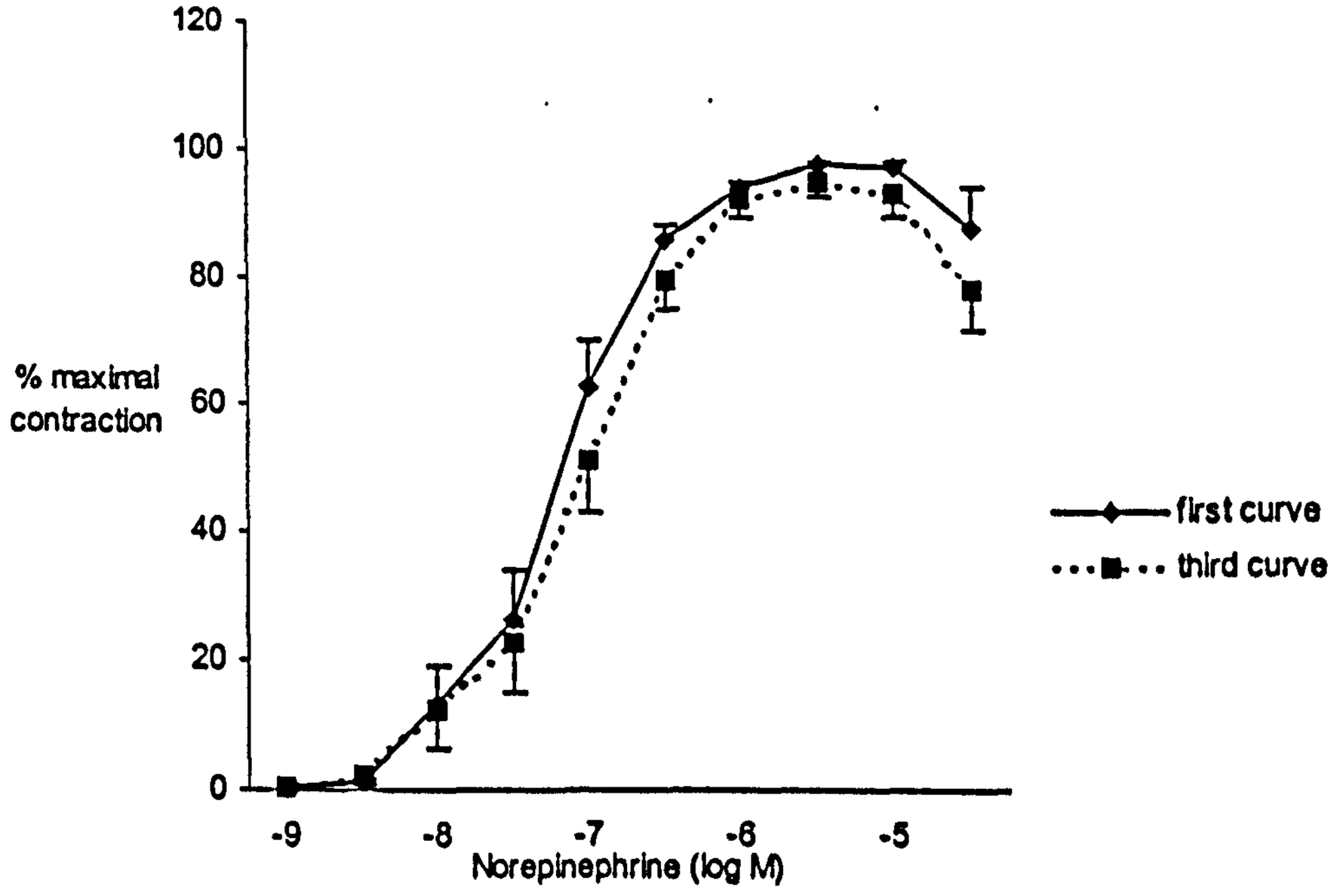


**Figure 6.5: Norepinephrine-induced vasoconstriction during placebo and dexamethasone.**

NE induced vasoconstriction in human resistance vessels (n=32) dissected from subjects (n=12) after five days of dexamethasone was compared with that after an equivalent period of matched placebo. No statistically significant difference in the $pD_2$ for NE induced vasoconstriction was observed (p=0.1). Error bars represent standard errors.

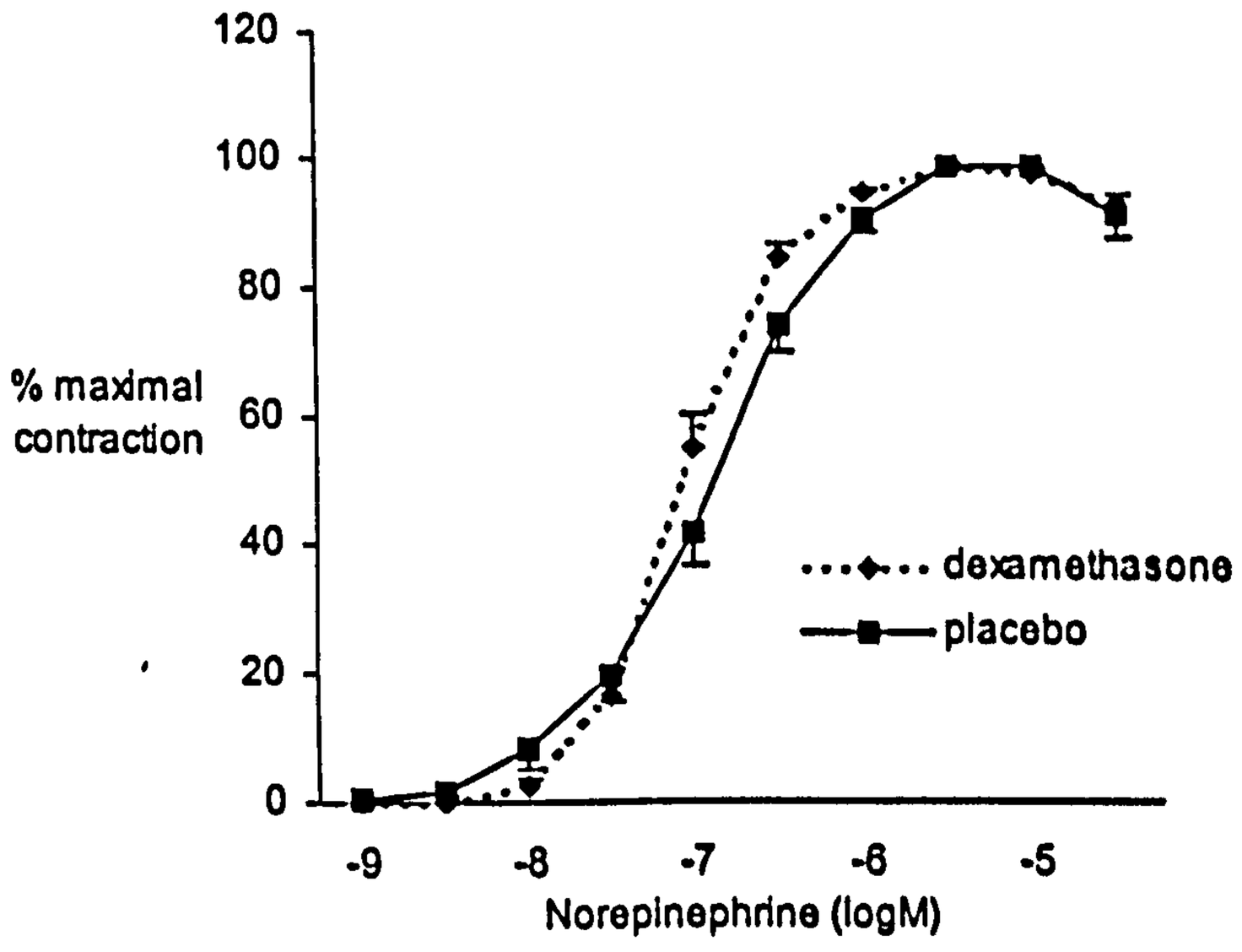


**Figure 6.6: Acetylcholine-mediated vasodilation during dexamethasone and placebo.**

Acetylcholine-mediated vasorelaxation in human resistance vessels was compared during placebo (n=11) (complete line) and dexamethasone (n=13) (interrupted line). Values are expressed as a percentage of the contraction generated by incubation with norepinephrine 10μM. No difference in the $pD_2$ of acetylcholine action was observed between phases. Error bars represent standard errors.

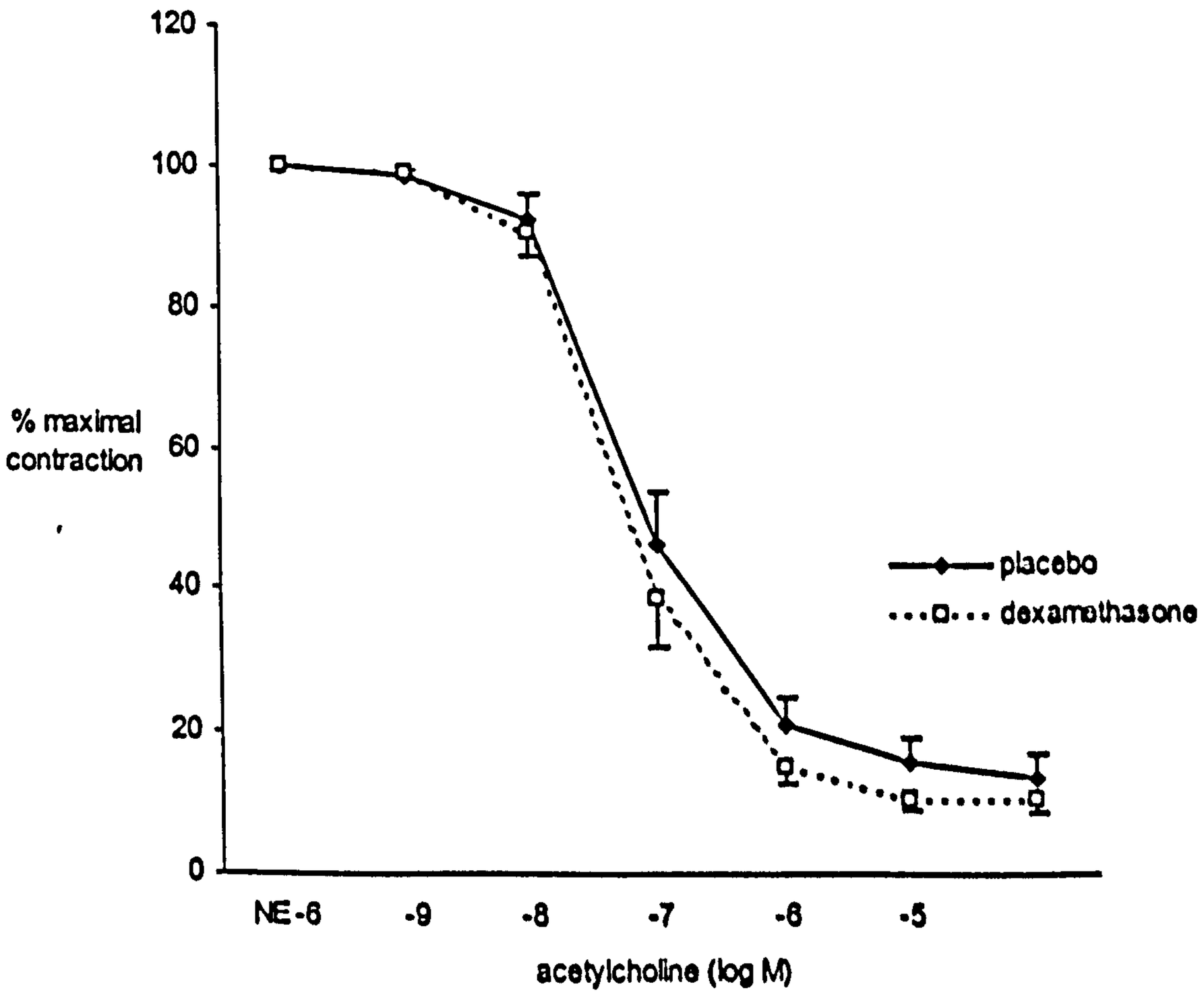


**Figure 6.7: The effect of insulin on norepinephrine-induced vasoconstriction during placebo phase.**

The effect of preincubation with insulin 1nM (n=12, figure 6.7.1), 100pM (n=13, figure 6.7.2) and 10pM n=6, figure 6.7.3) on NE induced vasoconstriction in human resistance vessels dissected during placebo phase was examined. A significant attenuation of the NE-induced vasoconstriction was seen at all three insulin concentrations, either in a change in $pD_2$ plus a reduction in calculated maximum active effective pressure (100pM) or as only a reduction in $pD_2$, (10pM) or maximum active effective pressure (1nM). Error bars represent standard errors.

**Figure 6.7.1**

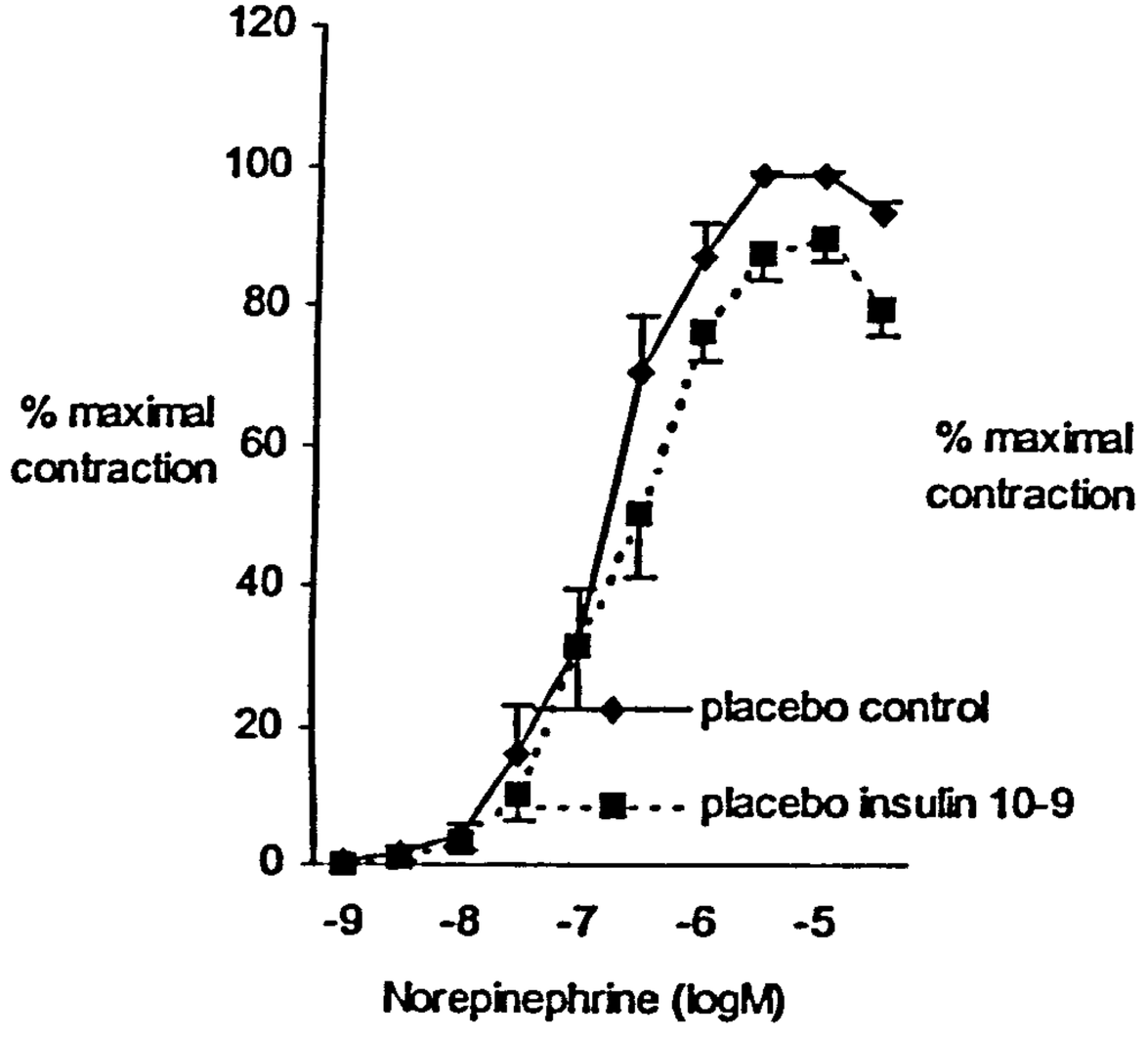



**Figure 6.7.2**

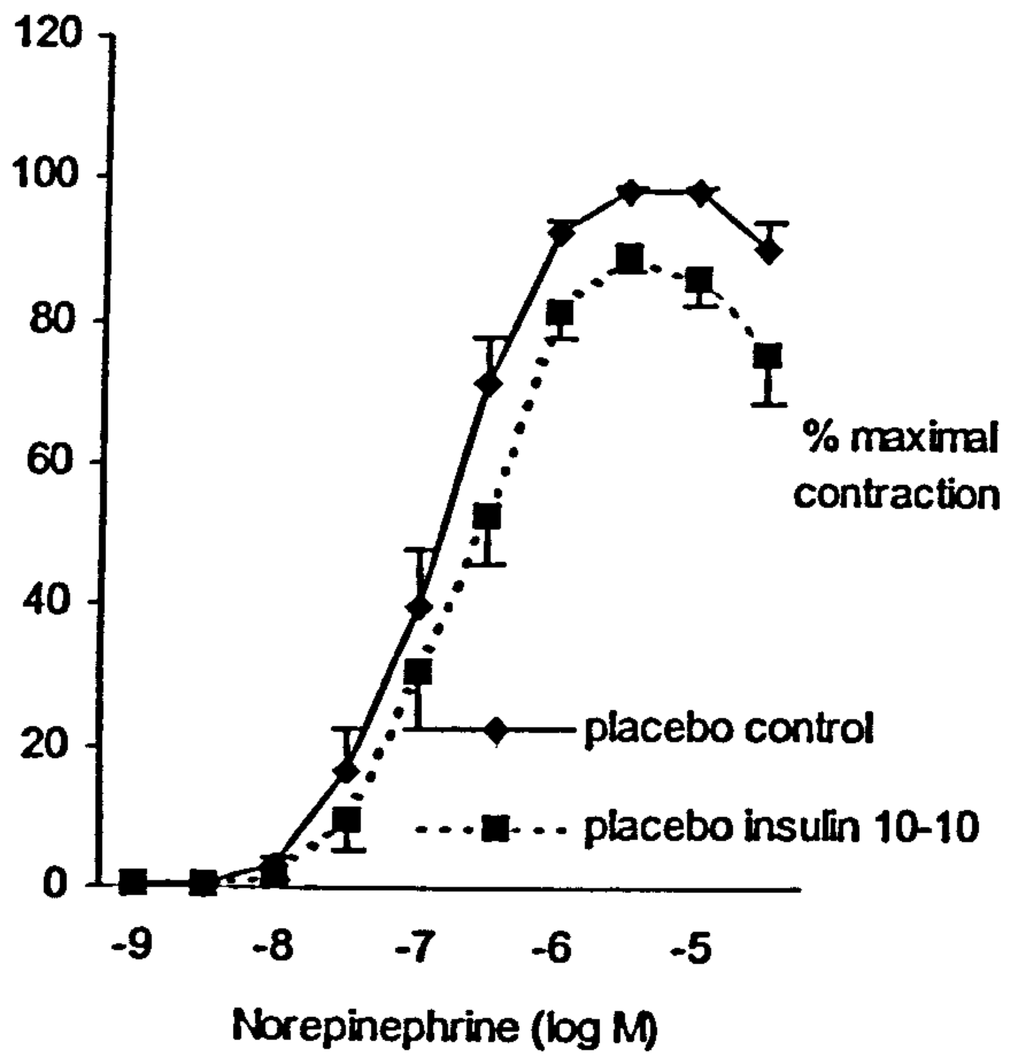



**Figure 6.7.3**

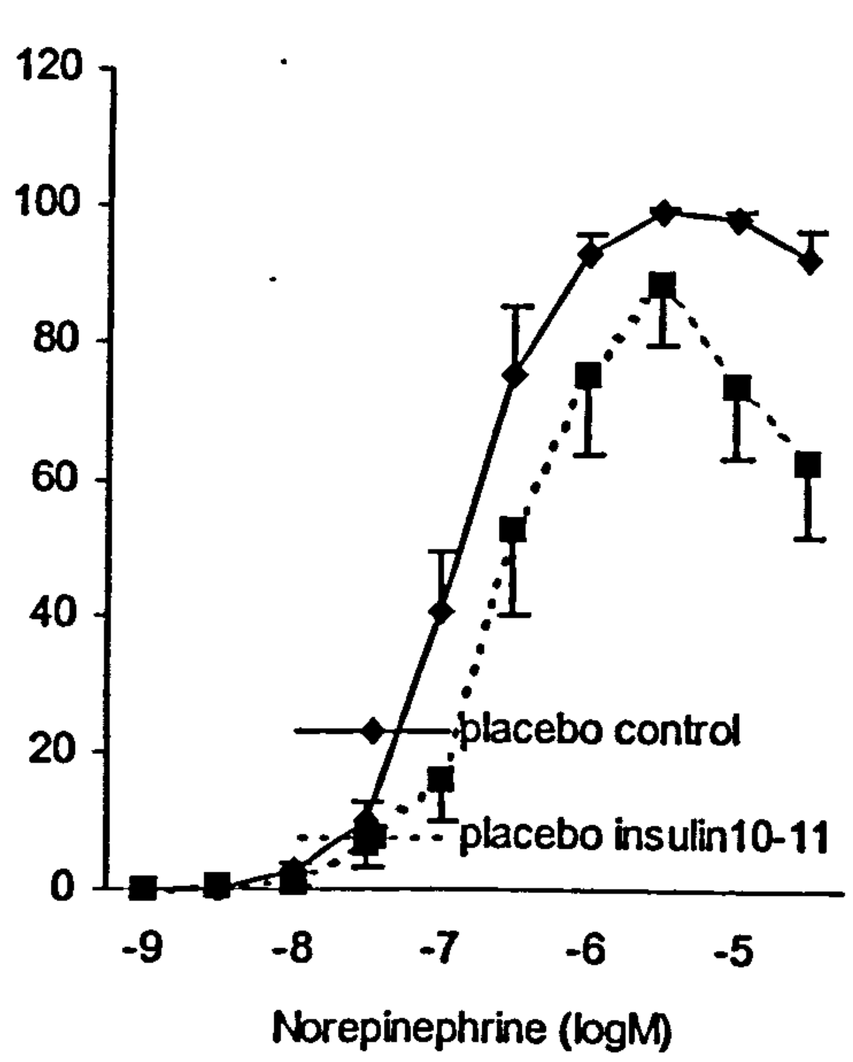


**Figure 6.8: The effect of insulin on norepinephrine-induced vasoconstriction during dexamethasone phase.**

The effect of preincubation with insulin 1nM n=14 (Figure 6.8.1), 100pM n=13 (Figure 6.8.2) and 10pM n=9 (Figure 6.8.3) on NE induced vasoconstriction in human resistance vessels dissected after five days of dexamethasone treatment was examined. A significant attenuation of the NE-induced vasoconstriction, measured as a change in $pD_2$ and a reduction in maximum active effective pressure was observed at insulin concentrations 1nM and 100pM ($p<0.05$ for $pD_2$ and maximum active effective pressure). No change in either parameter was observed in response to insulin 10pM ($p>0.05$ for $pD_2$ and maximum active effective pressure). Error bars represent standard errors.

**Figure 6.8.1**

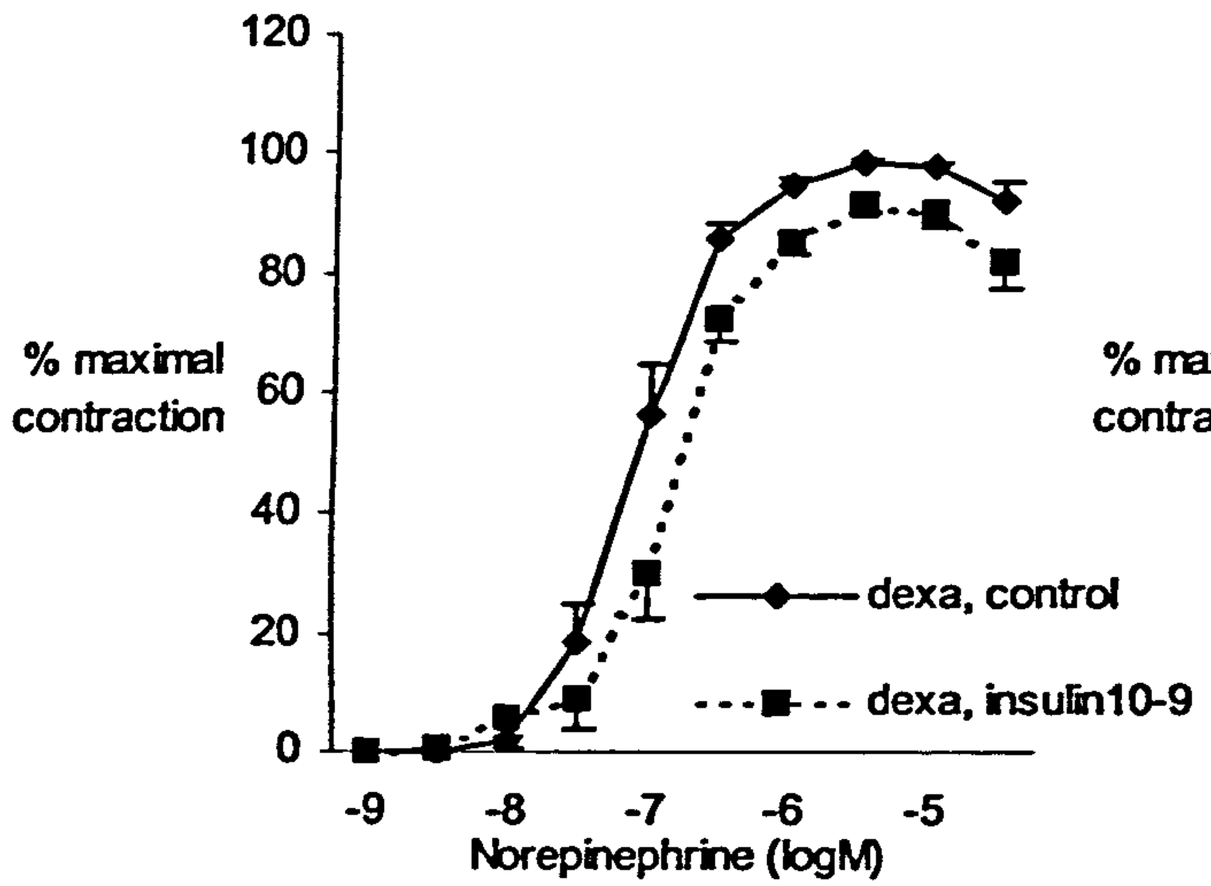



**Figure 6.8.2**

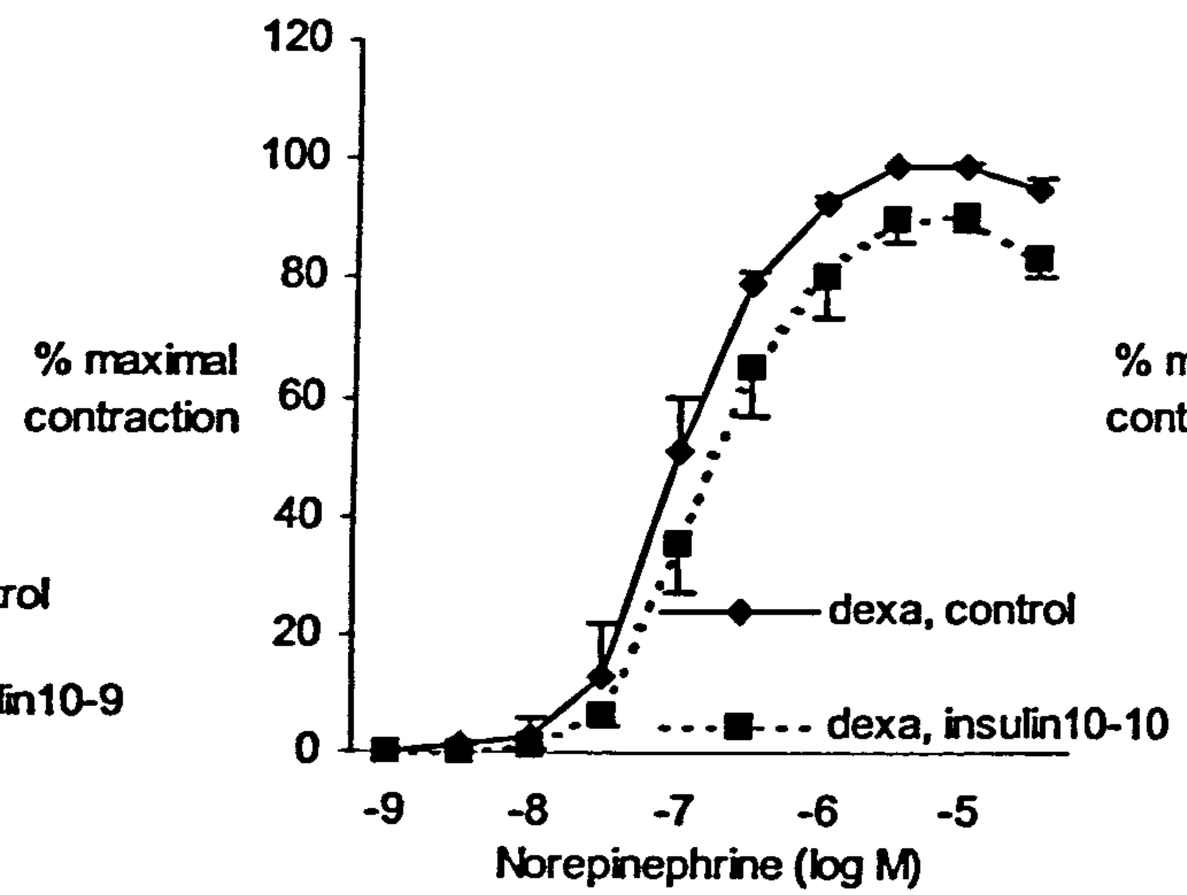



**Figure 6.8.3**

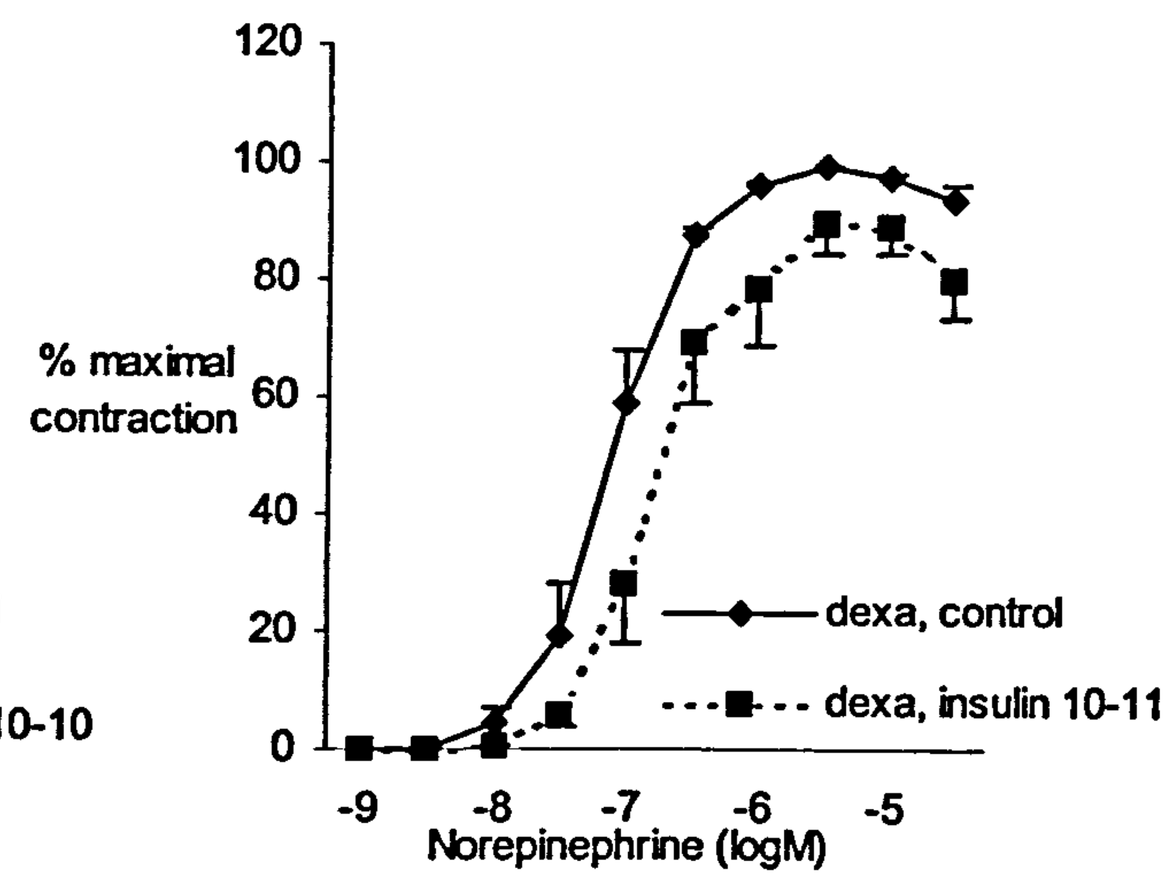


**Figure 6.9: Comparison of insulin action between placebo and dexamethasone phases.**

Insulin action, measured as the $pD_2$ in isolated human resistance vessels, was compared between placebo and dexamethasone phases. No difference ($p>0.05$) in $pD_2$ or in maximum active effective pressure was observed at either 1nM (n=11) (Figure 6.9.1) or 100pM (n=11) (Figure 6.9.2) insulin concentrations. Intermittent line = placebo (square = baseline NE concentration response curve, triangle = insulin pre-treatment), solid line = dexamethasone (square = baseline NE concentration response curve, triangle = insulin pre-treatment). Error bars represent standard errors.

**Figure 6.9.1**

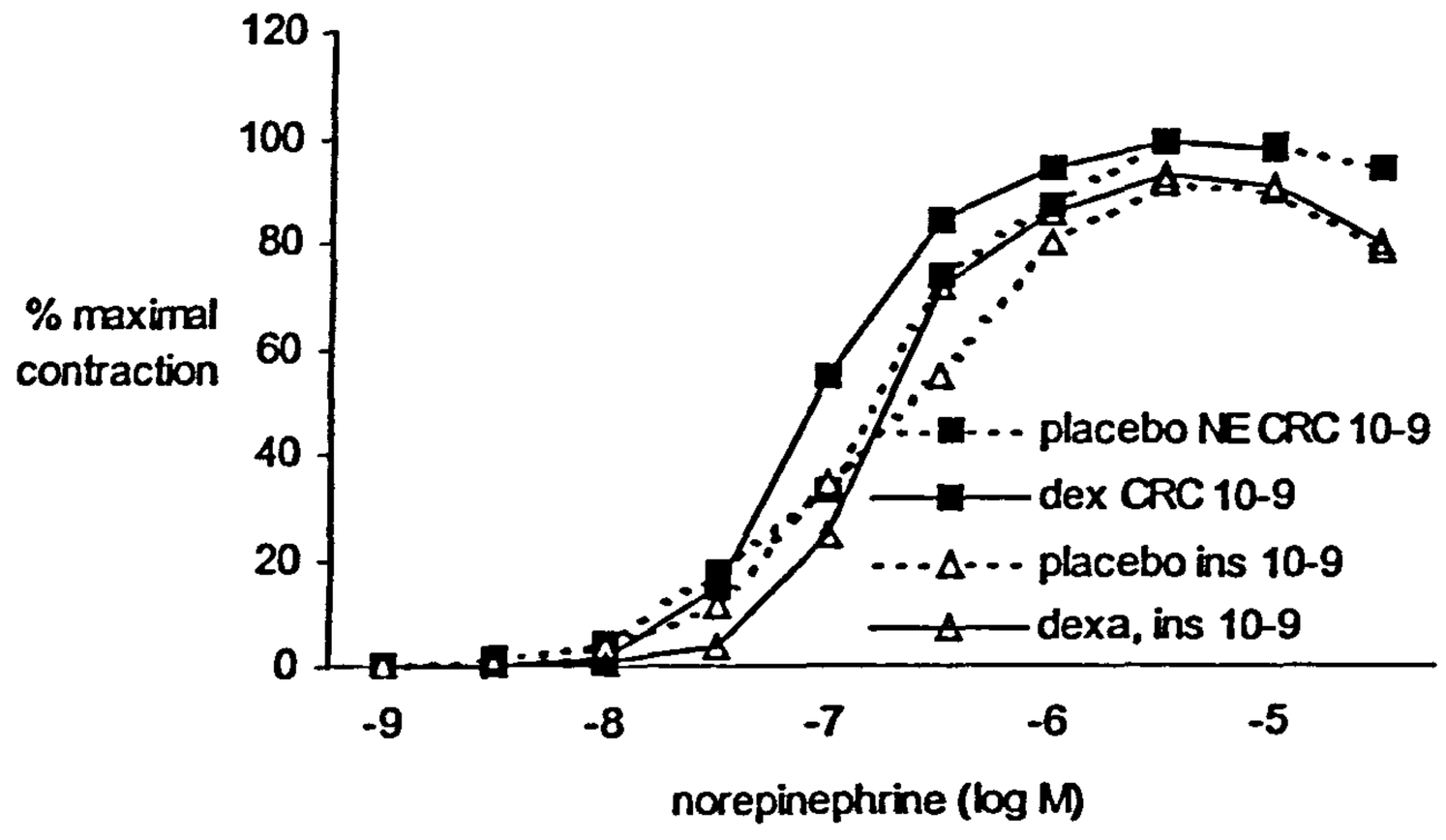



**Figure 6.9.2**

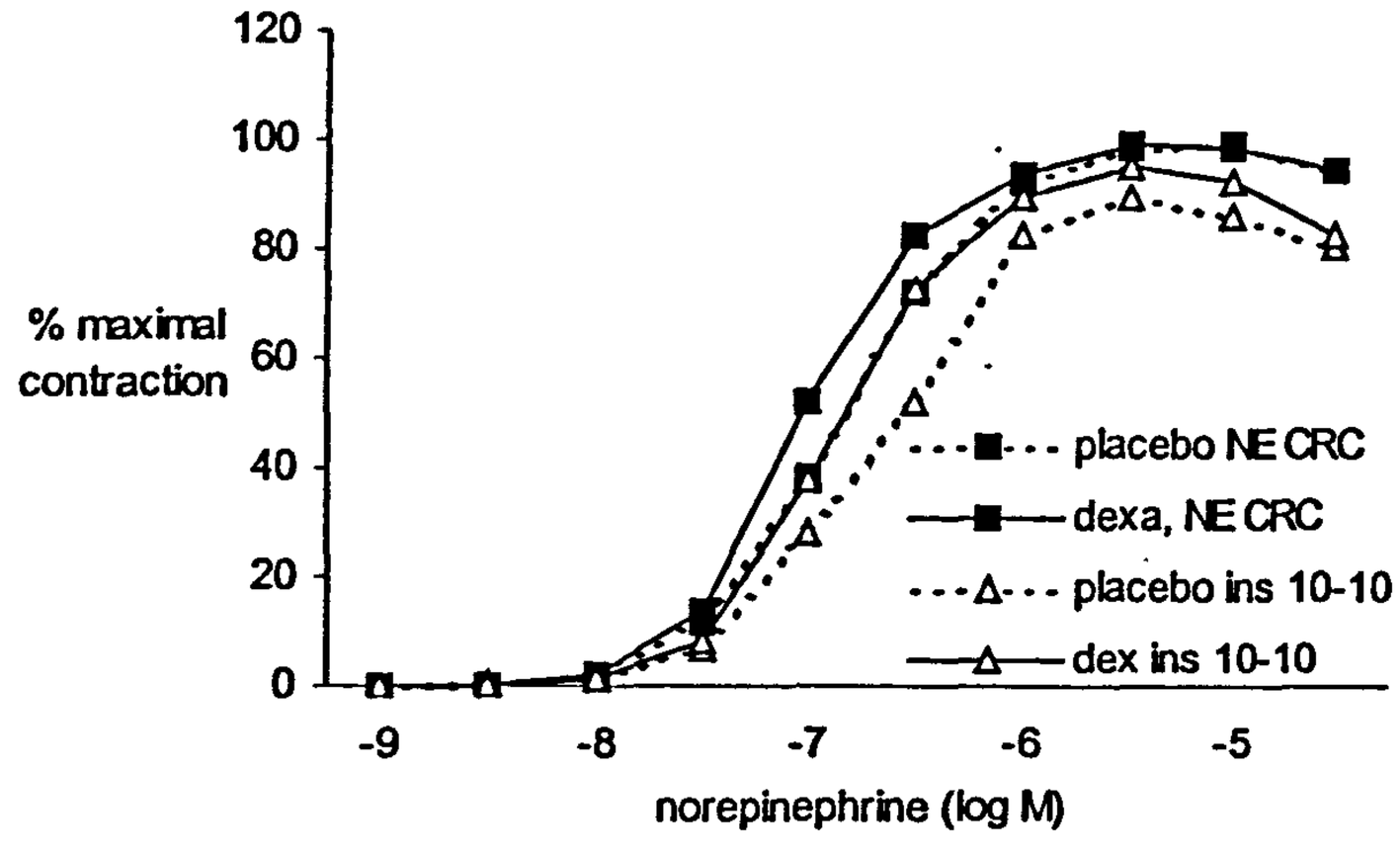


**Figure 6.10: Comparison of insulin action between placebo and dexamethasone phases (paired data)**

The $pD_2$ and maximum active effective pressure generated in response to a norepinephrine concentration response curve during placebo and dexamethasone, after incubation with 1nM (n=11) and 100pM (n=10) insulin, were compared in volunteers with myography data from both phases. Error bars represent standard errors.

**Figure 6.10.1** (p=0.75)

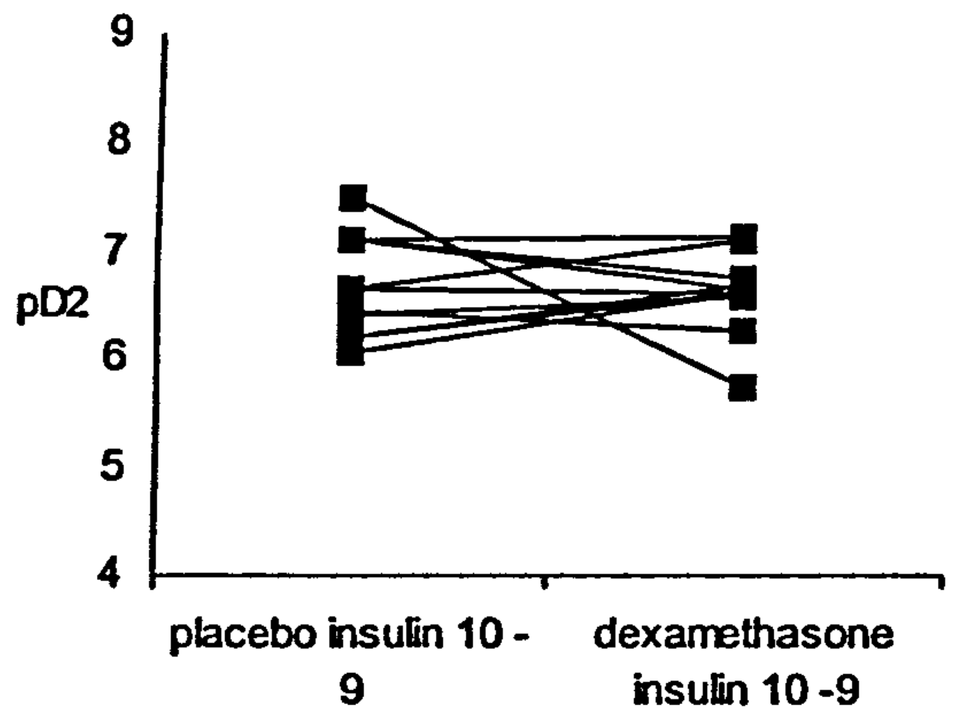



**Figure 6.10.2** (p=0.64)

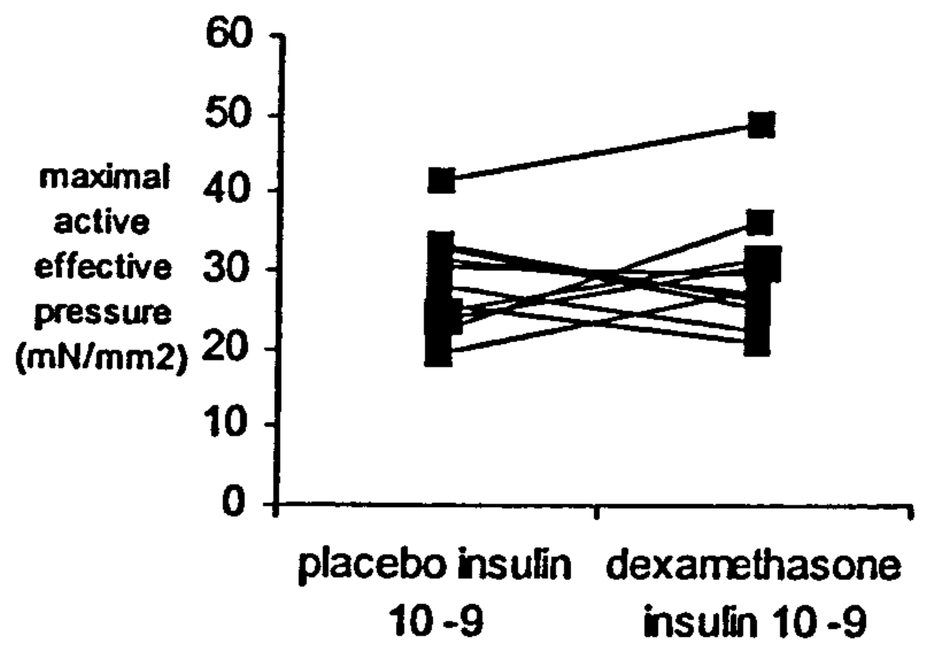



**Figure 6.10.3** (p=0.45)

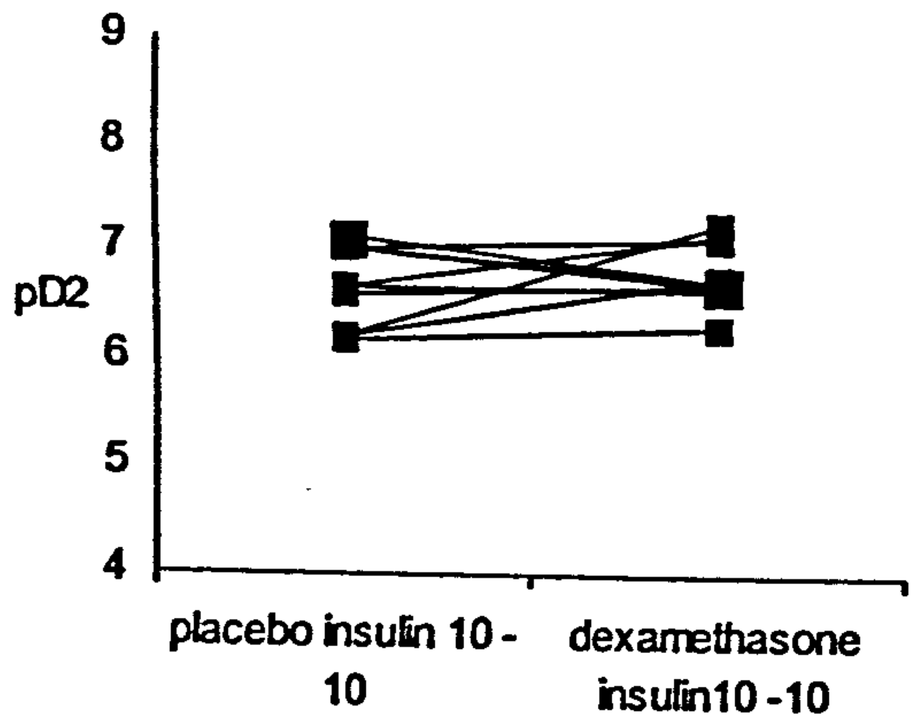



**Figure 6.10.4** (p=0.91)

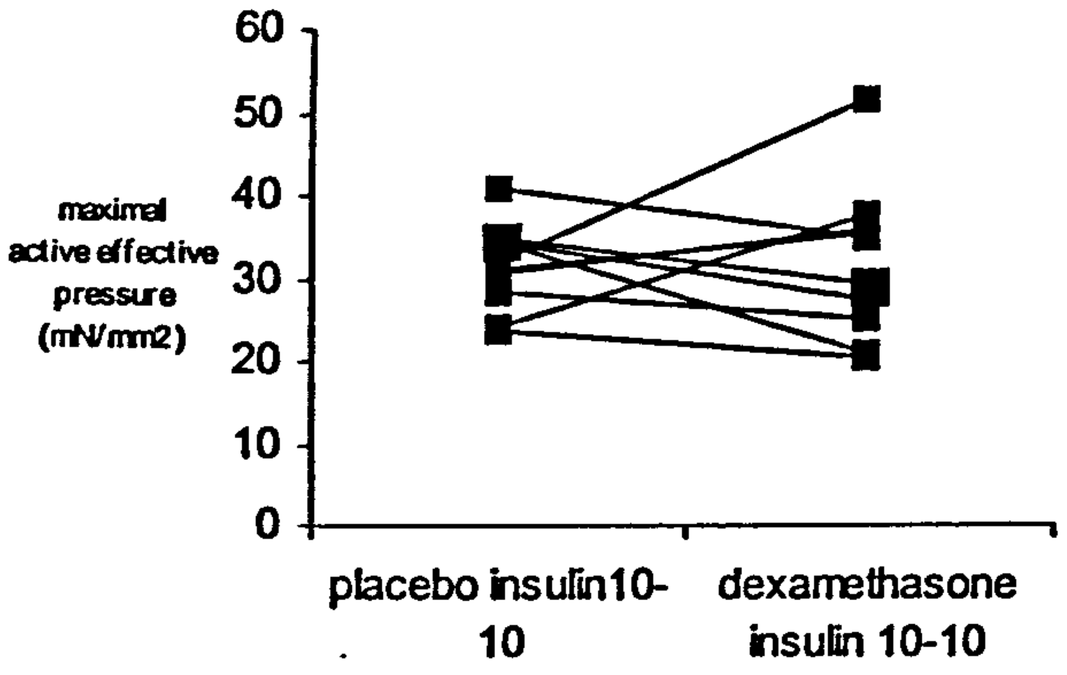


**Figure 6.11: The effect of L-NMMA on insulin-mediated attenuation of NE induced vasoconstriction during placebo**

The effect of L-NMMA on insulin mediated-attenuation of NE induced vasoconstriction during placebo was examined. Insulin concentrations a) 1nM (n=8, $p<0.05$ for effect of L-NMMA+ insulin+NE on maximum active effective pressure, p=ns for effect on $pD_2$ when compared with insulin+ NE) (Figure 6.11.1), b) 100pM (n=7, $p<0.05$ for effect of LNMMA+ insulin+NE on maximum active effective pressure, p=ns for effect on $pD_2$ when compared with insulin+ NE) (Figure 6.11.2) and c) 10pM (n=5, p=ns for effect of L-NMMA on $pD_2$ and on maximum active effective pressure compared with insulin + NE) (Figure 6.11.3) were examined (solid line with hollow circle=control NE concentration response curve, broken line with filled square = insulin + NE concentration response curve, solid line with filled square = L-NMMA + insulin + NE concentration response curve). Error bars represent standard errors.

**Figure 6.11.1**

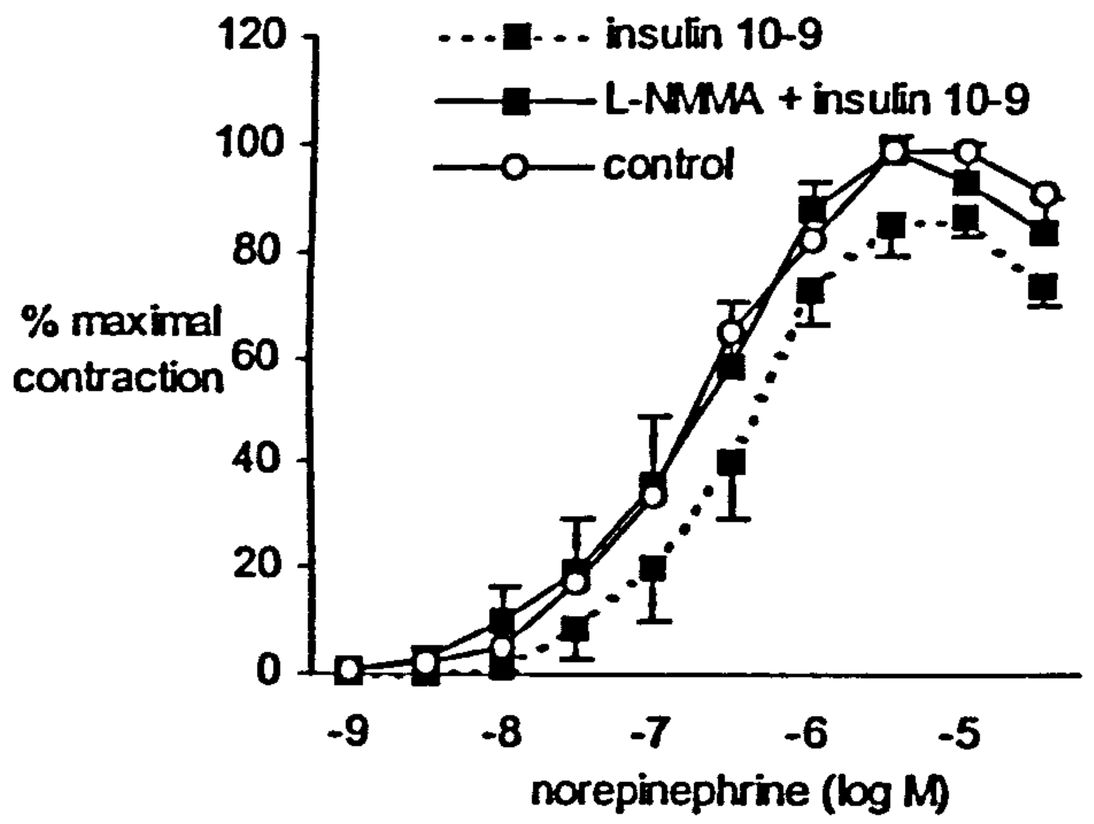



**Figure 6.11.2**

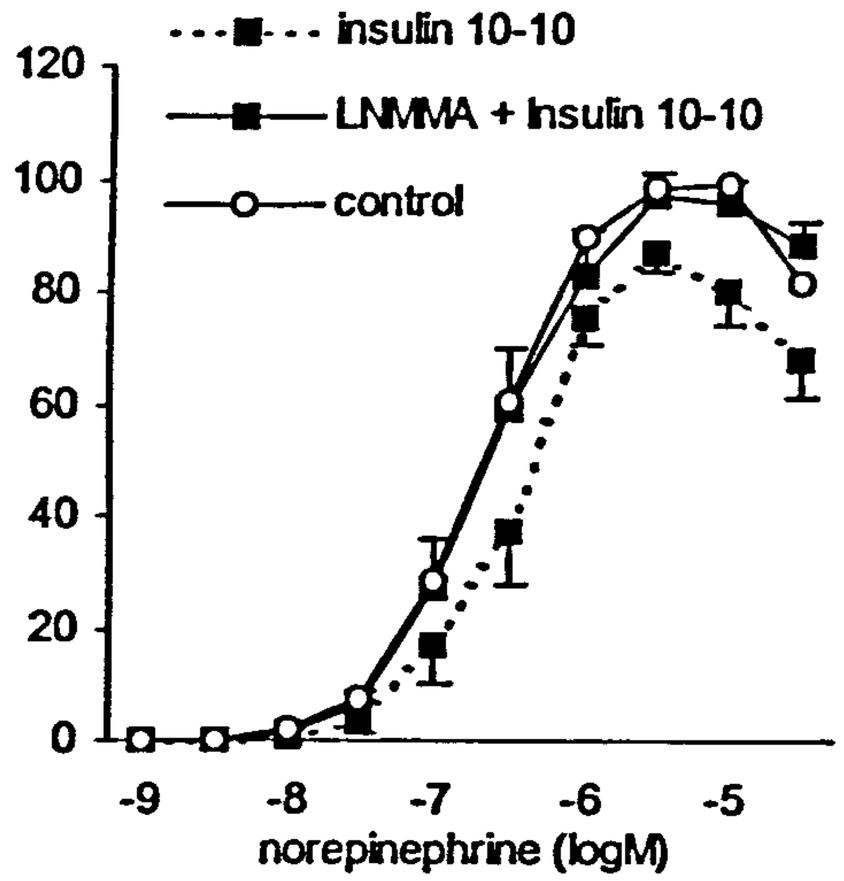



**Figure 6.11.3**

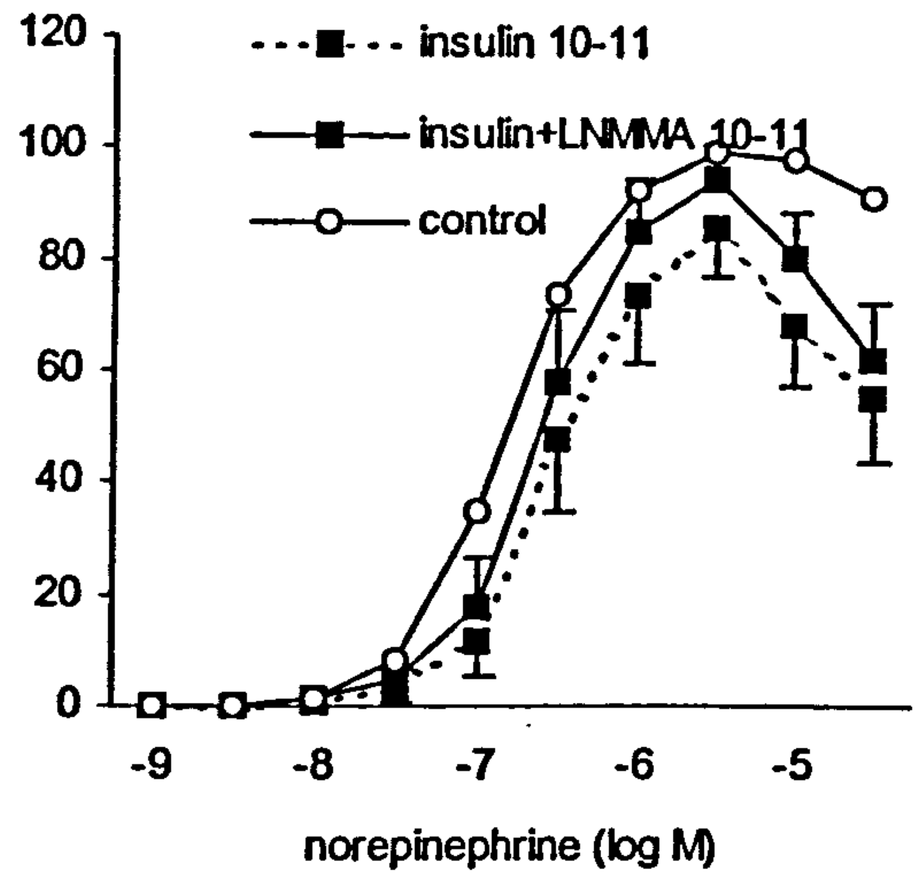


**Figure 6.12: The effect of L-NMMA on insulin-mediated attenuation of NE induced vasoconstriction during dexamethasone**

The effect of L-NMMA on insulin mediated-attenuation of NE induced vasoconstriction during dexamethasone was examined. Insulin concentrations a) 1nM (n=8, p=0.024 for effect of L-NMMA+ insulin+NE on $pD_2$, p=0.4 for effect on maximum active effective pressure when compared with insulin+ NE) (Figure 6.12.1), b) 100pM (n=8, p=0.14 for effect of L-NMMA+ insulin + NE on maximum active effective pressure, p=0.14 for effect on $pD_2$ when compared with insulin+ NE) (Figure 6.12.2) were examined (solid line with hollow circle=control NE concentration response curve, broken line with filled square = insulin + NE concentration response curve, solid line with filled square = L-NMMA + insulin + NE concentration response curve). Error bars represent standard errors.

**Figure 6.12.**

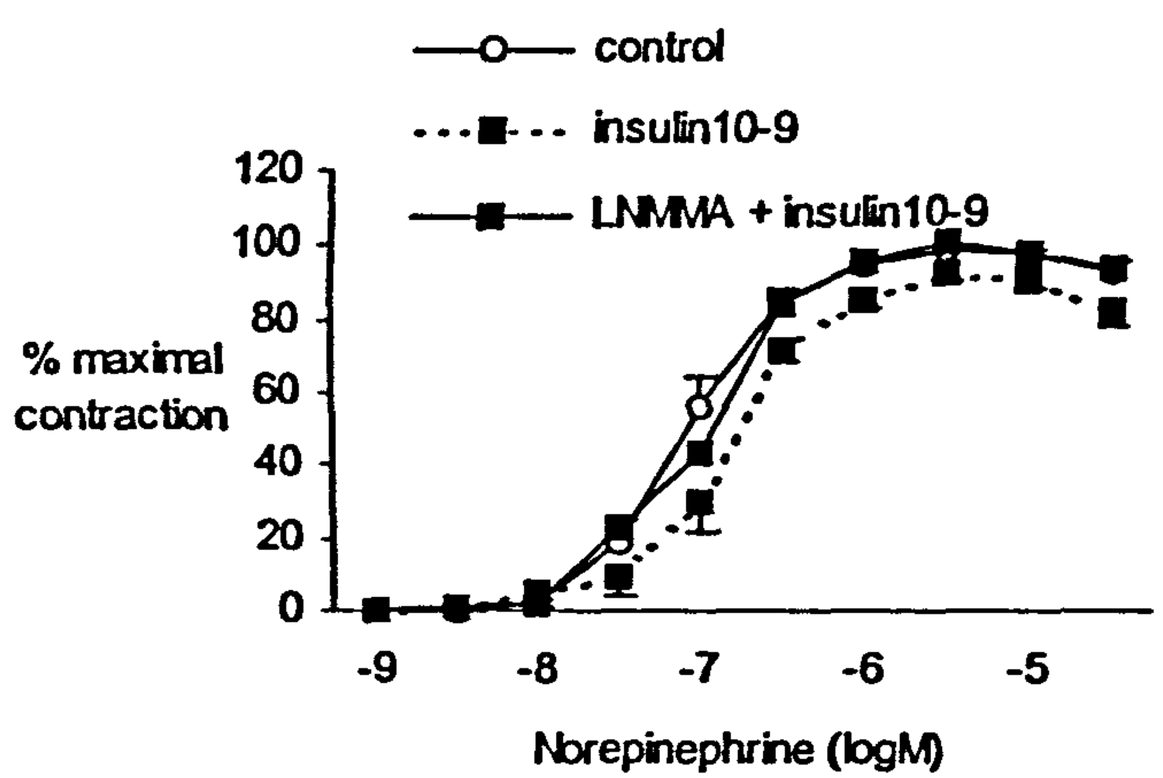



**Figure 6.12.2**

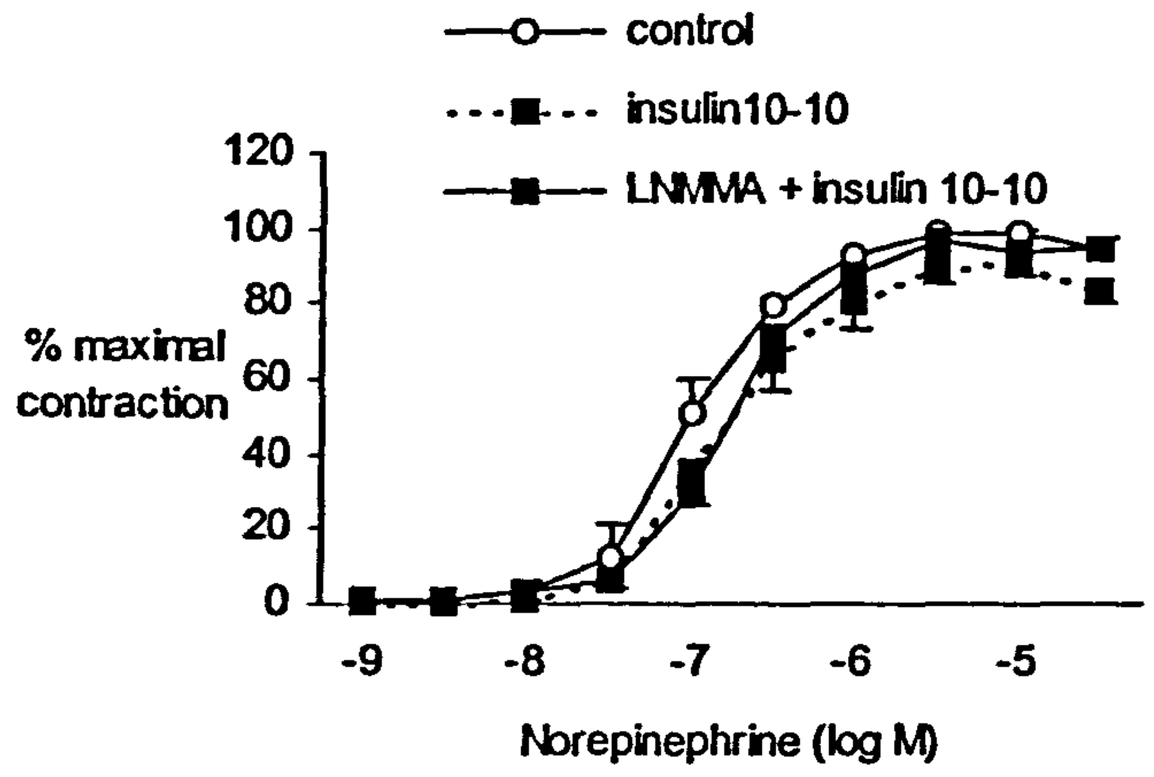


**Table 6.2: Metabolic characteristics of subjects during both study phases**

All values are expressed as mean and one standard deviation

| | Placebo | Dexamethasone | p-value (paired t-test) |
|---|---|---|---|
| **Weight (kg)** | 76.0±9 | 76.7±9 | 0.18 |
| **Systolic blood pressure (mmHg)** | 120.6±10 | 120.3±14 | 0.92 |
| **Diastolic blood pressure (mmHg)** | 60±9 | 61.7±9 | 0.45 |
| **Fasting insulin (μU/ml)** | 17.4±9 | 26.1±11 | 0.001 |
| **Insulin sensitivity (M-mg/kg/min)** | 10.13±2 | 7.2±2 | <0.001 |
| **Urinary free cortisol (/mol creatinine)** | 6.8 ±4 | 0.33 ± 0.3 | <0.001 |
| **Serum cortisol (nmol/l)** | 322.5 (median) | <27 (median) | |
| **Total cholesterol (mmol/l)** | 4.0± 0.9 | 4.1± 0.9 | 0.22 |
| **Triglycerides (mmol/l)** | 1.0± 0.7 | 1.2± 0.8 | 0.04 |
| **HDL cholesterol (mmol/l)** | 1.13± 0.2 | 1.3± 0.2 | 0.003 |

**Table 6.3: Insulin-mediated vasodilation in human resistance vessels during placebo and dexamethasone phases**

Errors represent standard error of the mean

| | **Placebo** | | | | **Dexamethasone** | | | |
|---|---|---|---|---|---|---|---|---|
| | *$pD_2$* | *p value vs control* | *Maximum active effective pressure* | *p value vs control* | *$pD_2$* | *p value vs control* | *Maximum active effective pressure* | *p value vs control* |
| **Control** | 6.8±0.1 | | 31.1±1.6 | | 7.1±0.1 | | 31.3±2.4 | |
| **Insulin $10^{-9}$** | 6.6±0.4 | 0.15 | 28.7±1.6 | <0.01 | 6.7±0.2 | 0.02 | 29.2±2 | 0.02 |
| | | | | | | | | |
| **Control** | 6.8±0.1 | | 33.8±1.2 | | 6.9±0.1 | | 33.3±2.6 | |
| **Insulin $10^{-10}$** | 6.6±0.1 | <0.01 | 30.7±1.5 | <0.001 | 6.7±0.1 | 0.01 | 31.1±2.6 | 0.02 |
| | | | | | | | | |
| **Control** | 6.8±0.1 | | 36.8±5.3 | | 7.1±0.1 | | 33.5±4.2 | |
| **Insulin $10^{-11}$** | 6.4±0.1 | <0.01 | 32.8±5.1 | 0.21 | 6.6±0.2 | 0.1 | 31.5±3.8 | 0.08 |

**Table 6.4: Comparison of insulin-mediated vasodilation during placebo and dexamethasone phases**

Errors represent standard error of the mean.

| | Parameter | Placebo | Dexamethasone | p value |
|---|---|---|---|---|
| **Insulin $10^{-9}$** | $pD_2$<br>Maximum active effective pressure | 6.6±0.1<br>28.7±2 | 6.7±0.1<br>29±2 | 0.75<br>0.64 |
| | | | | |
| **Insulin $10^{-10}$** | $pD_2$<br>Maximum active effective pressure | 6.6±0.1<br>30.7±2 | 6.7±0.1<br>31.4±1 | 0.45<br>0.91 |

**Table 6.5: The effect of L-NMMA on insulin-mediated vasodilation during placebo and dexamethasone phases**

Errors represent standard error of the mean

| | Placebo | | | | Dexamethasone | | | |
|---|---|---|---|---|---|---|---|---|
| | *$pD_2$* | *p value* | *Maximum active effective pressure* | *p value* | *$pD_2$* | *p value* | *Maximum active effective pressure* | *p value* |
| **NE + insulin$10^{-9}$** | 6.5±0.2 | 0.2 | 28±2.5 | 0.04 | 6.6±0.1 | 0.02 | 30.1±3 | 0.4 |
| **NE + insulin$10^{-9}$ + L-NMMA** | 6.8±0.2 | | 31±3 | | 7.0±0.2 | | 31.7±3.4 | |
| | | | | | | | | |
| **NE + insulin$10^{-10}$** | 6.4±0.1 | 0.08 | 29.9±3 | <0.01 | 6.5±0.1 | 0.14 | 36±3 | 0.31 |
| **NE + insulin$10^{-10}$ + L-NMMA** | 6.6±0.1 | | 33±3 | | 6.7±0.1 | | 37±3 | |
| | | | | | | | | |
| **NE + insulin$10^{-11}$** | 6.4±0.1 | 0.2 | 34±3 | 0.4 | | | | |
| **NE + insulin$10^{-11}$ + L-NMMA** | 6.6±0.1 | | 36±2 | | | | | |

## 6.4 Discussion

In this chapter I have demonstrated that six days of dexamethasone treatment was associated with a 29% reduction in metabolic insulin sensitivity, measured by the euglycaemic hyperinsulinaemic clamp. Despite this, there was no associated reduction in insulin action in small resistance arteries, measured using wire myography. Thus metabolic and vascular insulin responses, in this model, do not appear to be coupled. This observation may be of importance in unravelling the relationship between insulin's metabolic and vascular actions.

That glucocorticoids reduce insulin sensitivity is not a novel observation, though the underlying mechanisms are unclear. Rizza *et al* (325) suggested that the defect lies in both peripheral and hepatic insulin action, and is located downstream of the insulin receptor. Candidate "hepatic" mechanisms include an increase in phosphoenolpyruvate carboxykinase (PEP-CK) activity, the rate-limiting step in hepatic gluconeogenesis(326), a reduction in pancreatic $\beta$-cell insulin secretion (327) and an increase in $\alpha$-cell glucagon secretion (328). The defect in "peripheral" tissues may be secondary to an increase in circulating free fatty acids, or a more direct cellular effect on insulin binding at the plasma membrane (330), (331) or the insulin-signalling cascade (335) (370). It has also been proposed by several groups that the reduction in insulin–stimulated glucose transport is secondary to abnormalities of GLUT4, either in its amount (338) or distribution (336), (337), (371) within the cell.

Rooney and colleagues (326) confirmed that a 28-hour cortisol infusion reduced both hepatic and peripheral insulin sensitivity by nearly 50%. Similarly, Paquot *et al* (329) observed a 51.5% reduction in glucose uptake in healthy volunteers during a

hyperinsulinaemic euglycaemic clamp, and a 54% reduction in glucose oxidation after 2 days of dexamethasone 1mg bd. In the present study, a 29% reduction in peripheral insulin sensitivity was observed, in association with an elevation in fasting insulin. The reason for this more modest reduction is unclear; compliance with study medication was good as evidenced by all volunteers reducing serum cortisol to <27nmol/l and suppressing urinary free cortisol during the dexamethasone phase. It may be of relevance that the population studied consisted of young healthy volunteers who were largely insulin sensitive.

Clearly there was no attempt in this work to identify the mechanism responsible for the reduction in insulin sensitivity, however it is of note that there was no change in either body weight or blood pressure between phases.

There is good evidence to support a relationship between insulin sensitivity and lipid profile. In conditions associated with insulin resistance such as obesity and type 2 diabetes, insulin resistance is associated with elevated serum triglycerides and reduced HDL-cholesterol, while even in the healthy population, there is a continuous relationship between insulin resistance and triglycerides, and an inverse relationship with HDL-cholesterol. As such, the observation that dexamethasone exposure was associated with insulin resistance, an increase in triglycerides and an *increase* in HDL-cholesterol is intriguing. There is no obvious explanation for this elevation in HDL. This is most likely a direct effect of dexamethasone that has "overridden" the endogenous mechanism coupling insulin sensitivity and lipid profile. It may, of course, also be a chance observation. It is not in keeping with the notion that tissue corticosteroid levels may be involved in the development of the metabolic syndrome.

It is now accepted that insulin acts in the vasculature as an endothelium dependent vasodilator (described more fully in Chapter 1), the action of which is mediated at least partly by activation of the endothelial isoform of nitric oxide synthase. This process may be effectively inhibited by the addition of the stereospecific substrate inhibitor of eNOS - L-NMMA. Several authors have demonstrated a relationship between insulin resistance and endothelial function. Petrie (177) showed a correlation between metabolic insulin sensitivity and basal nitric oxide production (measured by the reduction in forearm blood flow following intra-arterial infusion of L-NMMA). Using similar techniques, Cleland *et al* (185) extended this observation across groups with varying phenotype, comparing three groups - healthy normotensives, non-diabetic hypertensives and type 2 diabetics - and found a relationship between metabolic insulin sensitivity and endothelial function on combining the data from all three groups. Thus, using these techniques, those with the lowest metabolic response to insulin seem also to have the lowest vascular insulin response. Observations in obese (176) hypertensive (180), and diabetic (176) cohorts have shown that acetylcholine-mediated vasodilation is also impaired, suggesting that a more generalised endothelial dysfunction is present in these metabolically insulin resistant groups.

Despite these observations, much remains unknown about the nature of this relationship, in particular the molecular mechanisms that mediate endothelial dysfunction and insulin resistance. The culture of human endothelial cells has led to a greater understanding of insulin action in vascular cells. Zeng *et al* (186) demonstrated an insulin-stimulated increase in NO release from cultured HUVECs, and found properties that were shared with the better-characterised insulin signal in muscle and adipose cells; insulin-stimulated release of NO was reduced by inhibition of PI3'-kinase. Similar findings were subsequently reported in bovine aortic endothelial cells (187), where insulin stimulated increases in

eNOS activity were sensitive to PI3'-kinase inhibitors. Furthermore, overexpression of insulin receptors into HUVECs (188) results in an increase in insulin stimulated NO production. Thus, insulin acts as an endothelium-dependent vasodilator, acting via the insulin receptor and activation of PI3'-kinase. Thus there are clear parallels with the insulin signal in metabolically active tissue, such as muscle and fat. This raises the possibility that there may a single proximal signalling defect in both vascular and metabolic cells which would account for two of the fundamental components of the insulin resistant phenotype- insulin resistance and vascular dysfunction. In keeping with this, in vascular tissue from animal models of insulin resistance (189), insulin stimulated tyrosine phosphorylation of the insulin receptor, IRS-1 and –2 is reduced.

Analysis of the myography data supports the role of insulin as an endothelium dependent vasodilator. Significant vasodilation was observed at physiological insulin concentrations during both placebo and dexamethasone phases. In addition, the effect of insulin was at least partially reversed by the preincubation of vessels with 1$\mu$M L-NMMA. The objective of this study was not to define the mechanism of insulin action in the vasculature; however, these data are in keeping with recent observations that while insulin action in partly NO mediated, there may be other endothelial relaxant factors that mediate insulin action in the vasculature; in a recent publication it was even suggested that the main vasorelaxant in resistance arteries is independent of nitric oxide(372). Indeed, the investigators demonstrated that only 20% of acetylcholine mediated vasodilation may be nitric oxide mediated, and proposed a considerable contribution from an endothelial dependent hyperpolarizing factor, possibly an arachidonic acid metabolite.

The primary hypothesis of this study was to determine whether the metabolic reduction in insulin sensitivity that may accompany dexamethasone treatment would be associated with a reduction in vascular insulin responsiveness. Clearly, if this were to be the case, this would be of fundamental importance in attempting to clarify the nature of the relationship between insulin response in vascular and metabolic tissue, as it would reinforce the coupling of the two variables, even after pharmacological manipulation, and would support a common mechanism. Despite the 29% reduction in metabolic insulin sensitivity associated with dexamethasone exposure, I demonstrated no reduction in insulin action in the vasculature, implying uncoupling of these two mechanisms. This may be explained by several possibilities. Firstly, previous studies examining insulin-mediated vasodilation have been undertaken *in vivo*, with vascular response to insulin measured using forearm plethysmography. While this technique also reflects small vessel responses, there are no data comparing these techniques; future studies need to address the correlation between measurements of insulin action in the vasculature, comparing wire myography with forearm plethysmography. In the only published study to have examined the vascular and metabolic effects of dexamethasone, Tappy and colleagues (373) showed that 48 hours of dexamethasone 2mg b.d. reduced metabolic insulin sensitivity, and, in association, demonstrated no increase in calf blood flow, measured by venous occlusion plethysmography, that would normally accompany the hyperinsulinaemic clamp. Mechanistically, it was suggested that the main metabolic defect was in glucose oxidation, and the main site of insulin resistance was peripheral rather than hepatic. As discussed above, the conflicting nature of these results with my own may reflect the different techniques used to measure increases in flow; it must also be considered that the phenomenon of dexamethasone action in the endothelium may be evanescent and so an effect may be missed in *ex vivo* models such as the one used in this chapter.

An effect of dexamethasone on vascular responsiveness was also considered as a confounding factor that may have masked differences in insulin sensitivity. No difference in either measurement of vessel contraction was observed between the NE concentration response curves during placebo and the dexamethasone phase, allowing the use of $pD_2$ and maximum active effective pressure to compare insulin action between phases.

This is one of the first studies to test the hypothesis that an exogenous stimulus can alter insulin response in more than one insulin responsive tissue. It may be that the coupling of endogenous insulin sensitivity is the result of a more complex interplay of circulating and tissue-based factors that is overwhelmed by the crude insult dealt to the metabolism by administering pharmacological glucocorticoid doses. Finally, it must be acknowledged that despite the clear vasodilatory effect of insulin in this vessel system, its effect was modest, and so subtle changes in vascular insulin response may not have been detected. The sensitivity of vessels to acetylcholine-mediated vasodilation was, however, also measured; again there was no difference between phases. Acetylcholine had a more marked vasodilatory action than insulin in these vessels, and as such, more minor changes in endothelial function would perhaps be more evident between phases in response to this vasodilator. This was not, however, the case.

By convention, myography studies comparing two groups have required groups of 16 subjects. While 20 subjects were recruited to this study, paired values of insulin action at a given dose were only available in 11 subjects. No difference was observed between phases, as was also the case on analysis of unpaired data (maximum n=14 on dexamethasone phase, n=13 on placebo p=ns for difference in $pD_2$ or maximum active effective pressure). Thus,

one criticism of the study is that it may be underpowered to detect a small difference in insulin action in the vasculature; in defence of this, the crossover design of the study does permit the analysis of paired data and as such increases statistical power.

The results of this trial are against the notion that cortisol excess underpins the insulin resistance syndrome based on the observations that vascular insulin resistance was not demonstrated and that the lipid profile did not change in the typical pattern associated with this syndrome. There is no doubt however that the metabolic effects of exogenous glucocorticoid are considerable, as encountered in patients with syndromes of cortisol excess.

In summary, glucocorticoid exposure for five days was associated with a reduction in metabolic, although not vascular insulin sensitivity, thus raising further questions about the nature of the vascular insulin resistance.

## Chapter 7: Conclusion

Insulin sensitivity, defined as a subnormal biological response to insulin at physiological concentrations, is the classical feature of type 2 diabetes. In addition, many authors have also described reduced insulin action in conditions such as hypertension, dyslipidaemia and obesity, all of which are clustered with insulin resistance in large epidemiological studies, even on exclusion of subjects with type 2 diabetes. As such, the study of insulin sensitivity, and factors that may determine or modify this biological variable, assumes increasing importance from both a basic scientific and clinical standpoint. In this thesis I have used several techniques to measure insulin action in the whole body and in isolated tissues, and considered endogenous factors that may modify insulin sensitivity.

Initial studies were undertaken in isolated subcutaneous adipocytes. Insulin sensitivity, measured as the suppression of isoproterenol-induced lipolysis, was measured in a small cohort of healthy females. The most phenotypically insulin resistant subjects, characterised primarily by their body habitus though also by fasting serum insulin and lipid profile, showed the least response to insulin in isolated cells. While this observation seems intuitive, it has considerable relevance to current scientific debate within this area. Some groups propose that resistance to insulin-stimulated glucose uptake may be largely influenced by the supply of substrate to that particular vascular bed. In addition, measurements of *in vivo* insulin sensitivity may be influenced by circulating and locally secreted factors, providing a further confounding effect on insulin sensitivity at the cellular level. The fundamental observation in Chapter 3, that the behaviour of adipose tissue after

dissection and digestion continues to reflect the host phenotype, suggests that there is a defect present at the cellular level, perhaps in the insulin-signalling cascade.

This assay was then used in association with clinical studies of dietary sodium restriction to investigate the role of the renin angiotensin system in determining insulin sensitivity. While several publications have investigated the effect of dietary sodium restriction on insulin sensitivity, the lack of uniformity in their findings is a reflection of the variation in methodology employed. As such, a randomised, placebo-controlled, double-blinded crossover study was designed, which included careful monitoring of urinary sodium excretion, and measurement of insulin sensitivity using the gold standard euglycaemic hyperinsulinaemic clamp. A modest, though significant, reduction in insulin sensitivity was observed in association with activation of the renin angiotensin system. Even a small reduction in insulin sensitivity may have an important role in determining future cardiovascular morbidity and mortality; the IRAS investigators demonstrated a continuous relationship between insulin resistance and intima media thickness of the internal carotid artery. As such, any reduction in insulin sensitivity may be anticipated to translate into cardiovascular morbidity and mortality. This observation seems at odds with the vogue for sodium restricted diet; there seems little doubt that sodium restriction is an effective non-pharmacological means of reducing blood pressure, however until there is good clinical trial evidence of a reduction in mortality in association with sodium restriction, the possibility of creating an adverse metabolic environment will continue to hamper efforts to introduce widespread sodium restriction.

In an attempt to explain the reduction in insulin sensitivity associated with sodium restriction and activation of the renin angiotensin system, the effect of angiotensin II on

insulin action in human adipocytes was then studied. Previous studies have shown that AII may interfere with insulin action at the level of intracellular second messengers, thereby providing a mechanism through which activation of the renin-angiotensin system would reduce insulin sensitivity in isolated tissue. Despite confirming that angiotensin II does indeed bind to adipocytes, there was no change in insulin action when adipocytes were co-incubated with angiotensin II. This implies that if angiotensin II is responsible for the reduction in insulin sensitivity seen in the clamp experiments, the mechanism is not mediated in adipose tissue. There is some evidence to suggest that a reduction in IRS-1 associated PI3'-kinase activity induced by angiotensin II may be compensated for by a heightened intracellular role of IRS-2. Alternatively, other components of the RAS, such as aldosterone, may have an effect on insulin sensitivity at the cellular level.

In the final results chapter, the effects of a short period of glucocorticoid exposure on the metabolic and vascular action of insulin are described. Despite data suggesting that the action of insulin in these tissues is coupled, in this system, a 29% fall in the metabolic action of insulin was not associated with any change in insulin action in the vasculature. Interpretation of these findings may offer insight into the nature of insulin action in different tissues. They support the intriguing possibility that the coupling of insulin action lies in the commonality of the *proximal* insulin-signalling cascade; there may be shared mechanisms of disrupted activation of such key intermediates as PI3`kinase that transcend tissue type. An example of this is the IRS-1 $^{-/-}$ mouse which displays both metabolic insulin resistance and endothelial dysfunction. In contrast, it seems that dexamethasone induced insulin resistance at the cellular level may be the result of defects that lie further downstream, such as the trafficking of GLUT4. This type of abnormality may be more tissue specific, and result only in metabolic abnormalities. Clearly this hypothesis requires

further investigation, however one potential model may be that of intracellular crosstalk between such circulating factors as TNF-$\alpha$ and insulin-signalling; if this relationship is mediated at the level of IRS-1/2, then wider effects than those seen in the dexamethasone model be manifest.

In summary, I have measured insulin action in the whole body, adipose tissue and vascular tissue. Insulin sensitivity in isolated tissues is closely related to the clinical phenotype, however activation of endocrine systems such as the RAS and the hypothalamic pituitary adrenal axis result in insulin resistance. In addition, while endogenous insulin action is coupled in different tissues, this delicate relationship may be disrupted by activation of such systems. As reduced insulin sensitivity has a relationship with type 2 diabetes, hypertension, obesity and dyslipidaemia, these results may have important clinical implications for the assessment and management of patients at risk of developing vascular disease.

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