Serum insulin concentrations, insulin sensitivity, and endothelial function in essential hypertension and non-insulin-dependent diabetes mellitus

by

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Declaration

I declare that this thesis has been composed by myself and 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 in the Department of Medicine and Therapeutics at the Western Infirmary Glasgow.

John R Petrie

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Summary

A series of studies is described in which specific and conventional insulin immunoassays, the hyperinsulinaemic clamp technique and forearm venous occlusion plethysmography with local intra-arterial infusions have been used to investigate:

- the effect of insulin assay specificity on the relationships among serum insulin concentrations, insulin sensitivity, and blood pressure in diabetic and non-diabetic subjects with and without essential hypertension (Chapter 5)
- the effect of sustained physiological activation of the renin-angiotensin system induced by moderate dietary sodium restriction on insulin sensitivity in patients with non-insulin-dependent diabetes mellitus (Chapter 6)
- the relationship between endothelial function and insulin sensitivity in healthy subjects (Chapter 7)

Prior to these investigations, preliminary studies (Chapters 3 and 4) were performed in order to validate aspects of the clinical physiological techniques required for the measurement of blood flow and insulin sensitivity.

(i) The reproducibility of bilateral forearm venous occlusion plethysmography

Studies using this technique to measure changes in forearm blood flow (FBF) during intra-arterial infusions of vasoactive substances often report changes in blood flow ratio (expressing responses in the intervention arm as a ratio of responses in the control arm) rather than absolute values for flow. However, unilateral measurements are reported by other investigators, and the possibility was considered that the method used for expressing responses might influence the conclusions reached. A reproducibility study was performed (Chapter 3) which demonstrated that the between-day intra-subject variability of bilateral forearm venous occlusion plethysmography (FBF ratios) was less than that of unilateral FBF measurements. The bilateral technique was used thereafter where possible.

(ii) The effect of hand-warming on insulin sensitivity and forearm blood flow

Given that investigation of haemodynamic influences on insulin-mediated glucose uptake were a major theme of these studies, validation studies were performed (Chapter 4) to investigate whether arterialisation of venous blood by the

commonly-used technique of hand-warming during the hyperinsulinaemic euglycaemic clamp had systemic haemodynamic effects or effects on the measurement of insulin sensitivity. The results of these studies in healthy subjects showed that hand-warming (by the heated-air hand box technique) has the potential to confound the results of studies using the euglycaemic clamp technique, particularly those with a case-control design and those assessing the haemodynamic effects of insulin.

(i) The effect of insulin assay specificity on the relationships among serum insulin concentrations, insulin sensitivity, and blood pressure

Serum insulin concentrations have been used as markers of insulin resistance in population studies examining the relationship between insulin resistance and blood pressure. It was hypothesised that differences in cross-reactivity of the antibodies used in conventional insulin immunoassays with proinsulin and proinsulin-like molecules might account for variations in the reported relationship between insulin and blood pressure. The relationships were therefore examined among fasting and post-glucose load serum insulin concentrations

(determined by both specific and conventional assays), insulin sensitivity (measured by the euglycaemic clamp technique), and blood pressure, in a group of 56 diabetic (NIDDM) and non-diabetic subjects (Chapter 5). The results demonstrated that the relationships among serum insulin concentrations, insulin sensitivity and blood pressure were independent of insulin assay specificity.

(ii) The effect of dietary sodium restriction on insulin sensitivity in non-insulin-dependent diabetes mellitus

Previous studies conducted in the CIRU had demonstrated an insulin-sensitising effect of acute infusion of the potent vasoconstrictor hormone angiotensin II (ANG II) in patients with NIDDM. The effect on insulin sensitivity of sustained physiological activation of the renin-angiotensin system was therefore examined in patients with NIDDM using sodium restriction (40 mmol/day) in a randomised, double-blind, placebo-controlled crossover protocol (Chapter 6). The results demonstrated a 12% reduction in insulin sensitivity associated with moderate dietary sodium restriction in these patients, providing further insights into the

effects of the renin-angiotensin system on glucose metabolism in patients with NIDDM.

(iii) The relationship between insulin sensitivity and endothelial function

There is evidence that insulin-mediated vasodilatation is reduced in states of insulin resistance. Observation of the co-existence of endothelial dysfunction and insulin resistance in a number of cardiovascular disease states generated the hypothesis of the existence of a physiological link between endothelial nitric oxide production and insulin-mediated glucose uptake. Forearm vascular endothelial function was therefore measured in healthy subjects across a range of insulin sensitivity (Chapter 7). The results demonstrated, for the first time, a positive relationship between basal endothelial nitric oxide production and insulin sensitivity in healthy young males. The hypothesis is discussed that physiological insulin sensitivity may in part be determined by an endothelium-dependent effect of insulin to promote glucose uptake by increasing blood flow (and hence delivery of insulin and glucose) to insulin-sensitive tissues.

Chapter 1.

Introduction and background

1.0 Introduction

Hormone resistance can be defined as a subnormal biologic response to a given concentration of that hormone. Insulin resistance is thought to be the most prevalent form of hormone resistance, and diabetes mellitus was differentiated into insulin-sensitive and insulin-insensitive types nearly 60 years ago (Himsworth 1936). As insulin has important and diverse effects on carbohydrate, lipid, and protein metabolism (Table 1.1), mediated by a complex receptor and second messenger system (Section 1.6), it is not surprising that insulin resistance has been implicated in the pathogenesis of a broad spectrum of disorders with metabolic features including obesity, diabetes mellitus, ovarian hyperandrogenism, and, possibly, essential hypertension (Moller and Flier 1991). By convention, the term "insulin resistance" usually refers to decreased sensitivity of tissues to the actions of insulin on glucose homeostasis, i.e. resistance to insulin-mediated glucose uptake. Conversely, "insulin sensitivity" refers to sensitivity of tissues to insulin-dependent, as opposed to insulin-independent, glucose disposal.

1.1 Insulin resistance and disease

The most important recognised physiologic determinants of insulin sensitivity, which varies up to three-fold in non-obese individuals with normal glucose

Table 1.1 Physiological actions of insulin

Action	Tissue	Mechanism
Carbohydrate metabolism		
Glucose transport (uptake) enhanced	All tissues except brain, enterocytes, hepatocytes, renal tubular cells, pancreas	Recruitment of glucose transport proteins from intracellular pool to plasma membrane
Glucose phosphorylation (initial step for entry of glucose into glycolysis)	Liver, muscle	Induction of glucokinase
Glucose oxidation enhanced	Liver, muscle	Pyruvate dehydrogenase activated
Glycogen synthesis enhanced	Liver, muscle	Induction of glycogen synthase
Pentose phosphate shunt stimulated	Adipose tissue	-
Glycogenolysis inhibited	Liver	Inhibition of phosphorylase
Gluconeogenesis inhibited	Liver	Indirect via decreased availability of acetyl coA (decreased allosteric stimulation of pyruvate carboxylase)
Protein metabolism		
Amino acid transport enhanced	Liver, muscle	Direct
Protein synthesis enhanced	Liver, muscle	Direct
Protein degradation inhibited	Liver, muscle	-
Lipid metabolism		
Fatty acid synthesis	Liver	Induction of acetyl CoA carboxylase, fatty acid synthetase
Triglyceride synthesis	Liver, adipose tisse	Increased availability of α-glycero-phosphate from glycolysis promotes esterification of fatty acids
Lipolysis inhibited	Adipose tissue	Hormone-sensitive lipase
Uptake of very-low-density lipoproteins (VLDL) and free fatty acids enhanced	Adipose tissue	Lipoprotein lipase
Reverse cholesterol transport by high-density lipoproteins (HDL) promoted	Adipose tissue	Hepatic triglyceride lipase
Ketone body synthesis inhibited	Liver	Increased malonyl CoA inhibits carnitine acyl transferase I
Electrolytes		
Cellular potassium uptake enhanced		Na^+-K^+ ATPase

tolerance, are age, body mass index, gender, regional fat distribution, and physical fitness (Hollenbeck and Reaven 1987; Yki-Jarvinen 1995). In addition, there are a number of pathophysiological states which are associated with an impaired sensitivity to insulin-mediated glucose uptake.

1.1.1 Insulin resistance in non-insulin dependent diabetes mellitus

A small subset of patients with non-insulin dependent diabetes mellitus (NIDDM) have clearly defined molecular defects resulting in insulin resistance. These are inherited, often in an autosomal dominant manner (Kadowaki et al 1988, Maasen and Kadowaki 1996, Bell 1996). However, it is fairly clear that resistance to insulin-mediated glucose uptake (both peripheral and hepatic) also plays a major role in the pathogenesis of common forms of NIDDM (DeFronzo et al 1992), and that the relationship is independent of obesity (Ludvik et al 1995). Considerable controversy remains regarding the mechanism of hormone resistance in NIDDM (Section 1.6), and its relative importance with respect to abnormalities of insulin secretion (Hales 1994).

The controversy reflects in part a philosophical difficulty in disentangling the complex and mutually-perpetuating relationship between the two metabolic processes: peripheral insulin resistance may lead initially to a compensatory insulin secretory response and eventually to ß -cell "exhaustion" (Del Prato et al 1994); conversely, hypoinsulinaemia and consequent hyperglycaemia lead to secondary insulin resistance (Hales 1994). In addition, perturbations of either process may affect hepatic glucose production. The debate also reflects a lack of

longitudinal studies, and methodological problems with measurement of both circulating serum insulin concentrations (Section 1.3.1; Robbins et al 1996) and of insulin sensitivity (Section 1.5).

1.1.2 Insulin resistance in essential hypertension

In an early study conducted in a small group of patients with essential hypertension, the presence of hormone resistance was inferred from normal or elevated blood glucose profiles in the presence of high serum insulin concentrations (Welborn et al 1966). Nineteen years later an association between serum insulin concentrations and blood pressure was reported in a large Israeli population (Modan et al 1985), supporting the notion that essential hypertension was an insulin resistant state. Since then, using more direct metabolic measurements of hormone resistance (see Section 1.4), American and Italian groups have reported decreased insulin-mediated glucose uptake insulin in non-obese essential hypertensive patients (Ferrannini et al 1987; Shen et al 1988). Such data have led to speculation that hyperinsulinaemia and/or insulin resistance might play a role in mediating the atherosclerotic complications of hypertension (Modan et al 1985, Reaven 1988). Furthermore, as the abnormality did not appear to be present in secondary hypertension (Shamiss et al 1992), it was proposed that it might be important in the pathogenesis of essential hypertension. This is discussed in detail in Section 1.2.

Much recent scientific attention has focused on the clustering of hyperinsulinaemia, insulin resistance, hypertension, glucose intolerance and

abnormal lipid metabolism within individuals of a population. Data from the Framingham Heart Study indicate that up to 50% of patients with diabetes have elevated blood pressure, and the incidences of both diabetes and hypertension increase progressively with the degree of obesity (Kannel and McGhee 1979). The constellation of subclinical cardiovascular risk factors in an individual patient has been termed "syndrome X" (Reaven 1988), "the insulin resistance syndrome" (Haffner et al 1992), and "the deadly quartet" (Kaplan 1989). However, the existence, mechanism and pathophysiological significance of hyperinsulinaemia/insulin resistance in essential hypertension remain controversial.

1.1.3 Insulin resistance, hyperlipidaemia and atherosclerosis

There is considerable evidence that insulin resistance plays a role in the promotion of atherosclerosis, the principal macrovascular complication of both hypertension and NIDDM. Lipoproteins are central to atherogenesis (Ross 1993), and insulin has a complex interaction with their metabolism (Reaven and Chen 1988, Ginsberg 1991). Under physiological circumstances, insulin suppresses free fatty acid (FFA) release from adipose tissue and promotes peripheral uptake of very-low-density lipoproteins (VLDL) and FFAs by activating lipoprotein lipase (LPL). Reduced availability of FFAs favours a decline in hepatic VLDL synthesis, and insulin-mediated activation of hepatic triglyceride lipase promotes reverse cholesterol transport by high-density lipoproteins (HDL). In obesity and NIDDM, there is a shift in the net balance of lipolysis and re-esterification of FFAs, producing a rise in their circulating concentration (Groop et al 1991); this results

from subnormal adipose tissue LPL activity (Taskinen et al 1982). FFAs in turn aggravate resistance to insulin-mediated glucose disposal in skeletal muscle, by competing for oxidation with glucose in mitochondria (Randle et al 1963), shifting hepatic glucose metabolism in favour of gluconeogenesis (via allosteric activation by acetyl CoA of pyruvate carboxylase), promoting hepatic VLDL production, raising serum triglyceride concentrations (Frayn et al 1996), and enhancing oxidative stress (Paolisso et al 1996).

There is evidence that both serum insulin concentrations (Stalder et al 1981, Orchard et al 1983, Laakso et al 1987), and directly-measured resistance to insulin-mediated glucose uptake (Laakso et al 1990b) are correlated with serum triglyceride concentrations in population studies. Reaven included dyslipidaemia, (low concentrations of plasma HDL cholesterol and high concentrations of LDL cholesterol) in his definition of "Syndrome X" (Reaven 1988). Interestingly, however, it has been reported that isolated hypercholesterolaemia (familial or otherwise) is not associated with insulin resistance (Karhapaa et al 1993, Sheu et al 1993).

Several prospective epidemiological studies have examined the relationship between serum insulin concentrations, as a marker of insulin resistance, and coronary events. The 5 year analysis of data from 1059 policemen in Helsinki revealed that the combined incidence of fatal and non-fatal myocardial infarction was greater in those who had the highest fasting insulin and post-glucose load insulin concentrations (Pyorala 1979). Similarly, in the Paris Prospective study of 7500 male civil servants, fasting serum insulin concentrations were related to the

incidence of coronary artery disease at 11 years follow-up independent of glucose tolerance and blood pressure, and was greater in obese rather than non-obese subjects (Ducimetiere et al 1980). A recent Canadian case-control study based on a population of 2103 men in whom 114 ischaemic events occurred over 15 years also reported an independent association between baseline fasting serum insulin concentrations and subsequent coronary events (Despres et al 1996). Similar results were reported in males, but not in females, in a large Western Australian population (Welborn and Wearne 1979).

However, in a large Welsh population (2512 men), the initial cross-sectional association between fasting serum insulin concentrations and ischaemic heart disease (Lichtenstein et al 1987) was not sustained in the longitudinal phase of the study: a univariate association was wholly accounted for by serum triglyceride concentrations and body mass (Yarnell et al 1994). Similar results have been reported in follow-up studies of smaller populations in Gothenburg (Welin et al 1992; n=644), California (Ferrara et al 1994; n= 1244), and in a subgroup analysis of cases and controls in the Multiple Risk Factor Intervention Trial (Orchard et al 1994). However, the epidemiological evidence in favour of a role for insulin in the pathogenesis of coronary artery disease is supported by numerous experimental studies showing that insulin accelerates atherosclerosis *in vitro* (Stout 1980; Stout 1989), and clinical data from the Bypass Angioplasty Revascularisation Investigation implicating direct deleterious effects of insulin on balloon-injured vessels after percutaneous transluminal angioplasty (Sobel 1996).

1.1.4 Genetic determinants of insulin resistance

A number of studies have demonstrated insulin resistance in offspring (Gulli et al 1992; Haffner et al 1988) or first degree relatives (Vaag et al 1992) of patients with NIDDM, as well as in identical twins discordant for NIDDM (Vaag et al 1995). Insulin resistance has also been reported in the normotensive offspring of hypertensive parents (Ferrari et al 1991). However, such studies have included only small numbers of subjects, and may be confounded by inadequate assessment of variables such as physical fitness (Yki-Jarvinen 1995); in addition, twin studies are potentially confounded by effects of the shared intra-uterine environment (Barker et al 1993a, Barker et al 1993b).

Insulin resistance is clearly modifiable by environmental factors, and the extent to which it is genetically determined remains controversial. In the case of NIDDM, genetic investigations are hampered by: a) the heterogeneity of the phenotype (a problem shared with essential hypertension); b) confusion over the relative primacy of insulin resistance and ß-cell dysfunction (Section 1.1.1); c) the arbitrary diagnostic thresholds of NIDDM and IGT, which are based on risk of diabetic complications in populations amongst whom glucose levels are continuously rather than categorically distributed.

In the case of Maturity Onset Diabetes of the Young (MODY), a specific phenotype of NIDDM characterised by early age of onset, autosomal dominant inheritance, and β-cell dysfunction, it has been possible to establish linkage with genes on chromosomes 12q, 20q, and 7p - the latter gene codes for glucokinase, a

key regulatory enzyme in determining insulin secretion by the pancreatic β-cell (Froguel et al 1992, Vionnet et al 1992, Froguel 1996). In addition, despite the difficulties of phenotypic heterogeneity in the common variant of NIDDM, a genome-wide search in affected sib-pairs from an isolated Finnish population has identified a major susceptibility locus *(D2S125)* on chromosome 2, although the mechanism by which it predisposes to the phenotype is unknown at present (Mahtani et al 1996).

1.1.5 Insulin resistance and non-pharmacological interventions

Obesity is clearly associated with insulin resistance in both diabetic and non-diabetic subjects (Bonadonna et al 1990, Cambien et al 1987, Ludvik et al 1995), particularly when associated with an upper-body fat distribution (Kissebah 1982; Peiris et al 1988); weight reduction results in an improvement in insulin sensitivity (Olefsky et al 1974) which is correlated with blood pressure reduction (Ikeda et al 1996). Physical exercise is associated with an increase in insulin sensitivity (Rodnick et al 1987), even when training bouts are too brief to be associated with weight reduction (Rogers et al 1988). The mechanisms of these effects remain poorly defined, but may relate to skeletal muscle capillarisation (Section 1.6.3).

In addition, insulin sensitivity can be impaired by both acute (Attvall et al 1993) and chronic (Facchini et al 1992) cigarette smoking.

1.1.6 Insulin resistance and drug treatment

As insulin resistance may be important in mediating the atherosclerotic complications of essential hypertension, it has been claimed that the effects of antihypertensive treatment on insulin sensitivity may be clinically important, independently of their effects on blood pressure (Rett et al 1986, Pollare et al 1989a). In particular, it has been suggested that the "shortfall" in expected cardiovascular mortality reduction resulting from antihypertensive treatment (Collins et al 1990) may be attributed to deleterious effects of commonly-used antihypertensive agents such as thiazide diuretics on cardiovascular risk factors, including effects on insulin sensitivity. In this context it is noteworthy that in NIDDM insulin sensitivity improves during treatment with any agent that chronically decreases blood glucose (sulphonlyureas, biguanides, chronic insulin therapy), but to date it has not been possible to demonstrate reductions in cardiovascular mortality attributable to such treatments (University Group Diabetes Program 1970).

The effects of antihypertensive drugs on insulin sensitivity have been widely studied but many of the studies in the literature are compromised by poor design: use of indirect measures of insulin sensitivity, before-and-after design, and lack of placebo data (Donnelly 1992). The insulin-sensitising effect of the ACE-inhibitor captopril reported by Pollare et al (1989a) remains widely-cited, but the study was compromised by carry-over effects in its intended crossover comparison of captopril and hydrochlorothiazide. In the consequent parallel-group analysis, the treatment groups were poorly matched at baseline, and insulin sensitivity data may

simply have regressed towards the mean. Several recent double-blind, placebo-controlled studies have demonstrated that neither calcium antagonists nor ACE inhibitors have significant effects on insulin sensitivity (Morris et al 1994b, Heinemann et al 1995, Giordano et al 1995; Wiggam et al 1996), although studies demonstrating an effect continue to be reported (Vuorinen-Markkola and Yki-Jarvinen 1995). In contrast, α-blockers may lead to small improvements in insulin sensitivity in patients with NIDDM (Giorda et al 1995). ß-blockers primarily affect insulin secretion (Kendall et al 1988), but decreases in insulin sensitivity have been reported in studies in which weight gain was not taken into consideration (Pollare et al 1989b). Thiazide diuretics, although known to cause deteriorations in glucose tolerance in high dose (Murphy et al 1982), have no short-term effect on insulin sensitivity when administered in low dose (1.25 mg daily) to non-diabetic subjects (Harper et al 1994) or patients with NIDDM (Harper et al 1995). However, data from these short-term studies (three months) must be taken in the context of data from longer term studies. In the MRC trial in mild hypertension (bendrofluazide 10 mg daily), the risk of developing glucose intolerance over 3 years was four times higher in the diuretic group than in the placebo group (Medical Research Council 1985); a similar trend was observed in a nine year follow-up study of 73 treated hypertensive patients and 65 normotensive control subjects (Skarfors et al 1989).

Standard therapies for NIDDM, sulphonylureas and biguanides, have their primary effects on insulin secretion and hepatic insulin resistance respectively, rather than on peripheral insulin sensitivity (DeFronzo et al 1991, Groop 1992). However, interesting observations have emerged from early clinical use of "insulin-

sensitising agents," such as the thiazolidinedione derivatives, in the treatment of NIDDM (Petrie and Donnelly 1994). These novel agents exert transcriptional effects on fatty acid metabolism (see Section 1.6.3) by activating a specific subclass of a recently-described nuclear receptor family of the steroid/thyroid hormone superfamily, peroxisome proliferator-activated receptors (PPARs) (Schoonjans et al 1996). Troglitazone, which increases peripheral insulin sensitivity by 28% in obese subjects, has been reported to cause reductions in blood pressure in man (Nolan et al 1994, Ogihara et al 1995). However, these studies were parallel group in design; only the study by Nolan et al was placebo-controlled, and baseline systolic blood pressure was poorly matched. In a larger study examining combination treatment with troglitazone and sulphonylureas, no difference in blood pressure was observed between placebo and control groups (Iwamoto et al 1996).

Interestingly, the structure of the troglitazone molecule is similar to that of vitamin E. A randomised, double-blind placebo-controlled trial conducted in healthy elderly subjects examining the effect of a pharmacological dose of vitamin E on insulin sensitivity demonstrated a 44% increase in insulin sensitivity as measured by the euglycaemic clamp technique (Paolisso et al 1994). It is not clear whether this action of vitamin E is related to its antioxidant properties (Section 1.6), or to PPAR-mediated effects on fatty acid metabolism.

Although hypercholesterolaemia in itself is not thought on the basis of the available information to be associated with insulin sensitivity (Section 1.1.3), standard therapies for hypercholesterolaemia (Shepherd et al 1995) such as

pravastatin appear to lower insulin levels in patients with hypercholesterolaemia and hypertension (Chan et al 1996). Fish oil, which is reported to have beneficial effects on lipid profiles (Goh et al 1997), appears to have a neutral or adverse effect on insulin sensitivity (Rivellese et al 1996).

In summary, the effects of standard antihypertensive drugs on insulin sensitivity, appear to be small and of little clinical significance. The effects of traditional oral hypoglycaemic agents on peripheral insulin sensitivity in NIDDM are largely indirect. Newer agents and antioxidant vitamins appear to have more profound effects on peripheral insulin sensitivity, and there are interesting parallels with their effects on endothelial function (Section 1.7.3).

1.2 Mechanisms by which hyperinsulinaemia/ insulin resistance might raise blood pressure

Central to the biological plausibility of any theory implicating insulin resistance in hypertension is knowledge of the mechanism by which a rise in blood pressure might be produced. The candidate mechanisms most frequently postulated are effects of pancreatic ß-cell compensatory hyperinsulinaemia on tissues that remain sensitive to insulin. Chronic hyperinsulinaemia in rats (Brands et al 1991b) results in hypertension; however, this is not the case in dogs (two weeks) (Hall et al 1990; Brands et al 1991a) or in patients with insulinoma (Fujita et al 1992). If such a mechanism does exists, it is clearly not a simple one.

1) Insulin induced antinatriuresis: Urinary sodium excretion falls by as much as 50% during euglycaemic hyperinsulinaemia (DeFronzo 1975; DeFronzo 1981a; Baum 1987); there appears to be a direct effect of insulin on renal tubular sodium reabsorption (Gupta et al 1992). However, although patients with NIDDM have an increased total body sodium (O'Hare et al 1985, Weidmann et al 1993), this abnormality is not present in young patients with essential hypertension (Beretta-Piccoli et al 1982).

2) Activation of the sympathetic nervous system: Acute hyperinsulinaemia activates the sympathetic nervous system in man, as evidenced by data from microneurography (Anderson et al 1991; Vollenweider et al 1994) and measurements of plasma catecholamines (Rowe et al 1981; Lembo et al 1992) and noradrenaline spillover (Landsberg and Krieger 1989). However, the pressor response is balanced by vasodilatation in healthy subjects, and there is no overall increase in blood pressure (Anderson and Mark 1993). One might speculate, however, that in subjects with essential hypertension there is attenuation of insulin-mediated vasodilatation with preserved sympathetic activation during hyperinsulinaemia (Sections 1.7.1 and 1.7.3).

3) Altered vascular smooth muscle structure and function: In pharmacological concentrations, insulin acts *in vitro* as a growth factor via insulin-like growth factor receptors (Banskota et al 1989) and may potentiate the effects of angiotensin II on DNA synthesis (Ko et al 1993). Consequent vascular hypertrophy, if produced *in vivo*, could act to promote and maintain high blood pressure (Folkow 1979), but effects on growth have not been demonstrated at physiological or pathophysiological concentrations of insulin.

4) Ion transport: Na^+-H^+/Na^+-Li^+countertransport is one of several transmembrane exchange systems (Section 1.6.3) modulated by insulin (Moore 1983). Increased activity of maximal Na^+-H^+/Na^+-Li^+ countertransport is a feature of essential hypertension (Canessa et al 1980) and is associated with insulin resistance (Doria et al 1991). Such abnormalities of transmembrane ion exchange may have effects on intracellular Ca^{++} and thus resting vascular tone (Section 1.6.3). It has been proposed that both insulin resistance and increased maximal Na^+-H^+/Na^+-Li^+ countertransport may be caused by increased membrane fluidity, via effects on enzyme activity and receptor binding (Tong et al 1995).

The arguments in favour of an important role for insulin in the pathogenesis of essential hypertension are weakened by variations in the relationship between serum insulin concentrations and blood pressure among studies (Section 1.3). Furthermore, insulin sensitivity varies up to three-fold in healthy non-obese individuals with normal glucose tolerance (Hollenbeck and Reaven 1987), while insulin resistance is present in conditions such as polycystic ovarian disease which are not associated with hypertension (Zimmerman et al 1992). Neither acute insulin infusion in man (Anderson and Mark 1993), nor chronic insulin infusion in animals (Hall et al 1990), leads to an elevation in blood pressure. Although the three most common conditions in which insulin resistance has been described (obesity, NIDDM, essential hypertension) frequently co-exist within the same patient, all three exist separately in the absence of the other two in some individuals.

1.3 Relationship between serum insulin concentrations and blood pressure

Although the "insulin hypothesis" of hypertension was generated initially from a small clinical study (Welborn et al 1966), it has received considerable support from cross-sectional epidemiological studies reporting correlations between radioimmunoassay measurements of serum insulin concentrations (fasting and post-load) and blood pressure in a variety of populations; there have been few longitudinal studies. In the presence of normal glucose concentrations, a high serum insulin level may reflect insulin resistance (acting as a surrogate measure), but this relationship breaks down when ß-cell "exhaustion" supervenes. For this reason, epidemiological studies examining the relationship between serum insulin concentrations and blood pressure usually exclude subjects with frank diabetes, or stratify for levels of glucose tolerance. The interpretation of correlations between serum insulin concentrations and blood pressure is generally that hyperinsulinaemia is a compensatory response to insulin resistance and that this may (or may not) mediate a rise in blood pressure.

Modan studied over 2000 non-diabetic individuals (male and female) in an Israeli population study, and reported higher "sum" insulin after a 100g glucose load (60 minute plus 120 minute serum insulin concentration) in hypertensive subjects than in normotensive subjects, independent of body mass index (Modan et al 1985). In a study of 247 healthy, non-obese, normotensive Italian factory workers, higher blood pressure was reported in a hyperinsulinaemic subgroup, matched with controls for age, sex, and body-mass index (Zavaroni et al 1989). In the Bogalusa Heart Study, conducted in Louisiana in over 3000 young adults, fasting serum

insulin concentrations were related to blood pressure within tertiles of body mass index in all four age groups (Jiang et al 1993a; Jiang et al 1993b). Cross-sectional data from the San Antonio Heart Study, a biethnic population study (Mexican Americans and non-Hispanic whites) of over 2000 individuals (male and female) in Texas, demonstrated that baseline insulin concentrations were higher in hypertensive (treated and untreated) than in normotensive subjects, even after adjustment for age, sex, ethnicity, body-mass index, and central adiposity (Morales et al 1993). Similar results have been reported in 708 Finnish and Dutch males in a recent cross-sectional analysis (Feskens et al 1995).

In longitudinal data from the San Antonio Heart study, non-diabetic subjects who were hypertensive (and hyperinsulinaemic) at baseline had a higher incidence of impaired glucose tolerance and NIDDM at eight year follow-up (Morales et al 1993). In a further longitudinal study, subjects with impaired glucose tolerance at baseline had an increased risk of hypertension at 20 years follow-up (Salomaa et al 1991).

However, a relationship between insulin and blood pressure has not been replicated in all studies. In a small clinic-based study (n=36), Mbanya reported no difference in serum insulin concentrations between hypertensive and normotensive subjects; although patients with NIDDM were hyperinsulinaemic, there was no correlation between blood pressure and serum insulin concentrations (Mbanya et al 1988). In a study of similar design to that of Modan et al. (1985) conducted in a Californian population, the significance of the association of post-load insulin with blood pressure was lost after stratification for body mass index and glucose

tolerance (Asch et al. 1991). Indeed, body fat distribution is the major determinant of blood pressure in many population studies (Chiang et al 1969, Weinsier et al 1985). In a population study conducted in Mauritius (n=5080), where the population comprises three main ethnic groups, only very weak associations between insulin and blood pressure were reported after stratification for confounding variables including ethnicity (Dowse et al 1993). Similar results were reported in a population of 649 individuals (male and female) in Baltimore (Muller et al 1993). In a Swedish population, elevated post-load insulin concentrations in 106 untreated hypertensives as compared to 41 controls were reported, but no correlation was detected between blood pressure and fasting insulin concentrations (Berglund et al 1976). Furthermore, in an additional longitudinal study, subjects with impaired glucose tolerance and hyperinsulinaemia at baseline did not have higher blood pressure than control subjects at 12 years follow-up (Vaccaro et al 1996).

1.3.1 Insulin assay methodology as a possible explanation for discrepancies in the relationship between serum insulin concentrations and blood pressure

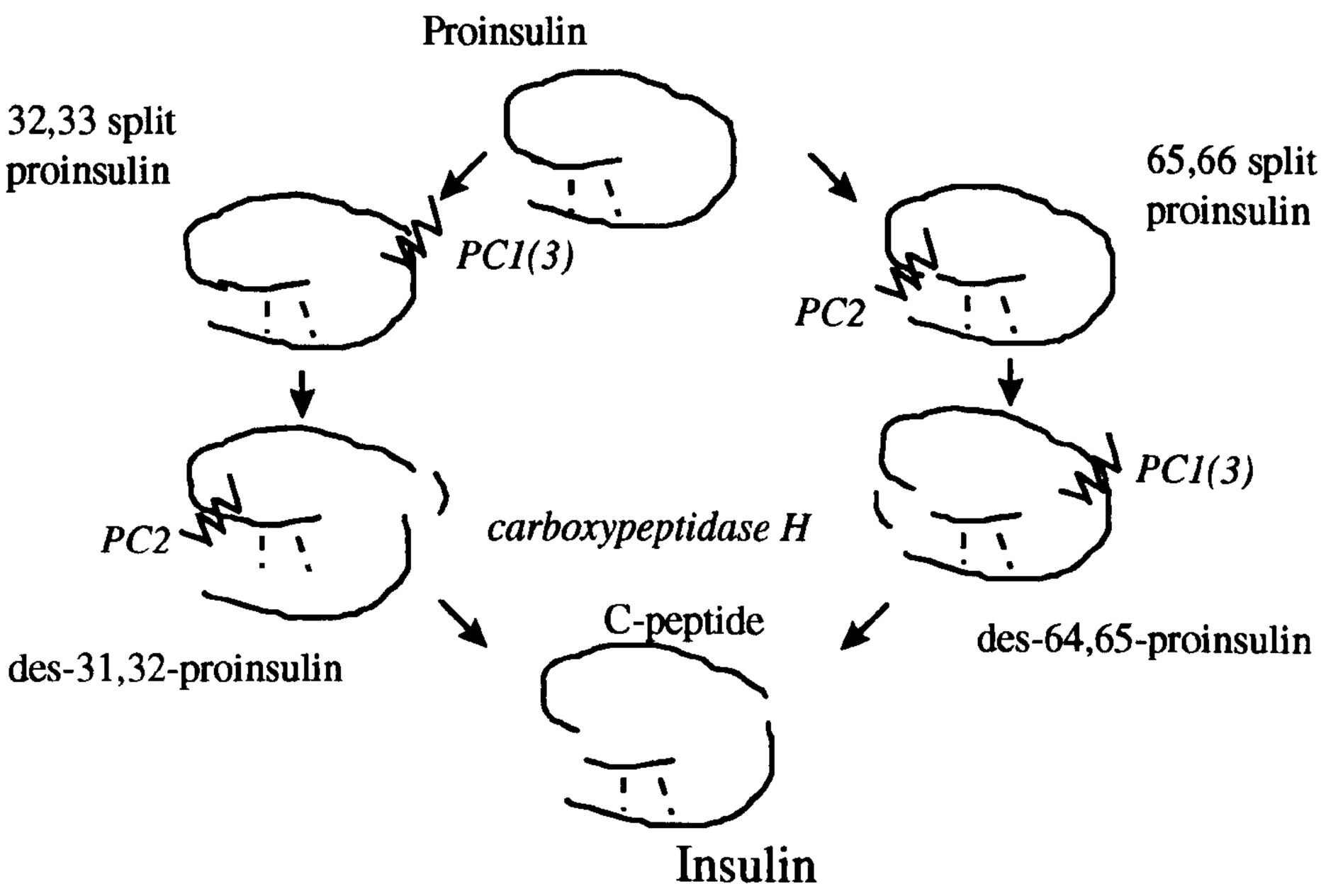
As already stated, obesity, or regional adiposity, may be a significant confounding variable in epidemiological studies of the relationship between insulin and blood pressure (Caro 1991). In addition, ethnicity may explain some of the differences between studies; for example, there is no evidence of a relationship between hyperinsulinaemia and blood pressure in the Pima Indians in the United States, in whom insulin resistance and hyperinsulinaemia are common (Saad et al 1991).

An alternative hypothesis for discrepancies between studies in the relationship between serum insulin concentrations and blood pressure is cross-reactivity of conventional insulin assays with proinsulin and its partially-processed split and des amino forms (Figure 1.1). The insulin portion of the proinsulin molecule is very similar in structure to that of the free insulin molecule (Frank et al 1972, Crowther et al 1994). Thus, any antibody which is able to differentiate insulin from proinsulin or split forms must have epitope specificity for residues near the split sites (for example a free NH_2-terminus on the A-chain), or must induce conformational change.

Circulating insulin derives from processing of proinsulin in the pancreatic islet ß-cell by three endopeptidase enzymes: the prohormone convertases (PC1(3) and PC2), and carboxypeptidase H. Thus, proinsulin is cleaved initially at either the 32-33 site (PC1(3)) or the 65-66 site (PC2) to form either 32-33 split proinsulin or 65-66 split proinsulin respectively. A two-amino acid fragment (31,32 or 64,65) is then cleaved (carboxypeptidase H) from the split forms to form either des 31,32 split proinsulin (which is the major metabolite) or des 64,65 split proinsulin respectively. These products are then further cleaved by the other prohormone convertase to form insulin and inactive C-peptide. Glucose strongly stimulates the production of both proinsulin and PC1(3), but not PC2: this is thought to result in the processing of des 31,32 to insulin becoming rate-limiting as ambient glucose concentrations rise (Hales 1994).

Figure 1.1 The proinsulin processing pathway

PC = prohormone convertase



Radioimmunoassays (RIAs) currently in use for human insulin are descended from those available since 1959, before the discovery of proinsulin (Steiner and Oyer 1967), and its detection in the human circulation (Mako et al 1977). Since use of these assays has mainly been limited to pathophysiological studies in small numbers of subjects, there have been few attempts at inter-laboratory standardisation. As conventional radioimmunoassays have different cross-reactivity with intact proinsulin (30-100% on a molar basis), they may variably overestimate true serum insulin concentrations (Heding 1977; Robbins et al 1996). The first more specific immunoradiometric assay (IRMAs) for human insulin was developed in 1968, but did not become widely available, mainly due to the requirement for relatively large amounts of immunoaffinity-purified antibody for iodination (Miles and Hales 1968). More recently, monoclonal antibody based sensitive and specific immunoradiometric assays for insulin, intact proinsulin, 65-66 split, and 32-33 split proinsulin products have been developed (Sobey et al 1989; Alpha et al 1992).

Use of these specific insulin assays has yielded some unexpected results. Temple et al reported that patients with NIDDM were in fact insulin deficient, rather than hyperinsulinaemic, and had relative hyperproinsulinaemia (Temple et al 1988). Another group of investigators could not replicate these results using an indirect RIA method in which sum proinsulin and proinsulin split-product levels were measured (using a non-specific RIA) and subtracted from total immunoreactive serum insulin concentrations (also measured using a non-specific RIA); they found "true" serum insulin concentrations to be elevated in NIDDM patients (Reaven et al 1993) This was the case even when results were checked in a sub-group by

immunoaffinity chromatographic extraction and reverse-phase HPLC. In a subsequent study published jointly by the two groups of investigators, insulin, proinsulin, and 32-33 split proinsulin were measured by both HPLC and immunoradiometric assays in the same samples from diet-treated patients with NIDDM and control subjects (Ostrega et al 1995). The results demonstrated that in the fasting state serum concentrations of all three analytes were elevated in the patients, independent of ethnicity; at 30 minutes after an oral glucose load serum insulin concentrations were lower in the patients, but proinsulin and 32-33 split proinsulin concentrations were higher; unfortunately, samples were not taken at 120 minutes post-load. Such data underline the potential significance of *hyperproinsulinaemia* in conditions previously assumed to be associated with hyperinsulinaemia.

1.3.2 Relationship between specific serum insulin concentrations and blood pressure

There are few published data using specific assays to measure serum insulin and proinsulin concentrations in essential hypertension. In 500 self-selected patients attending for health screening in Newcastle, serum insulin concentrations were measured using a relatively specific in-house assay with 5.3% molar cross-reactivity with intact proinsulin and 5.0% molar cross-reactivity with 32,33 split proinsulin (Winocour et al 1991). In this study, both fasting and two hour post-load serum insulin concentrations were only weakly and inconsistently associated with blood pressure. In a further study, 365 randomly-selected subjects from a Caucasian population underwent an oral glucose tolerance test, and serum insulin

concentrations were measured by both a conventional radioimmunoassay (cross-reactivity of 80% with both proinsulin and split proinsulin) and by an "insulin-specific" radioimmunoassay (Grootenhuis et al 1994). In a multiple regression analysis, after adjustment for age, gender, body mass index, waist hip ratio, and 2-hour blood glucose, only insulin concentrations as measured by the specific assay were significantly related to diastolic blood pressure. In the Phase II cohort of the San Antonio Heart study (Morales et al 1993), serum insulin concentrations were measured by both a specific (cross-reactivity with proinsulin 0.2%) and a non-specific assay (cross-reactivity with proinsulin 70-100%). Serum insulin concentrations were weakly correlated with blood pressure in non-diabetic subjects after adjusting for age, body mass index, waist-to-hip ratio, gender, and ethnicity, but there was essentially no difference in the relationship attributable to assay specificity; confidence in this finding was diminished somewhat by the reporting of higher insulin concentrations with the specific than with the conventional assay (Haffner et al 1994).

In summary, while it has been suggested that insulin assay specificity may influence the relationship between serum insulin concentrations and blood pressure, the few published studies that have examined this relationship measuring serum insulin concentrations using specific insulin assays have produced conflicting results. Insulin sensitivity was not measured in any of these studies.

1.3.3 Proinsulin-like molecules and cardiovascular risk

The A and B chains of intact proinsulin have 50% structural homology with insulin-like growth factor-1 (Peavy et al 1985). Proinsulin-like molecules form between 5 and 20% of fasting immunoreactive insulin in normal subjects, and circulate in disproportionate quantities with respect to insulin in patients with impaired glucose tolerance (Davies et al 1992; Krentz et al 1993), but not obesity (Shiraishi et al 1991), NIDDM (Schmidli et al 1993), or polycystic ovarian syndrome (Conway et al 1993). The proportion of proinsulin-like molecules secreted with respect to insulin by the the pancreatic islets can be reduced by dietary therapy in NIDDM (Davies et al 1994). Intact proinsulin has only 7% of the activity of insulin in stimulating glucose disposal in humans, but partially-processed split and des amino products have higher biological activities and are thought to bind to the insulin receptor (Peavy et al. 1985). Proinsulin has a longer elimination half-life than insulin and the endopeptidase converting enzymes required for processing to insulin are not present outwith the pancreatic islets (Section 1.3.1).

Many of the large-scale studies reviewed above (Section 1.3) examining the relationship between insulin concentrations and blood pressure in non-diabetic subjects used insulin concentrations derived from conventional radioimmunoassays with a high degree of cross-reactivity with proinsulin and its partially-processed intermediates (Berglund et al 1976, Modan et al 1985, Morales et al 1993, Jiang et al 1993, Feskens et al 1995). Several lines of evidence support the hypothesis that cross-reacting proinsulin-like molecules (rather than

insulin molecules) might play a role in the pathogenesis of insulin resistance and/ or hypertension/ atherosclerosis. These include observations of adverse events during the clinical use of proinsulin (Galloway et al 1992), the association between serum concentrations of proinsulin-like molecules and birth weight (Hales et al 1991, Barker et al 1993a, Fall et al 1995) and the association between serum proinsulin and angiographic severity of coronary atherosclerosis (Bavenholm et al 1995). In both diabetic (Nagi et al 1990), and non-diabetic subjects (Haffner et al 1994) there is epidemiological evidence that proinsulin concentrations are related to blood pressure. A potential mechanism for a causal relationship between proinsulin and atherosclerosis is provided by the observation that proinsulin-like molecules promote *in vitro* synthesis of plasminogen activator type-1 (PAI-1) - a potent risk factor for coronary artery disease (Aznar et al 1988; Negri et al 1993) - in cultured cells (Nordt et al 1994). Clinical studies suggest that PAI-1 activity is related to insulin resistance in patients with NIDDM (Panahloo et al 1995), but not in larger population-based groups (Mykannen et al 1994).

Such data raise the possibility that circulating proinsulin-like molecules may have biologically significant effects on either blood pressure or atherogenesis. However, the case has been reported of an individual patient with extremely high levels of proinsulin and split proinsulin products (secondary to a prohormone convertase mutation) with no evidence of cardiovascular disease (Chan et al 1987; Steiner et al 1990). Further investigation into the extent to which cross-reactivity with proinsulin-like molecules affects the relationship between serum insulin concentrations and blood pressure is clearly required.

1.4 Hyperinsulinaemia or insulin resistance?

It has been suggested that insulin resistance and hypertension may be directly linked independent of hyperinsulinaemia (Sowers 1990). If this was the case, examining the relationship between serum insulin concentrations and blood pressure might be misleading as serum insulin concentrations (being determined by a compensatory response), would have to be regarded as surrogate measures of insulin resistance (Morris et al 1994b; Nilsson et al. 1994). Evidence in favour of this hypothesis is provided by studies showing a higher degree of correlation between insulin sensitivity and blood pressure than between hyperinsulinaemia and blood pressure in both non-diabetic (Nilsson et al 1994; Yokota et al 1995) and diabetic subjects (Pinkney et al 1994).

Systematic investigation of this hypothesis, and in particular the impact of potentially confounding variables (obesity, ethnicity) when insulin resistance rather than hyperinsulinaemia is measured, has been hampered by the cumbersome nature of more direct means of measuring insulin sensitivity, making large studies extremely labour-intensive (Section 1.5). In the largest study to examine the association between insulin resistance (rather than hyperinsulinaemia) and atherosclerosis, insulin sensitivity was measured using minimal model analysis in a total of 1397 subjects from a multi-ethnic population, along with intima-media thickness of the carotid artery as an index of atherosclerosis (Howard et al 1996). Insulin resistance and intima-media thickness were positively associated in both Hispanic and non-Hispanic whites, and the association was independent of serum

insulin concentrations; full blood pressure data from this population have not yet been reported. In the largest study (n = 88) to examine the relationship between directly-measured insulin resistance and hypertension, and the effect of obesity on this relationship, the association between hypertension and insulin resistance appeared to be independent of body mass index (Maheux et al. 1994, Figure 1.2).

Thus, evidence to date suggests that insulin resistance *per se* may be more important than hyperinsulinaemia in the pathogenesis of hypertension. However, the paucity of data regarding the relationship between insulin resistance and blood pressure, taken together with the abundance of data already available on the relationship between serum immunoreactive insulin and blood pressure, indicate the need for closer examination of the effect of assay specificity on the relationships among serum insulin concentrations, insulin sensitivity, and blood pressure.

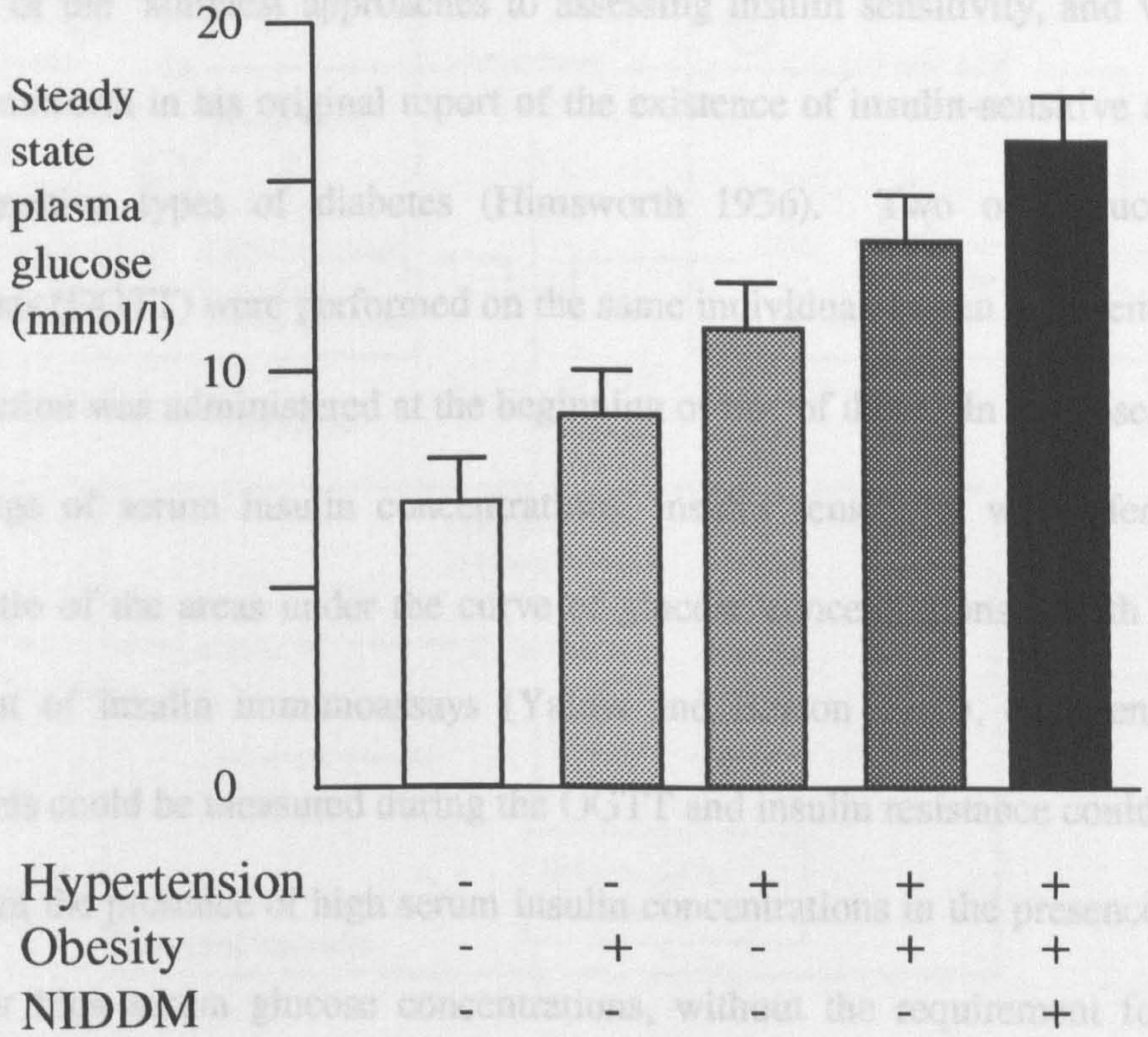
1.5 Measurement of insulin sensitivity

As discussed above, fasting serum insulin concentrations can be regarded as surrogate measurements of insulin sensitivity, and are dependent on an intact ß-cell insulin secretory response. It may, however, be desirable to measure insulin sensitivity more directly:

1) in order better to understand the pathophysiology of disease processes

2) in order to distinguish potential insulin-sensitising effects of interventions from effects on insulin secretion

Figure 1.2 Steady state plasma glucose (an index of insulin sensitivity) in 88 lean, obese, hypertensive and NIDDM subjects



Adapted from Maheux et al , 1994

3) in the evaluation of the efficacy of novel treatments

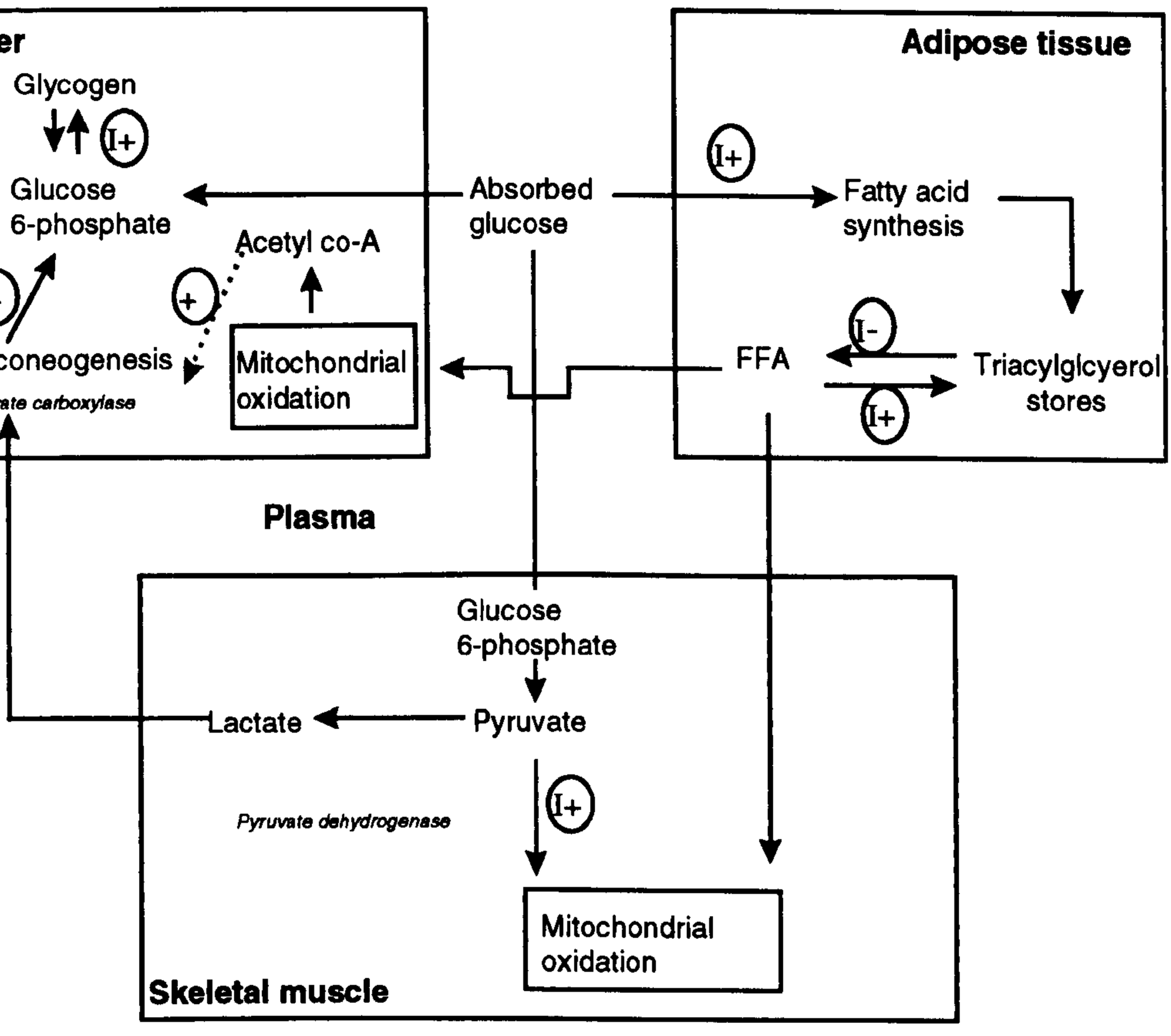
There is no overall consensus regarding methodology, and a number of different methods are in current use.

1.5.1 The oral glucose tolerance test

This is one of the simplest approaches to assessing insulin sensitivity, and was used by Himsworth in his original report of the existence of insulin-sensitive and insulin-insensitive types of diabetes (Himsworth 1936). Two oral glucose tolerance tests (OGTT) were performed on the same individual, but an intravenous insulin injection was administered at the beginning of one of these. In the absence of knowledge of serum insulin concentrations, insulin sensitivity was inferred from the ratio of the areas under the curve of glucose concentrations. With the development of insulin immunoassays (Yalow and Berson 1960), endogenous insulin levels could be measured during the OGTT and insulin resistance could be inferred from the presence of high serum insulin concentrations in the presence of a normal or high serum glucose concentrations, without the requirement for a second study day or an intravenous injection; this approach (Welborn et al 1966) has been used by many investigators since.

However, carbohydrate metabolism involves a series of complex and inter-related metabolic processes (Figure 1.3) none of which are interrupted in this procedure.

Figure 1.3 Insulin-regulated glucose and lipid metabolism



Abbreviations: FFA, free fatty acids; I+, pathway stimulated by insulin;
I- pathway inhibited by insulin

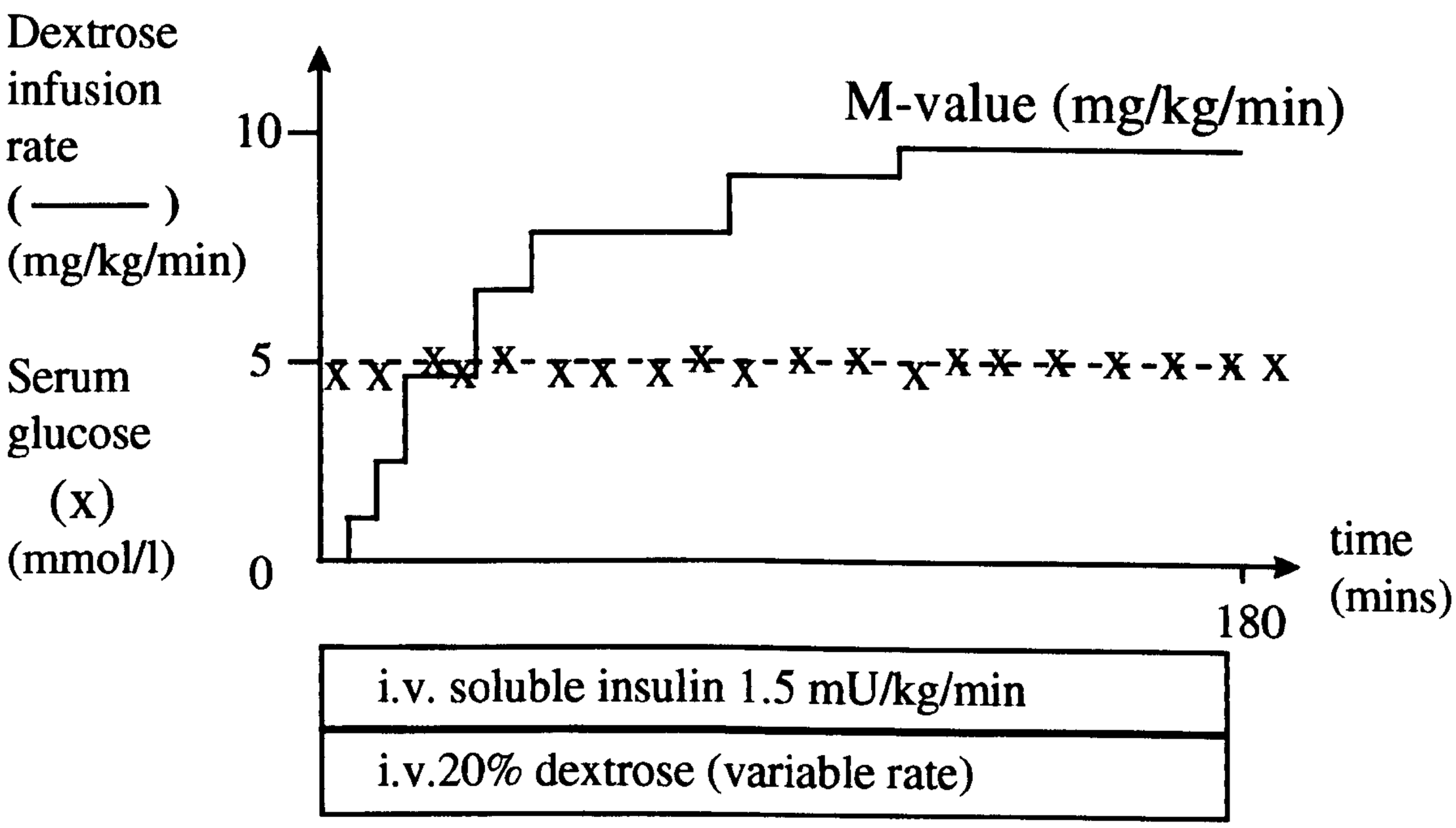
When a patient undergoes an experimental intervention, and an OGTT is performed both before and after, it is impossible to draw conclusions regarding insulin sensitivity from the changes in glucose and insulin profiles unless the intervention is known to have no effects on insulin secretion and hepatic glucose production. In addition, there is substantial test-retest biological variation (Mooy et al 1996).

1.5.2 Hyperinsulinaemic euglycaemic clamp

For the reasons cited above, more sophisticated methods of measurement of insulin sensitivity have been developed. From first principles, measurement of hormone responsiveness requires a stable relationship between serum hormone concentrations and a measurable hormone-dependent metabolic response; ideally, the relationship should be examined at different hormone concentrations and a dose-response curve constructed.

The most direct method of measuring insulin sensitivity, and arguably the "gold standard" (Keen 1994) is the hyperinsulinaemic euglycaemic clamp technique (Andres et al 1966, DeFronzo et al 1979). In brief (Section 2.4.1), plasma insulin is raised acutely to a steady-state level, usually 100μU/ml above fasting, and the investigator infuses sufficient glucose to maintain euglycaemia (usually 5.2 mmol/l) (see Figure 1.4). Steady state insulin concentrations are usually achieved within 30 minutes, but the maximal hypoglycaemic stimulus usually occurs at 60-120 minutes: the glucose infusion rate requires constant incremental adjustments,

Figure 1.4 The hyperinsulinaemic euglycaemic clamp



on the basis of frequent bedside arterial serum glucose measurements, until steady state euglycaemia is achieved.

Thus, the glucose-insulin feedback loop, central to the regulation of ambient glucose concentrations, is disrupted and placed under the control of the investigator. In most healthy subjects, endogenous secretion of insulin is suppressed by about 40% (Service et al 1978) and endogenous (hepatic) production of glucose (EGP) is suppressed by 85-90% (DeFronzo et al 1978). At steady state, the rate of glucose uptake by tissues (rate of disappearance) is equal to the rate of glucose infusion. The calculation of insulin sensitivity (insulin-mediated glucose uptake) reflects the amount of glucose metabolised, (M value = mg of infused glucose per kg of body weight per minute) and is based upon the glucose infusion rate over the last 60 minutes of the 180 minute procedure. If steady state serum insulin concentrations vary significantly between individuals, it may be necessary to adjust M by calculating the insulin sensitivity index (S_{IP}) (Bergman 1989) (Section 2.4.2):

There are a number of additional considerations regarding the euglycaemic clamp technique:

1) Adjustment of glucose infusion rate: The glucose infusion rate can be adjusted either manually or using a computerised (Biostator) glucose-controlled infusion system (Ponchner et al 1984). It has been claimed that with manual adjustment the investigator can influence the glucose infusion rate (Greenfield et al 1981), and it may therefore be preferable if the investigator is blinded to the treatment codes if manual adjustment is used in clinical pharmacological studies.

2) *Level of glycaemia:* Total glucose uptake can be sub-divided into insulin-dependent glucose disposal (hormone-mediated increase in fractional extraction of glucose), and insulin-independent glucose disposal (glucose mass action, or glucose effectiveness). The effect of glucose *per se* to enhance glucose disposal can be demonstrated in studies in which insulin secretion is blocked by infusion of somatostatin during infusion of exogenous glucose (Capaldo et al 1986); insulin-independent glucose disposal is thought to account for about 80% of basal glucose uptake in the fasting state (Kahn et al 1994). In individuals with higher fasting glucose levels, the distinction between insulin-dependent and -independent glucose disposal becomes more important, as the proportion of glucose disposal that is insulin-independent may be higher (Cherrington et al 1978). When assessing insulin sensitivity there are two potential solutions: subjects can either be brought to euglycaemia prior to the start of the procedure, or the procedure can be performed at the fasting glucose concentration and an adjustment made for glucose pool size (DeFronzo et al 1979, Greenfield et al 1981). The disadvantage of the former approach is that the process of normalising blood glucose concentrations may in itself affect insulin sensitivity by activating counterregulatory systems, and this effect may be larger for higher blood glucose concentrations; however, the latter procedure ("isoglycaemic clamp") requires a mathematical correction if comparisons are to be made with data from euglycaemic clamp studies. In practice, where insulin sensitivity is to be compared between individuals, and none have a fasting glucose concentration of > 10 mmol/l, it seems reasonable to clamp serum glucose at euglycaemia (Section 2.4.1). Where the effect of an intervention is to be assessed in individuals with varying fasting glucose levels, an isoglycaemic clamp may be preferable.

3) Cellular potassium uptake: Infusion of insulin and glucose *in vivo* causes a dose-dependent fall in plasma potassium by promoting cellular uptake; however, at a threshold level, splanchnic potassium exchange switches from net uptake to net release and clinically significant hypokalaemia has not been described (DeFronzo et al 1980). It has been argued that serum potassium should also be "clamped", as differential effects of interventions on potassium metabolism may have secondary effects on glucose metabolism (Heinemann et al 1995).

4) Arterial and venous sampling: Arterial blood glucose concentrations, which represent an average of the processes occurring in the various tissues of the body, were used in the original description of the hyperinsulinaemic clamp technique (DeFronzo et al 1979). Venous blood glucose data are more easily obtained in man but depend upon metabolism in the organ drained; since there is a high tissue glucose extraction rate during hyperinsulinaemia (resulting in a large arteriovenous glucose gradient), the use of venous glucose concentrations to adjust the glucose infusion rate would be expected to lead to an overestimation of insulin sensitivity. Most investigators now adjust the glucose infusion rate on the basis of "arterialised" venous blood from the superficial dorsal veins of the hand (Liu et al 1992). The assumption is that hand-warming (by a warm blanket or a heated-air hand box) increases blood flow through digital arteries, creating a functional arteriovenous shunt (Ferrannini et al 1987, Yki-Jarvinen 1987). There is some concern, however, that the heated-air hand box may itself confound the measurement of insulin sensitivity. This is examined in Chapter 4.

5) Endogenous glucose production: Endogenous (hepatic) glucose production (EGP) may not be completely suppressed, particularly in insulin resistant subjects, in which case M provides an underestimation of whole-body glucose uptake.

Some interventions may affect hepatic and peripheral insulin sensitivity differentially; EGP, an index of hepatic insulin sensitivity, can be estimated using radiolabelled tracer techniques (Section 2.5.2).

6) Hypoglycaemia: This is potentially a problem if the procedure is not carefully supervised, and the investigator must remain at the bedside throughout the procedure. Rebound hypoglycaemia can effectively be prevented by continuing the glucose infusion for 20 minutes after the insulin infusion is completed.

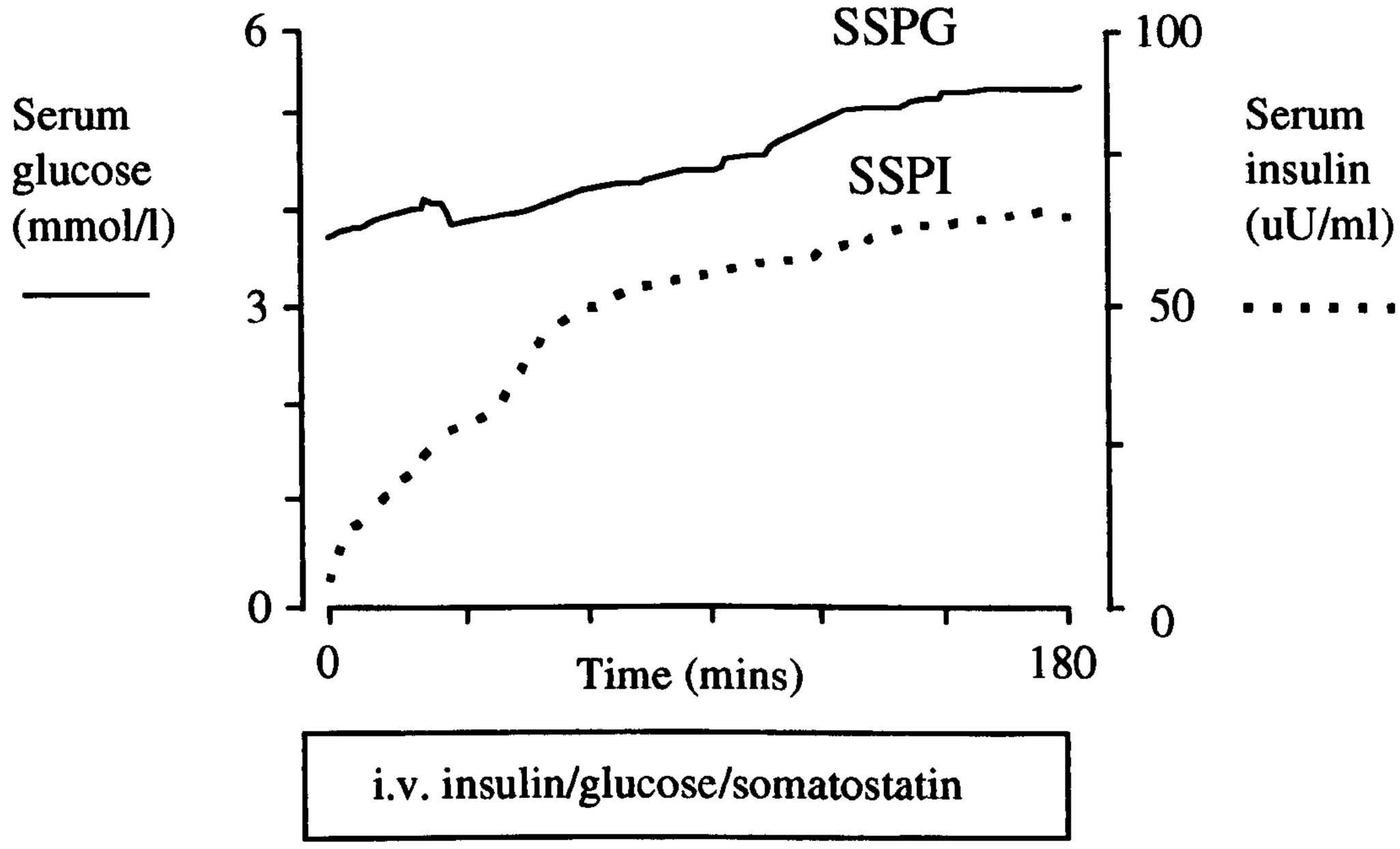
7) Reproducibility: In the original report by DeFronzo et al (1979), euglycaemic clamps were repeated in only four individuals. Reproducibility data from the clamp technique as performed in the current thesis are quoted in Section 2.4.2 (Morris et al 1994a).

In summary, the clamp provides a direct reproducible measurement of insulin sensitivity. It is not critically dependent on insulin assay data and can be combined with other metabolic techniques. However, it is labour intensive, expensive, and requires special equipment.

1.5.3 Insulin suppression test (IST)

This method of assessing insulin sensitivity was developed in parallel with the clamp (Shen et al 1970). Endocrine suppression is produced pharmacologically during a similar level of exogenous hyperinsulinaemia to that of the clamp, but the glucose infusion rate is not varied by the investigator (Figure 1.5). Thus the steady state glucose concentration (SSPG) is used as an index of insulin

Figure 1.5 The insulin suppression test



Abbreviations: SSPG, steady state plasma glucose; SSPI steady state plasma insulin

sensitivity: *high* SSPG indicates *low* insulin sensitivity. In earlier studies, adrenaline and propanolol were infused to inhibit endogenous insulin secretion, but this method has now been superseded by infusion of somatostatin (Harano et al 1978) or its commercially available analogue, octreotide (Mimura et al 1994). Use of octreotide allows the study of subjects in whom the technique was previously contra-indicated. There are a number of theoretical disadvantages of the insulin suppression test. Like the euglycaemic clamp technique, no account is taken of insulin-independent glucose disposal. In addition, because individuals reach steady state at different glucose levels, SSPG is an unpredictably non-linear function of insulin-dependent glucose disposal, and there may be inaccuracies at extremes of insulin resistance. In insulin resistant subjects, who have higher levels of SSPG, the renal threshold for glucose will be exceeded, leading to an overestimation of insulin sensitivity; conversely, in more insulin sensitive subjects, glucose levels may fall into the hypoglycaemic range and induce (partially-inhibited) counter-regulatory responses, a decrease in measured insulin sensitivity, and even unpleasant symptoms. In addition, the endocrine suppression agents themselves may, in theory, induce differential changes in insulin sensitivity between subjects, and no account is taken of hepatic glucose production.

Despite these considerations, a high degree of correlation between SSPG (derived from the insulin suppression test) and M (as measured by the euglycaemic clamp technique) has been reported by both the original authors (Greenfield et al 1981) and an independent group of investigators using the octreotide-modified version (Mimura et al 1994). In addition, the insulin suppression test appears to be

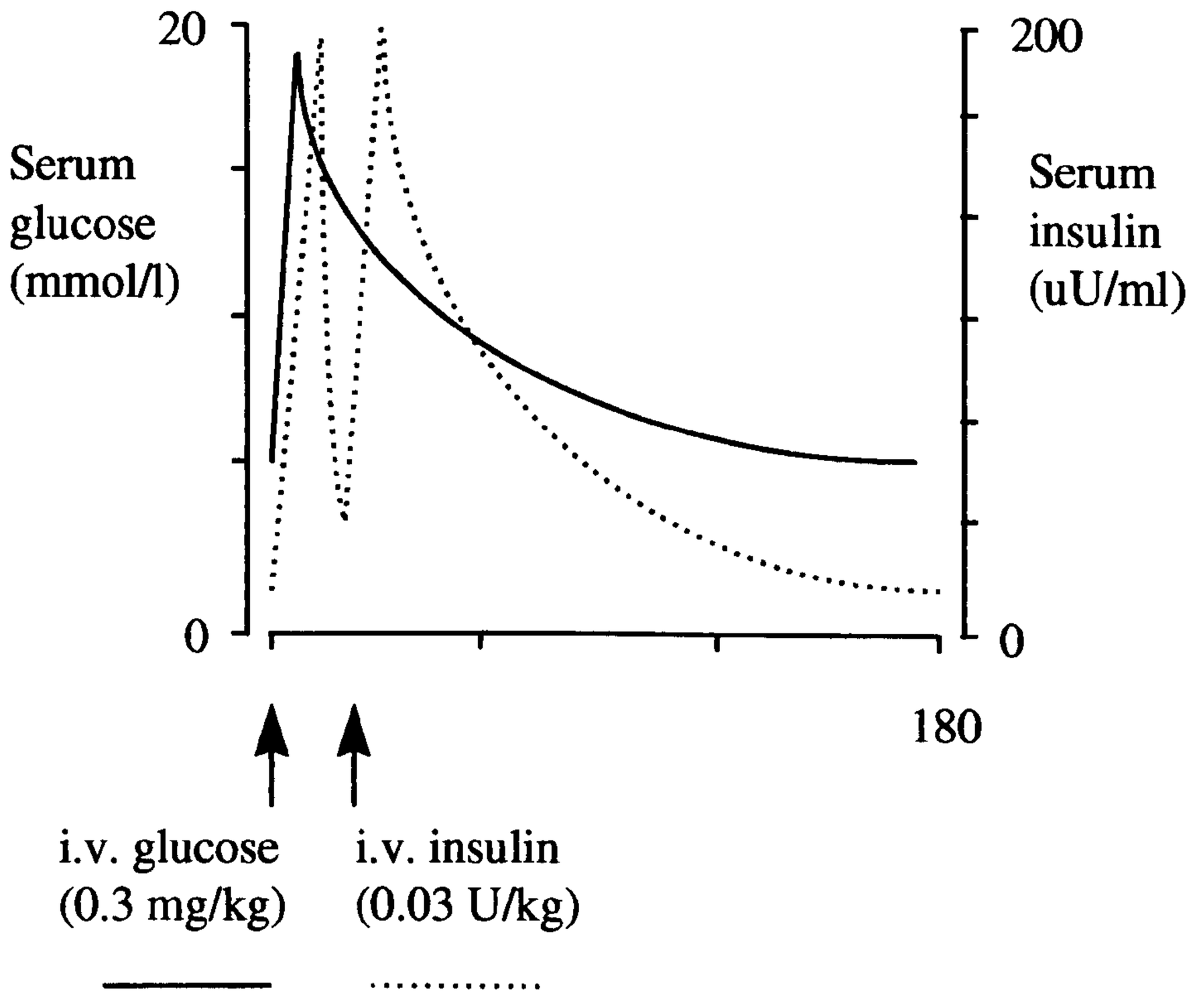
reproducible (Greenfield et al 1981), and has been used to examine insulin resistance in a wide variety of physiological and pathophysiological states. It has the advantages of being cheap and simple to perform and interpret, and requires little special equipment.

1.5.4 Intravenous glucose tolerance test (IVGTT) with minimal model analysis

Using complex mathematical modelling, this approach was developed in order to estimate the relationship between serum insulin concentrations and insulin-dependent glucose disposal without the need to break the glucose-insulin feedback loop (Bergman et al 1987). The clinical procedure is simple to perform (Figure 1.6): an intravenous glucose load (usually 0.3 mg/kg) is administered and frequent blood samples (22 over three hours) are withdrawn for determination of glucose and insulin. Data can be analysed using commercially available computer software (MINMOD, Pacini et al 1986).

In the analysis, insulin sensitivity is derived from a model which was found to provide the simplest and most accurate mathematical simulation of glucose metabolism in dogs. Measured insulin concentrations are entered into the model, which compares the actual rate of decline of glucose concentrations after the initial peak with the predicted concentrations in order to derive an estimate for insulin-dependent glucose disposal (S_i). A measure of insulin-independent glucose disposal is also derived (S_G), along with estimates of first and second-

Figure 1.6 The intravenous glucose tolerance test



phase insulin secretion. S_i was originally reported to be highly correlated with the insulin sensitivity index as measured by the hyperinsulinaemic euglycaemic clamp (Bergman et al 1987). Nevertheless, a number of improvements to the model and modifications of the clinical procedure have been introduced by the authors since its original description. For example, it may be necessary to administer an intravenous bolus of tolbutamide (300 mg) (Yang and Bergman 1987), or subcutaneous insulin 0.03 U/kg (Finegood et al 1990), at t=20 mins in order to produce adequate insulinaemia for estimation of S_i in patients with a poor second-phase insulin response. However, in the hands of an independent group of investigators, assessing the validity of the technique prior to the large Insulin Resistance Atherosclerosis Study (Howard et al 1996), S_i from minimal model analysis had only a reasonable correlation with clamp-derived insulin sensitivity when investigated across the spectrum of glucose tolerance (r = 0.5-0.6). In addition, there was a considerable discrepancy in the absolute values derived from the two techniques (Saad et al 1994). The reproducibility of minimal model analysis appears to be dependent upon the frequency of blood sampling: in one study, use of only 12 as opposed to 22 samples in the IVGTT resulted in an unacceptable increase in the intra-subject coefficient of variation of S_i from 20% to 28% (Steil et al 1994).

In effect, the IVGTT with minimal model analysis replaces more complex experiments yielding direct measurements with simple experiments requiring complicated analysis. It can be used more easily than the clamp for larger studies and has gained widespread acceptance. The disadvantage of using a model is that, by definition, only an approximate description of the real system is provided.

1.5.5 Other methods

As even the most complex techniques for the measurement of insulin sensitivity have significant disadvantages, some investigators have chosen to compromise by deriving alternative indices of insulin sensitivity. There is a clear need for a generally-accepted and readily calculated index of insulin sensitivity which can be applied to large populations of non-diabetic subjects and patients with NIDDM from easily collected data. A number of indices have been proposed including HOMA (homeostasis model assessment; Matthews et al 1985), FIRI (fasting insulin resistance index; Duncan et al 1996), fasting insulin, 2-hour insulin, "sum" insulin, and AUC insulin (Modan et al 1985; Scheen et al 1995). These variables have been validated against clamp-derived measurements of insulin sensitivity only in small groups of subjects and, although all incorporate serum insulin concentrations, the effect of cross-reactivity with proinsulin-like molecules in the insulin assays used to determine these concentrations has not previously been evaluated.

1.6 Mechanisms of insulin resistance

Under physiological circumstances, skeletal muscle is the main site of insulin-mediated glucose disposal in man (DeFronzo et al 1981b, Yki-Jarvinen et al 1983). Physiological insulin resistance, occurring in response to stress, pregnancy or puberty, may be explained by increased circulating concentrations of counter-regulatory hormones with insulin antagonist properties (catecholamines, cortisol, growth hormone, sex steroids). Mechanisms for resistance to insulin action in common pathophysiological states remain unknown. In essential hypertension (Natali et al 1991; Capaldo et al 1991), NIDDM (DeFronzo 1992), and obesity

(Olefsky 1981) the main site of insulin resistance is skeletal muscle tissue, but in NIDDM adipose tissue is also resistant to insulin action (Kasigawi et al 1983, Groop et al 1991). Despite these similarities, however, it is worth noting that the insulin resistance associated with each of these common disease states may not share a common mechanism.

In obesity, in the absence of hypertension or glucose intolerance, insulin resistance is global with decreased oxidative and non-oxidative glucose disposal (measured by indirect calorimetry), increased lipid oxidation, impaired suppression of lipolysis and decreased potassium uptake (Olefsky and Kolterman 1981; Bonadonna et al 1990). In NIDDM, lipid oxidation and potassium uptake are relatively unaffected, but the other abnormalities are present (Olefsky and Kolterman 1981; Groop et al 1991). In essential hypertension, limited data suggest that the defect is pathway-specific i.e. there is selective impairment of non-oxidative glucose metabolism (glycogen synthesis) (Ferranini et al 1987).

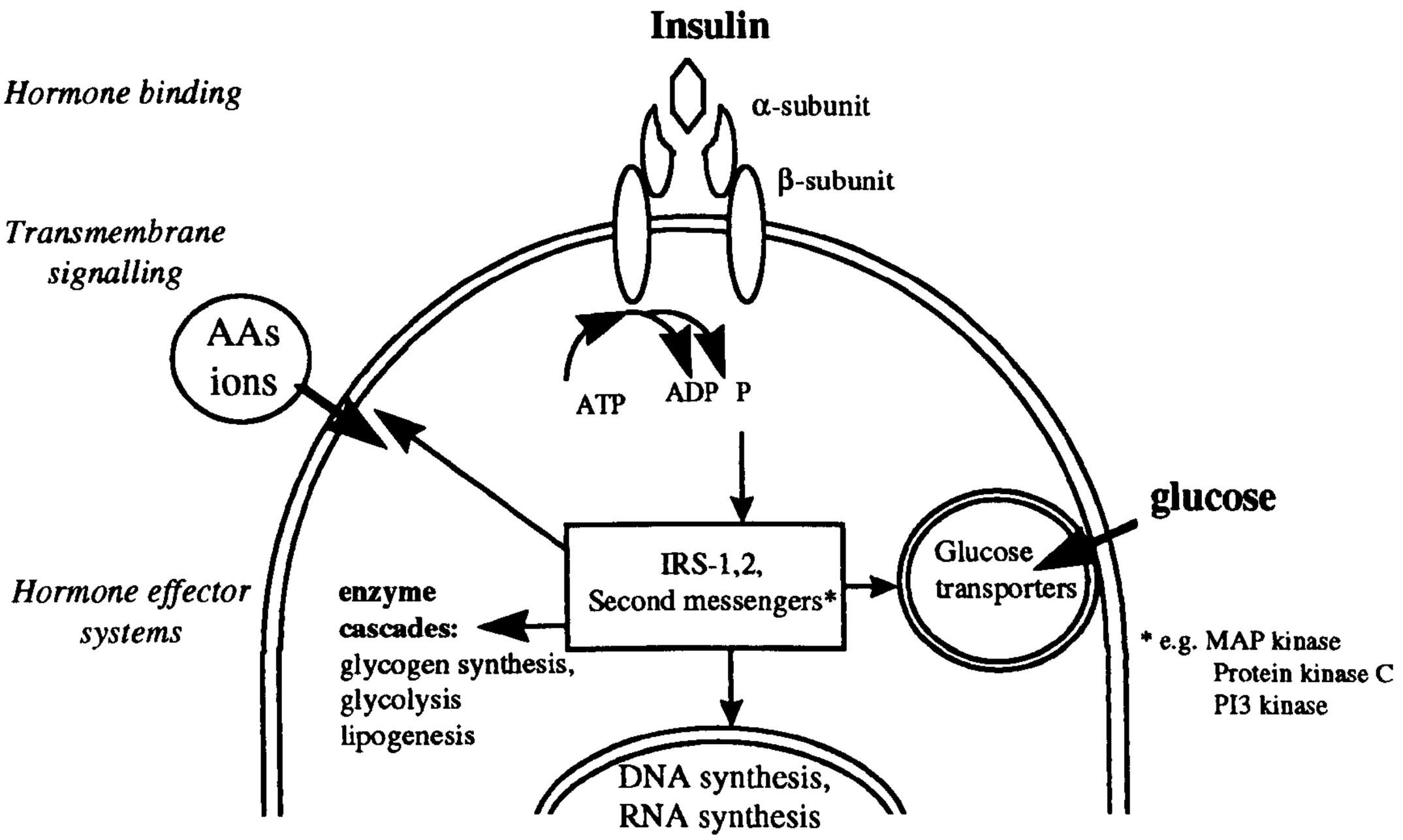
Once established, powerful mechanisms exist by which insulin resistance is perpetuated. For example, in obese subjects, an enlarged fat mass results in increased serum concentrations of free fatty acids (FFAs), which aggravate resistance to insulin action on hepatic glucose and lipid metabolism and skeletal muscle glucose metabolism (Section 1.1.3) (Groop et al 1991). Indeed, it has been argued that the relationship between insulin resistance, serum insulin concentrations, and glucose tolerance in apparently healthy subjects is mediated to a significant degree by changes in ambient serum FFA concentrations (Reaven 1988).

In the search for specific defects in insulin action, much attention has been given over the last decade to the investigation of events involving the interaction of insulin with its receptor, and to post-receptor events in insulin action. However, in conditions associated with more global resistance to the actions of insulin such as obesity and NIDDM, pre-receptor factors may be important. Selective impairment of particular pathways is easier to reconcile with a post-receptor mechanism.

1.6.1 Receptor defects in insulin action

The insulin receptor is a tetrameric transmembrane protein which has been cloned (Ebina et al 1985), and whose three-dimensional structure is known (Hubbard et al 1994) (Figure 1.7). After binding of insulin to the α-subunit, autophosphorylation of tyrosine kinase in the ß-subunit initiates further intracellular events. Various structural mutations in the insulin receptor locus leading to receptor binding abnormalities have been described in individual patients with extreme insulin resistance (leprechaunism, type A insulin resistance, Rabson-Mendenhall syndrome), but most of these have been found in one family only (Accili et al 1992, Bell 1996, Taylor 1996). In addition, receptor down-regulation can develop as a consequence of hyperinsulinaemia: in obesity there are fewer insulin receptors in both adipose tissue and skeletal muscle (Caro 1991); by the time of development of NIDDM, there is also evidence of altered receptor expression in skeletal muscle (Haring and Mehnert 1993) and monocytes (Rizza et al 1981). Insulin receptor number and binding are normal in spontaneously hypertensive rats

Figure 1.7 The insulin signalling pathway



Abbreviations: AAs, amino acids; ADP, adenosine diphosphate; ATP, adenosine triphosphate; IRS, insulin receptor substrate; P, phospate; PI, phosphatidyl inositol

(Reaven et al. 1989), and although there are few human data in essential hypertension, changes in receptor number and function are usually assumed to be secondary.

1.6.2 Post-receptor defects in insulin action

Defects in tyrosine kinase activation by the activated insulin receptor (Freidenberg et al 1987) have been reported in NIDDM. Following tyrosine kinase activation, intracellular molecules such as insulin receptor substrate (IRS-1) and serine-threonine kinases are phosphorylated. IRS-1 is thought to act as an adaptor molecule, linking the receptor kinase to the various cellular activities regulated by insulin, including PI3-kinase (White and Kahn 1994): no direct phosphorylation of the proteins involved in glycogen synthesis or glucose transport has been described. Two genetic polymorphisms of IRS-1 have been described with a combined prevalence of 12% in a Danish population; one of these may interact with obesity in determining insulin sensitivity, as assessed by the IVGTT (Clausen et al 1995). To date, none of the obvious candidate genes from the insulin signalling pathway have pointed to a major locus of mutation (Kahn 1995), and this has led to a search for molecules which may actively inhibit insulin action (Maddux et al 1995). Membrane glycoprotein PC-1 may play a part in insulin resistance in some individuals with NIDDM, but there are no data in essential hypertension.

Further candidates for defects in insulin action are glucose transporters, a family of structurally related proteins (classified as GLUT 1-5 and 7), which accelerate

glucose movement down a concentration gradient through energy independent mechanisms (Klip et al 1994; Gould 1994). In skeletal muscle, it appears that only the insulin-sensitive facilatative transporter protein GLUT-4 is translocated to the plasma membrane in response to the phosphorylation cascade initiated by insulin (Hirshman et al 1990; Livingstone et al 1995). Functionally important mutations of glucose transporter proteins have not yet been described in man, and there is no consistent decrease in GLUT-4 protein levels in skeletal muscle of diabetic humans or animals (Pedersen et al 1990; Handberg et al 1990), although reduced levels of GLUT-4 (Garvey et al 1988, Bastard et al 1995) and GLUT-5 (Bastard et al 1995) have been reported in adipose tissue from obese patients with NIDDM. There is one report of reduced levels of GLUT-4 in skeletal muscle tissue from morbidly obese patients with NIDDM (Dohm et al 1991)

A number of intrinsic defects in intracellular pathways of glucose metabolism have been identified in NIDDM. For example, glycogen synthase activity in skeletal muscle has been shown to correlate with insulin sensitivity in a population of obese patients with variable degrees of glucose tolerance (Bogardus et al 1984). However, if such defects were primary, it should be possible to demonstrate an accumulation of free intracellular glucose, and this does not appear to be the case (Garvey and Birnbaum 1993).

The description of multiple abnormalities of insulin signalling, glucose transport, and intracellular glucose metabolism in NIDDM suggests either that some of these may be secondary to hyperglycaemia, or that there is heterogeneity in the condition.

1.6.3 Pre-receptor defects in insulin action

In order to access its specific cell-surface receptor in target tissues, insulin secreted into the portal vein must travel through the vascular space, undergo transcapillary transport, and diffuse through the interstitial space. Therefore it has been suggested that factors governing delivery of insulin and substrate glucose to insulin-sensitive tissues may be important in determining overall sensitivity to insulin-mediated glucose uptake (Laakso et al 1990a). Studies in dogs (Yang et al 1989) measuring thoracic duct lymphatic insulin concentrations, and in lean and obese volunteers (Jansson et al 1993, Castillo et al 1994) measuring interstitial insulin concentrations in microdialysis fluid and lymph during hyperinsulinaemia, suggest that transport of insulin across the endothelial barrier is saturable, and may be rate-limiting for insulin action. In addition, there is evidence that insulin sensitivity may be determined in part by the ability of insulin to promote its own passage through the vascular space. This may be limited by structural or functional defects.

Microvascular changes: Lillioja et al. (1987) demonstrated a correlation between capillary density of skeletal muscle and insulin sensitivity as measured by the euglycaemic clamp technique, suggesting a role for microvascular haemodynamic factors in determining insulin-mediated glucose uptake (Julius et al 1991). In support of this hypothesis, there is evidence that individuals with predominantly type I (red, aerobic, slow twitch, insulin sensitive) skeletal muscle fibres have a higher capillary density than individuals with a higher proportion of type IIB (white, anaerobic, fast twitch, insulin resistant) skeletal muscle fibres (Juhlin-

Dannfelt et al 1979). Physical training can alter the relative proportions of these fibres by 5-10% in either direction, mainly via the plasticity of type IIA fibres (Tonino 1989).

Haemodynamic effects of insulin: Insulin given systemically has been shown to cause vasodilatation by most investigators (Laakso et al 1990a, Anderson et al 1991, Vollenweider et al 1993). In addition, locally-administered insulin in pharmacological doses has a similar effect (Creager et al 1985). However, studies which have examined the direct effects on vascular tone of more physiological local hyperinsulinaemia in man have reported either weak vasodilatation (Neahring et al 1993), no effect (Yki-Jarvinen et al 1987, Capaldo et al 1990, Natali et al 1990, Natali et al 1991; Utriainen et al 1995), or no effect combined with an ability to attenuate the vasoconstrictor effects of sympathetic nervous system activation (Lembo et al 1994) or vasoactive peptides (Sakai et al 1993). It should be noted, however, that local intra-arterial administration of insulin alone causes relative hypoglycaemia in the relevant vascular bed; co-infusion of D-glucose, to achieve local euglycaemic hyperinsulinaemia amplifies the vasodilating effect of insulin from 20% to 60% (Ueda et al 1995). Further insights into the physiological haemodynamic effects of insulin can be gleaned from studies conducted in the post-prandial state: while a high fat meal is a relatively poor stimulant of both insulin secretion and calf vasodilatation, co-infusion of insulin produces a threefold amplification of the response (Kearney et al 1996). In support of these findings, insulin-mediated vasodilatation *in vitro* appears to be dependent on local glucose uptake (Kahn et al 1995). It appears from these studies that insulin may have not only systemically-mediated but also locally-

acting vasodilator properties, and that impairment of either of these responses could result in a functional defect in the ability of insulin to promote its own passage through the vascular space, leading to decreased access of insulin to its receptors in target tissues such as skeletal muscle and adipose cells.

There are a number of mechanisms by which insulin might cause physiological vasodilatation or attenuation of vasoconstriction:

1) Direct effect on vascular smooth muscle relaxation: Vascular smooth muscle cells contract in response to an increase in intracellular Ca^{++}, which is increased basally in NIDDM and essential hypertension in a variety of human tissues (Levy and Gavin 1994). Physiological vasodilatation may occur via decreased intracellular Ca^{++}, either as a result of increased calcium efflux or decreased calcium influx. Conversely, increased vascular reactivity may occur because of decreased basal calcium efflux or increased basal calcium influx. Activity of Ca^{++}ATPase, the major cation pump system responsible for calcium efflux, is reduced in erythrocytes of insulin resistant obese Zucker rats (Zemel et al 1990), but there are few data concerning Ca^{++}ATPase activity in relevant human tissues (Levy et al 1994). In addition, insulin has the ability to decrease calcium influx by stimulating ouabain-sensitive vascular smooth muscle Na^{+}-K^{+}ATPase, causing cellular hyperpolarisation and closure of voltage-gated calcium channels (Kahn et al 1993; Levy and Gavin 1994; Tack et al 1996b); this action of insulin may also be affected by insulin resistance, resulting in increased vascular reactivity.

2) Indirect effect on vascular smooth muscle relaxation via release of or inhibition of a local vasoactive substance: Insulin might vasodilate by stimulating release of endothelially-released vasodilator substances (nitric oxide, prostacyclin), or

inhibiting the release of endothelial vasoconstrictor substances (thromboxane, endothelin) (Section 1.7.3).

3) Indirect effect coupled to tissue metabolic activity: Vasodilatation may occur via a mechanism linked to metabolic demand i.e. increased insulin-mediated cellular glucose uptake may result in production of a signal which couples substrate delivery with fuel requirement by increasing blood flow. If this is the case, decreased insulin-mediated vasodilatation is a consequence of decreased insulin-mediated glucose uptake. In accordance with this hypothesis, it has been reported that insulin-mediated vasodilatation in hamster cremaster muscle is inhibited by adenosine receptor antagonism (McKay and Hester 1996).

4) Antagonism of vasoconstrictors: Insulin may antagonise the vasoconstrictor effects of the sympathetic nervous system and circulating vasoconstrictor substances (Section 1.7.1). It is possible that more than one of these mechanisms are important in producing insulin-mediated vasodilatation, or that there are interactions between different mechanisms.

Assuming that insulin has a physiological vasodilator action, it is conceivable that impairment of this response might result in increased vascular resistance i.e. a potential direct link between insulin resistance and hypertension, independent of hyperinsulinaemia (Sowers et al 1990). Studies based on the Fick principle (tissue glucose uptake as the product of blood flow and arteriovenous glucose difference) conducted using the leg perfusion technique during systemic hyperinsulinaemia have examined the relative contributions of decreased substrate (glucose) delivery and decreased tissue glucose extraction to insulin-sensitive tissues in insulin resistant states. The findings of these studies have been consistent in NIDDM

(Baron et al 1991a), IDDM (Baron et al 1991b) and obesity (Baron et al. 1990) in showing that insulin resistance in these subjects is closely paralleled by a decreased ability of insulin to stimulate skeletal muscle blood flow (decreased substrate delivery). Furthermore, blood pressure is poitively correlated with the ability of insulin to cause vasodilatation in healthy subjects (Baron et al 1993).

The notion that attenuation of insulin-mediated vasodilatation and decreased capillary recruitment, limiting the ability of insulin to promote delivery of itself and its substrate glucose to target tissues, are important in the pathophysiology of insulin resistance has been termed the "haemodynamic hypothesis." In support of this, sensitivity to insulin-mediated venodilatation is highly correlated with insulin-mediated glucose uptake in healthy individuals (Feldman et al 1995). Extending the hypothesis to a pathophysiological setting, a paradoxical forearm vasoconstrictor effect of systemic insulin infusion has been demonstrated at physiological concentrations in a small group of obese hypertensive subjects; this effect was associated with a small rise in blood pressure (Gudbjornsdottir et al 1995). However, these observations were made in the context of inhibition of endogenous insulin secretion by somatostatin, which may have had other effects.

The haemodynamic hypothesis of insulin resistance implies that in the presence of insulin resistance the proportion of insulin-mediated glucose uptake which is facilitated under physiological conditions by insulin-mediated vasodilatation is diminished. However, the relative importance of the vascular effects of insulin (increased delivery of substrate) over its endocrine effects (increased fractional extraction of glucose) remains the subject of controversy. Baron et al have

reported that intra-arterial infusion of the endothelium-dependent vasodilator methacholine into the leg vascular bed during hyperinsulinaemia increases glucose uptake (Baron et al 1994b), and have presented calculations demonstrating that insulin-stimulated glucose uptake would be improved by up to 40% if the blood flow response to insulin was normal in obesity (Laakso et al 1990a). It has also been reported that increases in forearm blood flow *per se* mediated by exogenous bradykinin result in increased glucose uptake (Dietze et al 1996). However, some studies in hypertensive patients have demonstrated reduced skeletal muscle glucose uptake in the absence of any difference in insulin-stimulated blood flow (Capaldo et al 1991; Natali et al 1991). In addition, not all groups have demonstrated an increase in insulin-mediated glucose uptake as a consequence of increasing muscle blood flow. In one study, a 100% augmentation of forearm blood flow (achieved by infusing adenosine) in insulin-resistant patients with essential hypertension resulted in no change in muscle glucose uptake during hyperinsulinaemia (Natali et al 1994), while in another study a bradykinin-induced 58% increase in leg blood flow during hyperinsulinaemia resulted in no change in femoral muscle glucose uptake (Nuutila et al 1996). Furthermore, a study in healthy volunteers using positron emission tomography to measure both leg blood flow and tissue glucose uptake during insulin-mediated vasodilatation demonstrated no change in glucose uptake (Raitakari et al 1996). It has been argued that whereas blood flow appears to contribute to glucose disposal after an oral glucose tolerance test (Baron et al 1990), it makes little contribution after a more physiological mixed meal (Mijares and Jensen 1995), and that the ability of insulin to stimulate leg blood flow is not impaired after such a meal in NIDDM (Dela et al 1995). It should be noted that volunteers are essentially immobilised

in these experiments, which may in itself be unphysiological: studies in the isolated perfused rat hind-limb demonstrate that, in the context of muscle contraction, blood flow is a more potent determinant of glucose uptake (Hespel et al 1995).

Haemodynamic determinants of blood flow are an attractive hypothesis for the more global forms of insulin resistance found in obesity and NIDDM, but it is difficult to reconcile a haemodynamic mechanism with the reported pathway-specific nature of insulin resistance in essential hypertension (Ferrannini et al 1987; Natali et al 1991). While this is an important consideration, it should be noted that data on pathway specificity are based entirely on the technique of indirect calorimetry and have been extensively studied by only one group of investigators.

1.7 Haemodynamic influences on insulin sensitivity

From the above considerations, it appears that in some conditions, insulin sensitivity may be determined, at least in part, by blood flow to insulin-sensitive tissues. While structural phenomena such as capillary rarefaction and vascular smooth muscle hypertrophy (Folkow 1979) might account for haemodynamic differences in insulin sensitivity between essential hypertensives and control subjects, blood flow to skeletal muscle is also regulated by neural, hormonal, and paracrine mechanisms. For example, a change in the basal functional activity of the sympathetic nervous system or the circulating renin-angiotensin system, or a change in the basal endothelial release of locally-acting vasoactive substances (for example angiotensin II, endothelin, or nitric oxide) might result in an acute change in insulin sensitivity. In addition, functional impairments in insulin-mediated

vasodilatation might ultimately be causally related to structural abnormalities: chronic up- or down-regulation of any of these systems might result in capillary rarefaction and microvascular structural changes, potentially leading to more stable insulin resistance (Julius et al 1991).

1.7.1 Sympathetic nervous system

It has already been mentioned that acute euglycaemic hyperinsulinaemia concomitantly stimulates peripheral sympathetic efferent outflow and vasodilatation (Anderson et al 1991 - Section 1.2): the net effect in healthy volunteers is no change in blood pressure. However, there does appears to be "cross-talk" between insulin and the sympathetic nervous system: insulin resistance and consequent hyperinsulinaemia may activate the sympathetic nervous system (Rowe et al 1981); alternatively, activation of the sympathetic nervous system (by lower body negative pressure) may cause acute insulin resistance (Jamerson et al 1992). Adrenaline, and to a lesser extent noradrenaline, can reduce insulin sensitivity acutely partly by stimulating hepatic gluconeogenesis but predominantly by blocking peripheral glucose uptake (Sacca et al 1980; Rizza et al 1979). In addition, it has been reported that physiological vasoconstrictor effects of both specific α2-agonists and reflex sympathetic activation (probably mediated by both α1- and α2-adrenoceptor mechanisms) in the forearm can be attenuated by intra-arterial infusion of insulin (Lembo et al 1993; Lembo et al 1994).

Loss of insulin-mediated attenuation of α-adrenergic vasoconstriction could potentially occur in insulin resistant states, and result in both decreased skeletal

muscle blood flow and decreased insulin-mediated glucose uptake. An alternative perspective comes from work by another group (Scherrer et al 1993; Vollenweider et al 1994), who have suggested that insulin-mediated vasodilatation may predominantly be a *central* effect of insulin to increase sympathetic outflow occurring via non-cholinergic non-adrenergic *vasodilator* fibres of the sympathetic nervous system, and that attenuation of this mechanism, rather than loss of peripheral insulin-mediated attenuation of α-adrenergic vasoconstriction, may lead to decreased insulin-mediated vasodilatation in obese subjects.

Such data are consistent with a haemodynamic hypothesis of the physiological regulation and pathophysiological impairment of insulin sensitivity. However, neither increased sympathetic nervous system activation (Jones et al 1979; Lindqvist et al 1993; Floras and Hara 1993) nor decreased forearm blood flow are universal features of essential hypertension, and the area is replete with contradictory findings. For example, it has been reported that systemic hyperinsulinaemia *augments* rather than attenuates, noradrenaline pressor responsiveness in healthy volunteers (Gans et al 1991) and obese subjects (Baron et al 1994a). Furthermore, another group of investigators have reported α-adrenergic sympathetic effects of systemic hyperinsulinaemia in patients with NIDDM which become attenuated as the disease progresses (Tack et al 1996a).

If a physiological balance exists in normal subjects between insulin-mediated vasodilatation and sympathetic nervous system mediated vasoconstriction, it would be predicted that euglycaemic hyperinsulinaemia in subjects with spinal cord injuries would result in a depressor response. However, in such a study,

unexplained mild insulin *resistance* associated with an absence of a rise in plasma adrenaline was reported in a small group of such subjects compared with their healthy siblings; no blood pressure data were presented, but the authors did not comment on any hypotensive responses (Karlsson et al 1995).

In summary, data regarding the interaction between the sympathetic nervous system and insulin sensitivity both in physiological and pathophysiological states are conflicting, and it is difficult to dissect out the hormonal and haemodynamic effects of particular interventions on individual aspects of the complex homeostatic mechanisms in play. While most work has concentrated on the vasoconstricting effects of α-adrenergic stimulation, as induced experimentally by lower body negative pressure, physiological sympathetic system activation is more likely to include concurrent vasodilating $ß_2$-adrenergic stimulation. A complicating factor is that many euglycaemic clamp investigations carried out in humans utilise the hand-warming technique for arterialisation of venous blood (Ferrannini et al 1987; Yki-Jarvinen et al 1987). This in itself may have independent haemodynamic effects and may influence sympathetic nervous system activity (Moan et al 1995; Section 1.5.2(4) and Chapter 4).

1.7.2 Renin-angiotensin system

The renin-angiotensin (RAS) system is a complex neuroendocrine system with circulating, tissue, and tissue-bound components (Ganten et al 1989). It is important in the physiological regulation of blood pressure in response to changes in dietary sodium. The main effector hormone angiotensin II (ANGII), formed from angiotensin I by angiotensin converting enzyme (ACE) has diverse effects in

the heart, vasculature, and kidneys (Weidmann et al 1993). Hyperinsulinaemia promotes sodium re-absorption in the proximal renal tubule in patients with insulin resistant states (Rowe et al 1981), so that insulin resistant patients have a high total body sodium (O'Hare et al 1985). Failure of suppression of the RAS in response to this insulin-mediated sodium retention is a potential mechanism by which sodium accumulates in diabetes (Weidmann et al 1993).

Interest in the interactions between the RAS and insulin-mediated glucose uptake originated from reports of effects of ACE inhibitors on glucose metabolism (see Section 1.1.6) and evidence of enhancement by insulin of ANGII-mediated aldosterone release (Rocchini et al 1990). There are few data on the effects of individual components of the RAS on glucose transport, but it is often assumed that ANGII, like other counter-regulatory hormones, down-regulates pathways of insulin-mediated glucose metabolism: it has been shown to have gluconeogenic and glycogenolytic properties (DeWitt and Putney 1983; Kneer and Lardy 1983), and may enhance release of norepinephrine, an insulin antagonist, from sympathetic nerves (Taddei et al 1995).

Although most available data suggest that ACE inhibitors have very little effect on insulin sensitivity (Section 1.1.6), some investigators have reported decreases in fasting glucose or increases in insulin action. This has led to speculation regarding whether such effects are either hormonal or haemodynamic, and whether they are mediated by angiotensin II withdrawal or bradykinin potentiation (Jauch et al 1987, Dietze et al 1996). Previous studies have examined whether any insulin-sensitising effect of ACE inhibitors is likely to be due to angiotensin II

withdrawal by administering systemic infusions of angiotensin II to healthy volunteers during euglycaemic clamp studies (Morris et al 1993; Widgren et al 1993; Fliser et al 1993; Buchanan et al 1993). Data from these studies indicate that angiotensin II *increases* insulin sensitivity when given in pressor doses, and has a small insulin-sensitising effect (detected only in larger studies) when given in weakly pressor doses.

The mechanism for such unexpected insulin-sensitising effects of ANGII has been elucidated in studies in which leg blood flow was measured during systemic ANGII infusions: at high doses of this potent vasoconstrictor hormone, blood flow to the leg vascular bed actually *increased* as a result of redistribution from the renal vascular bed (Buchanan et al 1993). Thus the effect of angiotensin II on insulin sensitivity was accounted for by a haemodynamic rather than hormonal mechanism. In a similar study in patients with NIDDM, using subpressor and weakly pressor doses of angiotensin II (calculated to have no effect on renal blood flow), a similar increase in insulin sensitivity was observed (Morris et al 1994c). Since this effect was larger at the lower dose, was not associated with an increase in blood pressure, and was not associated with a change in insulin-mediated disposal of other substrates (potassium, triglycerides), it was postulated that there may be an additional non-haemodynamic effect of angiotensin II on insulin-mediated glucose disposal. One potential candidate mechanism for such an effect is ANGII-mediated activation of protein kinase C via receptor-linked formation of diacylglycerol (Smrcka et al 1991, Considine and Caro 1993). Alternatively, there is recent evidence that ANGII inhibits insulin-stimulated PI 3-kinase activity, and it is reasonable to speculate that this may in turn inhibit insulin-stimulated

translocation of glucose transporter proteins to the plasma membrane (Velloso et al 1996).

These observations were derived from acute experiments and are difficult to reconcile with the reported effect of ACE inhibition on insulin sensitivity, unless such effects are mediated by potentiation of bradykinin (Dietze et al 1996). Studies examining the effect of longer-term activation of the renin-angiotensin system (by dietary salt depletion) on glucose metabolism have been limited to healthy volunteers, and the findings have been conflicting (Section 6.4). All three of these studies were open in design, and all compared a diet higher in sodium than would be achieved in most populations with a diet lower in sodium than is easily achievable outwith a clinical research environment.

As far as can be ascertained, the effect of dietary salt depletion on insulin sensitivity has not previously been investigated in patients with NIDDM.

1.7.3 Endothelial release of nitric oxide and other paracrine substances

Endothelial cells form a monolayer lining the luminal surface of all blood vessels. Over the last fifteen years, the importance of the vascular endothelium in the regulation of peripheral vascular tone has been recognised (Furchgott and Zawadzki 1980), in addition to its important effects on the regulation of haemostasis, the control of cell growth, and the transport of plasma molecules (Henderson 1996). Endothelial cells synthesise and secrete vasodilator (endothelium-derived relaxing factor, endothelium-derived hyperpolarising factor,

prostacyclin) and vasoconstrictor (endothelin, thromboxane, prostaglandin H_2) substances (Boulanger and Vanhoutte 1994). It is now clear that endothelium-derived relaxing factor (EDRF) is the inorganic gas nitric oxide (NO) (Palmer et al 1987) which has multiple effects in co-ordinating and regulating the cardiovascular system. In particular, NO causes relaxation of vascular smooth muscle by activating guanylate cyclase. It is synthesised from the amino acid L-arginine by three isoforms of NO synthase: endothelial (eNOS), neuronal (nNOS), and inducible (iNOS): eNOS is constitutive in nature, but can be activated by 5-hydroxytryptamine, substance P, or bradykinin.

Endothelial damage may be an early step in the pathogenesis of atherosclerosis (Ross 1993) and it is has been proposed that asymptomatic endothelial dysfunction may be an early marker for this condition (Henderson 1996). One mechanism by which endothelial damage may occur is local generation of superoxide anions, either by endothelial cells or by monocytes attached to the endothelium (Chin et al 1992, Keaney 1993). Although the precise mechanism by which superoxide anions are generated is unknown, it may relate to dyslipidaemia, particularly high concentrations of VLDL, low concentrations of HDL (see Section 1.1.3), and high concentrations of small, dense LDL or oxidised LDL (Witzum 1993). The presence of superoxides causes enhanced destruction of nitric oxide, limiting the availability of NO to promote vasodilation.

Although it may be an oversimplification to envisage a global syndrome of endothelial dysfunction, there have been two main approaches towards quantifying endothelial function *in vivo* for prospective studies. The first of these is

measurement of various endothelial cell products released during injury, including von Willebrand factor (Stehouwer et al 1991), thrombomodulin (van den Berg 1995, Blann et al 1996), and tPA (Jensen et al 1989). This is a simple approach and can be easily applied to large groups of subjects; however, many of the markers are not specific to endothelial cells. The second approach is to use dynamic tests, which are more sophisticated and labour-intensive. Venous occlusion plethysmography (Section 2.7) is used to measure forearm blood flow (which is mainly determined by small skeletal muscle resistance vessels) in response to endothelium-dependent and -independent vasodilators and vasoconstrictors. Endothelial muscarinic receptors (Panza et al 1990), or bradykinin receptors (Kelm et al 1996), can be stimulated to cause NO release by infusing acetylcholine or bradykinin locally into the brachial artery; while the blood flow response is measured distally. Sodium nitroprusside, which is an endothelium-independent donor of NO, can be used as an experimental control for sensitivity of adjacent vascular smooth muscle to NO, emulating *in vivo* the above-mentioned experiments of Furchgott in the organ bath (Furchgott and Zawadzki 1980). An alternative, less invasive, test of endothelial function, involving measurement of flow-mediated vasodilatation, has been described using high resolution doppler ultrasound with wall-tracking following release of a wrist cuff inflated to suprasystolic pressures (Celermajer et al 1992, Celermajer et al 1993).

Inhibition of endothelial nitric oxide synthase (eNOS) in the forearm using local infusion of its stereospecific substrate inhibitor N_G-monomethyl-L-Arginine (L-NMMA) results in a 30-40% decrease in blood flow (Vallance et al 1989). This

demonstrates the importance of endothelial NO production in determining basal forearm blood flow, and provides the basis for an alternative dynamic test of endothelial function. The vasoconstrictor response to L-NMMA is an index of basal endothelial nitric oxide production, i.e. the proportion of resting vasodilator tone which can be attributed to NO production (Calver et al 1992). In this situation, vasoconstrictor responses to noradrenaline, or another endothelium-independent vasoconstrictor, may be used as an experimental control.

Decreased basal endothelial nitric oxide synthesis has been reported using this technique in essential hypertension (Calver et al 1992), although responses to endothelium-dependent agonists vary between studies (Panza et al 1990; Cockcroft et al 1994). In addition, impaired endothelium-dependent vasodilatation has been demonstrated in normotensive subjects with a family history of essential hypertension (Taddei et al 1992). Other conditions characterised by impairment of agonist-stimulated or basal endothelial function include hypercholesterolaemia (Creager et al 1990; Chowienczyk et al 1992), atherosclerosis (Chester et al 1990, Egashira et al 1993, Anderson et al 1995), NIDDM (McVeigh et al 1992), obesity (Steinberg et al 1996) and cigarette smoking (Jacobs et al 1993; Kiowski et al 1994). It is striking that many of these conditions are also characterised by impairment of insulin-mediated glucose uptake (Section 1).

Like insulin sensitivity, endothelial function is modifiable by non-pharmacological and pharmacological interventions. Exercise (Hornig et al 1996) and lipid-lowering treatment (Leung et al 1993; Treasure et al 1995) can improve

endothelial function. L-arginine, the substrate of NO synthase, either co-infused with endothelium-dependent vasodilators intra-arterially (Creager et al 1992; Imaizumi et al 1992) or orally (Clarkson et al 1996) is reported to normalise endothelial function in hypercholesterolaemic subjects. Both ACE-inhibitors and calcium antagonists may improve endothelial function in hypertensive subjects (Lyons et al 1994), but there are also negative studies (Kiowski et al 1993).

The antioxidant vitamin E has been shown to prevent the onset of defective endothelium-dependent relaxation in streptozotocin diabetic rat aorta (Keegan et al 1995), and vitamin C improves endothelial function in patients with NIDDM (Ting et al 1996). The beneficial effect of the antioxidant lipid-lowering agent probucol on coronary endothelium-dependent relaxation in man was greater than that produced by a similar degree of lipid lowering therapy using another agent (Anderson et al 1995). There is some evidence that positive effects on endothelial function may translate into actual outcome benefits: in the Cambridge Heart Antioxidant Study study, pharmacological doses of vitamin E were associated with decreased coronary events compared with placebo when administered to patients following myocardial infarction (Stephens et al 1996).

Most of the agents above which alter endothelial function are reported to produce similar effects on insulin sensitivity. One of the few agents which appears to produce a dissociated effect is fish oil, which has a neutral or adverse effect on insulin sensitivity (Section 1.1.6), and a beneficial effect on endothelial function (Chin and Dart 1994).

The similarities between the conditions in which endothelial dysfunction and insulin resistance are observed, and the improvements produced in both abnormalities by similar interventions, raise the hypothesis that they are physiologically linked. It has recently been demonstrated in experiments co-infusing insulin and L-NMMA in human skeletal muscle vascular beds, that insulin-mediated vasodilatation is dependent on endothelial nitric oxide synthesis/release (Scherrer et al 1994; Steinberg et al 1994). This is corroborated by *in vitro* data demonstrating inhibition of insulin-mediated vasodilatation in rat cremaster muscle by L-NMMA (Chen and Messina 1996). Thus, endothelial dysfunction may cause insulin resistance either indirectly, or by attenuating insulin-mediated endothelium-dependent vasodilatation. Alternatively, decreased insulin sensitivity may cause decreased basal production of eNOS, perhaps by limiting availability of L-arginine substrate or one of the other cofactors required for NO synthesis. A third possibility is that decreased basal endothelial nitric oxide production and impaired insulin sensitivity are manifestations of a common genetic or environmental antecedent, such as impaired vascular development. As far as can be ascertained, endothelial function has not previously been measured in a group of subjects characterised for insulin sensitivity using the euglycaemic clamp technique.

1.8 Aims of thesis

To assess:

1) the effect of insulin assay specificity on the relationships among serum insulin concentrations, insulin sensitivity, and blood pressure in diabetic and non-diabetic subjects with and without essential hypertension.

2) the effect of sustained physiological activation of the renin-angiotensin system induced by moderate dietary sodium restriction on insulin sensitivity in patients with NIDDM.

3) To assess the relationship between endothelial function and insulin sensitivity in healthy subjects.

Chapter 2

Methods

2.0 Summary

In this chapter, the general protocols for the clinical techniques used in the studies presented in the thesis are described.

2.1 Patients and healthy volunteers

All the clinical assessments were performed in the Clinical Investigation and Research Unit (CIRU), Department of Medicine and Therapeutics, Western Infirmary.

Non-diabetic hypertensive patients were recruited from patients attending either the Glasgow Blood Pressure Clinic or the Cardiovascular Risk Factor Clinic at the Western Infirmary, while subjects with NIDDM were identified from patients attending the Diabetes Clinics at Gartnavel General Hospital. Non-diabetic normotensive control subjects and healthy volunteers were recruited by advertisement (medical and nursing students were excluded). A total of 113 healthy volunteers and patients with essential hypertension and/or NIDDM completed the protocols, all of which were individually approved by the Ethics Committee of the West Glasgow Hospitals University NHS Trust.

Hypertensive subjects were either newly diagnosed and previously untreated, or patients in whom current antihypertensive therapy was ineffective or poorly

tolerated. Previously-treated patients discontinued their medication and were assessed for recruitment against the predefined entry criteria after a treatment-free period of at least four weeks.

NIDDM was defined according to WHO criteria (World Health Organisation 1980). Newly-diagnosed patients were stabilised on diet for two months prior to study entry, and patients with retinopathy (by direct fundoscopy), microalbuminuria, and those whom had been previously treated with insulin or oral hypoglycaemic agents were excluded.

Healthy volunteers who were taking any form of medication were excluded. Patients whom had been treated with thiazide diuretics in the previous six months were excluded, as were patients with: secondary forms of hypertension, co-existing disease requiring regular medication, a raised serum creatinine, a history of myocardial infarction, angina pectoris, intermittent claudication or stroke, or alcohol intake > 20 units weekly.

All subjects participating in these studies underwent an oral glucose tolerance test in order to screen for impaired glucose tolerance or confirm the previous diagnosis of diabetes.

2.2 General clinical protocol

Before study entry, all patients and volunteers underwent a health questionnaire and full clinical screening including physical examination, routine biochemistry,

haematology, dip-stick urinalysis, and an electrocardiogram as a screening test for significant cardiovascular disease or end-organ damage.

After informed consent was obtained, each individual was asked to refrain from any strenuous exercise for the duration of the study and to maintain his or her usual diet. Patients with NIDDM were instructed to adhere to an isocaloric diet throughout the study consisting of approximately 55% carbohydrate, 25% fat, and 20% protein.

On each day on which a clinical assessment was to be performed, subject were transported by taxi to the CIRU at 0800hrs after an overnight fast from 2200hrs. All subjects were asked to avoid alcohol and caffeine in the 24 hours prior to an assessment; in those studies in which smokers were not excluded, subjects were asked to refrain from smoking for 24 hours prior to attendance. A light meal was provided at the end of each assessment prior to taxi transport home.

2.3 Clinical and morphometric measurements

2.3.1 Body mass index

Body weight and height were measured with subjects in light clothes and without shoes to the nearest 0.5 kg of weight and to the nearest 0.5 cm of height. The same equipment was used throughout the study, and the calibration of the weighing scales (Seca, Germany) was checked regularly. Body mass index (BMI, kg/m^2) was calculated as:

$$BMI = \frac{\text{Body weight (kg)}}{(\text{height (m)})^2}$$

2.3.2 Waist-to-hip-ratio

Waist circumference (midway between the lowest rib margin and the iliac crest at the end of gentle expiration), and hip circumference (at the widest level of the greater trochanters), were measured to the nearest 0.5 cm while the subject was standing.

2.3.3 Blood pressure and heart rate

During all the clinical studies the technique of blood pressure and heart rate measurement was uniform. Systolic and diastolic blood pressure, and heart rate were measured after 10 minutes supine rest by an oscillometric technique using a Dinamap Critikon (Johnson and Johnson Professional Products Ltd., U.K.) semi-automatic sphygmomanometer, maintained and calibrated at regular intervals by the Department of Clinical Physics, Western Infirmary. Small (22cm x 10 cm), medium (31cm x 12 cm), and large (39cm x 16 cm) blood pressure cuffs were available at all times in order to comply with the recommendations of the British Hypertension Society (Petrie et al 1986). Hypertension was defined as a mean supine diastolic BP of $\geq$ 95 mmHg, or systolic BP $\geq$ 160 mmHg, on three readings after 10 minutes supine rest (Sever et al 1993).

2.3.4 Oral glucose tolerance test

Subjects attended the CIRU according to the usual protocol (Section 2.2). An intravenous cannula (Venflon, Helsinborg, Sweden) with a three-way tap was placed in an antecubital vein. After 20 minutes of semi-recumbent rest, a blood sample was withdrawn from the cannula. Subjects ingested 75 grams of powdered glucose dissolved in 100mls of water orally at 0 minutes, and further blood samples were withdrawn at 30, 60, 90, and 120 minutes for later analysis of plasma glucose and serum insulin concentrations.

2.4 Hyperinsulinaemic clamp technique

Insulin sensitivity was assessed using a modification (180 rather than 120 minutes) of the hyperinsulinaemic clamp described by DeFronzo et al (1979).

2.4.1 General clinical procedure

Subjects attended the CIRU according to the usual protocol (Section 2.2). Two 18 gauge intravenous cannulae were inserted: the first retrogradely into the right dorsal hand vein for blood sampling, and the second antegradely into the left antecubital fossa for administration of infusions. Three-way taps enabled easy sampling and simultaneous infusion of insulin and dextrose.

The infusion of soluble human insulin (Actrapid, NovoNordisk A/S, DK2880 Bagsvaerd, Denmark) was prepared in 45mls (10% vol/vol) of each patient's own

blood in order to minimise adsorption of insulin to the plastic surfaces of syringes and infusion lines. It was administered using a Braun Perfusor pump as a primed, constant rate infusion for 180 minutes, with the aim of achieving a steady-state serum insulin concentration approximately 120μU/ml above the basal fasting level. The priming regime was as follows:

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

20 minutes of supine rest were allowed after placement of cannulae. Blood pressure and heart rate were measured (Section 2.3.3) and baseline blood samples withdrawn before insulin infusion commenced at time(t) = 0 mins. A variable rate infusion of 20% dextrose (Baxter Healthcare, Norfolk, U.K.) was administered via an IMED infusion system (IMED, Abingdon, UK) from t = 2-180 mins (Figure 1.4). Serum glucose concentrations were maintained at euglycaemia (target serum glucose 5.2 mmol/l) in studies comparing insulin sensitivity between groups of subjects with heterogeneous fasting glucose levels (i.e. patients with NIDDM and non-diabetics) (Chapter 5) and in studies within groups of subjects of normal glucose tolerance (Chapters 4 and 7). For the euglycaemic clamps in patients with NIDDM, serum glucose concentrations were gradually normalised with an infusion of soluble insulin (2 U/hr) prior to commencing the procedure. In studies assessing the effect of an intervention on insulin sensitivity in patients with NIDDM (Chapter 6), serum glucose concentrations were maintained at isoglycaemia [target serum glucose = fasting

glucose (Greenfield et al 1981; Saad et al 1994)] in order to avoid the potentially confounding effect of inter-individual variations in fasting serum glucose concentrations (Section 1.5.4). The infusion rate was adjusted for body weight for each individual and expressed as mg glucose/kg body weight/minute (mg/kg/min). For example, the infusion rate of 20% dextrose equal to 1mg/kg/min for a 70 kg individual would be calculated as:

$$\frac{60 \text{ minutes}}{200\text{g glucose}} \times 70\,\text{kg} = 21\ \text{ml/hour}$$

At 5 minute intervals, 2 ml blood samples were withdrawn from the cannulated dorsal hand vein. Cannula patency was maintained using a slow infusion of 0.9% saline; a total of approximately 100 ml was administered during each procedure.After centrifugation at the bedside, the serum glucose concentration was determined (Section 2.8.2), and the dextrose infusion rate was manually adjusted to maintain target serum glucose concentration. The dorsal hand vein was surrounded by a heated box (55 ° C) with the aim of arterialising venous blood (Section 2.6 and Chapter 4).

2.4.2 Calculation of insulin sensitivity from the euglycaemic hyperinsulinaemic clamp

During hyperinsulinaemia with steady-state plasma glucose concentrations (usually during the last 40-60 minutes of the procedure), the rate of glucose infusion is equal to that of glucose removal from the glucose space (i.e. glucose metabolised, **M**). Assuming suppression of endogenous glucose production, the

M-value is an estimate of total body glucose metabolism, and reflects the ability of insulin to enhance tissue glucose disposal (Section 2.5).

In practice, the glucose infusion rate must be modified by two factors before it can be equated with M:

$$M = I - UC + SC$$

where:
- I = glucose infusion rate (mg/kg/min)
- UC = correction for urinary glucose loss (usually negligible during a euglycaemic clamp)
- SC = "space correction" (mg/kg/min) (for inevitable deviations from euglycaemia)

The space correction is calculated as follows (DeFronzo et al 1979):

$$SC = (5.2 - G) \times 17.86 \times 0.095$$

where:
- G = ambient glucose concentration over last 40 minutes of clamp (mmol/l)
- 17.86 = unit conversion factor (mmol/l to mg/dl)
- 0.095 = glucose space constant

When the achieved ambient glucose concentration at steady state is less than the desired value of 5.2 mmol/l (too little glucose has been infused) the space correction will be positive: M will therefore be greater than I; the converse also applies.

In some groups of subjects, particularly those who are obese, use of a weight-adjusted insulin infusion protocol may result in higher steady-state serum insulin concentrations, and in this situation, the M-value underestimates the degree of insulin resistance. The insulin sensitivity index (S_{IP} x 10^4 dl/(min.kg) per mU/l) is therefore calculated from the glucose infusion rate and ambient insulin and glucose concentrations at steady state (Bergman et al 1987):

$$S_{IP} = \Delta R_d / (\Delta I \times G)$$

where:

ΔR_d = increment in glucose uptake (basal to steady state)

ΔI = increment in insulin concentration (basal to steady state)

G = steady state glucose concentration

The reproducibility of M has previously been evaluated in the Clinical Investigation and Research Unit: between-day intra-subject coefficient of variation is 6% in healthy subjects (Morris et al 1994a).

2.5 Measurement of endogenous (hepatic) glucose production using tracer infusates

Hepatic glucose production (both basally and during hyperinsulinaemia) was measured using tracer infusates.

2.5.1 Clinical procedure

The protocol was identical to that given above (Section 2.4.1) except for the following modifications. A primed continuous infusion of HPLC purified [3-^{3}H]glucose was given during a 2 hour equilibration period (t = -120 to 0 mins, and continued throughout the procedure). In order to avoid the underestimation of endogenous glucose production associated with inter-compartmental tracer fluxes, a variable rate infusion of exogenous 20% dextrose pre-labelled with [3-^{3}H] glucose aiming to match basal plasma glucose specific activity was administered from 2-150 minutes (Finegood et al 1987, Levy et al 1989, Finegood et al 1990, Hother-Nielsen and Beck-Nielsen 1990, Hother-Nielsen et al 1992, Neely et al 1992) .

2.5.2 Calculation of endogenous glucose production

The specific activity of glucose in plasma is measured in disintegrations per minute (dpm) per μmol of plasma glucose (Section 2.8.3). At steady state, the total rate of glucose appearance (Tra, μmol/min) can be calculated by dividing the rate of tracer administration (dpm/min) by the glucose specific activity (SA = dpm/μmol) of plasma. Endogenous (hepatic) glucose production (EGP) is determined by subtracting the known rate of exogenous glucose infusion (I) [per kg of body weight] from the rate of glucose appearance ($R_{a)}$ [per kg of body weight].

$$EGP = R_a - I$$

In practice, steady state assumptions may not be fully met, and the non-steady state equations of Steele et al (1956), as modified by DeBodo (1963) may be used as follows to determine rates of glucose appearance (R_a) and disappearance (R_d) during t = -30 to 0 mins and t = 120 to 150 mins, assuming a pool fraction of 0.65 and an extracellular volume of 190 ml/kg.

The calculation of endogenous glucose production (EGP) can be summarised as follows:

i) Assuming steady state (ss):

$$Tra_{(ss)} = Ftotal/SA \ (\mu mol/min)$$

where:

SA = glucose specific activity (dpm/min)

F_{total} = $(R_{const} \times F_{const}) + (R_{dex} \times F_{dex})$ (dpm/min)

F_{const} = specific activity of constant [3-^{3}H]glucose infusion (dpm/min)

R_{const} = rate of constant [3-^{3}H]glucose infusion (ml/min)

F_{dex} = specific activity of variable [3-^{3}H]glucose infusion (dpm/min)

R_{dex} = rate of variable [3-^{3}H]glucose infusion (ml/min)

ii) Adjusting for deviations from steady state:

$Tra_{(nss)}$ = $Tra_{(ss)}$ - error term

where:

Error term = (p.V.G.dSA/dt.weight)/SA (μmol/min)

and:
- p = pool fraction (0.65)
- V = volume of distribution (190 ml/kg)
- G = plasma glucose concentration (mmol/l)
- SA = glucose specific activity of plasma (dpm/min)
- weight = weight (kg)

iii) Final steps:

$Ra_{(ss)}$ = Tra(ss)/weight (μmol/kg/min)

$Ra_{(nss)}$ = Tra(nss)/weight (μmol/kg/min)

R_d = R_a - $d_{(pool)}$

where:

$d_{(pool)}$ = p.V.dG/dt

(see above)

GIR = (R_{dex} x C_{Dex})/weight (μmol/kg/min)

(see above)

where:

GIR = rate of exogenous glucose infusion (μmol/kg/min)

C_{dex} = 20% dextrose concentration in μmol/ml

EGP = $Ra_{(nss)}$ - GIR (μmol/kg/min)

(divide by conversion factor 5.55 for mg/kg/min)

2.6 Arterialisation of venous blood

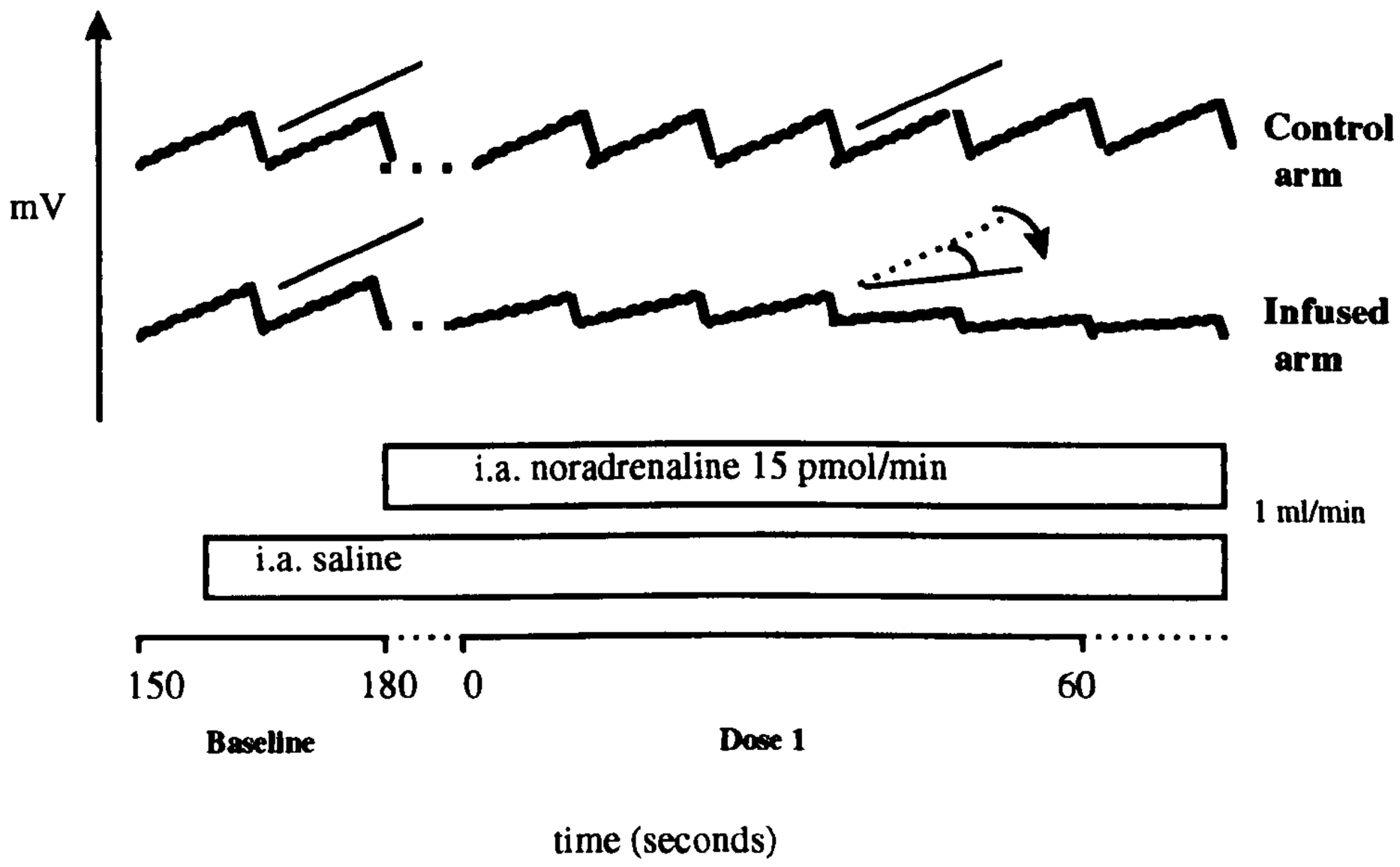
In the original description of the euglycaemic clamp, the glucose infusion rate was adjusted according to glucose values in arterial blood (DeFronzo et al 1979). However, in order to avoid cannulation of systemic arteries, many investigators now adjust the glucose infusion rate on the basis of "arterialised" venous blood withdrawn from the retrogradely cannulated dorsal hand vein; this technique was examined in the study described in Chapter 4, and was used in the clinical studies described in Chapter 5, 6 and 7. Thus, the ipsilateral hand was placed in a cylindrical perspex heated-air hand box at the beginning of the period of supine rest prior to the start of the procedure (Liu et al 1992). Under thermostatic conditions, the heated box maintains the ambient temperature surrounding the hand at 55°C (University of Nottingham, Department of Physiology and Pharmacology, UK).

2.7 Forearm venous occlusion plethysmography

Forearm blood flow (FBF) was measured in the studies described in this thesis using forearm venous occlusion plethysmography (Chapters 3,4, and 7). In this technique, the measurement of flow is derived from the rate of change in forearm volume during intermittent occlusion of venous return (with continued arterial inflow). The original technique, dating from the late nineteenth century, required the use of cumbersome water jackets to measure changes in forearm volume (reviewed in Greenfield et al 1963), but has been more readily applicable since the advent of mercury strain gauges together with a mathematical method for deriving

changes in forearm volume (expressed in ml of blood flow/100ml forearm/ minute) from changes in circumference (Whitney 1953). The development of an electrical calibration technique for strain gauges (Hokanson 1975), and the availability of computerised chart recorders, has led to increasing use of the technique to assess the effects on resistance vessel tone of agents infused directly into the brachial artery at subsystemic doses (Barcroft et al 1954, Collier and Robinson 1974, Benjamin et al 1989, Benjamin et al 1995, Calver et al 1992). An additional refinement is the simultaneous measurement of forearm blood flow in both arms: measurements in the experimental arm are adjusted for systemic changes unrelated to the local stimulus by expressing the two measurements as a ratio:

Figure 2.1 Plethysmographic traces: a schematic representation



$$\frac{\frac{F(i)_d}{F(ni)_d} - \frac{F(i)_v}{F(ni)_v}}{\frac{F(i)_v}{F(ni)_v}} \times 100\%$$

where:

F = flow

i = infused arm

ni = non-infused arm

v = vehicle

d = drug

The reproducibility of FBF measurement by this technique is evaluated in Chapter 3.

2.7.1 General clinical procedure

Temperature was maintained at 25-26°C and lighting was dimmed; the room was quiet and sealed. Subjects lay supine with arms supported on foam blocks in the same position on each occasion. Paediatric arterial occlusion cuffs (Hokanson SC5, PMS instruments, Maidenhead, Berkshire) were placed around the wrists and inflated to 200 mmHg for three minutes during each set of recordings. Collecting cuffs were placed around the upper arms, and inflated and deflated (40

mmHg) in a 15 second cycle (Hokanson SC10). Rapid cuff inflation was achieved using a commercially available air source (Hokanson AG101), coupled to rapid cuff inflators (Hokanson E20). On the first study day, the left forearm was measured at the largest circumference, and a strain gauge (Hokanson forearm set) 2 cm shorter was selected. The distance from the olecranon was measured and recorded in order to standardise strain gauge position from day to day. The strain gauge was calibrated electrically on the arm to the chart recorder programme (MacLab, AD instruments, UK) and test readings were recorded.

In studies examining the effect of local intra-arterial infusions on FBF, a 27G unmounted steel needle (Cooper's Needleworks, Birmingham, U.K.) was inserted under local anaesthesia into the brachial artery of the non-dominant arm for drug infusion. Cannula patency was maintained using an infusion of 0.9% saline (1 ml/min). All solutions were prepared in 0.9% sodium chloride in the Pharmacy Sterile Productions Unit, Western Infirmary.

Blood flow recordings began 45 seconds after wrist cuff inflation, and continued for 135 seconds. Subjects were allowed to acclimatise to inflation and deflation of the wrist and upper arm cuffs for 30 minutes before two baseline measurements 10 minutes apart: the mean of these was used as the baseline FBF. Local incremental doses of drugs (see Chapter 7) were dissolved in 0.9% saline and infused intra-arterially at a constant rate of 1ml/min.

2.7.2 Calculation of forearm blood flow

Data were acquired via a MacLab II chart recorder (AD instruments, Hampstead, London). Forearm blood flow (FBF) was derived from the slope of the plethysmographic traces (Figure 2.1) on the chart recorder according to the equation of Whitney (1953). Each blood flow measurement was the mean of five sequential recordings. Slopes were calculated from data points by acquiring co-ordinates using MacChart software (AD instruments), and pasting them into a customised spreadsheet.

2.8 Laboratory methods

Venous blood samples for laboratory assay were withdrawn from the right dorsal hand vein cannula and collected into plain (insulin, electrolytes, triglycerides), lithium heparin (aldosterone), potassium EDTA (renin), or heparinised tubes (blood gas analysis). Samples for AII measurement were collected into tubes containing a mixture of enzyme inhibitors (25 mM σ-phenanthroline, 125 mM EDTA, 2% ethanol, 0.2% neomycin sulphate, and 20 μM human renin inhibitor H142). All samples were immediately placed in crushed ice prior to centrifugation (3000 rpm, 4 °C), decanting, and storage at -20 °C, except those for insulin (which were kept at room temperature for 20 minutes prior to processing). Samples for AII measurement were stored at -70 °C. Blood gas, lipid, electrolyte, and glucose analyses were performed in the hospital routine laboratory on the day of sampling. Other assays were performed in batches in the laboratories of the Department of Medicine and Therapeutics.

2.8.1 Serum insulin concentrations

Serum insulin concentrations were determined using three commercially available insulin assay kits, with different degrees of cross-reactivity with proinsulin and its partially processed split and des- amino products. The Lifescreen Insulin EASIA, (Watford, U.K) is an immunoenzymometric assay specific for insulin (inter-assay coefficient of variation (CV) = 6.7% at 4.2 mU/l, 3.5% at 9.8 mU/l, and 3.3% at 81 mU/l). The Incstar insulin assay (Stillwater, Minnesota, USA) is a radioimmunoassay with a molar cross-reactivity with intact proinsulin of 30% (inter-assay CV 8-11%). The Pharmacia insulin RIA 100 (Uppsala, Sweden) is a radioimmunoassay with a molar cross-reactivity with intact proinsulin of 62% (inter-assay CV = 5.8% at 11.6 mU/l, 6.4% at 32.7 mU/l, and 6.5% at 65.2 mU/l). Serum insulin concentrations were expressed in uU/ml. The assay used in each study was dictated by the experimental design: the Incstar assay was used to measure the level of hyperinsulinaemia achieved at steady state during clamp procedures, while serum insulin concentrations measured with both the Lifescreen and Pharmacia assays were used as "specific" and "conventional" assays in the assessment of the importance of assay specificity (Chapter 5).

2.8.2 Serum and plasma glucose concentrations

During the hyperinsulinaemic clamp procedures, serum glucose was measured at the bedside by the glucose oxidase method using a Beckman 2 glucose analyser (Beckman Instruments, Fullerton, CA, USA; inter-assay coefficient of variation = 2%). Results were expressed in mmol/l. For assessment of glucose levels during

oral glucose tolerance tests, samples were collected in as plasma in fluoride-oxalate tubes and analysed in the hospital routine biochemistry laboratory.

2.8.3 Glucose specific activity

Plasma for glucose specific activity was deproteinized using $Ba(OH)_2$ and $ZnSO_4$ by the method of Somogyi (Somogyi 1945); after centrifugation, the supernatant was passed sequentially through anion and cation exchange columns to remove charged molecules. ^3H activity was detected in an automatic liquid scintillation counter. Results were expressed in dpm/μmol (Section 2.5.2). Aliquots of tracer infusate and labelled exogenous dextrose infusion were spiked into fasting venous plasma and were processed in parallel with plasma samples to allow calculation of [3-^3H]glucose infusion rates.

2.8.4 Plasma aldosterone concentrations

Plasma aldosterone concentrations were measured in batches by a commercially available radioimmunoassay kit (Biodata, Milan) according to the technique described by MacKenzie and Clements (1974). Results were expressed in ρmol/l. The intra- and inter-assay coefficents of variation were 5.0 and 5.4% respectively.

2.8.5 Plasma renin activity

Plasma renin activity was determined by radioimmunoassay of angiotensin I formation from angiotensinogen using a commercially available kit (Biodata, Milan); results were expressed as ng AI/ml/hour. Intra- and inter-assay coefficients of variation were 4.9% and 7.8% respectively.

2.8.6 Plasma angiotensin II concentrations

Plasma ANG II was assayed after HPLC separation by direct radioimmunoassay according to the technique described by Ducsterdieck and McElwee (1971). Results were expressed in ρmol/l. The intra- and inter-assay coefficients of variation were 6.4% and 8.8% respectively (Morton and Webb 1985).

2.8.7 Serum potassium concentrations

Potassium (mmo/l) was measured in the hospital routine biochemistry laboratory by diluting ion-selective electrode on an Olympus AU5200 auto-analyser.

2.8.8 Blood gas analysis

Blood gas analysis, including partial pressure of oxygen (pO_2, kPa), was performed in the hospital routine biochemistry laboratory using a Corning 288 blood gas analyser (Ciba Corning Diagnostics, Halsted, UK).

2.8.9 Serum lipid concentrations

Cholesterol and triglycerides (mmol/l) were measured in the hospital routine biochemistry laboratory using an enzymatic technique in a Multistat I/LS centrifugal analyser.

2.9 Statistical analysis

All data were checked for normality using the Shapiro-Wilks test (Minitab statistical package, Minitab Inc, Pennsylvania, USA), and skewed data were logarithmically transformed (log_{10}). Where appropriate, summary measures including area-under the curve (AUC) were calculated (Matthews et al 1990). The following procedures were performed using Minitab and are described in the individual chapters: Bland-Altman plots (Altman 1991), paired and unpaired t-tests; simple correlation (Pearson); multiple linear regression; one-, two-, and three-way analysis of variance (subject, treatment, time and their interactions fitted as terms). 95% confidence intervals are given in most cases. Unless otherwise stated, the level of statistical significance (α) was taken to be $p < 0.05$; where comparison of repeated measures made over time between groups was required, three-way analysis of variance was performed with Bonferroni-corrected t-tests at individual time-points. Coefficients of variation were calculated from the square root of the total error term of the adjusted mean squares from two-way ANOVA (Bland and Altman 1996). Where normally distributed data are grouped, the error bars show mean ± standard deviation.

Chapter 3

The reproducibility of bilateral forearm venous occlusion plethysmography

3.0 Summary

Forearm venous occlusion plethysmography is frequently used to assess the effects on vascular tone of agents infused directly into the brachial artery at subsystemic doses. In the studies described in this thesis, the technique was used both in the assessment of forearm blood flow (FBF) responses to hand-warming (Chapter 4), and in the assessment of endothelial function (Chapter 7). Prior to commencing these studies, a clinical study examining the reproducibility of forearm plethysmography was conducted.

3.1 Introduction

Forearm plethysmography is frequently used to assess the effects on vascular tone of agents infused directly into the brachial artery at subsystemic doses. Most investigators now use electrically-calibrated strain gauges to derive forearm blood flow (FBF) from changes in forearm circumference induced by intermittent venous occlusion (Whitney 1953, Greenfield et al 1963, Hokanson 1975, Benjamin et al 1995). Despite adequate assessment of the accuracy of strain gauge plethysmography when compared with other techniques (Clarke and Hellon 1957, Dahn and Hallbrook 1970), the reproducibility of the technique is less well established (Roberts et al 1986, Altenkirch et al 1990).

Roberts et al. (1986) studied between-day intra-subject variability of baseline unilateral FBF in six subjects on six occasions using unilateral measurements.

They reported reasonable reproducibility of baseline FBF with intra-subject coefficients of variation (CV) ranging between 7.8 and 15.6% (mean 10.5%), but poor reproducibility after systemic exercise (CV 13.0-29.0%). In contrast, Altenkirch et al. (1990), studied intra-subject variability of baseline unilateral FBF in twelve subjects on three occasions. They reported a pooled CV of 25% and concluded that the technique was "poorly suitable" for pharmacological trials. In a further study, between-day intra-subject FBF reproducibility data were quoted for only a subgroup of subjects, of whom several were excluded from analysis because of poor within-day baseline blood flow reproducibility (Egan et al 1988).

Simultaneous bilateral measurement of FBF as a method of reducing variability was first proposed over 40 years ago in reports of experiments using water-filled plethysmographs (Greenfield and Patterson 1954). The same method has been adopted by many investigators when performing strain-gauge plethysmography: in theory, measurements are adjusted for systemic changes unrelated to the local stimulus by expressing flow in the experimental arm as a ratio of concurrent flow in the non-infused (control) arm (FBF ratio) (Calver et al 1992, Cockcroft et al 1993). However, some investigators simply report data from the experimental arm along with a general statement that blood flow did not change significantly in the control arm (McVeigh et al 1992); some continue to use the unilateral technique (Panza et al 1990, Sakai et al 1993, Clarke et al 1989); and others at times report measurements of forearm vascular resistance (FVR = mean arterial pressure/ FBF)(Benjamin et al 1989, Panza et al 1990). It is possible therefore that use of suboptimal methods for expressing responses may influence the conclusions reached; alternatively relatively invasive measurements may be made on an unnecessarily large number of subjects to detect a difference between groups.

In view of the lack of adequate published information on the reproducibility of this widely used technique, the aims of the present study were to determine: 1) the intra-subject variability of bilateral forearm venous occlusion plethysmography (FBF ratios) in untrained healthy male subjects at rest, after standardised unilateral forearm exercise, and during intra-arterial infusions of vasoconstrictor substances; 2) whether bilateral plethysmography is more reproducible than unilateral plethysmography; and 3) the reproducibility of forearm vascular resistance (unilateral and bilateral).

.

3.2 Methods:

All studies were performed after several months of initial training in plethysmography.

3.2.1 Subjects

All subjects were male non-smokers, normotensive (BP < 140/90 mmHg), with no family history of hypertension or diabetes mellitus.

a) *Study 1.* Nine healthy male subjects were studied on three separate days one week apart at the same time of day, at least three hours after their last meal.

b) *Study 2.* Five healthy subjects were studied fasting at 0900 on two occasions, one month apart.

3.2.2 Clinical procedures

The general procedures for intra-arterial infusions and for measurement and calculation of forearm blood flow are detailed in Section 2.7.1.

a) *Study 1.* The protocol is illustrated in Figure 3.1. Experimental conditions were designed to resemble as closely as possible those reported by other groups (and which were to be used in the studies in this thesis).

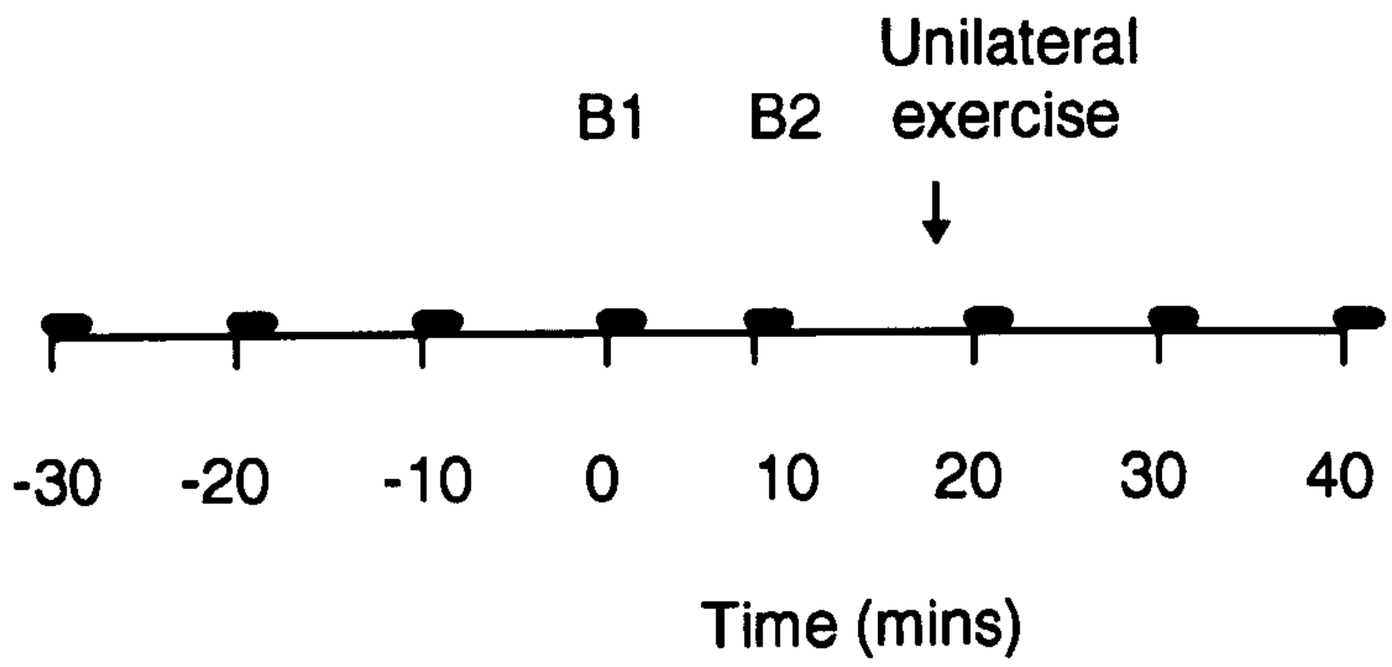
b) *Study 2.* The protocol is illustrated in Figure 3.1. Infusions were: 1) noradrenaline (Levophed, Sanofi-Winthrop) 15, 30, 150, 300 pmol/min; and 2) angiotensin II (Hypertensin, Ciba-Geigy) 1, 5, 10, 50 pmol/min (six minutes at each dose); the order of infusions was randomised between subjects, but each subject received the infusions in the same order on the two study days.

3.2.3 Statistical analysis and assessment of intra-subject variability

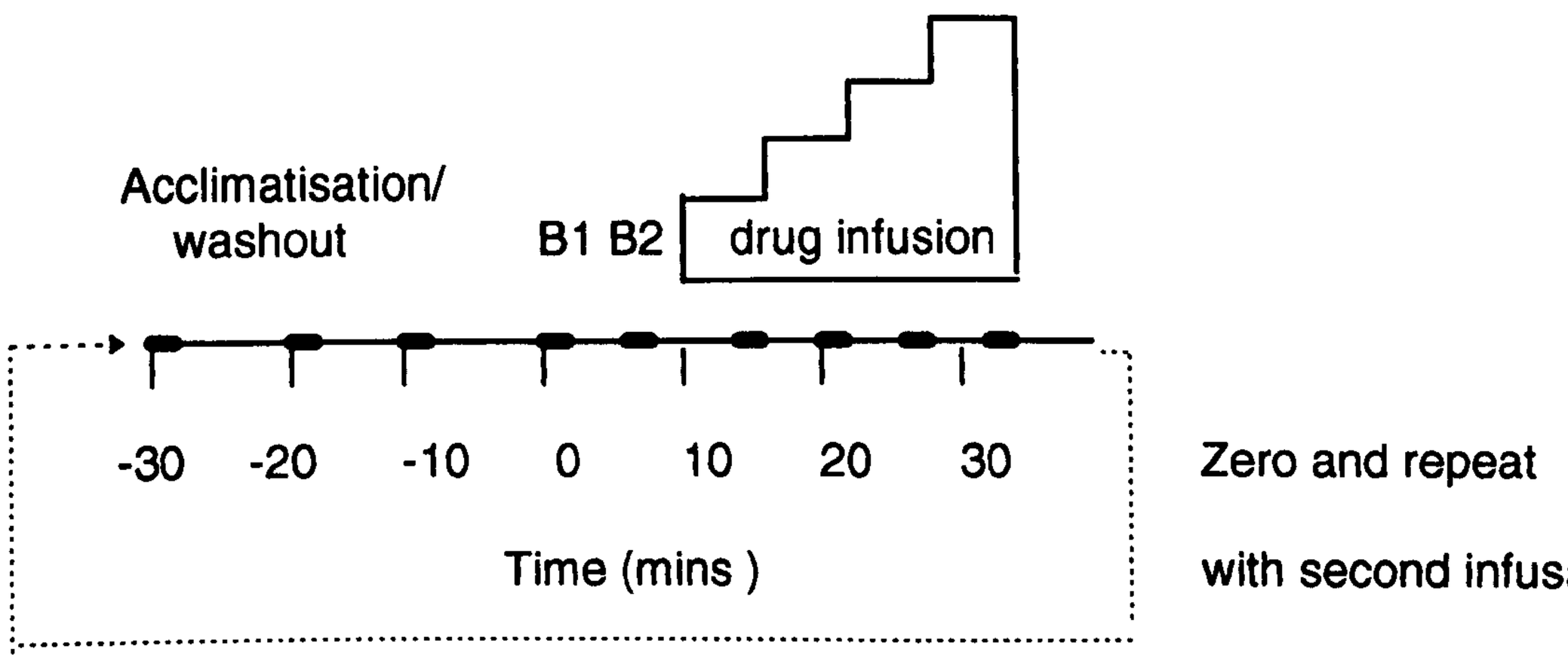
In *Study 1*, intra-subject variability was expressed as coefficients of variation (CV), which were calculated using two-way ANOVA (subject and day as terms) using MINITAB (Pennsylvania, USA): the square root of the total error term of the adjusted mean squares from ANOVA was divided by the mean and expressed as a percentage (Bland and Altman 1996). The Shapiro-Wilks test was used in order to check that residuals were normally distributed. A standard statistical test for comparing co-efficients of variation derived in this way has not been described. In *Study 2*, following Bland and Altman (Altman 1991), intra-subject variability was expressed as the difference between responses on days 1 and 2 plotted against the mean response on days 1 and 2 for each individual subject at each dose: as the differences were not normally distributed, the interquartile range of the differences was calculated as a summary statistic.

Figure 3.1 Experimental protocols for *Study 1* (upper panel) and *Study 2* (lower panel). Bold bars represent 3 minute periods of FBF measurements (see Methods). B1 = baseline 1, B2 = baseline 2.

Study 1



Study 2



3.3 Results

3.3.1 Baseline and post-exercise measurements

Baseline and post-exercise unilateral FBF and FBF ratios for each subject on the three study days (*Study 1*) are shown in Figure 3.2. Coefficents of variation (CV) are summarised in Table 3.1.

a) Forearm blood flow: At rest, intra-subject variability of baseline FBF ratios was 19% compared with 39% and 31% for left and right unilateral FBF measurements respectively. After ipsilateral exercise, unilateral readings became more reproducible (32 vs 17%); by 20 minutes after exercise, the previous pattern had been re-established (19 vs 27%).

b) Forearm vascular resistance: At rest, intra-subject variability of baseline FVR ratios was 14% compared with 29% and 27% for left and right unilateral FVR measurements respectively. After ipsilateral exercise, unilateral readings became more reproducible (40 vs 14%); by 20 minutes after exercise, the previous pattern had been re-established (17 vs 32%).

3.3.2 Responses to intra-arterial infusions

Blood flow responses to both sets of intra-arterial infusions (angiotensin II and noradrenaline) on days 1 and 2 are shown conventionally as grouped data (mean±SEM) in Figure 3.3; the individual data are shown in Figure 3.4 (angiotensin II) and Figure 3.5 (noradrenaline). Plots of the differences between responses on days 1 and 2 (Figure 3.6), indicated that variability of unilateral FBF between study days was higher than that of FBF ratios for both infusions. This was confirmed by the inter-quartile ranges of the differences (FBF ratios vs FBF): angiotensin II 14 vs 18%; noradrenaline 16 vs 27%.

Table 3.1

Intra-subject variability of baseline and post-exercise responses expressed independently in each arm and as a ratio of left/right arms (*Study 1*)

		Left	Right	ratio
		CV (%)	**CV(%)**	**CV(%)**
Baseline	Blood pressure	7	-	-
	Heart rate	9	-	-
	FBF	39	31	**19**
	FVR	29	27	**14**
Post-exercise	FBF	**17**	29	32
	FVR	**14**	32	40
10 minutes post-exercise	FBF	25	26	28
	FVR	33	30	24
20 minutes post-exercise	FBF	25	27	**19**
	FVR	32	27	**17**

CV, coefficient of variation (%) - calculated using two-way ANOVA (see Methods); those less than 20% are highlighted in bold.

Figure 3.2 Individual FBF (left and right) and FBF ratios in 9 healthy male subjects on days 1, 2 and 3. Data are shown for Baseline 1 (left panel), Unilateral exercise (middle panel), and 20 minutes post-exercise (right panel).

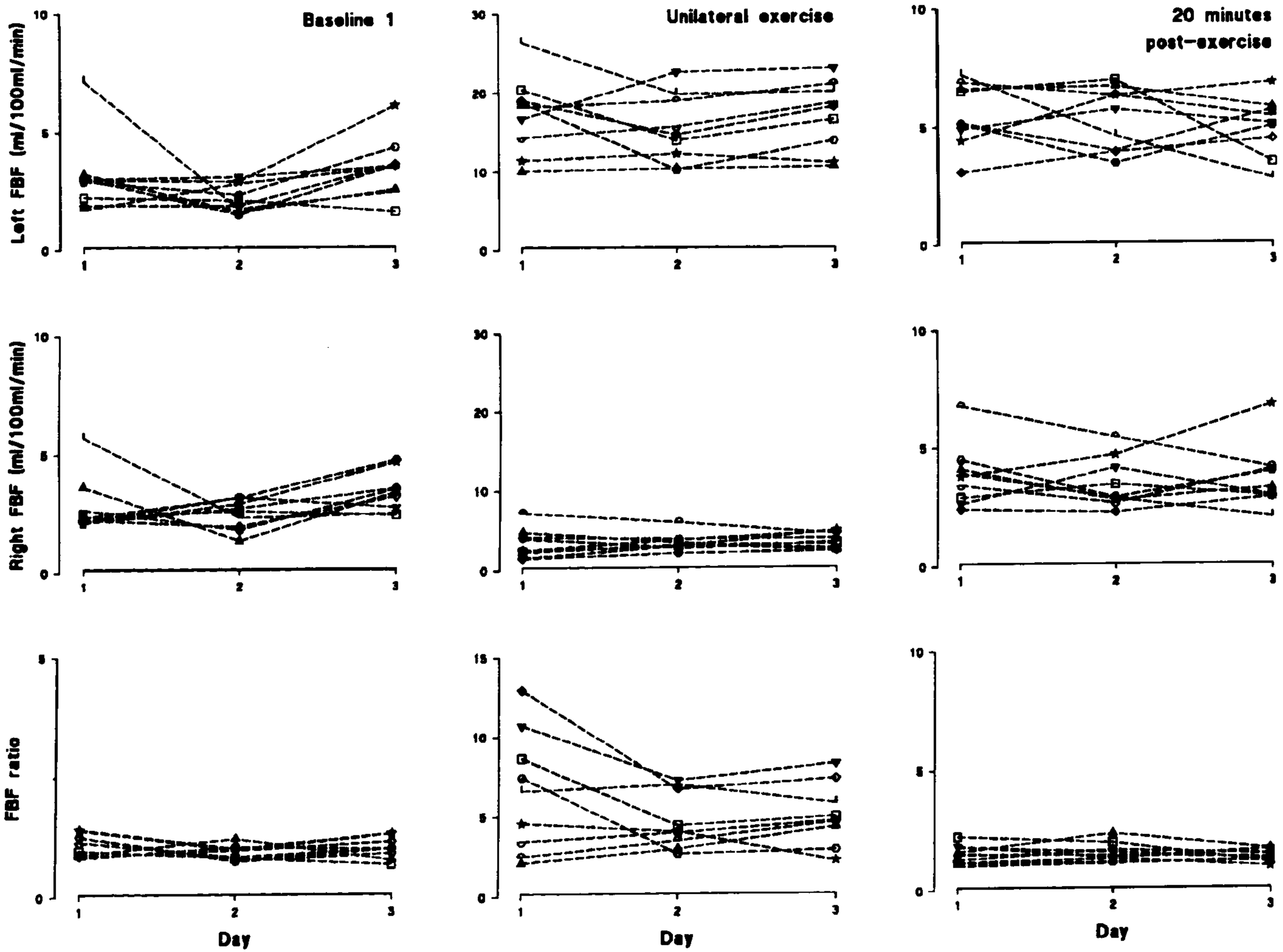


Figure 3.3 Grouped (mean±SE, % change from baseline) FBF (left and right) and FBF ratios in 5 healthy male subjects during intra-arterial infusions of angiotensin II (left) and noradrenaline (right) on days 1 and 2.

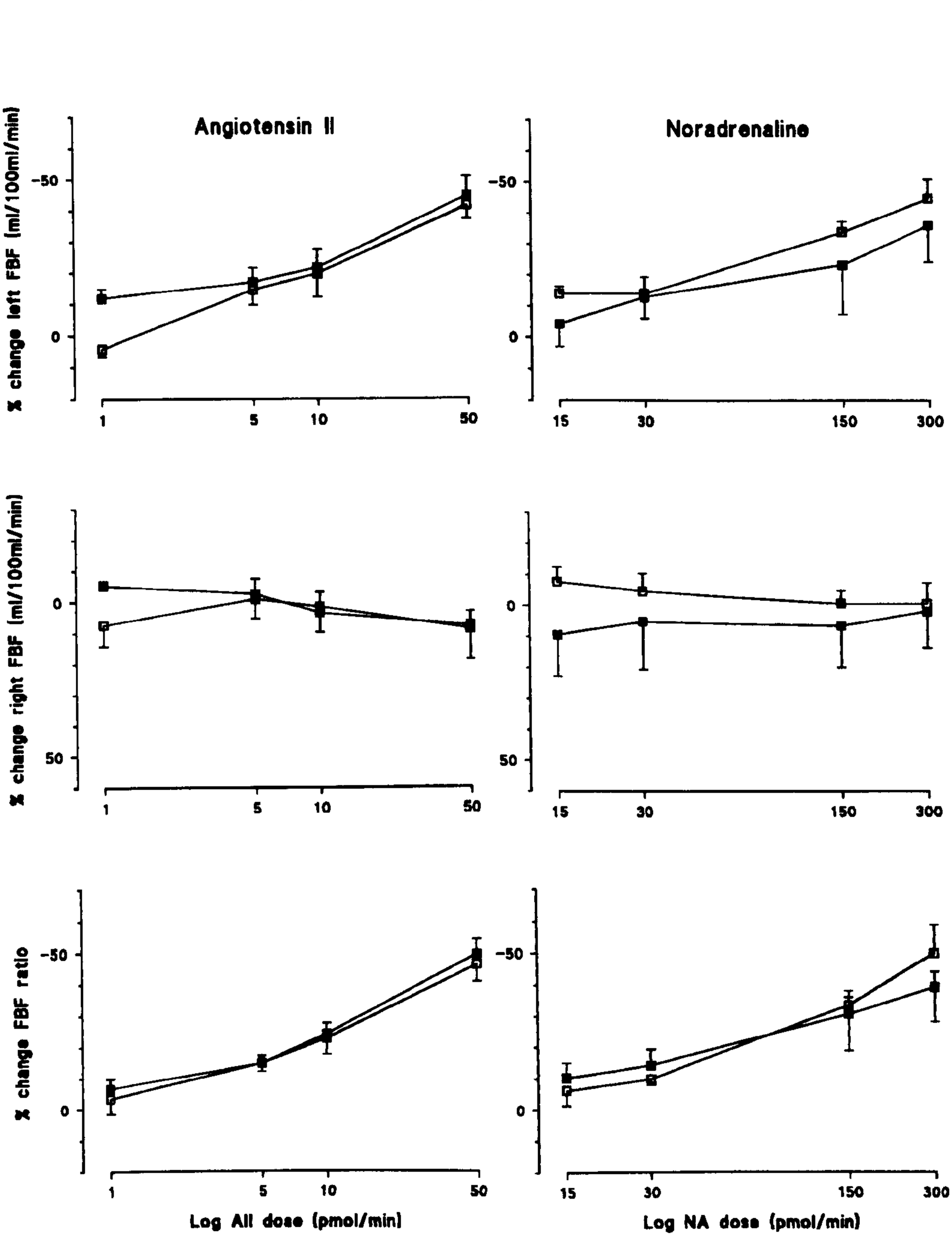


Figure 3.4 Individual FBF (left and right) and FBF ratios (%change from baseline) in 5 healthy male subjects during intra-arterial infusions of angiotensin II on day 1 (closed symbols) and day 2 (open symbols).

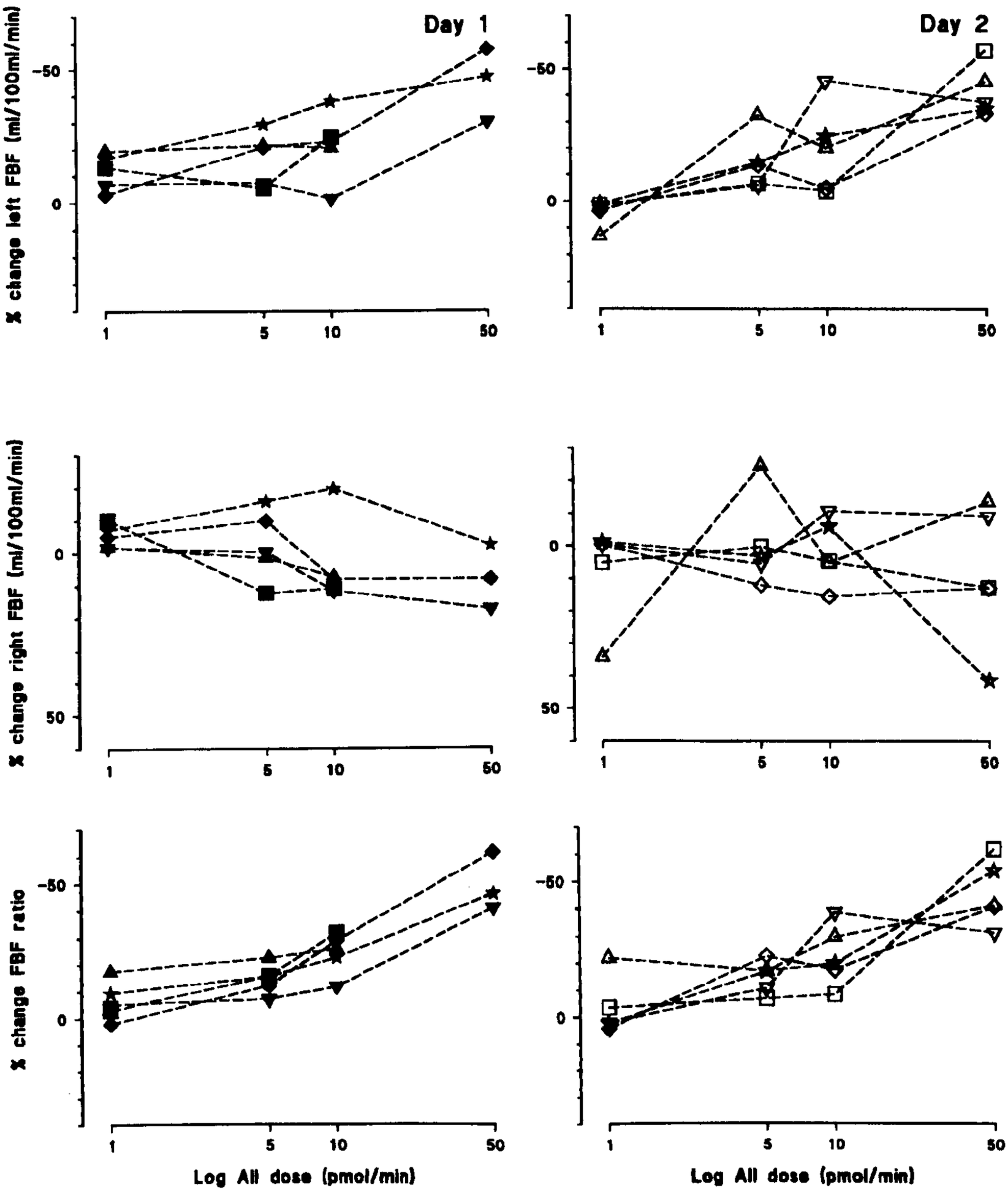


Figure 3.5 Individual FBF (left and right) and FBF ratios (%change from baseline) in 5 healthy male subjects during intra-arterial infusions of noradrenaline on day 1 (closed symbols) and day 2 (open symbols)

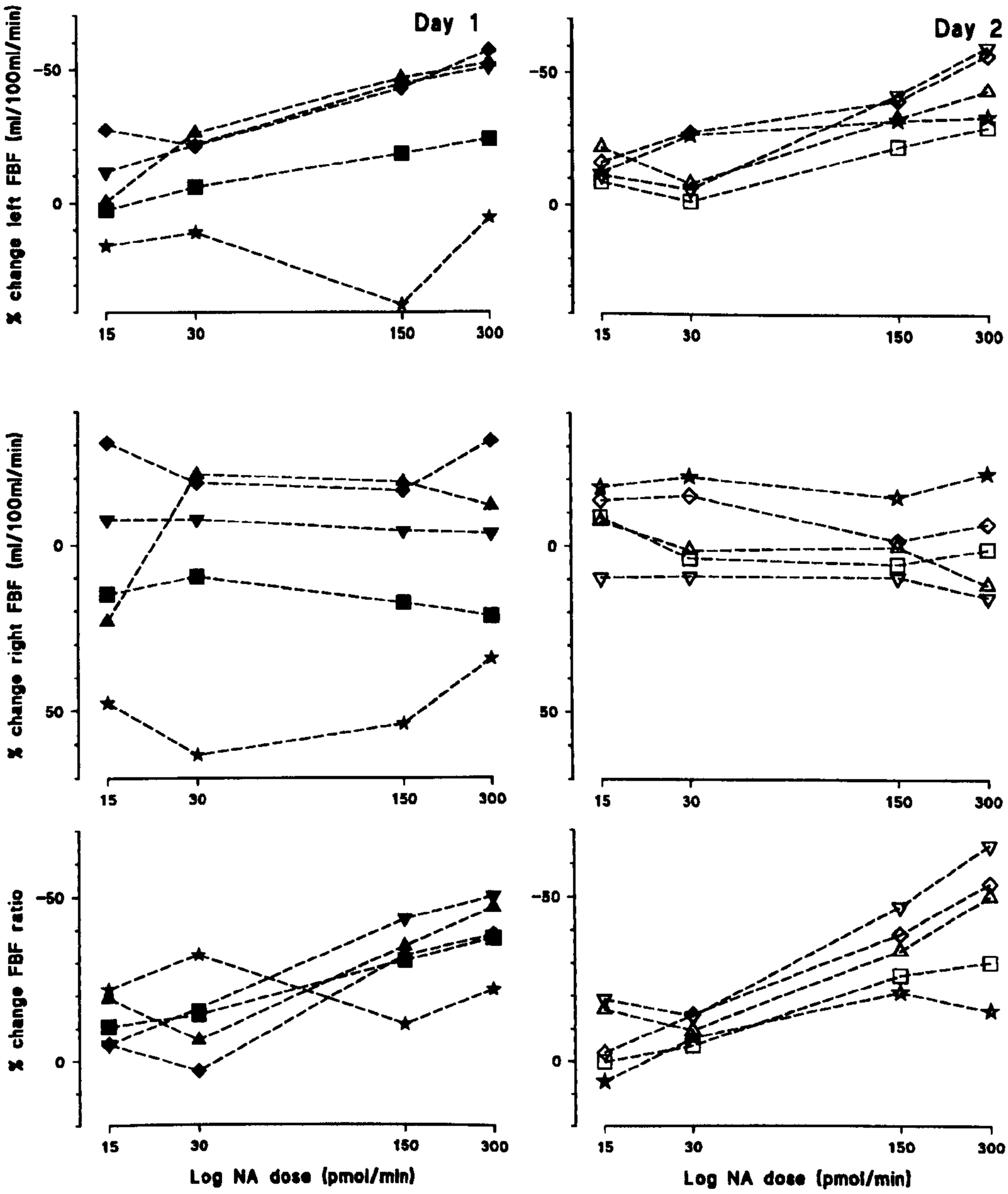
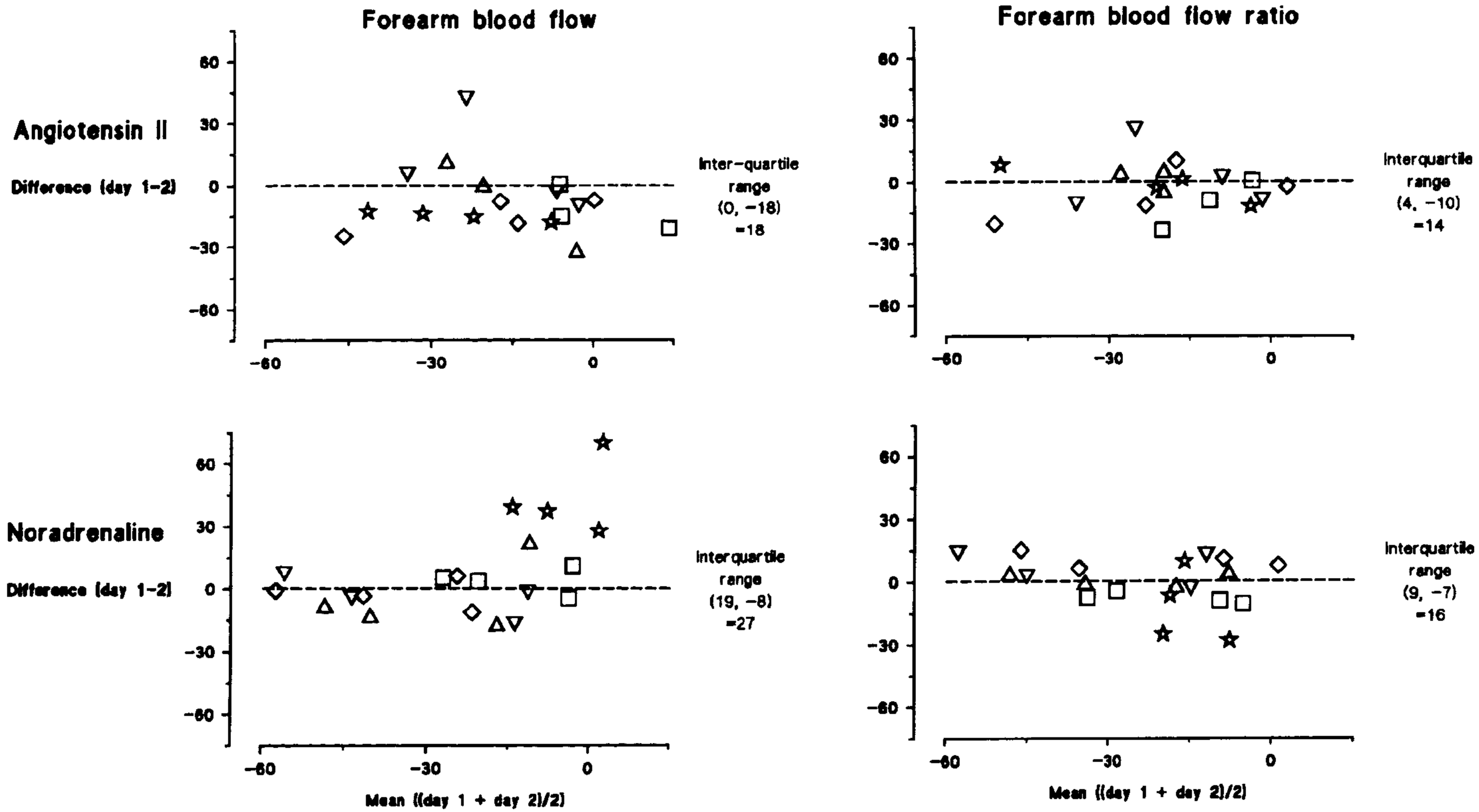


Figure 3.6 Difference between responses on days 1 and 2 to angiotensin II (upper panel) and noradrenaline (lower panel) plotted against mean response for each individual subject at each dose for both FBF and FBF ratios (see Methods); plotted for each dose received by each subject.



3.4 Discussion

In the subjects studied: 1) intra-subject variability (CV) of bilateral FBF measurements (FBF ratios) was 19% at rest; 2) FBF ratios were more reproducible than unilateral FBF measurements (CV 31-39%) at rest and during intra-arterial infusions, but not after unilateral forearm exercise; 3) forearm vascular resistance (FVR) as an alternative method of expressing responses resulted in a small improvement in reproducibility over FBF at rest and after exercise. These findings have implications for the interpretation and design of studies in which venous occlusion plethysmography is used to measure responses to local intra-arterial infusions.

Bilateral plethysmography using FBF ratios to express results was first proposed in the context of water plethysmography over 40 years ago as a means of controlling for systemic changes unrelated to the local stimulus (Greenfield and Paterson 1954). However, intra-subject variability data have not previously been reported for FBF ratios, which are frequently and currently used to express results of studies using strain gauge plethysmography. In the present study, FBF ratios at rest were reasonably reproducible for physiological measurements, albeit with a higher coefficient of variation (19%) than concurrent blood pressure (7%) and heart rate (9%) measured by semi-automatic sphygmomanometer. Previous studies have addressed intra-subject variability only of *unilateral* measurements of FBF and have reported coefficients of variation of 7.8-15.6% (Roberts et al 1986) and 25% at rest (Altenkirch et al 1990). It is noteworthy that studies reporting unilateral FBF measurements have produced conflicting findings (Panza et al 1990, Cockcroft et al 1994); In the present study, intra-subject variability of unilateral measurements at rest was 31-39%.

When data from the intra-arterial infusion studies are plotted in the conventional manner as grouped data (mean±SEM)(Figure 3.3), they appear similar to those reported by other investigators, suggesting that similar reproducibility was achieved. However, inspection of Figures 3.4 and 3.5 reveals the considerable intra-subject variability that is concealed by such plots. This variability was considerably reduced when results were expressed as FBF ratios (Figure 3.6).

Given that similar reproducibility was achieved to that of other investigators in the intra-arterial infusion studies, how can the higher coefficients of variation in the resting measurements in *Study 1* be explained? Between-day variations in FBF measurements must derive either from intrinsic variability in blood flow or from experimental error. Even at rest, FBF constantly changes in response to changes in sympathetic nervous system activity, mental arousal, and ambient temperature. It is possible that the relatively high variability in the present study in comparison with that of Roberts et al. (1986) might be explained on the basis of inadequate control of the experimental environment and the general level of arousal of the subjects. However, it is unlikely that the subjects in the present study experienced a more variable environment than that experienced by subjects in other studies, since they were studied in a quiet room and were allowed to become acclimatised for a full 30 minutes not just to room temperature, which was stable, but also to cuff inflation and deflation. In many published studies, acclimatisation periods are of 10 minutes or less (Cockcroft et al 1993). Concurrently-measured blood pressure and heart rate in the present study were reproducible between days, suggesting reasonably consistent experimental conditions.

A further possible source of day-to-day variability may have been in positioning of strain gauges on the forearm. However, strain gauges of exactly the same length were used on each occasion with each subject and care was taken with positioning. In further studies carried out since the completion of this study

similar between-day coefficients of variation for baseline FBF and FBF ratio to those reported have been observed, suggesting that the data presented in this study do not simply reflect the early portion of a "learning curve" on the part of the investigators. Some variability in *Study 1* could be attributed to the non-fasting state of the subjects, but the aim was to evaluate the technique in the conditions reportedly used by most investigators (Benjamin et al 1989, Calver et al 1992).

An alternative possibility is that intra-subject variability was in part subject-dependent: the subjects in the present study were "naive" to plethysmography, but many studies are carried out using subjects who have undergone such measurements before. The importance of subject-dependent factors was acknowledged by Greenfield and Patterson (who mostly used each other as experimental subjects): "the results of these experiments *(in other subjects)*. . . were similar . . . but they were rather more scattered" (Greenfield and Patterson 1954). The study by Roberts et al. (Roberts et al 1986) may have been conducted in "trained" subjects. While such subjects may be suitable for pharmacological studies, investigation of pathophysiological mechanisms in man depends upon studies in patients, and reproducibility data in naive healthy volunteers may be more relevant.

The final aim of the present study was to assess whether expressing responses as forearm vascular resistance (FVR)(i.e. corrected for changes in mean arterial pressure as measured by a semi-automatic sphygmomanometer) would decrease intra-subject variability. There was a tendency for FVR and FVR ratios to be more reproducible than FBF and FBF ratios for baseline and post-exercise readings, but it is doubtful whether the magnitude of the improvement in reproducility would be relevant in practice. It should be noted that invasive measurements of arterial pressure were not performed, and it remains possible that this would result in a larger improvement.

After unilateral forearm exercise, simple unilateral FBF measurements were more reproducible than FBF ratios. It is likely that this was due to a combination of two factors. Firstly, the unilateral exercise task may have been sufficiently vigorous to produce a near maximal response; there are previous data to demonstrate the reproducibility of maximal FBF (minimal FVR) (Takeshita et al 1980). Secondly, blood flow in the contralateral arm during this task appears to have varied in an inconsistent manner between days.

In conclusion, the present study provides evidence that bilateral plethysmography, expressing responses in the intervention arm as a ratio of responses in the control arm, is more reproducible than unilateral plethysmography. In addition, the data demonstrate that correcting measurements of blood flow for small variations in mean arterial pressure (forearm vascular resistance) results in only a small improvement in reproducibility. These findings have clear implications for the design of clinical studies, in particular the calculation of sample size, and sound a note of caution for investigators setting up the technique in a new centre. It is important that the method of expressing responses is considered before the uncritical acceptance of findings of studies using plethysmography to examine arterial responses in cardiovascular disorders.

Chapter 4

Potential confounding haemodynamic effect of hand-warming on the measurement of insulin sensitivity

4.0 Summary

Hand-warming is frequently-employed during the hyperinsulinaemic euglycaemic clamp procedure in order to arterialise venous blood. The effect of hand-warming on the value for insulin sensitivity derived from the clamp was assessed in two clinical studies, which are described in this chapter.

4.1 Introduction

In the clinical studies described in Chapters 5,6, and 7, insulin-mediated glucose uptake (insulin sensitivity or M) was assessed using the hyperinsulinaemic euglycaemic clamp technique (Section 1.5.2). Adjustment of the glucose infusion rate on the basis of venous glucose concentrations would be expected to lead to an overestimation of insulin sensitivity, and most investigators now use glucose concentrations measured in "arterialised" venous blood (Liu et al 1992). Hand-warming (by a warm blanket or a heated-air hand box) is used to increase blood flow through digital arteries, creating a functional arteriovenous shunt; blood is sampled from the superficial dorsal veins of the hand (Ferrannini et al 1987, Yki-Jarvinen 1987)

However, in contrast to predictions made from the above theoretical considerations, there is evidence from several groups of investigators that M-values as measured by the euglycaemic clamp are similar when the glucose

infusion rate is adjusted according to arterialised blood or mixed venous blood (Andrews et al 1984, Wahab et al 1992, Nauck et al 1996). In addition, it has been observed that hand-warming, at least by the warm blanket technique, affects body temperature and contralateral forearm subcutaneous blood flow during oral glucose tolerance testing (Astrup et al 1988), and may cause sympathetic nervous system activation during the euglycaemic clamp (Moan et al 1995). Although the heated-air hand box is reported to have lesser systemic effects than the warm blanket (Gallen and MacDonald 1990), concern remains regarding the appropriateness of hand-warming during metabolic studies, particularly those in which concurrent measurements of blood flow have been used to draw conclusions about insulin-mediated vasodilatation and forearm glucose uptake (Yki-Jarvinen et al 1987, Hunter et al 1994, Walker et al 1995).

The aim of *Study 1* was to determine if hand-warming by the heated-air hand box affected the calculated value for insulin sensitivity (M) derived from the euglycaemic clamp technique. It was therefore assessed whether there was any difference in M when the procedure was performed with the box *in situ* but switched off and the glucose infusion rate adjusted in the usual manner according to ipsilateral dorsal hand vein samples (*effect of arterialisation*). In order to control for effects of hand-warming other than those resulting simply from a change in local ipsilateral glucose concentrations, M was also compared when the procedure was performed with the box switched either on or off with the glucose infusion rate adjusted according to samples from the contralateral antecubital vein. It was reasoned that, as blood glucose concentrations in this deep vein were unlikely to be affected by warming the other hand (Roddie et al 1956), any

difference in M-values obtained with the box switched on as opposed to off would be attributable to *systemic haemodynamic effects* of hand-warming. It was anticipated that M-values obtained from antecubital vein clamps (box-on and box-off) would be higher than those obtained from dorsal hand vein clamps because of the relatively minor extraction of glucose in the capillary beds of the hand compared with those of the forearm during euglycaemic hyperinsulinaemia. Direct arterial puncture to obtain true arterial glucose measurents was not performed, as the primary aim was to examine whether hand-warming affected derived insulin sensitivity: this was addressed with minimum invasiveness using the above design.

In the light of the findings of the first study, the aim of *Study 2* was to assess the effect of hand-warming (by the same method) on contralateral forearm blood flow (FBF), using venous occlusion plethysmography.

4.2 Methods

4.2.1 Subjects

a) *Study 1.* Eight healthy normotensive fasting male subjects aged 21-35 years with normal glucose tolerance attended four study days, one week apart.

b) *Study 2.* Five healthy normotensive fasting subjects (one female) with normal glucose tolerance attended two study days (random order).

4.2.2 Clinical procedures

a) *Study 1.* On each study day, one of four modified euglycaemic clamps (Section 2.4) was performed, according to a random order Latin Square design. After 20 minutes of supine rest with the right hand placed in a heated-air hand box at 55°C (University of Nottingham, Department of Physiology and Pharmacology), a primed infusion of soluble human insulin (1.5 mU/kg/min; Actrapid, NovoNordisk A/S, DK2880 Bagsvaerd, Denmark) along with a variable rate infusion of 20% dextrose (Baxter Healthcare, Norfolk, U.K.) was administered via a right antecubital vein for three hours to maintain euglycaemia at 5.2 mmol/l. The infusion rate was adjusted on the basis of samples (every 5 minutes) from an ipsilateral right dorsal hand vein or a contralateral left arm antecubital vein according to study day (random order) as follows: 1) heated box, ipsilateral samples; 2) box *in situ* but switched off, ipsilateral samples; 3) heated box, contralateral samples; 4) box *in situ* but switched off and contralateral samples. Care was taken to place cannulae in the same veins on each study day for each subject. Patency of the main sampling cannula was maintained using a slow infusion (0.5 ml/minute) of 0.9% sodium chloride. Blood samples were withdrawn at 0, 60, 120, and 180 minutes for measurement of serum insulin (Section 2.8.1), potassium (Section 2.8.7), and triglycerides (Section 2.8.9). Blood samples were withdrawn simultaneously from both the right dorsal hand vein and the left antecubital vein for measurement of pO_2 (Section 2.8.8) and serum glucose (Section 2.8.2) at 0, 60, 120, and 180 minutes. Blood pressure (left arm) and heart rate were measured every 10 minutes (Dinamap Critikon).

b) *Study 2.* Temperature was maintained at 25-26°C and lighting was dimmed; the room was quiet and sealed. Subjects lay supine with arms supported on foam blocks in the same position on each occasion. A paediatric cuff (Hokanson SC5, PMS instruments, Maidenhead, Berkshire) was placed around the left wrist and inflated to 200 mmHg for three minutes during each set of measurements. A collecting cuff was placed around the upper arm, and inflated (40 mmHg) and deflated in a 15 second cycle (Hokanson SC10). Rapid cuff inflation was achieved using a commercially available air source (Hokanson AG101), coupled to a rapid cuff inflator (Hokanson E20). On the first day, the left forearm was measured at the largest circumference, and a strain gauge (Hokanson forearm set) 2 cm shorter was selected. The distance from the olecranon was measured and recorded in order to standardise strain gauge position from day to day. The strain gauge was calibrated electrically on the arm to the chart recorder programme. Blood flow measurements began 45 seconds after wrist cuff inflation, and continued for 135 seconds. Subjects were allowed to acclimatise to inflation and deflation of the wrist and upper arm cuffs for 30 minutes before the recording of two sets of baseline measurements, ten minutes apart: the mean FBF during these two sets of measurements was used as the baseline forearm blood flow. After the baseline measurements were completed, the right arm was placed in a heated-air hand box at either 55°C or room temperature. Unilateral left arm FBF measurements were continued at 5 minutes, and subsequently at 10 minute intervals, up to 125 minutes.

4.2.3 Statistical analysis

a) *Study 1.* The primary end-points (M-values) in conditions 1 vs 2 (right dorsal hand vein), and 3 vs 4 (left antecubital vein), were compared using paired t-tests (Minitab statistical package, Minitab Inc, Pennsylvania, USA). Differences within and between treatments (box-on vs. box-off) in blood glucose, pO_2, and other metabolic variables were assessed by three-way analysis of variance (Section 2.9).

b) *Study 2.* All data were acquired via a MacLab II Chart recorder (AD instruments, Hampstead, London). Percentage change in blood flow from mean baseline in the left arm was analysed using three-way analysis of variance.

4.3 Results

4.3.1 Insulin sensitivity

When clamps were performed according to samples taken from the contralateral antecubital vein, derived insulin sensitivity (M) was significantly lower on box-on vs. box-off days (mean±SD 10.2±3.0 vs. 13.0±3.8 mg/kg/min, $p < 0.05$, 95% C.I.-0.2, -5.3; Figure 4.1, right panel). However, when clamps were performed on the basis of samples from the ipsilateral dorsal hand vein, there was no difference in calculated insulin sensitivity between box-on and box-off days (9.2±2.1 vs. 9.0±1.7mg/kg/min, p=0.57, 95% C.I. -0.5, +0.9; Figure 4.1, left panel).

4.3.2 Arterialisation of venous blood

a) Oxygenation: In samples of blood taken from the right dorsal hand vein, there was a mean difference of 2.0 kPa in arterialised-venous pO_2 between box-on and

box-off days: this was statistically significant [$p < 0.01$, interaction between treatment (box-on vs box-off) and time, Figure 4.2 upper panel]. However, there were no statistically significant differences in pO_2 values in the left antecubital vein between box-on and box-off days (not shown).

b) Glucose levels: There were no significant differences in glucose levels between box-on and box-off days in samples from either the ipsilateral dorsal hand vein (Figure 4.2, lower panel) or the contralateral antecubital vein. Blood glucose during the final 40 minutes of the clamps was less variable in box-on conditions: coefficients of variation for right dorsal hand vein studies were 4.8% (box on) and 7.9% (box-off); for left antecubital vein conditions they were 7.9% (box-on) and 10.0% (box-off).

3) Haemodynamic effects

a) *Study 1.* Mean heart rate throughout the procedure was 6 beats per minute higher in conditions in which the heated box was used [$p < 0.05$, interaction between treatment (box-on vs. box-off) and time, Figure 4.3 upper panel]. There were no significant differences in blood pressure between study days (Figure 4.3, lower panel).

b) *Study 2.* Baseline left forearm blood flow (mean±SD) was similar in both conditions: 2.63 ± 1.05 vs. 2.72 ± 1.25 ml/100ml forearm/min for box-on vs. box-off day. Left FBF expressed as percentage change from baseline was higher during right hand-warming than during the control condition [$p < 0.05$ for the interaction between treatment (box-on vs. box-off) and time, Figure 4.4]. There

Figure 4.1 Insulin sensitivity (M, euglycaemic clamp technique) during hand-warming (box-on) and control (box-off)
Left panel: Glucose infusion rate adjusted according to ipsilateral (right dorsal hand vein) samples.
Right panel: Glucose infusion rate adjusted according to contralateral (left antecubital vein) samples.
* = $p < 0.05$ (paired t-test). Figures in brackets are 95% confidence intervals.

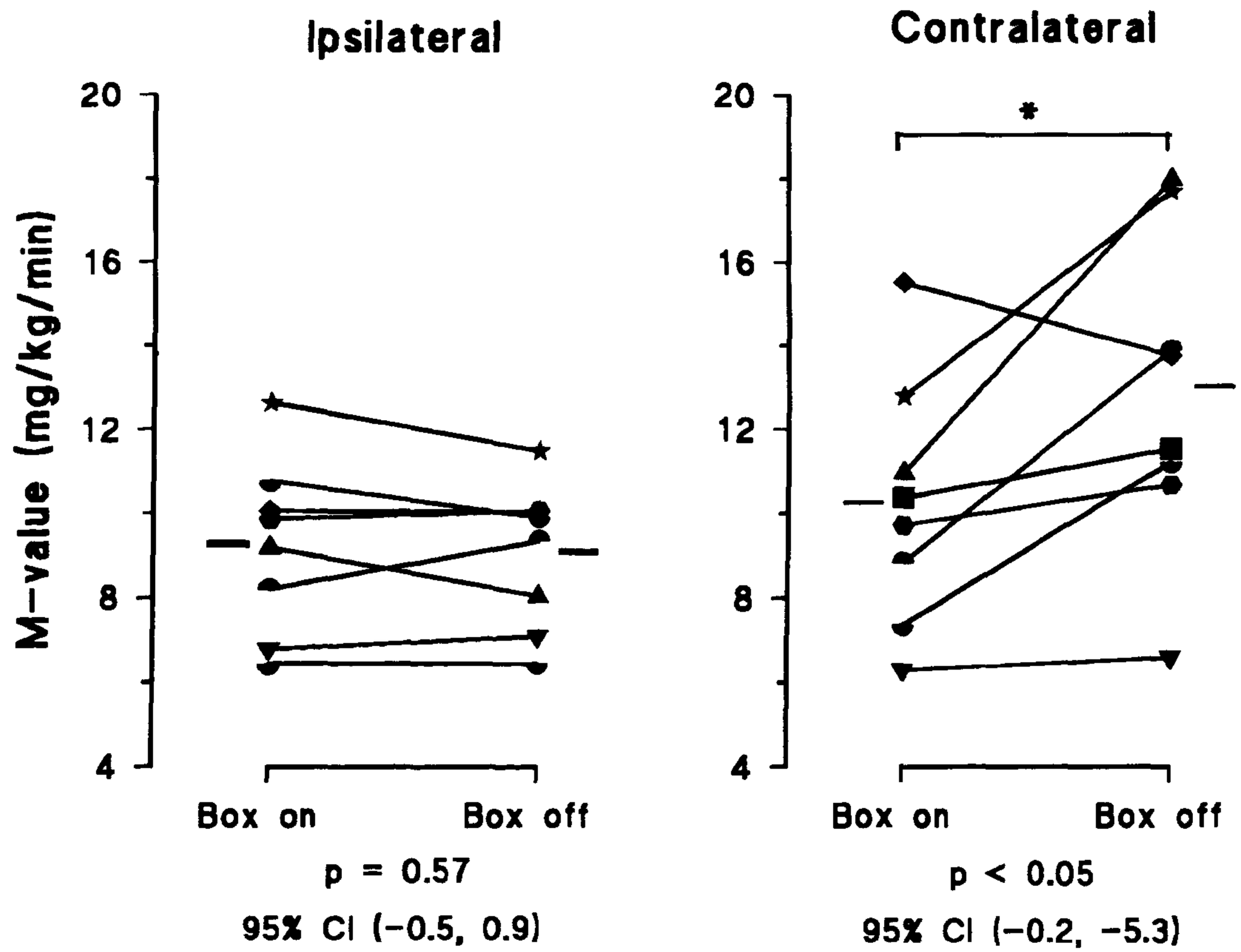


Figure 4.2 Arterialisation of venous blood during euglycaemic clamps. Oxygen tension (*upper panel, pO_2, kPa)*, glucose levels *(lower panel, mmol/*l) measured in the ipsilateral dorsal hand vein in box-on and box-off conditions.

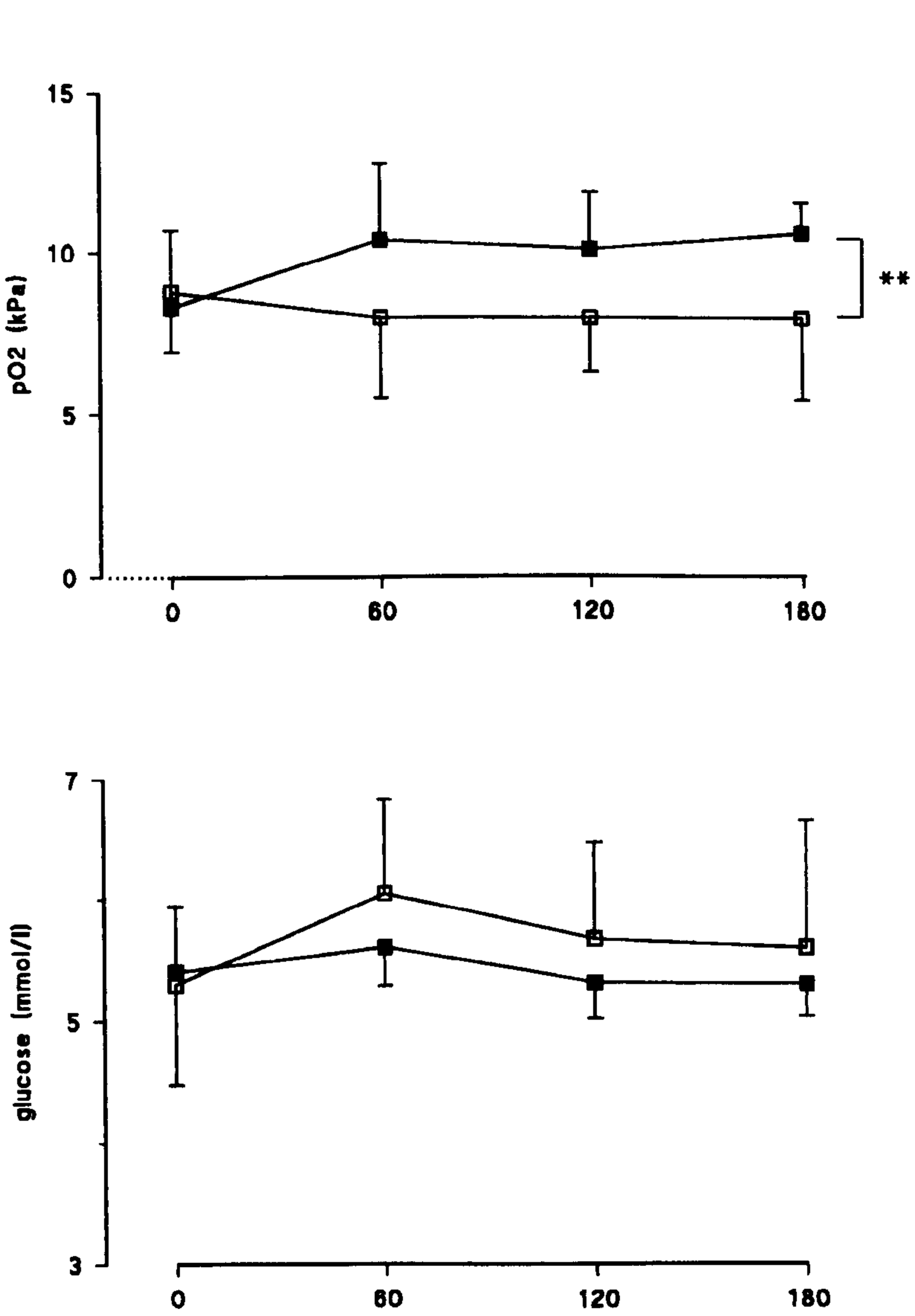


Figure 4.3 Heart rate and blood pressure during euglycaemic clamps.
■ box-on, ipsilateral; □ box-off, ipsilateral;
▲ box-on contralateral; △ box-off contralateral. * = $p < 0.05$

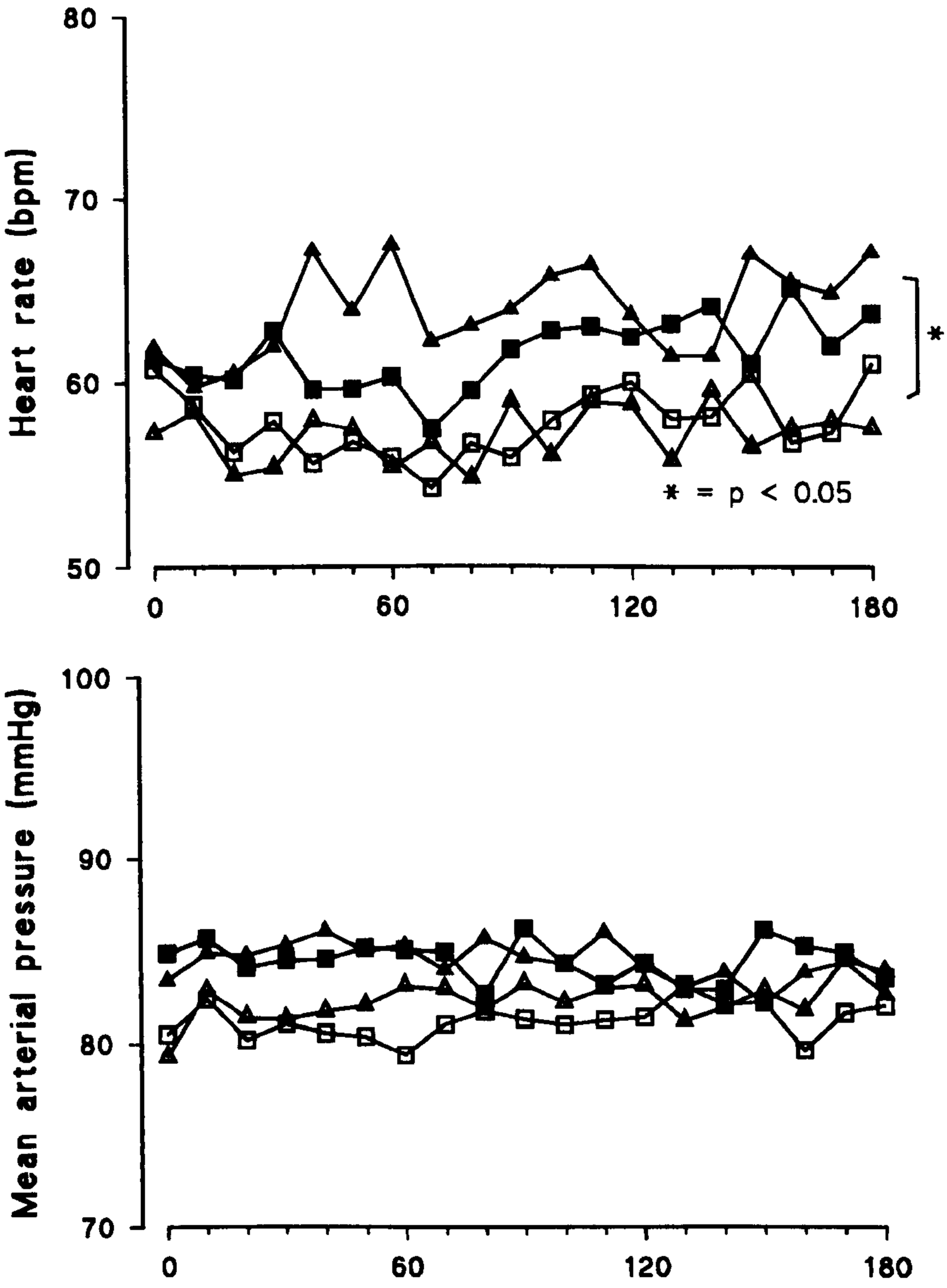


Figure 4.4 Contralateral forearm blood flow during hand-warming
Change in left forearm blood flow expressed as a percentage of baseline (mean±SD) during right hand-warming and control.

■ box on; □ box off * = $p < 0.05$

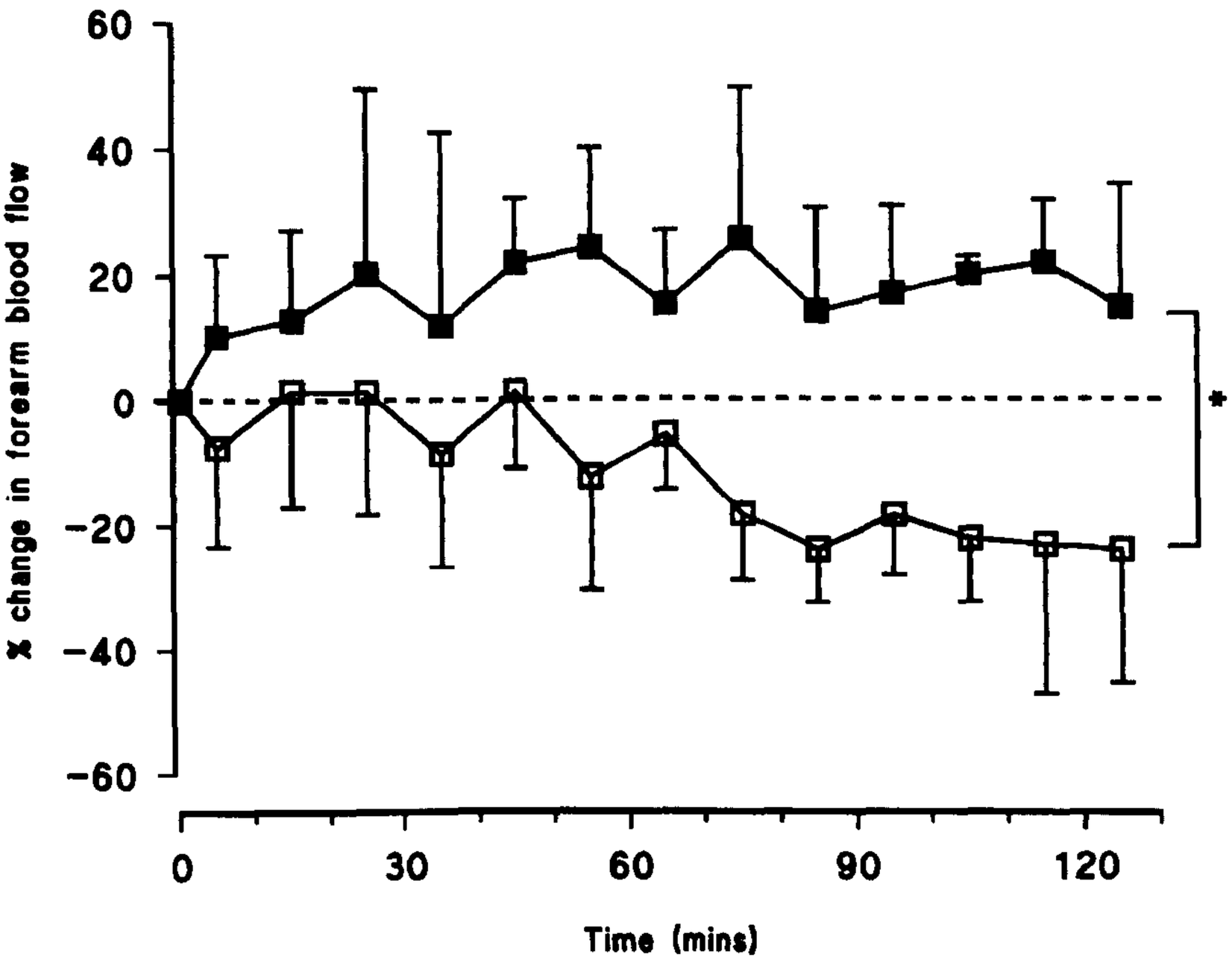
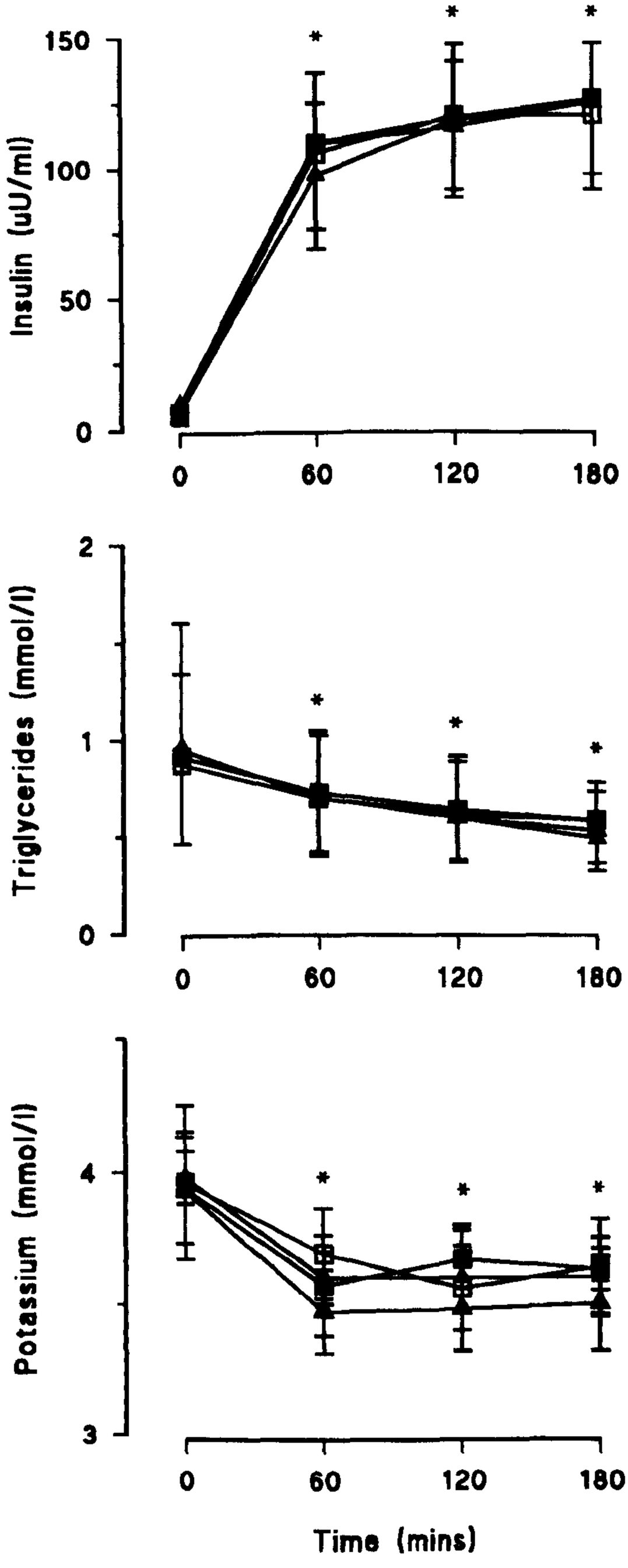


Figure 4.5 Insulin, triglycerides, and potassium levels during euglycaemic clamps.

■ box-on, ipsilateral; □ box-off, ipsilateral;
▲ box-on contralateral; △ box-off contralateral

**= $p < 0.01$; * = $p < 0.05$ (change from baseline)



was no difference in heart rate between the two conditions (e.g. at 65 minutes 63.6±8.65 vs 63.2±11.4 beats per minute, p=0.85 for the interaction between treatment and time).

4) Insulin, triglycerides and potassium

Mean±SD steady-state insulin concentrations for the four study days were 127±21.4μU/ml, 121±28.8μU/ml, 122±29.4μU/ml, and 126±27.8μU/ml respectively; there were no significant differences in insulin levels between days (Figure 4.5). There were no significant differences between study days in potassium or triglycerides levels, which had decreased significantly from baseline by 60 minutes ($p < 0.05$, Figure 4.5).

4.4 Discussion

Hand-warming is widely used in metabolic investigation, and euglycaemic clamps are almost invariably performed using glucose levels in arterialised blood to determine the required rate of glucose infusion. In addition, hand-warming has been used in studies measuring blood flow and forearm glucose uptake during hyperinsulinaemia (Yki-Jarvinen 1987, Hunter et al 1994, Walker et al 1995). The principal aim of the present controlled studies was to determine if hand-warming by the heated-air hand box method affected the value for insulin sensitivity (M) derived from the euglycaemic clamp technique. It was hypothesised that hand-warming might have two independent effects on the derived M-value: (1) the usually accepted mechanism, i.e. improving the approximation of heated superficial dorsal hand vein glucose concentrations to arterial glucose

concentrations (thereby decreasing the required infusion rate and reducing over-estimation of M) - i.e. *arterialisation*; and (2) causing thermoregulatory alterations in cardiovascular regulation outwith the warmed arm - i.e. *systemic haemodynamic effects*. It was felt important to define the effect of hand-warming on blood flow, as such an effect might potentially confound investigations of the cardiovascular effects of systemic hyperinsulinaemia.

In order to dissect out which of the two putative effects was predominant (arterialisation or systemic haemodynamic effects) three control conditions were used: one in which the heated box was switched off and the clamp was performed according to ipsilateral samples in the usual manner, and a further two in which the box was switched either on or off and the clamp was performed according to deep venous glucose levels in a site (the contralateral arm) unlikely to be affected by arterialisation (Roddie et al 1956). It was reasoned that if arterialisation was the predominant mechanism by which hand-warming affected the measurement of insulin sensitivity, then the derived M-value should have been lower when the box was switched on and the clamp was performed according to ipsilateral samples; conversely, there should have been no difference in derived M-values with hand-warming when clamps were performed according to samples from the contralateral antecubital vein.

In contrast with these expectations, hand-warming resulted in no detectable difference in derived M-value when clamps were performed according to ipsilateral samples. However, when clamps were performed according to samples from a site that was not affected directly by hand-warming (the contralateral

antecubital vein), a lower M-value resulted. As anticipated *a priori*, M-values (box-on and box-off) tended to be higher when clamps were performed using blood sampled from the antecubital site, because of the higher extraction of glucose in the capillary beds of the forearm compared with the hand during euglycaemic hyperinsulinaemia. Although clamps were not performed according to directly-sampled arterial blood as a "gold standard," the study design (using contralateral antecubital vein clamps) provided a measure of the potential impact of hand-warming on derived insulin sensitivity, while avoiding excessive invasiveness.

The absence of any detectable effect of warming the sampling hand on calculated M is consistent with the reported findings of a number of other groups, using different methods of hand-warming (Andrews et al 1984, Wahab et al 1992, Nauck et al 1996). However, two of these groups did not confirm the degree of arterialisation by measuring oxygenation of sampled blood (Andrews et al 1984, Nauck et al 1996) and the other used a design in which arterialised and mixed venous blood were both withdrawn from the same (heated) arm, thereby reducing any arterialised-venous glucose gradient (Wahab et al 1992). Data from the present study indicate that there was effective arterialisation (in terms of oxygen tension) when the box was switched on but that this, surprisingly, did not affect the simultaneously-measured glucose levels or the derived values for M.

What might be the mechanism by which hand-warming decreased derived insulin sensitivity in clamps performed according to samples from the contralateral antecubital vein? Heart rate was significantly higher during prolonged hand-

warming and hyperinsulinaemia (three hours) than during the control conditions; as far as can be ascertained, this response has not previously been described. An increase in heart rate with hand-warming was not observed under normoinsulinaemic conditions (Study 2), but there was an increase in contralateral forearm blood flow. This response has been previously described (Abramson et al 1965), although a previous validation study of the heated air hand box - with measurements over a period of one hour only - reported no statistically significant effect of heating on contralateral forearm blood flow compared with control (Gallen and MacDonald 1990). From the results of Study 2, it would clearly be possible for the unwary investigator to confuse the vasodilator effects of hand-warming with those directly attributable to hyperinsulinaemia in studies assessing the importance of blunting of insulin-mediated vasodilatation in insulin-resistant states.

The forearm vasodilator response to leg heating is thought to be mediated by the sympathetic nervous system, probably by a reciprocal decrease in vasoconstrictor tone and an increase in vasodilator tone (Lewis et al 1931, Barcroft et al 1947, Moan et al 1995). The increase in heart rate and contralateral forearm blood flow observed in the present study might therefore be attributed to activation of vasodilator fibres of the sympathetic nervous system. Although the two studies were not directly comparable, and forearm blood flow was not measured during hyperinsulinaemia, the present data suggest that the haemodynamic effects of hand-warming are more pronounced during hyperinsulinaemia. Increased skin blood flow accounts for most of the forearm vasodilator response to leg-warming (Roddie et al 1956, Edholm et al 1956), and Astrup and colleagues (using ^{133}Xe

clearance) have reported that hand-warming during a glucose tolerance test increases *subcutaneous* blood flow leaving total forearm blood flow unaffected (Astrup et al 1988). One might speculate that, in the present study, haemodynamic effects observed during hand-warming mediated the associated changes in insulin sensitivity: during hyperinsulinaemia, hand-warming may have resulted in increased skin blood flow in the contralateral arm (and possibly other sites) diverting glucose delivery away from the insulin-sensitive skeletal muscle vascular bed.

Despite the concerns raised by the present study, it is reassuring that the heated-air hand box had negligible effects on derived M-values when clamps were performed in the usual manner. Haemodynamically-mediated effects of hand-warming on M-values may occur in the same direction as effects of true "arterialisation" i.e. reducing overestimation of M with respect to arterial clamps.

The observations reported are limited to healthy male volunteers, and measurements of sympathetic nervous system activation have not been performed; if all subjects were equally sensitive to such haemodynamic effects, and clamps were conducted in the usual manner, such effects would be of little practical importance. However, it may not be safe to extrapolate this assumption to metabolic studies of patients with cardiovascular disease. The findings would be of potential importance in a case-control comparison of insulin sensitivity if one group of subjects were more sensitive to sympathetic nervous system activation than the other (Goldstein 1983). It is a testable hypothesis that a false positive finding of reduced insulin sensitivity in patients with essential hypertension might

arise as a result of hand-warming, even if clamps were conducted in the conventional manner.

Small baseline differences in glucose levels between superficial and deep veins (Baltzan et al 1962) are known to exist, but these do not account for the reported findings as sampling sites were not compared; rather, the effect of hand-warming on derived M-value *within* each sampling site was assessed. Contralateral antecubital vein clamps were used purely as an experimental control, and it should be emphasised that adjusting the glucose infusion rate on the basis of contralateral arm glucose values is not recommended in clinical studies. The coefficient of variation of glucose levels was higher for box-on than for box-off conditions, but this applied for both sampling sites and is therefore unlikely to account for the findings.

In summary, in these controlled studies, hand-warming by the heated-air hand box decreased derived insulin sensitivity (M) as measured by the euglycaemic clamp technique in healthy subjects when clamps were performed according to samples from a site unlikely to be affected by arterialisation (the contralateral antecubital vein). However, there was no difference in M-value with hand-warming when clamps were based on samples from a site in which arterialisation could be demonstrated (the ipsilateral dorsal hand vein). In addition, hand-warming appeared to cause an increase in heart rate during hyperinsulinaemia and an increase in contralateral forearm blood flow under basal conditions (systemic haemodynamic effects). These findings sound a note of caution for the interpretation of studies which have used the euglycaemic clamp technique to

investigate metabolic aspects of cardiovascular disease: hand-warming has the potential to confound euglycaemic clamp studies with a case-control design, particularly those assessing the haemodynamic effects of insulin.

Chapter 5

Serum insulin concentrations, insulin sensitivity, and blood pressure: is assay specificity important?

5.0 Summary

In the clinical study described in this chapter, the effect of insulin assay specificity on relationships among serum insulin concentrations, insulin sensitivity and blood pressure were examined in a group of 56 diabetic (NIDDM) and non-diabetic subjects characterised for insulin sensitivity using the euglycaemic clamp technique.

5.1 Introduction

In non-diabetic obese and hypertensive individuals, normal glucose tolerance is maintained, at least in the short term, by increased pancreatic ß-cell secretion of insulin. Serum insulin concentrations in such individuals are raised in proportion to the degree of insulin resistance and the resulting hyperinsulinaemia has been implicated in the pathogenesis of cardiovascular disease (Reaven 1988, Pyorala 1979, Ducimitiere et al 1980, Stout 1989, Després et al 1996) (Section 1.1). Blood pressure appears to be more closely related to insulin sensitivity than to serum insulin concentrations (Pinkney et al 1994), but measurement of insulin sensitivity is relatively labour-intensive and circulating insulin concentrations (fasting and post-glucose load) have been used as surrogate measurements in many of the large-scale studies which have implicated insulin resistance in the

pathogenesis of essential hypertension (Berglund et al 1976, Modan et al 1985, Morales et al 1993, Jiang et al 1993a, Jiang et al 1993b, Feskens et al 1995) (Section 1.3). However, the relationship between insulin concentrations and blood pressure is variable among studies and ethnic groups, particularly after adjustment for confounding variables such as body mass index, and its existence and significance remain controversial (Mbanya et al 1988, Asch et al 1991, Saad et al 1991, Dowse et al 1993, Muller et al 1993).

Commercially-available radioimmunoassays for insulin cross-react with intact proinsulin and its partially-processed split and des-amino products. Sensitive and specific assays have now been developed for these insulin precursor hormones (Sobey et al 1989, Alpha et al 1992, Hales 1994) (Section 1.3.1). While partially-processed proinsulin products have decreased biological activity in terms of glucose disposal when compared with insulin, they have longer half-lives and are not converted to insulin in the circulation (Galloway et al 1992) (Section 1.3.3). It has been reported that total proinsulin rather than insulin concentrations are more strongly related to cardiovascular risk factors in both non-diabetic (Haffner et al 1993, Mohamed-Ali et al 1995) and NIDDM (Nagi et al 1990) populations, and adults who were of low birth weight appear to have abnormal proinsulin processing (Hales et al 1991). It is possible that the cardiovascular risk attributed to hyperinsulinaemia might in part reflect cross-reactivity of proinsulin-like molecules in conventional insulin assays.

The study was designed to clarify for the first time whether the relationships among insulin concentrations, insulin sensitivity (measured using the "gold

standard" euglycaemic clamp technique), and blood pressure are affected by the specificity of the assay used to determine serum insulin concentrations and hence whether some of the variability between studies in the relationship between insulin concentrations and blood pressure might be accounted for by use of insulin assays with different degrees of cross-reactivity with proinsulin and its split/ des-amino products.

5.2 Methods

5.2.1 Patients

56 Caucasian subjects (26 patients with NIDDM, 30 non-diabetic subjects) (Table 5.1) were recruited (Section 2.1) and gave informed consent to participate in the study. Subjects with a body mass index of < 30 kg/m^2 were classified as "lean"; otherwise they were deemed "obese." Hypertension and NIDDM were diagnosed according to the criteria stated in Section 2.1.

5.2.2 Clinical Procedure

The study design was such that patients and controls were studied concurrently: for each lean hypertensive subject recruited (NIDDM or non-diabetic), two age- and sex-matched controls were recruited: one lean normotensive and the other obese hypertensive. All subjects recruited attended an initial screening visit, when baseline characteristics were recorded, followed by two further study days. On the first of these, a standard 75g oral glucose tolerance test was performed (Section 2.3.4); fasting cholesterol and triglycerides were measured on the baseline sample only (Section 2.8.9). On the second day, a euglycaemic clamp was performed (Section 2.3.5).

5.2.3 Insulin assays

Serum insulin concentrations were determined by a commercially available assay specific for insulin (Lifescreen, Insulin EASIA, Watford, U.K.) and also by a conventional assay with a quoted 62% molar cross-reaction with intact proinsulin (Section 2.8.1).

5.2.4 Statistical analysis

All data were checked for normality using the Shapiro-Wilks test (Minitab statistical package, Minitab Inc, Pennsylvania, USA), and skewed data were logarithmically transformed (log_{10}) (Section 2.9). A Bland-Altman plot of insulin concentrations as measured by the two assays was performed (Altman 1991). Area-under-the-curve (AUC) insulin was calculated as a summary measure for each individual (Matthews et al 1990). Log_{10} serum insulin and glucose concentrations were back-transformed to give the geometric mean for presentation of oral glucose tolerance test data. One-way ANOVA was used in the analysis of steady state serum insulin concentrations and insulin sensitivity index by subgroup. Unpaired t-tests were used for comparisons between subgroups; in order to adjust for the use of data from the lean hypertensives in both comparisons, 97.5% confidence intervals were used, and $2p < 0.05$ was taken to indicate statistical significance.

In univariate analysis, simple correlations were plotted of the relationships among serum insulin concentrations, insulin sensitivity, and blood pressure for the specific and conventional assays. The relative contributions of other variables (age, sex, fasting and post-load glucose concentrations, BMI, waist-to-hip ratio,

smoking status, and fasting cholesterol and triglyceride concentrations) to the relationships observed were examined by multiple regression analyses (all subsets and forward stepwise). These analyses were performed separately for both specific and conventional assays.

5.3 Results

56 subjects were recruited and completed the protocol without complication. Two of the non-diabetic subjects screened (one lean normotensive, one lean hypertensive) were excluded as they were found on the basis of their post-load glucose concentrations to have impaired glucose tolerance (IGT). In addition, four of the nine obese hypertensive non-diabetic subjects screened were found to have post-load IGT; these subjects were not excluded. Satisfactory matching was achieved between comparable subgroups for age, sex, and body mass index although within the NIDDM group the lean normotensive and obese hypertensive subgroups were significantly younger than the lean hypertensive subgroup (Table 5.1).

5.3.1 Simple assay comparison

Insulin concentrations as measured by the two methods were highly correlated ($r=0.97$, $p<0.0001$, Figure 5.1a). A Bland-Altman plot revealed that AUC insulin concentrations by the conventional method were greater than or equal to those by the specific method in 52 of the 56 subjects (Figure 5.1b). The largest differences detected were in the obese NIDDM subjects and in those obese non-diabetic subjects who were glucose intolerant (Figure 5.1b). In the four subjects

Table 5.1 Characteristics of 56 non-diabetic and NIDDM subjects (mean±SD)

	Lean normotensive	**Lean hypertensive**	**Obese hypertensive**
Non-diabetic subjects	n=11	n=10	n=9
age (years)	38±8.0	44±8.8	49±10.0
BMI (kg/m^2)	24.8±2.93	26.5±2.35	32.9±2.79$^{\#\#\#}$
M/F	10/1	8/2	9/0
MAP (mmHg)	92±10.2	119±7.0***	121±11.6***
Glucose intolerant	-	-	4
Fasting glucose (mmol/l)	5.4±0.44	5.3±0.55	5.9±0.56
Cholesterol (mmol/l)	4.91±0.80	5.64±1.41	5.03±0.61
Duration hypertension (months: median (range))	-	3 (2-300)	3 (2-36)
NIDDM subjects	n=11	n=7	n=8
age (years)	54±7.5	67±6.2**	57±11.9
BMI (kg/m^2)	25.4±2.91	25.8±2.74	34.4±4.11$^{\#\#\#}$
M/F	9/2	6/1	7/1
MAP (mmHg)	96±6.3	118±8.0***	123±5.6***
Fasting glucose (mmol/l)	9.1±2.49	8.0±1.42	8.2±2.09
Cholesterol (mmol/l)	5.87±1.04	4.92±0.50	6.21±0.95
Duration hypertension (months: median (range))	-	3(3-36)	3(3-41)
Duration diabetes (months: median (range))	19 (4-98)	10 (2-60)	16 (2-84)

* = 2p < 0.05, ** = 2p < 0.01, *** 2p = < 0.001 lean hypertensive vs lean normotensive
# = 2p < 0.05, ## = 2p < 0.01, ### 2p = < 0.001 obese hypertensive vs lean hypertensive

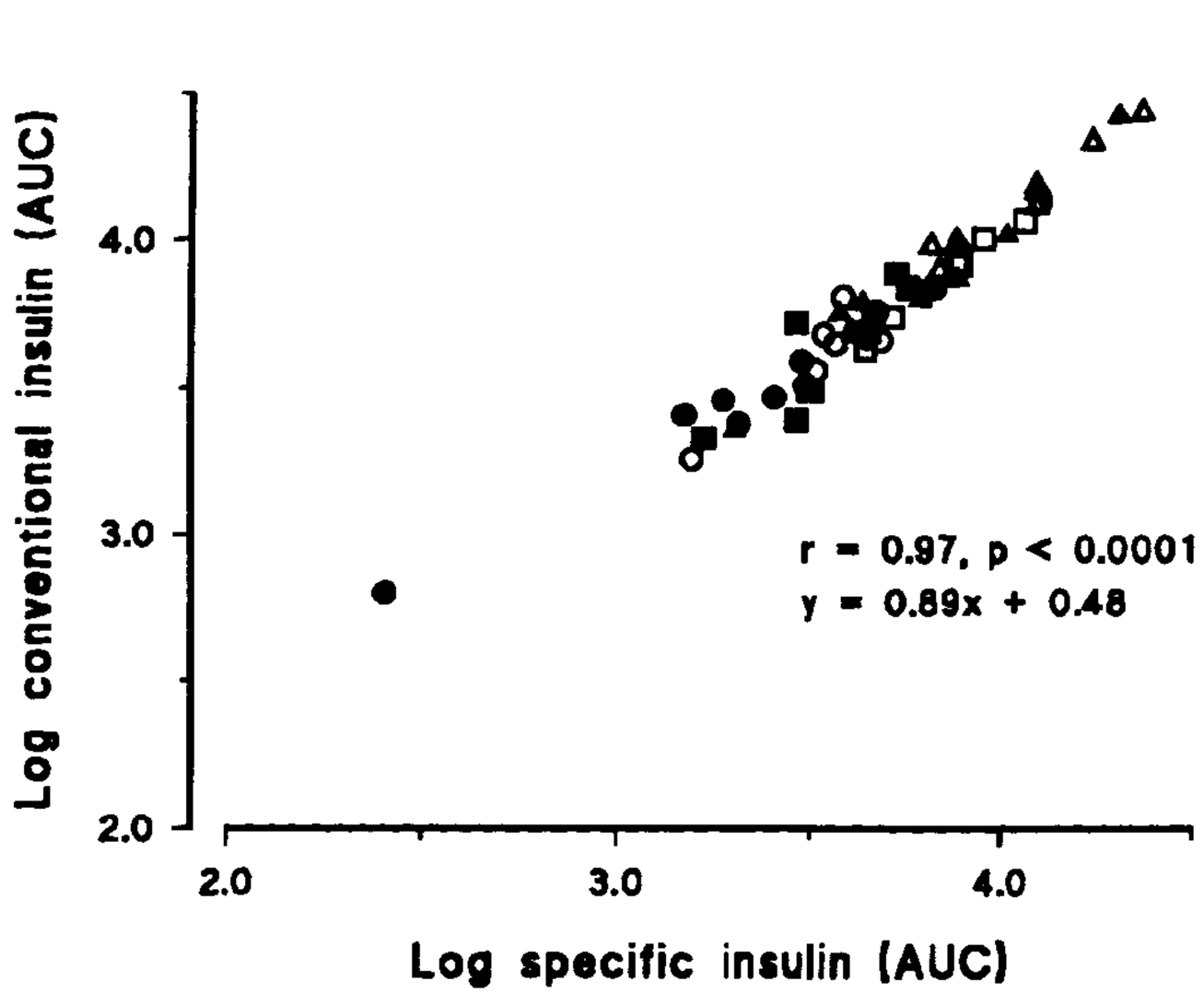
in whom specific insulin concentrations were apparently greater than conventional insulin concentrations, the magnitude of the difference was small and reflected a difference at a single time-point in the later part of the OGTT.

5.3.2 Insulin and glucose concentrations

a) Insulin: Fasting and AUC insulin results were similar for both specific and conventional assays. In the non-diabetic subjects, AUC insulin was greater in obese hypertensive compared with lean hypertensive subjects ($2p < 0.05$, Figure 5.2); there was a tendency for AUC insulin to be greater in lean hypertensive compared with lean normotensive subjects, but this did not reach statistical significance ($2p = 0.14$, Figure 5.2). In NIDDM subjects there was a trend for AUC insulin to be greater in obese hypertensive compared with lean hypertensive subjects, whom in turn tended to have higher AUC insulin than lean normotensive subjects; however, these trends did not reach statistical significance (Figure 5.2).

b) Glucose: There were no statistically significant differences in fasting glucose concentrations within the non-diabetic and NIDDM groups (Table 5.1). However, within the non-diabetic group, AUC glucose was higher in obese hypertensive compared with lean hypertensive subjects ($2p < 0.05$), and in lean hypertensive compared with lean normotensive subjects ($2p < 0.05$, Figure 5.2).

Figure 5.1 Insulin assay comparison:
a) Simple correlation: log10 AUC insulin from OGTT data in all subjects (n=53, missing data in 3 cases) determined using conventional and specific assays
b) Bland-Altman plot: the difference between AUC insulin as measured by the conventional and specific assays plotted against log10 specific AUC insulin.



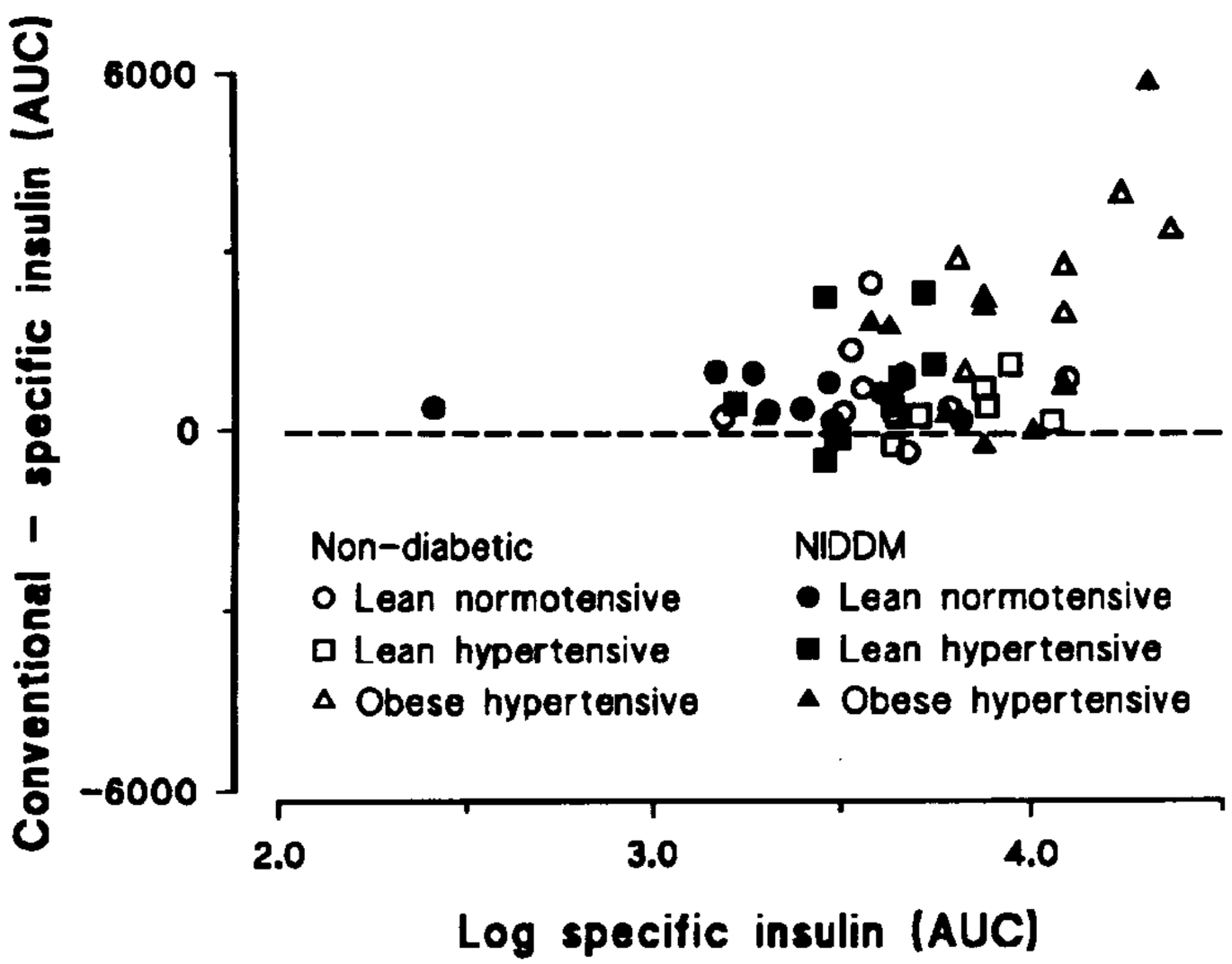
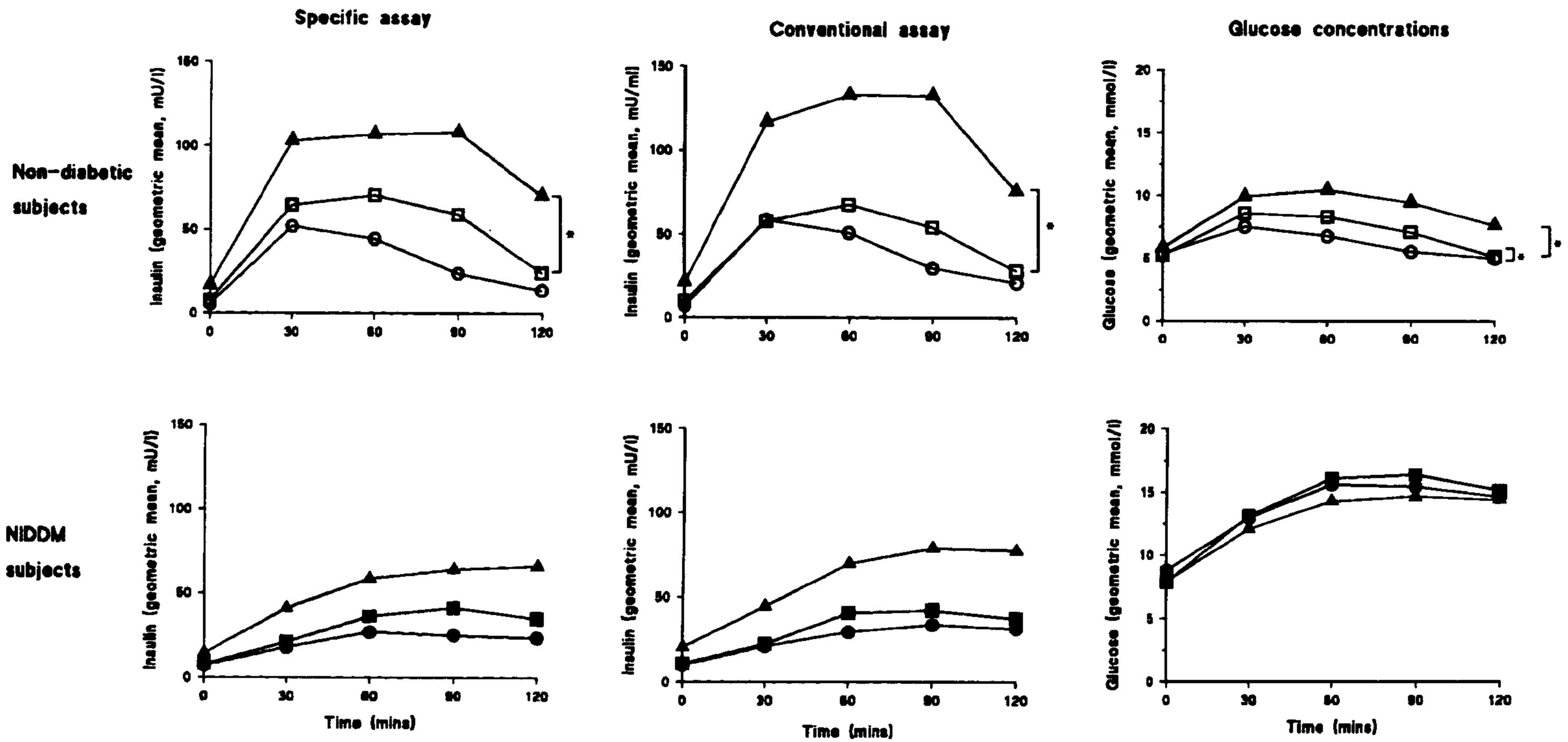


Figure 5.2 Insulin and glucose concentrations (geometric means).
* = 2p < 0.05 for comparison of log10 AUC between groups.



Non-diabetic subjects: ○ = lean normotensive, □ = lean hypertensive, △ = obese hypertensive.
NIDDM subjects: ● = lean normotensive, ■ = lean hypertensive, ▲ = obese hypertensive.

Figure 5.3 Insulin sensitivity index (SIP x 104 dl/(min.kg) per U/ml) for each subject (n=56) as measured by a three hour euglycaemic clamp.
Upper square brackets, one-way ANOVA by subgroup;
lower square brackets. 97.5% confidence intervals for comparisons between individual subgroups (see text).

* = p < 0.05, ** = p < 0.01, *** = p < 0.001

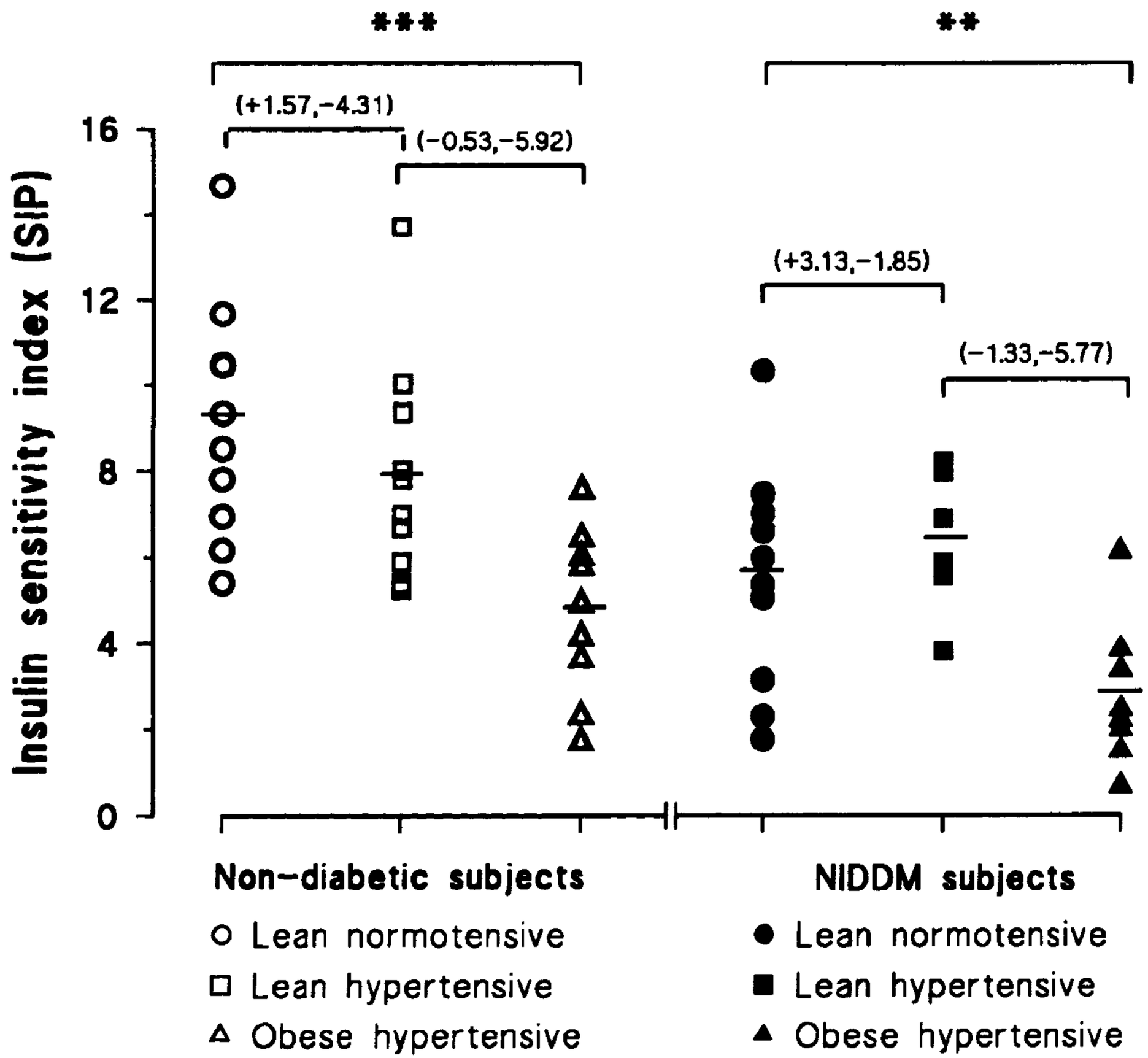
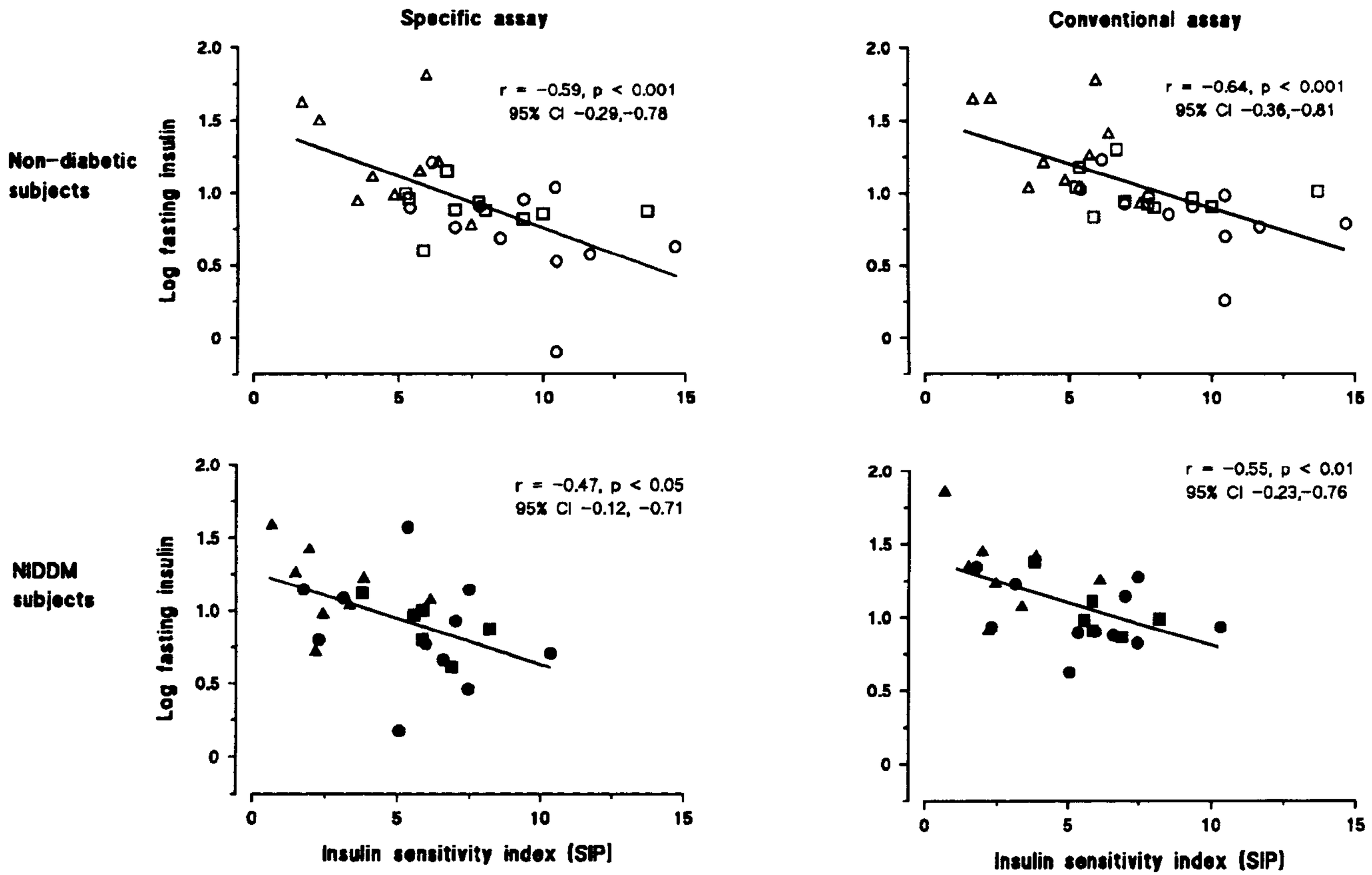
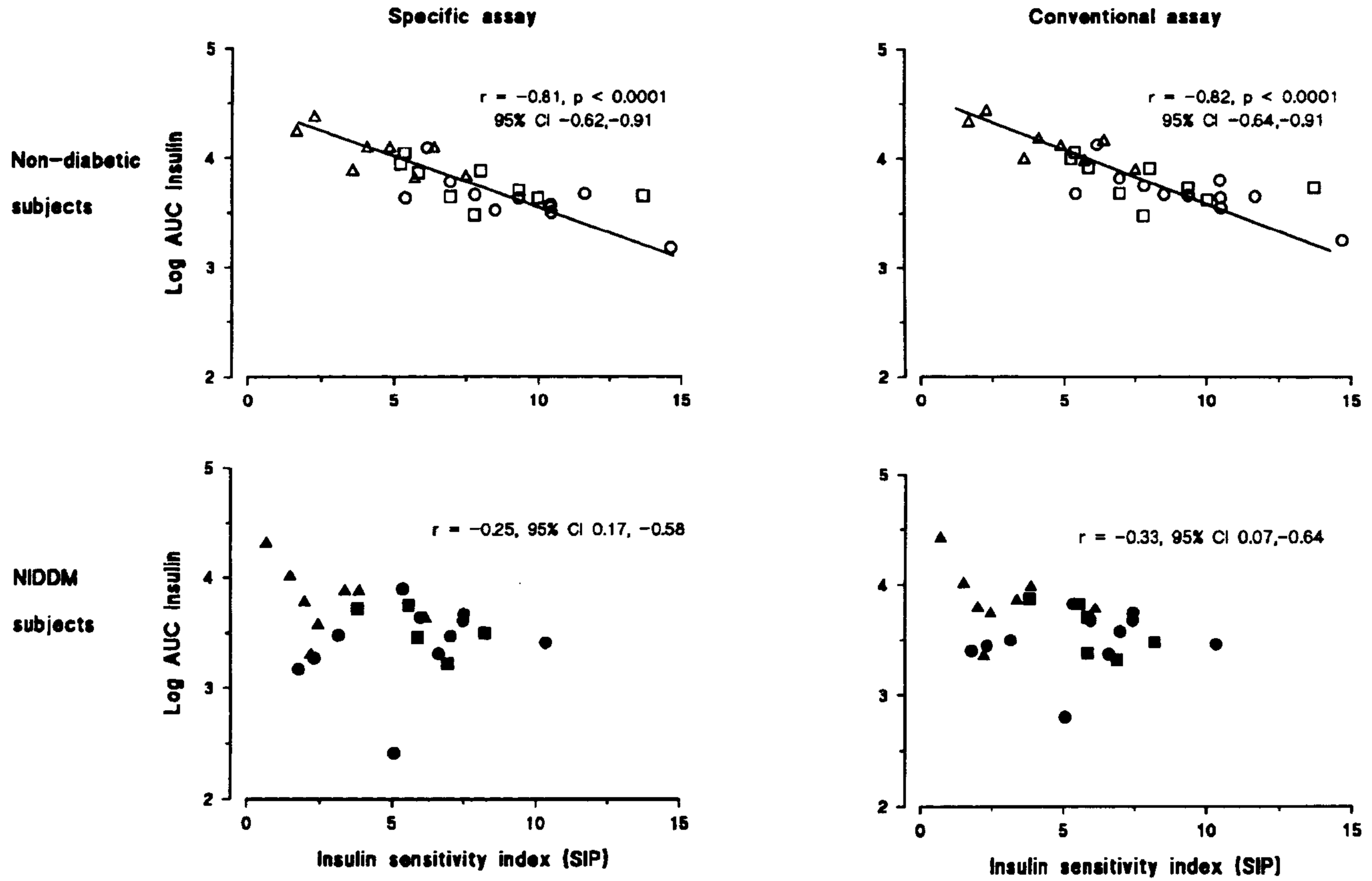


Figure 5.4 Log10 fasting insulin plotted against insulin sensitivity index (SIP x 104 dl/(min.kg) per U/ml) as measured by a 3 hour euglycaemic clamp. Pearson's r is shown together with 95% confidence intervals. *Upper panels:* Non-diabetic subjects (n= 30). *Lower panels:* NIDDM subjects (n= 25, 1 subject with missing data).



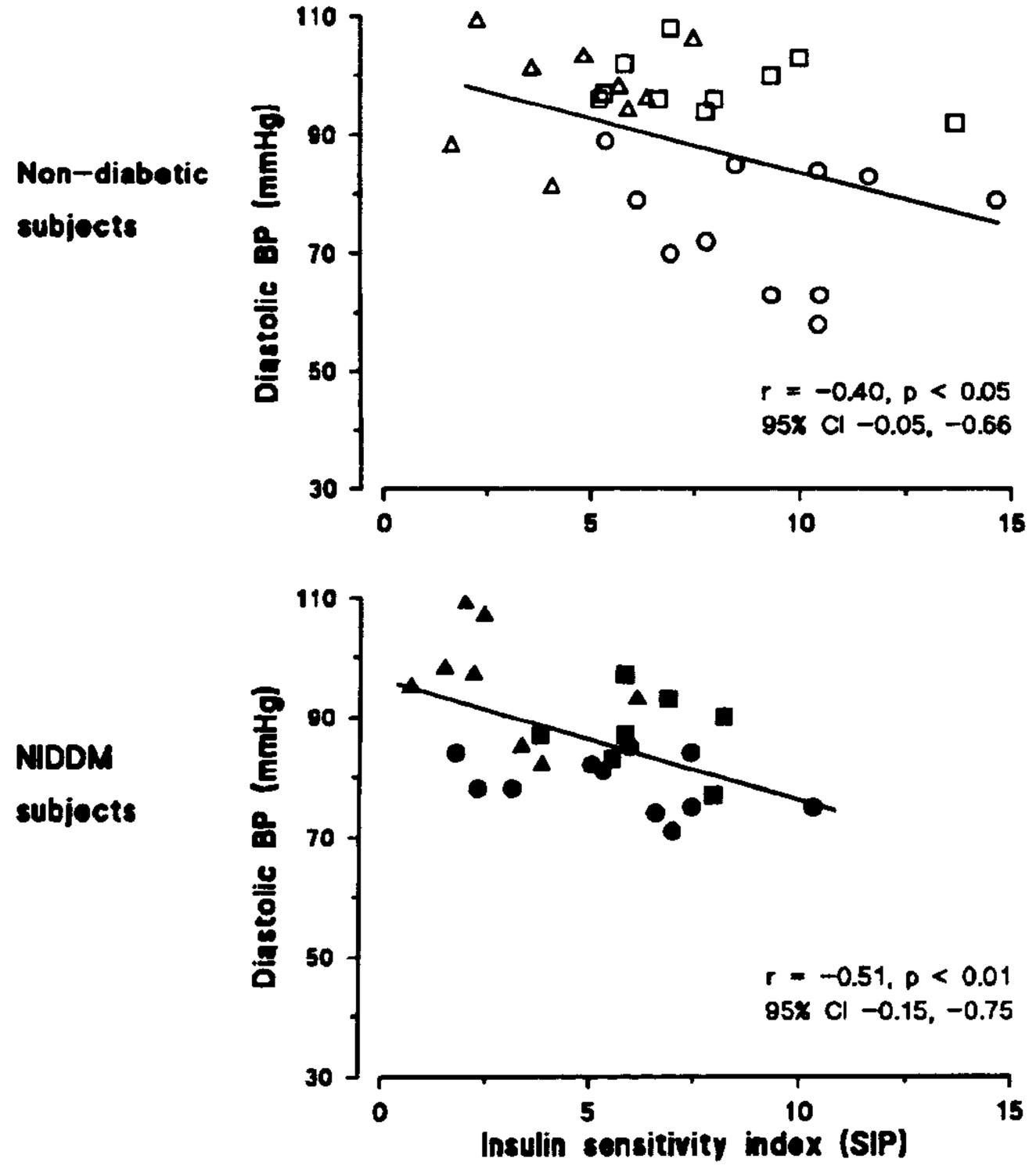
Non-diabetic subjects: ○ = lean normotensive, □ = lean hypertensive, △ = obese hypertensive.
NIDDM subjects: ● = lean normotensive, ■ = lean hypertensive, ▲ = obese hypertensive.

Figure 5.5 Log10AUC insulin plotted against insulin sensitivity index (SIP x 104 dl/(min.kg) per U/ml) as measured by a 3 hour euglycaemic clamp. Pearson's r is shown together with 95% confidence intervals.
Upper panels: Non-diabetic subjects (n= 28, 2 subjects with missing data).
Lower panels: NIDDM subjects (n= 25, 1 subject with missing data).



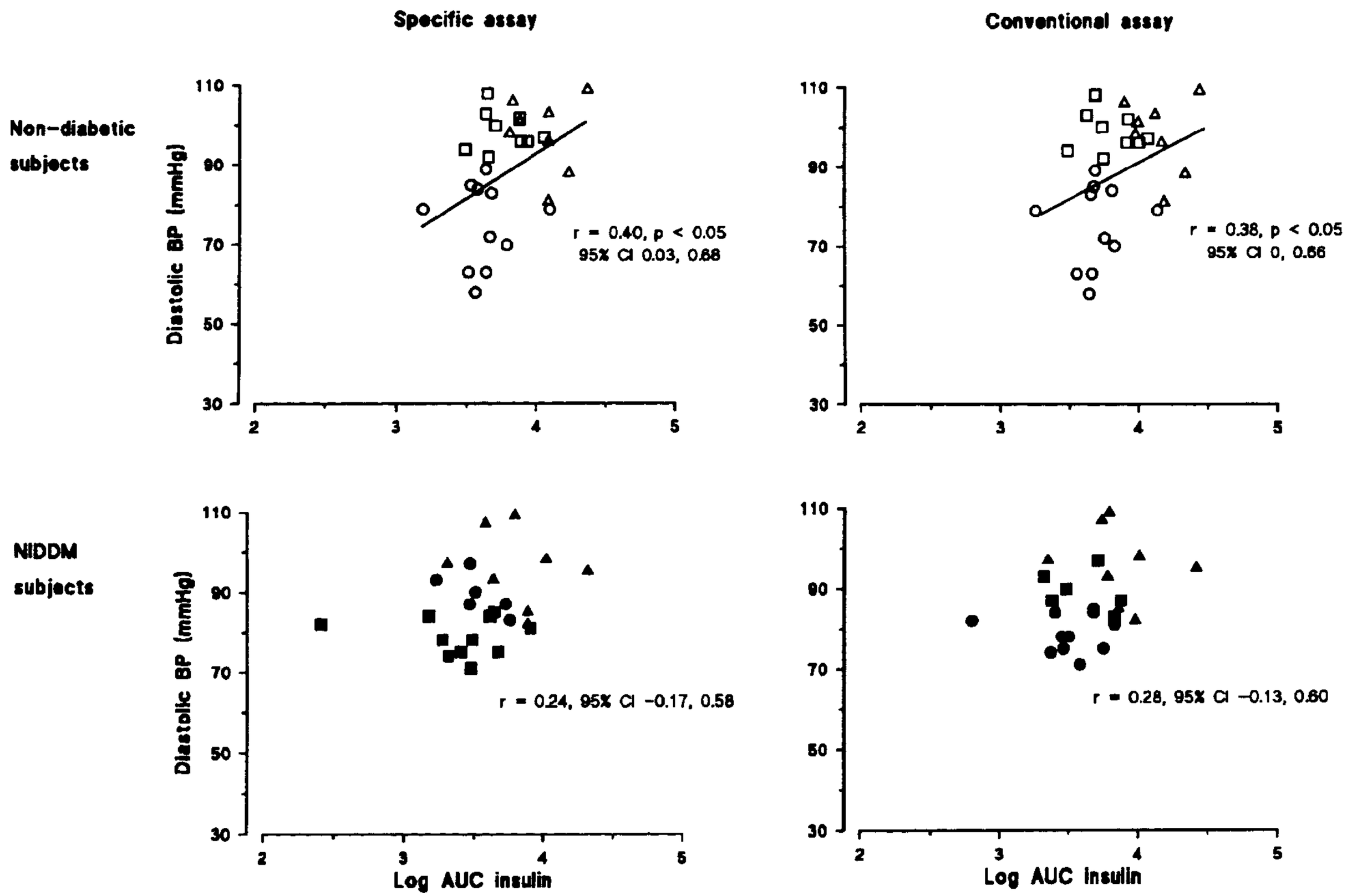
Non-diabetic subjects: ○ = lean normotensive, □ = lean hypertensive, △ = obese hypertensive.
NIDDM subjects: ● = lean normotensive, ■ = lean hypertensive, ▲ = obese hypertensive.

Figure 5.6 Blood pressure and insulin sensitivity index. Diastolic blood pressure (mmHg) plotted against insulin sensitivity index (SIP x 104 dl/(min.kg) per U/ml) as measured by a euglycaemic clamp. Pearson's r is shown together with 95% confidence intervals.
Upper panel: Non-diabetic subjects (n=30). *Lower panels:* Subjects with NIDDM (n= 26).



Non-diabetic subjects: ○ = lean normotensive, □ = lean hypertensive, △ = obese hypertensive.
NIDDM subjects: ● = lean normotensive, ■ = lean hypertensive, ▲ = obese hypertensive.

Figure 5.7 Blood pressure and AUC insulin. Diastolic blood pressure (mmHg) plotted against log10AUC insulin concentrations. Pearson's r is shown together with 95% confidence intervals.
Upper panel: Non-diabetic subjects (n= 28, 2 subjects with missing data).
Lower panels: Subjects with NIDDM (n= 25, 1 subject with missing data).



Non-diabetic subjects: ○ = lean normotensive, □ = lean hypertensive, △ = obese hypertensive.
NIDDM subjects: ● = lean normotensive, ■ = lean hypertensive, ▲ = obese hypertensive.

5.3.3 Insulin sensitivity

Mean±SD steady-state insulin concentrations (μU/ml) achieved during the euglycaemic clamp procedures were higher in obese subjects [non-diabetic subjects 113±20.6 (lean normotensive), 115±27.2 (lean hypertensive), 163±38.0 (obese hypertensive); NIDDM subjects 109±19.0 (lean normotensive), 121±27.9 (lean hypertensive), and 159±60.6 (obese hypertensive)], $p < 0.01$, ANOVA. The insulin sensitivity index (S_{IP}), correcting for steady state insulin concentrations, was therefore calculated for each subject (Figure 5.3). Coefficients of variation of blood glucose during the final 40 minutes of the clamps were 3.3% (non-diabetic subjects) and 4.7% (NIDDM subjects).

In both the non-diabetic subjects and subjects with NIDDM, insulin sensitivity index (Figure 5.3) was lower in the sub-groups with higher blood pressure and increased weight (non-diabetic subjects, $p < 0.001$; NIDDM subjects $p < 0.01$, ANOVA). When subgroups were compared, with adjustment for multiple comparisons, insulin sensitivity index was lower in obese than lean hypertensive subjects (non-diabetic subjects, $2p < 0.05$; NIDDM subjects, $2p < 0.01$).

5.3.4 Insulin concentrations and insulin sensitivity

The univariate relationships between insulin sensitivity and serum insulin concentrations were independent of assay specificity in both non-diabetic subjects and subjects with NIDDM (Table 5.2).

a) Fasting insulin

i) Non-diabetic subjects: There was a negative correlation (Figure 5.4) between $\log_{10}$ fasting serum insulin and insulin sensitivity index (r=-0.59, p< 0.001, 95% CI -0.29, -0.78 (specific assay), r=-0.64, p<0.001, 95% CI -0.36, -0.81 (conventional assay).

ii) *Subjects with NIDDM:* There was a negative correlation (Figure 5.4) between $\log_{10}$ fasting serum insulin and insulin sensitivity index [r=-0.47, p<0.05, 95% CI -0.12, -0.71 (specific assay), r=-0.55, p<0.01, 95% CI -0.23, -0.76 (conventional assay)].

b) AUC insulin:

i) Non-diabetic subjects: There was a negative correlation (Figure 5.5) between $\log_{10}$ AUC insulin and insulin sensitivity (r=-0.81, p<0.0001, 95% CI -0.62, -0.91 (specific assay), r=-0.82, p<0.0001, 95% CI -0.64, -0.91 (conventional assay)). In multiple regression analysis, AUC insulin (measured by either specific or conventional assay) was a significant predictor of insulin sensitivity even when waist-to-hip ratio was included in the model (Table 5.3).

ii) NIDDM subjects: There was no significant relationship between $\log_{10}$ AUC insulin and insulin sensitivity index (r=-0.24, 95% CI +0.17, -0.58 (specific assay), r=-0.33, 95% CI +0.07, -0.64 (conventional assay). In multiple regression analysis, BMI was the only significant predictor of insulin sensitivity (adjusted R^2 0.46, t=-4.71, p < 0.001).

Table 5.2: Univariate correlations (Pearson's r) between demographic, metabolic and haemodynamic measurements and insulin sensitivity index (S_{IP}).

	Non-diabetic subjects (n=30)	**NIDDM subjects (n=26)**
	r-value	**r-value**
age	-0.40*	-0.22
BMI	-0.77***	-0.69***
Systolic BP	-0.29	-0.16
Diastolic BP	-0.40*	-0.51**
Waist-to-hip ratio	-0.75***	-0.66***
Fasting glucose (log_{10})	-0.23	-0.21
Fasting triglycerides (log_{10})	-0.42*	-0.47*

	Specific insulin assay	**Conventional insulin assay**	**Specific insulin assay**	**Conventional insulin assay**
	r-value	**r-value**	**r-value**	**r-value**
Fasting insulin (log_{10})	-0.59***	-0.64***	-0.47*	-0.55**
30-minute insulin (log_{10})	-0.67***	-0.68***	-0.23	-0.35
120 minute insulin (log_{10})	-0.49**	-0.44*	-0.19	-0.28
AUC insulin (log_{10})	-0.81***	-0.82***	-0.24	-0.33

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

5.3.5 Blood pressure and insulin sensitivity

a) Non-diabetic subjects: Diastolic blood pressure was negatively correlated with insulin sensitivity index (Figure 5.6) (r=-0.40, $p < 0.05$, 95% CI -0.05, -0.66). However, in multiple regression analysis, age was the only significant predictor of blood pressure (adjusted R^2 0.23, $t = 3.10$, $p < 0.01$).

b) NIDDM subjects: Diastolic blood pressure was negatively correlated with insulin sensitivity index (Figure 5.6) (r=-0.51, $p < 0.01$, 95% CI -0.15, -0.75). However, in multiple regression analysis, the relationship was not statistically significant after body mass index was included in the model (Table 5.4).

5.3.6 Insulin concentrations and blood pressure

a) Non-diabetic subjects: The univariate relationship between $\log_{10}$ AUC insulin and diastolic blood pressure was similar with both assays (Figure 5.7): non-diabetic subjects r=0.40, $p < 0.05$, 95% CI 0.03, 0.68 (specific assay); r=0.38, $p < 0.05$, 95% CI 0, 0.66 (conventional assay). However, in multiple regression analysis, AUC insulin was not a significant predictor of blood pressure.

b) NIDDM subjects: No significant relationship was detected with either assay: r=0.24, p=0.18, 95% CI -0.17, 0.58 (specific assay), r=0.28, p=0.17, 95% CI -0.13, 0.60 (conventional assay).

Table 5.3 Multiple regression models with insulin sensitivity index (S_{IP}) as dependent variable in non-diabetic subjects

Model	AUC insulin[1] *(specific assay)*	Waist-to-hip ratio	Adjusted R^2	*t* for AUC insulin	*p*
1	-9.90 (-12.42 to -7.08)		0.65	-7.15	<0.001
2	-6.9 (-9.56 to -4.24)	-20.9 (-30.35 to -9.95)	0.80	-5.26	<0.001

Model	AUC insulin[1] *(conventional assay)*	Waist-to-hip ratio	Adjusted R^2	*t* for AUC insulin	*p*
1	-9.66 (-12.27 to -7.05)		0.67	-6.33	<0.001
2	-6.86 (-9.41 to -4.31)	-21.0 (-30.66 to -11.34)	0.83	-5.16	<0.001

[1]AUC insulin indicates area-under-the-curve insulin, measured during a standard oral glucose tolerance test (see text).

The table shows prediction of insulin sensitivity index from AUC insulin and waist-to-hip ratio by multiple regression analysis in 30 non-diabetic subjects. Changes are shown as adjusted R^2 and *t* for AUC insulin, with forward stepwise addition of waist-to-hip ratio. AUC insulin remains a significant predictor of S_{IP} after waist-to-hip ratio is included in the model. Partial regression coefficient (β) and 95% confidence intervals are shown. Predictor variables: model 1, AUC insulin; model 2, AUC insulin plus waist-to-hip ratio.

Table 5.4 Multiple regression models with diastolic blood pressure as dependent variable in subjects with NIDDM

Model	S_{IP}	Body mass index (BMI)	Adjusted R^2	*t* for S_{IP}	*p*
1	-2.02 (-3.34 to -0.65)		0.23	-2.90	<0.01
2	-1.00 (-2.84 to 0.84)	0.72 (-0.18, 1.62)	0.27	-1.06	0.3

[1]S_{IP} indicates insulin sensitivity index, as measured by the isoglycaemic clamp technique (see text).

The table shows prediction of diastolic blood pressure from S_{IP} and BMI in 26 subjects with NIDDM. Changes are shown as adjusted R^2 and *t* for S_{IP}, with forward stepwise addition of BMI. S_{IP} is not a significant predictor of diastolic BP after BMI is included in the model. Partial regression coefficient (β) and 95% confidence intervals are shown. Predictor variables: model 1, S_{IP}; model 2, S_{IP} plus BMI.

5.4 Discussion

The major finding of this study was that, in the subjects studied, the specificity of the insulin assay used to measure serum insulin concentrations had no detectable effect on the relationships observed amongst serum insulin concentrations, insulin sensitivity, and blood pressure. Serum insulin concentrations measured by specific and conventional assays were highly correlated, and the relationships among lean, obese, and hypertensive NIDDM and non-diabetic subgroups in terms of serum insulin concentrations were almost identical irrespective of the assay method used. The present study is the first, as far as can be ascertained, to examine the relationships among serum insulin concentrations as measured by both conventional and specific insulin assay methods, euglycaemic-clamp derived insulin sensitivity, and blood pressure.

Many of the large-scale studies which have examined the relationship between insulin concentrations and blood pressure in non-diabetic subjects measured insulin using conventional radioimmunoassays with a high degree of cross-reactivity with proinsulin and its partially-processed intermediates (Berglund et al 1976, Modan et al 1985, Morales et al 1993, Jiang et al 1993a, Jiang et al 1993b, Feskens et al 1995). Three of these studies (Modan et al 1985, Jiang et al 1993a, Jiang et al 1993b, Feskens et al 1995) used the same commercially available conventional assay kit (Pharmacia RIA 100) that was used in this study. In

contrast, there are few published data on the relationship between specific insulin concentrations and blood pressure, and previous data are conflicting (Section 1.3.2).

The detailed nature of the present study was such that only a relatively small number of subjects could be studied (n=56). Insulin sensitivity was measured by a three hour euglycaemic clamp technique which is the acknowledged current "gold standard" (Keen 1994), and the patients studied were not taking potentially-confounding drug treatment. In non-diabetic subjects, the relationship between serum insulin concentrations (as determined by either assay method) and blood pressure was almost identical to that between euglycaemic clamp-derived insulin sensitivity and blood pressure. Taken with the findings from the San Antonio group (Haffner et al 1994), the present data suggest that assay specificity is unlikely to account for discrepancies between epidemiological studies in the strength of the relationship observed between serum insulin concentrations and blood pressure. It is likely that such discrepancies are accounted for either by differences in the populations studied (Dowse et al 1993), or by lack of standardisation of insulin assays between centres (Robbins et al 1996). Proinsulin and partially-processed proinsulin products were not measured in the present study, but the relationships examined were almost identical when insulin concentrations were measured with insulin assays of both relatively high and no cross-reactivity with these molecules. Disproportionately elevated insulin concentrations measured with the conventional as opposed to specific insulin

assay, suggesting high levels of proinsulin-like molecules, were observed mainly in obese (NIDDM or non-diabetic glucose intolerant) subjects. However, this does not explain the lack of a univariate relationship between serum insulin concentrations and blood pressure in these subjects as similar correlation coefficients and confidence intervals were observed when insulin concentrations were measured using the specific assay.

In the subjects studied, the relationships among insulin/insulin sensitivity and blood pressure were confounded by age in non-diabetic subjects, and by body mass index in subjects with NIDDM. The importance of confounding variables in this relationship has been discussed elsewhere (Asch et al 1991, Jarrett 1992, Haffner 1993); however, with respect to the main aim of the present study, it can be concluded that addition of potentially confounding variables to multiple regression models had similar effects on partial regression coefficients irrespective of the method used to determine serum insulin concentrations.

In summary, this study showed no differences attributable to insulin assay specificity in the relationships among serum insulin concentrations, clamp-derived insulin sensitivity, and blood pressure in a group of 56 diabetic and non-diabetic subjects with widely-varying degrees of blood pressure and body mass index. Use of specific assays which have recently become commercially available in future epidemiological studies is unlikely to alter the relationships detected between serum insulin concentrations, insulin sensitivity and blood pressure.

Chapter 6

Dietary salt restriction impairs insulin sensitivity in non-insulin dependent diabetes mellitus

6.0 Summary

In the clinical study described in this chapter, the effect on insulin sensitivity and endogenous glucose production of activation of the renin-angiotensin system (RAS) by dietary sodium restriction was evaluated in patients with NIDDM using a double-blind, placebo-controlled design.

6.1 Introduction

The insulin-sensitising effect of acute angiotensin II infusion on insulin sensitivity (Section 1.7.2) in patients with NIDDM (Morris et al 1994c) is difficult to reconcile with the reported insulin-sensitising effect of chronic ACE inhibitor therapy in the same patients (Vuorinen-Markkola and Yki-Jarvinen 1995)(Section 1.1.6). There are no data on the effect of more sustained activation of the RAS on insulin-mediated glucose uptake in patients with NIDDM, and data from open studies of dietary sodium restriction in healthy volunteers are conflicting (Sharma et al 1993, Donovan et al 1993, Fliser et al 1995).

The aim of this study was to examine the effect of physiological activation of the RAS by dietary sodium restriction on insulin-mediated glucose uptake in patients with NIDDM. This question is not only of metabolic importance but also of

clinical relevance. Total body sodium is elevated in patients with diabetes, and thus 'normal' levels of renin and angiotensin II may be inappropriately high (O'Hare et al 1985, Weidmann et al 1993). Pharmacological and non-pharmacological interventions which influence insulin sensitivity may have an impact on glycaemic control and ultimately the incidence of diabetic complications. Current dietary recommendations suggest dietary sodium restriction for both hypertensive and normotensive patients with diabetes (Nutrition Study Group, European Association for the Study of Diabetes 1988, American Diabetes Association 1996), but the impact of this intervention on insulin sensitivity in NIDDM has not been formally evaluated.

6.2 Methods

6.2.1 Patients

Nine diet-controlled Caucasian patients with NIDDM (one female), mean±SD 57±9.7 years, body mass index (BMI) 29±3.9 kg/m^2, fasting plasma glucose 9.0±2.2 mmol/l (range 5.7 to 12.4 mmol/l), $HbA1_c$ 5.7±0.8 % (normal range 3.4-4.9%), total cholesterol 5.76±1.25 mmol/l with median duration of diabetes 12 months (range 4-92) gave written informed consent to participate in this study. NIDDM was confirmed according to WHO criteria (World Health Organisation 1980), and none had evidence of retinopathy (as determined by direct fundoscopy) or microalbuminuria. Mean blood pressure on screening was 148±25/82±7 mmHg; two were cigarette smokers. Subjects were maintained on an isocaloric diet throughout the study consisting of 55% carbohydrate, 25% fat, and 20%

protein. They were asked to refrain from tobacco or strenuous exercise, and none were receiving any proprietary or prescription medication.

6.2.2 Diets

The sodium replete (160 mmol/day) and sodium deplete (40 mmol/day) diets were allocated in a randomised double-blind placebo-controlled design for four days each (Figure 6.1) (McFadyen et al 1993). On day 1, each patient commenced a 40 mmol/day diet for 4-days prior to the first study day (day 5); on day 8 they began an identical 4-day period prior to the second study day (day 12). During these two periods of 40 mmol/day sodium diet, patients were randomised in a crossover design to receive a) slow sodium tablets (Ciba-Geigy, Horsham, UK) 120 mmol/day, and b) matching placebo slow sodium tablets (double-blind) in random-order. On the first day of the sodium deplete regime, patients received a single oral dose of frusemide (40 mg); this was matched by an identical placebo on the first day of the sodium replete regime.

6.2.3 Clinical Procedure

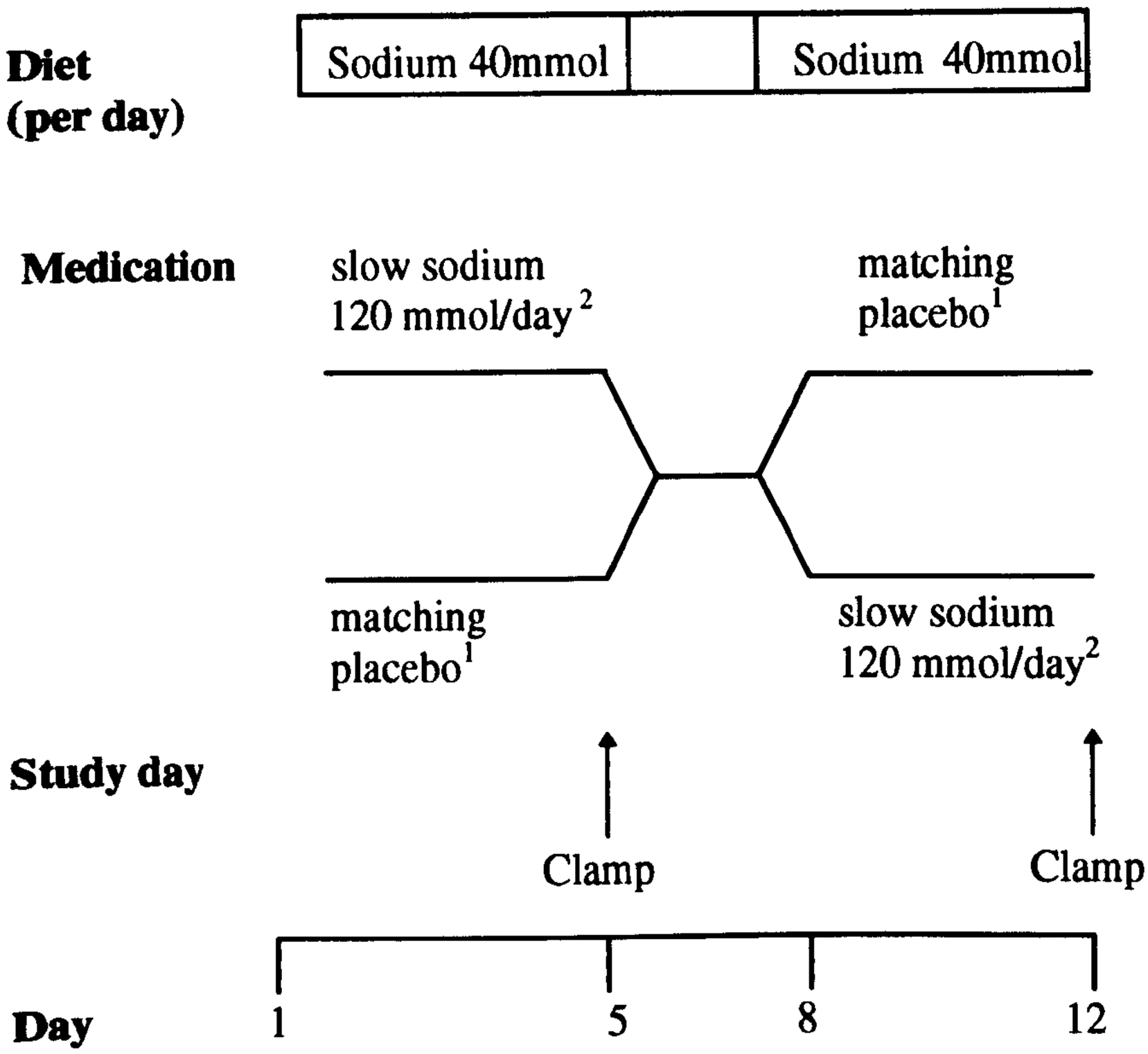
Each patient attended two 5-h study days in the Clinical Investigation and Research Unit to evaluate the effects of the sodium deplete and sodium replete diets on insulin-mediated glucose uptake and endogenous glucose production. On each occasion, after an overnight fast (water was permitted), patients attended at 0800 hrs and completed a 24 hour urine collection on arrival. Insulin sensitivity (M-value) and endogenous glucose production were assessed using the

hyperinsulinaemic clamp technique (DeFronzo et al 1979) with [3-^{3}H]glucose (Levy et al 1989, Hother-Nielsen and Beck-Nielsen 1990, Hother-Nielsen et al 1992) (Sections 2.4 and 2.5). In order to avoid potentially confounding higher rates of insulin-independent glucose disposal in patients with higher fasting serum glucose concentrations, clamps were performed at each patient's own fasting glucose level as determined at the screening visit (i.e. clamps were isoglycaemic rather than euglycaemic) (Saad et al 1994). The total activity of ^{3}H administered to each subject was 5.2 MBq. Blood pressure and heart rate were measured every 10 minutes throughout the procedure. At -120, -60, -30, -20, -10, 0, 60, 120, 130,140 and 150 minutes, additional blood samples were withdrawn for measurement of glucose specific activity, serum insulin, C-peptide, triglycerides, and electrolytes and plasma renin activity (PRA) and aldosterone (Section 2.8). Plasma ANG II was measured at -120, 0 and 150 minutes.

6.2.4 Statistical analysis.

Insulin sensitivity (M and S_{IP}), and endogenous glucose production, were compared on salt replete vs salt replete days using paired t-tests. Measurements of serum insulin, C-peptide, sodium, potassium, PRA, angiotensin II and aldosterone at individual time points were compared between study days by repeated measures analysis of variance (ANOVA) using the Bonferroni method to correct for multiple comparisons (Section 2.9).

Figure 6.1 Study design



[1]sodium deplete diet (single dose frusemide 40 mg given on day 1/day 8)
[2]sodium replete diet (single dose placebo matching frusemide 40 mg given on day 1/day 8)

6.3 Results

The hyperinsulinaemic clamps and diets were well tolerated and there were no adverse events. Nine patients gave informed consent to enter the study, but one patient was withdrawn owing to failure to comply with diet.

6.3.1 BP and weight.

There were no significant differences in systolic BP, diastolic BP, or weight on the sodium replete and deplete diets (Table 6.1).

6.3.2 PRA, plasma ANG II, plasma aldosterone, serum electrolyte and triglyceride concentrations.

24 hour urinary sodium excretion was significantly lower (Table 6.1), and the corresponding PRA, angiotensin II, plasma aldosterone levels and serum sodium concentrations were significantly higher on the sodium deplete diet, both at baseline and throughout the clamp studies (Figures 6.2, 6.3). There were no significant differences in baseline or insulin-mediated reductions in serum potassium or triglycerides between the two study days (Figure 6.3).

6.3.3 Serum insulin, glucose, and C-peptide concentrations.

There were no significant differences in fasting serum insulin or glucose levels with dietary sodium manipulation (Table 6.1). Similarly, there were no significant difference in steady state serum insulin concentrations during the two study days (Figure 6.4).

6.3.4 Insulin sensitivity and endogenous glucose production.

The coefficients of variation of serum glucose at steady state were 3.1% (sodium replete) and 2.4% (sodium deplete). Insulin sensitivity (M-value) was 7.8±2.0 mg/kg/min and 6.5±2.2 mg/kg/min on the sodium replete and sodium deplete diets respectively, p = 0.04, 95% CI (-1.82, -0.04) (Figure 6.5). Insulin sensitivity index (S_{IP}) was 5.8±2.69 and 4.6±2.04 x10^4 dl/min.kg per mU/l on the sodium replete and sodium deplete diets respectively, p = 0.02, 95% CI (-2.14, -0.29). Fasting endogenous glucose production (EGP) was similar on the two diets (1.64±0.85 vs 1.48±0.90 mg/kg/min p = 0.74, 95% CI -0.91, 1.22), but there was a trend towards blunting of insulin-mediated EGP suppression on the sodium deplete diet which failed to reach statistical significance (-0.63±0.94 vs -0.17±1.14 mg/kg/min, p = 0.09, 95% CI -1.04, 0.10) (Figure 6.6).

Table 6.1: Haemodynamic, metabolic and hormonal measurements at baseline

Mean±SD	Sodium deplete	Sodium replete	p value
Systolic blood pressure (mmHg)	130±21	128±12	0.66
Diastolic blood pressure (mmHg)	78±11	73±10	0.15
Weight (kg)	79.4±12.2	80.0±12.1	0.15
Urinary sodium (mmol/day)	67±19.5	197±76.0	0.03*
Fasting glucose (mmol/l)	8.7±2.44	8.5±1.99	0.55
Fasting serum insulin (pmol/l)	93±53.8	82±53.9	0.13

Figure 6.2. MeanSD profiles of PRA, ANGII, and aldosterone for 2 hours before and during clamp.

■ sodium replete; □ sodium deplete.

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (sodium replete vs. deplete)

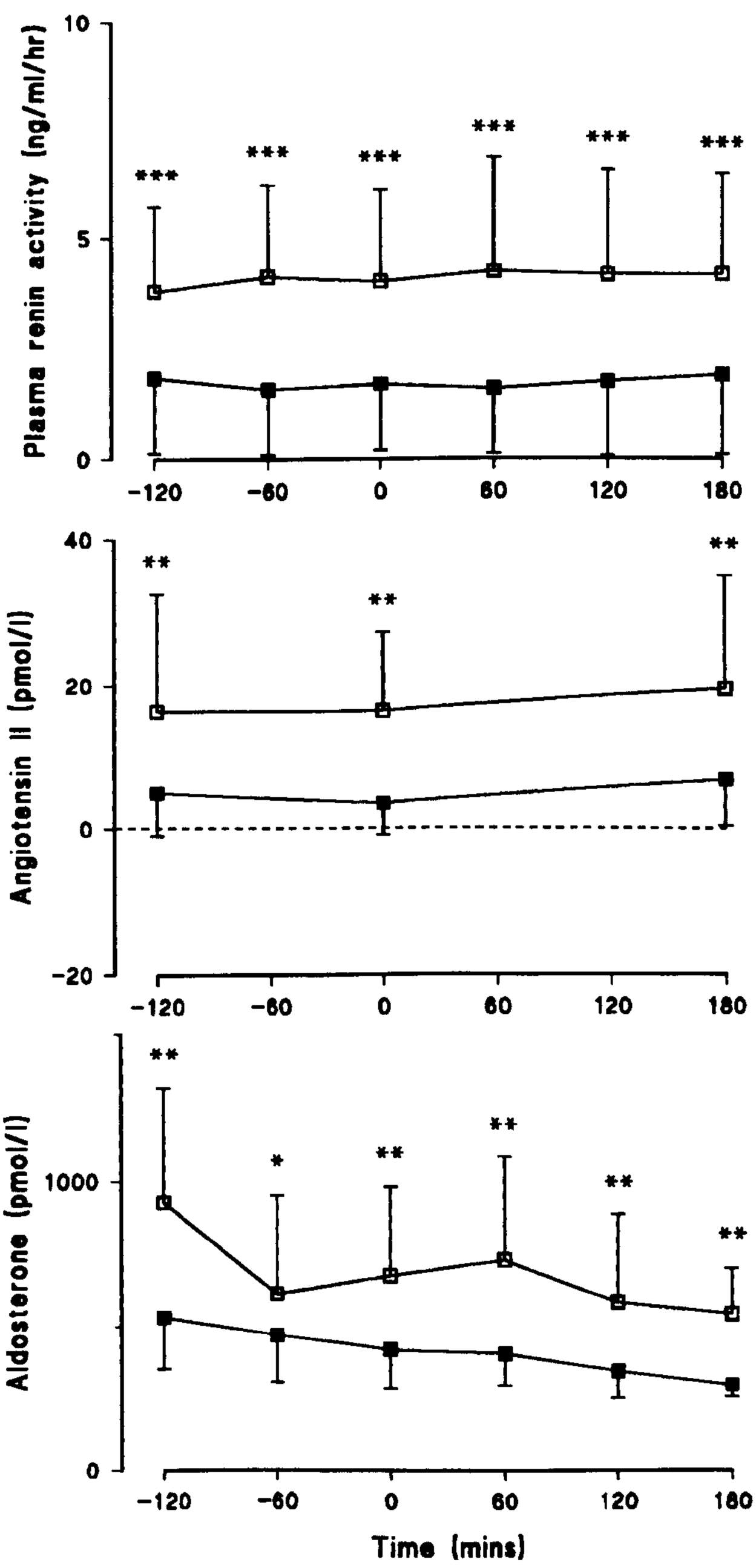


Figure 6.3 MeanSD profiles of sodium, potassium, and triglycerides for 2 hours before and during clamp.

■ sodium replete; □ sodium deplete.

* $p < 0.05$ (sodium replete vs. deplete)

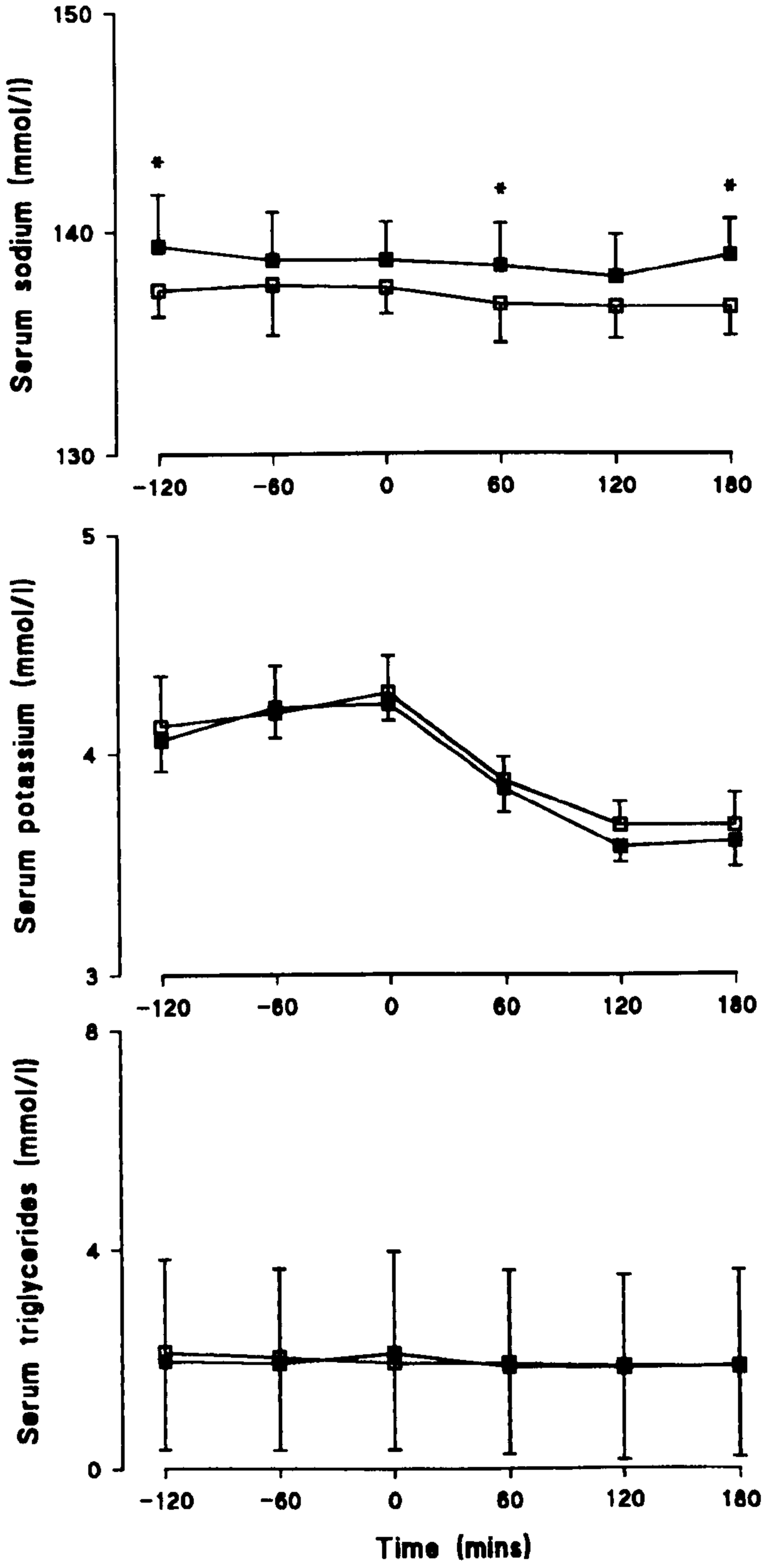


Figure 6.4 Mean SD profiles of insulin and C-peptide for 2 hours before and during clamp.

■ sodium replete; □ sodium deplete.

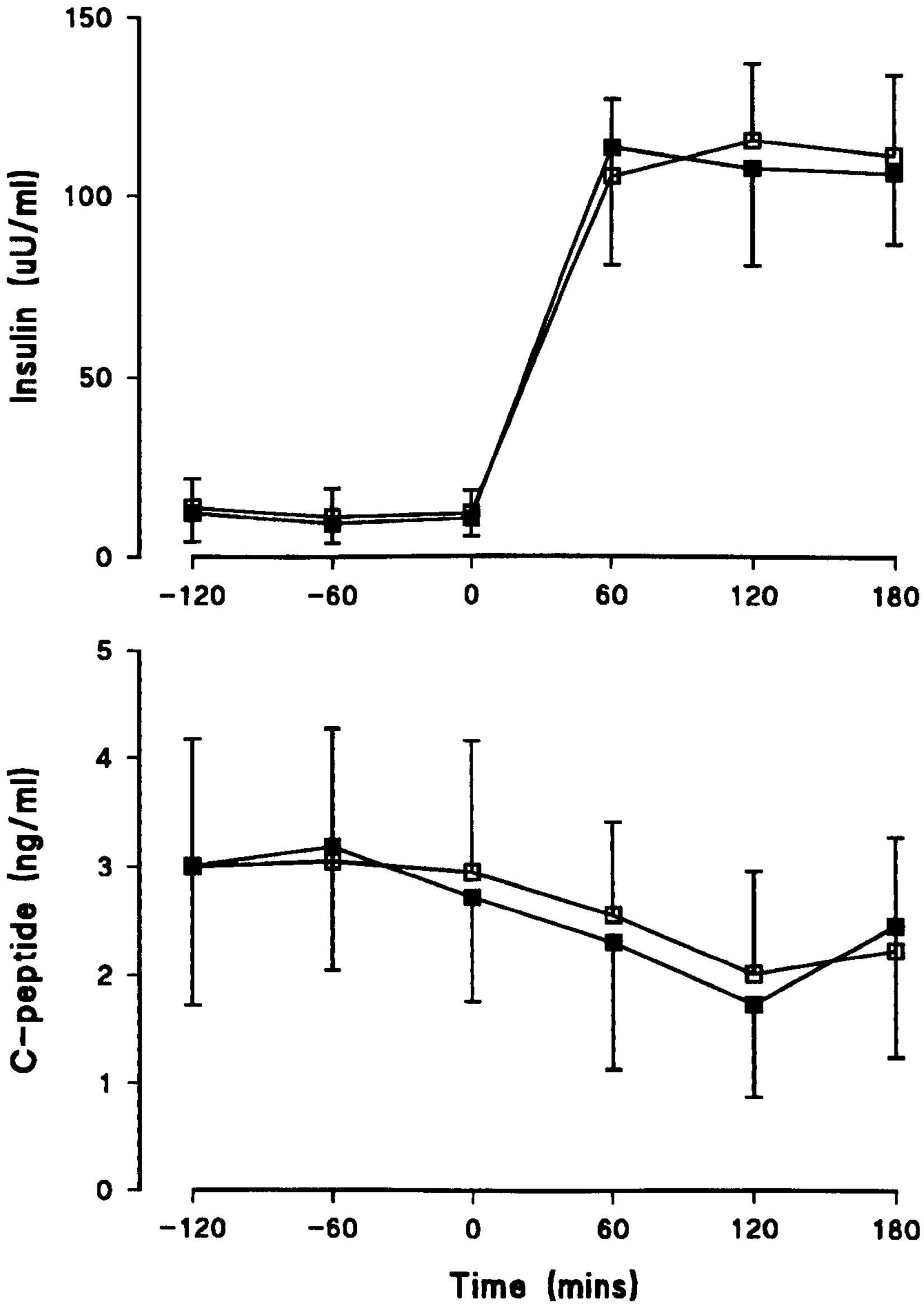


Figure 6.5 Insulin sensitivity (M, mg glucose/kg/min) on sodium replete and sodium deplete diets. * $p < 0.05$.

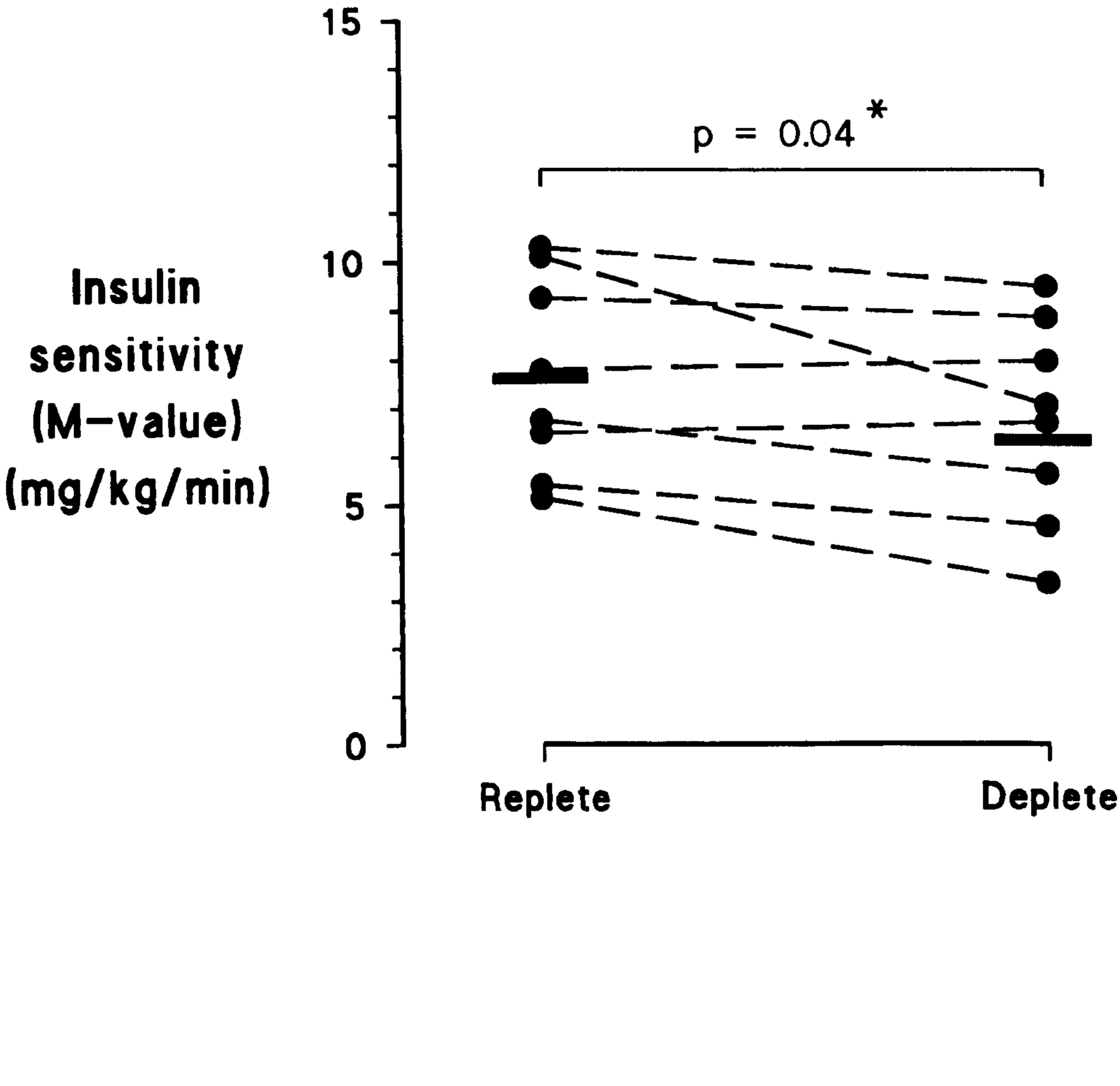
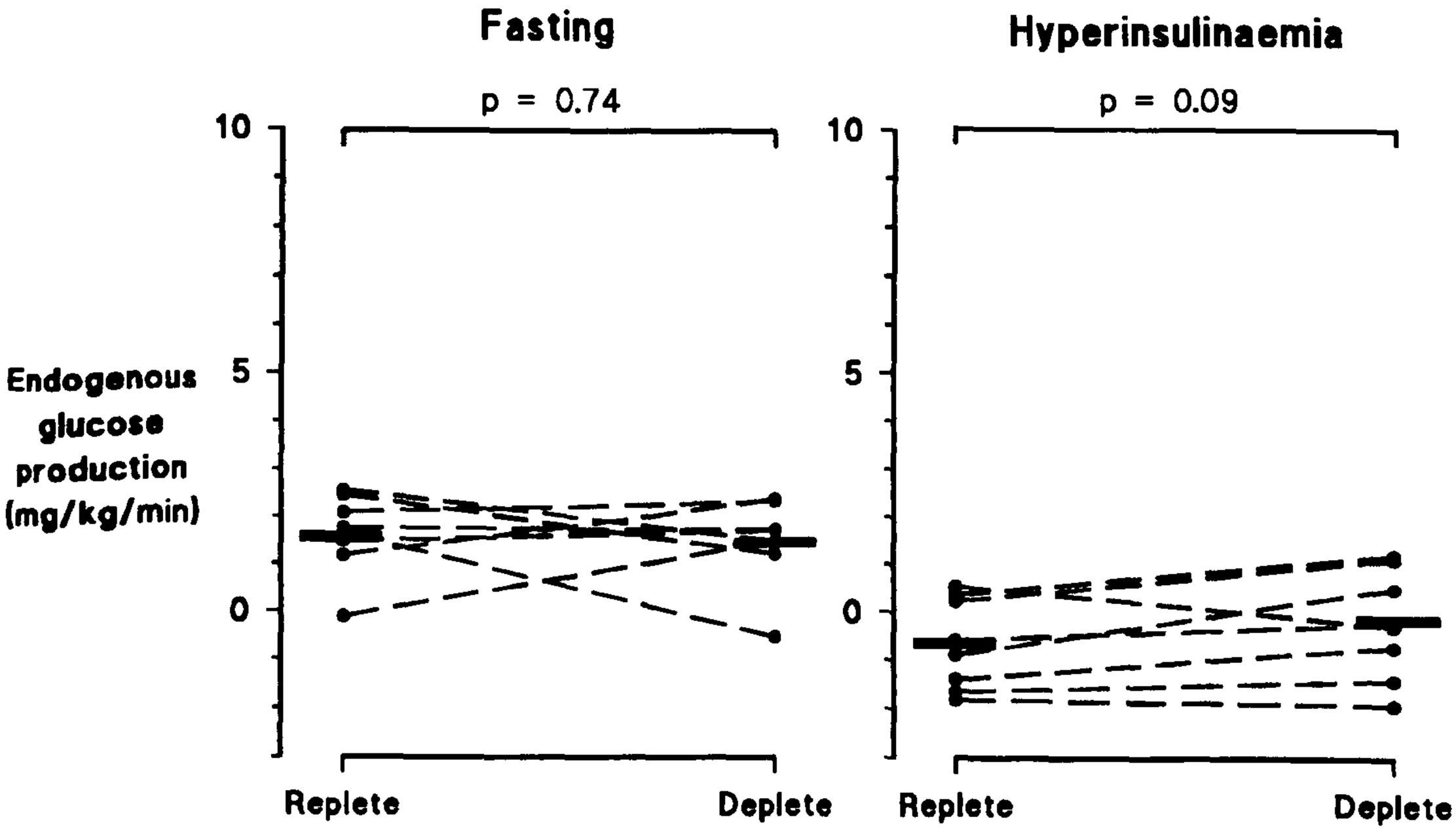


Figure 6.6 Endogenous glucose production (mg glucose/kg/min) fasting and at steady-state hyperinsulinaemia on sodium replete and sodium deplete diets.



6.4 Discussion

The novel finding of this double-blind randomised placebo-controlled study was that moderate 4-day dietary sodium restriction, accompanied by biochemical evidence of activation of the renin-angiotensin system (RAS), was associated with a 12% reduction in insulin-mediated glucose uptake in patients with NIDDM. This effect was predominantly on peripheral glucose metabolism, but an additional trend was observed towards an attenuation of hepatic insulin sensitivity.

As far as can be ascertained, the effect of dietary sodium restriction on insulin-mediated glucose uptake in patients with diabetes has not previously been reported. However, the present data are consistent with limited previously-available information on the insulin antagonist effects of components of the RAS (Section 1.7.2), and the potentiation by ANGII of sympathetic neurotransmission in sodium-restricted subjects (Taddei et al 1995). The overall effect of ANGII on insulin sensitivity is complex, and the present findings are difficult to reconcile with previous reports of *increased* insulin sensitivity during intravenous infusions of ANGII in both patients with NIDDM (Morris et al 1994c) and healthy volunteers (Buchanan et al 1993, Widgren et al 1993). While it has been suggested that insulin-sensitising effects of acute ANGII infusion may be predominantly due to macrovascular haemodynamic mechanisms (redistribution of blood flow from visceral to skeletal muscle beds (Buchanan et al 1993), the observation of insulin-sensitising effects at subpressor doses requires an

alternative explanation. For example, acute ANGII infusion may enhance glucose disposal by a microvascular haemodynamic mechanism (redistribution of blood flow from type 2 to more insulin-sensitive type 1 skeletal muscle fibres) or by a direct metabolic effect.

In contrast to these acute studies, increased ANGII levels were associated with a decrease in insulin sensitivity in the current study. The major difference between this study using dietary sodium restriction and studies involving acute infusions of ANGII was a more sustained physiological elevation of plasma ANGII concentrations. Down-regulation of vascular angiotensin receptors occurs at high endogenous levels of ANGII (Hollenberg et al 1974), and thus a temporal effect may account for the discrepancy between the effects of acute and chronic elevation of ANGII. Aldosterone release in response to ANGII is enhanced during hyperinsulinaemia (Rocchini et al 1990) and sodium restriction (Dawson-Hughes et al 1981), but the decrease in insulin sensitivity observed in the present study cannot readily be attributed to aldosterone itself as measured concentrations were comparable to those observed during acute ANGII infusion, which was associated with an increase in insulin sensitivity. Steady-state serum insulin levels achieved during the clamp procedures were similar on both dietary regimes, despite the theoretical possibility of plasma volume contraction on the sodium deplete diet.

The effect of activation of the RAS on insulin sensitivity has previously been examined in studies of dietary sodium depletion in healthy volunteers, but results

have been conflicting. For example, Sharma et al reported no change in insulin sensitivity, as measured by the insulin suppression test, when subjects were maintained on 240 mmol/day vs 20 mmol/day dietary sodium for 7 days (Sharma et al 1993). Donovan et al reported *increased* insulin sensitivity measured by the euglycaemic clamp technique when subjects were maintained on very low (10 mmol/day) compared with very high (200 mmol/day) sodium diets for 5 days (Donovan et al 1993). In contrast, in a further study using the euglycaemic clamp, insulin sensitivity was higher after 3 days on a high sodium diet (Fliser et al 1995). All three of these studies were open in design and used either very high or very low sodium diets. These are only easily administered in a clinical research setting and are not generally applicable to the management of patients with NIDDM.

The effect of inhibition of the RAS, and hence withdrawal of angiotensin II, on insulin-mediated glucose uptake has been more widely investigated than activation of the RAS in studies of the metabolic effects of ACE inhibitors. However, as already discussed (Section 1.1.6), many studies in the literature are compromised by use of indirect measures of insulin sensitivity, before-and-after design, and lack of placebo data (Donnelly 1992). In addition, in those studies in which an effect is reported, inhibition of kinin degradation cannot be dissociated from the withdrawal of ANGII (Dietze et al 1996).

From the present data it is not possible to be certain of the mechanism for the consistent decrease in peripheral insulin sensitivity observed during sodium

depletion in the subjects studied. However, RAS activation with elevated plasma ANGII concentrations is a strong candidate mechanism; given that plasma concentrations were elevated only moderately, and that blood pressure did not change, a metabolic rather than haemodynamic effect seems most likely. For example, ANGII has recognised effects on hepatic glucose metabolism (DeWitt et al 1983, Kneer et al 1983), consistent with the trend observed towards blunting of insulin-mediated suppression of endogenous glucose production. In addition, there is evidence that the hormone alters the intracellular signalling pathway initiated by activation of the insulin receptor. ANG II activates protein kinase C via receptor-linked formation of diacylglycerol (Smrcka et al 1991), and inhibits insulin-stimulated PI3-kinase activity (Velloso et al 1996); inhibition of the phosphorylation cascades initiated by insulin may result in decreased translocation to the plasma membrane of the facilatative transporter protein GLUT-4. In the present study, plasma catecholamines were not measured, but a further possible mechanism for the decrease in insulin sensitivity observed would be RAS-mediated activation of the sympathetic nervous system.

Measurements of endogenous glucose production showed the expected inhibitory effect of hyperinsulinaemia. In the technique used, the aim of labelling both constant and variable rate glucose infusions when measuring endogenous glucose production in clamp studies was to prevent a fall in glucose specific activity during the procedure, and thereby to reduce the intercompartmental fluxes which are thought to be responsible for negative values for hepatic glucose production (Levy et al 1989). The technique is known not to abolish the occurrence of such

values entirely, even when specific activity matching is achieved (Hother-Nielsen and Beck-Nielsen 1990, Hother-Nielsen et al 1992). In the present study, despite a *rise* in glucose specific activity between baseline and steady-state hyperinsulinaemia, some of the EGP values during hyperinsulinaemia were negative. However, reassuringly, mean values were very similar to those reported by other investigators in similar groups of patients (Harper et al 1995).

Regardless of the underlying mechanism for the deterioration in insulin sensitivity associated with dietary sodium depletion in these patients, the result may have clinical as well as metabolic relevance. Current dietary recommendations for diabetes (in both Europe and the US) are for normotensive patients to restrict sodium intake to 100 mmol/day (6g), and for patients with co-existing hypertension to restrict intake to 50 mmol/day (3g) (Nutrition Study Group, European Association for the Study of Diabetes 1988, American Diabetes Association 1996). The regimen used in this study, in normotensive patients with NIDDM, allowed 40 mmol/day (mean sodium excretion 67 mmol/day) and resulted in impaired insulin sensitivity; moderate dietary sodium restriction may not therefore be optimal for metabolic function in these patients.

In conclusion, dietary sodium restriction decreases insulin sensitivity in patients with NIDDM, and this may be a result of RAS activation. These findings provide further insights into the effects of the RAS on glucose metabolism and, if confirmed, have implications for dietary sodium intake recommendations for these patients.

Chapter 7

Insulin sensitivity and endothelial function: a physiological link with implications for pathogenesis of cardiovascular disease

7.0 Summary

The clinical study described in this chapter was designed to examine *in vivo* in man the relationship between basal and stimulated endothelial nitric oxide production, as assessed by forearm vasoconstrictor responses to intra-arterial infusions of N_G-monomethyl L-arginine (L-NMMA) and acetyl-choline, and insulin-mediated glucose uptake (insulin sensitivity), measured using the euglycaemic clamp technique.

7.1 Introduction

The elucidation of the mechanism of insulin resistance in common insulin resistant states has been complicated by the threefold variation in insulin sensitivity observed within groups of apparently healthy non-obese individuals (Section 1.1.2) (Hollenbeck et al 1987). The similarities between the conditions in which endothelial function and insulin sensitivity are impaired, and between the interventions which are thought to improve them, raise the hypothesis that they are physiologically linked (Section 1.7.3).

Insulin is an arterial vasodilator in skeletal muscle vascular beds, and there is evidence that insulin-mediated vasodilatation is reduced in states of insulin resistance (Baron et al 1990, 1991a, 1993) (Section 1.7.3). It has recently been

reported that the vascular effects of insulin are dependent on endothelial nitric oxide synthesis/release (Scherrer et al 1994; Steinberg et al 1994), while decreased basal endothelial nitric oxide synthesis has been reported in essential hypertension (Calver et al 1992).

If endothelial function and insulin sensitivity are physiologically linked, there should be a correlation between these two variables across the range of insulin sensitivity found in healthy man. The aim of this study was to examine this hypothesis.

7.2 Methods

7.2.1 Subjects

Nineteen healthy normotensive male volunteers aged 21-35 years with normal glucose tolerance participated in this study. At a screening visit, supine blood pressure was measured in triplicate (Section 2.3.3). In order to quantify habitual physical activity as a potentially-confounding variable, all subjects were asked to complete the self-reported section of a previously described questionnaire assessing moderate and vigorous physical activity level (Sallis et al 1985). In addition, age, BMI (body mass index), MAP (mean arterial pressure), plasma glucose level, serum cholesterol level, alcohol intake, and family history of cardiovascular disease were recorded.

7.2.2 Clinical procedures

i) Hyperinsulinaemic euglycaemic clamp. On the first study day, subjects underwent assessment of sensitivity to insulin-mediated glucose uptake using a three hour euglycaemic clamp (Section 2.4.1).

ii) Forearm venous occlusion plethysmography. On two further study days, subjects attended for measurement of basal and stimulated endothelial nitric oxide production. Forearm blood flow (FBF) was measured using venous occlusion plethysmography. A 30 minute stabilisation period was allowed prior to baseline measurements, and a 30 minute washout period was intercalated between drug infusions.

iii) Intra-arterial drug infusion. A 27G unmounted steel needle (Cooper's Needleworks, Birmingham, U.K.) was inserted under local anaesthesia into the brachial artery of the non-dominant arm for drug infusion (Section 2.7). Local incremental doses of drugs [acetylcholine 20, 40, 80, 160 ρmol/min (Miochol, Cibavision, Southampton, U.K.); sodium nitroprusside 3, 10, 30 ρmol/min (Roche, Basel, Switzerland); noradrenaline 15, 30, 150, 300 ρmol/min (Levophed, Sanofi-Winthrop, U.K.); N^G-monomethyl-L-arginine 1, 2, 4 μmol/min (L-NMMA; Clinalfa AG, Läufelfingen, Switzerland)] were dissolved in 0.9% saline and infused intra-arterially at a constant rate of 1ml/min.

7.2.3 Experimental protocol

i) Day 1. Subjects received ascending doses of acetylcholine, an endothelium-dependent stimulator of nitric oxide synthase, and sodium nitroprusside, an endothelium-independent donor of nitric oxide.

ii) Day 2. Subjects received ascending doses of noradrenaline, a "control" vasoconstrictor, and L-NMMA, a substrate inhibitor of nitric oxide synthase. Blood flow was measured in both forearms and each value was the mean of five sequential measurements. Percentage change from basal values in the ratio of blood flow between infused and non-infused arms was calculated with blood flow in the non-infused arm as a concurrent control (Benjamin et al 1995).

7.2.4 Statistical analysis

Insulin sensitivity and drug response data were normally distributed (Section 2.9). Because serial measurements were made, and log dose-response plots were linear, each subject's mean response to all administered doses of each drug was calculated as a summary measure (Matthews et al 1990). Data were initially examined using simple correlation; multiple regression analysis was performed in order to examine potential confounders.

7.3 Results

19 subjects completed acetylcholine and sodium nitroprusside infusions; 15 completed noradrenaline and L-NMMA infusions (arterial cannulation could not be repeated in two subjects, and two withdrew for personal reasons). Characteristics of the subjects are shown in Table 7.1.

7.3.1 Forearm blood flow (FBF) responses to intra-arterial drug infusion

Vasodilator responses to acetylcholine and sodium nitroprusside, and vasoconstrictor responses to noradrenaline are shown in Figure 7.1.

7.3.2 Insulin sensitivity

Insulin sensitivity (M) ranged from 6.2 to 11.6 mg/kg/min (Figure 7.2).

7.3.3 Insulin sensitivity and FBF responses to intra-arterial drug infusion

In univariate analysis, individual measurements of insulin sensitivity (M) were positively related to individual mean L-NMMA responses (r=0.52, p<0.05, 95% CI 0.01, 0.81)(Figure 7.2). However, no relationships were observed between M-values and noradrenaline responses (r=0.14, p=0.62, 95% CI -0.40, 0.61) acetylcholine responses (r=0.15, p=0.53, 95% CI -0.33, 0.56) or sodium nitroprusside responses (r=0.31, p=0.20, 95%CI -0.17, 0.67)(Figure 7.2). Similar results were obtained when the area under the curve was used as an alternative summary measure of drug response (Matthews et al 1990).

7.3.4 Multiple regression analysis

One subject, identified by the Minitab statistical package (Section 2.9) as an "unusual observation," was excluded from this analysis. The results, taking into account age, BMI (body mass index), MAP (mean arterial pressure), plasma glucose level, serum cholesterol level, alcohol intake, family history and physical activity level, are shown in Table 7.2.

Table 7.1 Haemodynamic and metabolic characteristics of the subjects (n=19)

	(mean±SD)
Age (years)	27.1±5.3
Body mass index ($kg/m^{2)}$	24.6±3.0
Fasting glucose (mmol/l)	5.1±0.3
Fasting cholesterol (mmol/l)	4.40±0.74
Blood pressure (mmHg)	130±10/69±7
Smokers	3/19
Family history of cardiovascular disease or diabetes	6/19
Median alcohol consumption (units)	16 (interquartile range 6-20)

Figure 7.1 Grouped (meanSD) vasoconstrictor and vasodilator responses (percentage change in forearm blood flow ratio) to ascending doses of a) L-NMMA, b) noradrenaline, c) acetyl-choline, d) sodium nitroprusside.

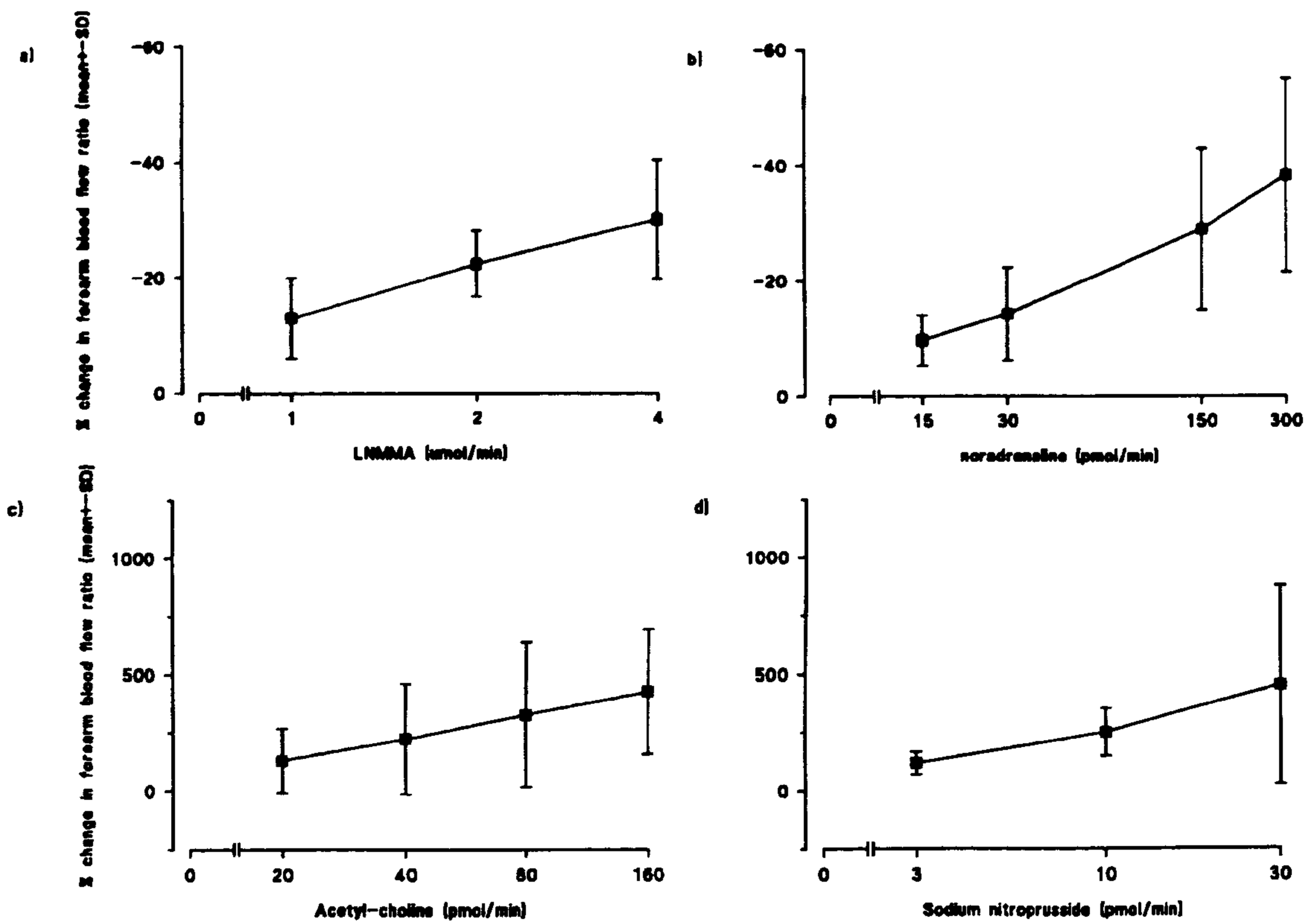


Figure 7.2 Mean response (percentage change in forearm blood flow ratio) of each individual to a) L-NMMA, b) noradrenaline, c) acetyl-choline, d) sodium nitroprusside plotted against M (insulin sensitivity, mg/kg/minute).

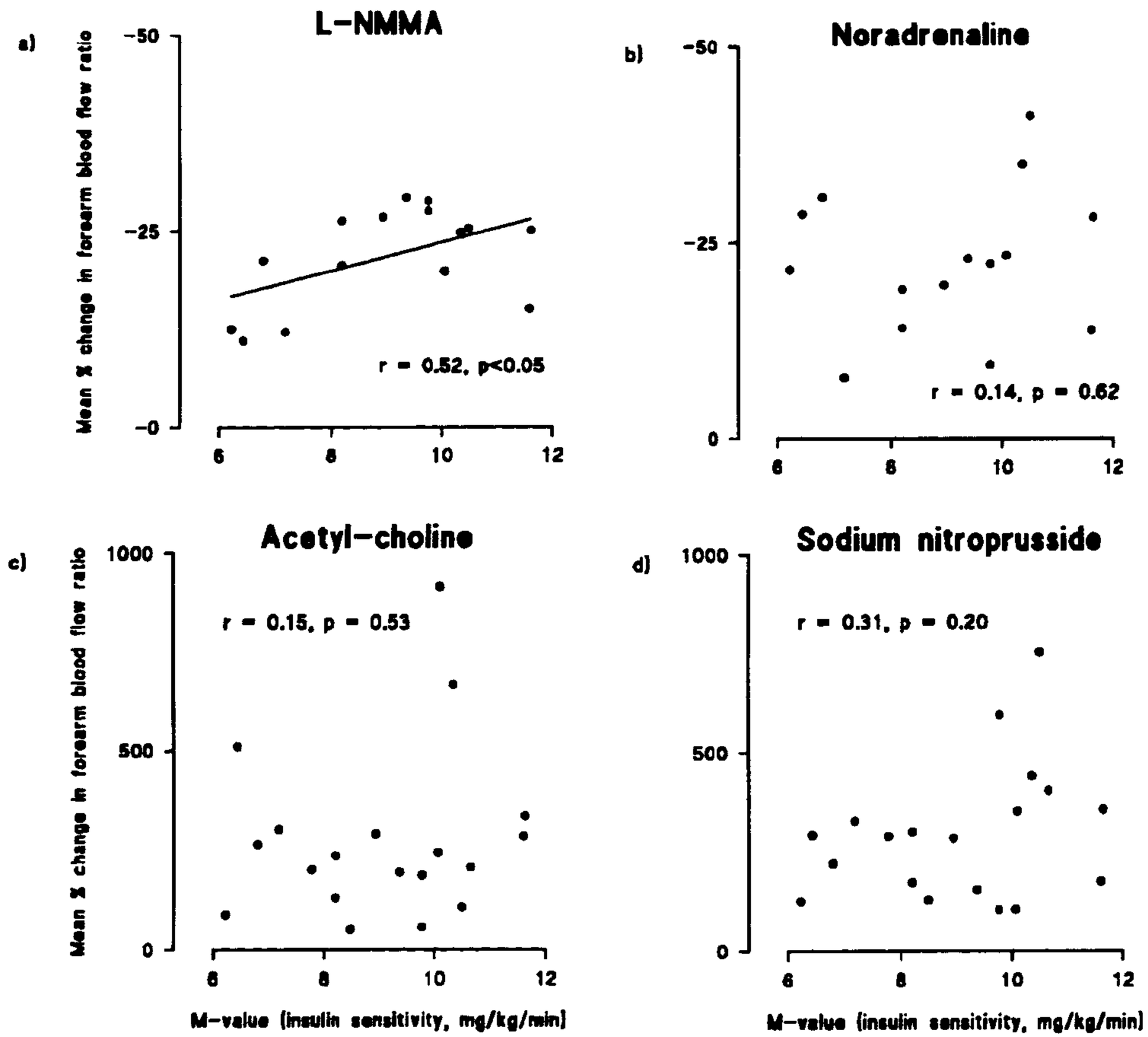


Table 7.2 Multiple regression model with insulin sensitivity (M) as dependent variable

Model	L-NMMA response	Mean arterial pressure	BMI	Non-smoker/ smoker	Adjusted R^2	*t* for L-NMMA	*p*
1	0.19 (0.09, 0.30)				0.48	3.62	<0.01
2	0.18 (0.07, 0.28)	0.10 (0.02, 0.18)			0.58	3.25	<0.01
3	0.13 (0.04, 0.23)	0.14 (0.02, 0.25)	-0.24 (-0.45, -0.03)		0.64	2.72	<0.05
4	0.15 (0.06, 0.247)	0.18 (0.06, 0.29)	-0.26 (-0.46, -0.06)	1.21 (-0.26, 2.68)	0.69	3.27	<0.05

The table shows prediction of insulin sensitivity (M) from mean forearm vasoconstrictor response during intra-arterial infusion of L-NMMA, mean arterial pressure (MAP), BMI, and smoking status. Changes are shown as adjusted R^2 and *t* for L-NMMA response, with forward stepwise addition of MAP, BMI, and smoking status. L-NMMA response remains a significant predictor of M after potentially confounding variables are included in the model. Other variables which were entered were: fasting cholesterol and glucose concentrations, physical activity level, and family history of cardiovascular disease (positive or negative). Partial regression coefficient (β) and 95% confidence intervals are shown. Predictor variables: model 1, L-NMMA; model 2, L-NMMA plus MAP; model 3, L-NMMA plus MAP plus BMI; model 4, L-NMMA plus MAP plus BMI plus smoking status.

7.4 Discussion

The results of this study demonstrate, for the first time, a positive relationship between basal vascular endothelial nitric oxide production and insulin sensitivity in healthy young males. Under physiological circumstances, insulin causes arterial vasodilatation in skeletal muscle vascular beds (Section 1.7.3). By increasing its own delivery, and that of glucose, to insulin-sensitive tissues it may amplify its own action in promoting glucose uptake into skeletal muscle. If local insulin-mediated vasodilatation insulin is impaired, a reduction in insulin sensitivity may occur.

It has previously been reported that insulin-mediated vasodilatation is impaired in insulin resistant states (Baron et al 1990, 1991a, 1993). The present data, along with recent evidence that endothelial nitric oxide may mediate insulin's vascular effects (Scherrer et al 1994, Steinberg et al 1994), suggest a direct physiological link between vascular endothelial function and insulin sensitivity i.e. individuals who are relatively insensitive to insulin-mediated glucose uptake appear also to have a corresponding decrease in basal endothelial nitric oxide production.

No relationship was observed between noradrenaline responses and insulin sensitivity: this argues against a non-specific decrease in vascular reactivity in subjects who are less sensitive to insulin-mediated glucose uptake. Furthermore, the absence of a relationship between either acetylcholine or

sodium nitroprusside responses and insulin sensitivity suggests that decreased insulin sensitivity is not associated with a reduced ability of the vascular endothelium to synthesise nitric oxide when stimulated, or with a reduction in sensitivity of vascular smooth muscle to nitric oxide released from the endothelium.

Insulin sensitivity has been shown to be related to age, body mass index, and maximal aerobic capacity in normal subjects, but may vary up to threefold in subjects apparently similar in these respects (Hollenbeck et al 1987). The present study group was as homogeneous as possible for these variables, with only a twofold variation in insulin sensitivity. The relationship between L-NMMA response and insulin sensitivity was stronger than the relationships between all of the other variables and insulin sensitivity, accounting for 48% of the variance, and was not explained by a known confounding variable. Age was not associated with insulin sensitivity, perhaps because of the homogeneity of the group.

The relationship identified in this study is compatible with three hypotheses: 1) decreased basal endothelial nitric oxide production results in decreased insulin sensitivity, possibly due to reduced insulin-mediated vasodilatation in skeletal muscle; 2) decreased insulin sensitivity results in decreased basal production of nitric oxide by the vascular endothelium; 3) decreased basal endothelial nitric oxide production and impaired insulin sensitivity are manifestations of a common genetic or environmental antecedent.

The present data demonstrate an association between insulin sensitivity an endothelial function, but do not provide evidence of causality. However, there are a number of putative mechanisms by which endothelial dysfunction might cause impairment of insulin action. Insulin must cross the endothelial barrier in order to exert its metabolic effects in target tissues; dysfunction of an active transport mechanism might result in a decrease insulin sensitivity. Alternatively, reduced activity of an endothelial enzyme such as lipoprotein lipase could result in dyslipidaemia with secondary effects, perhaps via oxidation of LDL (DiCorleto and Soyombo 1993), on insulin-mediated glucose metabolism (Ahn et al 1993).

It is also possible to hypothesise mechanisms by which insulin resistance might cause endothelial dysfunction. The substrate for endothelial nitric oxide synthase is the amino-acid, L-arginine; although availability of L-arginine is not thought to be rate-limiting for nitric oxide synthesis under physiological conditions, it is conceivable that L-arginine transport, which is activated in human endothelial cells by glucose and insulin (Sobrevia et al 1996), might be impaired in conditions of insulin resistance.

Lastly, both endothelial function and insulin resistance may arise as a result of a common antecedent. For example, high concentrations of free fatty acids in insulin resistant states (Section 1.1.3) are known to compete with glucose for peripheral uptake (Randle et al 1963), and in addition appear to inhibit endothelial nitric oxide synthesis (Davda et al 1995). Other potential common antecedents include low skeletal muscle capillary density (Section 1.6.3), and oxidative stress

(Section 1.7.3). Alternatively, both phenotypes may occur as a result of abnormal intra-uterine development (Barker et al 1993a, 1993b).

Important candidate mechanisms for physiological processes potentially linking insulin-mediated vasodilatation, insulin-mediated glucose metabolism and endothelial function may be gleaned from investigation of the mechanisms of local insulin-mediated vasodilatation in skeletal muscle vascular beds. Clearly, further studies are required: a) to determine whether a relationship between endothelial function and insulin sensitivity is observed in insulin resistant pathophysiological states; b) to investigate the effects of manipulating endothelial function on insulin-mediated glucose uptake; and c) to investigate the effects of therapeutic interventions on insulin sensitivity and endothelial function in the same subjects.

Chapter 8

General discussion

Variations in sensitivity to insulin treatment in patients with diabetes were observed by Himsworth over 60 years ago (Himsworth 1936), and hyperinsulinaemia was reported in patients with essential hypertension (Welborn et al 1966) only 6 years after the introduction of early immunoassays for insulin (Yalow and Berson 1960). It is now recognised that hyperinsulinaemia in essential hypertension occurs as a pancreatic compensatory response to resistance to insulin-mediated glucose uptake in skeletal muscle tissue (Ferrannini et al 1987, Natali et al 1991). However, the relationship between serum insulin concentrations and blood pressure is variable between studies (Modan et al 1985, Asch et al 1991), and insulin sensitivity varies up to threefold in apparently healthy individuals (Hollenbeck and Reaven 1987). The mechanism and pathophysiological significance of insulin resistance in common cardiovascular disease states such as non-insulin dependent diabetes mellitus, essential hypertension, dyslipidaemia, and obesity remain obscure, although hyperinsulinaemia appears to be an independent risk factor for ischaemic heart disease (Despres et al 1996), and may be directly modifiable by non-pharmacological and pharmacological interventions.

Investigation of the mechanisms of insulin resistance has focused on the insulin receptor and post-receptor signalling mechanisms. However, abnormalities recognised to date in these processes do not account for insulin resistance in

common pathophysiological states and there is increasing recognition of a possible role for pre-receptor haemodynamic factors in the physiological regulation of insulin sensitivity and in the pathogenesis of insulin resistance in cardiovascular disease. Considerable controversy surrounds the phenomenon of insulin-mediated vasodilatation and whether attenuation of the physiological vasodilator effect of insulin might account directly for decreased tissue glucose uptake during hyperinsulinaemia in skeletal muscle vascular beds in insulin resistant states (Baron et al 1993).

This thesis is comprised of a series of studies which have used specific and conventional insulin immunoassays, the hyperinsulinaemic clamp technique and forearm venous occlusion plethysmography with local intra-arterial infusions to address some of these issues. The major emphases are on the relationship between serum insulin concentrations and blood pressure, and the role of haemodynamic factors in determining insulin sensitivity.

The following hypotheses were investigated:

1) are variations in specificity for insulin (vs. proinsulin-like molecules) among conventional insulin immunoassays responsible for variations in the reported relationships among serum insulin concentrations, insulin sensitivity, and blood pressure?

2) does sustained physiological activation of the renin-angiotensin system induced by moderate dietary sodium restriction affect insulin sensitivity in patients with non-insulin-dependent diabetes mellitus?

3) is there a relationship between endothelial function and insulin sensitivity in healthy subjects?

(i) The reproducibility of venous occlusion plethysmography

Venous occlusion plethysmography was chosen as a method of blood flow measurement for studies investigating putative haemodynamic factors in the physiological regulation of insulin sensitivity. It was noted that studies using this technique to report changes in forearm blood flow (FBF) during intra-arterial infusions of vasoactive substances often quoted changes in flow ratio between infused and control arms rather than absolute values for flow, although unilateral measurements and forearm vascular resistance data were quoted by other investigators. The possibility was considered that the method used for expressing responses might influence the conclusions reached, or lead to performance of a relatively invasive technique on an unnecessarily large number of subjects to detect small differences between groups. A reproducibility study was therefore performed which demonstrated:

1) that the between-day intra-subject variability of bilateral forearm venous occlusion plethysmography (FBF ratios) was 19% at rest;

2) FBF ratios were more reproducible than unilateral FBF measurements (CV 31-39%) at rest and during intra-arterial infusions;

3) expressing results as forearm vascular resistance resulted in only a small improvement in reproducibility over FBF at rest.

These findings suggested that bilateral plethysmography, expressing responses in the intervention arm as a ratio of responses in the control arm, was more reproducible than unilateral plethysmography: the bilateral technique was therefore used where possible in further studies.

(ii) The effect of hand-warming on insulin sensitivity and forearm blood flow

The methodology for the reproducible measurement of insulin sensitivity using the hyperinsulinaemic euglycaemic clamp technique had previously been established in the Clinical Investigation and Research Unit. However, given that investigation of haemodynamic influences on insulin-mediated glucose uptake were a major theme of these studies, further validation studies were performed to investigate whether vasodilatation produced during arterialisation of venous blood by the commonly-used technique of hand-warming had systemic haemodynamic effects or effects on the measurement of insulin sensitivity. The results of these controlled studies in healthy subjects showed that hand-warming by the heated-air hand box was associated with a decrease in derived insulin sensitivity when clamps were performed according to samples from a site unlikely to be affected by arterialisation (the contralateral antecubital vein). However, no difference in insulin sensitivity associated with hand-warming was demonstrated when the infusion rate was adjusted on the basis of samples from a site in which arterialisation could be demonstrated (the ipsilateral dorsal hand vein). In addition, hand-warming appeared to cause an increase in heart rate during hyperinsulinaemia and an increase in contralateral forearm blood flow under basal conditions. It was

therefore concluded that the systemic haemodynamic effects of hand-warming have the potential to confound the results of studies using the euglycaemic clamp technique, particularly those with a case-control design and those assessing the haemodynamic effects of insulin. It was reassuring that hand-warming had negligible effects on derived insulin sensitivity when clamps were performed in the usual manner, but it was decided that future measurements of forearm blood flow would be conducted in the absence of hand-warming.

(iii) The effect of insulin assay specificity on the relationships among serum insulin concentrations, insulin sensitivity, and blood pressure

Serum insulin concentrations have been used as markers of insulin resistance in population studies examining the relationship between insulin resistance and blood pressure, but the relationship between insulin and blood pressure is variable among studies (Modan et al 1985, Asch et al 1991). Insulin assay methodology is not standardised between centres (Robbins et al 1996), and epidemiological studies have used a variety of commercially-available assay kits for the measurement of insulin concentrations. It was hypothesised that variability in the reported relationship between insulin and blood pressure might be accounted for by variability in cross-reactivity of antibodies used in insulin assay kits with intact proinsulin and its split and des-amino products. It appeared that epidemiological studies which had used assay systems which were relatively specific for insulin had observed a weaker relationship betwen hyperinsulinaemia and blood pressure than those which had used antibodies with a higher degree of specificity (Winocour et al 1991). Indeed, reports of a (statistically non-significant)

excess of cardiovascular deaths during early clinical use of proinsulin in man gave rise to the hypothesis that circulating proinsulin-like molecules might be associated with cardiovascular disease (Galloway et al 1992).

The relationships were therefore examined among fasting and post-glucose load serum insulin concentrations (determined by both specific and conventional assays), insulin sensitivity (measured by the euglycaemic clamp technique), and blood pressure, in a group of 56 patients with NIDDM and non-diabetic subjects. Insulin concentrations as measured by the two methods were highly correlated ($r=0.97$, $p < 0.0001$), and the relationships among serum insulin concentrations, insulin sensitivity and blood pressure were independent of assay method; for example, in non-diabetic subjects the univariate correlation between $\log_{10}$ AUC insulin and insulin sensitivity index was similar with both methods [$r=-0.81$ vs $r=-0.82$, $p < 0.0001$ (specific assay vs. conventional assay)]. It was concluded that variations among insulin assay kits in cross-reactivity for insulin vs proinsulin-like molecules were unlikely to account for discrepancies in the relationship between serum insulin concentrations and blood pressure, and that these were therefore likely to be accounted for either by other non-standardised aspects of insulin assay methodology, or by genuine differences among the populations studied.

Although proinsulin-like molecules were not measured in this series of studies, a study published during the period that this work was conducted has reported similar correlations between proinsulin-like molecules and cardiovascular risk factors as those previously reported between insulin and the same risk factors

(Mohamed-Ali et al 1995). In their discussion of their findings, the authors of this report suggested that the detection of correlations between circulating concentrations of proinsulin-like molecules and cardiovascular risk factors is evidence against a modulating role for insulin in cardiovascular disease, as proinsulin-like molecules are equally strongly related to cardiovascular risk factors, yet circulate at less that 10% of the concentration of insulin.

(iv) The effect of dietary sodium restriction on insulin sensitivity in non-insulin-dependent diabetes mellitus

Angiotensin II is a potent vasoconstrictor hormone with counter-regulatory metabolic effects. A haemodynamic hypothesis of insulin sensitivity would predict that acute infusion of ANGII would result in an exacerbation of the decrease in insulin-mediated glucose uptake that would be expected from its metabolic effects. However, previous studies conducted in the CIRU demonstrated an insulin-sensitising effect of acute ANG II infusion in patients with NIDDM (Morris et al 1994c). Data from studies in which acute infusions of ANGII were administered to healthy male subjects allowed this effect to be reconciled with a haemodynamic hypothesis: acute systemic ANGII infusion is associated with a redistribution of blood flow away from visceral and splanchnic vascular beds towards insulin-sensitive skeletal muscle beds (Buchanan et al 1993).

The apparent haemodynamically-mediated insulin-sensitising effects of acute ANGII infusion remained at odds with its counter-regulatory metabolic effects

and the reported insulin-sensitising effect of inhibitors of angiotensin-converting enzyme (Pollare et al 1989a, Vuorinen-Markkola and Yki-Jarvinen 1995). It was therefore hypothesised that the effect of ANGII on insulin sensitivity was temporal, with a discrepancy between acute and chronic responses. The effect on insulin sensitivity of more sustained physiological activation of the renin-angiotensin system was therefore examined in patients with NIDDM using sodium restriction (40 mmol/day) in a randomised, double-blind, placebo-controlled crossover protocol. Investigation of the effect of sodium restriction on insulin sensitivity was of both metabolic importance and clinical relevance as current dietary recommendations suggest dietary sodium restriction for both hypertensive and normotensive patients with diabetes.

The results of the study demonstrated that moderate dietary sodium restriction was associated with a 12% reduction in insulin sensitivity in patients with NIDDM. This decrease in insulin sensitivity, associated with sustained elevation of plasma ANGII levels, is more consistent with the expected metabolic and haemodynamic effects of ANGII and with the reported effect of ACE inhibition on glucose metabolism than the effect observed during acute ANGII infusion. Although there is clear *in vitro* evidence for cross-talk between the insulin and angiotensin post-receptor signalling mechanisms (Velloso et al 1996), a recently-reported study has provided direct evidence for a haemodynamic mechanism: sodium depletion resulted in increased vascular reactivity in normotensive subjects and hypertensive patients (Feldman et al 1996).

(v) The relationship between insulin sensitivity and endothelial function

The importance of the vascular endothelium in determining regional blood flow has been increasingly recognised over the last fifteen years (Furchgott and Zawadzki 1980). The balance of evidence is in favour of a local arterial vasodilator action of insulin in skeletal muscle vascular beds (Section 1.7.3). There is both *in vivo* (Scherrer et al 1994; Steinberg et al 1994) and *in vitro* (Chen and Messina 1996) evidence that insulin-mediated vasodilatation is endothelium dependent, and that it is attenuated in states of insulin resistance (Baron et al 1990, Baron et al 1991a, Baron et al 1991b, Baron et al 1993). Observation of the co-existence of endothelial dysfunction and insulin resistance in a number of cardiovascular disease states (essential hypertension, non-insulin dependent diabetes mellitus, obesity, dyslipidaemia, and atherosclerosis) generated the hypothesis of the existence of a physiological link between endothelial nitric oxide production and insulin-mediated glucose uptake. The threefold variation in insulin sensitivity reported within groups of apparently healthy non-obese individuals (Hollenbeck and Reaven 1987), afforded the opportuniy to examine forearm vascular endothelial function in a series of healthy subjects across a range of insulin sensitivity.

The results of this study demonstrated, for the first time, a positive relationship between basal endothelial nitric oxide production and insulin sensitivity in healthy young males. These data raise a further hypothesis that insulin sensitivity may in part be determined by an endothelium-dependent effect of insulin to promote glucose uptake by increasing blood flow (and hence delivery of insulin and its substrate glucose) to insulin-sensitive tissues. It might be

speculated that attenuation of insulin-mediated vasodilatation in insulin resistant states may at least in part be a result of endothelial dysfunction, although there are some data to suggest that the metabolic effect of insulin to increase fractional glucose extraction is more important in determining glucose uptake than its effect on blood flow within physiological ranges of insulinaemia (Utriainen et al 1995, Raitakari et al 1996; Nuutila et al 1996).

An independent group of investigators have recently attempted to replicate the relationship demonstrated in the present thesis between insulin sensitivity and endothelial function. Interestingly, their study demonstrated a positive relationship between insulin-mediated vasodilatation and endothelial function in 30 healthy male subjects, although the data are compromised to some extent by measurements of blood flow conducted during hand-warming (Utriainen et al 1996a). No relationship was detected between insulin sensitivity and forearm vasoconstrictor response to L-NMMA (Utriainen et al 1996b). The major difference between the protocol used by this group of investigators and the protocol used in the present study is that L-NMMA was infused at the end of a long study morning, after infusions of acetylcholine and sodium nitroprusside, rather than after a simple infusion of a control vasoconstrictor.

Although it is attractive to hypothesise mechanisms by which endothelial dysfunction can result in insulin resistance, it is also possible to suggest mechanisms by which insulin resistance might cause endothelial dysfunction. The substrate for endothelial nitric oxide synthase is the amino-acid, L-arginine; availability of L-arginine is not thought to be rate-limiting for nitric oxide

synthesis under physiological conditions, but it is conceivable that L-arginine transport, which is activated in human endothelial cells by glucose and insulin (Sobrevia et al 1996), might be impaired in conditions of insulin resistance. Alternatively, both endothelial function and insulin resistance may arise as a result of a common antecedent. For example, low skeletal muscle capillary density (Section 1.6.3), abnormal free fatty acid metabolism (Section 1.1.3), or oxidative stress (Section 1.7.3) might be associated with both phenotypes, perhaps as a consequence of abnormal intra-uterine development (Barker et al 1993a, 1993b).

Data from the studies described in this thesis do not allow a decision to be made amongst these possibilities. Important candidate mechanisms for the physiological processes linking insulin-mediated vasodilatation, insulin-mediated glucose uptake and endothelial function may be gleaned from investigation of the mechanisms of local insulin-mediated vasodilatation in skeletal muscle vascular beds.

Conclusions

This series of studies has used specific and conventional immunoassays, the hyperinsulinaemic clamp technique, and forearm venous occlusion plethysmography with local intra-arterial infusions to demonstrate that:

1) discrepancies between studies in the relationship between serum insulin concentrations and blood pressure in diabetic and non-diabetic subjects are likely to be due to factors other than cross-reactivity of conventional insulin assays with proinsulin-like molecules.

2) moderate dietary sodium restriction (which is associated with sustained elevation of plasma angiotensin II) results in a 12% reduction in insulin sensitivity in patients with non-insulin dependent diabetes mellitus

3) a physiological relationship may exist between insulin sensitivity and vascular endothelial function.

Publications containing work undertaken for this thesis:

i) Reviews

Petrie J.R., Donnelly R. (1994) New pharmacological approaches to insulin and lipid metabolism. Drugs, 47, 701-710.

Morris A.D., Petrie J.R., Connell J.M.C. (1994) Insulin and hypertension. J Hypertension, 12, 633-642.

ii) Papers

Petrie J.R., Ueda S., Webb D.J., Elliott H.L., Connell J.M.C. (1996) Endothelial nitric oxide production and insulin sensitivity: a physiological link with implications for pathogenesis of cardiovascular disease. Circulation, 93, 1331-1333.

Petrie J.R., Ueda S., Morris A.D., Elliott H.L., Connell J.M.C. (1996) Potential confounding effect of hand-warming in the measurement of insulin sensitivity.
Clin Sci, 91, 65-71.

Petrie J.R., Ueda S., Morris A.D., Elliott H.L., Connell J.M.C. (1997) How reproducible is bilateral forearm venous occlusion plethysmography in man?
Br J Clin Pharm (in press)

Petrie JR, Morris AD, Dorrian CA, Small M, Connell JMC.
Insulin levels, insulin sensitivity, and blood pressure: is assay specificity important?
Quarterly Journal of Medicine (in press)

iii) Abstracts

Petrie J.R., Morris A.D., Minamisawa K., Elliott H.L., Small M., Connell J.M.C. (1996) Salt depletion impairs insulin sensitivity in NIDDM.
Diabetes, 45 (Suppl 2), 306A, A1140.
(full paper submitted to Journal of Clinical Endocrinology and Metabolism)

Oral presentations to Learned Societies containing work undertaken for this thesis

Caledonian Clinical Pharmacology Society, September 1993.
The reproducibility of forearm venous occlusion plethysmography in man.

British Hypertension Society, Dublin, September 1994.
Is hand-warming an unnecessary and confounding factor in the measurement of insulin sensitivity?

British Hypertension Society, Glasgow, September 1995
Basal endothelial nitric oxide synthesis and endothelial function are positively correlated in man.

British Diabetic Association, Harrogate, October 1995
Salt depletion impairs insulin sensitivity in NIDDM.

European Association for the Study of Diabetes "Hypertension in Diabetes" Study Group Padua, Italy, March 1996.
Endothelial nitric oxide production and insulin sensitivity.

Caledonian Society for Endocrinology, Dalmahoy, May 1996
Specific insulin levels, insulin sensitivity, and cardiovascular phenotype: is assay specificity important?

10th Anniversary Meeting Anglo-Danish Dutch Diabetes Group
Aberfoyle, May 1996.
Measurement of insulin sensitivity: gold standards and good surrogates.

Investigator-led satellite of International Society of Hypertension "Blood pressure and insulin resistance: can we reconcile clinical and epidemiological evidence?"
Naples, Italy, June 1996.
Insulin sensitivity and endothelial function: a physiological relationship with potential pathophysiological significance.

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Abbreviations:

ACE	angiotensin-converting enzyme
ANGII	angiotensin II
ANOVA	analysis of variance
AUC	area-under-the-curve
BMI	body mass index
BP	blood pressure
CIRU	Clinical Investigation and Research Unit
CI	confidence interval
CV	coefficient of variation
EGP	endogenous glucose production
eNOS	endothelial nitric oxide synthase
EDRF	endothelium-derived relaxing factor
FBF	forearm blood flow
FFA	free fatty acid
FVR	forearm vascular resistance
FIRI	fasting insulin resistance index
GLUT	glucose transporter
HDL	high-density lipoprotein
HOMA	homeostasis model assessment
HPLC	high performance liquid chromatography
IGT	impaired glucose tolerance
IST	insulin suppression test
IVGTT	intravenous glucose tolerance test

L-NMMA	N_G-monomethyl-L-arginine
LDL	Low-density lipoprotein
LPL	Lipoprotein lipase
M	insulin sensitivity (glucose metabolised)
MAP	mean arterial pressure
NIDDM	non-insulin-dependent diabetes mellitus
NO	nitric oxide
OGTT	oral glucose tolerance test
PAI-1	Plasminogen Activator Type-1 (PAI-1)
PRA	plasma renin activity
R_a	rate of appearance
R_d	rate of disappearance
RAS	renin-angiotensin system
RIA	radioimmunoassay
SSPG	steady state plasma glucose concentration
SC	space correction
UC	urinary correction for glucose loss
VLDL	very-low-density lipoprotein
WHR	waist-to-hip ratio