Insulin resistance, hypertension and the insulin-responsive glucose transporter, GLUT-4

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BY

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Abstract

Insulin resistance is a disease state characterised by the reduced ability of insulin to exert its effects in peripheral tissues, skeletal muscle and adipose tissue. This condition has been associated with a number of other disease states including obesity and hypertension.

The hypertensive Milan rat has previously been shown to be insulin resistant. Unlike any other hypertensive, insulin resistant model, the Milan rat has a normotensive, isogenic control which responds normally to insulin. As GLUT-4, the insulin-stimulated glucose transporter, had been implicated in insulin resistance I examined the levels of GLUT-4 present in the Milan rat. Results suggest that the insulin resistance experienced by this hypertensive strain may be due to a reduction in GLUT-4 within the intracellular membranes of skeletal muscle. This is due to the nature of insulin-stimulated glucose transport, which arises as a result of GLUT-4 translocating to the cell surface from an intracellular pool, and therefore increasing the rate of glucose uptake. Consequently, any reduction in intracellular GLUT-4 may account for the insulin resistance observed.

Further studies examined the stroke-prone spontaneously hypertensive rat, and the stroke-prone spontaneously hypertensive rat F2 generation. The F2 generation contains individuals that are extremely hypertensive and others which are normotensive. This was done to determine if the decrease in GLUT-4 observed in the hypertensive Milan rat correlated with increasing blood pressure. The results suggest that GLUT-4 levels in the stroke-prone spontaneously hypertensive rat are not altered by an increase in blood pressure. This result is in agreement with most studies on skeletal muscle GLUT-4, and highlights the complex nature of insulin resistance associated with hypertension.

The concluding chapter discusses the effects of oestrogen, and derivatives, on insulin-stimulated glucose uptake in 3T3-L1 adipocytes. Previous studies have shown that females taking steroid hormones, either by means of the contraceptive pill, of hormone replacement therapy, tend to suffer from insulin resistance. In 3T3-L1 adipocytes a 48 hour treatment with 30nM oestrogen significantly reduces insulin-stimulated glucose transport. This demonstrates that the cells have developed insulin resistance. However,

these cells do not have reduced GLUT-4 levels and the insulin resistance, induced by oestrogen, occurs by an as yet unknown mechanism.

Acknowledgements

Initially, I would like to thank the British Heart Foundation for funding my research. Thanks must go to the Division of Biochemistry and Molecular Biology for providing me with the facilities to complete my project.

For three years Gwyn Gould had the dubious pleasure of supervising me. Until I arrived the lab was a quiet, sedate place - what a change!!!! For his non-stop enthusiasm and encouragement I would thank him very much. I would like to thank the remainder of C36, but especially Mikey, who along with Gwyn made the visits to the Stevie building interesting to say the least!!!! Also, Callum for his help with the preps, (what fun!), and Derek for his expert proof-reading skills. If I have missed anyone I'm sure you'll let me know!!!

Special thanks must go to my family. Firstly my wife, Angela, whose love and support means so much to me. Also my old Ma' ; Bazza, Rona, Myra, and Neil who have encouraged me so much over my whole academic career. Finally, my dad, John, and brother, Craig, who will be forever in my thoughts.

Finally, as a release from lab work, as everyone who knows me will agree, Kilmarnock Football Club are my pride and joy. KFC deserve a mention for giving me something to bore everyone with!!!!

Dedication

I dedicate this thesis to my wife, Angela,
and the remainder of Clan Campbell

Quotations

"Football is not a matter of life and death.....it is much more important than that"

Bill Shankly

"Kilmarnock-0, Dundee United -2, and to be quite frank, Kilmarnock were fortunate to get nil"

Alex Cameron
Daily Record

CONTENTS

Figures

Chapter 4 GLUT levels in stroke-prone spontaneously hypertensive rats: Comparison of parental and F2 generation animals.

Tables

Abbreviations

ATP	Adenosine triphosphate
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
cDNA	Complementary deoxyribonucleic acid
DeGlc	2-Deoxy-D-glucose
DFP	Diisopropylfluorophosphate
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
E1	oestrogen
E2	Oestradiol
E3	Oestriol
ECL	Enhanced chemiluminescence
EDTA	Diaminoethanetetra-acetic acid, disodium salt
Ep 64	Trans-epoxysuccinyl-L-leucylamido(4-guanidino))-butane
FCS	Foetal calf serum
GDM	Gestational diabetes mellitus
GK	Glucokinase
GLUT	Glucose transporter
Glu-6-Pase	Glucose-6-phosphatase
HDM	High density microsome
HEPES	H-2-hydoxyethylpiperazine-N'2-ethanse sulphonic acid
HRP	Horseradish peroxidase
HRT	Hormone replacement therapy
IgG	Immunoglobulin gamma
IGT	Impaired glucose tolerance
IRS-1	Insulin receptor substrate-1
kDa	Kilodalton
KRH	Krebs ringer HEPES
KRP	Krebs ringer phosphate
LDM	Low density microsome
MAP kinase	Mitogen activating protein kinase
mRNA	Messenger ribonucleic acid
NAD	Nicotinamide adenine dinucleotide
NCS	Newborn calf serum
NIDDM	Non insulin-dependant diabetes mellitus

NPY	Neuropeptide Y
O.D.	Optical density
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCOS	polycystic ovary syndrome
Pi	Inorganic phosphate
PI-3' kinase	Phosphatidyl inositol-3' kinase
PM	Plasma membrane
PPi	Pyrophosphate
SDS	Sodium dodecyl sulphate
SHBG	sex hormone-binding globulin
SHR	Spontaneously hypertensive rat
SHRSP	Stroke-prone spontaneously hypertensive rat
SH2	Src homology 2
TEMED	N,N,N',N'-tetramethylenediamine
Tris	Tris(hydroxymethyl)aminoethane
U.V.	Ultraviolet
WKY	Wistar Kyoto rat

Chapter 1

Introduction

1.1 General introduction: The facilitative glucose transporter family (GLUTs).

The transport of glucose across the plasma membrane and into the cytosol of a cell is arguably the most important transport function a cell performs as D-glucose is required for cellular growth, homeostasis and metabolism. In 1982 Lienhard and his colleagues purified and partially sequenced the first member of the family now known as the facilitative glucose transporters, or GLUTs [Lienhard *et al.* (1982)]. These transporters allow the movement of glucose across the plasma membrane down its chemical gradient, either into or out of cells. Furthermore this family of proteins are specific for the D-enantiomer of glucose and are not coupled to any energy-requiring components, such as ATP hydrolysis or a H^+ gradient. Initially, GLUT-1 was purified from erythrocytes and antibodies raised against the polypeptide. Together the antibodies and the partial sequence obtained from the purified protein assisted in the isolation of cDNA clone in 1985 and subsequently the isolation of the gene coding for GLUT-1 [Fukumoto *et al.* (1988)]. GLUT-1 is expressed at the highest levels at the blood-brain barrier and erythrocytes, but it is also found in almost every mammalian cell at low levels. GLUT-1 is located on the plasma membrane, is not enriched in any intracellular compartment and is widely regarded as the "house-keeping" glucose transporter [Flier *et al.* (1987)].

Subsequent to the isolation of the GLUT-1 cDNA more members of the family were discovered by screening cDNA libraries' of several tissues with the GLUT-1 cDNA under conditions of low stringency, thus allowing the identification of cDNAs similar to GLUT-1. Using this method, the cDNA for GLUT-2 was discovered [Thorens *et al.* (1988); Fukumoto *et al.* (1988)]. Experimental analysis of this transporter revealed that it is a high-capacity,

high K_m transporter. These properties are ideally suited to allow the liver to function as one of the key tissues involved in glucose homeostasis; the high K_m and transport capacity endows GLUT-2 with properties enabling the rapid efflux of glucose from the liver following gluconeogenesis [Gould *et al.* (1991); Thorens *et al.* (1988)].

GLUT-3, the next isoform identified, was found to be the prominent isoform in the brain and neural tissue. GLUT-3 is expressed at high levels in human foetal muscle, but Northern blot analysis of the adult skeletal muscle revealed no trace of GLUT-3. In contrast high GLUT-3 mRNA is present in a range of other tissues such as the placenta, liver, heart and kidney. The mRNA for GLUT-3 has been found at high levels in these tissues, but the sites of protein expression are more restricted, indicating that perhaps the GLUT-3 may undergo negative post-translational regulation [Gould & Holman (1993)].

GLUT-5, the major facilitative glucose transporter of the small intestine was also identified by library screening with the GLUT-1 cDNA probe [Kayano *et al.* (1988)]. It is localised to the apical brush border on the luminal side of absorptive epithelial cells [Davidson *et al.* (1992)]. This transporter has a high affinity for fructose and a low affinity for glucose, therefore it seems reasonable to assume that it plays a major role in the uptake of dietary fructose [Burant *et al.* (1992)]. This explanation may be the reason that GLUT-5 is found at lesser levels in many tissues including muscle, brain and adipose tissue [Shepherd *et al.* (1992].

Using the same method of library screening another transporter-like transcript was identified [Kayano *et al.* (1990)]. The GLUT-6 cDNA was found to contain multiple stop codons and several frame shifts, and therefore is unlikely to encode a functional transporter. The cDNA is similar to that of

GLUT-3 and it has been suggested that the glucose transporter-like region of GLUT-6 may have arisen from the insertion of a reverse transcribed copy of GLUT-3 into a non-coding region of a universally expressed gene [Kayano *et al.* (1990)].

GLUT-7 was the last known member of the facilitative glucose transporter family to be discovered [Waddell *et al.* (1992)]. This transporter is at expressed at high levels in hepatic microsomes where it is a component of the G-6-Pase system. It is the least well characterised of all the members of the GLUT family, and will not be discussed further here. For a summary of the tissue distribution of the facilitative glucose transporters see table 1.1.

The final member of this family, GLUT4, will be considered in detail below and is the main isoform studied in this thesis.

1.1.1 Structure and membrane topology of the GLUTs

After the purification and cloning of the GLUTs work began on the analysis of the predicted amino acid sequences, and modelling of the membrane topology of the transporters. This work revealed that mammalian glucose transporters are highly homologous with one another. There is also a high level of sequence identity between that of the mammalian transporters and those of other species including *Eschericia coli*, yeast, algae and protozoa. This high degree of similarity probably relates to a common mechanism of transport catalysis, a common type of substrate and is suggestive of a

Table 1.1.

Major sites of expression of the different glucose transporters in human and rodent tissues.

Isoform.	Tissue.
GLUT-1	Placenta; brain; blood-tissue barrier; adipose and muscle tissue (low levels); tissue culture cells; transformed cells.
GLUT-2	Liver; pancreatic β-cell; kidney proximal tubule and small intestine (basolateral membranes).
GLUT-3	Brain and nerve cells in rodents. Brain, nerve; low levels in placenta, liver and heart (humans).
GLUT-4	Muscle, heart and adipose tissue.
GLUT-5	Small intestine (apical membranes), kidney, testis, brain, muscle adipose tissue, muscle and brain at low levels (humans). Small intestine (apical membranes), kidney (rat).
GLUT-7	Microsomal glucose transporter; liver

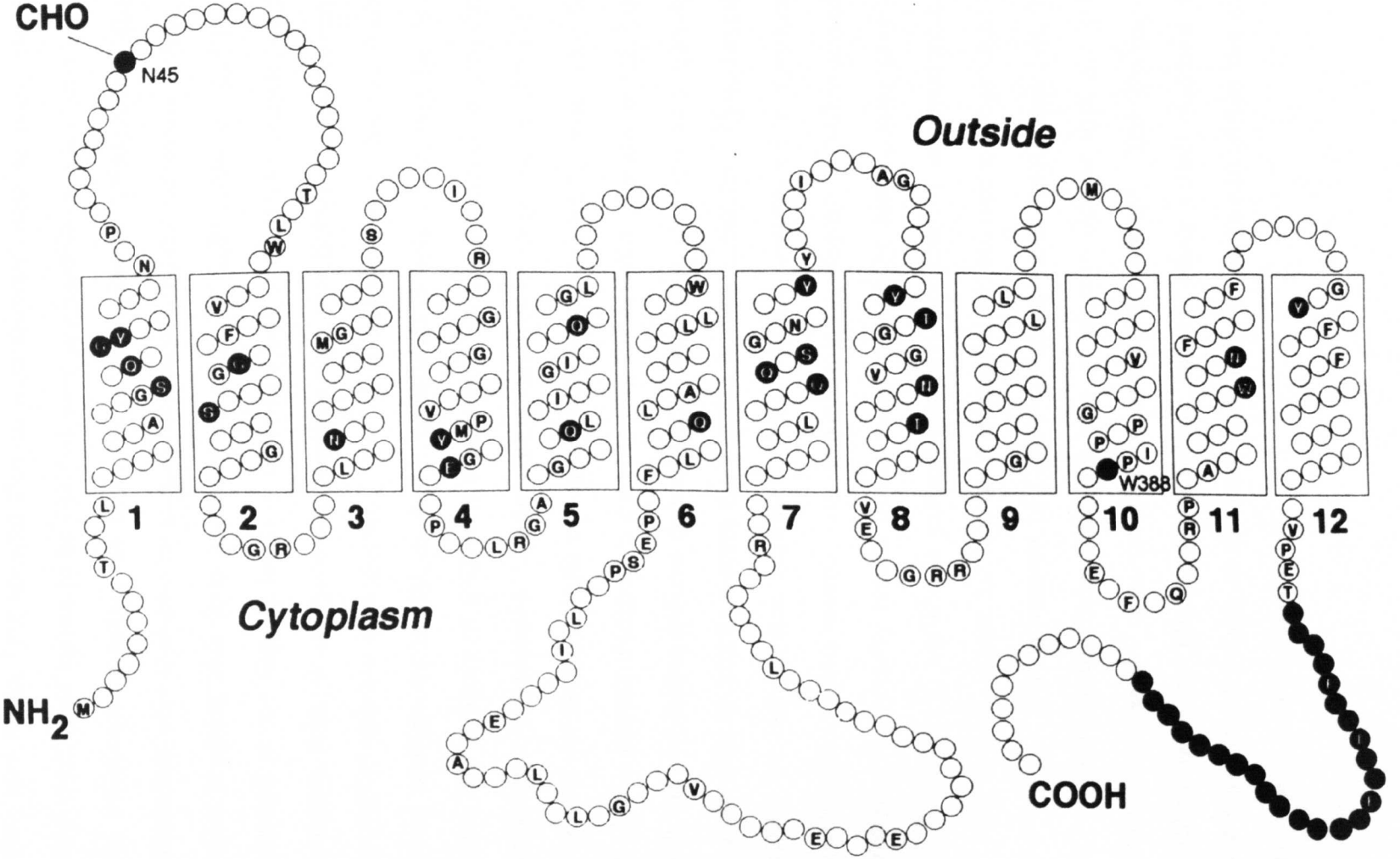
common evolution from a single ancestral gene. The common features revealed by sequence alignment and analysis of the transporters include 12 predicted amphipathic helices arranged so that both the N-and C-termini are at the cytoplasmic surface. Also predicted are large loops between helices 1 and 2 and between helices 6 and 7. The loop between helices 6 and 7 divides the structure into two halves, usually referred to as the N-terminal domain and the C-terminal domain. The loops between the remainder of the helices at the cytoplasmic surface are very short and the length of these loops (about 8 residues) is a conserved feature of the entire family. The short loops place severe constraints on the possible tertiary structure and suggest very close packing of the helices at the inner surface of the membrane in each half of the protein. The length and sequence identity of the loops at the extracellular surface of these proteins are very varied but are generally longer than the loops at the cytoplasmic surface. This may potentially result in a less compact helical packing at the external surface. The two dimensional topography, shown in figure 1.1, with the N- and C-termini on the cytoplasmic surface. This has been examined using anti-peptide antibodies which react only when the inner surface of the transporter in exposed, as in inverted vesicles containing human erythrocyte GLUT-1. This work supports the overall nature of this model. The general features of this model including the membrane spanning topology have been confirmed by experimental analysis (reviewed in Gould & Holman (1993)).

1.2 The insulin-stimulated glucose transporter - GLUT-4.

Fat and muscle are unique tissues with respect to glucose transport, as they exhibit acute insulin-stimulated increases in transport of up to 30-fold (fat)

Figure 1.1

Predicted membrane topology of the facilitative glucose transporter family


CHO
N45
Outside
W388
1
2
3
4
5
6
7
8
9
10
11
12
Cytoplasm
NH2
COOH

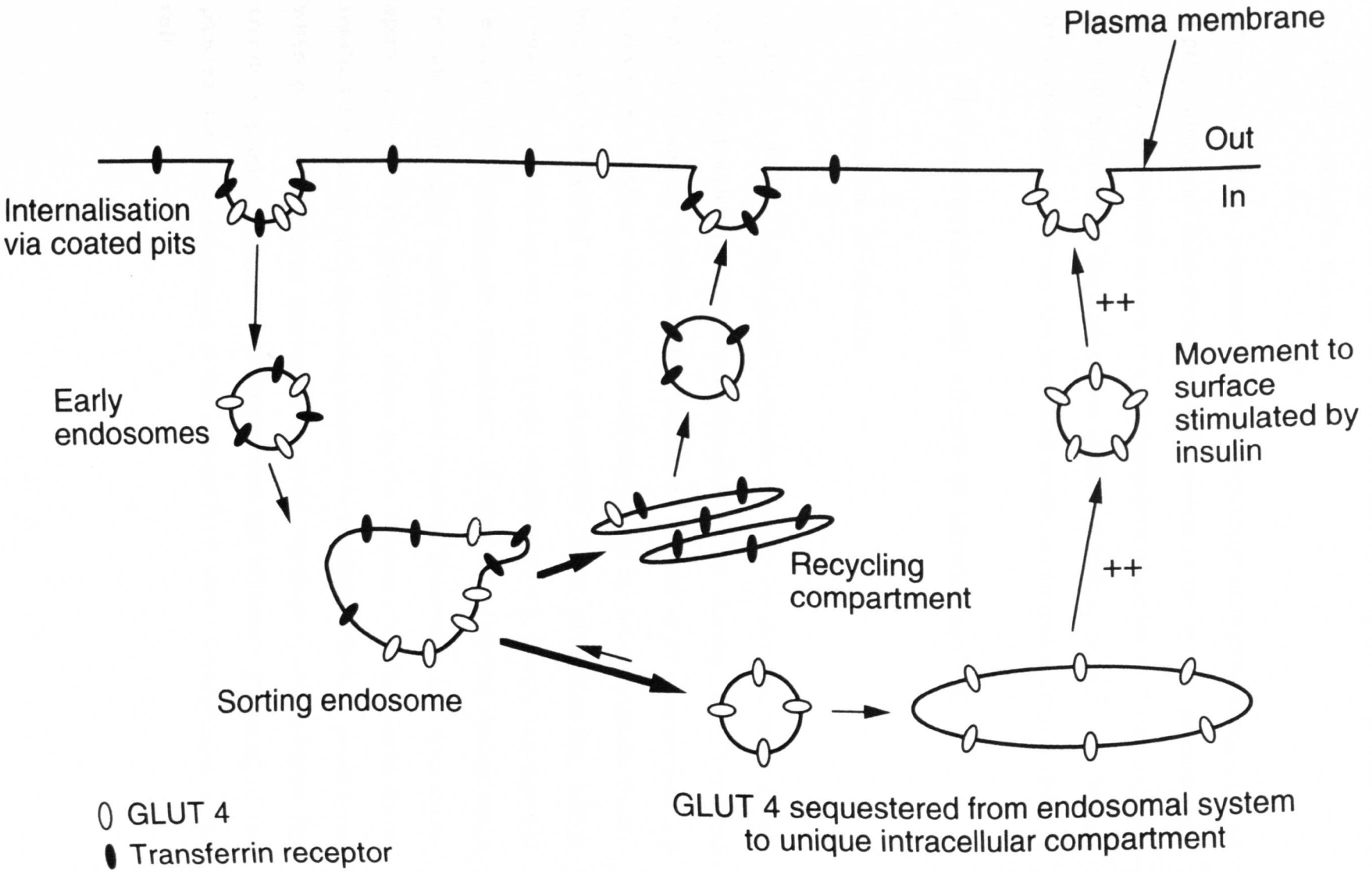
and 10-fold (muscle). In 1980, Cushman and Wardzala, and independently Suzuki and Kono, first showed that in unstimulated adipose cells, glucose transporters (now known to be GLUT-4) were predominantly associated with a light microsome fraction of the cells. Upon insulin stimaulation these transporters were recruited or translocated to the plasma membrane. Thus, it seemed probable that these tissues express a novel GLUT isoform. GLUT-4 is expressed at the highest levels in adipose tissue and skeletal muscle, and a substantial body of evidence now suggests that this isoform is the "insulin-responsive" glucose transporter. GLUT-4 is unique within the facilitative glucose transporter family, as under conditions of low circulating levels of insulin, the majority of GLUT-4 is sequestered within a unique intracellular membrane compartment. Under these conditions only 5% of GLUT-4 is present at the surface of the cell and available to transport glucose into the cell. Thus, glucose transport is rate-limiting for cellular glucose metabolism. When the circulating glucose concentration increases, this acts as a stimulus for pancreatic β-cells to secrete the peptide hormone, insulin. Upon insulin binding to the insulin receptor, the receptor undergoes a tyrosine-autophosphorylation event. An intracellular pathway is subsequently activated which finally results in the movement of the intracellular pool of GLUT-4 to the cell surface, a process generally referred to as translocation (see section 1.3). Figure 1.2 is a diagrammatical model representing GLUT-4 translocation. The movement of GLUT-4 to the plasma membrane has the effect of dramatically increasing the rate of glucose uptake into the cell, thereby reducing the concentration of circulating glucose. The glucose is phosphorylated to glucose-6-phosphate before being either utilised to produce metabolic energy in the form of ATP, or being stored within the cell as glycogen or triacylglycerides.

This GLUT-4-rich intracellular pool is the subject of much debate and is currently being characterised by a number of laboratories. It is hoped that by understanding the targeting and translocation of GLUT-4 it may then be possible to fully comprehend the mechanism by which insulin stimulates the movement of

Figure 1.2

Diagrammatic representataion of GLUT-4 translocation in skeletal muscle and adipose tissue.

This figure illustrates the slow, constitutive recycling of the insulin-stimulated glucose transporter, GLUT-4, through the classical endosomal membrane recycling pathway. Upon insulin stimulation the intracellular GLUT-4 pool is quickly translocated to the cell surface, resulting in a rapid increase in glucose uptake.


Plasma membrane
Out
In
Internalisation
via coated pits
Early
endosomes
++
Movement to
surface
stimulated by
insulin
Recycling
compartment
++
Sorting endosome
GLUT 4
Transferrin receptor
GLUT 4 sequestered from endosomal system
to unique intracellular compartment

this glucose transporter, and therefore increases the uptake of glucose into the insulin-responsive tissues.

A further benefit in understanding the mechanism of insulin action may be in pin-pointing the exact locations of the defects in the system characterised by disease states such as non insulin-dependent diabetes mellitus (NIDDM) and insulin resistance. Detailed review of the cell biology of GLUT-4 is beyond the scope of this thesis, and the interested reader is referred to Gould (1997).

1.3 The mechanism and effects of insulin action.

1.3.1 Production of insulin

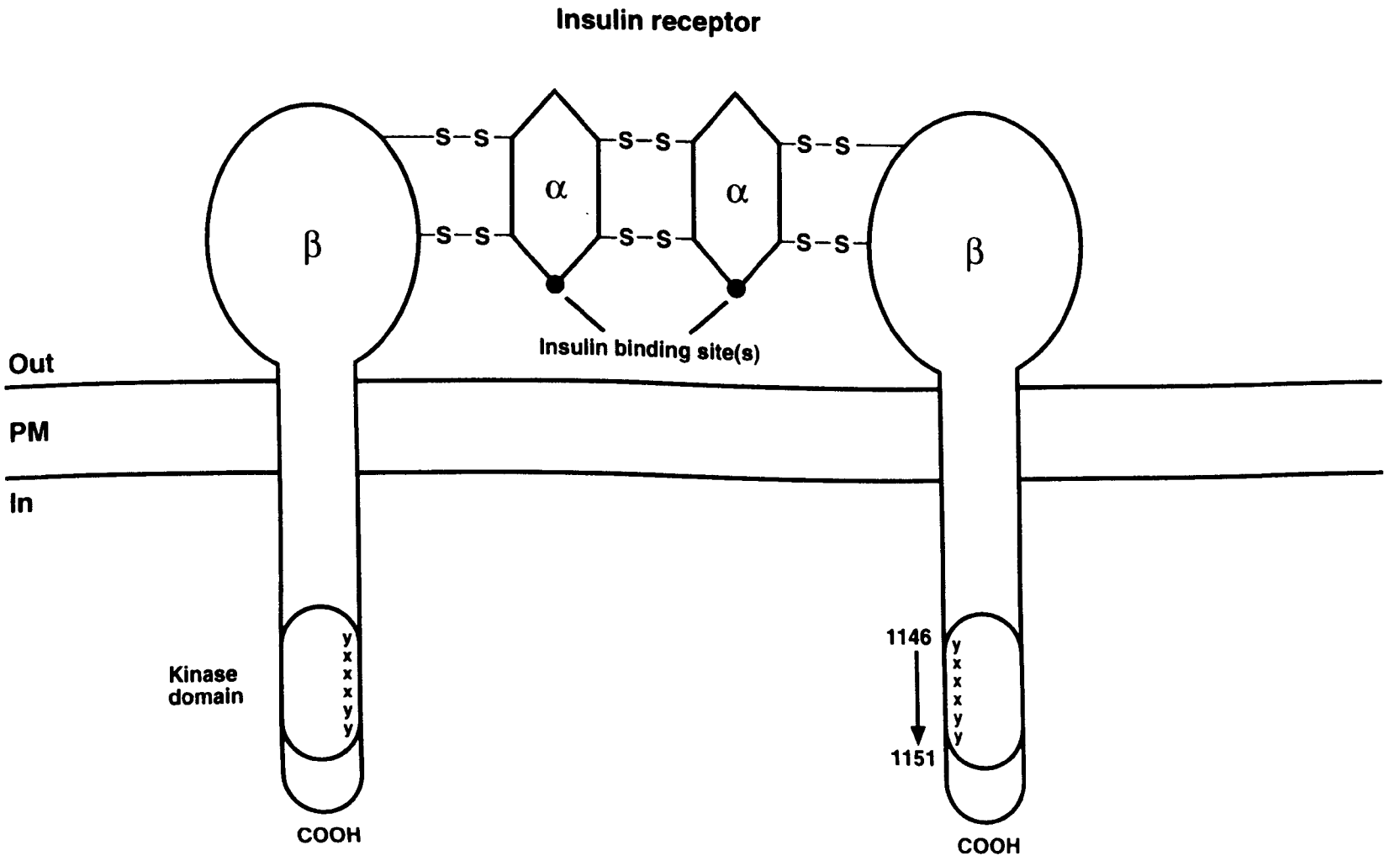
Insulin is a 5.8kDa polypeptide hormone secreted by the pancreatic ß-cells and is an important regulator of anabolic metabolism. Sanger in 1953 showed that bovine insulin consists of two chains, an α chain of 21 residues and a β chain of 30 residues, which are covalently linked by two disulphide bonds. Insulin is synthesised as a single polypeptide chain, preproinsulin, which contains a nineteen amino acid signal sequence which directs the nascent chain to the endoplasmic reticulum. In the lumen of the endoplasmic reticulum the signal peptide is cleaved to form proinsulin which contains approximately thirty residues absent in the mature peptide. These thirty residues are called the connecting peptide (C-peptide), which is proteolysed when proinsulin passes through the Golgi apparatus and reaches the secretory granules. Insulin is secreted from the secretory granules in the pancreas when the membrane of the granule fuses with the membrane of the cell.

1.3.2 The insulin receptor

The insulin receptor is an integral membrane protein which resides in the plasma membrane of the cell and is a tetrameric protein composed of two α-subunits that are each linked to a ß-subunit and to each other by di-sulphide bonds. The receptor is a member of the tyrosine kinase family and behaves like a classical allosteric enzyme. The α-subunits are located entirely outside the cell and contain the insulin binding site(s) whereas the intracellular part of the ß-subunit contains the insulin regulated tyrosine protein kinase. From stoichiometric studies 1-2 insulin molecules bind per receptor with the binding sites displaying negative co-operativity, and the ligand binding determinants being mapped to a distinct region within the first 500 amino acids of the α-subunit. The unoccupied α-subunit of the insulin receptor inhibits the tyrosine kinase activity of the ß-subunit. Several functional regions have been defined in the ß-subunit including the ATP binding domain and autophosphorylation sites in the intracellular juxtamembrane region, a regulatory region and the COOH terminus. Tyrosine phosphorylation appears to occur through a *trans* mechanism in which insulin binding to the α-subunit of one αß-dimer stimulates the phosphorylation of the adjacent covalently linked ß-subunit [for review, see White & Khan (1994)]. Phosphorylation of all three tyrosine residues within the YxxxYY motif of the regulatory region increases kinase activity 10-20 fold. Mutations of tyrosines 1146, 1150 and 1151 either singularly or collectively within this domain of the ß-subunit result in a progressive reduction in insulin-stimulated kinase activity and additionally a parallel loss in biological activity. The structure of the insulin receptor is illustrated in figure 1.3.

Figure 1.3

The structure of the insulin receptor.


Insulin receptor
S–S
S–S
S–S
S–S
S–S
S–S
α
α
β
β
Insulin binding site(s)
Out
PM
In
Kinase domain
y
x
x
x
y
y
COOH
1146
1151
y
x
x
x
y
y
COOH

1.3.3 Control of the insulin receptor

As well as being under the influence of tyrosine phosphorylation, the insulin receptor is also phosphorylated on serine and threonine residues in the basal state in response to stimulation by insulin, cAMP analogues and phorbol esters. The combination of ligand binding, tyrosine phophorylation, and serine/threonine phosphorylation, provide three levels of control which are sensitive to extracellular messengers and intracellular events. Another mechanism of control may be tumour necrosis factor-α (TNF-α) which has been shown to cause insulin resistance in adipose tissue, possibly through inhibition of autophosphorylation and kinase activity [Hotamisligil *et al.* (1993)].

The intracellular juxtamembrane region of the insulin receptor ß-subunit is essential for signal transmission as replacement of tyrosine 960 with alanine impairs receptor signal transmission even though autophosphorylation in the other regions is normal and the kinase activates fully *in vitro*. This may be due to the inability of the mutant receptors to phosphorylate exogenous receptor substrates, such as insulin receptor substrate-1 (IRS-1). Some mutations in the juxtamembrane region may be rescued with overexpression of IRS-1.

As well as signal transduction, the insulin receptor mediates insulin internalisation. Internalisation of the receptor, with bound insulin, via endocytosis leads to the degradation of insulin by its targeting to the lysosommal system, with the majority of unoccupied receptors being recycled back to the plasma membrane. The site of insulin receptor signalling in this recycling is not clear.

1.3.4 Insulin receptor signalling

After the tyrosine and serine threonine phosphorylation events the "activated" receptor has the capacity to phosphorylate target proteins. The most well documented of the target proteins is the Insulin Receptor Substrate-1 (IRS-1). IRS-1 has a predicted molecular weight of 131 kilodaltons and is a specific substrate for the insulin and IGF-1 receptors. Initially it was detected with the use of high affinity anti-phosphotyrosine antibodies and has since been purified and cloned from several sources. IRS-1 contains multiple sites for tyrosine and serine/threonine phosphorylation in motifs recognised by various kinases and these multiple phosphorylation sites result in the protein migrating with an apparent molecular weight of 180 kDa. At least eight tyrosines in IRS-1 are phosphorylated by the activated insulin receptor. The phosphorylated IRS-1 is recognised by the src homology 2 (SH2) domains of a plethora of signalling proteins via specific phosphorylated tyrosine residues.

1.3.5 Insulin receptor signalling - PI-3' kinase

Phosphatidyl inositol-3' kinase (PI-3' kinase) was the first SH2 containing protein that was found to associate with IRS-1. PI'-3 kinase is composed of a 110-kDa catalytic subunit and an 85-kDa regulatory subunit which contains the two SH2 domains. The SH2 domains have been shown to specifically bind phosphorylated IRS-1 *in vivo* and *in vitro*. [Sun *et al.*, (1993)] The activation of PI'-3 kinase occurs when both SH2 domains are occupied with a phosphotyrosine in a YxxM or YMxM motif (where x is any amino acid). This sequence corresponds to an immunoreceptor tyrosine activation motif (ITAM). Such motifs are common in IRS-1. The biological role of IRS-1 will be discussed in later chapters of this thesis.

1.3.6 Insulin receptor signalling - Syp

SH-PTP2 (syp) is a protein tyrosine phosphatase which also contains two SH2 domains and binds to tyrosine 1172 of IRS-1. This association of SH-PTP2 with phosphoproteins such as IRS-1 activates the phosphatase, and suggests a role for IRS-1 in the activation of this signalling pathway [Kahn(1994)].

1.3.7 Insulin receptor signalling - MAP kinase

Another protein that binds to IRS-1 is the small cytoplasmic protein, GRB-2 which contains two Src homolgy 3 (SH3) domains, which recognise proline-rich amino acid sequences, and one SH2 domain. GRB-2 binds to tyrosine 895 in IRS-1, and is thought to act as an "adaptor molecule" linking the guanidine nucleotide exchange factor for $P21^{ras}$ termed mSOS to tyrosyl phosphoproteins, such as IRS-1. The binding of GRB-2/mSOS to IRS-1 may mediate the insulin stimulation of $p21^{ras}$. Ras has been shown to bind to Raf-1 serine/threonine kinase, which in turn activates MAP kinase via phosphorylation and activation of MAP kinase kinase. Thus the binding of GRB-2 to IRS-1 might provide a pathway for insulin regulation of Ras.

The MAP kinase pathway results in the activation of several other kinases, such as p90 S6 kinase. S6 kinase activates a phosphatase which dephosphorylates and therefore activates glycogen synthatase, the rate limiting enzyme in glycogen synthesis.

1.3.8 Insulin receptor signalling - synopsis

Insulin action is mediated by a cascade of covalent and non-covalent interactions that centre around IRS-1. Insulin binding to the receptor on the cell surface leads to the phosphorylation of IRS-1 which serves as the docking protein for several intracellular enzymes and docking proteins. It is the docking of these proteins that leads to the multiple signals generated by the action of insulin [Khan (1994)]. The events listed above involving IRS-1 indicate the importance of IRS-1 in most, if not all, of insulin's biological actions. The insulin receptor signalling pathway is summarised in figure 1.5.

1.3.9 Insulin receptor signalling - mitogenesis

The role of insulin as a mitogen has been studied in Chinese Hamster Ovary (CHO) cells. In cells overexpressing IRS-1 there was a two-fold increase in thymidine incorporation upon insulin stimulation. However, when IRS-1 levels were reduced using antisense cDNA, there was a reduction in the response and sensitivity of the mitogenesis following insulin stimulation. Such studies indicate a key role for IRS-1 in mitogenesis.

IRS-1 is absent from the 32D myeloid progenitor cell line, which is also unable to produce a mitogenic response to an insulin stimulus. Ectopic expression of IRS-1 in the 32D line results in insulin stimulated IRS-1 phosphorylation and DNA synthesis, thus proving that IRS-1 is required for the mitogenic response produced by insulin.

Although IRS-1 plays a key role in bringing about the biological actions of insulin, the signalling pathways downstream of IRS-1 remain unclear. The main role of insulin is to play a key role in glucose homeostasis. This is achieved by the translocation of the facilitative glucose transporter, GLUT-4,

and the subsequent increase in uptake of circulating glucose, thereby reducing the concentration of blood glucose.

Figure 1.4

Diagrammatic representation of insulin receptor re-cycling.

* - indicates that it has not been determined if the receptor is still signalling at these intracellular locations.

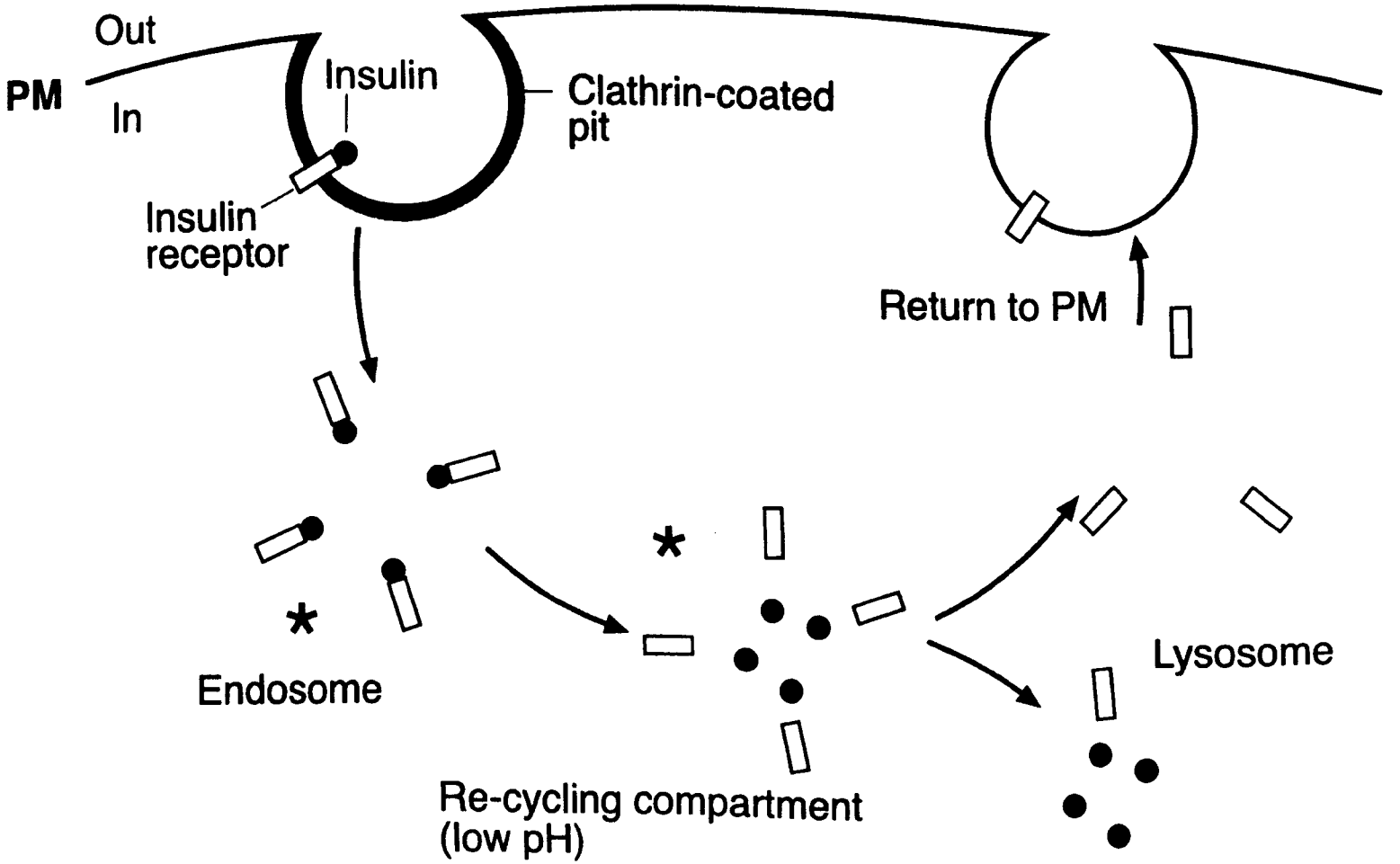
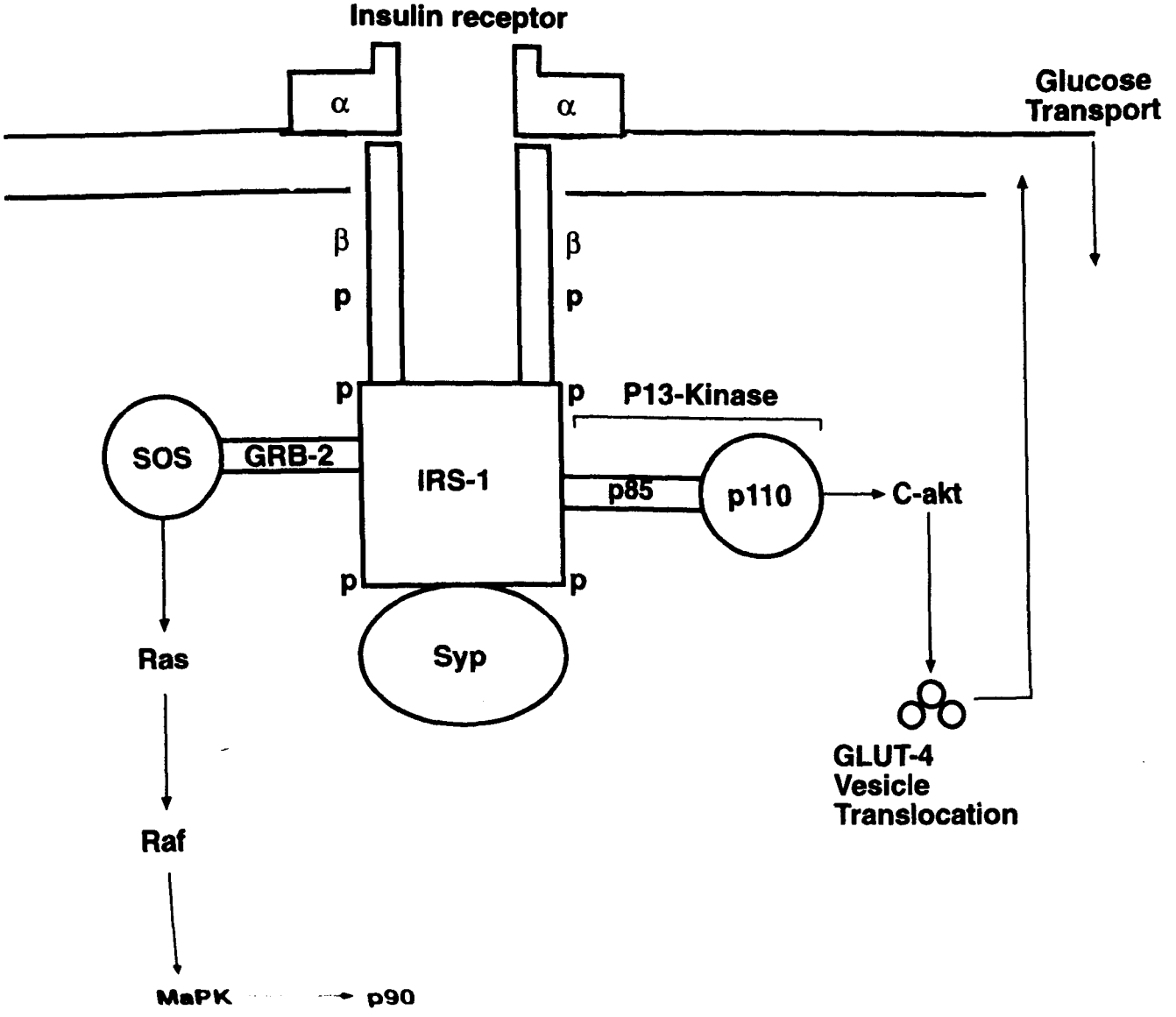

Out
PM
In
Insulin
Clathrin-coated pit
Insulin receptor
Return to PM
Endosome
Re-cycling compartment (low pH)
Lysosome

Figure 1.5

The insulin receptor signalling pathway through IRS-1 and associated proteins.

Insulin receptor signalling



As mentioned above insulin also produces a variety of other biological responses. It has been noted that insulin is a mitogen, inducing growth in a variety of tissues. Furthermore insulin has been shown to stimulate the sympathetic nervous system. Finally insulin plays a key role in insulin metabolism. It is widely acknowledged that insulin resistance mainly occurs with respect to glucose transport in peripheral tissues, with the remaining properties of insulin unaffected (see below).

1.3.10 Cation transport

Insulin stimulates the Na^+/K^+ ATPase, Ca^{2+} ATPase and the Na^+/H^+ antiport as well as potential and receptor mediated Ca^{2+} channels.. The net effect of insulin is to reduce the concentration of intracellular Na^+, Ca^{2+}, and H^+, and also to elevate Mg^{2+}. Vascular smooth muscle cells will show diminished responses under these circumstances. The role of cation transport in insulin resistance will be discussed in later chapters of this thesis. Experimental evidence suggests that insulin resistance extends to both the Na^+/K^+ ATPase and the Ca^{2+} ATPase. This leads to a reduction in the activity of these enzymes in insulin resistant states such as hypertension and obesity. Decreased activity of the Na^+/K^+ ATPase and Ca^{2+} ATPase could lead to increased intracellular Ca^{2+} concentration, which is a determinant of vascular smooth muscle contractility. If on the other hand the Na^+/H^+ antiporter retains normal sensitivity to insulin, the resultant hyperinsulinaemia will augment Na^+/H^+ exchange. This antiporter is a known genetic marker for primary hypertension, and is a ubiquitous transport system invovled in the regulation of cell volume, growth, intracellular pH and also Ca^{2+} exchange.

1.4 Insulin resistance

Insulin resistance is a disease characterised by the reduced ability of insulin to exert its effects in peripheral tissues, skeletal muscle and adipose tissue. The disease is characterised by the patient displaying hyperglycaemia in the face of normal or even elevated circulating concentrations of insulin. One effect of insulin resisance is the marked decrease in insulin-stimulated glucose uptake into skeletal muscle and adipose tissue. If severe, insulin resistance can lead to overt Non-Insulin Dependent Diabetes Mellitus (NIDDM) and has been associated with a number of other disease states including obesity, Impaired Glucose Tolerance (IGT), NIDDM, hypertension, pregnancy and steroid therapy. The fact that NIDDM, obesity and essential hypertension often occur together in a clinical situation implies that there may be common factors in the underlying pathology of these conditions [Reaven *et al.*, (1989)].

The mechanism of insulin resistance is poorly understood and one of the aims of my project was to try and elucidate the role of the facilitative glucose transporters, and in particular GLUT-4, in insulin resistance associated with hypertension.

1.5 Insulin resistance and the glucose transporter GLUT-4.

As previously stated GLUT-4 is the "insulin-responsive glucose transporter" which translocates to the cell surface under the stimulus of insulin. The rapid recruitment of GLUT-4 to the muscle and fat cell surface greatly increases the capacity of the cell to import glucose, convert it into glucose-6-phosphate and finally glycogen, to decrease circulating levels of blood

glucose. In section 1.4 insulin resistance was characterised as the failure of insulin to result in efficient glucose disposal, and in particular a failure of insulin to produce its normal increase in glucose transport in target tissues. Although skeltal muscle is the main site of glucose disposal, the majority of work on insulin resistance has been performed on adipose tissue. Adipose tissue only accounts for 5-20% of whole body glucose disposal but is a suitable tissue to study as it contains GLUT-4 and is readily isolated or grown in culture. Muscle tissue is difficult to work with and in particular skeletal muscle is very difficult to isolate and separate into subcellular membrane fractions.

The most common disease linked with insulin resistance is diabetes mellitus. This disease is responsible for a great deal of morbidity and mortality in the western world. However, the majority of patients suffering from this ailment are not dependant upon insulin treatment, i.e. they suffer from NIDDM. NIDDM is characterised by hyperglycaemia in the face of normal or elevated levels of circulating insulin. It has been established that the abnormality impairing insulin's ability to stimulate glucose disposal into peripheral tissues in such individuals generally lies distal to the binding of insulin to its plasma membrane receptor [Reaven *et al.*, (1989). The full signalling pathway from binding of the receptor to the uptake of glucose has not been fully elucidated, see Kahn (1994), so the abnormality could lie anywhere from the generation of the signal to the glucose uptake itself. Research efforts are currently focused on the three main areas best understood in the process: firstly activation of the insulin receptor-associated tyrosine kinase, secondly glucose uptake in the cell and finally GLUT-4 trafficking and targeting.

Within whole body glucose homeostasis, GLUT-4 is the most important facilitative glucose transporter [Gould & Holman (1994)]. As stated previously

(section 1.2), expression of this isoform is restricted to tissues which exhibit acute, insulin-stimulated glucose transport (skeletal muscle, brown and white adipose tissue and cardiac muscle) and is unique by virtue of it's almost exclusive intracellular location in the absence of insulin. During postprandial hyperglycaemia, circulating insulin signals this transporter to translocate from intracellular locations to the plasma membrane, where its presence is associated with a rapid and massive increase in glucose uptake. Following the cloning of the insulin-regulatable glucose transporter by James and co-workers in 1989 there has been an exponential growth in research within this topic. The cloning and purification of the transporter led to the production of anti-GLUT-4 antisera enabling the protein to be quantified in tissues. Furthermore a considerable amount of research has been directed towards the understanding of GLUT-4 translocation. The signal which recruits GLUT-4 to the cell surface remains unclear but it is now becoming more apparent that GLUT-4 functioning is defective both in animal models and human insulin resistance.

1.5.1 The nature of GLUT-4 defects in insulin resistance

Patients with NIDDM have been examined extensively for mutations of the GLUT-4 gene which might result in a dysfunctional transporter. Point mutations have been noted but these occur in too small a percentage of cases for them to be considered an important aetiological factor in insulin resistance [reviewed in Gould, (1997)]. Furthermore, gene defects would be expected to underlie an irreversible form of insulin resistance, however the phenomenon of insulin resistance appears to be subject to a number of influences, and is not always irreversible eg. gestational diabetes mellitus (GDM).

It is unsurprising then that functional impairment of insulin-stimulated transport in NIDDM patients has emerged as being more important than genetic defects and the basis for this impairment is becoming apparent.

The types of defect found in insulin-regulatable glucose transport can be divided into two groups,

i) where there is cellular depletion of GLUT-4

and/or

ii)where the protein is apparently expressed at normal levels but fails to translocate effectively to the cell surface in response to insulin, owing to a defective signal pathway following binding of insulin to its receptor.

1.5.2 Cellular depletion of GLUT-4 levels and insulin resistance in adipose tissue

In adipose tissue from obese and NIDDM patients there is a clear and well documented reduction of GLUT-4 expression compared to that of lean controls. Furthermore, insulin-stimulation of GLUT-4 translocation has been shown to be impaired in adipocytes from NIDDM patients [reviewed in Gould, (1997)]. These patients have lower than normal levels of GLUT-4 mRNA, indicative of a pre-translational effect. A similar picture is observed in GDM [Garvey *et al.* (1993)]. GLUT-4 levels were shown to be severly depleted in half the GDM patients studied but in addition all the GDM cohort in this study exhibited abnormal targetting of the protein to an intracellular compartment from which it could not be effectively translocated to the cell surface upon insulin stimulation [Garvey *et al.*, (1988).

As yet the influences upon GLUT-4 expression are poorly understood as are the factors underlying transporter depletion in diabetes. However, the signalling nucleotide cyclic 5'-monophosphate (cAMP) has been found to have a repressive effect on the GLUT-4 gene. It is possible that elevated levels of cAMP known to be present in diabetic adipocytes may contribute to poor expression of the transporter [Shepherd & Kahn (1993)]. Secondly, hyperinsulinaemia present in the early stages of NIDDM may play a causative role in in the down-regulation of insulin-stimulated glucose transport. The recent identification of the GLUT-4 promoter and the ability to use transgenic technology to engineer tissue-specific expression of reporter genes should provide key insights into the regulation of this gene, and thus alow the identification of the factors which control GLUT-4 expression [reveiwed in Gould, (1997)]

1.5.3 Insulin resistance in skeletal muscle

In skeletal muscle the nature of insulin resistance has proven to be different, implying that there are tissue-specific diffferences in the pathology of insulin resistance as well as in GLUT-4 expression under normal circumstances [Pedersen *et al.*, (1990)]. Most studies have shown no change in muscle cellular GLUT-4 expression at both the level of mRNA and protein concentrations in insulin resistant patients [Pedersen *et al.*, (1990)].. Only the plasma membrane-associated GLUT-4 level was lower, suggesting an abnormality in the translocation of GLUT-4 in response to insulin. The majority of studies present in literature suggest that altered GLUT-4 expression does not contribute to skeletal muscle insulin resistance [Shepherd & Kahn, (1993)]. Despite some reports of decreased GLUT-4 expression in skeletal muscle of individuals with NIDDM, it is now generally accepted that skeletal muscle GLUT-4 levels are unchanged in individuals

with NIDDM compared to age-and weight-matched control subjects [Pedersen *et. al.* (1991)]. This was recently borne out by the study of Bjorback *et al.* (1994) which analysed the GLUT-4 promoter of insulin resistant subjects. Although some genetic variants were discovered, none were considered likely to impair expression of the gene and therefore cannot be implicated in insulin resistance.

The overwhelming number of reports present in the literature suggest that reduced expression of GLUT-4 does *not* contribute to skeletal muscle insulin resistance [Shepherd & Kahn, (1993)]. It further suggests that the defect in this tissue may lie either on the pathway of insulin-signalling or transporter translocation. Figure 1.6 illustates the potential sites of insulin resistrance in relation to insulin-stimulated GLUT-4 translocation. Thus, understanding GLUT-4 trafficking and targeting remain key goals for future reasearch. This point will be further discussed in chapters 3 and 4 of this thesis.

1.6 Insulin resistance and the relationship with hypertension.

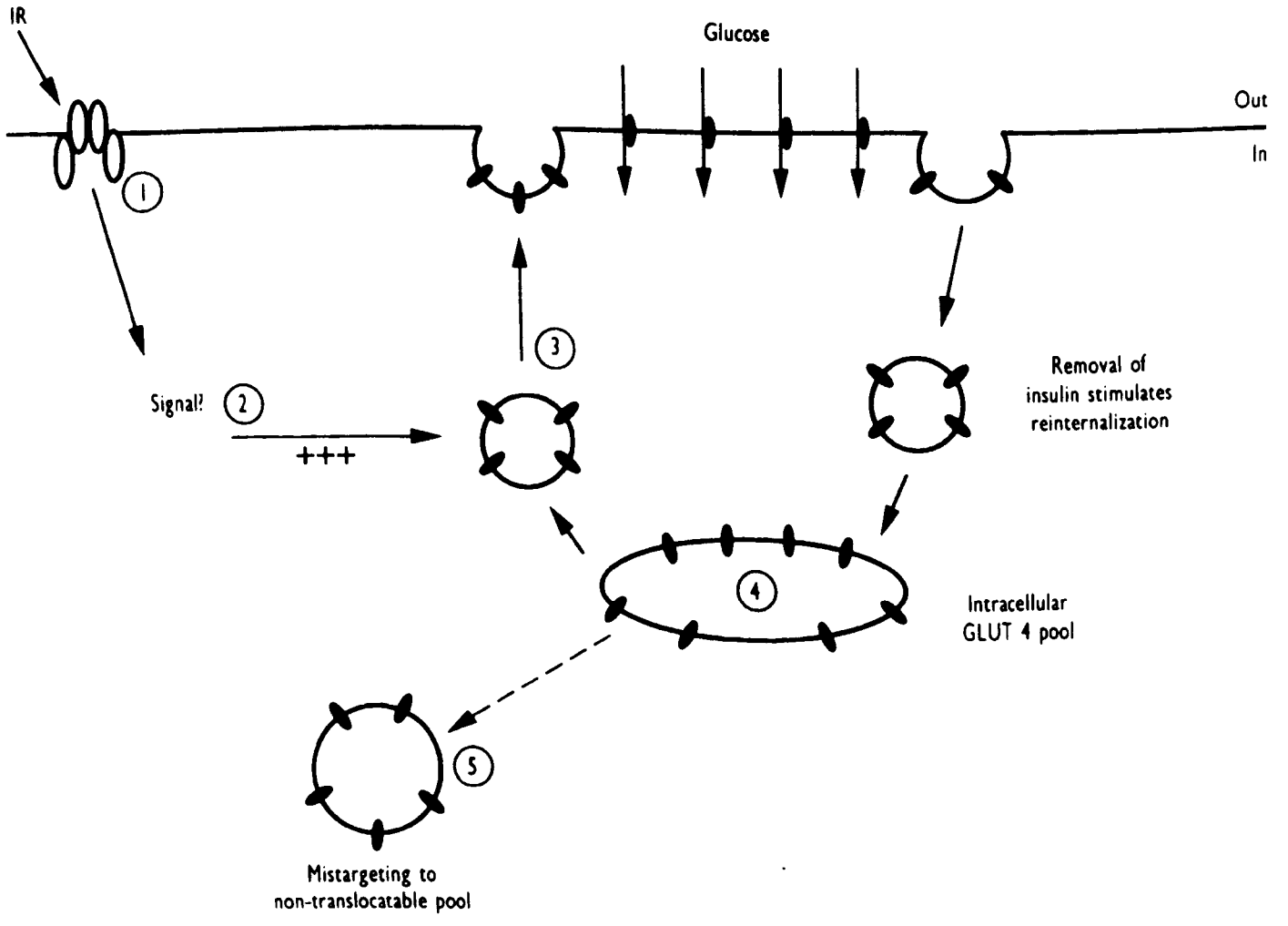
Insulin resistance is characterised by hyperglycaemia and hyperinsulinaemia. This disease state is known to occur in tandem with other conditions such as obesity and non-insulin dependant diabetes mellitus (NIDDM). One other disease state that commonly occurs with insulin resistance is hypertension. The relationship between these two disease states is not straightforward. Rocchini *et al.* (1995) state that approximately 50% of hypertensive subjects have insulin values within 1 standard deviation of the normotensive population and were therefore not insulin resistant. Nevertheless, he argued that the two situations are closely linked, as evidenced by 50% of this group exhibiting some hypertension. The link

between hypertension and insulin resistance is complex and multifactional, and has been extensively reviewed recently so it will not be re-iterated here. However, it should be noted that insulin resistance and hypertension often occur in the same patient, and are in many cases inextricably linked.

Figure 1.6

The potential sites of insulin resistance in adipose tissue.

Under basal conditions, approximately 95% of GLUT-4 is located intracellularly in a pool of vesicles, the nature of which is poorly understood. GLUT-4 undergoes a slow rate of constitutive recycling between the plasma membrane and this intracellular site, a process which is thought to occur through coated pits and entry into the endosomal system. Upon insulin-stimulation, 40-50% of the intracellular pool translocates rapidly (within minutes), giving rise to 20-30 fold increases in the cell surface GLUT-4 levels, and so accounting for the large increase in glucose transport observed under such conditions. as circulating glucose and insulin levels fall, there is a reversal of this situation, with the GLUT-4 becoming sequestered in the intracellular pool. This model is based on studies on adipocytes. Potential sites of insulin resistance shown are as follows: (1) Reduced binding of insulin to its plasma membrane receptor or impaired activation of the receptor-associated tyrosine kinase. (2) Impaired intracellular insulin signalling pathway. Both defects would render cells insulin-resistant for glucose transport independently of any defects in transporter expression or function. (3) Defective translocation of GLUT-4 to the cell surface. In this case, normal levels of GLUT-4 are present in the intracellular pool, but a defect in the mechanism responsible for moving this pool to the cell surface results in blunted insulin-stimulated transport. (4) Reduction in the intracellular pool of GLUT-4. In this scenario, translocation of GLUT-4 occurs as normal, but there is a reduced level of insulin-stimulated glucose transport due to a profound reduction in the GLUT-4 available for translocation. (5) Mistargeting of GLUT-4 to a non-translocable pool. This mislocalisation of GLUT-4 to a site from which it cannot be targeted would have the effect of reducing insulin-stimulated glucose transport.


IR
Glucose
Out
In
1
Signal?
2
+++
3
Removal of
insulin stimulates
reinternalization
4
Intracellular
GLUT 4 pool
5
Mistargeting to
non-translocatable pool

Hypertension can precede insulin resistance, but the converse is also true, with insulin resistance preceding hypertension. However, note that not all insulin resistant patients become hypertensive nor do all hypertensive patients become insulin resistant, hence the relationship between these clinical conditions is complex [Reaven *et al.*, (1989, 1991)].

1.6.1 Insulin resistance may be pathway specific

Although insulin resistance is characterised the impaired ability of insulin to cause whole body glucose disposal, some or all other physiological actions of insulin may be preserved. Potentially the most important of these with respect to hypertension is the ability of insulin to induce renal sodium retention. It has been demonstrated that obese adolescents have "selective" insulin resistance as they are resistant with respect to glucose uptake by skeletal muscle yet are still sensitive to the renal sodium-retaining effects of insulin. Also, Ferrannini *et al.* (1987) have demonstrated in non-obese essential hypertensives that insulin resistance is selective, tissue specific and pathway specific. The selectivity predominantly involves glucose metabolism, but also amino acid and fatty acid metabolism to a smaller degree. The main site of insulin resistance is skeletal muscle, with the liver and adipose tissue affected to a lesser extent. In terms of pathway specificity it appears that only the glycogen synthesis pathway is usually affected although in cases of diabetic ketoacidosis all anabolic pathways are resistant to the effects of insulin. Therefore in any individual the degree to which the insulin resistance is tissue and pathway specific may determine whether hypertension will develop.

Insulin resistance may result in hypertension through changes in vascular structure and function, activation of the sympathetic nervous system and

enhanced renal sodium retention. Aspects of these points will be further discussed in subsequent chapters as necessary.

1.7 Animal models of hypertension and insulin resistance.

The use of rodent models to study insulin resistance and hypertension has produced conflicting results regarding the aetiology and underlying pathology responsible. This may in part be due to the model systems displaying varying degrees of insulin resistance, perhaps reflective of the heterogeneity of the disease, but it may also be accounted for by difficulty in the preparation of sub-cellular fraction of tissues such as skeletal muscle. Furthermore the employment of different techniques to study the tissues under investigation may produce inconsistent results.

1.7.1 GLUT-4 in animal models of insulin resistance

GLUT-4 levels in animal models of insulin resistance have been extensively studied and reviewed [Livingstone *et al.*, (1994)]. This thesis concerns GLUT levels in animal models of insulin resistance associated with hypertension. Some of these models are discussed below in brief.

1.7.2 GLUT-4 in rodent models of genetic hypertension

Obese Zucker Rat.

The obese Zucker (fa/fa) rat model was initially employed as a model of insulin resistance associated with obesity. This model has been extensively studied with respect to insulin signalling and glucose uptake into the insulin-sensitive tissues. The results proved that this model was indeed insulin resistant as the animal displayed normoglycaemia with elevated circulating levels of insulin and diminished insulin-stimulated glucose uptake, in adipocytes and all major muscle groups. Further studies revealed that this animal was hypertensive when compared with control strains and therefore also represents a useful model for this condition.

Skeletal muscle from these animals exhibit no change in GLUT-4 expression compared with lean control animals, and impaired insulin-stimulated GLUT-4 translocation to the plasma membrane is again a likely possibility to account for the insulin resistance observed.

Male rats of a related strain, the ZDF/drt/fa Zucker rat, are also obese and insulin-resistant, but go on to develop overt diabetes. Interestingly, in this strain, GLUT4 levels have been found to be significantly reduced in both skeletal muscle and adipose tissue, accounting for the insulin resistance. The level of GLUT-4 expression in the obese animal has been restored by combating the hyperglycaemia, suugesting that this may be contributing to the impaired expression of the transporter. Reduced GLUT4 in this animal model appears to be decisive in causing the diabetes, since in other respects the animals are similar to their respective non-diabetic strains.

Spontaneously Hypertensive Rat (SHR).

The SHR is the most studied rodent model of genetic hypertension. As well as hypertension, the SHR exhibits insulin resistance and hyperinsulinaemia compared with control strains. Insulin stimulation of glucose uptake into adipocytes isolated from these animals is impaired, and there is also reduced uptake of glucose into skeletal muscle. The site of the insulin resistance appears to be distal to the insulin receptor in the signalling process and Reaven *et al.* suggest that this defect lies at the level of glucose transporter function. However it is important to note that doubt has been expressed about the existence of insulin resistance in this strain. Some have interpreted the appearance of normoglycaemia and hyperinsulinaemia to imply insulin resistance in SHRs, and have provided evidence for reduced maximal response to insulin. However, this is controversial as another study has demonstrated increased sensitivity and unchanged maximal responses to insulin, using hyperinsulinaemic euglycaemic clamp techniques. This conflict in the published literature awaits resolution.

It is of interest that skeletal muscle from SHRs exhibit similar levels of GLUT-4 to two control strains. A further point of note is that the study clearly demonstrates the GLUT-4 content of the intracellular pool is unchanged in the hypertensive strain. This resembles the situation in skeletal muscle from human studies on insulin resistance, and suggests that translocation of the transporter may be defective.

SHR/N-cp.

A relative of the SHR, the obese diabetic SHR/N-*cp*, exhibits hyperinsulinaemia and is also hyperglycaemic. It inherits obesity as an autosomal recessive trait and develops NIDDM along with characteristic diabetic complications. In the obese SHR/N-*cp*, GLUT-4 levels in the skeletal muscle are significantly reduced when compared to the lean SHRs. This change is likely to be significantly involved in the dramatic insulin resistance associated with these obese animals. At present little information is available regarding the relative levels of hypertension between the two strains, but it is interesting to note that the obese SHR/N-*cp* model exhibits marked hyperinsulinaemia and is diabetic, as evident by 20-fold higher serum glucose levels and 2-fold higher insulin levels than the lean SHR/N-*cp* control animals. This observation is strikingly similar to results noted with the obese diabetic ZDF/*drf-fa* rat, which exhibited decreased levels of GLUT-4 in muscle coincident with hyperglycaemia.

Dahl rat

The Dahl rat is another model of genetic hypertension which has been found to be insulin-resistant and hyperinsulinaemic compared with its control strain; the Sprague-Drawley rat. Two strains of the Dahl rat have been studied, the Dahl-S rat which becomes hypertensive when fed on a high salt diet, and the Dahl-R rat which does not develop hypertension but still exhibits insulin resistance. Isolated adipocytes from both these strains show impaired glucose uptake in response to insulin; therefore the insulin resistance is not dependent on the animal being hypertensive, nor is it a direct function of its salt intake. As yet, glucose transporter isoforms have not been quantified in insulin-sensitive tissues from this animal model. It will also be of interest to

assess changes in skeletal muscle GLUT-4 in the context of the glycaemic and insulinaemic status of these animals.

Milan rat

The Milan hypertensive strain is an inbred strain of rat which spontaneously develops hypertension, unlike its genetic control, the Milan normotensive strain, which exhibits normal blood pressure. As with the other animal models discussed above, development of hypertension in these animals is associated with hyperinsulinaemia and insulin resistance, but these two strains exhibit identical fasting glucose levels. In the course of this project I have shown that the male Milan hypertensive strain has reduced levels of GLUT-4 in skeletal muscle compared with the normotensive strain, and that this decreased expression is primarily associated with a decrease in the levels of GLUT-4 in the insulin-sensitive intracellular pool. Interestingly no change was observed in the level of expression of GLUT-4 in the adipocyte membranes from these animals.

Stroke-prone Spontaneously hypertensive rat (SHRSP)

In chapter 4 of this thesis we undertook a novel study examining the levels of the facilitative glucose transporters in the SHRSP rat strain, looking at the SHRSP F2 progeny. The SHRSP originally come from Okamoto's group in Japan around 1973. The F2 generation is a classical reciprocal cross with cross 1 = WKY male x SHRSP female and cross 2= WKY female x SHRSP male. The reason for this cross was to investigate the importance of the Y chromosome inheritance and to segregate the WKY and SHRSP genome. Throughout the study the rats have unlimited access to rat chow and water.

They are kept in a regime of 12hr light 12hr darkness. light 7am to 7pm, night 7pm to 7am.

The blood pressures are variable in the parentals with the max systolic being 220-230 for SHRSP and 140-150 for WKY.(Pressures are in mm mercury). The pressures were all measured by radiotelemetry ie a probe was inserted into the abbominal aorta. The probe was linked to a battery
which was sutured to the abdominal wall. Telemetry was used to reduce the stress on the animals during blood pressure (bp) measurements. The F2 progeny display blood pressures which range from normotensive to extremely hypertensive. Using this data we wished to examine the correlation between blood pressure and facilitative glucose transporter levels.

1.7.3 The use of hypertensive, insulin resistant rodent model systems

Insulin resistance is a heterogeneous condition with different characteristics in different pathophysiological states. Thus, the insulin resistance associated with essential hypertension is subtly different from that observed in obesity or type II diabetes, as the former is confined mainly to peripheral tissues (muscle and adipose tissue) and not the liver, and also seems to be limited to non-oxidative pathways of intracellular glucose utilisation [Livingstone & Gould(1995)].

The development of insulin resistance in both type I and type II diabetes has been extensively studied in both human and rodent models of the disease. It is now well established that defects in the glucose transport systems present in either peripheral tissues or the pancreatic b-cells are potentially important

in both the onset and development of the disease [James & Piper (1994); Kahn (1992)].

Glucose transport across the plasma membrane of animal cells is mediated by a family of glucose transporter proteins of the facilitative diffusion type. Each transporter is the product of a distinct gene and is expressed in a highly tissue specific fashion [see section 1]. Within the context of diabetes, the most important transporter would appear to be GLUT-4. It is clear from a variety of studies that defects in GLUT-4 expression, translocation or targeting (or a combination of all three) may underlie the inability of insulin to stimulate effectively glucose transport in adipocytes and muscle in a range of diseases [Livingstone *et al.*, (1995). Thus, understanding the regulation of GLUT-4 expression and function represent questions of considerable importance, not only in the field of diabetes, but also within the context of hypertension, and other pathophysiological states associated with insulin resistance. This is one of the central themes of this thesis.

However, the contribution of other GLUT isoforms to dysregulated glucose homeostasis should not be overlooked. GLUT-2 (liver and pancreatic β-cells) and GLUTs 1 and 3 (in brain/nerve) may also be defective in certain clinical conditions, and their expression has been poorly studied in hypertensive model systems to date.

1.8 Aims of the study.

The main aim of the study is to examine the sub-cellular distribution of the insulin-stimulated facilitative glucose transporter, GLUT-4, in the mildly hypertensive, insulin resistant Milan rat strain. The focus will therefore be placed upon the insulin-sensitive tissues, fat and muscle. The reasoning behind this is to determine if there are any defects either in expression or localisation, or both, that may offer an explanation of insulin resistance in this strain.

Furthermore, GLUTs 1, 2 and 3 will be studied to build up a profile of the facilitative glucose transporter distribution within the brain (GLUTs 1 and 3) and hepatic tissue (GLUT-2) of the Milan rat.

As a follow up to the initial aims I also examined the GLUT-4 content of the tissues named above in the progeny of an F2 generation of SHRSP strain. The purpose of this was to determine if this insulin resistant, hypertensive strain also possessed a defect in the amount of GLUT-4 located in the intracellular membrane pool. The SHRSP F2 progeny are animals with a wide range of blood pressures, therefore I also wanted to examine whether there was a correlation between the degree of hypertension from normotensive to extremely hypertensive, and the level of GLUT-4 present within each of the membrane fractions.

GLUTs 1, 2 and 3 were also investigated in the SHRSP F2 progeny, GLUT-2 in liver and GLUTs 1 and 3 neuronal tissue. This gives detailed information on the amount of facilitative glucose transporters present in the SHRSP rat strain.

Additionally I examined the amount of PI-3' kinase present in the soluble fraction of SHRSP muscle. As stated previously PI-3' kinase is a key molecule in the signalling pathway of the insulin receptor and any defect, or reduction in expression, may play an important role in insulin resistance.

My final study looked at models of insulin resistance and the role of sex steroids. It has been acknowledged for some time that women on hormone replacement therapy or those on the contraceptive pill are more susceptible to insulin resistance. Therefore, I examined what effect treating 3T3-Ll adipocytes with oestrogen and its derivatives would have upon insulin-stimulated glucose transport. If any defects were noticeable I would then examine the GLUT-4 content of cells treated with oestrogen or any of the derivatives.. This system was chosen in the hope of developing an *in vitro* model of insulin resistance.

Chapter 2

Materials and Methods

2.1 Materials

All reagents used in the course of the project were of a high quality and were obtained from the following suppliers:

2.1.1 General Reagents

Bio-Rad Laboratories Ltd, Hemel Hempstead, Hertfordshire, UK

cellophane membrane backing

N, N, N', N'-tetramethylethylenediamine (TEMED)

pre-stained SDS-PAGE Standards (myosin, β-galactosidase, BSA and ovalbumin)

Diversified Biotech, Boston, MA, U.S.A

Quantigold

Fisons, Loughborough, Leicestershire, UK

acrylamide

ammonium persulphate

Folin and Ciocalteau's phenol reagent

glucose

glycerol

glycine

Hepes

hydrochloric acid

methanol

N, N' methylene-bis-acrylamide

potassium chloride

sodium dodecyl sulphate (SDS)

sodium chloride

sodium dihydrogen orthophosphate dihydrate

sodium diaminoethanetetra-acetic acid (EDTA)

sodium hydrogen carbonate

trichloroacetic acid

Gibco BRL, Paisley, Lanarkshire, UK

Tris base

Kodak Ltd, Hemel Hempstead, Hertfordshire, UK

RP X-Omat liquid fixer/replenisher

RP X-Omat liquid developer/replenisher

X-Omat AR film

X-Omat S film

Merck Ltd (BDH), Lutterworth, Leicestershire, UK

calcium chloride hexahydrate

calcium nitrate tetrahydrate

dimethyl sulphoxide

magnesium chloride hexahydrate

magnesium sulphate heptahydrate

Packard Instrument B.V. -Chemical Operations, Groningen, The Netherlands

Ultima-Flo scintillation fluid

Premier Brands UK, Knighton Adbaston, Staffordshire, UK

Marvel powdered milk

Schleicher & Schuell, Dassel, Germany

nitrocellulose membrane (0.45μM)

Sigma Chemical Company Ltd, Poole, Dorset, UK

aprotinin

BSA (A-7030)

bromophenol blue

cytochalasin B

2-deoxy-D-glucose

diisopropyl fluorophosphate (DFP)

DL-dithiothreitol

E64

β-estradiol

estriol

estrone

insulin (porcine monocomponent)

Pepstatin A

sodium deoxycholate

trichloroacetic acid

Triton X-100

Whatman International Ltd, Maidstone, UK

Whatman 3mm filter paper

2.1.2 Antibodies

Amersham International Plc, Aylesbury, Buckinghamshire, UK

horseradish peroxidase (HRP)-conjugated donkey anti-rabbit IgG antibody

East Acres Biologicals, Southbridge, Massachusetts, USA

rabbit anti-human GLUT-1 antibody

2.1.3 Cells

American Type Culture Collection, Rockville, USA

3T3-L1 fibroblasts

2.1.4 Cell culture media and reagents

Gibco BRL, Paisley, Lanarkshire, UK

Dulbecco's modified Eagle's medium (without sodium pyruvate, with 4500mg/L glucose) (DMEM)

10000U/ml penicillin, 10000U/ml streptomycin

trypsin

Flow Laboratories, Irvine, Ayrshire, UK

new born calf serum (NCS)

foetal calf serum (FCS)

2.1.5 Cell Culture Plastics

AS Nunc, DK Roskilde, Denmark

50 ml centrifuge tubes

6 cm^2 cell culture plates

10 cm^2 cell culture plates

80cm^2 cell culture flasks

6-well cell culture plates

Bibby Sterilin Ltd, Stone, Staffordshire, UK

sterile pipettes

13.5 ml centrifuge tubes

2.1.6 Radioactive materials

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

2-deoxy-D-[2,6-^{3}H] glucose

^{125}I-conjugated goat anti-rabbit IgG antibody

2.2 Buffers and media

2.2.1 Cell culture media

Serum-free DMEM

100U/ml penicillin, 100U/ml streptomycin in DMEM

10% NCS/DMEM

100U/ml penicillin, 100U/ml streptomycin, 10%(v/v) NCS in DMEM

10% FCS/DMEM

100U/ml penicillin, 100U/ml streptomycin, 10%(v/v) FCS in DMEM

Sterile trypsin solution for cell passage

25%(w/v) trypsin in PBS (see General Buffers) was syringe filtered through a sterile 2μM membrane and stored in 10ml aliquots in 50 ml sterile centrifuge tubes at -20°C.

2.2.2 General buffers

Phosphate buffered saline (PBS)

150mM sodium chloride, 10mM sodium dihydrogen orthophosphate dihydrate, (pH 7.4).

KRP buffer

64mM NaCl, 2.5mM KCl, 2.5mM $NaH_2PO_4.2H_2O$, 0.6mM $MgSO_4$.& H_2O, 0.6mM $CaCl_2$, (pH 7.4).

Protein concentration assay: Reagent A

0.025% (w/v) copper sulphate pentahydrate/ 0.05% (w/v) sodium potassium tartrate/ 0.25% (w/v) 2.5% (w/v) SDS/ 0.2M sodium hydroxide.

Protein concentration assay: Reagent B

A 1:5 dilution of Folin and Ciocalteau's phenol reagent in water.

2.2.3 SDS-PAGE Buffers

Electrode buffer

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

Sample buffer

93mM Tris hydrochloride pH6.8, 20mM dithiothreitol, 1mM sodium EDTA, 10% (w/v) glycerol, 2% (w/v) SDS, 0.002% (w/v) bromophenol blue.

The dithiothreitol was added immediately before use.

2.2.4 Protease inhibitor stocks

Pepstatin A

1mg/ml in DMSO.

E 64

10mM in 2mM sodium EDTA.

DFP

200mM in isopropanol.

All protease inhibitor stocks were kept at -20°C.

2.2.5 Western blot buffers

Blot buffer

25mM sodium dihydrogen orthophosphate dihydrate, (pH 6.5).

First wash buffer

150mM sodium chloride, 5mM sodium dihydrogen orthophosphate dihydrate, 1mM sodium EDTA, 0.1%(v/v) Triton X-100, (pH 7.4).

Second wash buffer

790mM sodium chloride, 5mM sodium dihydrogen orthophosphate dihydrate, 1mM sodium EDTA, 0.1% (v/v) Triton X-100, (pH 7.4).

Towbin buffer

25mM tris-base, 192mM glycine, 20% (v/v) methanol, (pH 8.3).

2.2.6 Muscle buffer

10mM $NaHCO_3$, 0.25M sucrose, 5mM NaN_3.

2.2.7 5'-nucleotidase assay buffer A

150mM KCl, 20mM Hepes, 2mM $MgCl_2$.

2.2.8 SH Buffer

0.25M sucrose, 5mM Hepes, (pH 7.4).

2.2.9 KRH buffer

0.1M NaCl, 5mM KCl, 1mM KH_2PO_4, 0.1mM $MgSO_4.7H_2O$, 25mM Hepes, 50mM glucose, 1mM $CaCl_2$, (pH 7.4).

2.3 3T3-L1 Fibroblast culture

2.3.1 Growth of 3T3-L1 Fibroblasts

3T3-L1 fibroblasts were grown on cell culture flasks and plates containing 10% (v/v) NCS/DMEM (section 2.2.1). The medium was replaced every 2 days, with the cells being kept in an incubator at 37°C in a humidified atmosphere containing 10% CO_2.

2.3.2 Trypsinisation of 3T3-L1 Fibroblasts

When the cells were 70-80% confluent they were removed from the flasks using trypsin. The medium was aspirated from each flask and 5ml of 0.25% (w/v) trypsin in PBS was added. The flask was placed in the incubator for 5 minutes, allowing the cells to float, before trypsinisation was stopped by the addition of the cells to a volume of 10% NCS/DMEM. The diluted cells were

subsequently seeded onto new cell culture dishes. On average the cells from one $10cm^2$ plate were seeded onto ten $10cm^2$ plates.

2.3.3 Preparation of 3T3-L1 Fibroblast Differentiation Medium

Differentiation medium containing 10% FCS (v/v), 0.5mM methyl isobutylxanthine, 0.25mM dexamethasone, and insulin (1μg/ml) was prepared as outlined below.

A 500X stock solution of dexamethasone was prepared by a 1:20 dilution of 2.5mM dexamethasone in ethanol with 10% FCS/DMEM prior to use. A 500X sterile stock solution of methyl isobutylxanthine (IBMX) was prepared by dissolving 55.6 mg IBMX in 1.0 ml of 0.35M KOH and passing the solution through a 0.22 micron filter. Insulin (1mg/ml)was prepared in 10mM HCl, and again filtered by passing through a 0.22 micron filter.

3T3-L1 fibroblast differentiation medium was prepared by diluting both the dexamethasone and IBMX solutions to a 1X concentration in 10% (v/v) FCS/DMEM and finally adding insulin to a concentration of 1μg/ml.

2.3.4 Differentiation Protocol for 3T3-L1 Fibroblasts

Cells seeded in 6-well plates were grown and maintained in 10% (v/v) NCS/DMEM until 48 hours post confluence. At this time the medium was aspirated and replaced with 2ml per well of differentiation medium which is described above in 2.3.3. After a further 2 days this medium was aspirated and replaced with 10% (v/v) FCS/DMEM containing 1μg/ml insulin. The cells were incubated in this medium for a further 2 days, then the media was aspirated and replaced with 10%(v/v) FCS/DMEM. Cells were fed every 2 days

thereafter in this medium. Cells were used between 8-13 days post differentiation, at which time insulin-stimulated 2-deoxy-D-glucose transport was maximal.

2.3.5 Storage of 3T3-L1 Fibroblasts in liquid nitrogen

Confluent cells were removed from a 75cm^2 flask as in Section 2.3.2 and resuspended in 5ml of 10% (v/v) NCS/DMEM. The suspension was centrifuged at 1000 x g at room temperature for 5 minutes; the supernatant was then removed by aspiration. 10% NCS/DMEM containing 10% (v/v) glycerol was equilibrated in 10% CO_2 for 1 hour, and the cell pellet was resuspended in 1ml of this medium. Aliquots of the suspension were put into cryotubes, packed in cotton wool and frozen overnight at -80°C. The tubes were then transferred to liquid N_2 for long term storage.

2.3.6 Resurrection of frozen cell stocks from liquid nitrogen

The cryotube containing the cells was removed from liquid nitrogen and placed in a 37°C water bath. The tube was then transferred to the cell culture sterile flow hood where the cells were titurated gently with a sterile Pasteur pipette to disperse any large aggregates of cells. The 3T3-L1 fibroblasts were seeded onto 10cm^2 cell culture plates containing 10% (v/v) NCS/DMEM medium. The cells were then maintained in an incubator at 37°C in an atmosphere of 10% CO_2.

2.3.7 Insulin-stimulated 2-deoxy-D-glucose uptake in 3T3-L1 adipocytes

Uptake of 2-deoxy-D-glucose was measured using the method of Gibbs *et al.* (1988) and Frost and Lane (1985).

In all experiments cells were used between 8 and 13 days post differentiation. For transport assays 3T3-L1 adipocytes grown on 6-well plates were used. Prior to assay, cells were incubated in serum-free Dulbecco's Modified Eagles Medium for 3 hours prior to use. The cell monolayer in each well was washed three times in 3ml of KRP buffer (see section 2.2.2) at 37°C and then incubated on a hot plate at 37°C in 950µl of KRP. Insulin was added at the required concentration for the time indicated in the figures and tables.

The uptake of the sugar was initiated by the addition of 50µl of [^{3}H] 2-deoxy-D-glucose to a final concentration of 100µM and 0.5µCi/ml. The reaction was terminated 3 minutes later by rapidly decanting the KRP buffer, (containing the 2-deoxy-D-glucose) and immediately immersing the 6-well plate sequentially into 3 beakers containing ice-cold PBS (see section 2.2.2 for PBS details). After air-drying the plates, 1ml of 1% Triton X-100 in water was added to each well and the plates were left shaking for 1 hour. The cell associated radioactivity was measured by scintillation counting. To determine the specific uptake of 2-deoxy-D-glucose, the inhibitor cytochalasin B, was added to three of the 6-wells in each plate containing the KRP and insulin, at a concentration of 10µM, at the beginning of each assay.

The radioactivity associated with the wells in the presence of cytochalasin B is taken as a measurement of non-specific deoxy-glucose association with the monolayer. This value was subtracted from counts obtained in the abscence

of cytochalasin B to provide a figure which was used to calculate the specific transport rate.

It has been calculated from experiments in this lab and others that one well of a 6-well plate contains 2.1 million cells and as a result the rate of 2-deoxy-D-glucose transport is expressed as pmol/min/million cells which is the standard unit used within the field of glucose transport.

2.3.8 Hormonal treatment of 3T3-L1 adipocytes

48 hours prior to the assaying the insulin-stimulated 2-deoxy-D-glucose uptake, the adipocytes were fed with 10% (v/v) FCS/DMEM containing the concentration of steroid hormone indicated in the figures and tables presented in subsequent chapters. All other procedures involved in the assay are exactly as described in section 2.3.7.

2.4 Protein Concentration Assays

2.4.1 Lowry method of protein concentration determination

The protein concentration of the membranes in the studies presented were assayed after trichloroacetic acid precipitation [Lowry *et al.*, (1951); Peterson, (1977)]. The volume of each protein sample was brought to 1ml using distilled water, and 100µl of 0.15% (w/v) sodium deoxycholate was added to each tube. The contents of each tube were mixed, and incubated at room temperature for 10 minutes. After addition of 100µl of 72% (w/v) trichloroacetic acid the contents of each tube were mixed again, then centrifuged at 3000 x g for 15 minutes at 4°C and the precipitates collected. The supernatants were discarded and the precitates air dried. The pellets were resuspended in 200µl

of distilled water and 1ml of Reagent A (Section 2.2.2) added, the contents of each tube mixed, then incubated at room temperature for 10 minutes. 0.5ml of Reagent B (Section 2.2.2) was added, the contents of each tube mixed and the absorbance (A_{750}) measured after a 30 minutes incubation at room temperature.

The concentration of the sample proteins were determined from a standard curve (0 to 200µg) constructed from 1mg/ml BSA and treated in the same manner as the protein samples of unknown concentration

2.4.2 Quantigold Protein Determination

This method was used to determine the protein concentrations of sub-fractionated muscle preparations from individual animals, where concentrations were very low and samples at a premium.

Using BSA as a standard, 10µl of sample was added to 1ml of Quantigold solution, mixed, and incubated at 37°C for up to one hour in a plastic cuvette. The absorbance was then read in a spectrophotometer at 595nm. The protein concentration was determined after plotting the standard curve of concentration of BSA against absorbance. This method of protein determination is accurate to 5ng of protein and is linear up to 200ng protein.

2.5 SDS/polyacrylamide gel electrophoresis

SDS/polyacrylamide gel electrophoresis was carried out using Bio-Rad mini-PROTEAN II or Hoefer large gel apparatus.

The Bio-Rad mini-PROTEAN II slab gels had a stacking gel of 2cm and the Hoefer stacking gel was 5cm. The stacking gel was composed of 5% acrylamide/0.136% bisacrylamide in 125mM Tris-HCl, (pH 6.8), 0.1% SDS, polymerised with 0.1% (w/v) ammonium persulphate and 0.05% N, N, N', N'-tetramethylethylenediamine (TEMED).

The separating gel consisted of 10% acrylamide/0.28% bisacrylamide in 0.383mM Tris-HCl, (pH 8.8), 0.1% SDS and polymerised with 0.1% (w/v) ammonium persulphate and 0.019% N, N, N', N'-tetramethylethylenediamine (TEMED).

The protein samples were solubilised in sample buffer (see section 2.2.3) and loaded onto the wells in the stacking gel. The gel was then immersed in electrode buffer (see section 2.2.3) and the gel electrophoresed until the tracking dye had migrated to the bottom of the gel. For Bio-Rad mini-PROTEAN gel a constant voltage of 150 volts for 90 minutes ensured adequate separation of the pre-stained SDS/PAGE markers. Large gels were electrophoresed overnight at 60-70 volts in a Hoefer gel tank.

2.6 Western blotting of proteins

After separation of the proteins by SDS/PAGE as described in section 2.5, the gels were removed from the plates and equilibrated in blot buffer (see section 2.2.5) at room temperature for 30 minutes. Each gel was then placed on top of a piece of nitrocellulose paper (0.45μm pore size) which had been cut to the size of the gel and pre-soaked in blot buffer. This was then "sandwiched" between 2 layers of 3mm filter paper which had also been pre-soaked with blot buffer. The sandwich was then placed in a cassette and transfer of the proteins onto the nitrocellulose was performed using either a Bio-Rad trans-

blot tank, for large gels, or the Bio-Rad mini trans-blot tank for the mini-gels.

Transfer was achieved at a constant current of 250mA for 3 hours at room temperature. The nitrocellulose membranes were then removed and the efficiency of transfer was determined by staining the nitrocellulose with Ponceau S solution prior to blocking.

2.6.1 Immunodetection of GLUT-1 on nitrocellulose membranes

To block non-specific binding sites on the nitrocellulose, the membrane was shaken in 3% (w/v)BSA/1st wash buffer (see section 2.2.5) for 1 hour. The nitrocellulose was then placed into 3% (w/v)BSA/1st wash buffer containing the GLUT-1 antibody at a concentration of 10μg/ml and incubated overnight at room temperature on an orbital shaking platform. Following this, the nitrocellulose was washed five times at 10 minute intervals with 1st wash buffer, and a further three times in 2nd wash buffer (see section 2.2.5) at the same interval. The membrane was then placed in 3% (w/v)BSA/1st wash buffer containing ^{125}I-labelled goat anti-rabbit IgG (1μCi/20ml) for 3 hours. After this the nitrocellulose was the washed as before and dried between two sheets of cellophane. Finally the nitrocellulose was exposed to Kodak X-Omat S film in an autoradiography cassette for up to 48 hours before being developed using an X-Omat processor.

2.6.2 Immunodetection of GLUT-2, 3 & 4 on nitrocellulose membranes

The non-specific binding sites on the nitrocellulose membrane were blocked by shaking for 1 hour in 5% (w/v) non-fat milk/1st wash buffer. The

membrane was then transferred into 1% (w/v) non-fat milk/1st wash buffer containing affinity-purified antibody at 10μg/ml or serum at a 1 : 200 dilution and incubated overnight at room temperature on an orbital shaking platform. The nitrocellulose was then washed five times in 1st wash buffer followed by three times in 2nd wash buffer with 10 minutes between each wash. After this the membrane was incubated in 1% non-fat milk/1st wash buffer containing either ^{125}I-labelled goat anti-rabbit IgG (1μCi/20ml) or HRP-linked goat anti-rabbit IgG (1 : 10000) for 3 hours or 1 hour respectively. If the ^{125}I-labelled IgG method was used then the blot was washed and developed as in section 2.6.1.

However if the second antibody used was HRP-linked goat anti-rabbit IgG, after washing as before the membrane was submerged in a solution containing equal volumes of Amersham "detection reagent 1" and "detection reagent 2" for 1 minute. The nitrocellulose was then removed from the solution, wrapped in cling-film and exposed to Kodak X-Omat S film and developed in an X-Omat processor.

2.7 Antibody Preparations

2.7.1 Purification of anti-peptide antibodies

A series of peptide columns have been prepared by others which were used to prepare affinity purified antibody stocks. The protocol for use is as follows. The column was pre-washed with 10ml of 25mM sodium phosphate, (pH 6.5). 5.5ml of anti-GLUT peptide rabbit serum, was mixed with 0.5ml of 250mM sodium phosphate (pH 6.5) and added to the column, allowed to run through it by gravity, and subsequently passed through the column 4 more times. The column was then washed with 25mM sodium phosphate (pH 6.5) until the

eluant gave an OD_{280nm} reading of less than 0.1. The column was then allowed to almost run dry before the bound IgG was eluted by the addition of 10ml of 0.1M glycine, (pH 2.4).

The eluant was collected in 1ml fractions and the OD_{280nm} readings were taken for each fraction. The fractions with the highest OD_{280nm} were pooled and 100µl of 0.5M Tris (pH 6.8) was added to neutralise the pool. The pooled sample was dialysed against PBS, (pH 7.4) overnight at 4°C. Finally the dialysed samples were then aliquoted into 1.5ml microfuge tubes, snap frozen and stored at -80°C until use.

2.8 Animal upkeep, feeding and blood pressure monitoring

2.8.1 Milan rat upkeep

Male Milan rats (body weight 250g) were purchased from the University of Sheffield Field Laboratory (Sheffield, United Kingdom). Animals were housed in the Joint Animal Facility, at the University of Glasgow, on a 12h day/night cycle with unrestricted access to water and standard laboratory chow. 24 hours after delivery of a group of animals, blood pressure measurements were made using tail-cuff plethysmography [Evans *et al.* (1993)]. This procedure was carried out on our behalf by the staff at the Joint Animal Facility. All other strains of rat mentioned in the study were part of a controlled breeding programme by the animal house at the Western Infirmary (Glasgow) and the animals were subject to the same conditions mentioned above. Aspects of these species will be discussed in subsequent chapters.

2.8.2 SHRSP F2 upkeep

The animals were housed in the animal facility at the Western Infirmary. All rats were housed in conditions of temperature (21°C) and a 12 hour day/night cycle (7 am to 7 pm)and maintained on normal rat chow and water *ad libitum*.

2.9 Hindlimb skeletal muscle dissection and sub-cellular fractionation

The method of Klip *et al.*, (1987) was used in the preparation and sub-cellular fractionation of hindlimb skeletal muscle membranes. The animals were sacrificed by cervical dislocation and the hindlimb muscle was dissected. All subsequent procedures were carried out on ice or at 4°C. The skeletal muscle was minced and diluted 1g/10ml in muscle buffer (see section 2.2.6) before being homogenised in a Polytron PT20 for 1 minute at setting 4-5. The homogenate was then centrifuged at 1,200 x g for 10 minutes. The pellet was re-homogenised exactly as above and the centrifugation repeated. The supernatants from both steps were pooled before being further centrifuged at 9000 x g for 10 minutes. The pellet was discarded and the supernatant was centrifuged at 190000 x g for 60 minutes. The pellet from this latest step contained the "crude membranes" which were subsequently hand homogenised for 20 strokes in 1ml of muscle buffer.

The crude membrane fraction was then applied to a discontinuous sucrose gradient of 25%, 30% and 35% (w/v) sucrose and centrifuged at 150,000 x g for 16 hours at 4°C. The 25% (w/v) sucrose fraction contained the purified plasma membranes and the 35% (w/v) sucrose fraction contained the intracellular membranes. These fractions were collected, diluted 5-fold in muscle buffer then further centrifuged at 190,000 x g for 60 minutes to wash

out the sucrose. The pellets from the plasma membrane and intracellular membrane fractions were then resuspended and homogenised in 100µl of muscle buffer before being snap-frozen in liquid nitrogen and stored at -80°C until use.

2.10 Dissection and "crude membrane" preparation of liver tissue

All the procedures were performed on ice or at 4°C and in accordance with the method of Arion *et al.*, (1984). The liver was dissected from the animal which had been sacrificed by cervical dislocation. The tissue was weighed and placed in a beaker with SH buffer (see section 2.2.8) at 10 ml per gram of tissue. Next the liver was homogenised with 20 strokes in a motorised 30ml teflon homogeniser, clearance 0.4mm. The homogenate was centrifuged at 10000 x g for 15 minutes at 4°C. The pellet from this centrifugation was resuspended in SH buffer. The pellet was then homogenised, snap-frozen in liquid nitrogen and stored at -80°C until use. This fraction contains approximately 80% of the liver 5' nucleotidase activity and was therefore used as an crude membrane fraction in subsequent studies.

2.11 Dissection and sub-cellular fractionation of adipocyte tissue

The epididymal fat pads were removed from male rats which had been sacrificed by cervical dislocation. All subsequent steps in the procedure follow an amended protocol from Simpson *et al.* (1991). The tissue was rinsed in KRH buffer, pH7.4, containing 1%(w/v) BSA at 37°C before being minced through a plastic tea strainer into a 100ml siliconised flask containing 10 ml KRH/BSA, 20mg collagenase and 5mg trypsin inhibitor. The flask was

incubated at 37°C with continuous gassing with nitrogen and gentle agitation for 1 hour. The contents of the flask were again filtered through a tea strainer and washed in KRH buffer pH 7.4, containing 1%(w/v) BSA at 37°C. The solution was centrifuged at 10000 x g for 1 minute to compact the adipocytes which float, and all the buffer below the cells was removed using a 20ml syringe and needle. This procedure was repeated twice more to obtain a pure cell suspension. The cells were resuspended in KRH buffer, (pH 7.4), containing 3%(w/v) BSA before being homogenised by 20 up and down strokes of a hand-held homogeniser. The homogenate was centrifuged in an SS34 rotor for 15 minutes at 16000 x g at 4°C and the pellets were resuspended in 1ml of KRH buffer (pH 7.4). This pellet represents a crude plasma membrane preparation which was homogenised, snap-frozen in liquid nitrogen and stored at -80°C until use.

The supernatant from the first spin was further centrifuged at 41000 x g at 4°C for 15 minutes. The pellet from this spin contained the "Heavy Microsomes" which were resuspended in 1ml of KRH buffer, (pH 7.4), homogenised, snap-frozen in liquid nitrogen and stored at -80°C until use. The supernatant from the previous spin was transferred to a clean tube and centrifuged at 100000 x g for 75 minutes at 4°C. After this, the pellets were resuspended in 2ml KRH buffer, (pH 7.4), homogenised and re-centrifuged at 100000xg for another 75 minutes at 4°C. The pellet from this step contained the "Light Microsomes" which were resuspended in 1ml KRH buffer, homogenised, snap-frozen in liquid nitrogen and stored at -80°C until use.

2.12 Brain region dissection and membrane preparation

Brain regions were individually dissected as described in Brant *et al.* (1993). The regions from four rats were combined and homogenised in ice-cold 5 mM

sodium phosphate, (pH 7.4), 150 mM NaCl as described Brant *et al.* (1993). A membrane pellet was obtained by centrifugation at 100000 x g for 1 hour. The membranes were washed and aliquoted before being snap frozen and stored at -80°C prior to use.

2.13 5'-Nucleotidase assay

5' Nucleotidase is a marker enzyme for plasma membranes and was used to determine the distribution of the plasma membrane fraction in the procedure above. An aliquot of the membrane fraction was added to buffer A to a final volume of 1ml in the presence of 0.3% (v/v) Triton-X-100, 5.0mM 2'3'-AMP, 0.2mM 5'-AMP and 2.0mM (2μCi) [^{3}H] 5'-AMP. The solution was mixed and incubated at 37°C. Duplicate 100μl aliquots were removed at 0, 10, 20, 30, 60 and 90 minutes after addition of membranes. Immediately after removal, 50μl 0.25M zinc sulphate was added and the tube vortexed. 100μl 0.125M barium chloride was then added and the sample was again vortexed. The samples were incubated on ice for 10 minutes then centrifuged in a microfuge at 14500 x g for 5 minutes. Duplicate 100μl aliquots of the supernatant were removed and counted.

The supernatant contains the product of the 5' nucleotidase and the slope of product/time used to calculate rates as mole product/hour/mg protein in duplicate assays.

2.14 Statistical analysis

Results presented in this thesis were subject to statistical analysis using the StatView Package on a Macintosh computer. Unless stated the student's T-test in the model used.

Chapter 3

Analysis of the glucose transporter complement of metabolically important tissues from the Milan hypertensive rat.

3.1 Aims

The aims of this chapter are:

1. To examine the glucose transporter complement of muscle and fat of the mildly hypertensive Milan rat compared to the isogenic normotensive control strain.

2. To examine changes in the expression of GLUT-2 in liver of the Milan hypertensive rat compared to the normotensive control, and to also examine the activity of glucokinase and glucose-6-phosphatase in this tissue.

3. To study the glucose transporter distribution in selected brain regions of these animals.

3.2 Introduction

The inability of peripheral tissues to respond to insulin has been identified in a number of clinical conditions, many of which are associated with an increase in blood pressure, these include ageing, obesity and non-insulin dependent diabetes mellitus (Type II diabetes) [Ferrani *et al.* (1987); Natali *et al.* (1991)]. Insulin resistance has also been reported in several model systems for hypertension, including the spontaneously hypertensive rat (SHR) and Milan rat. The insulin resistance associated with essential hypertension may be subtly different from that observed in obesity or type II diabetes in that it is confined mainly to peripheral tissues, and is limited to non-oxidative pathways of intracellular glucose utilisation [Ferrani *et al.* 1987; Ferrani & Weidmann (1990); Natali *et al.* (1991)].

Dall'Aglio *et al.* (1991) first demonstrated the abnormalities in insulin and lipid metabolism in the normotensive and hypertensive Milan rat strains which were generated by Professor G. Bianchi in his laboratory in Milan. These two rat strains are genetically related and have been developed from the same Wistar stock colony with some cross-mating between the rats of these two strains during the first two generations. [Bianchi *et al.* (1984). In the study by Dall'Aglio *et al.* (1991) the rats were sacrificed by decapitation and blood was collected for analysis. The blood was subsequently assayed for glucose, insulin, triglyceride and total cholesterol. The Milan hypertensive rat strain was shown to have elevated blood pressure, with a mean blood pressure of 118mm Hg, compared to 95mm Hg for the normotensive strain. Furthermore the hypertensive rat is hyperinsulinaemic and hypertriglyceridaemic. However both the normotensive and hypertensive strains had the same circulating glucose concentration. Dall'Aglio proposed that the association of hypertension, hyperinsulinaemia and

hypertriglyceridaemia demonstrate a defect in the ability of insulin to stimulate glucose uptake. The compensatory hyperinsulinaemia results in the increase of both blood pressure and plasma triglyceride concentration. This theory is substantiated by Reaven *et al.* (1991) in a study examining insulin-stimulated glucose transport in adipocytes from SHR and WKY rats. The adipocytes from the SHR rat were shown to be insulin resistant compared to those from the normotensive WKY rat strain. Furthermore, the greater the defect in insulin action, the higher the plasma insulin concentration, the plasma triglyceride concentration, and the blood pressure.

The development of insulin resistance in both type I and type II diabetes has been extensively studied in both human and rodent models of the disease. It has been well established that defects in the glucose transport systems present in either peripheral tissues or the pancreatic β-cells are potentially important in both the onset and development of the disease [Gould & Bell (1990); Bell *et al.*, (1990)]. Glucose transport across the plasma membranes of animal cells is mediated by a family of glucose transporter proteins of the facilitative diffusion type. Within the context of diabetes, the most important transporter would appear to be the so-called insulin-responsive glucose transporter, GLUT-4. This transporter is expressed in adipose tissue, muscle and heart; tissues which exhibit an acute increase in their rates of glucose transport within minutes of exposure to insulin. This increase is mediated by the movement of a pool of GLUT-4 from an intracellular location to the plasma membrane; a process termed translocation. Defects in the level of expression, function and/or targeting of GLUT-4 have been reported associated with insulin resistant states [Gould & Holman (1993); Shepherd & Kahn (1993); James & Piper (1994); Garvey *et al.* (1989); Kahn & Flier (1990); Pedersen *et al.* (1990)]. Hence, it will be of interest to examine changes in the pattern of expression of this and other transporter isoforms in models of hypertension.

Here, a panel of immunological reagents have been used to determine the relative levels of the glucose transporter isoforms expressed in adipose tissue, muscle and liver, together with several brain regions in the hypertensive Milan rat compared to its control normotensive relative. This study has shown that there are profound decreases in the level of GLUT-4 in the intracellular membranes of skeletal muscle from hypertensive animals. This may play a key role in the observed insulin resistance in this species. In addition, several potentially important changes have been observed in brain regions from the hypertensive animals. These results suggest that defects in glucose transporter expression may participate in the observed insulin resistant states associated with hypertension.

3.3 Methods

3.3.1 Animals

Male Milan rats (body weight ~250g) were purchased from the University of Sheffield Field Laboratory (Sheffield, United Kingdom). Animals were housed on a 12h day/night cycle with unrestricted access to water and standard laboratory chow. 24 hours post delivery of a group of animals, blood pressure measurements were made using tail-cuff plethysmography [Evans *et al.* (1993)]; the characteristics of the animals used in this study are outlined in Table 3.1. Blood pressure measurements were made on each animal while conscious; five recordings were obtained from each animal and the average value calculated. The mean blood pressure of the animals within each of four groups analysed are presented (Table 3.1). Animals were sacrificed the following morning, and individual tissues immediately dissected and maintained on ice. In all cases, homogenisation and subcellular fractionation of the individual tissues according to the protocols outlined in Chapter 2 was undertaken within 1 hour of sacrifice.

3.3.2 Glucose-6-phosphatase assay reagents

Glucose-6-phosphate (monosodium salt), potassium pyrophosphate, mannose-6-phosphate (disodium salt) and Histone 2A were all obtained from Sigma Chemical Co., Poole, UK. The following solutions were prepared:

Substrate stock solutions:

Glucose-6-phosphate (monosodium salt), 0.3 M stock

Mannose-6-phosphate (monosodium salt), 12.5 mM stock

Both of the above solutions were adjusted to pH 6.5

Histone, 10 mg/ml.

0.1 M HEPES, 4.776g in 200 ml H_2O, pH 6.5.

0.1 M EDTA, 3.72g in 100 ml H_2O, pH 6.5.

Stopping reagent. (Made fresh daily.)

6 parts 0.42% Ammonium molybdate/0.5M H_2SO_4.

2 parts 10% SDS.

1 part dH_2O

The assay measured phosphate produced, with determination by 4 micromodifications of the Ames method (Ames 1966) where the reaction of inorganic phosphate with ammonium molybdate and sulphuric acid produces a phosphomolybdate complex. The absorbance of this at 820 nm is proportional to the amount of inorganic phosphate produced. The absorbance is related to the amount of phosphate released using a standard curve constructed using stock inorganic phosphate. One unit of activity represents 1 mmol of phosphate released/min.

3.3.3 Glucose-6-Phosphatase assay

Glucose-6-phosphatase activity was measured in a 100µl final reaction volume in disposable plastic tubes, in order to eliminate the possibility of trace phosphate contamination from glass tubes washed in detergents. Final

concentrations were 20 mM HEPES, 2.5 mM EDTA and final glucose-6-phosphate concentrations of 1, 1.4, 2, 2.6, 5 and 30 mM for kinetic analysis.

The reaction was initiated by addition of 20µl of appropriately diluted microsomes in Sucrose-HEPES buffer to 80µl of substrate at 30°C. The assay was performed in duplicate. Disruption of microsomal vesicles was carried out by inclusion of 10 mg/ml Histone 2A in the assay (Blair and Burchell, 1988). Substrate without the inclusion of any microsomes was termed 'blank' and was a measure of any free, non-specific inorganic phosphate which may naturally be present in the assay. After a 10 minute period the reaction was stopped by addition of 900µl stopping reagent. 20µl of the diluted microsomes was then added to the 'blank' tube. The assay was then placed in a 42°C water bath for 20 minutes to allow full colour development.

The assay was measured at 820 nm on a Phillips PU8670 spectrophotometer.

3.3.4 Pyrophosphatase assay

Pyrophosphatase activity was measured as described above using HEPES adjusted to pH 6.0 and potassium pyrophosphate as the substrate. The final concentration of pyrophosphate used was 0.5, 1, 1.4, 2, 2.6 and 5 mM. Colour was allowed to develop for 10 minutes at 30°C prior to measurement of the absorbance at 820 nm.

3.3.5 Mannose-6-phosphatase assay

One unique feature of glucose-6-phosphatase is that it is situated with its active site inside the lumen of the endoplasmic reticulum, whereas all the other enzymes involved in gluconeogenesis and glycogenolysis are

elsewhere in the cell. This means that for glucose-6-phosphatase activity *in vivo*, in addition to the glucose-6-phosphatase enzyme itself, it is also necessary to allow the substrates and products of the enzyme to cross the endoplasmic reticulum membrane.

Untreated microsomes are heterogeneous preparations composed of (a) intact vesicles (intact microsomes) in which the membrane acts as a selective permeability barrier, and (b) disrupted structures in which selective permeability is lacking and the enzyme has free access to ionic substrates and inhibitors.

When glucose-6-phosphatase activity is assayed in microsomal samples in the test-tube, the enzyme appears more active in disrupted microsomal vesicles than in intact microsomes as the active site has unrestricted access to the substrate. The enzyme activity is therefore described as latent. Latency may be expressed as the percentage of the enzyme activity of fully disrupted microsomes that is not expressed in untreated microsomes. In other words, latency is a measure of the intactness of the microsomes.

The proportion of the two forms (intact and disrupted) may be quantified by assays of the low K_m mannose-6-phosphatase activity that is expressed only in disrupted structures (Arion *et al*, 1976). Therefore the latency of the enzyme in the disrupted component of untreated microsomes can be calculated using the following equation:

$$V_{IM} = V_{UM} - [(V_{DM}.M_{UM}/M_{DM}) / (1-M_{UM}/M_{DM})]$$

Where IM, UM and DM added to the reaction velocity (V) or mannose-6-phosphatase activity (M) represent the values for intact, untreated and disrupted microsomes respectively.

3.3.6 Glucokinase and Hexokinase assays

Hexokinase and glucokinase were determined by the method of Agius and Tosh (1990). Hexokinase and glucokinase were determined in the cytosolic fractions of rat liver samples. The following reagents were prepared:

Medium	100 mM KCl	1.0 ml
	50 mM HEPES	0.5 ml
	7.5 mM $MgCl_2$	0.75 ml
	1 mM EDTA	0.1 ml
	5 mM (DTT)	0.50 ml
	Dithiothreitol	

7.15 ml of H_2O was added and the pH of the solution adjusted to 7.4.

Main Reagent	100 mM KCl	2 ml
	50 mM HEPES	1 ml
	100 mM EDTA	0.2 ml
	7.5 mM $MgCl_2$	1.5 ml
	0.5 mM NAD*	1.0 ml
	5 mM DTT	1.0 ml
	G6PDH (1 U/ml)	20 µl
		6.7 ml

*Nicotinamide adenine dinucleotide.

The preparation of the main reagent was based on the method of Davidson and Arion (1987). The volume was made up to 20 ml.

Starting reagent: 2.5 mM adenosine triphosphate (ATP).

Main reagent --- 10 ml + 0.5 mM glucose

Main reagent --- 10 ml + 100 mM glucose

The supernatant fraction was diluted (1:3) in the medium as detailed above. The reactions were then started by the addition of ATP. Blanks without cell extract were run for each cocktail (low and high glucose, with or without ATP). Sample blanks contained cell extracts and complete cocktail except for ATP. Sample blanks (without ATP) were subtracted for both high and low glucose concentrations. Glucokinase activity was determined from the difference in corrected rates between 100 mM and 0.5 mM glucose.

The assays were performed at 30°C using a Cobas Fara analyser (Department of Clinical Biochemistry, University of Dundee Ninewells Hospital and Medical School) and rates were determined by a linear search and regression using a PC directly coupled to the analyser for this purpose.

3.4 Results

3.4.1 Subcellular fractionation of skeletal muscle

We wished to examine the relative levels of GLUT-4 in skeletal muscle from Milan rats. A subcellular fractionation procedure employing sucrose gradient centrifugation has been published which described a method for the preparation of plasma membranes and intracellular membranes enriched for insulin-responsive glucose transporters [Klip *et al.*, (1987)]. Our first objective was to establish that this procedure was appropriate and reproducible for the Milan rat strain to be employed. To establish this, 5' nucleotidase activity was assayed as it is a well established plasma membrane marker. The fractions obtained from the sucrose gradient were analysed and confirmed the results of [Klip *et al.*, (1987)], in that the 25% sucrose fraction (proposed to be enriched in plasma membranes) was greatly enriched in 5' nucleotidase activity, and the 35% fraction (which contains the insulin-sensitive glucose transporter pool) exhibited no enrichment of activity (Table 3.2). At the time of this work, there were no suitable markers for the intracellular membrane fraction; however, as shown below, the intracellular fraction does contain the majority of the GLUT-4, suggesting that the fractionation procedure is appropriate.

Two further sets of control experiments were performed. We assayed the protein content of the fractions from the sucrose gradients prepared from normotensive and hypertensive animals. As shown in Table 3.2, no significant changes in protein content were observed when comparing the two strains. Similarly, these two strains showed identical subcellular distribution of 5' nucleotidase activity, further suggesting that the

Table 3.1

Characteristics of animals.

	Body weight (g)			
Group No:	1	2	3	4
Normotensive	243±9	255±12	n.d.	238±10
Hypertensive	251±9	245±7	n.d	246±8
	Blood pressure (mmHg)			
Normotensive	133±7	n.d.	137±5	138±4
Hypertensive	164±4*	n.d.	168±3*	163±9*

All experiments were performed on four separate groups of littermates, purchased over a twelve month period. Within each group, 4 normo- or 4 hypertensive Milan rats from the same litter were analysed coincidentally. Blood pressure measurements were performed on each rat using plethysmography as described in section 2.8, and an average value of 5 determinations for each rat was obtained. Values above are mean ± s.d. (n=4) of the average values for each animal within each group (1 - 4). *Indicates a statistically significant difference between normo- and hypertensive animals within the given group ($p<0.05$). n.d.=not determined.

Table 3.2

Subcellular fractionation of skeletal muscle from normo- and hypertensive Milan rats.

	Protein (μg)		5' Nucleotidase activity (nmol/mg/h)	
	MNS	MHS	MNS	MHS
Fraction				
Total membranes	n.d.	n.d.	35 ± 6	38 ± 8
25%	612 ± 20	523 ± 25	221.5 ± 23	238.5 ± 41
30%	1013 ± 55	1003 ± 135	105.3 ± 14	107.4 ± 12
35%	1587 ± 255	1700 ± 215	30.3 ± 4.5	20.5 ± 6.7

Shown above are data from a representative group of animals, purchased at the same time. Each value is the mean ± s.d. of at least triplicate determinations. MHS = Milan hypertensive strain and MNS = Milan normotensive strain.

subcellular fractionation procedure produced consistent fractions when applied to the two animal strains used.

We therefore measured GLUT-4 levels in the plasma membrane fraction (25% sucrose) and intracellular membrane fractions (35% sucrose). No significant changes were observed in the plasma membrane GLUT-4 content of normo- and hypertensive animals (Fig. 3.1); note that the small increase in plasma membrane GLUT-4 content is not significant, as determined using Students t-test. In contrast, a significant reduction in the level of GLUT-4 in the 35% sucrose fraction (corresponding to the intracellular, insulin-responsive GLUT-4 pool) was consistently observed in the hypertensive group [53 ± 4%, $n=4$, $p<0.01$] (see Fig. 3.1 panels A & B). These results are unlikely to be an artefact of different subcellular fraction profiles from the normotensive and hypertensive animals, as (i) the protein recoveries were similar in each fraction from both normo- and hypertensive animals (Table 3.2), (ii) SDS-PAGE analysis indicated no gross change in polypeptide composition of the intracellular membrane fraction between normo- and hypertensive preparations (data not shown), and (iii) the relative recoveries and enrichments of the plasma membrane marker enzyme 5' nucleotidase were unchanged between the two groups (see Table 3.2). The reduction in GLUT-4 levels was observed in four independent groups of animals, and the data from the four sets is presented graphically in Fig. 3.1 panel B. Note that the levels of GLUT-4 observed in the plasma membrane represent about 5% of the total levels of GLUT-4 in muscle membranes. Thus, the statistically insignificant increase in GLUT-4 levels in the plasma membrane fraction cannot account for the large decrease in the intracellular membrane fraction observed in the hypertensive animals.

Figure 3.1a

Skeletal muscle GLUT-4 levels in Milan normo- and hypertensive rats.

Shown is a representative immunoblot of sucrose gradient fractions of skeletal muscle membranes. Fractions from the 25%, 30% and 35% w/v sucrose fractions were analysed from groups of normo- (N) or hypertensive (H) Milan rats. 25μg of membrane protein were electrophoresed on 10% gels, prior to transfer to nitrocellulose membranes as described in section 2.6. Immunoblots were developed with anti-GLUT-4 antibodies exactly as described [Brant *et al.* (1993)], the position of the 50kDa molecular weight marker is indicated.

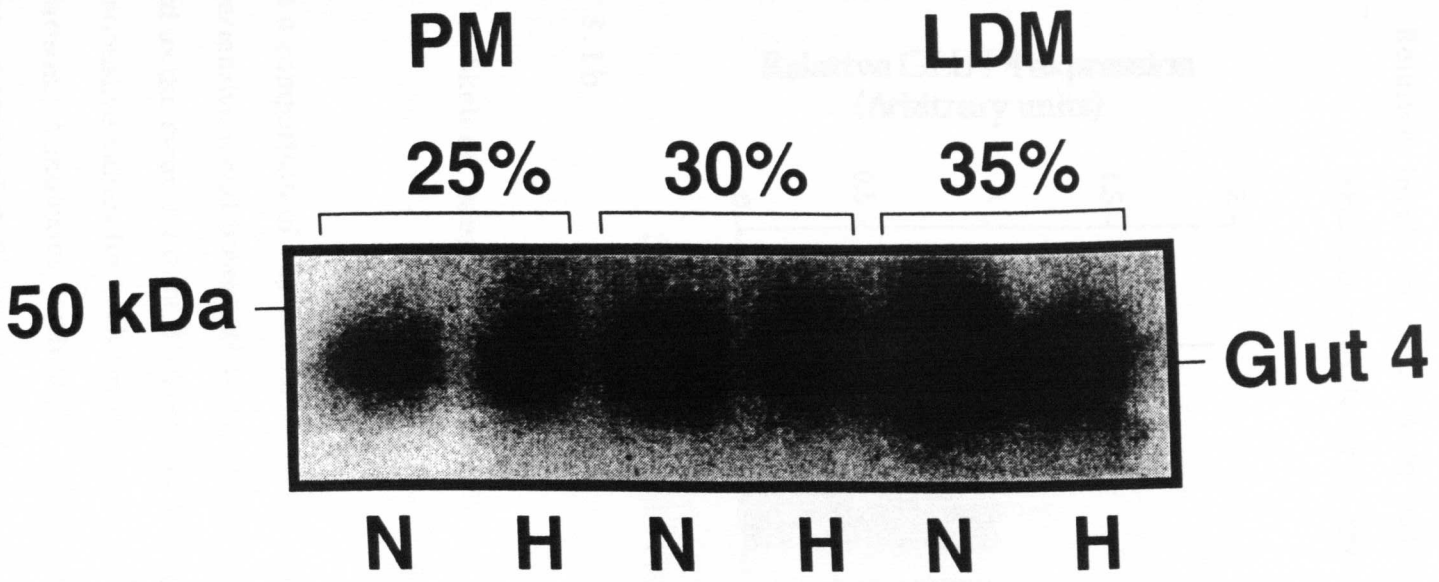

PM
LDM
25%
30%
35%
50 kDa
Glut 4
N
H
N
H
N
H

Figure 3.1b

Relative levels of GLUT-4 in sub-cellular fractions of skeletal muscle fron the Milan rat.

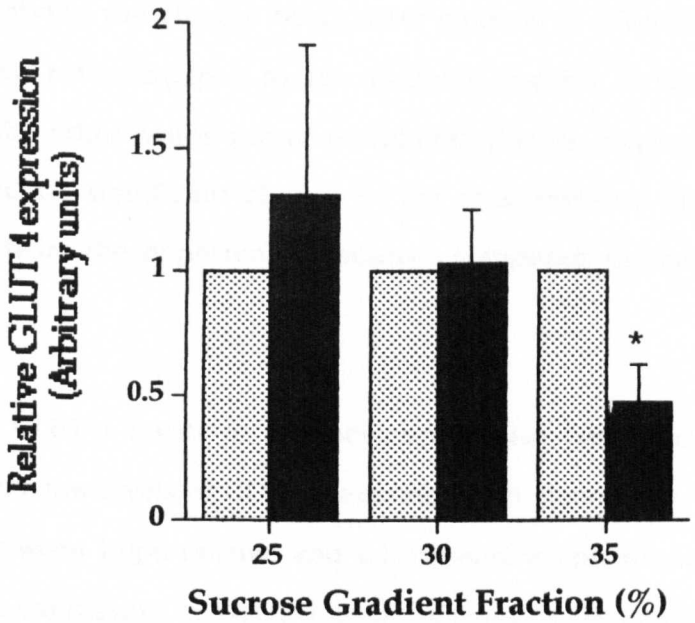


Figure 3.1b

Skeletal muscle GLUT-4 levels in Milan normo- and hypertensive rats.

Shown is a comparison of the GLUT-4 levels in normotensive (stippled boxes) and hypertensive (solid boxes) Milan rat muscle membrane fractions. Data is presented as the mean ± s.d. of 4 independent groups of animals, in each case the normotensive values for each fraction have been set at 100% for purposes of comparison. * indicates statistically significant differences, $p<0.01$. The percentage distribution of levels of GLUT-4 in these fractions were: 25% fraction 6.5% total GLUT-4, 30% fraction 14.6% total GLUT-4, 35% fraction 74% total GLUT-4 (mean values of four normotensive animals).

Levels of GLUT-1 in subcellular fractions were, in our hands, too low to reliably quantitate (data not shown).

3.4.2 Subcellular fractionation of Adipocytes

We have analysed the glucose transporter content of adipocytes of the Milan hypertensive rat compared to the isogenic control strain, as adipocytes constitute the other major site of peripheral glucose disposal. Surprisingly, we observed no significant change in the total level of GLUT-4 protein in adipocytes from the hypertensive animals compared to controls as seen in Fig. 3.2.

Analysis of GLUT-1 levels in adipocytes was also performed. However we observed very low levels of GLUT-1 expression in these cells, with no change apparent between hypertensive and normotensive animals (not shown). The low level of expression of GLUT-1 in adipocytes is consistent with previous studies from this and other laboratories [Gould & Holman (1993); Baldwin (1993)].

3.4.3. GLUT-2 expression in liver

Glucose transport in hepatocytes is mediated via the GLUT-2 isoform [Gould & Bell (1990)]. Since GLUT-2 is predominantly expressed at the plasma membrane, we employed the method cited in section 2.10 to prepare of hepatocyte plasma membranes for immunological analysis. Figure 3.3 is a representative immunoblot which demonstrates that there is no significant change in hepatic GLUT-2 expression in the hypertensive state compared to the normotensive control group.

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IN
ORIGINAL

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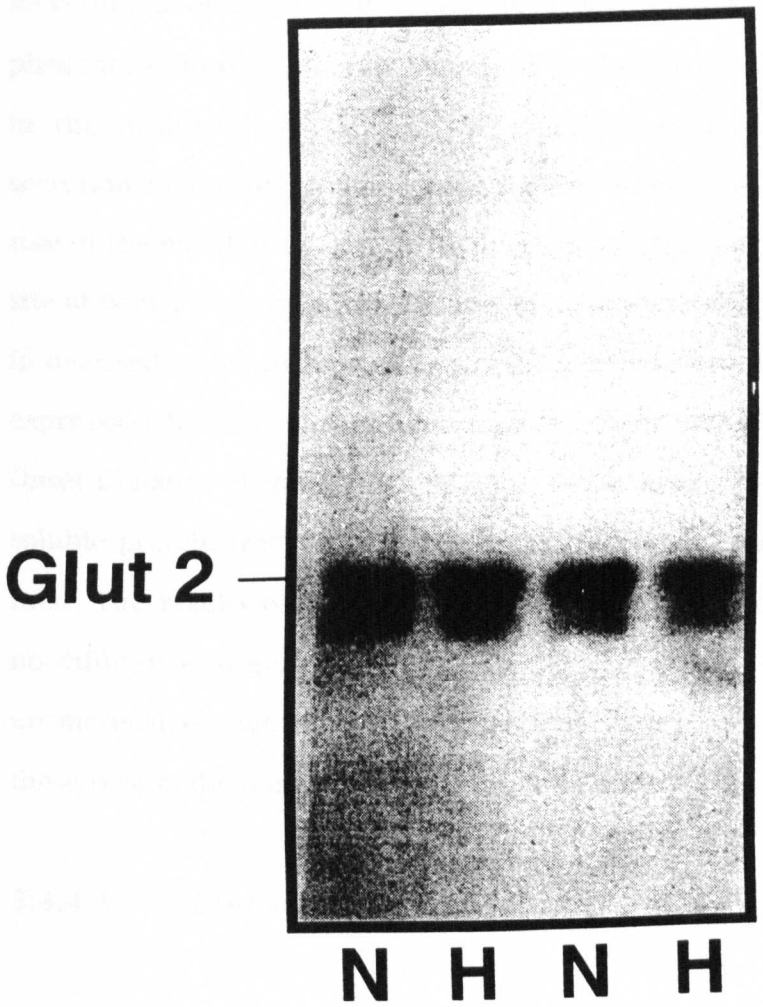
IN
ORIGINAL

Figure 3.3

GLUT-2 expression in hepatocyte plasma membranes.

25μg of plasma membranes isolated from liver homogenates were subject to electrophoresis and immunoblotting as described in the materials and methods section. Membranes from two groups of normotensive (N) and hypertensive (H) rats are presented for comparison. The blots were probed with antiserum against the liver-type glucose transporter, GLUT-2, and the entire running gel is shown.


Glut 2
N H N H

3.4.4. Glucokinase and glucose-6-phosphatase levels in liver

3.4.4.1 Glucokinase

Glucokinase (GK) is an isozyme of hexokinase that is expressed in the insulin-secreting pancreatic β-cells and hepatocytes. GK catalyses the high-K_m phosphorylation of glucose, functions as the proximal and rate-limiting step in the utilisation of glucose and plays a crucial role in regulating the secretion of insulin by pancreatic β-cells. Therefore GK plays an important role in the metabolism of glucose in the liver. This enzyme may be a potential site at which defects in sub-cellular glucose metabolism may be dysregulated in diseased states such as diabetes. Indeed, defective glucokinase activity and expression have been identified in some cases of diabetes, notably Maturity Onset Diabetes of the Young (MODY). We assayed glucokinase activity in the soluble protein fraction of hepatocytes from normotensive and hypertensive rats. The results of this analysis although preliminary suggest that there is no difference in glucokinase activtiy between the hypertensive strain and its normotensive counterpart. Furthermore, due to the preliminary nature of these results the data is not included in this thesis.

3.4.4.2 Glucose-6-phosphatase

Glucose-6-phosphatase (Glu-6-Pase) is a key enzyme in the homeostatic regulation of blood glucose concentration, especially in gluconeogenesis, as it catalyses the terminal step of this pathway. Glucose-6-phophatase is located in the lumen of the endoplasmic reticulum, and has been established to be a multicomponent enzyme, with polypeptides involved in the phosphatase reaction, and polypeptides which catalyse the transport of substrates in and products out of the endoplasmic reticulum lumen. Liver Glu-6-Pase is

increased in starved, diabetic and glucocorticoid-treated adrenalectomized rats. Re-feeding of starved rats and insulin administration to diabetic rats decreases Glu-6-Pase activity. Defects in glucose-6-phosphatase have been shown to underlie several other human pathophysiological conditions, including the glycogen storage diseases. The abberant expression of one or many of the polypeptides of the glucose-6-phosphatase complex have also been shown to be responsible for some cases of sudden infant death syndrome (SIDS) associated with hyper- or hypoglycaemic episodes. Hence, given the established role of this enzyme in carbohydrate metabolism, we measured the activity of this enzyme in hepatocyte membranes from normotensive and hypertensive rats. The assay set-up for measuring the activity is complicated. In the intact hepatocyte, glucose-6-phosphate has to get into he lumen of the cell and become de-phosphorylated. Subsequently both the glucose and the inorganic phosphate have to exit the lumen. If any component in this system fails the overall activity for G-6-Pase is low. To take that into consideration, mannose-6-phosphate is used as a substrate,to determine if the mannose-6-phosphate is active. Another substrate, pyrophosphate (PPi), is used to determine whether the Pi transporter is functioning. Histone is used to disrupt the lumen to detemine the activity in intact versus disrupted hepatocytes. Thus, if there is no activity with mannose-6-phosphate, but ther is with PPi, then ther is a defect in the glucose-6-phosphate transporter. Also if activity is measured with mannose-6-phosphate, but not PPi, then the defect lies in the phosphate transporter. Once again our very preliminary results indicate that glucose-6-phosphatase activity is normal in both the normo- and hypertensive Milan rat. Our data is not shown due to the experiments only being completed once.

3.4.5. GLUT distribution in brain regions.

Since glucose is the main energy source for brain, we have examined the relative levels of GLUTs 1, 3 and 4 in normotensive and hypertensive rat brain regions (specifically the frontal cortex, medulla, pituitary, hypothalamus, thalamus, caudate putamen and hippocampus). The data from this analysis are presented in Figs. 3.4. (GLUT-1), 3.5 (GLUT-3) and 3.6 (GLUT-4).

In the detection of GLUTs 1 and 3 we performed the electrophoresis and immunoblotting exactly as described in sections 2.5 and 2.6. Quantitation of these transporters was made possible by the detection of primary antibody using [^{125}I]-goat anti-rabbit, followed by gamma counting of the nitrocellulose. For the levels of GLUT-4 found in the brain this method proved insensitive, therefore the primary antibody was detected using the ECL method which uses an HRP-linked IgG.

Analysis of Fig. 3.4 shows that GLUT-1 levels are increased in the hypothalmus and parietal cortex in the hypertensive Milan rat. The remainder of the regions analysed appear to display no change in GLUT-1 levels. Fig. 3.5 displays the amount of GLUT-3 detected after gamma counting. Again, the hypothalamus contains an increased amount of GLUT-3 relative to the normotensive strain. The thalamus also shows an increase with both the hippocampus and parietal cortex showing a decrease in the GLUT-3 levels. Of these discoveries the most interesting observation is the change of transporter level in the hypothalamus.

Figure 3.4a

Analysis of brain GLUT-1 levels.

A representative immunoblot from 4 independent experiments is presented. 25μg of membrane protein from the indicated brain regions were electrophoresed, transferred to nitrocellulose and the blot developed with anti-GLUT-1 antiserum. (N=normotensive animals, H=hypertensive animals).

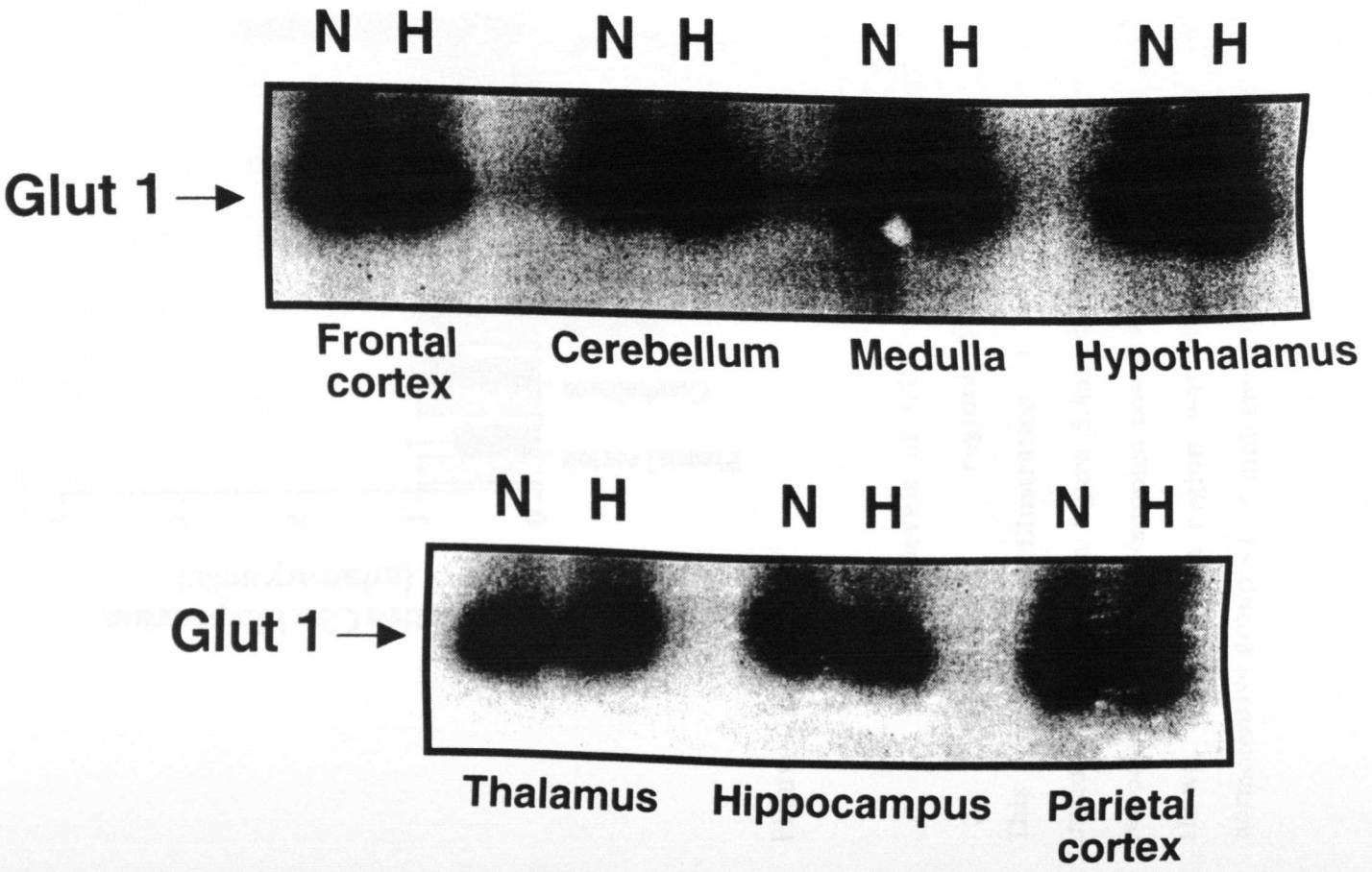

N H
N H
N H
N H
Glut 1
Frontal cortex
Cerebellum
Medulla
Hypothalamus
N H
N H
N H
Glut 1
Thalamus
Hippocampus
Parietal cortex

Figure 3.4b

Relative levels of GLUT-1 expressed in distinct brain regions of the Milan rat.

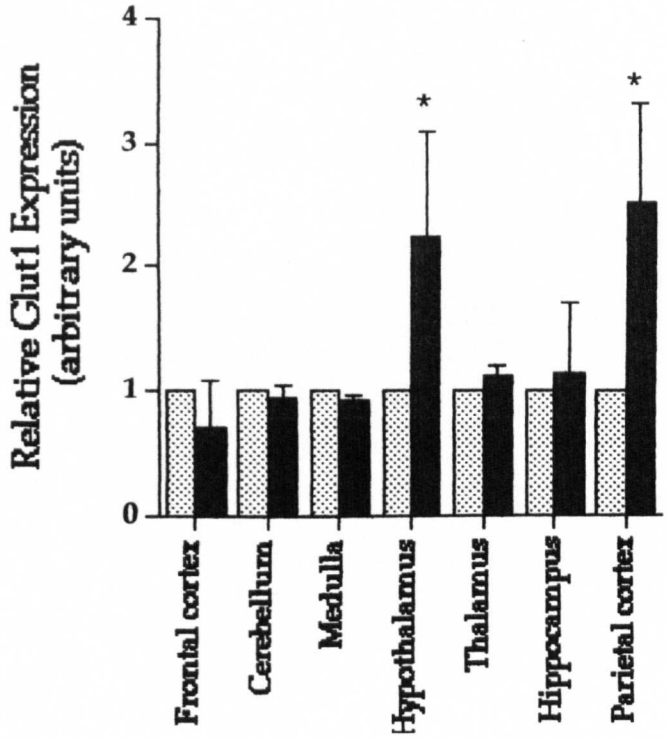


Figure 3.4b

Relative levels of GLUT-1 expressed in distinct brain regions of the Milan rat.

This is a graphical illustration of the level of GLUT-1 present in the immunoblot shown in figure 3.4a, with the mean and S.D. (n=4) for each region in the normo- (stippled boxes) and hypertensive (solid boxes) animals shown. Each brain region was adjusted such that the value in the normotensive group =1. * indicates a statistically significant change, p=0.05.

Figure 3.5a

Analysis of brain GLUT-3 levels.

A representative immunoblot from 4 independent experiments is presented. 25μg of membrane protein from the indicated brain regions were electrophoresed, transferred to nitrocellulose and the blot developed with anti-GLUT-3 antiserum. (N=normotensive animals, H=hypertensive animals).

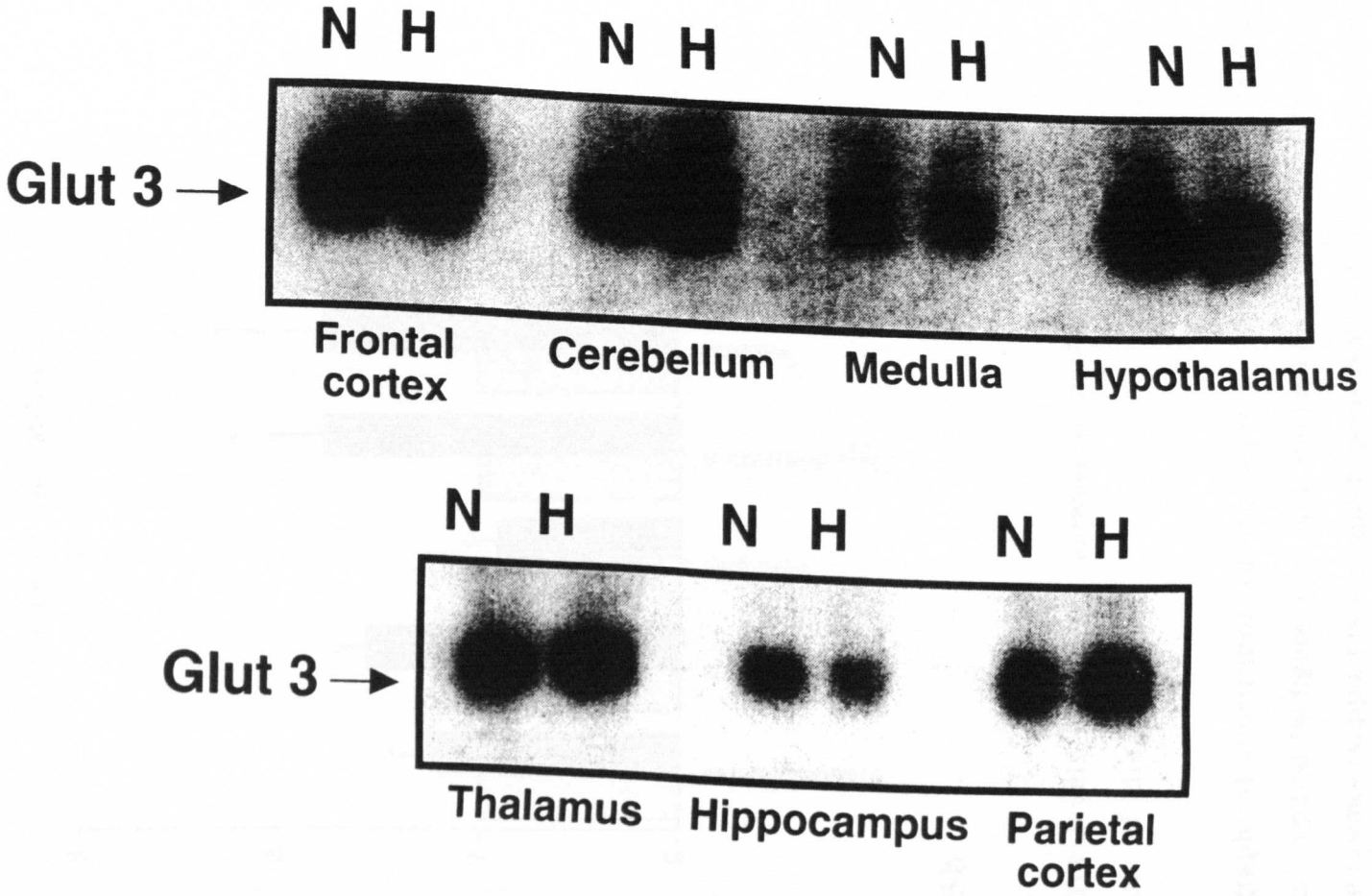

N H
N H
N H
N H
Glut 3
Frontal cortex
Cerebellum
Medulla
Hypothalamus
N H
N H
N H
Glut 3
Thalamus
Hippocampus
Parietal cortex

Figure 3.5b

Relative levels of GLUT-3 expressed in distinct brain regions of the Milan rat.

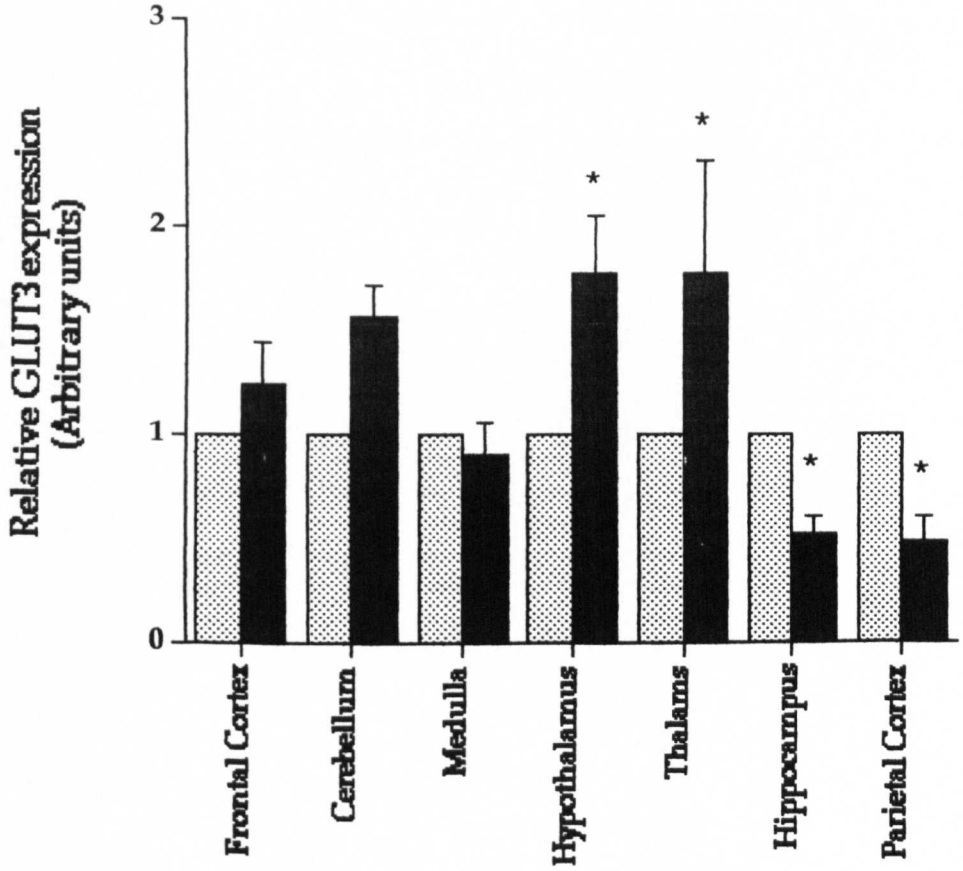


Figure 3.5b

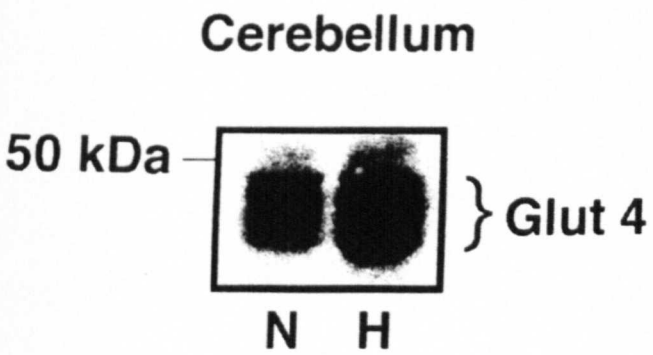
Relative levels of GLUT-3 expressed in distinct brain regions of the Milan rat.

This is a graphical illustration of the level of GLUT-3 present in the immunoblots , typified by figure 3.5a with the mean and S.D. (n=4) for each region in the normo- (stippled boxes) and hypertensive (solid boxes) animals shown. Each region was adjusted such that the value in the normotensive group =1. * indicates a statistically significant change, $p<0.05$.

Figure 3.6.

Analysis of brain GLUT-4 levels.

25μg of cerebellum membrane protein from normotensive (N) or hypertensive (H) Milan rat brain was analysed using anti-GLUT-4 antiserum as described. Shown is a representative immunoblot, the position of the 50kDa molecular weight standard is indicated. Note that this blot was developed using ECL. In several independent experiments, the increase in GLUT-4 levels associated with the hypertensive state was ~2- to 3-fold (data not shown).


Cerebellum
50 kDa
Glut 4
N
H

It is known that leptin and NPY are synthesised in the hypothalamus and both these peptides play a role in energy metabolism. It has also been postulated the the hypothalamus acts as a "blood glucose sensor", therefore any change in insulin sensitvity may result in altered expression of the transporters within the hypothalamus. It must be noted that this is speculation at the moment and the role of the hypothalamus in overall glucose homeostasis must be analysed further. As stated previously the levels of GLUT-4 present within the regions of the brain were too low to quantitate directly but by visual examination of Fig. 3.6 it appears that GLUT-4 is expressed at higher levels in the cerebellum of the hypertensive animal.

3.5 Discussion

The molecular basis of diminished insulin-stimulated glucose transport associated with insulin resistance may be the result of depletion of cellular glucose transporters, mis-targetting of glucose transporters to a non insulin-sensitive intracellular location or alternatively may lie on the signalling pathway from the insulin receptor to the transporter protein [Gould & Holman (1993); Shepherd & Kahn (1993)]. Hypertension is frequently accompanied by peripheral insulin resistance, and studies using the spontaneously hypertensive rat model have indicated that adipocytes from this species are insulin resistant and exhibit a reduced level of expression of GLUT-4 protein [Reaven *et al.*, (1989)]; similarly, the Milan rat strain is also known to exhibit insulin resistance [Dall'Aglio *et al.*, (1991)], but to date, no information of the relative GLUT-4 levels in peripheral tissues of this strain have been reported, and moreover studies of other tissues (notably brain and liver) have not been performed. Hence, we wished to study the changes in glucose transporter expression in the Milan hypertensive rat compared to the normotensive isogenic control strain with a view to determining what, if any, effects on glucose transporter expression were associated with the hypertensive state.

3.5.1 Glucose transporter content in peripheral tissues.

Skeletal muscle is the major site of postprandial glucose disposal. We therefore examined the relative levels of GLUT-4 in subcellular fractions of skeletal muscle from hypertensive rats, and compared the levels to those observed in control, normotensive animals. We felt it appropriate to undertake subcellular fractionation of skeletal muscle into plasma membrane and low density microsomal fractions, since changes in the level of

expression in the intracellular GLUT-4 pool would have profound implications for insulin-stimulated glucose transport [Shepherd & Kahn (1993); James & Piper (1994)]. Subcellular fractionation of skeletal muscle membranes was performed exactly as described in [Klip *et al.*, (1987)]. In skeletal muscle, we consistently observed a large decrease [53 ± 4%, n=4, $p<0.01$] in the level of GLUT-4 protein in the low density microsomal fraction of hypertensive animals compared to age- and weight-matched controls (Fig. 3..1 A & B). Such a large decrease in the intracellular pool of GLUT-4 is likely to have profound implications for insulin-stimulated glucose transport in these cells, and suggest that the peripheral insulin-resistance observed by others [Dall'Aglio *et al.* (1991)] in the Milan hypertensive rat may be a consequence of decreased GLUT-4 expression.

Interestingly, we observed no changes in adipocyte GLUT-4 expression in these animals. The insulin resistance observed in adipocytes is therefore likely to be the result of a defect in an as yet unidentified intracellular signalling pathway, as adipocyte GLUT-4 levels are similar in the normo- and hypertensive states (data not shown). It is unlikely that mis-targetting of GLUT-4 accounts for adipocyte insulin resistance, as no gross changes in subcellular distribution of GLUT-4 were observed.

It is interesting to note that in an analysis of adipocytes from SHR rats, Reaven *et al.*, (1989) clearly demonstrated a decrease in GLUT-4 expression compared to normotensive animals. In addition, Häring *et al.*, (1992) report that GLUT-4 expression in muscle from the SHR strain is unchanged compared to control (normotensive) strains. These results are distinct from those observed here in the Milan hypertensive rat. However, a relative of the SHR, the obese, hypertensive SHR/N-cp, exhibits significantly reduced skeletal muscle GLUT-1 levels [Marette *et al.*, (1993)]. Such data add further weight to

the polygenic nature of the syndrome of insulin resistance and hypertension.

Given the variability of the results observed for Milan, SHR, ZDF Zucker rats *etc.*, and the observation that GLUT-4 levels are modulated by the glycaemic status of the animal, it is not possible to draw any firm conclusions regarding the relevance of the decrease in GLUT-4 expression in the Milan hypertensive rat to the aetiology of hypertension. As will be discussed in Chapter 4, one way to address this issue is to perform genetic linkage studies of in-bred populations and to determine whether decreased GLUT-4 levels co-segregate with the hypertensive phenotype.

3.3.2 Glucose transporter expression in liver.

Glucose transport in hepatocytes is mediated via the GLUT-2 isoform [Gould & Bell. (1990); Bell *et al.*, (1990)]. Our results show that there is no significant change in hepatic GLUT-2 expression in the hypertensive state (Fig. 3.2). This result is perhaps unsurprising since previous studies of the insulin-resistance associated with hypertension has indicated that the insulin resistance is localised exclusively to the peripheral tissues. Given the high capacity of the liver-type glucose transporter, it would in any case be unlikely that small changes in the level of expression would exert anything other than relatively minor effects on whole body glucose homeostasis. Nevertheless, here we show for the first time that the expression of the hepatocye glucose transporter is not altered in the Milan hypertensive rat model.

3.5.3 Glucokinase and glucose-6-phosphatase.

Glucokinase and glucose-6-phosphate are hepatic enzymes that play a key role in the maintainance of whole body glucose homeostasis. Preliminary reults of experiments carried out in Dundee in collaboration with Dr. Ann Burchell would suggest that there is no decrease in the specific activity of glucokinase, in the Milan hypertensive rat strain,. This enzyme acts as the rate-limiting step in the utilisation of glucose and plays a crucial role in regulating the secretion of insulin by pancreatic β-cells. In insulin resistant states circulating blood glucose levels are elevated. A defect in glucokinase would result in a decrease in the rate of glucose phosphorylation possibly resulting in hyperglycaemia. These results are preliminary and further work would be required to confirm the GK activity as *bona fide*. However, they do suggest that in the Milan strain, defective liver GK activity does not contribute to the hyperglycaemia.

Glucose-6-phosphatase is a main enzyme in the homeostatic regulation of blood glucose concentration, as it catalyses the terminal step in the gluconeogenesis pathway. The preliminary results of the Glu-6-pase assays show that there is no difference in the V_{max} in the hypertensive rat strain.

3.5.4 Glucose transporter expression in brain regions and changes with hypertension.

Glucose is the main energy source for mammalian brain. A previous study from this laboratory has indicated that different brain regions exhibit distinct levels of GLUT-1 and GLUT3- transporter isoforms [Brant *et al.* (1993)]. Moreover, certain brain regions express the insulin-responsive glucose transporter, GLUT-4; raising important questions regarding the function of

this protein in these regions, and how its activity may be regulated by insulin and the subsequent neuronal consequences of increased glucose transport [Livingstone *et al.*, (1994)].

The results of this analysis indicate that changes in the patterns of glucose transporter expression are coincident with the hypertensive state, but these changes are restricted to defined brain regions. For example, the parietal cortex exhibits a large (~2-fold) increase in GLUT-1 expression, with a corresponding decrease in GLUT-3 expression. In contrast, the hippocampus exhibits a decrease in GLUT-3 levels independently of any change in GLUT-1 levels, and the cerebellum exhibits markedly increased GLUT-1 and GLUT-3 levels, together with an increase in GLUT-4 content. It has been established that in certain brain regions, neuronal output may be modulated by glucose levels in the cerebrospinal fluid [Marfaing-Jallat & Penicaud (1993a); Marfaing-Jallat *et al.* (1993b)], thus the changes in glucose transporter levels reported here may have a significant impact on brain function in the hypertensive state. It will be of interest to determine whether such changes in transporter levels are localised to specific nuclei or regions within these affected areas.

Although the metabolic consequences of expression of GLUT-4 in the cerebellum remain to be determined, these results are interesting in so far as GLUT-4 expression in this region is markedly elevated. Thus, in different tissues of the same animal group (hypertensives), GLUT-4 expression is either profoundly decreased (muscle), unaffected (adipose tissue) or increased (cerebellum), suggesting that the GLUT-4 gene is under precise tissue-specific control.

3.5.5 The role of leptin and Neuropeptide Y (NPY) in circulating glucose levels.

The brain was also examined as it plays a key role in the control of feeding and hormonal regulation of glucose metabolism both directly and indirectly. The first insight into the brain controlling feeding came in 1958 when Hervey noted the presence of a hormone that regulated body weight through an interaction with the hypothalamus. This hormone is now known as leptin, the "fat-melting hormone". It has now been well established that leptin resistance accounts for some cases of human obesity. This phenomenon has been further investigated using several obese rodent strains [Caro *et al.* (1996)]. As with insulin resistance, the animal models studied differ in their phenotype. The db/db mouse and fa/fa mouse both contain mutations in the leptin receptor, and the ob/ob mouse strain does not produce leptin [Zhang *et al.* (1994)].

As discussed previously it has been proposed that most obese humans are resistant to their exogenous production of leptin [Caro *et al.*, (1996)]. Furthermore, it has been demonstrated that the product of the obese gene, leptin, is capable of inducing weight loss in the ob/ob mouse. The weight loss after leptin adminstration has been shown to due to decreased appetite and food consumption. Also leptin administration actually increased thermogenesis and activity levels. Additionally, it has been observed that there is a normalisation of hyperglycaemia and hyperinsulinaemia prior to any significant weight loss [Campfield *et al.*, (1995)].

Further studies by Campfield *et al.*, (1995) suggests that specific brain areas are the target for leptin, including high-affinity leptin binding in the rat hypothalamic plasma membrane. Subsequently it has been shown that

chronic leptin adminstration decreased hypothalamic neuropeptide Y (NPY) mRNA and directly suppressed NPY release from isolated perfused normal rat hypothalamus. Hypothalamic NPY stimulates food intake, decreases thermogenesis, and increases plasma insulin and corticosteroid levels. Therfore, NPY appears to be a logical transducer system for leptin action. Experiments involving leptin administration also proved the concept of leptin resistance. A model of diet-induced obesity required 5-10 fold more leptin to achieve the equivalent weight loss of that produced in the ob/ob mouse. Studies have also shown that in humans, serum leptin concentrations reflect the amount of adipose tissue in the body.

As leptin appears to be involved in the mechanism of conserving energy during food deprivation and preventing obesity during periods of energy excess, the relationship between insulin and leptin has been investigated. Acute and chronic administration of insulin *in vivo* and *in vitro* increases adipose tissue *ob* mRNA and therefore leptin synthesis in rodents [Saladin *et al.* (1995)]. Although ob mRNA synthesis increases, a rise in the secretion of leptin only occurs in the final 24 hours of a 3-day hyperglycaemic clamp. This delay in the insulin-stimulated secretion of leptin demonstrates that it may be the consequence of the trophic effect of insulin on adipocytes, rather than one of the classical metabolic responses to insulin. Little is known about the signalling pathways followed after leptin binds to the receptor. This area is currently a black box: it may arise that leptin transport, receptor and signalling are all normal and that the defect resides in the leptin transducer system.

3.5.6 Model of NPY action

Hypothalamic neuropeptide Y-ergic neurones control energy balance in part by stimulating feeding and inhibiting thermogenesis [Stephens *et al.* (1995)]. Previous studies have shown that leptin decreases NPY in normal animals, with a suppression of appetite through an unknown pathway. The increase in thermogenesis is likely due to an increase in sympathetic out-flow that ultimately activates the release of norepinephrine from sympathetic nerve terminals. This in turn activates the β3 adrenergic receptor in brown adipose tissue. When norepinephrine binds to the adrenergic receptor in fat cells, it raises their metabolic rate by increasing expression of the gene that encodes uncoupling protein. This protein, present in the inner membrane of the mitochondria, releases energy from fatty acids as heat. The relevance of leptin's regulation of thermogenesis in humans, if any, is unknown at this time. This is particularly true, since humans have little brown fat compared with rodents. The above paragraph represents a theoretical model of NPY acting as the leptin transducer system This model may be inaccurate and it may be that such a fundamental physiological function as maintainance of energy balance is regulated by a multi-dimensional system with overlapping control pathways.

It is clear that specific brain regions, primarily the hypothalamus, play a role in the controlling feeding and hormonal regulation of glucose metabolism. Leptin and NPY synthesised and secreted by the brain have been shown to be involved in insulin action. Therefore when discussing insulin resistance and hyperglycaemia it is important to remember the role played by leptin and NPY.

Our demonstration that glucose transporter levels are changed in defined brain regions may have profound consequences in terms of glucose-induced neuronal output, and also potentially in hormonal secretion (eg NPY, leptin). Clearly, on the basis of the data presented we can draw no firm conclusions in this regard. Nevertheless, the data suggest that such studies may offer fruitful lines of future research.

3.5.7 Limitations of the study

i) Due to the constraints of the laboratory's animal licence we were unable to perform any experiments to calculate the rate of glucose transport or the degree of insulin resistance in the Milan strain. This is unfortunate as it would have been desirable to back up our observations with a direct examination of glucose transport. Therefore, we can only speculate on the effects our observations have on glucose transport and insulin resistance.

ii) As stated previously this is a study of one rat strain and there is already information present in the literature that the degree of insulin resistance, levels of glucose transporters and degree of hypertension varies significantly between strains. It has been shown from many studies on this topic that both insulin resistance and hypertension are polygenic diseases with many variable parameters. This means that the conclusions drawn from this study may only be relevant to the Milan rat strain.

Further to this we decided to examine the F2 progeny of the SHRSP rat strain. This F2 population has blood pressures ranging from normotensive to extremely hypertensive, therefore we wished to examine the levels of glucose transporters to see how they correlate with blood pressure. This enables us to look at glucose transporters with blood pressure being the only variable.

3.6 Summary

We have shown that skeletal muscle from the Milan hypertensive rat exhibits a decreased level of expression of the insulin-regulatable glucose transporter GLUT-4, compared to its normotensive relative. This result is likely to account at least in part for the insulin resistance associated with this strain. No change in hepatic or adipocyte glucose transporter levels were observed. Marked changes in the expression of GLUTs 1, 3 and 4 were observed in distinct regions of the hypertensive rat brain, the significance of which remains to be elucidated.

These results suggest that a defect in the expression of GLUT-4 in muscle may account for the insulin resistance associated with the elevated blood pressure of the hypertensive group. However, in order to further address this, we decided to extend these studies into another animal model system, and to examine the levels of GLUT-4 expression in the Stroke Prone Spontaneously Hypertensive Rat, and in the progeny of a cross between these animals and a normotensive WKY control strain.

Chapter 4

GLUT Levels in Stroke-Prone Spontaneously Hypertensive Rats: Comparison of Parental and F2 Generation Animals.

4.1 Aims.

The aims of this chapter are as follows:

1. To examine facilitative glucose transporter levels in muscle of SHRSP animals compared to parental WKY strains.

2. To analyse the expression levels of GLUT-4 in skeletal muscle of SHRSP animals in the F2 generation of a cross of SHRSP with WKY animals, and to correlate GLUT-4 levels with blood pressure.

3. To examine sex-dependent expression of GLUT-4 in SHRSP animals.

4.2 Introduction.

In the previous chapter we showed that in hind-limb muscle hypertensive, insulin resistant Milan rats had decreased intracellular GLUT-4 levels in comparison with isogenic Milan normotensive controls [Campbell *et al.* (1995)]. We wished to determine whether this decrease was also evident in other rat models of hypertension. As a model system, in this section we used the stoke-prone spontaneously hypertensive rat (SHRSP). The SHRSP model is considered to be one of the most realistic experimental models of human essential hypertension [Clark *et al.*, 1996)]. As in humans, the male SHRSP display higher blood pressure than female. This is also true of the more commonly used spontaneously hypertensive rat (SHR). A further important aspect of the choice of SHRSP is the demonstration that crosses between parental SHRSP and control (WKY) animals produce off-spring with blood pressures which span the range from normotensive to extremely hypertensive. The F2 progeny provide an ideal model to examine the effect of blood pressure on relative levels of facilitative glucose transporters present in tissues, as the changes in GLUT expression can be directly correlated with blood pressure (measured concomitantly in the same animals). Furthermore, the potential for subsequent genetic analysis of the gene(s) which regulate GLUT expression has been amply demonstrated by recent studies of such animals [Jeffs *et al.* (1997)]. Any reduction in GLUT levels, particularly in skeletal muscle GLUT-4, may account for the insulin resistance observed in these animals, and the results may be used as an indicator as to whether or not blood pressure affects the levels of GLUTs present in insulin responsive tissues.

4.3 Methods.

The WKY and SHRSP rats were descendants of the two strains maintained at the University of Michigan. After arrival in Glasgow in 1991 the colonies of SHRSP and WKY were established by mating brother X sister. In order to establish the SHRSP F2 population two reciprocal crosses were then performed. In brief, 1 male SHRSP was mated with 2 WKY females (cross 1) and 1 male WKY was mated with 2 SHRSP females (cross 2) to generate the F1 progeny. Subsequently the F1 rats of each cross (3 males and 6 females) were brother X sister-mated to generate F2 rats. The pups are weaned and sexed after 3 weeks and housed according to sibling group and sex thereafter.

Blood pressure measurements were performed over long-periods using telemetry probes inserted into the animals. These probes send radio signals to detector units which record the diurnal variations in blood pressure in individual animals. Other parameters measured included age, sex, weight, and left ventricular volume. Blood pressure measurements were correlated by Dr Delyth Graham and Professor Anna F. Dominiczak (University Department of Medicine and Therapeutics, Western Infirmary, Glasgow) who generously made this data available to us. Statistical analysis was performed using ANOVA by the University of Glasgow Department of Biological Statistics.

GLUT-4 levels in both LDM and PM fractions of hind-limb skeletal muscle were measured using the sub-cellular fractionation and immunoblotting protocols outlined in chapter 2. In these experiments, GLUT-4 levels in LDM/PM fractions from individual rats were determined by quantitative immunoblotting. On each immunoblot, duplicate lanes of GLUT-4 standard were loaded, and GLUT-4 levels in fractions isolated from individual animals were compared to this standard. In the analysis of all the samples in this

chapter, this GLUT-4 standard was constant. Prior to analysis of GLUT-4 levels in these fractions, we showed that the immunoblot signal obtained for these standards was well within the linear response range of the antibody/detection system. Care was taken to ensure all 'experimental' samples were measured within the linear range for each antibody. All fractions were assayed at least three times and the values presented are mean ± s.d. for the three independent blots.

4.4 Results.

4.4.1 Analysis of blood pressure in SHRSP AND SHRSP F2

The first criteria to be analysed is the blood pressure measurements from all the strains examined in this chapter. Figure 4.1 illustrates that the SHRSP rat strain has by far the highest mean systolic blood pressure of 222.3 mmHg ± 26.7, as measured by telemetry probes inserted into the animals, compared to 156.8 mmHg ± 10.6 mmHg for the control group. Additionally treatment with losartan, which is a blocker of the AT1 receptor for angiotensin II, significantly lowers blood pressure to normotensive levels. Interestingly, the SHRSP F2 strain had an average cystolic blood pressure of 167.75 mmHg ± 22.75 mmHg, which is only slightly hypertensive. This is not altogether surprising as the SHRSP F2 generation is comprised of individual animals which display blood pressure levels from normotensive to extreme hypertensive.

As stated in the introduction of this chapter, the SHRSP rat strain is regarded as the hypertension model closest related to human essential hypertension, in particular that the severity of hypertension is gender associated. This is demonstrated in figure 4.2 which illustrate that the mean male blood pressure is significantly higher than that of its female counterpart. The males recorded an average blood pressure of 239.1 mmHg ± 16.9 mmHg, compare to the females 194.2 mmHg ± 5.2 mmHg. Furthermore, both the male and female SHRSP have blood pressures much greater than the normotensive WKY control. Additionally figure 4.3 highlights that male SHRSP rats have a greater average body weight than the female counterpart. The blood pressures from both sexes are much greater than that of the control normotensive WKY strain.

Figure 4.1

Differences in blood pressure between SHRSP, SHRSP F2 and WKY rat strains.

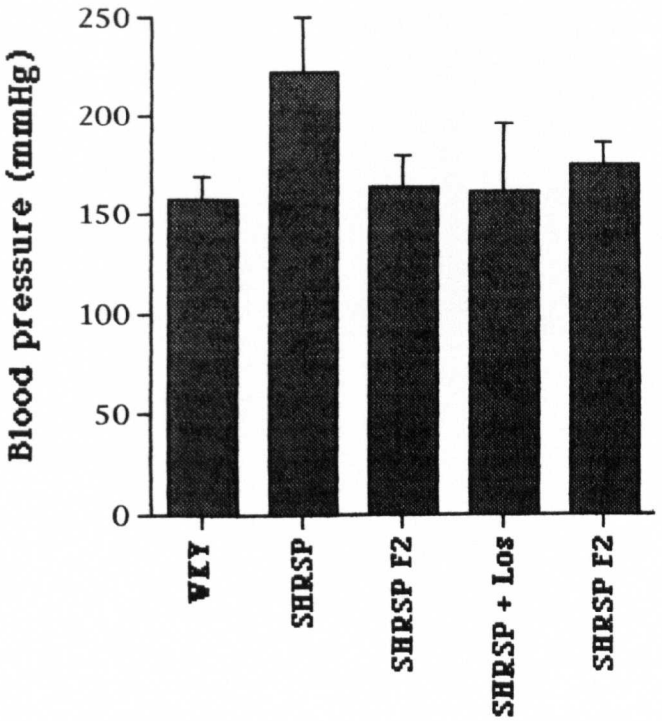


Figure 4.1

Differences in blood pressure between SHRSP, SHRSP F2 and WKY rat strains.

A graphical illustration of the differences in the blood pressure of SHRSP, SHRSP F2 and WKY rat strains. The blood pressure was measured by telemetry probes over a period of approximately 7 days.

Figure 4.2

Analysis of the relationship between blood pressure and gender in the SHRSP and WKY rat strains.

A graphical illustration of the relationship between blood pressure and gender in the SHRSP generation, compared to the blood pressure measured in the normotensive WKY strain.

Figure 4.2

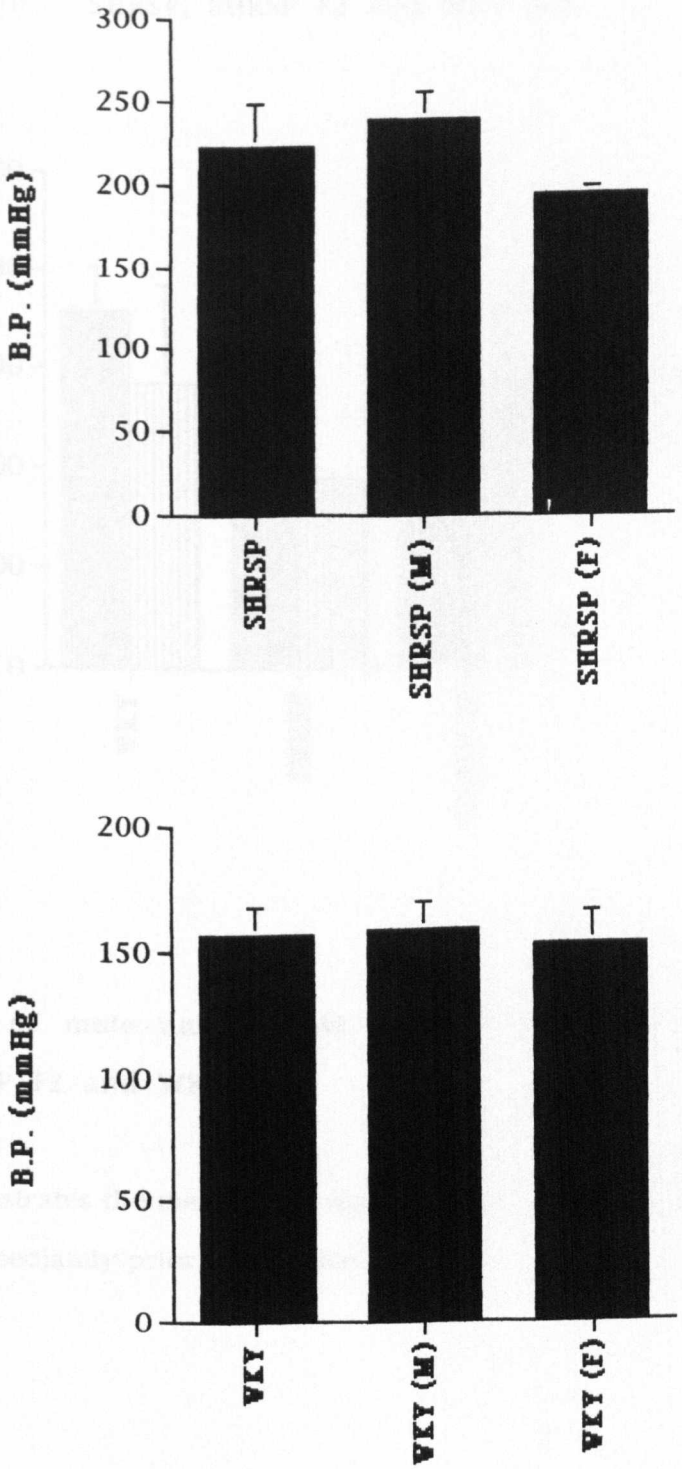

B.P. (mmHg)
300
250
200
150
100
50
0
SHRSP
SHRSP (M)
SHRSP (F)
B.P. (mmHg)
200
150
100
50
0
WKY
WKY (M)
WKY (F)

Figure 4.3

Comparison of male and female mean body weights from the SHRSP, SHRSP F2 and WKY rats.

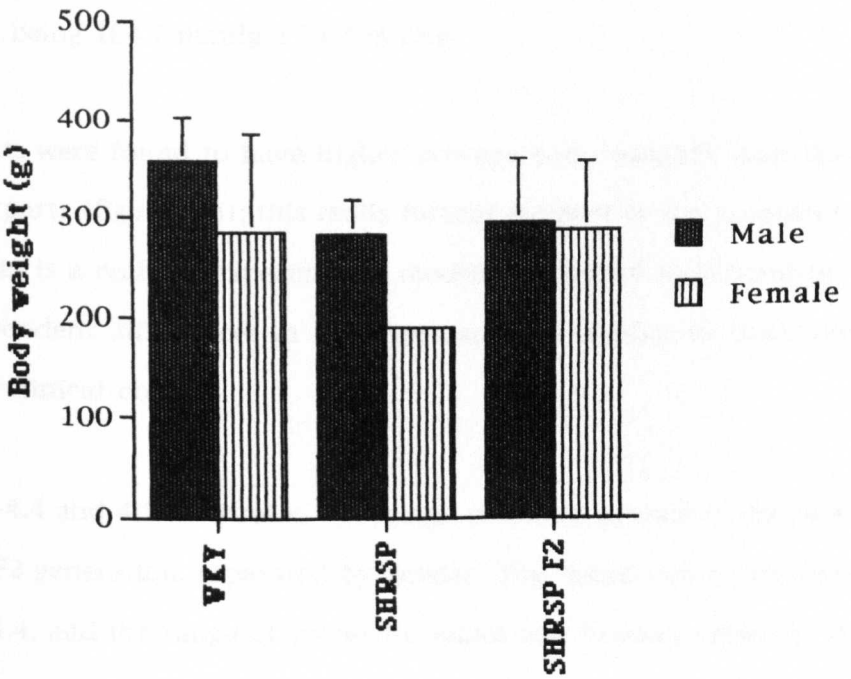


Figure 4.3

Comparison of male and female mean body weights from the SHRSP, SHRSP F2 and WKY rats.

The graph illustrates the mean body weights, in grammes (g), of the animals measured immediately prior to sacrifice.

The SHRSP F2 strain displays average blood pressure that does appear to be linked to gender. As stated earlier the average blood pressure for the total group was 167.75 mmHg ± 22.75 mmHg. Looking at the blood pressures for males and females separately, as is shown graphically in figure 4.4, it is apparent that the males have a higher mean blood pressure. For males the average blood pressure being 172.5 mmHg ± 8.6 mmHg, and the average for females being 164.7 mmHg ± 11.3 mmHg.

Male rats were found to have higher average body weights than their female counterparts (figure 4.3); this lends further support to the argument that this rat strain is a realistic experimental model of essential hypertension, as these sex-dependent differences in blood pressure are similar to those reported in human clinical cohorts.

Figures 4.4 and 4.5 illustrates the range of blood pressures displayed by the SHRSP F2 generation, separated by gender. The mean values are presented in figure 4.4, and the range of values for males and females shown in figure 4.5.

4.4.2 GLUT-4 levels in the SHRSP and SHRSP F2 skeletal muscle

After measuring the relative amounts of intracellular GLUT-4 from the skeletal muscle from individual animals (both WKY controls, parental SHRSP and F2 animals), I examined the possibility of GLUT-4 levels correlating with gender or blood pressure in these animals. Representative immunoblots of GLUT-4 in such animals are presented in figure 4.6.

Figure 4.4

Mean blood pressure measurements in the SHRSP F2 generation.

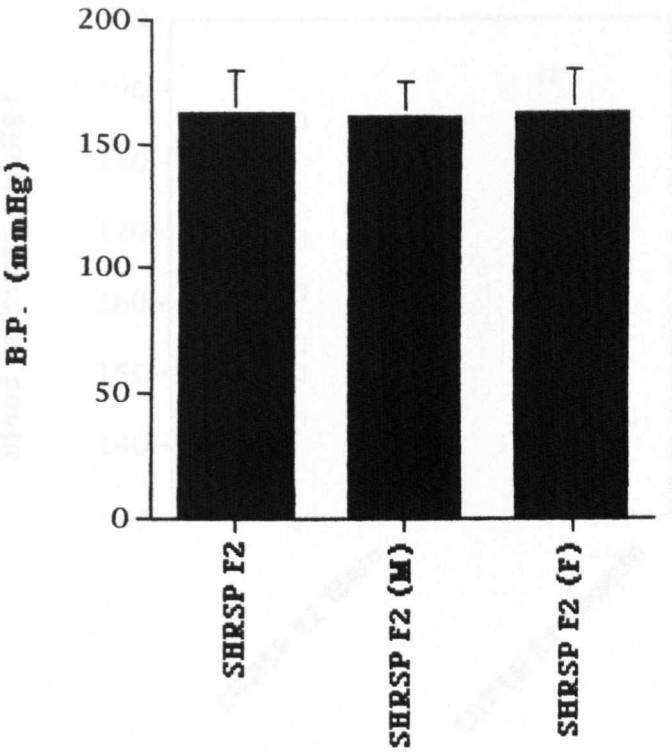


Figure 4.4

Mean blood pressure measurements in the SHRSP F2 generation.

Blood pressure measurements were made in individual rats over 5 days using radio telemetry probes. Shown are the mean BP values for the SHRSP F2 population, both as a total group, and separated be gender (Males M; Females F). The mean values for males and females are not statistically different.

Figure 4.5

Analysis of the relationship between blood pressure and gender in the SHRSP F2 progeny.

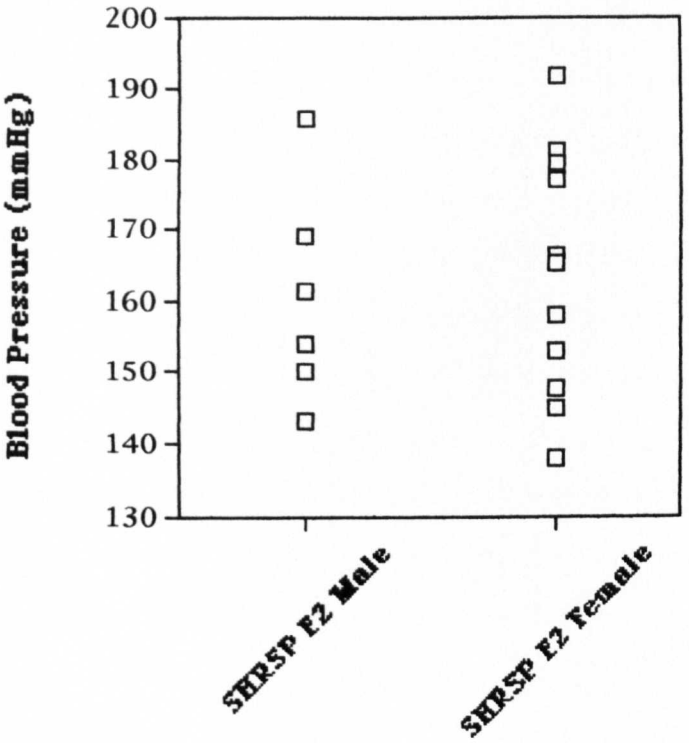


Figure 4.5

Analysis of the relationship between blood pressure and gender in the SHRSP F2 progeny.

A graphical illustration of the relationship between blood pressure, as measured by telemetry probes, and gender in the SHRSP F2 generation. The graph has been plotted in two columns, one male and the other female. Each point marks the average blood pressure for an individual animal.

Figure 4.6

Immunological analysis of intracellular GLUT-4 levels in SHRSP F2 skeletal muscle.

A representative immunoblot of the intracellular GLUT-4 levels present in the skeletal muscle of SHRSP F2 animals is shown. Low density microsomal membrane protein (25μg) from each individual was electrophoresed on 10% SDS-PAGE, prior to transfer onto nitrocellulose membranes. Immunoblots were developed with anti-GLUT-4 antibodies (Campbell *et al.* 1995). Shown is a representative blot from F2 animals of the SHRSP study group. The bands on the developed autoradiograph were cut out of the nitrocellulose and the 125-I labelled antibody was γ counted. A GLUT-4 standard was run on the SDS-PAGE in order to allow a comparison between each immunoblot. Blots were repeated at least twice for each muscle fraction, and the mean value calculated as a percentage of the signal obtained from the control GLUT-4 standard. These values were used to calculate the data for figure 4.7.

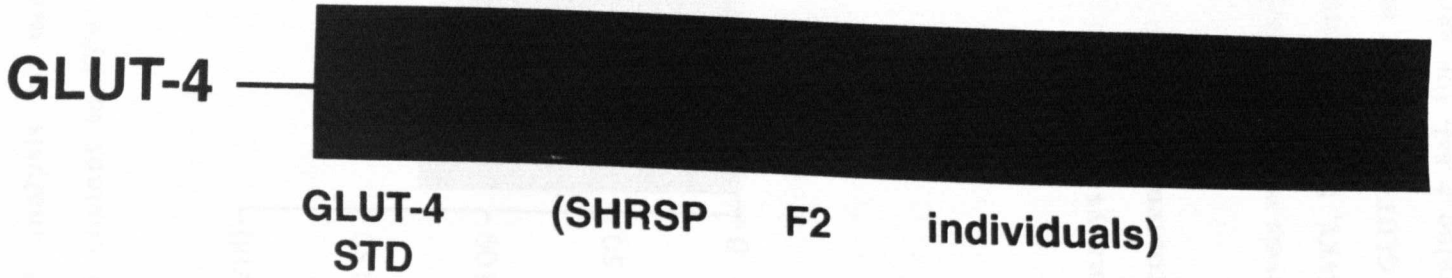

GLUT-4
GLUT-4
STD
(SHRSP
F2
individuals)

Figure 4.7

Immunological analysis of skeletal muscle intracellular membrane GLUT-4 content in SHRSP, SHRSP F2 and WKY strains.

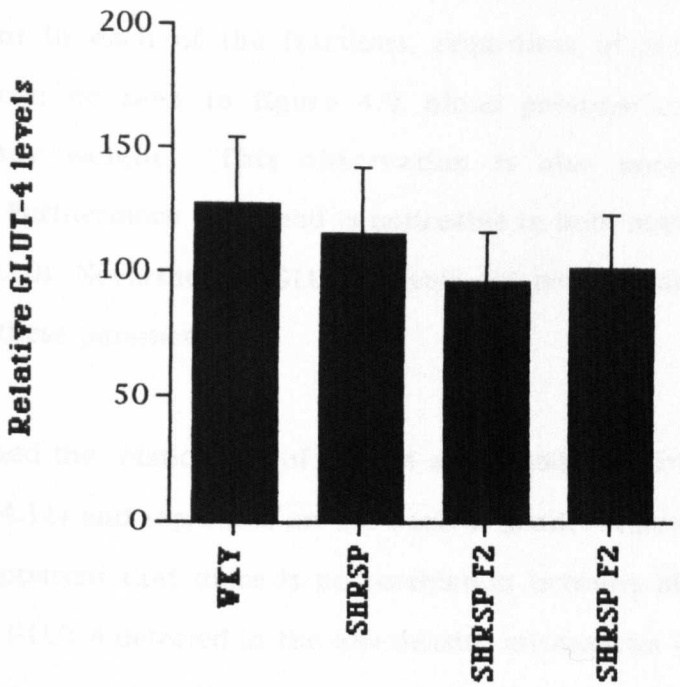


Figure 4.7

Immunological analysis of skeletal muscle intracellular membrane GLUT-4 content in SHRSP, SHRSP F2 and WKY strains.

Intracellular GLUT-4 levels were determined in triplicate for animals in each of the study groups (WKY, SHRSP parentals, SHRSP F2 etc.) as outlined in figure 4.6. These mean GLUT-4 value for each group was then calculated, and shown above is this value ± s.d. for each group. There was no statistical difference between any of these groups.

Surprisingly, we observed no statistical difference in GLUT-4 levels in either PM (not shown) or LDM fractions (fig 4.7) from SHRSP parental animals compared to control (WKY strains).

Despite male rats having higher blood pressure and greater body weight (see figure 4.3 above), no significant difference was observed in the amount of GLUT-4 present in each of the fractions, regardless of sex (figure 4.8). However, as can be seen in figure 4.9 blood pressure increases with increasing body weight. This observation is also noted in human hypertension. Furthermore this trend is noticeable in both male and females alike (figure 4.10). Nevertheless, GLUT-4 levels did not appear to correlate with either of these parameters.

We also examined the relationship of GLUT-4 and blood pressure in the total cohort (figure 4.11) and separated on the basis of gender (figure 4.12). From this data it is apparent that there is no correlation between blood pressure and the level of GLUT-4 detected in the low density microsomes (LDMs) of the hind-limb skeletal muscle. This finding highlights the diverse nature of insulin resistance and would appear to emphasise the differences between rodent models of hypertension and insulin resistance.

Another topic under investigation in the course of this chapter is the effect of gender upon skeletal muscle GLUT-4, body weight and hypertension. For the SHRSP F2 strain our findings reveal that there is no difference in GLUT-4 levels between male and female individual rats. Not surprising the males have a slightly higher mean blood pressure, but there is no significant difference in average body weight nor relative levels of the glucose transporter, GLUT-4. As stated in the previous

Figure 4.8

Analysis of the relationship between gender and skeletal muscle intracellular GLUT-4 SHRSP, SHRSP F2 and WKY strains.

This shows figure 4.7 separated into male and female average intracellular GLUT-4 levels (see figure 4.5 for explanation). Data calculated after running 25μg protein on SDS-PAGE, western blotted and the resultant band from the autoradiograph cut out of the nitrocellulose and γ-counted.

Figure 4.8

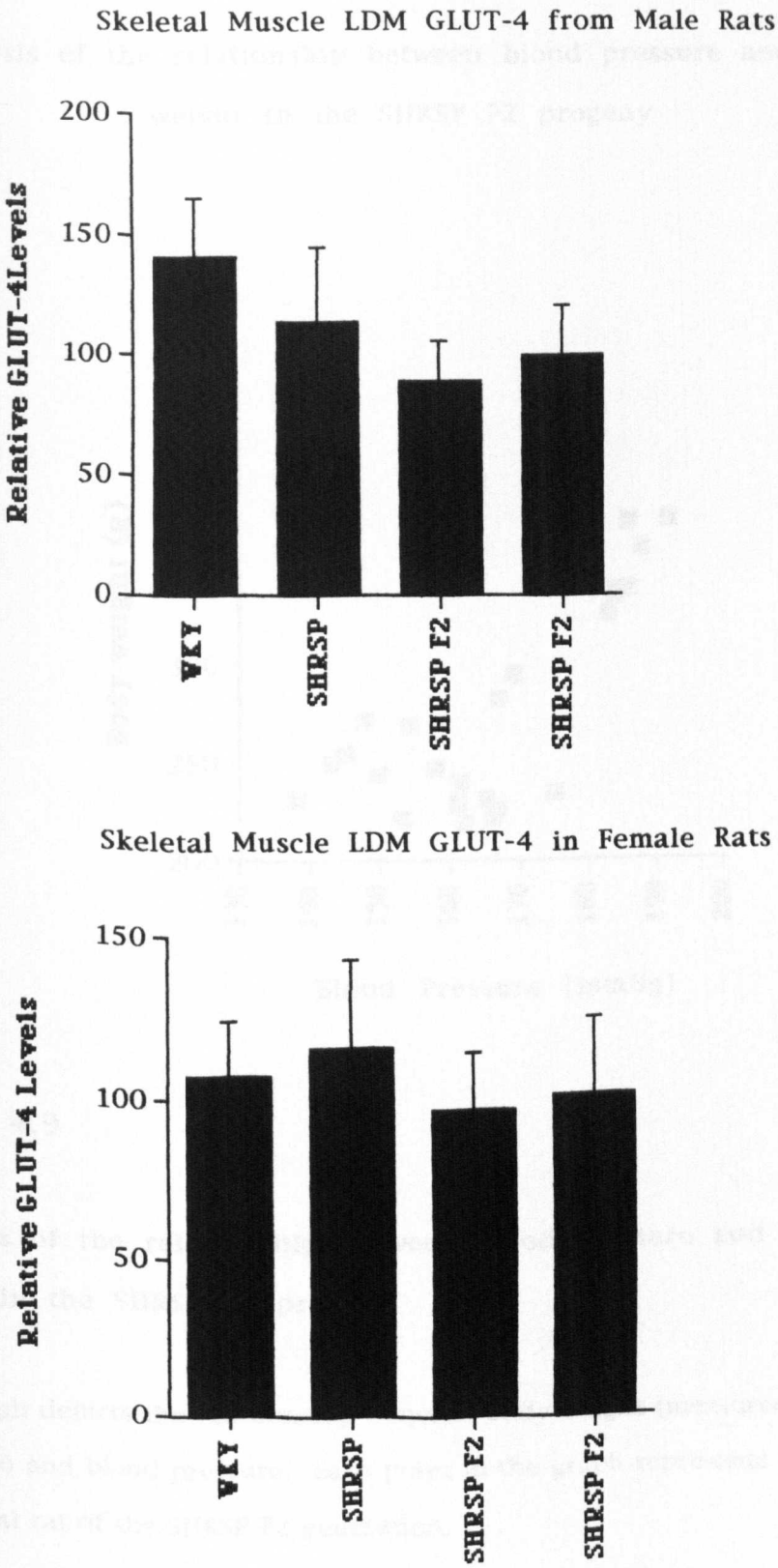

Skeletal Muscle LDM GLUT-4 from Male Rats
Relative GLUT-4Levels
200
150
100
50
0
WKY
SHRSP
SHRSP F2
SHRSP F2
Skeletal Muscle LDM GLUT-4 in Female Rats
Relative GLUT-4 Levels
150
100
50
0
WKY
SHRSP
SHRSP F2
SHRSP F2

Figure 4.9

Analysis of the relationship between blood pressure and body weight in the SHRSP F2 progeny

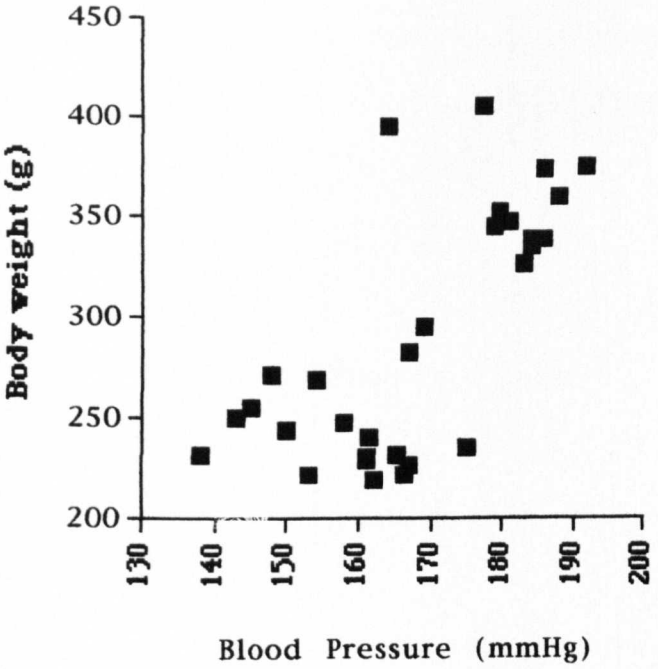


Figure 4.9

Analysis of the relationship between blood pressure and body weight in the SHRSP F2 progeny

This graph depicts the relationship between body weight (measured in grammes) and blood pressure. Each point in the graph represents one individual rat of the SHRSP F2 generation.

Figure 4.10

Gender and the relationship between blood pressure and body weight in the SHRSP F2 progeny

The SHRSP animals from the F2 generation (figure 4.9) were considered separated by gender. The results of this analysis are shown above. In both cases, there is a positive correlation between blood pressure and body weight, as has been reported by others (see text for details). Note that for clarity, not all points have been presented.

Figure 4.10

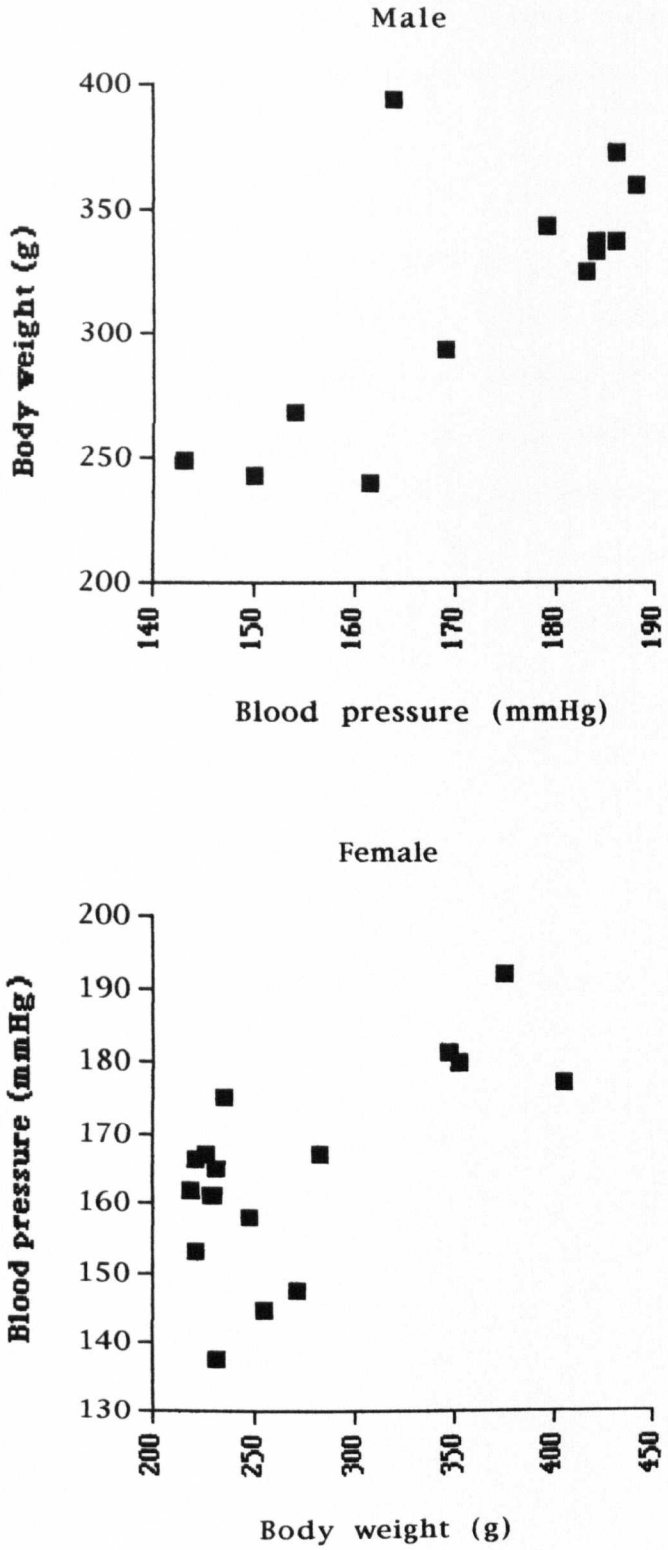

Male
Body weight (g)
400
350
300
250
200
140
150
160
170
180
190
Blood pressure (mmHg)
Female
Blood pressure (mmHg)
200
190
180
170
160
150
140
130
200
250
300
350
400
450
Body weight (g)

Figure 4.11

Analysis of the relationship between skeletal muscle intracellular GLUT-4 and blood pressure in the SHRSP F2 generation

A graphical illustration of the relationship between blood pressure, as measured by radio probe telemetry, and intracellular GLUT-4 levels, determined by quantitative immunoblotting, in the SHRSP F2 generation. Each point in the graph represents an individual animal. There is no relationship evident between these variables. Note that for clarity, not all points have been presented.

Figure 4.11

Analysis of the relationship between skeletal muscle intracellular GLUT-4 and blood pressure in the SHRSP F2 generation

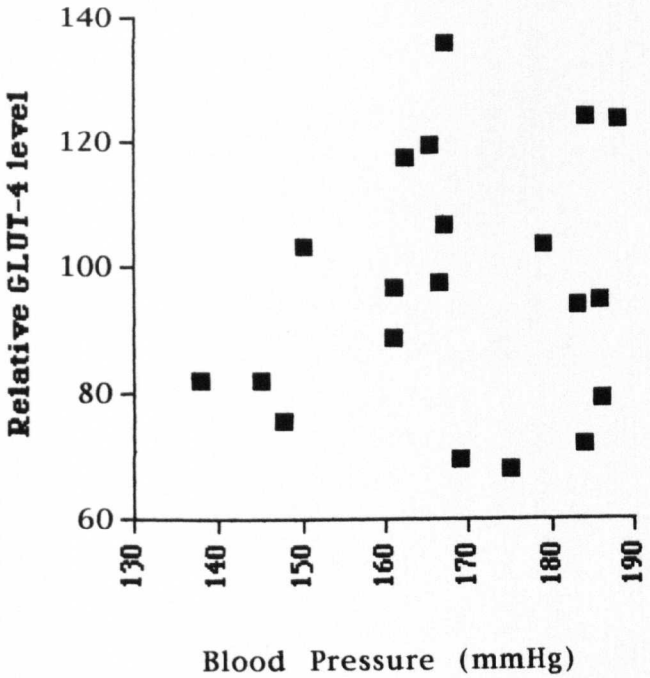
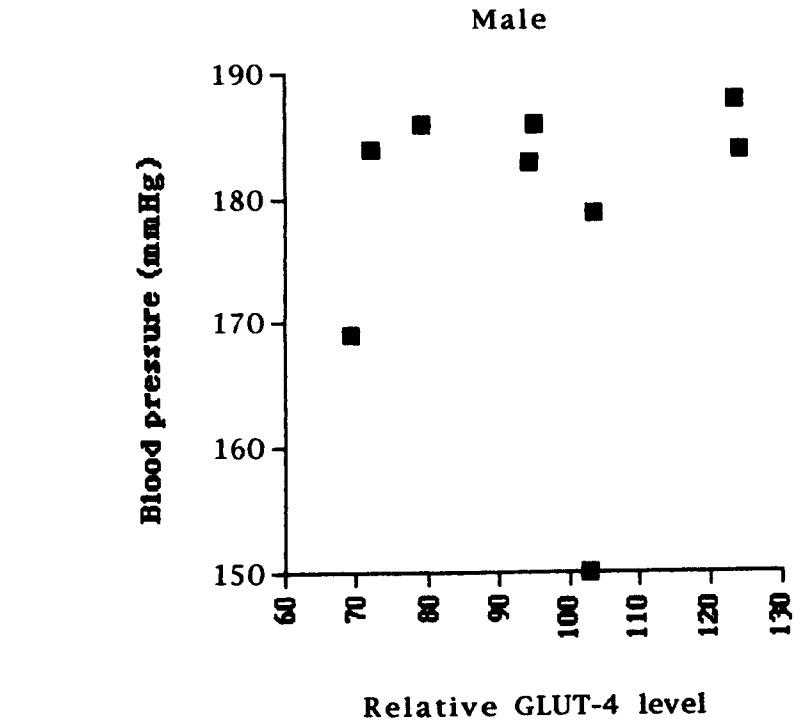


Figure 4.12

Gender and the relationship between intracellular GLUT-4 and blood pressure in the SHRSP F2.

This separates figure 4.11 into male and female individuals. However it still demonstrates a lack of correlation between GLUT-4 levels and blood pressure.

Figure 4.12


Male
Blood pressure (mmHg)
190
180
170
160
150
60
70
80
90
100
110
120
130
Relative GLUT-4 level

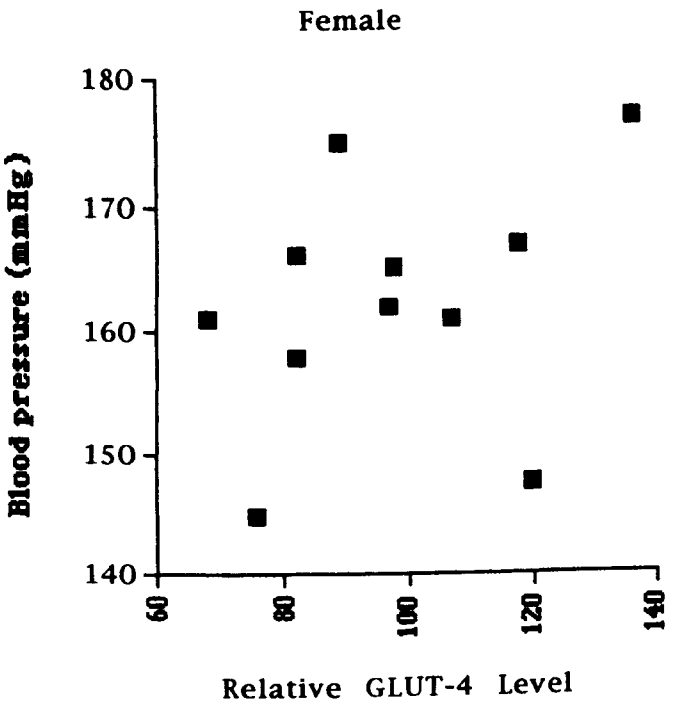

Female
Blood pressure (mmHg)
180
170
160
150
140
60
80
100
120
140
Relative GLUT-4 Level

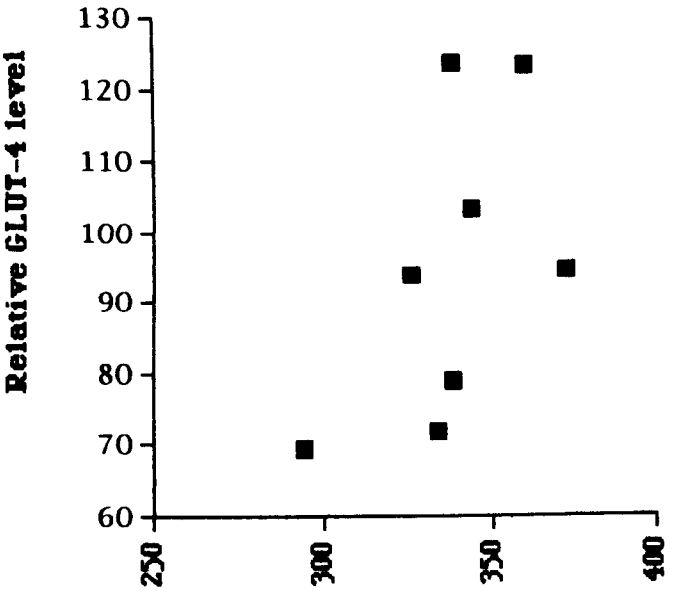
Figure 4.13

Analysis of the relationship between body weight and skeletal muscle intracellular GLUT-4.

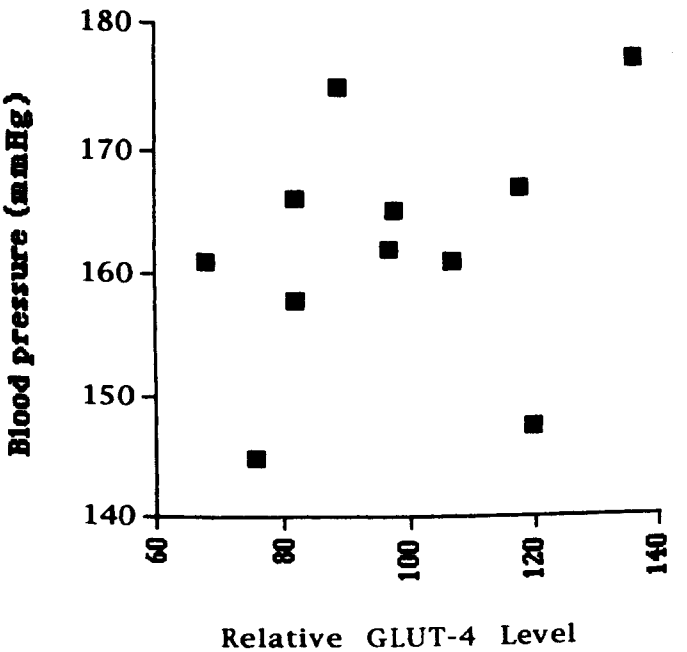
This illustration separates the SHRSP F2 generation into males and females and examines the relationship between body weight and GLUT-4. Linear correlation coefficients of these graphs do not reach statistical significance. Note that for clarity, not all points have been presented.

Figure 4.13

Male body weight Vs. LDM GLUT-4 levels



SHRSP F2 female blood pressure Vs. LDM GLUT-4



chapter, many insulin resistant rodent models do not express reduced levels of GLUT-4. Additionally there is no relationship between body weight and the level of GLUT-4(Fig 4.13). Previous work had shown that there is a reduction in GLUT-4 correlating with increasing obesity. However, many hypertensive rat models are obese and have normal GLUT-4 expression.

This study demonstrates that the decrease in muscle GLUT-4 levels observed in a related animal model of hypertension, the Milan rat, is not observed in this model of hypertension. Since the progeny of the SHRSP x WKY cross exhibit blood pressures which span the range from normotensive to hypertensive, then if a decrease in skeletal muscle GLUT-4 was tightly linked to the hypertensive state, this link should be manifest among this progeny. Our results clearly indicate that this is not the case. We conclude that decreased GLUT-4 in skeletal muscle is not tightly correlated with the hypertensive state in this model of hypertension.

4.5 Discussion.

In order to compare the relationship between blood pressure and GLUT-4 levels in the SHRSP F2 generation, we must initially consider the levels of GLUT-4 found in the parental SHRSP animal. WKY animals were chosen as control subjects as they are the closest related normotensive strain. However, it is important to note that the WKY, unlike the Milan normotensive strain, is not an isogenic control. When comparing skeletal muscle GLUT-4 levels between WKY and parental SHRSP animals, it is apparent that there are no differences in GLUT-4 levels between strains, either in the low density microsomes or the plasma membrane.

As was discussed in the previous chapter many rodent models of insulin resistance have been examined in recent years with many different defects in GLUT-4 levels and the insulin signalling mechanism being identified. These models have shown that no one defect is consistently displayed and therefore being pin-pointed as the causative "agent" in insulin resistance. Thus, it is perhaps not surprising that the SHRSP parental strain is not defective with regard to GLUT-4 levels. When looking at gender, although the male rats have significantly higher mean blood pressure, there was no difference in GLUT-4 levels in any sub-cellular fraction considered.

When looking at the SHRSP F2 generation the story is somewhat similar. As previously stated SHRSP F2 individuals display blood pressures ranging from normotensive to extremely hypertensive but there is no correlation between blood pressure and GLUT-4 levels in these animals, either as a total group or separated on the basis of gender. This result is further proof that rodent models with insulin resistance are not necessarily defective in GLUT-4. Furthermore, it confirms and extends our observations that there is no

relationship between GLUT-4 level and blood pressure in the hypertensive parentals (compared to the WKY control strain). Additionally, the SHRSP F2 GLUT-4 levels are not gender dependent, even though the males have a slightly higher average blood pressure, the relative levels of GLUT-4 are unchanged between males and females. We were unable to demonstrate a correlation between GLUT-4 levels and blood pressure values for the parental SHRSP animals compared to WKY controls. Hence , it is not possible to statistically analyse the data from the SHRSP F2 generation using ANOVA to definitively delineate the relationship of blood pressure to GLUT4 levels in these animals. However, inspection of the data would appear to not reveal any correlation between these variables.

.Although the lack of a correlation between blood pressure and GLUT-4 contradicts the data obtained from the Milan rat strain in chapter 3, it must be remembered that insulin resistance, hypertension and hyperglycaemia, although related, are independent disease states with multiple genes involved. Furthermore, as discussed in chapters 1 and 3 of this thesis there are many means of becoming insulin resistant besides a defect in GLUT-4 expression. Other avenues have to be explored for this particular rat model including inefficient targetting of GLUT-4 to the plasma membrane, defects in the insulin receptor signalling mechanism, and also the possibility of failings at the level of the insulin receptor itself. Altered expression of one or many of the genes involved in insulin -stimulated sugar transport and blood pressure control may produce the resultant phenotype.

With regard to insulin resistance, and GLUT-4 in particular, many different rodent models and human subjects have been studied. From these studies no clear pattern has been observed with GLUT-4 levels being increased, decreased or even unchanged depending upon which model and interestingly

which tissue was under examination. In a study of human patients with either obesity or NIDDM it was observed that altered levels of GLUTs 1 and 4 were not responsible for insulin resistance [Pedersen *et al.* (1990)]. However, the streptozotocin diabetic, insulin deficient rat GLUT-4 mRNA and protein are reduced in both skeletal muscle and adipose tissue. Furthermore the decrease is associated with insulin deficiency, as after insulin treatment , both the mRNA and protein content are normalised in fat tissue [Garvey *et al.* (1989); Kahn *et al.* (1989)]. Consequently, it is not possible to offer a single unifying mechanism for insulin resistance associated with all clinical conditions. Hence, our observation here that defective GLUT-4 expression is not associated with blood pressure suggest that at least for SHRSPs, defective GLUT-4 expression is not a contributing factor to insulin resistance.

In recent years the levels of GLUT-4 in diabetic patients has been examined closely, and in particular the effect exercise has on the amount of glucose transporter protein present. In 1993 Koivisto *et al.* reported that exercise reduces the muscle glucose transport protein (GLUT-4) mRNA in type 1 diabetic patients. However, in patients with NIDDM, physical training increases muscle GLUT-4 protein and mRNA [Dela *et al.* (1994)]. In young, healthy subjects, physical training increases insulin-stimulated glucose transport in skeletal muscle. Similar studies of training increasing whole-body insulin action and GLUT-4 protein content in aged people with impaired glucose tolerance have been published [Hughes *et al.* (1993)]. As previously mentioned the study of Dela *et al.* (1994) observed an increase in the level of GLUT-4 mRNA in the NIDDM patients. However, the level of GLUT-4 mRNA was lower in the NIDDM patient than in the control subjects, irrespective of training status. Other investigators have found that GLUT-4 mRNA in skeletal muscle is unchanged [Garvey *et al.* (1992); Pedersen *et al.* (1990)] or even higher [Eriksson *et al.* (1992)] in NIDDM than in age- and weight-matched

control subjects. Thus, data on GLUT-4 mRNA content in NIDDM patients is controversial.

There are further conflicts when looking at GLUT-4 protein levels in rodent models of diabetes. This probably reflects a combination of the use of different animal models with varying degrees of insulin resistance. A further problem in examining GLUT-4 levels in skeletal muscle is the difficulty in preparing sub-cellular fractions from this tissue. Taking all this into consideration the consensus view is that muscle GLUT-4 levels are unchanged, with some notable discrepancies. Male rats from the ZDF/*drt-fa* Zucker rat strain are obese and insulin-resistant, but go on to develop overt diabetes. In this strain GLUT-4 levels are reduced by 40% in both skeletal muscle and adipose tissue, accounting for insulin resistance. This reduction in GLUT-4 is restored by combating the hyperglycaemia, suggesting that this may be contributing to impaired expression of the transporter. Reduced GLUT-4 expression in this animal model appears to be decisive in causing diabetes, since in other respects these animals are similar to their respective non-diabetic strains [Friedman *et al.* (1991)].

We had hoped that analysis of the SHRSP animal for studies of GLUT4 and the link to hypertension may offer new insight into GLUT4 regulation. F2 animals such as those outlined above offer a powerful genetically malleable tool with which to address the segregation of genetic material with the hypertensive phenotype. We had hoped to utilise this to address the issue of the control of GLUT4 regulation in hypertension. Unfortunately, this analysis is impossible as the expected phenotype of reduced GLUT4 expression in the hypertensive state was not observed.

4.6 Summary

This chapter highlights the diverse nature of insulin resistance. From the data presented it is clear that we could not detect any reduction the facilitative glucose transporter, GLUT-4, in the SHRSP F2 generation. These results are consistent with many previous studies that have shown that GLUT-4 expression and activity is not reduced in insulin resistance. Further analysis of the insulin signalling pathway may elucidate the mechanism of insulin resisitance associated with hypertension in the SHRSP rodent model.

Chapter 5

The effect of oestrogen and derivatives upon insulin-stimulated glucose transport in 3T3-L1 adipocytes.

5.1 Aims.

The aims of this chapter are:

1. To examine the effect of the steroids oestrogen, oestriol and oestradiol upon insulin-stimulated glucose transport.

2. To compare these effects with those induced by dexamethasone.

3. To determine the nature of any defect in insulin-stimulated glucose transport, including analysing the levels of GLUT-4 and PI-3 kinase.

5.2 Introduction

5.2.1 Sex hormone effects on insulin-stimulated glucose transport

Cardiovascular disease in postmenopausal women is a major public health concern. A third of all women between the ages of 50 and 75 are affected, and one sixth ultimately die from the disease [Ettinger, B. (1990)]]. The use of oestrogen replacement therapy in postmenopausal women has been shown to reduce the risk of cardiovascular disease by about 50% compared to women who remain untreated. However, as a result of concerns regarding endometrial carcinoma and hyperplasia, a progestin is often used in combination with an oestrogen to protect the endometrium from the excessive stimulation which may occur as a consequence of unopposed oestrogen. Notelovitz (1982) and Diamond *et al.*, (1995) both note that the use of progestin has been reported to counteract some of the beneficial cardiovascular effects of the oestrogen, such as increased high-density lipoprotein cholesterol concentrations, improved vascular tone, and glucose metabolism.

Caefalu *et al.*, (1994) demonstrated using the surgically postmenopausal cynomolgus monkey that progestins alone, or in combination with oestrogens can induce insulin resistance. Surgically postmenopausal monkeys were fed a diet containing conjugated equine oestrogen or medroxyprogesterone acetate, tamoxifen, or a combination of those for a period of 12 weeks. Insulin sensitivity and circulating levels of glucose were determined by frequent intravenous-sampling and performing tolerance tests. The results of the study suggest that progestins alone, or in combination with oestrogens, can induce insulin resistance in postmenopausal monkeys.

A similar induction of insulin resistance is observed in women on HRT, although is should be pointed out that there is some ambiguity in the data regarding glucose tolerance tests in some of these studies which has perhaps underestimated the prevalence of this problem [Spellacy (1982)]. These discoveries have been further complemented with research into the effect of the oral contraceptive pill on carbohydrate metabolism. Patterns of insulin resistance have been observed in women on the oral contraceptive pill [Godsland *et al.*, (1992)], and the co-existence of insulin resistance and hyperandrogenism has been described frequently, most notably in women with polycystic ovarian syndrome [Dunaif *et al.*, (1992)]. Godsland *et al.*, (1992) examined insulin sensitivity in women on the oral contraceptive pill. The study looked at the effects of varying the levels of progestin while maintaining the oestrogen component. The progestin only formulation had no effect upon insulin sensitivity, but the combination of oestrogen and progestin induced insulin resistance. These results suggest that it is more likely that the oestrogen component of oral contraceptives induces insulin resistance. The progestin component is thought to modify the effects of oestrogen-induced insulin resistance, perhaps as a consequence of progestin prolonging the half-life of insulin action. This means that users of the combined oral contraceptive have elevated plasma insulin concentrations. Therefore this has the effect of enabling the insulin resistant subjects to sustain their rate of glucose disposal.

Dunaif *et al.*, (1992) examined insulin resistance in polycystic ovary syndrome (PCOS), a disease state characterised by hyperandrogenism, disordered gonadotropin secretion, and oligo- or anovulation. Women with PCOS display significant insulin resistance and hyperinsulinaemia. As previously stated insulin-stimulated glucose uptake is largely mediated by GLUT-4, hence a study was performed to determine if the reduced insulin

sensitivity might be attributable to diminished cellular GLUT-4 content. In adipose tissue from PCOS patients GLUT-4 content is reduced by 35%, a reduction which probably accounts for the reduced insulin responsiveness witnessed in this tissue.

These and other studies have provided a clear demonstration that insulin and sex hormones interact, but the mechanistic basis of this interaction is unclear.

5.2.2 Interactions of insulin and sex-hormones

There is a difference of opinion on the cause and effect relationship between insulin and the sex hormones. One hypothesis which has gained the most considerable support is that high insulin levels found in insulin-resistant states may stimulate the ovary to induce overproduction of the sex hormones. [Poretsky, L. (1991)]. However, what is not readily accounted for by this theory is why insulin should promote the excess production of androgens only, as other sex hormones are not affected by insulin.

In contrast, it has been suggested that androgens may induce insulin resistance by an as yet uncharacterised mechanism. This hypothesis has been based on several observations. Firstly, it has been demonstrated in some hyperandrogenic, insulin resistant women that improvement of insulin sensitivity is observed upon correction of the hyperandrogenism [Shoupe & Lobo (1984)]. Secondly, Landon J., *et al.* (1963) reported the induction of insulin resistance in normal subjects following administration of the androgen methandienone. They reported a decrease in glucose clearance from healthy men after 2 -4 weeks of methandienone ingestion. Interestingly, they reported normal insulin sensitivity, which was measured

by the decrease in serum glucose concentration after insulin administration. Additionally, the same group noted an increase in plasma insulin response to tolbutamide administration, which is indicative of impaired insulin sensitivity, post methandienone treatment. Further studies by Cohen & Hickman (1987) demonstrated a causal link between anabolic steroid abuse and insulin resistance in young athletes. Specifically they looked at the effect of ingesting anabolic steroids on insulin sensitivity in powerlifters. The subjects abusing the steroids ingested large doses of approximately 200 mg/day for a period of up to seven years. These athletes had diminished glucose tolerance when compared to a non-steroid using group, whilst having substantially higher post-glucose serum insulin concentrations. The diminished glucose tolerance is likely to be secondary to the insulin resistance. The reduction in glucose tolerance could be explained in two .ways. Firstly, there is the possibility that inadequate insulin is released in response to an increase in plasma glucose concentration, as is the case with type 1 diabetes mellitus. Another possibility is that there is a specific tissue resistance to insulin action, this situation is associated with insulin resistance in subjects suffering from obesity. The insulin resistant powerlifters displayed both elevated fasting insulin concentrations and very high post-glucose insulin concentrations indicating that the diminished glucose tolerance was the result of tissue-specific insulin resistance.

Further key evidence in favour of such a link has come from studies of male to female and female to male transsexuals [Polerdman *et al.*, (1994)]. The subjects were monitored before, during, and after 4 months of hormone administration. The females were treated with injections of testosterone ethers (250 mg/day); males participants were administered with ethinyl oestradiol (0.1 mg/day) alone, or a combination of ethinyl oestradiol and cyproterone acetate. Throughout the study blood glucose and plasma insulin

levels were monitored using hyperinsulinemic-euglycemic clamping. This study reached the conclusion that sex hormone administration, i.e. testosterone treatment in females and ethinyl oestradiol in males, can induce insulin resistance in healthy subjects.

Interestingly, a recent study using sensitive clamp techniques has shown that normal puberty is associated with a reduction in insulin stimulated glucose uptake in peripheral tissues [Cutfield *et al.* (1990)]; although growth hormone levels may in part modulate this response, elevated levels of androgens and oestrogens may also contribute. High levels of progesterone and oestrogens in normal pregnancy are also associated with decreased insulin sensitivity, this also provides some support, albeit anecdotal, to suggest that androgens and oestrogens may be involved in the aetiology of insulin resistance.

5.2.3 Insulin resistance: molecular pathology

The inability of peripheral tissues to respond to insulin i.e. insulin resistance, has been identified in a number of clinical conditions, many of which are associated with an increase in blood pressure [reviewed in Livingstone *et al.* (1995)]. These include ageing, obesity and non-insulin dependent diabetes mellitus (Type II diabetes). For example, it has recently been demonstrated that adipocytes from lean, non-diabetic essential hypertensives exhibit insulin resistance correlated with an inability of insulin to stimulate glucose transport into these cells. Insulin resistance has also been reported in several model systems for hypertension [see above and reviewed in Livingstone *et al.* (1995)].

Such studies have identified potential points of lesion in the action of insulin to stimulate glucose disposal, and have been discussed in detail in previous sections of this thesis. These type of studies have not, however, been extended into studies of sex-hormones and insulin resistance, but offer a suitable framework within which to explore the molecular pathophysiology of other insulin resistant states.

5.2.4 Insulin resistance and sex hormone-binding protein

As noted above, sex hormones may be involved in the determination of risk factors for non insulin-dependent diabetes mellitus (NIDDM). In vivo assessment of sex hormones and binding proteins in both premenopausal and post menopausal women has suggested that decreased sex hormone-binding globulin (SHBG) is associated with higher circulating levels of glucose and insulin concentrations [Haffner, S.M (1996)]. Further studies have shown that increased insulin resistance has been associated with decreased SHBG levels. SHBG is thought to be an indirect measure of androgenecity with its concentrations mainly determined by the level of free oestrogens and androgens [Anderson, D.G. (1974)]. Increased glucose and insulin concentrations were associated with increased testosterone and decreased SHBG in non-diabetic subjects in the San Antonio Heart Study. Obesity is also another factor linking insulin resistance and SHBG. Upper body adiposity has also been associated with increased free testosterone and decreased SHBG. While overall adiposity is associated with increased pancreatic insulin secretion, increased upper body adiposity is associated with decreased hepatic clearance of insulin. In addition, hepatic clearance of insulin shows a positive correlation with concentrations of SHBG. Women with polycystic ovary syndrome (PCOS) who have increased levels of free testosterone, have increased insulin and glucose concentrations. Finally, although few studies

have been done on the whether the alteration of androgen levels predict metabolic disease, two studies have shown that low levels of SHBG predict the incidence of NIDDM in women [Haffner *et al.*, (1993); Lindstedt *et al.*, (1991)] .

5.2.5 The known effects of steroids on adipose cells and glucose transport

Even before the isolation and characterisation of GLUT-4 Garvey *et al.*, (1989) noted that the glucocorticoid, dexamethasone regulates the glucose transport system in primary cultured adipocytes. They discovered these cells became insulin resistant following exposure to the steroid. Furthermore the mechanism of insulin resistance differed depending on the length of time the cells were exposed to dexamethasone. Short term treatment of between 90-120 minutes resulted in rapidly decrease glucose transport in both basal and maximally insulin-stimulated cells. This decrease was associated with a reduction in the number of glucose transporters present at the plasma membrane, evaluated using a cytochalasin B binding assay. This contrasts with an increase in the amount of glucose transporters present in the LDMs from both basal and insulin-stimulated cells. Dexamethasone treatment led to a 22% increase in intracellular glucose transporters. These observations indicate that dexamethasone acutely decreases insulin responsiveness by impairing the translocation efficiency of the intracellular glucose transporters to the cell surface.

Chronic glucocorticoid treatment led to more profound decrements in both basal and insulin-stimulated glucose transport rates. The relative decrease in stimulated transport was more marked at sub-maximal insulin concentrations. As dexamethasone did not impair insulin binding its receptor it was concluded that the reduction in response to insulin were distal to the interaction

between insulin and receptor. Furthermore, glucose transporter levels were reduced at both the plasma membrane and intracellular pool. This discovery led to the conclusion that chronic treatment with dexamethasone enhances insulin resistance by inducing a defect in the total number of cellular glucose transporters.

Further to these studies Saad *et al.*, (1994) examined the effect of dexamethasone on the regulation of the insulin receptor, insulin receptor substrate-1 (IRS-1) and phosphatidylinositol 3-kinase (PI 3-kinase) in 3T3-F422A adipocytes. Section 1.3 gives a detailed account of the involvement of IRS-1 and PI 3-kinase in the insulin receptor signalling pathway. Differentiation of these cells results in a 13-fold increase in the amount of the insulin receptor present, a 9-fold increase in IRS-1. Additionally there was a 3.5-fold increase in PI 3-kinase protein (85kDa sub-unit) and a 16.5-fold increase in IRS-1-associated PI 3-kinase activity.

24 hour treatment of the adipocytes with 1nM dexamethasone induced an increase in the level of insulin receptor in these cells. Analysis of the autophosphorylation of the insulin receptor revealed that there was a decrease of 31%. Simultaneously, IRS-1 protein levels are decreased by 56% resulting in a decrease in IRS-1 phosphorylation of 31%. The down regulation of IRS-1 was also observed in 3T3-L1 adipocytes after treatment with dexamethasone [Turnbow M.A. *et al.*, (1994)]. Conversely dexamethasone increased the level of PI 3-kinase by 69%, as determined by western blotting. The combination of reduced IRS-1 levels and increased PI 3-kinase resulted in a 15% decrease in the association/activation between IRS-1 and PI 3-kinase. A 24 hour treatment of these adipocytes with 100 nM insulin resulted in a decrease in insulin receptor, IRS-1 and PI 3-kinase levels. Thus three of the

early steps of insulin action may have an important role in hormone-induced insulin resistance.

Another putative insulin receptor signal protein, pp160 has also shown to be regulated by dexamethasone [Rice *et al.*, (1992)]. This 160kDa cytosolic protein is phosphorylated on tyrosine residues in response to insulin and was postulated to be involved in signalling from the insulin receptor. It is now established that this protein is the murine homologue of IRS-1. Chronic exposure of fully differentiated adipocytes to dexamethasone and/or insulin resulted in a 70-85% decrease in the expression of pp160. These results demonstrate that pp160 is expressed in 3T3-L1 adipocytes when insulin receptors are expressed in large numbers. Furthermore maintenance of pp160 concentrations can be regulated by insulin and dexamethasone. The decreased expression of pp160 caused by these factors may be related to post receptor insulin resistance. This compliments the data from Turnbow *et al.* outlined above.

Here we set out to examine the effect of sex steroids on insulin action in isolated adipose cells. This represents a useful model to directly examine the effects of steroids on fat cells. Furthermore, such a system may offer a powerful model with which to probe the molecular basis of insulin resistance.

5.3 Methods

3T3-L1 fibroblasts were grown in 10% (v/v) NCS/DMEM until 48 hours post confluence. The cells were then induced to differentiate by a method detailed in section 2.3.4.

The adipocytes were maintained for a further 8-10 days in 10% (v/v) FCS/DMEM. 48 hours prior to assay the cells were fed with 10% (v/v) FCS/DMEM containing one of the oestrogen derivatives at a final concentration of between 0 and 100 nM.

Finally the cells were assayed to determine the rate of insulin-stimulated ^{3}[H] 2-deoxy-D-glucose uptake using the procedure detailed in section 2.3.8.

5.4 Results

As stated previously in this thesis, insulin-responsive tissues, skeletal muscle and fat, exhibit rapid translocation of GLUT-4 from the intracellular membranes to the plasma membrane in response to insulin. This results in a rapid increase in glucose transport, and eventually an increase in glucose metabolism. This phenomenon can also be observed in the cultured clonal adipocyte cell line, 3T3-L1 adipocytes. Figure 5.1 shows that in response to an insulin stimulus, [^{3}H] 2-deoxy-D-glucose transport into these cells is increased between 10- to 15-fold. Figure 5.2 shows an immunoblot of sub-cellular fractions of 3T3-L1 adipocytes treated with or without insulin. Upon insulin stimulation there is a noticeable decrease in GLUT-4 in the low density microsomes. Furthermore, there is a corresponding increase in GLUT-4 at the plasma membrane. This re-distribution in GLUT-4 to the cell surface is sufficient to explain the subsequent increase in glucose transport. Additional studies have shown that there is a slight increase in GLUT-1 at the cell surface after stimulation by insulin, but this increase is not as dramatic as that for GLUT-4 (typically 3-fold for GLUT-1 compared to 12- to 15-fold for GLUT4; see REF). As shown in Figure 5.1, the increase in [^{3}H] 2-deoxy-D-glucose uptake is dependent on the concentration of insulin added. The EC_{50} for insulin-stimulated glucose uptake is between 0.5 and 3 nM.

As stated previously insulin resistance is associated with a decrease in insulin's ability to simulated glucose uptake via translocation of GLUT-4. Therefore it was decided to employ the uptake of [^{3}H] 2-deoxy-D-glucose after stimulation by insulin as an index of insulin responsiveness. In the experiments described in this chapter, we set out to examine the effects of sex-steroids on insulin responsiveness. Ultimately, we hoped to develop a

Figure 5.1

Dose-dependent insulin stimulated 2-deoxy-D-glucose uptake in 3T3-L1 adipocytes.

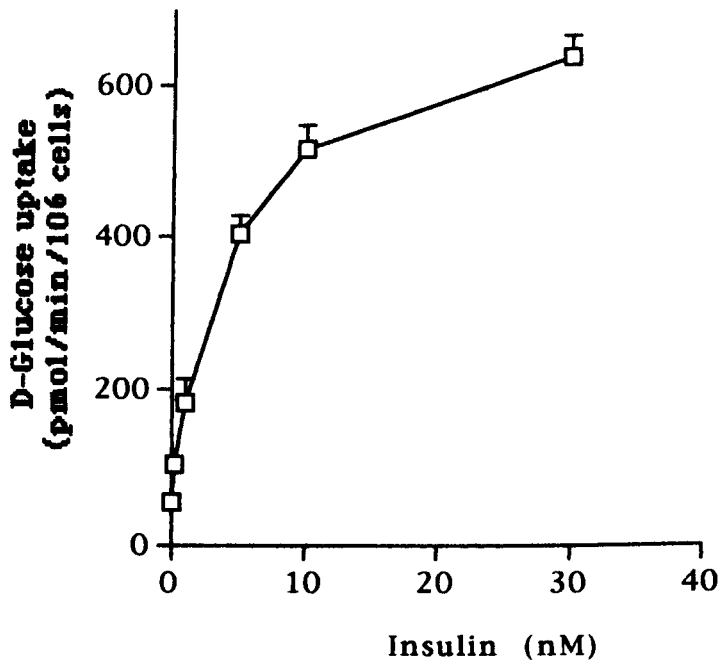


Figure 5.1

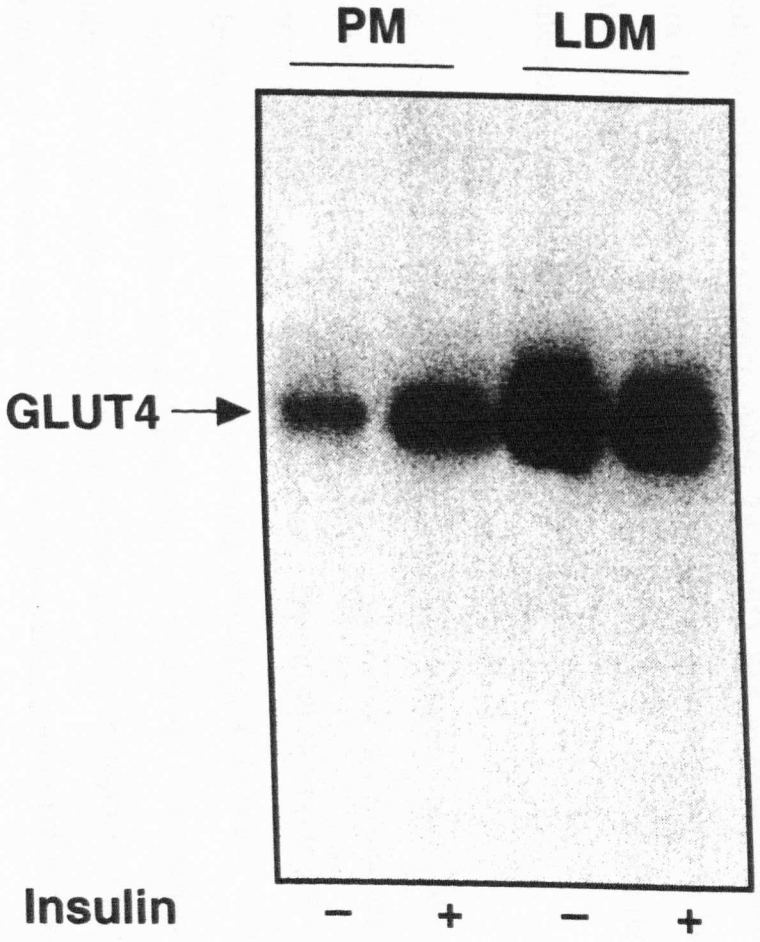
Insulin-stimulated 2-deoxy-D-glucose uptake in untreated 3T3-L1 adipocytes.

Insulin-stimulated 2-deoxy-D-glucose uptake in 3T3-L1 adipocytes was assayed as described in section 2.3.7 of this thesis. Insulin was added at the indicated concentration for 30 minutes prior to measurement of deoxyglucose transport. The data shown is from a representative experiment and was repeated four times with similar results.

Figure 5.2

Translocation of GLUT-4 to the plasma membrane from the low density microsomes following insulin stimulation.

This representative immunoblot depicts the level of GLUT-4 in the plasma membrane (PM) and low density microsomes (LDM) fractions of 3T3-L1 adipocytes, +/- insulin stimulation (1mM for 30 minutes). 25μg of protein for each fraction were separated by 10% SDS-PAGE and inmmunoblotted using anti-GLUT-4 antobdies as in accordance with section 2.6.


PM
LDM
GLUT4
Insulin
–
+
–
+

cell system with which to examine aspects of the mechanistic basis of insulin resistance.

Initial work performed by Garvey and co-workers demonstrated that exposure of primary cultured adipocytes to the glucocorticoid dexamethasone induced an inhibition of insulin-stimulated glucose transport [Garvey *et al.*, (1989)]. These studies concluded that 20 nM dexamethasone induced a progressive insulin resistance by acting at various stages of the insulin-stimulated glucose transport mechanism. Acute exposure (up to 2 hours) to dexamethasone impairs the ability of insulin to translocate intracellular GLUT-4 to the cell surface, resulting in a 35% decrease in maximal insulin-stimulated glucose transport. After 24 hours the dexamethasone-induced insulin resistance was shown to be the result of a 43% reduction in the total cellular GLUT-4 transporter level. In this instance the maximal insulin-stimulated glucose transport is reduced by 55%.

These workers also assessed whether the dexamethasone-induced insulin resistance is glucose dependent. The adipocytes were cultured in the presence and absence of dexamethasone or insulin in the presence of up to 20 mM D-glucose. Dexamethasone treatment produced a decrease in both basal and maximal insulin-stimulated transport (see above). In contrast, chronic insulin treatment was unable to decrease glucose transport activity in the absence of glucose, but led to a progressive, concentration dependent lowering of basal and maximal transport rates as the glucose level was raised to 20 mM. This data indicated that insulin-responsiveness can be impaired by at least two mechanisms, one glucose dependent and the other glucose-independent.

Finally, insulin can heterologously inhibit dexamethasone's effect on glucose transport at both early and late phases of desensitisation. Dexamethasone did not alter cell surface insulin binding and therefore the decrease in insulin sensitivity and maximal insulin responsiveness were due to effects distal to receptor binding. These studies reinforce the fact that the glucose transport system is subject to complex hormonal regulation.

We therefore set out to examine the effects of dexamethasone (as a control) and sex steroids, in the form of oestrogens, on insulin-stimulated glucose transport in 3T3-L1 adipocytes. Figure 5.3 shows that the chronic inhibition of insulin-stimulated glucose transport by dexamethasone is dose dependent with an IC_{50} of approximately 15 nM. These results are in good agreement with the data of Garvey et al. (1989).

Oestriol (E3) treatment of 3T3-L1 adipocytes for 48 h was observed to result in a profound decrease in cute insulin-stimulated glucose transport with an IC_{50} similar to that of dexamethasone (figure 5.4). In contrast, incubation of adipocytes with oestradiol (E2) for 48 h induced only a partial reduction in subsequent acute insulin-stimulated glucose transport. Furthermore, this inhibition was only observed at concentrations of greater than 30 nM (figure 5.5). Under these conditions, we observed a rightward-shift in the dose response curve for insulin-stimulated glucose transport (IC_{50} 3 nM *Vs* ~20 nM) (data not shown). Prolonged treatment with oestrogen (E1) (48 hours) also resulted in decreased insulin-stimulated glucose transport (IC_{50} 22 nM) (figure 5.6).

As, after steroid treatment, these cells displayed a decrease in the uptake of [^{3}H] 2-deoxy-D-glucose it was decided to examine the level of GLUT-4 expressed in sub-cellular fractions of the 3T3-L1 adipocytes.

Figure 5.3

Inhibition of insulin stimulated 2-deoxy-D-glucose uptake by dexamethasone.

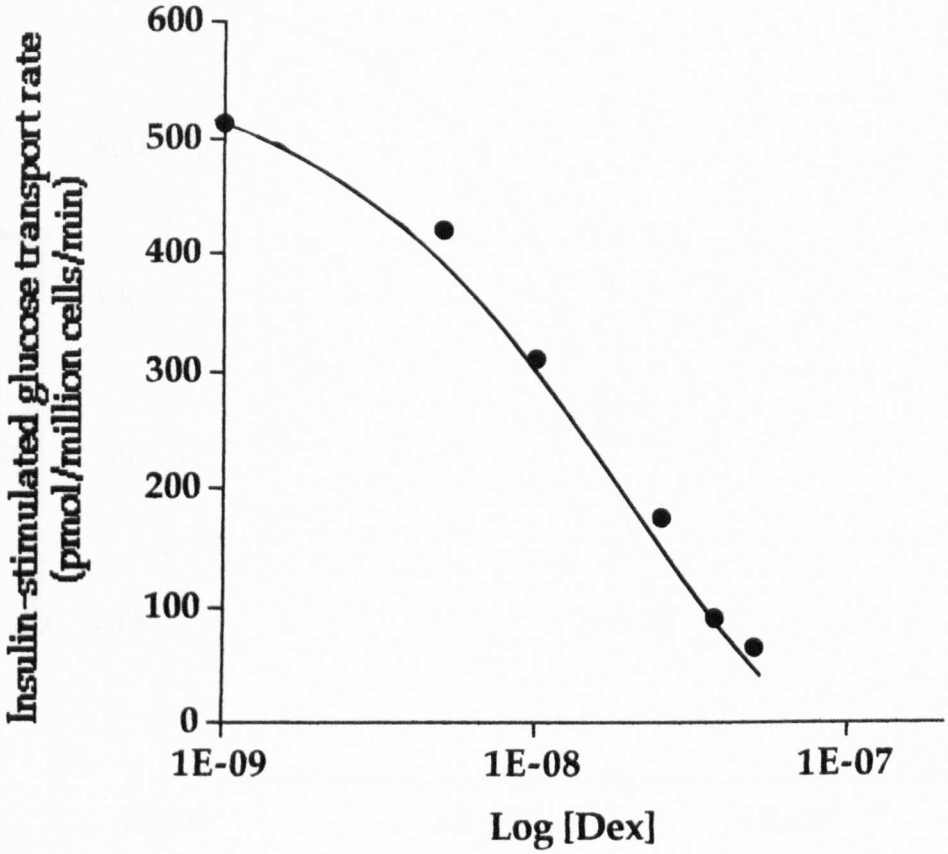


Figure 5.3

Inhibition of insulin stimulated 2-deoxy-D-glucose uptake by dexamethasone.

3T3-L1 adipocytes, at least 8 days post-differentiation, were treated with dexamethasone, at the concentrations shown, 48 hours prior assaying the cells for insulin-stimulated 2-deoxy-D-glucose uptake. This assay was carried out as in section 2.3.7 of this thesis on 3 occasions.

Figure 5.4

Inhibition of insulin-stimulated glucose transport by oestriol (E3) in 3T3-L1 adipocytes.

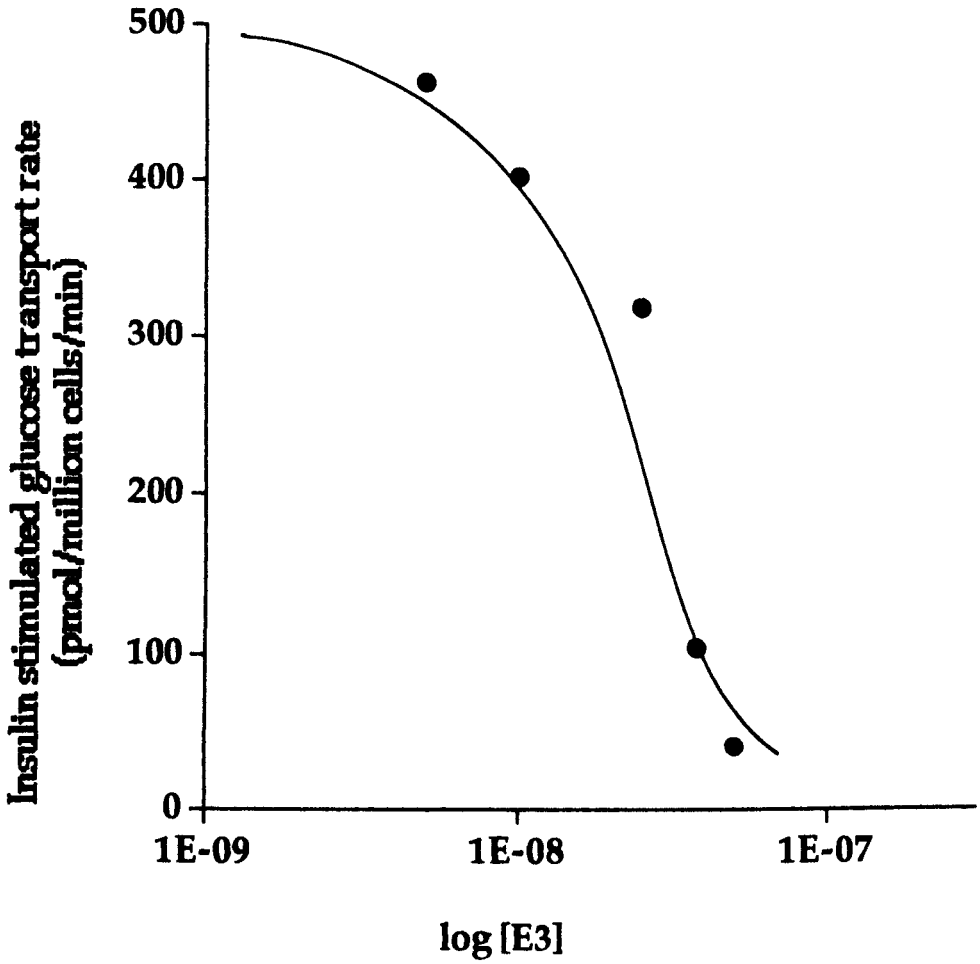


Figure 5.4

Inhibition of insulin stimulated 2-deoxy-D-glucose uptake by oestriol (E3).

3T3-L1 adipocytes, at least 8 days post-differentiation, were treated with E3, at the concentrations shown, 48 hours prior assaying the cells for insulin-stimulated 2-deoxy-D-glucose uptake. This assay was carried out as in section 2.3.7 of this thesis on 3 occasions.

Figure 5.5

Inhibition of insulin-stimulated glucose transport by oestradiol (E2) in 3T3-L1 adipocytes.

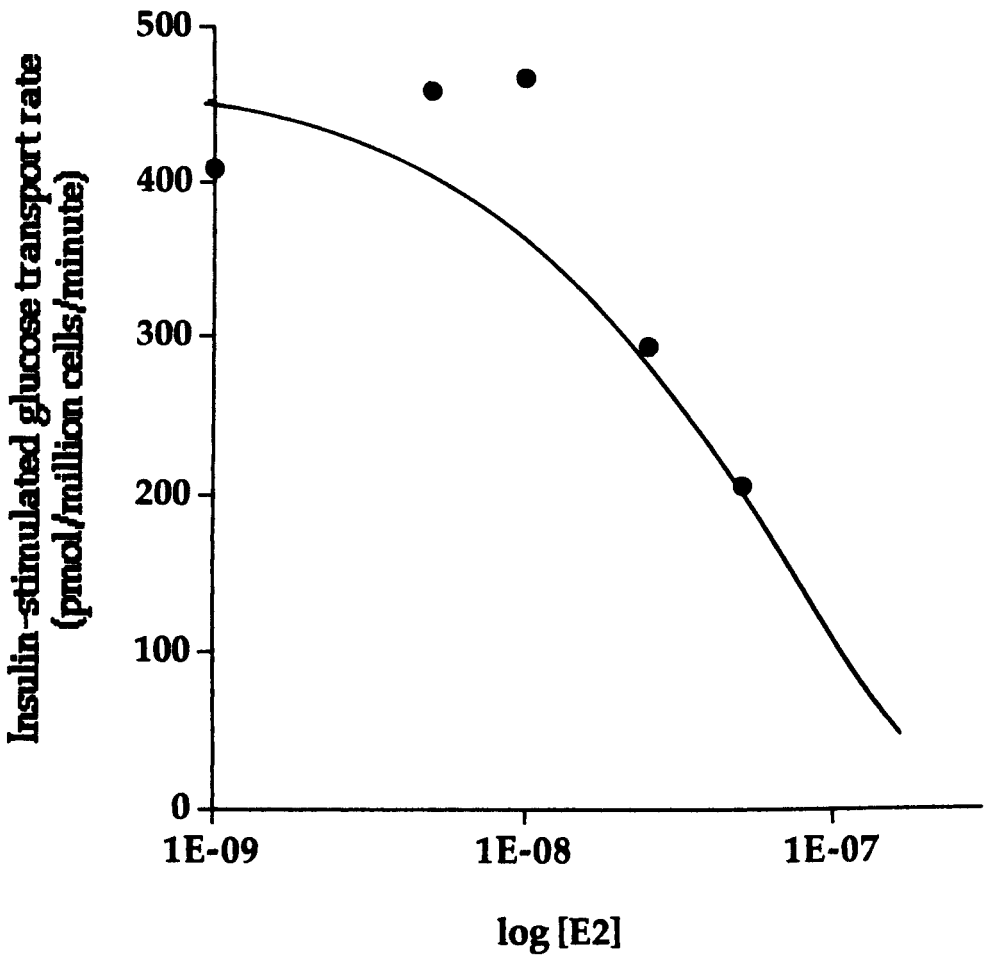


Figure 5.5

Inhibition of insulin stimulated 2-deoxy-D-glucose uptake by oestradiol (E2).

3T3-L1 adipocytes, at least 8 days post-differentiation, were treated with E2, at the concentrations shown, 48 hours prior assaying the cells for insulin-stimulated 2-deoxy-D-glucose uptake. This assay was carried out as in section 2.3.7 of this thesis on 3 occasions.

Figure 5.6

Inhibition of insulin-stimulated glucose transport by oestrogen (E1) in 3T3-L1 adipocytes.

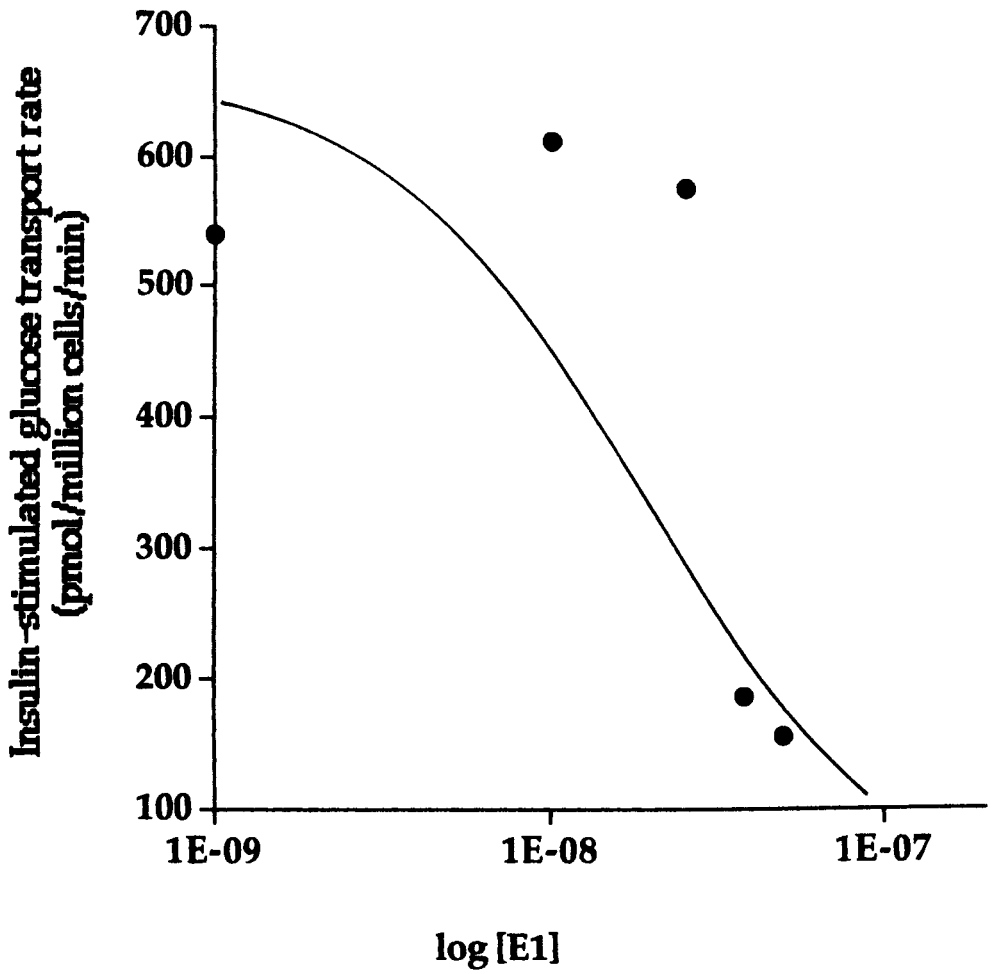


Figure 5.6

Inhibition of insulin stimulated 2-deoxy-D-glucose uptake by oestrogen (E1).

3T3-L1 adipocytes, at least 8 days post-differentiation, were treated with E1, at the concentrations shown, 48 hours prior assaying the cells for insulin-stimulated 2-deoxy-D-glucose uptake. This assay was carried out as in section 2.3.7 of this thesis on 3 occasions.

Such an analysis would be expected to reveal any decreases in total cellular GLUT-4 levels, or illustrate alterations in the subcellular distribution of GLUT-4. Figure 5.7 shows a representative immunoblot of GLUT-4 present in the low density microsomes (LDMs) of cells treated with either ethanol (vehicle control) or sex-steroids as indicated. The bands were then cut out of the nitrocellulose and γ-counted as described in section 2.6 of this thesis to allow quantification of the levels of GLUT-4 protein. Figure 5.8 illustrates that there is no decrease in GLUT-4 in the LDMs post steroid treatment.

Figure 5.7

An immunoblot illustrating the level of GLUT-4 present in low density microsomes of 3T3-L1 adipocytes.

Shown is a representative immunoblot of the 3T3-L1 adipocyte low density microsomes (LDM) fraction probed with antibodies against GLUT-4 post treament with the steroid indicated in the x-axis legend. 25μg of membrane protein were loaded on each lane. In this representative experiment 30nM E1, E2 and E3 were added for 48 hours. Eth represents ethanol as a vehicle control. Quantification of the data was as outlined and collated data is shown in figure 5.8.

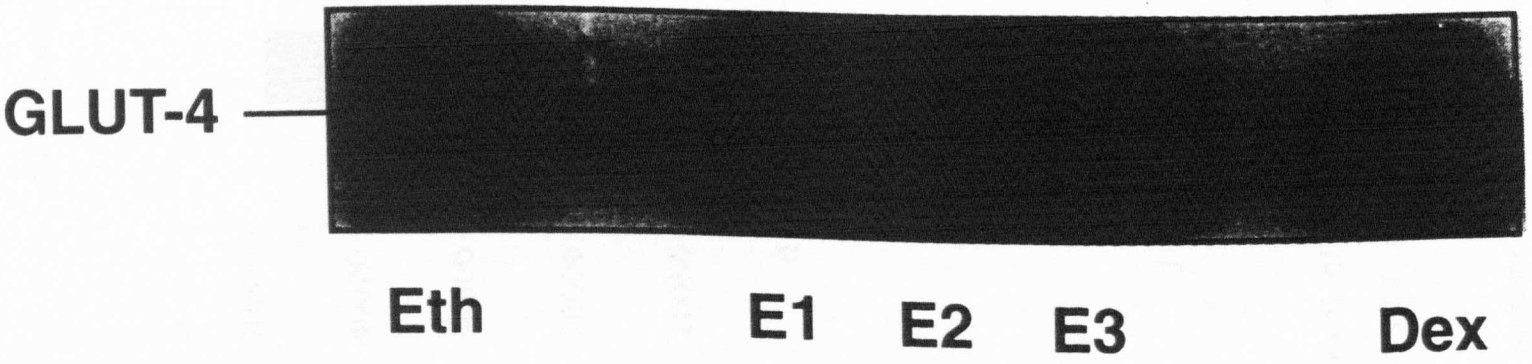

GLUT-4
Eth
E1
E2
E3
Dex

Figure 5.8

Relative level of GLUT-4 in low density microsomes of treated 3T3-L1 adipocytes.

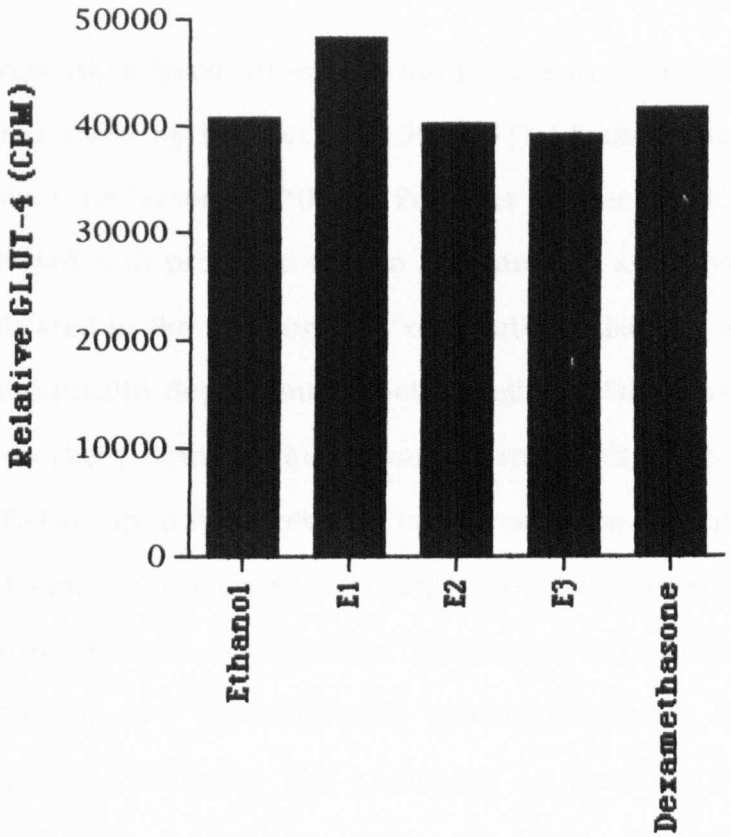


Figure 5.8

Relative level of GLUT-4 in low density microsomes of treated 3T3-L1 adipocytes.

Quantification of immunoblot data from figure 5.7.

5.5 Discussion

The induction of insulin resistance in 3T3-L1 adipocytes, by oestrogen and derivatives has generated a model which may be utilised to examine the aetiology of insulin resistance, such as that observed in post-menopausal women on hormone replacement therapy.

Other conditions have been shown to induce insulin resistance in 3T3-L1 adipocytes. In a study by Pilch et al. (1997), 3T3-L1 adipocytes were treated with tumour necrosis factor-α (TNF-α). Previous studies had identified TNF-α as being associated with profound insulin resistance in adipocytes, and TNF-α also been implicated in the development of insulin resistance associated with obesity and non-insulin dependent diabetes mellitus [Stephens et al. (1991)]. Pilch and co-workers demonstrated that a prolonged exposure of 3T3-L1 adipocytes to TNF-α (up to 96 h) resulted in the reduction in the levels of IRS-1 and GLUT-4 mRNA, and protein. Furthermore, the level of the insulin receptor is also reduced by between 30% and 50%. Interestingly, the remaining proteins are biochemically indistinguishable from those in untreated adipocytes. Despite the reduction in both GLUT-4 and other proteins, some degree of residual insulin-stimulated glucose transport and GLUT-4 translocation was still observed in TNF-α treated adipocytes. Pilch proposes that the reason for the observed insulin resistance is the reduction in the requisite proteins involved in insulin action. The insulin resistance caused by oestrogen treatment obviously occurs via an alternative mechanism as we see no reduction in the level of either GLUT-4 or PI3 kinase protein levels in comparison to untreated cells.

As has been stated many times during the course of this thesis, skeletal muscle is the most important insulin-sensitive system, accounting for

approximately 95% of total glucose disposal. Recently a study has been carried out looking at the mechanisms of glucocorticoid-induced insulin resistance in rat soleus muscle [Dimitriadis et al (1997)]. Male Wistar rats were injected with 0.5mg/day dexamethasone for 5 days prior to sacrifice. The soleus muscle was dissected and experiments demonstrated that dexamethasone decreased 3-*O*methylglucose transport, 2 deoxy-D-glucose phosphorylation, glycogen synthesis and glucose oxidation in response to insulin. However, the decrease in glucose transport is the not result of a decrease in the total GLUT-4 content as this remained unaltered in response to dexamethasone treatment. However, subsequent subcellular fractionation analysis showed that the insulin-stimulated increase in GLUT-4 at the plasma membrane was significantly diminished in dexamethasone treated animals compared to controls. These results suggest that glucocorticoid excess causes insulin resistance in skeletal muscle by directly inhibiting the translocation of the glucose transporter GLUT-4 to the plasma membrane following stimulation by insulin. It is possible that a similar mechanistic basis underlies the inability of insulin to stimulate glucose transport in adipocytes treated with steroids. The results obtained in these experiments highlight the limitation of using 3T3-L1 adipocytes. These cell can only be used 8-13 days post differentiation. It would be advantageous to study what effect the administering much lower doses of steroid for a longer period of time would have upon insulin-stimulated glucose transport. Furthermore, longer-term exposure to steroids at lower concentrations may induce the same responses observed in this chapter, but with more physiological relevance.

5.6 Future work

As stated throughout this thesis, and depicted in figure 1.6, there are many potential sites of insulin resistance, from the insulin receptor to mis-targeting of GLUT-4. Studies aimed at addressing each of these potential sites of lesion in the insulin-stimulated pathway of translocation would be useful. However, due to time constraints, it was not possible to complete such studies. Some of the experiments which could be undertaken are detailed below:

- It would be of interest to study the distribution of GLUT-4, after stimulation by insulin in order to determine if GLUT-4 is being translocated efficiently to the plasma membrane.
- Similarly, translocation of GLUT-4 containing vesicles to the plasma membrane but no subsequent fusion of these vesicles with the membrane should also be considered.
- Immunoblotting for members of the insulin signalling pathway to determine if one or more of these proteins are expressed at a lower level, therefore accounting for the reduction in insulin-stimulated glucose transport would be appropriate. Such proteins include PI-3 kinase, IRS-1 and c-AKT. Additionally, it is possible to assay for enzymes such as PI-3 kinase, this information would be invaluable in determining if such proteins function efficiently in treated adipocytes..

Another issue we would like to address is the exposure of lower concentration of steroids for a longer period of time. At the moment the concentration of oestrogen used is much higher than the physiological concentration used in the contraceptive pill and HRT, and is also a pharmacological dose, rather than a physiological one. Unfortunately, because cultured adipocytes have an effective window of only 4 days during which steroid administration can

be administered, it has not been possible to examine the effect of multiple lower doses of steroid (for example). This situation would be a more accurate reflection of the situation faced by patients on HRT.

Chapter 6

Overview

Insulin is the main anabolic and anti-catabolic hormone in mammals. In fulfilling this role, insulin induces large modification in carbohydrate, lipid and protein metabolism in hepatic, muscle and adipose tissues. Insulin resistance is said to occur whenever normal concentrations of insulin produce a subnormal biological response. Most commonly associated with insulin resistance is a decrease in glucose uptake, especially in adipose and more importantly skeletal muscle. It is important to note that insulin resistance may only involve one tissue within a mammal, with all other tissues responding normally to insulin stimulation. Furthermore, insulin resistance may affect pathways other than those of glucose uptake, such as lipid or potassium uptake, or sodium retention, which are other important actions of insulin.

Severe insulin resistance can lead to non-insulin dependent diabetes mellitus (NIDDM) in individuals where both fasting and postprandial plasma glucose levels are elevated. The fact the NIDDM, obesity and primary hypertension often occur together in the clinical situation implies that there may be some common factors in the underlying pathology of these conditions [Livingstone *et al.*, (1995)]. In general hypertensive subjects have a degree of insulin resistance. Furthermore, the insulin resistance which accompanies hypertension is distinct from the in obesity and NIDDM in that it only affects glucose uptake and subsequent glycogen synthesis, and is limited to peripheral, insulin-sensitive tissues. Most evidence suggests that insulin resistance precedes hypertension, this strongly suggesting that metabolic abnormalities contribute to the development of hypertension. Although many theories have been postulated, the mechanism of this remains unclear [Donnelly & Connell (1992)].

This thesis attempted to examine the reduction in insulin-stimulated glucose transport in the hypertensive Milan rat, and after identified a reduction in GLUT-4 in the LDMs of skeletal muscle. This discovery may explain the insulin resistance experienced by this rodent model. There may be additional defects in the insulin receptor signalling pathway that also contribute to the disease state in these animals. In contradiction to this the SHRSP rat strain shows no reduction in GLUT-4 levels in skeletal muscle or adipose tissue. This rodent model of hypertension is thought to best mimic human essential hypertension, therefore the results are not surprising, as a reduction in GLUT-4 is not commonly found in insulin resistant, hypertensive patients. An, as yet unidentified, alternative mechanism of insulin resistance produces the disease state in these animals. Due to the constraints of time and more importantly animal tissue we were unable to further elucidate the location of this defect. It is our belief that the many enzymes involved in the insulin receptor signalling pathway may be site of fruitful research in the forthcoming years.

Finally, we decided to examine the role of steroid hormones on insulin resistance in 3T3-L1 adipocytes. This was done based on the fact that women on steroid treatment, either by means of the contraceptive pill, or on HRT, became insulin resistant. Previous studies had shown that glucocorticoids induce insulin resistance in this cell line. Indeed, after 48 hours of treatment with oestrogen, or derivatives, the cells showed a reduction in insulin-stimulated 2-deoxy-D-glucose uptake. This demonstrated that the cells had become insulin resistant. Immunoblotting for GLUT-4 in sub-cellular fractions of the cells showed that there was no reduction in GLUT-4 protein levels. Once again the mechanism of this insulin resistance remains unclear. Further work on the proteins involved in insulin receptor signalling may again prove. In this case it is possible to measure the activity of PI-3 kinase, a

key enzyme in the signal transduction process. Additionally, it may be beneficial to determine the efficiency of GLUT-4 translocation from the intracellular membranes to the cell surface. A defect in the translocationary machinery, may also result in the reduction of insulin-stimulated glucose uptake.

It is clear from this study that insulin resistance is condition that arises in many different and unrelated disease states. Although the mechanism of insulin resistance is known in some cases, it is apparent that there are a variety of mechanisms that can induce insulin resistance, affecting many different proteins, at the level of both transcription and translation.

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