Insulin signalling in insulin resistance and cardiovascular disease syndromes

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Abstract

Although the relationship between insulin resistance and cardiovascular diseases is well established, nature and mechanism of the insulin resistance in peripheral tissues is unclear.

In this thesis I have demonstrated that a rodent model of genetic hypertension, the stroke-prone spontaneously hypertensive rat (SHRSP), displays resistance to the effects of insulin, characterised by defects at the level of both carbohydrate and lipid metabolism in primary epididymal adipocytes. Insulin-stimulated 2-deoxy-D-glucose uptake is reduced SHRSP compared to Wistar-Kyoto (WKY) control, and there is a profound reduction in the ability of insulin to inhibit isoprenaline-mediated lipolysis.

The SHRSP is a relative of the SHR, which develops a similar though not identical phenotype. The SHR defect has recently been attributed to aberrant expression of Cd36, a putative fatty acid transporter in adipocyte membranes. Thus, Cd36 has emerged as a potentially important link in cardiovascular and insulin resistance syndromes. Interestingly however levels of Cd36 mRNA and protein in SHRSP adipocyte membranes are comparable to those in WKY normotensive controls. This suggests that other molecules may contribute to the development of the SHRSP phenotype.

The potential for aberrant insulin signalling to contribute to the development of insulin resistance is also apparent in other syndromes such as Polycystic Ovarian Syndrome (PCOS). To that end I studied the effects of sex hormone treatment in 3T3-L1, as sex hormone levels are elevated in PCOS. In sex hormone treated 3T3-L1 adipocytes there is a down-regulation of IRS-1, IRS-2 and PI3'K. There is also a re-distribution of IRS-1 and IRS-2 from the membrane fraction to the cytosol in sex hormone treated cells. This type of movement has previously been implicated in the development of cellular insulin resistance, and indeed there is resistance to the effects of insulin to stimulate GLUT4 translocation and glucose uptake

in these cells. This is independent of any change in expression of GLUT4, although insulin-stimulated translocation is dramatically reduced. This data suggests a potential mechanism by which insulin resistance could develop in syndromes, such as PCOS, where adipocytes are exposed to an abnormal balance of sex steroids.

Assessing the contribution of insulin signalling to the development of insulin resistance in different disease states will be useful at several levels. At the most basic level it will reveal more about the functions of specific proteins, and importantly the diseases which result when these proteins are abnormally regulated. This will present new avenues for therapeutic treatment, aimed at both correcting apparent defects, or by alternative mechanisms which could by-pass dysfunctional signalling pathways.

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This thesis is dedicated to my late father, Mr John Collison, and to my mother, Mrs Mary Collison

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Abbreviations

ARF	ADP ribosylation Factor
ATP	Adenosine 5'-triphosphate
BN	Brown Norway Rat
BSA	Bovine Serum Albumin
cAMP	adenosine 3', 5'-cyclic monophosphate
cDNA	Complementary deoxyribonucleic acid
CHO	Chinese Hamster Ovary
cpm	Counts per minute
DeGlc	2-Deoxy-D-glucose
DFP	Diisopropyl fluorphosphate
DMEM	Dulbecco's modified Eagles medium
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
E1	Oestrogen
E2	Oestradiol
E3	Oestriol
ECL	Enhanced chemiluminescence
EDTA	Diaminoethanetetra-acetic acid, disodium salt
ER	Endoplasmic reticulum
E64	L-transepxoysuccinyl-leucylamido-4-guanidinobutane
FCS	Foetal calf serum
FSH	Follicle stimulating hormone
GLUT	Glucose transporter

GTP	Guanosine 5'-triphosphate
HEPES	*N*-2-hydroxyethylpiperazine-*N*-2'ethane sulphonic acid
HRP	Horseradish peroxidase
IBMX	Isobutylmethylxanthine
IDDM	Insulin-dependent diabetes mellitus
IgA	Immunoglobulin alpha
IgG	Immunoglobulin gamma
IRS-1	Insulin Receptor Substrate 1
IRS-2	Insulin Receptor Substrate 2
IRS-3	Insulin Receptor Substrate 3
IRS-4	Insulin Receptor Substrate 4
kDa	kilodaltons
KRP	Krebs Ringer Phosphate
LDL	Low density lipoprotein
LDM	Low density microsome
LH	Luitenizing Hormone
MAP kinase	Mitogen-activated Protein kinase
mA	milliamps
NCS	Newborn calf serum
NIDDM	Non-insulin-dependent diabetes mellitus
NSF	*N*-ethylmaleimide sensitive factor
PAGE	Polyacrylamide gel electrophoresis
PCOS	Polycystic Ovarian Syndrome
PDGF	Platelet Derived Growth Factor
PDGFR	Platelet-Derived Growth Factor Receptor

PH	Pleckstrin homology
Pi	inorganic phosphate
PI3'K	Phosphatidylinositide 3'kinase
PI	Phosphatidylinositol
PIP_2	Phosphatidylinositol 4,5-bisphosphate
PIP_3	Phosphatidylinositol 3,4,5-triphosphate
PM	Plasma membrane
PKA	cAMP-activated Protein kinase
PKB	Protein kinase B
PKC	Calcium and phospholipid-dependent Protein kinase
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SHR	Spontaneously Hypertensive Rat
SHRSP	stroke-prone Spontaneously Hypertensive Rat
SH2	Src homology 2
SNAP	Soluble NSF attachment protein
SNAP25	Synaptosome-associated 25kDa protein
SNARE	SNAP receptor
SP	Soluble protein
TBST-1	Tris buffered saline Tween
TEMED	*N,N,N',N'*-tetramethylenediamine
Tris	Tris(hydroxymethyl)aminoethane
VAMP	Vesicle-associated membrane protein
v/v	volume/volume ratio
w/v	weight/volume ratio

WKY	Wistar-Kyoto

List of Amino Acids

Amino acid residue	Three letter code	Oneletter symbol
Alanine	Ala	A
Cysteine	Cys	C
Aspartate	Asp	D
Glutamate	Glu	E
Phenylalanine	Phe	F
Glycine	Gly	G
Histidine	His	H
Isoleucine	Iso	I
Lysine	Lys	K
Leucine	Leu	L
Methionine	Met	M
Asparagine	Asp	N
Proline	Pro	P
Glutamine	Gln	Q
Arginine	Arg	R
Serine	Ser	S
Threonine	Thr	T
Valine	Val	V
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Any amino acid		X
Any amino acid with a phosphate attached		Xp

Publications

Collison M, Glazier AM, Aitman TJ, Scott JR, Graham D, Morton JJ, Dominiczak MH, Connell JMC, Dominiczak AF and Gould GW. Cd36 and the molecular mechanisms of insulin resistance in the stroke prone spontaneously hypertensive rat. Diabetes (in press).

Collison M, Glazier AM, Graham D, Morton JJ, Dominiczak MH, Aitman TJ, Connell JMC, Dominiczak AF and Gould GW. Cd36 and the molecular basis of insulin resistance in the SHRSP. Diabetes 2000, volume 49 SUPP 1, A211

James D, Collison M, Salt IP, Dominiczak AF, Connell JMC and Gould GW. 2000. Insulin- and AICA Riboside-stimulated glucose transport in primary muscle cultures from control and insulin resistant rats. Diabetes 2000, volume 49 SUPP 1, A240

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1 General Introduction

1.1 Glucose as an energy source

To function properly the human body requires consumption of adequate amounts of the three main food groups: proteins (composed of a variety of essential and non-essential amino acids), fats, and carbohydrates. Although all of the food sources are able to provide energy for both storage purposes and immediate use, carbohydrates are important as they represent the only source of anaerobic non-oxidative energy. The main product of carbohydrate breakdown is glucose and in aerobic organisms energy is generated from this molecule through glycolysis. Glycolysis plays a central role in energy metabolism by providing a substantial portion of the energy consumed by most organisms. Due to this ability to provide energy, glucose is the preferred source of energy for many tissues of the body. This is discussed below (Chapter 27, Zubay, 1998).

1.2 Glucose metabolism in different tissues

1.2.1 Glucose and the brain

The necessity for glucose as an energy source is perhaps best illustrated in the brain where there is an absolute requirement for glucose, which varies little whether active or at rest. Indeed blood glucose concentrations falling to less than half the optimum 5mmoles per litre can result in severe brain dysfunction, where subjects will exhibit symptoms such as lack of coordination and impairment in concentration. Only in situations of extreme starvation will the brain resort to utilising the one other energy source available to it, ketone bodies. Ketone bodies, which are essentially soluble forms of fatty acids, enter the circulation from the liver where they are generated in a process known as ketogenesis. Due to their solubility they are able to cross the blood brain barrier, unlike normal fatty acids which are unable to do this due to their structure. The brain is therefore

able to metabolise them and generate energy from them. This, however, is always a short term energy source and as soon as glucose becomes readily available brain metabolism of ketone bodies discontinues (Chapter 27, Zubay, 1998.).

1.2.2 Glucose and skeletal muscle

Muscle is not so selective as brain regarding its energy supply, and is able to utilise glucose (predominantly in the storage form, glycogen), fatty acids and ketone bodies as energy sources. Nevertheless skeletal muscle still represents a main glucose sink, and a large percentage of circulating glucose is taken up by this tissue. As soon as glucose enters muscle it is rapidly converted to the main storage carbohydrate; glycogen, and up to 2% of a resting well-fed muscle normally constitutes glycogen stores. This glycogen is readily utilisable within the muscle as an energy source as it can be converted into glucose-6-phosphate, and subsequently glycolysis. Muscle is however unable to carryout gluconeogenesis, as it lacks glucose-6-phosphatase, the enzyme necessary for hydrolysis of glucose-6-phosphate. Nevertheless muscle is able to act as an efficient energy reservoir when required in severe starvation, by the mechanism outlined over-page in Figure 1.1 (Chapter 27, Zubay, 1998.).

1.2.3 Glucose and adipose tissue

Adipose tissue, like skeletal muscle, is an important energy storage depot, and is mostly located just under the skin in the abdominal cavity, around skeletal muscle and in the mammary glands. The importance of fat in this regard is perhaps best demonstrated by the fact that an average 70 kg man will contain around 15kg of his total weight in fat, an amount sufficient to maintain life for around 3 months. Nevertheless fat does not function simply as a passive storage depot. The main storage components of adipose tissue are triacylglcerides (TAG), which are synthesised from fatty

acids obtained either from the liver or the dietary intake (Chapter 27, Zubay, 1998.). These TAG's are rapidly mobilised by hydrolysis into non-esterified fatty acids (NEFA's), and can be secreted into the circulation in response to a variety of lipolytic hormones, such as catecholamines. This release of fatty acids is nevertheless also closely coordinated with the carbohydrate energy available. If glucose is abundant in the blood then a percentage of it will enter the adipocyte, and then be converted to glucose-6-phosphate as in other tissues. This glucose-6-phosphate in a sense serves as meter to indicate whether the mobilisation of fatty acids into the circulation is necessary, and should levels of glucose-6-phosphate be low then more fatty acids are released (Chapter 27, Zubay, 1998.).

Thus the importance of glucose as an energy source is clearly illustrated in these three tissue types which are the main consumers of glucose, and indeed these tissues all play an important role in maintaining the energy balance of the whole body. However one other tissue plays a crucial role in energy, particularly glucose, homeostasis and that is the liver.

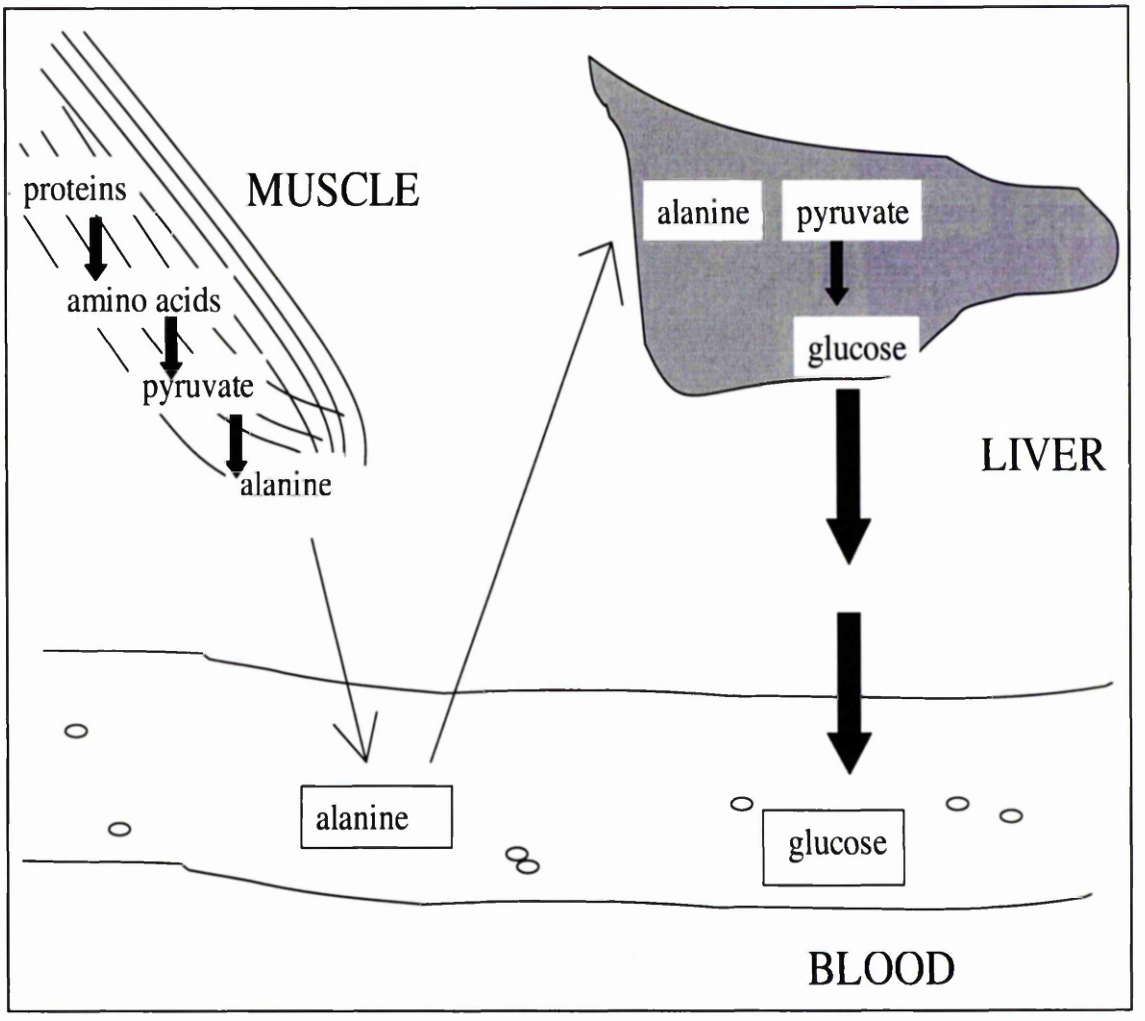


Figure 1.1 Indirect generation of glucose from Muscle Protein Stores

adapted from (Chapter 27, Zubay, 1998)

1.2.4 Glucose and the liver

In common with the tissues described above, the liver has the ability also to utilise and store glucose. Importantly however, unlike the other tissue types, liver can also generate glucose *de novo*, which can be rapidly mobilised into the blood stream when required. The liver is ideally situated for this dual function, served by the portal vein which carries all of the nutrients (except fatty acids) absorbed from the intestines into the blood stream. In the fasted state, where few nutrients reach the liver and the blood glucose is hence low, a concerted action involving the liver and the pancreas results in secretion of glucose into the blood stream. Hypoglycaemia results in the stimulation of pancreatic α cells to secrete the hormone glucagon. This glucagon travels through the blood stream to the liver, where it acts on glucagon receptors to stimulate the cAMP-mediated breakdown of glycogen. During hepatic glycogenolysis, the glycogen is broken down into glucose-1-phosphate and subsequently converted to glucose-6-phosphate. In the liver, unlike other tissues, this glucose-6-phosphate generated by glycogenolysis can be hydrolysed by a specific glucose-6-phosphatase, resulting in the generation of glucose. The presence of this hormone is central to the unique function of the liver, as it represents the means by which free glucose can be generated and re-directed back into the circulation. Obviously this then lets available glucose be directed to the tissues which require it for their survival (Chapter 27, Zubay, 1998.).

As mentioned above the liver can also take glucose up from the blood stream when required. However this process is always secondary to the consumption of glucose by the main energy consumers, brain, muscle and adipose tissue. This is achieved by the unique properties of the variant of hexokinase expressed in liver cells- glucokinase. Hexokinase enzymes in most cell types have a high affinity for glucose, with a K_m of less than 0.1mM. These enzymes are also inhibited by accumulation of the reaction product, glucose-6-phosphate. The liver glucokinase, however, is unique in that it has a much lower affinity for glucose, with a K_m of 5mM. In

other words the rate of transport of glucose varies proportionally with the blood glucose level over the physiological range. In contrast to the other main consumers of glucose, liver cells are less sensitive to the actions of insulin and freely permeable to glucose. Thus the liver will not consume glucose until the requirements of muscle and adipose have been saturated. Only then will it serve to 'mop-up' the excess and convert it to glycogen (Chapter 27, Zubay, 1998).

1.3 Hormonal Control of glucose metabolism

Although there is a variety of hormones that will affect how glucose is used within the body, the main control comes from the hormones glucagon and insulin. As discussed above, glucagon is released from the pancreatic α cells in response to low blood glucose concentrations, and in turn stimulates the breakdown of glucose in the liver and subsequent release into the blood stream. In the fed state however, when circulating glucose levels are high, it is necessary to promote the uptake of glucose into tissues. It has already been discussed above how this is mediated in the liver but in the other target tissues such as muscle and adipose, this process is stimulated by the action of the hormone insulin. High blood glucose stimulates the release of insulin from pancreatic β cells, and this promotes the uptake of glucose primarily into fat and muscle cells. Although many of the actions of glucagon can be partly compensated by other hormones such as catecholamines and cortisol, the actions of insulin are unique and therefore understanding the action of this hormone at the cellular level are very important. This is discussed in more detail below (Chapter 27, Zubay, 1998).

1.4 Insulin action at the cellular level

1.4.1 Glucose transporters and facilitative transport

As discussed in the beginning of this chapter, a primary biological function of insulin is to promote glucose uptake into tissues that are dependent on it for survival. Although monosaccharides can enter cells via diffusion or active transport, these processes are generally too slow to meet the energy requirements of glucose utilising tissues. For this reason glucose uptake into tissues is mediated by a family of facilitative glucose transporters (Gould, 1997).

Each of these mammalian glucose transporters are similar in basic structure, containing twelve alpha-helical trans-membrane spanning domains with both the carboxy and amino termini extending into the cytoplasm. This folded structure generates 6 exo-facial loops, with the first loop being largest and containing an asparagine-linked glycosylation site, and five endofacial loops including a large central hydrophillic loop (Hunter and Garvey, 1998).

To date ten transporter isoforms have been identified and named GLUT's 1-10. However the product of the GLUT6 gene was found to be a pseudo-gene soon after its discovery. Therefore no functional GLUT6 protein is expressed (Gould, 1997). The GLUT5 transporter isoform is unique in that, unlike the other transporters, it is a specific fructose transporter. GLUT's 8, 9 and 10 have only recently been discovered, and thus they are still being characterised and assigned to specific cellular functions (Hunter *et al.*, 1998; Carayannopoulous *et al.*, 2000; Doege *et al.*, 2000). Therefore, at least five transporter isoforms exist which are able to selectively promote glucose uptake into specific cell types in response to the appropriate physiological signals. Table 1.1 below illustrates the kinetic properties, tissue distribution and important functions of each glucose transporter.

Transporter Isoform	Tissue expression	Transports	Characteristics	Homology to GLUT1
GLUT1	placenta, brain, blood-tissue barrier, adipose, skeletal muscle, cardiac muscle, tissue culture cells, transformed cells	D-glucose D-galactose	basal energy requirements	100%
GLUT2	liver, pancreatic beta cell, kidney proximal tubule, basolateral membranes of small intestine	D-glucose D-fructose	high Km transporter	56%
GLUT3	brain and nerve cells in rodents, brain, nerve, placenta humans, liver, heart	D-glucose D-galactose	low Km transporter found in tissues metabolically-dependent on glucose	64%
GLUT4	adipose, skeletal muscle, cardiac muscle	D-glucose	insulin-stimulated glucose transport in insulin-sensitive tissues	65%
GLUT5	small intestine, kidney, testis, adipose, muscle, brain, small intestine, kidney (rat)	D-fructose	high affinity for fructose	42%
GLUT6	pseudogene	NA	NA	
GLUT7	Cloning artefact	NA	NA	
GLUT8	pre-pubertal testes (absent in testicular cancer), low amounts in insulin-sensitive tissues	D-glucose	probably involved in gonadotrophin control, possibly involved in glucose transport in mammalian embryo's	29%
GLUT9	spleen, leukocytes, brain	D-glucose	probably involved in tissue-specific roles	28.5%
GLUT10				37.1%

Table 1.1 The mammalian facilitative glucose transporters

adapted from Gould, 1997; Hunter and Garvey, 1998; Carayannopoulous *et al.*, 2000; Doege *et al.*, 2000

Within insulin responsive tissues (predominantly adipose and skeletal muscle) GLUT1 and GLUT4 are the predominantly expressed isoforms, each transporter performing a unique role. In the basal state almost 90% of GLUT1 is located at the plasma membrane, where it is responsible for maintaining basal glucose uptake (Gould, 1997; Hunter and Garvey; 1998). Under such conditions glucose transport is the rate-limiting step for glucose utilisation.

In contrast, the GLUT4 isoform is located primarily intracellularly in the basal state. Upon insulin stimulation however vesicles containing GLUT4 are re-distributed from this intracellular site and translocate to the plasma membrane where they are able to facilitate the entry of large amounts of glucose into the cell (Gould, 199; Holman and Kasuga, 1997). The precise molecular events and specific regulatory molecules involved in this process are still controversial. Some of the current ideas are discussed in Section 1.5.

Before insulin-stimulated glucose transport, and indeed any of the other biological effects of insulin, can occur it is necessary to activate a cascade of signalling proteins. These proteins are responsible for transducing the signal of insulin binding to its receptor to the inside of the cell. Although insulin is able to promote a variety of different cellular effects, many of the initial signalling proteins involved form part of more than one pathway. These early signalling steps will be discussed with regard to insulin signalling in general. The molecules involved in the specific processes of glucose transport, anti-lipolysis and the mitogenic effects of insulin in adipocytes will then be discussed with regard to each process.

1.4.2 Insulin Receptor

The insulin receptor is expressed relatively ubiquitously among tissues, although the classically insulin responsive-tissues express a much higher number of receptors. The receptor itself is synthesised as a single peptide

from a gene located on chromosome 19 (Gould, 1997). The individual 'pro-receptor' subunits generated from this gene are cleaved and glycosylated, where-upon they are joined to another identical subunit by disulphide bonds. This results in the generation of a hetero-tetrameric protein consisting of two alpha and two beta subunits (Gould, 1997). This structure is illustrated below in Figure 1.2.

Like other growth factor receptors, the hetero-tetrameric insulin receptor possesses ligand-activated intrinsic tyrosine kinase activity. In other words, binding of an insulin molecule to the extra-facial alpha subunit activates the intrinsic tyrosine kinase activity within the kinase domains of the beta subunits (Gould, 1997; Holman and Kasuga, 1997). This kinase activity results initially in the rapid tyrosine auto-phosphorylation of the beta subunits on specific tyrosine residues located within specific recognition motifs (Holman and Kasuga, 1997). The importance of the tyrosine kinase activity of the receptor is perhaps best illustrated by studies involving kinase inactive (KI) mutants. Using a rat adipocyte transfection system, Quon *et al* illustrated that transfection of wild type insulin receptors caused an enhancement in basal GLUT4 translocation and glucose transport, a main biological effect of insulin in adipocytes. However, when a KI mutant was similarly expressed there was a lack of GLUT4 translocation. Therefore, the kinase activity of the receptor is clearly intrinsic to its biological action (Quon *et al.*, 1994). With regard to ligand-induced tyrosine phosphorylation, it is emerging that phosphorylation on different tyrosine residues is important in mediating the different biological effects of insulin, presumably a reflection of the preferred interaction of down-stream effectors with differentially located tyrosine residues. Current studies suggest that mutation of the C-terminal tyrosine residues 1316 and 1322 has little effect on glucose transport, but shifts the dose response to the mitogenic effects (based on insulin-stimulated Ras activation) to the right by 5- 10 fold (Holman and Kasuga, 1997). This is in contrast to previous studies that reported a reduced metabolic signalling capacity of C-terminally truncated insulin receptors. Indeed C-terminal truncations do show a significantly defective

phosphorylation, but this appears less to affect the ability of the receptor to phosphorylate down-stream proteins, and more a reflection on ability to auto-phosphorylate (Holman and Kasuga, 1997). Divergence in signalling at the level of the insulin receptor is also evident in studies of mutations in the juxta-membrane region, with a tyrosine to alanine mutation at position 960 showing differential impairment in activation of downstream signalling molecules involved in different processes (Chaika *et al.*, 1999).

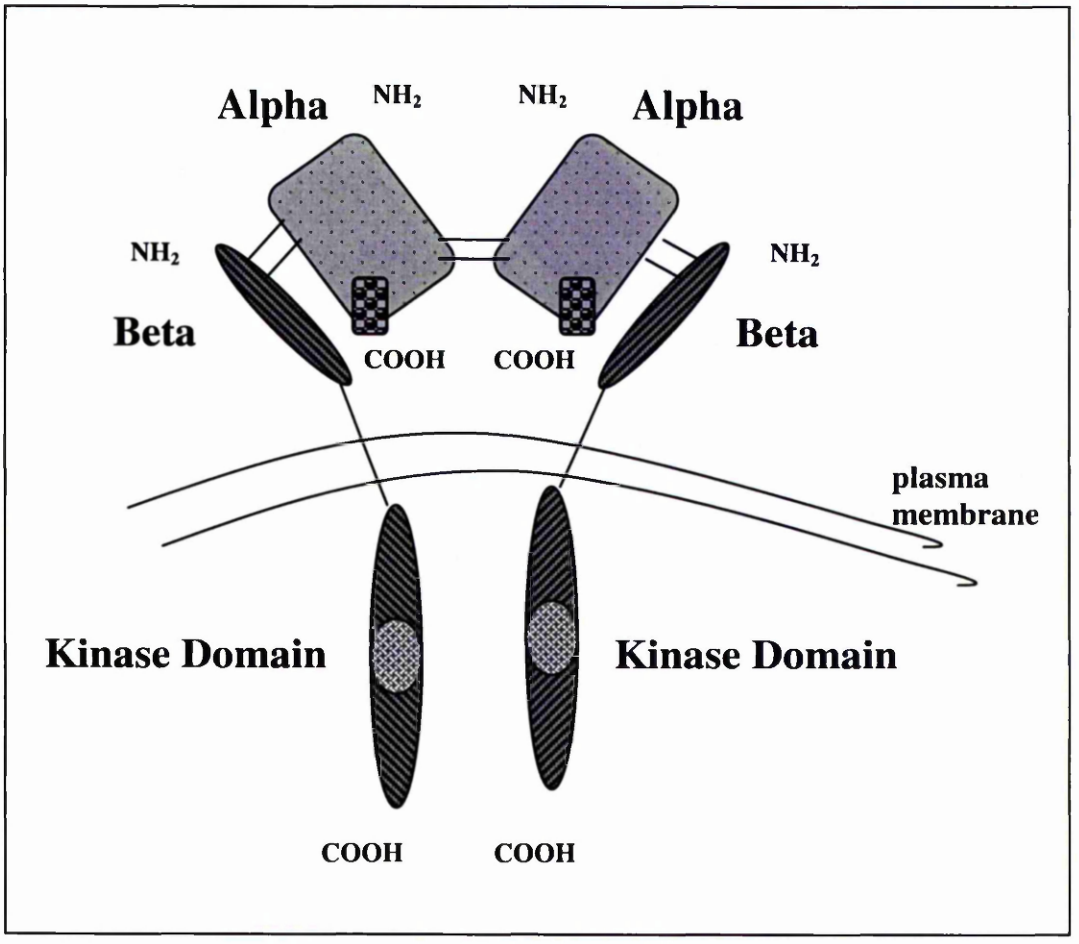


Figure 1.2 The insulin receptor

adapted from Gould, 1997

Therefore, we can see clearly how the kinase activity of the insulin receptor is very important for its function and, importantly, how the regulation of this kinase activity allows for selectivity and divergence at this level. A similar scenario is seen with other growth factor receptors, such as the PDGF receptor where many of the residues by which specific enzymes and proteins interact have been effectively mapped through mutation studies (Fantl *et al.*, 1992). Nevertheless signalling through the insulin receptor does vary from the general model of growth factor effector interactions. Although activation of the insulin receptor as described above generates potential sites where proteins such as PI3'K or Grb2 could interact, these interactions are much weaker than with other growth factor receptors (Malarkey *et al.*, 1995; Gould, 1997). Instead, the insulin receptor utilises adaptor proteins known as insulin receptor substrate (IRS) proteins which, as the name suggests, form a link between the phosphorylated insulin receptor and downstream signalling intermediates (Malarkey *et al.*, 1995). This is discussed in Section 1.4.3.

Signalling through the insulin receptor is unique from other growth factor receptors in one other important respect. Over the last few years, the importance of specific cellular location in insulin action has been revealed, and the one important protein identified with regard to insulin receptor function is caveolin (Yamamoto *et al.*, 1998). Caveolin is a major structural protein of caveolae, of which there are 3 sub-types, caveolins-1, -2 and -3. Caveolae are cell-surface invaginations, distinct from clathrin-coated pits, which have recently been shown to function as sub-cellular compartments to which specific signalling processes locate (Yamamoto *et al.*, 1998). This has been illustrated with a variety of signalling molecules such as the PDGF receptor and to date, the majority of interactions seem to be inhibitory to receptor signalling. The interaction with the insulin receptor, however, appears unique. Indeed Yamamoto's group illustrated that through binding to the scaffolding regions of Caveolin-1 or –3, the kinase activity of the insulin receptor towards downstream substrates such as IRS-1 is dramatically increased. This occured independently of any increase in auto-phosphorylation of the receptor. The precise mechanism

of this caveolin-induced enhancement in kinase activity is unclear. The interaction is thought to be of functional significance and caveolin functions somehow to localise and enhance signalling by the insulin receptor. Expression of caveolin is enhanced during adipocyte differentiation, in common with many of the components of the insulin signalling pathway (Kandror, 1995, Baumann *et al.*, 2000), and this therefore supports a positive role for the protein in insulin signalling.

1.4.3 Insulin Receptor Substrates

1.4.3.1 IRS-1

IRS-1 was the first IRS protein to be discovered. It was identified as a 185kDa protein in phosphotyrosine immunoprecipitates from insulin-stimulated Fao hepatoma cells (Sun *et al.*, 1993). This protein was subsequently purified and cloned from rat liver, and was found to be expressed in most cell and tissue types (Sun *et al.*, 1993). With regard to insulin sensitive tissues, IRS-1 is expressed in a regulated manner like the other proteins of the insulin signalling casade. Indeed in the 3T3-L1 adipocyte, a cell culture model of an insulin-sensitive tissue, expression of the insulin receptor, IRS-1 and GLUT4 are all dramatically increased throughout the differentiation from the fibroblast to adipocyte phenotype.

IRS-1 interacts with the insulin receptor by utilising structural motifs present within the IRS protein and the insulin receptor. The pleckstrin homology domain (PH domain), is responsible for targetting IRS-1 to the membrane and the insulin receptor where IRS-1 can then interact with specific phosphotyrosine residues located in NPXpY motifs (Sun *et al.*, 1991; Sun *et al.*, 1993; Virkamaki *et al.*, 1999). Interaction with these residues occurs through association of the IRS-1 phosphotyrosine binding domain (PTB domain) with the NPXpY sequence in the insulin receptor. This then allows phosphorylation of IRS-1 on specific tyrosine residues by the insulin receptor. Like the insulin receptor, IRS-1 possesses many of

these potential regulatory tyrosine phosphorylation sites which exist in YXXM/YMXM motifs or in hydrophobic areas. The activated insulin receptor phosphorylates at least 8 of these 21 residues, including residues 608, 628, 939 and 987 (Sun *et al.*, 1991; Sun *et al.*, 1993). Several unique signalling proteins are then able to interact with specific phosphotyrosine residues via Src homology collagen 2 domains (SH2 domains) located within the proteins (Virkamaki *et al.*, 1999). These include proteins such as PI3'Kinase, Src homology collagen (Shc), or Grb2, and these interactions are discussed in relation to the biological outcomes in Sections 1.5, 1.6 and 1.7.

Since the discovery that the insulin receptor activates and utilises IRS-1 to activate downstream signalling processes there has been considerable interest regarding whether this protein has a unique function within the cell. Although many *in vitro* studies, using over-expression, micro-injection or similar techniques, have illustrated the ability of IRS-1 to activate PI3'K and enhance insulin-stimulated glucose transport, there is question over the absolute requirement for this IRS protein in these processes (White and Kahn, 1994; Whitehead *et al.*, 2000). Perhaps the most conclusive evidence regarding the role of IRS-1 comes from studies using knockout mice. Tamemoto *et al* illustrated how IRS-$1^{-/-}$ mice surprisingly exhibit only a mild insulin resistance and do not develop overt diabetes since the secretion of insulin increases in order to compensate for the reduced tissue sensitivity to the hormone. In contrast, there is marked growth retardation in these knockout animals (Tamemoto *et al.*, 1994). This is therefore suggestive that although IRS-1 can undoubtedly mediate the effects of insulin to stimulate glucose transport, it is not absolutely necessary. This therefore initiated the search for other proteins which could function in a similar manner to IRS-1 and which were potential candidates for mediating the IRS-1-independent pathway in these animals

1.4.3.2 IRS-2

IRS-2 was first identified by Tobe *et al* as a 190kDa tyrosine-phosphorylated protein in insulin-stimulated livers from IRS-$1^{-/-}$ mice (Tobe *et al.*, 1995). When compared to wild type mice the tyrosine phosphorylation of this protein was greatly enhanced in the knockouts compared to wild type, suggesting that activity of this protein was somehow up-regulated in the absence of IRS-1 (Tamemoto *et al.*, 1994). Like IRS-1, IRS-2 can also associate with PI3'K and Grb2, suggesting that it is also able to mediate the cellular actions of insulin (Gould 1997; Holman and Kasuga, 1997). Subsequent work has also revealed that IRS-2 is able to promote GLUT4 translocation in a manner analogous to that previously demonstrated for IRS-1 (Zhou *et al.*, 1997).

Analysis of the expression of IRS-2 has revealed that it is also ubiquitously expressed, messenger RNA being found in haematopoietic cells, skeletal muscle, lung, brain, liver, kidney, heart, spleen, and adipose tissue (Sun *et al.*, 1995). Alignment of murine IRS-2 with mouse, rat or human IRS-1 has revealed the presence of areas of extensive homology located at the amino terminus- termed IRS homology domains (IH-1 and IH-2) (Sun *et al.*, 1995). Like IRS-1, IRS-2 possesses around 20 potential tyrosine phosphorylation motifs which are generally located in similar positions (Sun *et al.*, 1997). This therefore suggests that IRS-1 and IRS-2 are able to bind the same downstream SH2 domain containing proteins and that there may be some redundancy in function. Interestingly however IRS-2 does have some unique sites, suggesting also that there are some differences in how these proteins interact with the insulin receptor and in their ability to activate unique down-stream processes specifically (Sun *et al.*, 1997). IRS-$2^{-/-}$ knockout mice have also been generated, and characterisation of these animals has revealed a striking phenotype. From an early age (around 3 weeks) these animals displayed fasting hyperglycaemia which develops into marked glucose intolerance and eventually overt diabetes as the animals reach maturity. Male mice showed polydypsia, polyuria, ketosis and generally died from dehydration

and hyperosmolar coma (Withers *et al.*, 1998). Female mice also showed a similar disease progression but rarely died. The IRS-$2^{-/-}$ knockout mice displayed insulin resistance, with higher fasting insulin levels and higher insulin concentrations required to stimulate adequate glucose disposal. PI3'K stimulation in the IRS-$2^{-/-}$ knockout mice was also altered, reflecting the importance of IRS-2 in activation of this protein (Withers *et al.*, 1998). Although there are similar changes in the IRS-1 knockout mice this is largely compensated by the pancreas, where the tissue mass is markedly elevated in order to produce more insulin. In the IRS-$2^{-/-}$ knockout mice however the pancreas is actually reduced in size, and hence there are no adequate compensatory mechanisms to overcome the reduced tissue sensitivity to insulin (Withers *et al.*, 1998).

Thus, it appears that both IRS-1 and IRS-2 are involved in mediating the biological effects of insulin and undoubtedly there is considerable overlap in action. Nevertheless, it is apparent from the knockout studies that there are certain functions which one or other protein is able to perform more effectively.

1.4.3.3 Additional regulation in IRS-1 and IRS-2 function

It has recently emerged that, although the tyrosine phosphorylation steps described above are central to IRS function, the proteins are also regulated in other ways. Indeed, it appears that serine phosphorylation also represents an important means of controlling IRS action in cells. Much of this work has focused on IRS-1 and several studies have now illustrated mechanisms of serine phosphorylation of this protein. Putative serine kinases involved include PKC's, or MAPK which is able to phosphorylate IRS-1 on serine 612. Indeed DeFea *et al* have demonstrated that endothelin-1 is able to phosphorylate IRS-1 in a MAPK-dependent manner (DeFea *et al.*, 1997). Interestingly PDGF is also able to stimulate the serine phosphorylation of IRS-1. It does this through a rapamycin-sensitive pathway, phosphorylating serines 632, 662 and 731. This

therefore implicates the involvement of proteins such as mTOR. (Li *et al.*, 1999). PKB and PKC have also been suggested as potential candidates in this role (Li *et al.*, 1999). In all of these cases, the serine phosphorylation inhibited IRS-1 action and therefore functioned in a negative regulatory manner. Paz *et al* also observed IRS-1 phosphorylation and showed this to be PKB-dependent (Paz *et al.*, 1999). In contrast however, they concluded that the PKB-mediated phosphorylation was protective to IRS-1 in that it prevented the action of protein tyrosine phosphatases on the phosphotyrosine residues, thereby maintaining IRS-1 in the active state for longer. They conclude that the negative regulation must be mediated by some serine kinase other than PKB. This protective role of PKB is unique so far. Normally serine phosphorylation is associated with reduced signalling through IRS-1 and the development of insulin resistance (Paz *et al.*, 1999). Indeed, tumour necrosis factor alpha (TNFα) is reported to cause serine phosphorylation of IRS-1 by unknown mechanisms, resulting in the development of cellular insulin resistance (Wang *et al.*, 1998).

A second mechanism thought to be involved in the development of insulin resistance and important for normal IRS function is the correct compartmentalisation of signalling through the IR and IRS proteins. Recent work by Clark *et al* has illustrated that the IRS proteins appear to associate with a cytoskeletal fraction that is insoluble in non-ionic detergents and associates with the particulate fraction of cell membranes (Clark *et al.*, 2000). This complex is believed to perform a unique function in that it allows the IRS proteins to interact with the IR and also provides a location for IRS interaction with downstream target molecules. Subsequent release of IRS proteins from this scaffold occurs after insulin stimulation. Interestingly, release from this platform is also associated with development of insulin resistance, the IRS proteins failing to interact with or being abnormally released from the scaffold, hence preventing the normal interactions (Clark *et al.*, 2000).

1.4.3.4 IRS-3

Although IRS-3 is rapidly tyrosine-phosphorylated in response to insulin in rat adipocytes, unlike IRS-1 and IRS-2 it does not bind PI3'K to any significant extent (Anai *et al.*, 1998). Structurally IRS-3 is much smaller than the other IRS proteins (60kDa versus 165-180kDa), although it does also possess a PH and PTB domain that are very highly conserved among the proteins (Anai *et al.*, 1998). Additionally, many phosphotyrosine motifs through which the SH2 domains of proteins such as PI3'K bind are conserved. Interestingly, however, there are regions present within IRS-3 which show no sequence homology with any area present in IRS-1 or IRS-2 (Anai *et al.*, 1998). Perhaps the most striking data regarding the role of IRS-3 in insulin stimulated glucose transport comes from studies in IRS-3$^{-/-}$ knockout mice (Liu *et al.*, 1999). Data generated from this study indicates that these animals have a normal pattern of growth and normal insulin sensitivity (normal fasting glucose and insulin levels, and no alteration in adipocyte glucose transport in response to insulin). The importance of sub-cellular localisation in IRS-1 and IRS-2 action was discussed above. Interestingly it seems that IRS-3 is located in a different intracellular compartment from IRS-1 and –2 (Anai *et al.*, 1998). Indeed, Anai *et al* illustrated that most of IRS-3 is located in the plasma membrane (PM) fraction, in contrast to the majority of IRS-1 and IRS-2 residing within the low density membrane (LDM) fraction. It therefore appears that IRS-3 is not one of the major players mediating the metabolic actions of insulin and further work to characterise the proteins with which it interacts will ultimately reveal the cellular consequences of activation of this unique protein.

1.4.3.5 IRS-4

Although IRS-4 is of similar size to IRS-1 and IRS-2 and shares some structural similarities, it is unlikely to play a role in the metabolic actions of insulin. IRS-3 was originally identified in haematopoetic cells where it

was discovered to have a role in insulin-like growth factor 1 (IGF-1), interleukin-4 and insulin-induced cell proliferation (Fantin *et al.*, 1999; Qu *et al.*, 1999). Recent studies have suggested that transfection of this protein into rat adipose cells can cause translocation of GLUT4, but a physiological role for such a function has not been demonstrated (Zhou *et al.*, 1999). Like IRS-3, knocking out IRS-4 leads to mild impairments in growth, reproduction and glucose homeostasis (Fantin *et al.*, 2000), suggesting that role for IRS-4 in these processes is likely to be minimal.

1.4.4 Phosphatidylinositide-3'kinase

The first observations of insulin-induced PI3'K activity were in the 1990's when two independent groups observed enhanced PI3'K activity in phosphotyrosine immunoprecipitates from insulin treated cells (Endemann *et al.*, 1990). This enhanced activity was originally thought to be through an interaction within the IR. However it was subsequently revealed that this only accounted for a small proportion of the activity (Endemann *et al.*, 1990; Malarkey *et al.*, 1995). Indeed most of the enhanced activity was actually revealed to be a result of interaction with the IRS proteins (Malarkey *et al.*, 1995; Gould, 1997; Holman and Kasuga, 1997; Whitehead *et al.*, 2000).

The first characterised mammalian PI3'K was found to be a heterodimer, which consisted of an 85kDa regulatory subunit and a 110kDa catalytic subunit (Shepherd *et al.*, 1996, 1997a,b, 1998). From this a model of PI3'K action was developed, involving PI3'K interaction with phosphotyrosine residues on growth factor receptors (or other proteins such as the IRS proteins) through the SH2 domains present on the p85 regulatory subunit. This in turn activates the catalytic activity of the p110 subunit. As the name suggests this catalytic activity is directed towards phosphorylation of the 3 position of the inositol ring of phosphoinositides, resulting in the generation of the phosphatidylinositol phosphates PIP, PIP_2 and PIP_3 (Shepherd *et al.*, 1996, 1997a,b, 1998). Because of the emerging

importance of PI3'Ks in mediating the cellular effects of insulin, work focused on characterising these enzymes more fully. Subsequently, it was revealed that the original p85-p110 complex was part of a larger group of enzymes, comprising several different regulatory and catalytic subunits. The enzyme catalytic subunits can be grouped into 3 classes- class 1 which is subdivided into class 1a and class1b, class 2 and class 3 (Shepherd *et al.*, 1996, 1997a,b, 1998).

Class 3 PI3'K's are unique in that that they only possess catalytic activity against PI and none of the other phosphoinositides. It is not considered that these kinases play a role in mediating the metabolic actions of insulin since insulin does not increase the cellular levels of PI3P, the product of this enzyme (Shepherd *et al.*, 1996, 1997a,b,1998).

Similarly, it is not thought that Class 2 PI3'K's are involved in mediating the effects of insulin. This is based on the fact that, although insulin-mediated responses are sensitive to the effects of inhibitors such as wortmannin, this class of enzyme are relatively resistant to these effects (Shepherd *et al.*, 1996, 1997a,b,1998).

The class 1a and class 1b PI3'K's are differentially activated, the 1a enzymes being activated by binding to the p85 regulatory subunit and 1b as a result of interactions with G-protein $\beta\gamma$ sub-units. The class 1a and 1b catalytic subunits share a significant sequence homology. The class 1c subunits are activated in a similar manner to the 1a enzymes, although by different regulatory subunits (Shepherd *et al.*, 1996, 1997a,b,1998). The domain structures of each of the kinase subunits are illustrated below in Figure 1.3.

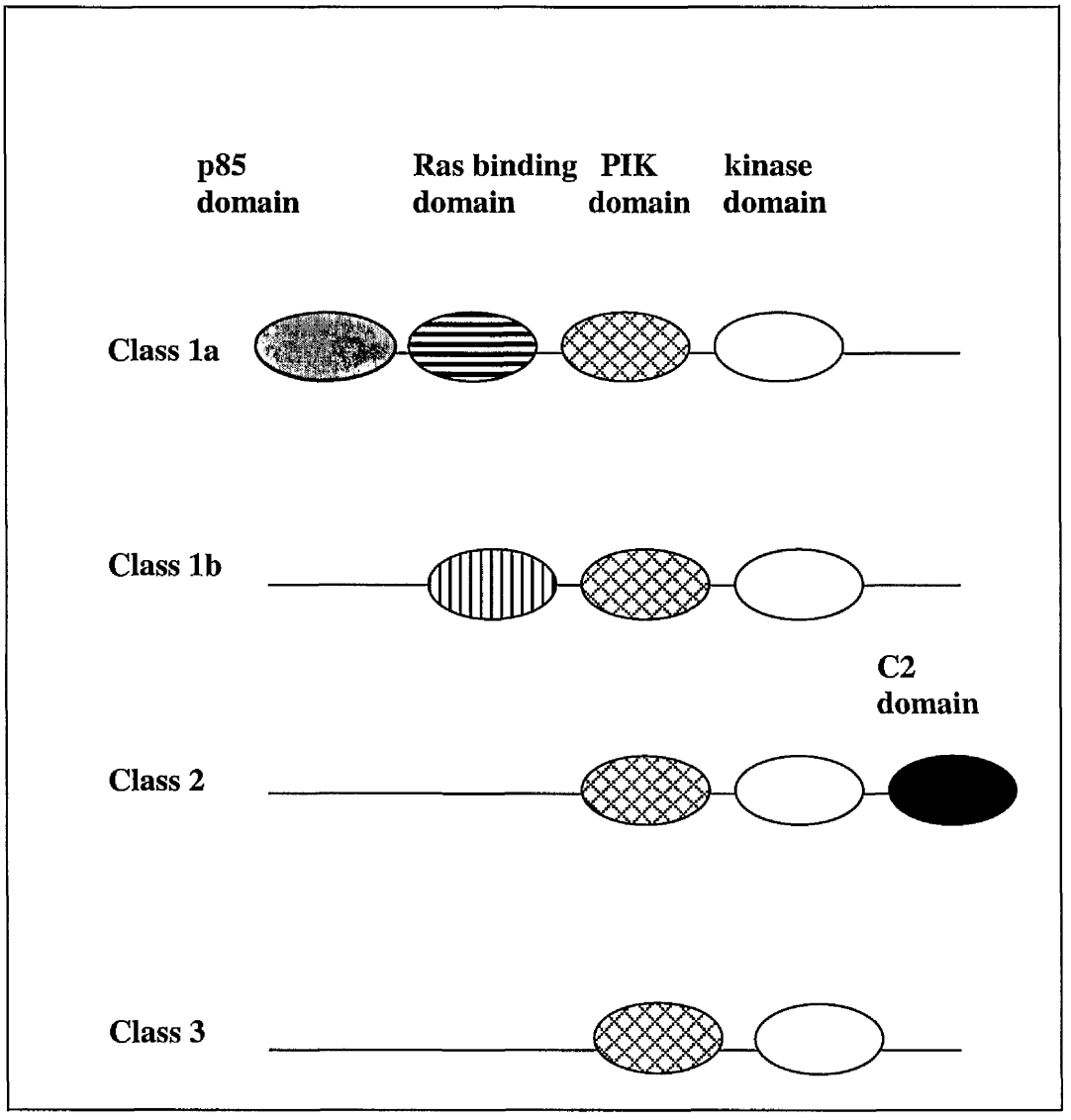


Figure 1.3 The domain structure of the PI3'K catalytic subunits
adapted from Shepherd *et al* 1996, 1997a,b,1998.

As outlined in Figure 1.3, the class 1a PI3'K's possess a p85 binding domain. As discussed earlier, this allows the interaction of the p110 catalytic subunits with the regulatory isoforms linking them to activated receptors or IRS proteins. Although the p85 regulatory subunit is the most well-characterised regulatory subunit, there are actually five such proteins p85α, p55α, p50α, p85β and p55γ (Shepherd *et al.*, 1996, 1997a,b,1998). There are at least three splice variants of the p85α subunit and these all share considerable structural homology with the p85β subunits. This includes the presence of two SH2 domains located at the N- and C-termini, an SH3 domain, a Bcr/Rac GTPase-activating protein (GAP) homology domain (BH domain) and two proline-rich domains either side of the BH domain (P1 and P2 domains) (Shepherd *et al.*, 1996, 1997a,b,1998). Although the two SH2 domains both have a high specificity for phosphorylated YXXM and YMXM motifs not shared by SH2 domains from other proteins, there is a slight variance in exact sequence preference of the two different SH2 domains. The relevance of this has not, however, been addressed. The *in vivo* roles of the other domains are also less well defined, although it is known that SH3 domains and proline-rich regions will interact. This is suggestive of an involvement of these two areas in an auto-regulatory mechanism (Shepherd *et al.*, 1996, 1997a,b,1998). The p55 isoforms also possess SH2 domains, but lack the SH3, P1 and BH domains. p55α, also known as AS53, is expressed in brain and muscle and is therefore unlikely to contribute to the actions of insulin in adipose tissue. p50α is also unlikely to contribute to insulin action in adipocytes as like p55α is not expressed in fat, although it is expressed in brain, liver, muscle and kidney. p55γ, also known as $p55^{PIK}$, has a similar domain structure to p55α (Shepherd *et al.*, 1996, 1997a,b,1998). Little is known about the expression of this isoform.

The significance of the many different catalytic and regulatory PI3'K subunits is unclear and some studies suggest that there may be functional redundancy at some levels. Further characterisation of the different isoform combinations may reveal specific functions and determine the

importance of factors such as cell specific expression. Nevertheless the importance of class 1a PI3'K's in insulin action cannot be ignored, and this is discussed in more detail in sections 1.5, 1.6 and 1.7.

1.4.5 Other Protein Kinases

1.4.5.1 PKB

PKB (also known as c-akt, RAC-PK) was originally identified due to it's homology with the viral oncogene product v-akt. Currently three isoforms are known to exist- α, β and γ (Walker *et al.*, 1998). Early work, mainly non-isoform specific or concentrating on PKBα, revealed that insulin can produce up to 10 fold elevations in PKB activity in both 3T3-L1 and rat adipocytes (Cross *et al.*, 1997; Walker *et al.*, 1998). Initial characterisation studies revealed this protein to be downstream of PI3'K in the signalling cascade (Bos, 1995), since its activity was abolished by PI3'K inhibitors such as wortmannin or by over-expression of dominant negative forms of PI3'K (Bos, 1995). Subsequently a mechanism of PKB activation was proposed which was dependent on PI3'K at two levels, and this model is now widely accepted. The importance of PI3'K activation is at the level of compartmentalisation. In other words, the polyphosphoinostide products are responsible for recruiting PKB to a specific cellular location, via an interaction with the PH domain of PKB (Bos, 1995; Cross *et al.*, 1997; Wijkander *et al.*, 1997; Walker *et al.*, 1998). Once in the correct location the enzyme is activated by phosphorylation on specific serine and threonine residues. This phosphorylation is carried out by a class of PI3'K – dependent enzymes called the phosphoinositide-dependent kinases (PDK's). PKBα is phosphorylated on serine 303 and threonine 473, and PKBβ is phosphorylated on serine 304 and threonine 474 by PDK1 (Cross *et al.*, 1997; Wijkander *et al.*, 1997; Walker *et al.*, 1998). Therefore the PI3'K–induced activation of PKB is a two-step process- involving subcellular relocalisation and specific phosphorylation events.

Unlike PI3'K however, a direct role for PKB in insulin-stimulated GLUT4 translocation has been less easy to demonstrate. Many of the early studies produced inconclusive results. Some groups have highlighted the ability of over-expressed or constitutively active PKB to promote GLUT4 translocation (Cong *et al.*, 1997; Tanti *et al.*, 1997; Foran *et al.*, 1999; Hill *et al.*, 1999), however other groups have failed to demonstrate such an effect (Kitamura *et al.*, 1998). Perhaps one of the most useful studies so far in this regard is an extensive study and review of the different PKB isoforms, and their potential roles in insulin action (Hill *et al.*, 1999). By using a micro-injection based technique they showed that a PKB substrate peptide or antibody was able to inhibit insulin-stimulated GLUT4 translocation by approximately 60% (Hill *et al.*, 1999). This agrees with some previous work to a certain extent, for example Cong *et al* demonstrated a 20% reduction in insulin-stimulated GLUT4 translocation using a kinase inactive PKBα, K179A (Cong *et al.*, 1997). In contrast however Hadjuch *et al* saw no such effects similar effects of this peptide in L6 myotubes (Hadjuch *et al.*, 1997). Interestingly, Hill *et al* suggest that much of this controversy may lie simply in the way in which these experiments are performed and interpreted. Indeed, they suggest that much may be resolved by studying the specific PKB isoforms individually, bearing in mind which are predominantly expressed in the tissue under question, and how specific the reagents in use are. Although much of the preliminary work suggested that PKBα is the primary isoform expressed in 3T3-L1 adipocytes, it is emerging that in fact PKBβ is the most abundant form in these cells. Although PKBα was the predominant form expressed in fibroblasts, there was a change over to PKBβ as adipogenesis proceeded (Hill *et al.*, 1999). Indeed the enhanced expression of PKBβ occurred in a manner parallel to the enhanced expression of GLUT4 (Hill *et al.*, 1999). They therefore conclude that PKBα is more likely to involved with the growth capacity of fibroblasts, whereas PKBβ has a role in mediating the metabolic effects of insulin in mature differentiated cells. This is reinforced by the observations that there are high levels of PKBβ mRNA in brown adipose tissue and that expression is increased as two different muscle cell lines (Sol8 and C2C12)

undergo differentiation (Hill *et al.*, 1999). Further evidence for the apparent differing roles of the PKB isoforms comes from studies using PDGF. PDGF is unable to activate many of the biological responses activated by insulin and, indeed, PDGF is also unable to significantly stimulate PKBβ, even though it is able to influence the activity of PKBα in non-differentiated cells (Hill *et al.*, 1999).

Although the rationale presented above is very convincing regarding the role of PKB in adipocytes there are certain caveats. With regard to PKBα, credible demonstrations of increased activity in response to insulin have been demonstrated in both rat and 3T3-L1 adipocytes (Cross *et al.*, 1997, Walker *et al.*, 1998). Clearly this contradicts Hill and colleagues work. Nevertheless they raise an interesting point in response. Indeed, they suggest that much of the response detected by PKBα antibodies may represent other PKB isoforms, since there is demonstrable cross-reactivity (Hill *et al.*, 1999). The fact that PKBβ appears to play more of a role may also explain why some groups such as Kitamura *et al* have demonstrated little effect of PKBα mutants on glucose transport (Kitamura *et al.*, 1998), since they are possibly concentrating their studies on the wrong isoform.

Therefore, it seems that although the evidence regarding a role for PKB in insulin-stimulated GLUT4 translocation is less than ideal, it is likely that the PKBβ isoform does play a role in this process. Undoubtedly however other proteins are of important in light of the fact that the process is not completely inhibited by PKBβ inhibition.

Although PKBγ can be activated by the PDK enzymes in the same manner as the others and has a similar catalytic activity, this protein is not detectable in adipocytes or skeletal muscle *in vivo*. Indeed the main role of this protein in insulin-sensitive responses appears to be in the stimulation of glucose transport in L6 myotubes (Walker *et al.*, 1998; Hill *et al.*, 1999).

PKB also appears to have a role in mediating some of the other effects of insulin. PKB mutated at the specific activating phosphorylation sites (Akt-AA), can effectively inhibit insulin-stimulated activation of p70s6kinase by around 30% (Kitamura *et al.*, 1998). Obviously this level of inhibition suggests that although PKB is involved in this process, there are probably other pathways and intermediates which are involved in this process. The role of PKB in mediating the anti-lipolytic actions of insulin is also emerging. This is discussed in Section 1.3.

1.4.5.2 PKC

Although a decisive role for Protein Kinase C (PKC) in insulin-stimulated glucose transport is still under debate, a wealth of evidence indicates that atypical PKC isoforms are able to induce GLUT4 translocation and do so in response to insulin (Standaert *et al.*, 1997; Standaert *et al.*, 1998; Standaert *et al.*, 1999). There are many PKC isoforms expressed in mammalian tissues and these are broadly grouped into three classes, the classical diacylglycerol (DAG)-sensitive isoforms (PKCα and PKCβ), the novel PKC's (PKCδ and PKCε), and the DAG-insensitive and polyphosphoinositide-sensitive PKC ι, ζ and λ (Standaert *et al.*, 1997; Standaert *et al.*, 1998; Bandyopadhyay *et al.*, 1999; Standaert *et al.*, 1999).

Both PKC ζ and λ are reported to be activated by insulin in several tissues including 3T3-L1 adipocytes, rat adipocytes and L6 myotubes. Bandyopadhyay and colleagues convincingly illustrated the involvement of both of these isoforms in insulin-stimulated GLUT4 translocation, using wild type and kinase inactive (KI) mutants and examining the ability of these proteins to stimulate translocation of tagged-GLUT4 (Bandyopadhyay *et al.*, 1999). In cells transfected with either KI PKC ζ or λ, there was a consistent inhibition of insulin-stimulated movement of tagged GLUT4. Interestingly both of these peptides were without effect

on basal or GTPγS-stimulated (insulin-independent) GLUT4 translocation. The role of PKCζ in insulin-stimulated GLUT4 translocation is further reinforced by studies demonstrating the ability of wild type PKCζ to enhance insulin-stimulated GLUT4 translocation. Standaert and colleagues have taken this a step further and have illustrated how insulin is able to influence the phosphorylation state of PKCζ and the catalytic activity of the enzyme (Standaert *et al.*, 1997). Because the enhanced phosphorylation could be inhibited by the PKC-pseudosubstrate (PKC-ps), it was concluded that this was likely to be an auto-phosphorylation step. The effect of insulin was demonstrated also to be PI3'K-dependent, by virtue of the fact that the effects of PIP_3 and insulin were non-additive (Standaert *et al.*, 1997).

In contrast however to the effects of the atypical PKC's, PKCα, β, δ, or ε appear not to be involved in GLUT4 translocation. Indeed Bandyopadhyay demonstrated a lack of effect of KI PKCα, β, δ, or ε on insulin-stimulated glucose translocation. This is reinforced by Standaert and colleagues work where they indicate a lack of effect of either PKCα, β1, β2 , δ, or ε depletion or conventional PKC inhibitors on insulin-stimulated GLUT4 translocation (Bandyopadhyay *et al.*, 1999). Nevertheless some studies have contradicted this, and there is some evidence that DAG-sensitive PKC's may also be involved in mediating the effects of insulin (Bandyopadhyay *et al.*, 1999). Activation is believed to be through PI3'K-activated PLD mediated generation of phosphatidic acid (PA) and DAG (Bandyopadhyay *et al.*, 1999). There is uncertainty regarding the mechanism by which PI3'K can activate PKB, although PDK1 has been suggested as the link. Certainly PDK1 is able to phosphorylate threonine 410 on PKCζ, although it is unclear whether this operates as a functional mechanism of activating the DAG-sensitive PKC's (Bandyopadhyay *et al.*, 1999).

1.4.6 Other putative signalling molecules involved in metabolic signalling by insulin

Although the majority of players in the insulin-signalling field have been discussed in the previous sections, there are a few other less well characterised molecules that require mentioning.

1.4.6.1 Fyn/Cbl/CAP complex

Fyn is a cytoplasmic tyrosine kinase, which can be activated either by association with other phosphotyrosine proteins through its SH2 domain, or by dephosphorylation of the carboxy terminal tyrosine (Vikramaki *et al.*, 1999). Although the IR does not activate this protein, it can be activated by IRS-1 or another tyrosine phosphorylated protein Cbl. Fyn activated by binding to Cbl will then translocate to caveolae where it is able to cause phosphorylation of caveolin (Baumann *et al.*, 2000). The ability of insulin to cause caveolin phosphorylation is unique and stimulation of other growth factor receptors does not have a similar effect (Vikramaki *et al.*, 1999; Pessin *et al.*, 2000).

Cbl is also able to associate with another caveolin-associated protein CAP, a protein which is specifically expressed in insulin-sensitive cells (Vikramaki *et al.*, 1999; Pessin *et al.*, 2000, Baumann *et al.*, 2000). Current lines of evidence suggest that this pathway may represent an alternative means of insulin-stimulated GLUT4 translocation, as illustrated below in Figure 1.4.

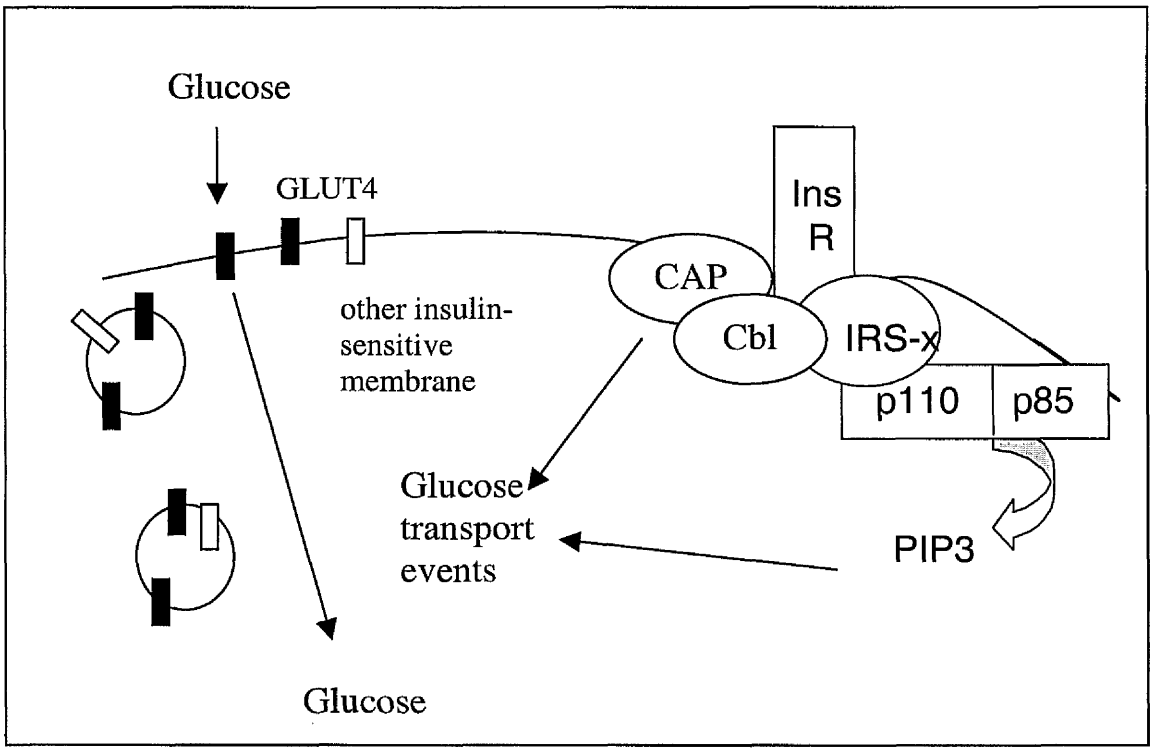


Figure 1.4 Other signalling molecules potentially involved in the steps leading to insulin-stimulated glucose transport

adapted from Vikramaki *et al.*, 1999; Pessin *et al.*, 2000

1.4.6.2 Csk

Csk is a cytoplasmic tyrosine kinase, responsible for the inactivation of the src family of kinases (Vikramaki *et al.*, 1999; Pessin *et al.*, 2000). This protein is also reported to associate with IRS-1through its SH2 domain (Vikramaki *et al.*, 1999). This interaction promotes dephosphorylation of Focal Adhesion Kinase (FAK) in an insulin-dependent manner. This is believed to form part of the pathway involved in the insulin-induced rearrangement of cytoskeletal elements, now widely accepted to be an intrinsic part of insulin-mediated signalling pathways (Vikramaki *et al.*, 1999).

1.4.6.3 SHIP

This protein is a 145kDa SH2-containing inositol 5-phosphatase, involved in the conversion of PIP_4 and PIP_3 to PIP_2 (Vikramaki *et al.*, 1999). Although conclusive evidence regarding the role of SHIP remains elusive, over-expression studies have illustrated that this protein will inhibit insulin-induced glucose transport and DNA-synthesis (Vikramaki *et al.*, 1999). It is unclear whether SHIP can associate with the IR or IRS proteins but it is known to possess a structure that consists of the following structure-

N	SH2	phosphatase	NPXYNPXY	proline rich domain	C

Aside from presenting a potential new target in studies of insulin action, using SHIP reveals information regarding PI3'K signalling in that it appears insulin-sensitive processes prefer PIP_3 over PIP_2, since SHIP reduces efficiency of signalling (Vikramaki *et al.*, 1999).

1.5 Signalling and trafficking steps leading to insulin-stimulated glucose transport

Although there is controversy regarding exactly how PI3'K stimulation leads to the movement of GLUT4 from its intracellular store to the plasma membrane and whether other signalling factors are involved, there is little doubt that PI3'K does play a crucial role (Gould, 1997; Holman and Kasuga., 1997). Studies using inhibitors such as wortmannin, or dominant negative mutant p85 subunits, clearly inhibit insulin-stimulated GLUT4 translocation and glucose transport (Gould, 1997; Holman and Kasuga, 1997). There is also considerable documentation regarding the ability of PI3'K's to influence vesicular transport. In yeast cells, the gene product Vps34p is a PI3'K homologue, required for sorting of newly synthesised proteins from the Golgi to the yeast vacuole (Corvera and Czech., 1998). Since many functions are conserved between yeast and mammalian cells this is suggestive of a similar role for PI3K's in mammalian cells. Another line of evidence in support of a role for PI3'K in membrane transport is from studies using the PI3'K inhibitor wortmannin. Treatment of mammalian cells with wortmannin generates a rapid and striking alteration in the morphology of endosomes, with the appearance of tubular structures which in some cases form an inter-connected network with enlarged perinuclear endosomes (Corvera and Czech., 1998).

Shortly after the discovery of GLUT4, and the realisation that insulin administration to adipocytes caused a translocation of this protein from intracellular stores to the plasma membrane, the search for molecules involved in regulating this process began. It soon emerged that this movement of GLUT4-containing vesicles utilised a pathway analogous to that of regulated trafficking in neuro-endocrine and yeast cells (Corvera and Czech., 1998). Although GLUT4 appears to travel in part through the normal endosomal recycling system in order to move from an intracellular area to the plasma membrane, the majority is packaged into discrete GLUT4 storage vesicles (GSVs) (Corvera and Czech., 1998; Hashimoto and James., 1998). Thus in order to understand the molecules and

processes regulating GLUT4 trafficking to and from the plasma membrane in insulin sensitive cells, it is necessary to consider membrane transport at the most basic level.

Many *in vivo* studies have illustrated that multiple fusion events can occur between individual components of the endosomal system, and isolated endosomes have also been observed to fuse *in vitro* (Hashimoto and James., 1998). To date however, the specific pathways from the early to late endosome are poorly understood. Nevertheless work has focused on trying to understand these processes and the mechanisms by which they are regulated, and consequently two models have been proposed. The first model is the 'Vesicular Transport Model' which works on the basis that the early and late endosomes are pre-formed entities and that proteins move between them via vesicular traffic (Hashimoto and James., 1998). The second model, the 'Maturation Model', is more fluid and transport through the endosomal system involves continuous fusion and fission of vesicles (Hashimoto and James., 1998). Nevertheless, regardless which of these models is closest to the actual situation, they both have one very crucial factor in common. In order for correct fusion events to occur at the correct time, these fusion events must be very specific. Like all specific interactions this relies on the presence of receptor-ligand mediated interactions. Within the recycling endosomal system, this interaction specificity is brought about by the SNARE proteins (Hashimoto and James., 1998).

1.5.1 The 'SNARE' hypothesis

The central dogma of the 'SNARE hypothesis' is essentially very simple and relies on the specific interactions between v-SNARE proteins on the vesicle membrane and t-SNARES on target membranes (Hashimoto and James., 1998). This specific interaction is believed to then act as a platform for the interaction with other proteins necessary for vesicle docking and fusion. This mainly involves the interaction with n-

ethylenemaleimide-sensitive factors (NSF's) and soluble NSF attachment proteins (SNAP's) (Hashimoto *et al.*, 1998).

Although the majority of these proteins were not originally identified with respect to GLUT4 translocation it is emerging that many of these proteins or homologues are actually involved in this process. Some of these are discussed below.

1.5.1.1 t-SNARE's

Two of the most important t-SNARE's found in neuronal cells are Syntaxin 1a and SNAP25 (Hashimoto and James., 1998). They are expressed on the pre-synaptic plasma membrane and bind to the v-SNARE VAMP (vesicle attachment membrane protein) present on vesicle membranes (Hashimoto and James., 1998). Although neither of these are expressed in insulin-sensitive cells, adipocytes do express two related t-SNARE's- Syntaxin 4 and SNAP23 (Hashimoto and James., 1998). Both SNAP23 and Syntaxin 4 are also able to bind VAMP molecules present on the vesicle membrane (Hashimoto and James., 1998). Both syntaxin 4 and SNAP23 both appear essential for GLUT4 translocation since inhibition of their action using either antibodies or recombinant proteins inhibits GLUT4 translocation (Hashimoto and James., 1998).

1.5.1.2 v-SNARE's

Two main v-SNARE's have been identified in insulin-sensitive cells, VAMP2 (synaptobrevin) and cellubrevin (Hashimoto and James., 1998). Both of these proteins have been found to co-localise with vesicles found to be positive for GLUT4 (Hashimoto and James., 1998), and information about their function in this regard had been found with the use of toxins which are able to modify these v-SNARE's. Two clostridial toxins, botulinum and tetanus, are able to selectively cleave VAMP2 and

cellubrevin at specific sites rendering them non-functional. Results from these studies indicate that both botulinum and tetanus toxin are able to inhibit GLUT4 translocation in permeabilised cells (Hashimoto and James., 1998), indicating that both VAMP2 and cellubrevin have a role in this process. More specific studies, involving the use of inhibitors which affect only one of these v-SNARE's, have yielded more useful information regarding the relative contribution of each protein. Treatment of cells with an IgA protease which cleaves VAMP2 but has no effect on cellubrevin blocks the majority of insulin-stimulated GLUT4 translocation (Hashimoto and James., 1998), indicating that VAMP2 has a major role in this process. Further work has confirmed this, with immuno-electron microscopy of rat adipocytes indicating that a large fraction of VAMP2 is targetted to the GLUT4 storage compartment (Martin *et al.*, 1996, Hashimoto and James., 1998). In contrast, the majority of the cellubrevin is observed to localise to endosomal structures (Hashimoto and James., 1998). Work using synthetic peptides with a unique VAMP2 sequence has also revealed that, although expression of this protein blocks GLUT4 exocytosis, it has little effect on constitutive GLUT1 trafficking in permeabilised adipocytes (Martin *et al.,* 1998). This has led to the conclusion that VAMP2 and cellubrevin possess different functions, with VAMP2 involved in the docking and fusion steps of GLUT4 regulated exocytosis whereas cellubrevin is primarily involved in constitutive endosomal recycling (Millar *et al.*, 1999).

One other v-SNARE, involved in synaptic vesicle exocytosis in neurons, is synaptotagmin (Hashimoto and James., 1998). Activation of this protein is triggered by a rise in intracellular calcium, and is thought to confer the calcium dependency of certain SNARE interactions in neurons (Hashimoto and James., 1998). There is controversy over whether calcium is involved in GLUT4 translocation, so a role for this protein in GLUT4 exocytosis is only speculative. Nevertheless other isoforms exist, and it is feasible that they may be regulated in a different way to the calcium-dependent isoform(s) (Hashimoto and James., 1998).

1.5.2 Specific movement of GLUT4 from intracellular stores to the plasma membrane

The information presented above clearly illustrates how there are multitudes of proteins involved in the recycling of proteins to and from the plasma membrane in mammalian cells. Undoubtedly GLUT4 recycles through this system like many other cellular proteins. Interestingly, however, the exocytic rate of GLUT4 is much less than that of other recycling proteins, suggesting that GLUT4 must somehow be sequestered away from the normal recycling system (Gould, 1997; Hashimoto and James., 1998). Also, in response to insulin, there is a rapid increase in the content of GLUT4 and several other specific proteins at the plasma membrane (Gould, 1997). This again suggests that these proteins must somehow be removed from the normal recycling system to some other pool to allow an increased movement to a specific cellular compartment when required. There are currently two different models proposed to explain how this unique sorting of GLUT4 is achieved (Hashimoto and James., 1998). This is illustrated below in Figure 1.5.

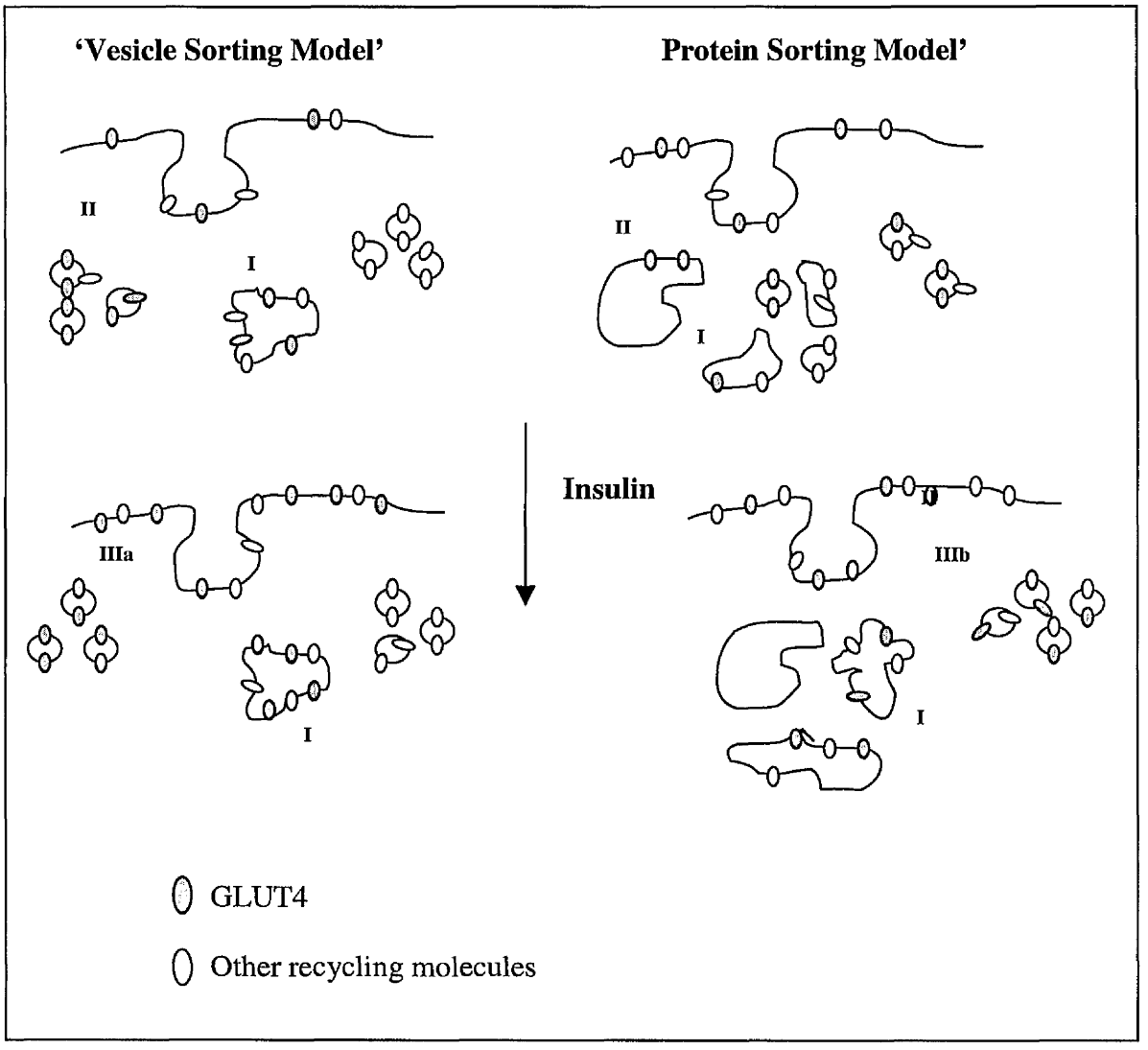


Figure 1.5 Proposed models for GLUT4 exocytosis

adapted from Hashimoto Hashimoto and James., 1998

see page 37 for explanantory notes

(I) In both of the models, GLUT4 is sequestered within in the cell away from the plasma membrane by an efficient internalisation process.

(II) The rate of GLUT4 recycling is low under these conditions, when compared to other recycling proteins.

(IIIa) The **vesicle sorting model** then works on the assumption that GLUT4 is sequestered into a unique population of vesicles away from the recycling pathway. These vesicles are then assumed to be highly enriched in GLUT4 with a good responsiveness to insulin (Hashimoto and James., 1998). Insulin stimulation will cause an increased flux through both this pathway and the recycling pathway, resulting in a dramatic increase in the amount of GLUT4 at the cell surface (Hashimoto and James., 1998).

(IIIb) In contrast the **protein sorting model** is based on differential affinity for different sorting proteins being responsible for the movement of proteins to specific micro-domains within the tubulo-vesicular network (Hashimoto and James., 1998). Insulin may act simply to alter the formation of different network connections, resulting in propagation of GLUT4 and other molecules to the plasma membrane.

Current lines of evidence suggest the former model to be more realistic, and a wealth of data supports the notion of specific compartments of vesicles highly enriched in GLUT4 (the GSVs- discussed in Section 1.5). This population of vesicles is highly sensitive to insulin, and will account for a large percentage of GLUT4 that translocates to the plasma membrane in response acute insulin stimulation.

Nevertheless although insulin-stimulated GLUT4 translocation and stimulation of glucose transport are an essential action of insulin, they are not the only important biological effect of the hormone.

1.6 Anti-lipolytic effects of insulin

As discussed in Section 1.2.3, a main action of insulin is in the control of fat metabolism in adipocytes. Insulin is an anti-lipolytic hormone, and is able to reverse the lipid hydrolysing effects of hormones such as adrenaline (Chapter 27, Zubay, 1996). Although the steps leading to anti-lipolysis are not as well understood as those leading to glucose transport, it is generally accepted that the early stages of this pathway also utilise the insulin receptor and the IRS proteins (Botion and Green., 1999 Van Harmelen *et al.*, 1999; Kasuga, 2000). Interestingly it is also emerging that the ant-lipolytic actions of insulin are PI3'K-dependent (Botion and Green., 1999; Kasuga, 2000).

1.6.1 Activation of lipolysis

In general, it is believed that the lipolytic action of different hormones is mediated by the Protein Kinase A (PKA) and cAMP pathway (Botion and Green., 1999). Binding of hormone to its G-protein coupled receptor results in the activation of the associated G-proteins. G-proteins are composed of three subunits- alpha, beta and gamma. It is generally accepted that the activated alpha subunit is responsible for the activation of

PKA, (although the beta gamma subunits which were originally thought to act as structural anchors are now known to also have a signalling ability independent of the alpha subunit). Activation of PKA results in the generation of cAMP, which in turn activates the hormone sensitive lipase (HSL). Although the precise mechanism of HSL activation is not completely understood, phosphorylation on specific serine residues has been demonstrated to be of importance (Botion and Green., 1999). Indeed activation involves multi-site phosphorylation, and serines 659 and 650 have been established as activity controlling sites with respect to activation by PKA (Botion and Green., 1999). Serine 563 is also phosphorylated although this protein phosphorylation site plays a minor role. The importance of Serine 565 has also been recognised, although phosphorylation on this residue is actually thought to be inhibitory to subsequent phosphorylation at Serine 563 and activation by PKA (Botion and Green., 1999). Studies have illustrated that the phosphorylation state of HSL is closely linked to its activity and that the phosphorylation state is very important at maximal lipolysis. It is also emerging that HSL will re-distribute within the cell in response to lipolytic stimuli- moving from the cytosol into the lipid droplet (Clifford *et al.*, 2000). Interestingly, some studies have suggested that control exerted by the HSL is not sufficient to explain the regulation of lipolysis, and some groups have suggested the involvement of additional control mechanisms. One such suggestion is the involvement of substrate activation by some interacting factor possibly present at the surface of the lipid droplet (Clifford *et al.*, 2000). Nevertheless, to date the HSL is the best characterised factor controlling the activation of lipolysis within fat cells. Once activated, HSL will catalyse the breakdown of triacylglycerides into the constituent glycerol and fatty acids, the fatty acids being exported and utilised as an energy source (Chapter 27, Zubay, 1998).

1.6.2 Activation of anti-lipolysis

It has long been appreciated that insulin is able to influence the activation of lipolysis, presumably by an action to inhibit the activity of the HSL. Nevertheless only recently have some of the pathways involved began to emerge. These are outlined below in Figure 1.6.

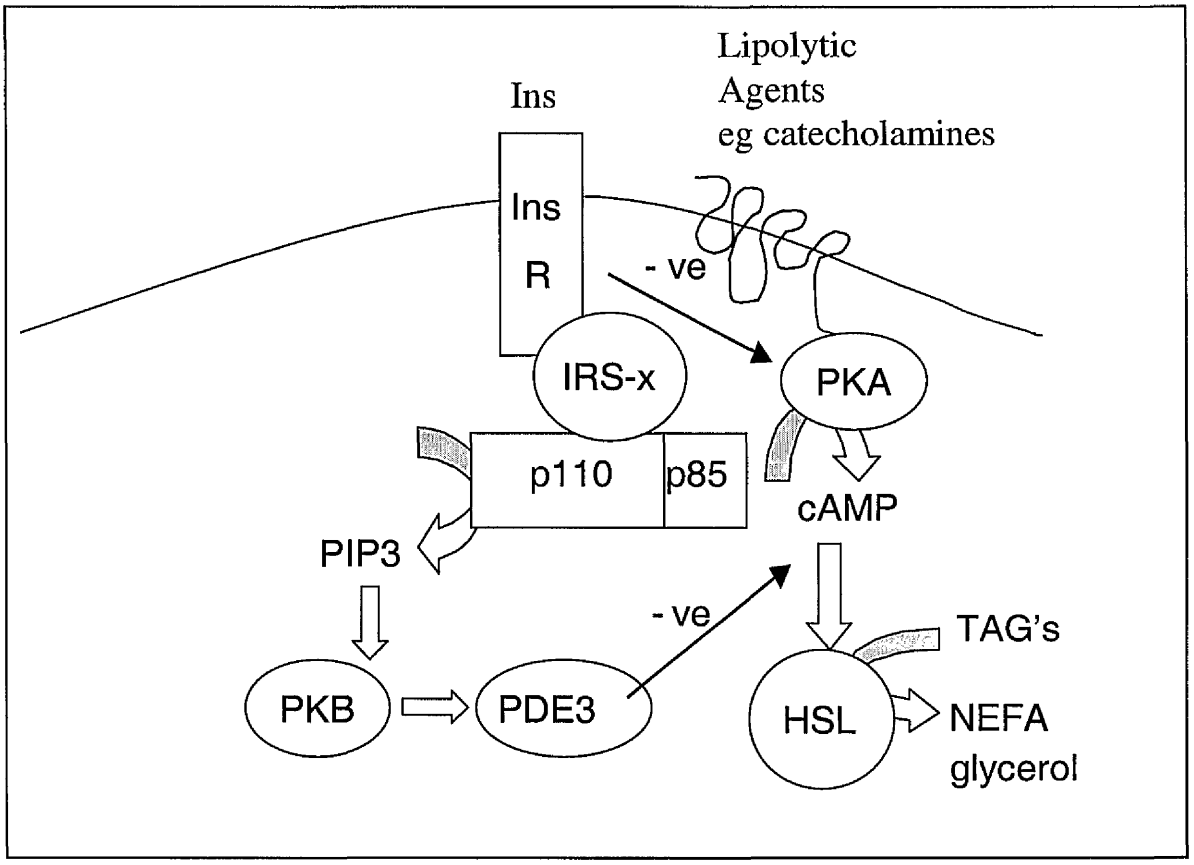


Figure 1.6 Potential mediators of the anti-lipolytic effects of insulin adapted from Botion and Green., 1999; Van Harmelen *et al.*, 1999; Kasuga, 2000

Insulin activates the class 3B phosphodiesterase enzyme, although it is only recently that the links between PKB and this enzyme have been appreciated (Botion and Green., 1999; Van Harmelen *et al.*, 1999; Kasuga, 2000). As discussed in Section 1.4.5.1, PKB is rapidly activated in response to insulin, whereupon it is phosphorylated on specific threonine and serine residues (Thr308 and Ser473 in the case of PKBα). By making PKB mutants in which both of these residues were mutated to alanine residues, Kasuga *et al* illustrated how the normal 2-fold insulin-dependent activation of PDE3B was completely inhibited (Kasuga *et al.*, 2000). This suggested PKB involvement in the insulin-dependent activation of PDE3B. In order to determine how PKB was activating PDE3B, several serine residues on PDE3B were mutated, and this revealed serine 273 to be important for insulin-induced activation. This was also nicely illustrated by immunoprecipitating the wild type and mutant PKB enzymes and determining which residues of PDE3B were being phosphorylated by PKB. Interestingly, further evidence for the links between PKB and PDE3B in mediating the anti-lipolytic effects of insulin were illustrated by the ability of mutant PKB to inhibit the normal effects of insulin to reduce cellular cAMP levels (Kasuga *et al.*, 2000). Therefore, although we have seen conflicting evidence regarding a role for PKB in insulin-stimulated glucose transport, it is likely that PKB does play a role in at least one of the important actions of insulin in adipocytes, that of anti-lipolysis. With regard to the phosphodiesterase enzymes, there are many different isoforms expressed in mammalian cells, the functions of many currently under debate. It is therefore feasible that isoforms other than PDE3B may have some role to play in the anti-lipolytic effects of insulin. Finally, insulin has been seen to reverse the translocation of HSL to the lipid droplet induced by lipolytic agents (Botion *et al.*, 1999).

All of the effects described in Sections 1.4 to 1.6 relate to the metabolic actions of insulin. Although these effects are undoubtedly the most important effects of insulin and the main focus of this thesis, it is also necessary to consider the other important biological effects. Perhaps the best understood of these effects are the growth-promoting actions.

1.7 Mitogenic actions of insulin

Aside from the metabolic actions of insulin it is a mitogenic hormone, able to stimulate protein synthesis in a variety of mammalian cells, including fat and muscle. Some of the pathways mediating these effects are outlined in Figure 1.7.

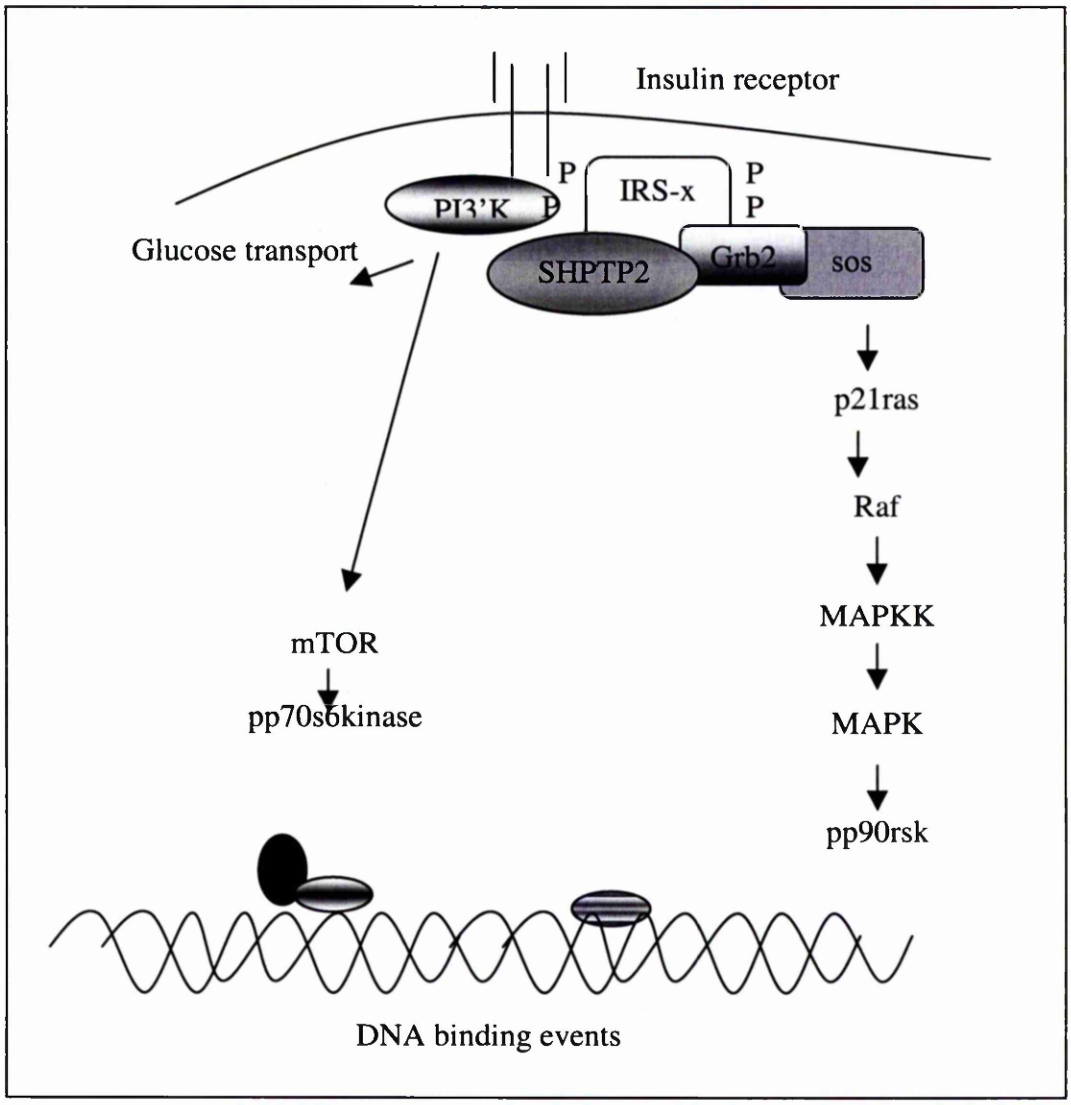


Figure 1.7 Potential pathways mediating the growth stimulatory actions of insulin

adapted from Malarkey *et al.*, 1995; Gould, 1997

The mitogen-activated protein kinase (MAPK, also known as extracellular regulated kinase (ERK)) pathway was one of the earliest and best characterised pathways mediating the growth promoting effects of many different growth factors (Malarkey *et al.*, 1995). Most growth factor receptors couple to this pathway by an interaction of phosphotyrosine residues on the activated growth factor receptor with the SH2 domain of the adaptor molecule Grb2 (Malarkey *et al.*, 1995). This links the receptor, via the nucleotide exchange factor, mammalian son of sevenless (mSOS, the homologue of the related protein first discovered in Drosophila), to the small G-protein Ras. Activated Ras then initiates the cascade of events outlined in Figure 1.7, culminating in the alteration of events at the DNA level (Malarkey *et al.*, 1995).

With regard to insulin, the receptor links to this pathway in a slightly different way from the other growth factors. The receptor links to this growth stimulatory pathway through the association of either IRS proteins or SHPTP2 with the Grb2 adaptor protein (Malarkey *et al.*, 1995). Downstream of Grb2 the insulin receptor then signals through this pathway in the same manner as the other growth factors. It is believed that MAPK-activated gene responses are involved in the early phases of activation of DNA synthesis in many cell types (Malarkey *et al.*, 1995). This process is also known to be of importance in the insulin-induced increase in GLUT1 expression (Gould, 1997).

However, the MAPK pathway is not the only pathway through which insulin is able to influence events at the DNA level. Instead many of the actions are mediated through PI3'K, mammalian target of rapamycin (mTOR) and p70 S6kinase-dependent pathways (Wang *et al.*, 2000). Indeed, many different transcription factors have been identified as important in mediating the effects of insulin, and most of these can be inhibited by inhibitors of the proteins mentioned above. These include eukaryotic initiation factors (eIF's), 4E binding proteins, and eukaryotic elongation factors (eEF2) (Proud and Denton., 1996; Proud and Denton.,1997; Wang *et al.*, 2000). Although there is considerable

complexity in the action of insulin on these translation factors, some of the general control mechanisms thought to operate are outlined below in Figure 1.8.

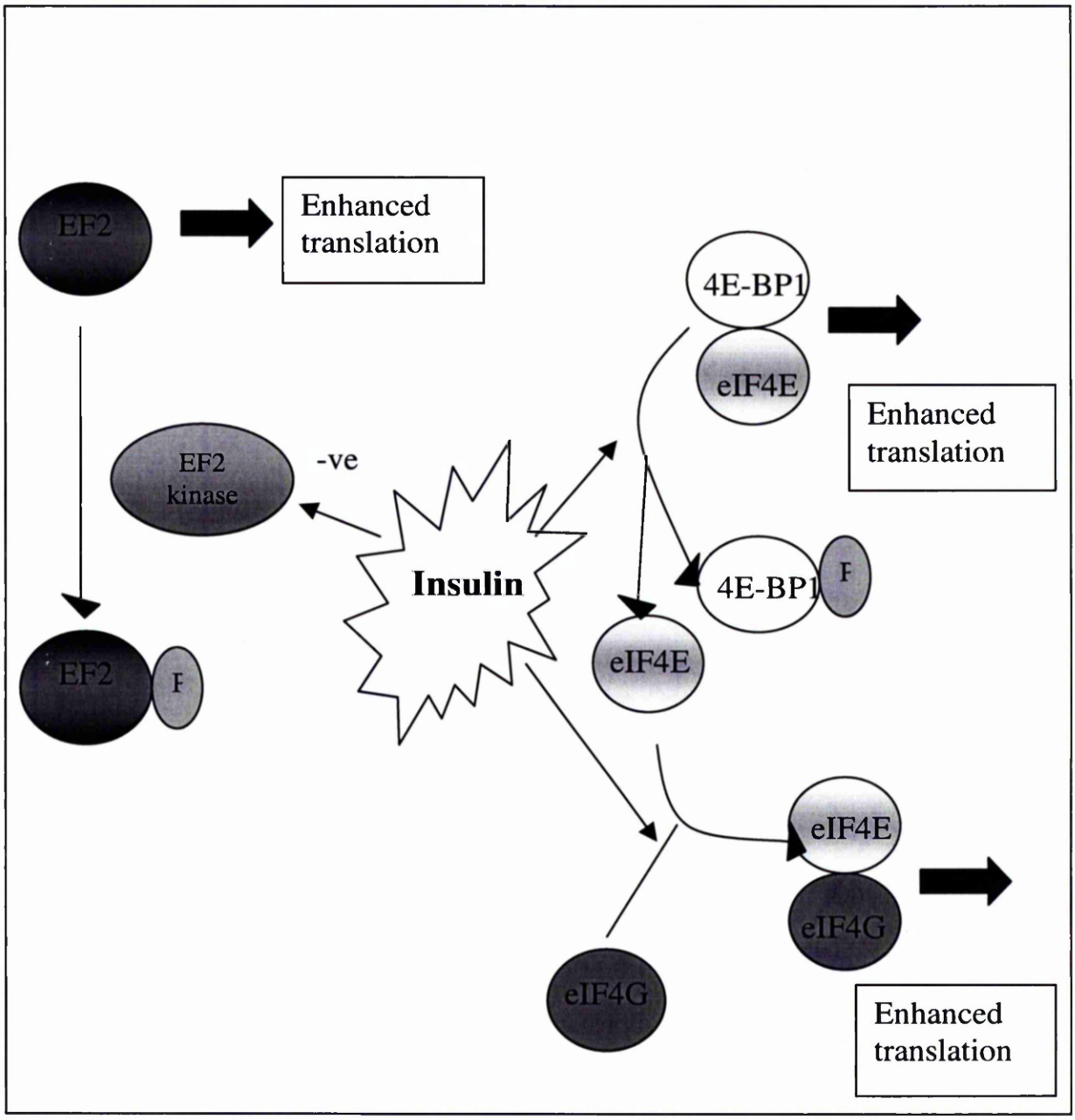


Figure 1.8 Some of the important translation factors mediating the growth promoting effects of insulin

adapted from Proud and Denton., 1996; Proud and Denton.,1997; Wang *et al.*, 2000

Interestingly, it is now emerging that although the metabolic and mitogenic effects of insulin appear to utilise similar early intermediates, there is clearly divergence in the pathways. This is of relevance in many situations, not least because it allows for selective activation of the different pathways as required. This is not only of interest with regard to understanding how the different processes occur at the biochemical level, but also in conditions such as insulin resistance, where there are likely to be selective impairments in different branches of the pathways.

1.8 Insulin Sensitivity and Insulin Resistance

In view of the complexities of insulin signalling to its biological effects as discussed above, it is not hard to comprehend why there are a multitude of disorders associated with dysregulation of these processes. Indeed it was displayed by Himsworth as early as the 1930's that even within a normal population there exists inter-individual differences in the hypoglycaemic response to insulin (Himsworth, 1936). In some individuals however, there is a marked loss of sensitivity to insulin: a phenomenon known as insulin resistance. In all cases of insulin resistance, there is a sub-normal biological response to a given dose of insulin in target tissues. This results in lower levels of uptake from the blood into the target tissues predominantly skeletal muscle and to a lesser extent adipose tissue. Therefore, the blood glucose levels become raised: a phenomenon known as hyperglycaemia. In a bid to overcome the hyperglycaemia, in the face of reduced tissue sensitivity to insulin, more insulin is secreted resulting also in a characteristic hyperinsulinaemia. In the majority of pre- or early-phase diabetics, this elevated secretion of insulin is enough to overcome the tissue insensitivity and patients can exist in a hyperinsulinaemic but euglycaemic state. Eventually, however, the hyperinsulinaemic response may become inadequate (due to pancreatic β cell dysfunction) to overcome the deficit and these subjects become both hyperinsulinaemic and hyperglycaemic. At this point, patients will then be classified as suffering from non-insulin dependent diabetes mellitus (NIDDM).

1.9 Insulin Resistance and NIDDM

As with many metabolic disturbances the severity of insulin resistance varies between different disease states and within distinct disease groups. Perhaps one of the best defined syndromes of insulin resistance is that present in NIDDM, where there is generally a profound reduction in the ability of insulin to stimulate glucose uptake (Dale *et al.*, 1996; Gould, 1997; Brady and Saltiel., 1999). Indeed the presence of insulin-resistance in an individual is accepted as an excellent marker of subjects highly likely to develop overt diabetes.

As a direct consequence of the high incidence of diabetes, and the complications associated with the disease, the search for the underlying cause(s) is of high priority.

1.9.1 GLUT4 in insulin resistance

Due to the central nature of GLUT4 in insulin-stimulated glucose transport a wealth of studies have focused on this protein, both in human studies and in animal models of insulin resistance and diabetes. The observation that there is a defective stimulatory effect of insulin in skeletal muscle from NIDDM patients suggests that either GLUT4, or another protein linked to its activation, is somehow dysfunctional (Dale *et al.*, 1996; Gould, 1997).

Interestingly levels of GLUT4 in adipocytes from NIDDM subjects do show a reduced GLUT4 protein content, due to a pre-translational suppression of GLUT4 gene expression (Gould, 1997). Although this can explain the resistance to insulin seen in isolated adipocytes, it is unclear exactly how this change contributes the insulin resistance seen at the whole body level. Although a similar reduction in GLUT4 content in muscle would explain this in part, no changes in GLUT4 expression are observed at the time when reduced insulin-stimulated glucose transport is manifest (Dale *et al.*, 1996; Gould, 1997). Interestingly GLUT4 gene

studies have revealed that no mutations were detectable in tissue from several diabetic subjects (Gould, 1997).

A similar picture has been generated in animal models of insulin resistance and diabetes. Although all of the different animal models undoubtedly have differing underlying physiology, they are still useful in ascertaining how GLUT4 may be altered in, or contribute to, states of cellular insulin resistance.

In the obese and spontaneously diabetic Rhesus monkey the first detectable signs of a pre-diabetic state are the hyper-secretion of insulin, followed by an obvious peripheral insulin resistance (Dale *et al.*, 1996). This is later followed by pancreatic insufficiency defined by a deficiency in insulin secretion (Dale *et al.*, 1996). These animals, however, exhibit no detectable change in GLUT4 content of fat or muscle (Dale *et al.*, 1996).

The genetically obese Zucker rat (fa/fa) is an animal model which is characterised by extreme obesity, insulin resistance, hyperinsulinaemia, hyperlipidaemia and a variable degree of glucose intolerance (Galante *et al.*, 1993). This rat therefore shares some of the characteristics of human NIDDM. The animals have impaired glucose uptake into isolated adipocytes and all major muscle groups (Livingstone *et al.*, 1995; Dale *et al.*, 1996). These changes are accompanied by a reduction in GLUT4 content of adipose, with little change in skeletal muscle (Livingstone *et al.*, 1995; Dale *et al.*, 1996). This is similar to the situation observed in many pre- or early- diabetes.

Interestingly, recent studies have highlighted the role of SNARE proteins in the development of insulin resistance. Maier *et al*, illustrated how the cellular levels of two v-SNARE's- VAMP-2 and cellubrevin, and one t-SNARE- Syntaxin 4, were significantly elevated in skeletal muscle from ZDF rats (Maier *et al.*, 2000). This elevation could be ameliorated by normalization of blood glucose levels using the thiazolidinedione anti-

diabetic agent, rosiglitazone. This is of significance to human diabetes in that it is the first observation of altered expression of proteins known to regulate GLUT4 function, and secondly because it highlights how these proteins can be regulated by agents which also regulate insulin sensitivity. Although it may initially seem surprising in that these proteins are increased in insulin resistant states, it suggests a compensatory mechanism aiming to overcome some other defect. This animal is also considerably obese, and this is believed to contribute to worsening of the phenotype, as indeed obesity worsens the insulin resistance syndrome in humans.

1.9.2 Other signalling molecules and insulin resistance

Although there are observations of mutations in the insulin receptor and the IRS proteins, none of these mutations appears to explain the presence of diabetes in the population as a whole. With the development of knockout mice, certain insights have been gained regarding the potential role certain proteins may play. Both IRS-1, IRS-2 and IRS-3 knockout mice have been studied. Intriguingly only the IRS-2 knockout develops overt diabetes, due to a peripheral insulin resistance and failure of β-cell compensatory mechanisms (Tamamoto *et al.*, 1994; Withers *et al.*, 1998; Liu *et al.*, 1999). The IRS-1 knockout has impaired growth and mild glucose intolerance whereas the IRS-3 knockout appears essentially similar to the wild type litter-mates (Tamamoto *et al.*, 1994; Liu *et al.*, 1999). Large scale naturally occurring defects such as these, however, have not been observed in animal models of insulin-resistance or in human studies. Similar studies have been carried out with phosphotyrosine phosphatase (PTP) knockout mice. Although these do develop insulin resistance to varying degrees, again defects in these proteins have not been observed in the diabetic population or animal models (Kahn, 2000). Perhaps one of the most interesting of the recent studies came from Jiang and colleagues, who illustrated the involvement of the IRS-1/2 PI3'K pathway in resistance to insulin signalling in the vasculature of Zucker (fa/fa) rats (Cusi *et al.*, 2000). Importantly this was a selective resistance,

and insulin mediated-signalling through the MAPK pathway was normal. Although this study does not use a classical insulin responsive tissue, it presents an interesting idea, in that selective resistance to the metabolic but not mitogenic actions could contribute to worsening of the disease phenotype.

1.10 Insulin Resistance and Hypertension

It was first observed in 1966 by Wellborn (Wellborn, 1966) that insulin levels in hypertensive patients are often raised, and thirty years later the association is now well established. More detailed studies have revealed that lean, non-diabetic essential hypertensives are hyperinsulinaemic compared with well-matched normotensive controls (Mitchell *et al.*, 1990). Data from the San Antonio Heart Study revealed that by the fifth decade of life eighty-five percent of diabetics are hypertensive and obese. Similar observations were made in a study by Modan *et al* (Modan *et al.*, 1985) examining the links between hypertension, obesity and glucose intolerance in an Israeli population. They concluded that there was both a higher prevalence of elevated blood pressure in subjects with impaired glucose tolerance and obesity, and increased post-prandial insulin levels in treated and non-treated hypertensives. Numerous other studies have clearly illustrated the close association between insulin sensitivity, elevated blood pressure and obesity; an association that increases with age (Morris and Connell., 1994). Furthermore, it now also well established that the relation between insulin resistance and hypertension exists independently of age, obesity and renal function (Morris and Connell., 1994). Elevated blood pressure and insulin resistance have also been seen to cluster with elevated circulating triacylglycerol (TAG) and diminished HDL-cholesterol levels in certain subjects. This condition, known as Syndrome X can often precipitate the development of certain cardiovascular conditions such as atherosclerosis (Morris and Connell., 1994).

Although a role for insulin in primary hypertension and associated diseases has been well established the exact relationship between the two syndromes is more controversial. Julius *et al* have proposed that hypertension may precede the development of insulin resistance and hyperinsulinaemia and may in fact be the cause (Julius *et al.*, 1993). They suggest that reduced muscle blood flow and microvascular disease leads to reduced glucose uptake. Certainly there is clinical evidence to support this (Donnelly *et al.*, 1992), but this theory fails to explain the wealth of convincing data from both human subjects and animal models which indicates that the opposite situation is true (Morris and Connell., 1994). Indeed, it has been shown that when primary hypertension and NIDDM co-exist, the hypertension is eight times more likely to antedate the development of NIDDM. The spontaneously hypertensive rat (SHR), a genetic model of hypertension, also develops insulin resistance (Reaven *et al.*, 1989). The insulin resistance, as documented by defective glucose transport into adipocytes, develops long before any elevations in blood pressure are apparent. Further evidence to reinforce this comes also from studies of primary and secondary hypertensives, in that insulin resistance is prevalent only in subjects with primary and not secondary hypertension (Donnelly *et al.*, 1992). The lack of association between secondary hypertension and insulin resistance provides evidence therefore that elevated blood pressure *per se* does not automatically lead to insulin resistance. This has also been shown in rodent models, where rats made hypertensive by renal artery clipping do not go on to develop insulin resistance or hyperinsulinaemia (Buchanan *et al.*, 1991). This, taken together, suggests that it is unlikely that the development of hypertension precedes insulin resistance in primary hypertensives.

1.10.1 How might Insulin Resistance lead to Hypertension ?

Insulin and hyperinsulinaemia can potentially lead to the activation of several systems which, alone or in co-operation, can promote elevations in blood pressure. These include the actions of insulin on the sympathetic

nervous system, sodium balance, and effects on vascular smooth muscle and endothelial cells (Morris and Connell., 1994; Donnelly *et al.*, 1992).

1.10.2 The Metabolic Syndrome

Clearly, hypertension and insulin resistance can co-exist in a variety of different clinical situations, and it is not a simple task to begin to classify these in any way. Nevertheless, many groups have observed that certain key metabolic and haemodynamic disorders appear to cluster together in a broad but coincident manner. For example, many insulin resistant primary hypertensives also display other metabolic disturbances such as disrupted blood lipid profiles (generally elevated circulating triglycerides, reduced HDL-cholesterol levels or both) and impaired catecholamine mediated free fatty acid uptake (Ginsberg, 2000). These disorders, often occurring in conjunction with abdominal obesity, are referred to as the Metabolic Syndrome (also known as Syndrome X or Reaven's Syndrome). It is generally believed that patients who fit this description are at greater risk of subsequently developing atherosclerotic lesions and related conditions.

1.10.3 Lessons from animal models

As discussed above, animal models have proved useful in understanding the development of insulin resistance and how insulin resistance may affect other associated diseases. Several different animal models have yielded valuable information regarding the potential relationships between insulin resistance and hypertension. This is discussed below.

The Milan Hypertensive (MHS) Rat is a well characterised rodent model of hypertension. The strain exhibits a markedly elevated blood pressure compared to the normotensive control strain (MNS), with blood pressures of around 120mm Hg in the MHS, compared to aroud 95mm Hg in the MNS (Dall'Aglio *et al.*, 1991). The MHS also exhibits an enlarged left

ventricular mass and a slower heart rate than weight matched controls (Ferrari *et al.*, 1987). In addition to the cardiovascular defects observed in these animals, there is an apparent defect in carbohydrate metabolism. Dall'Aglio et al have demonstrated that although blood glucose levels are similar between the two strains, the MHS display hyperinsulinaemia and hypertriglyceridaemia. (Dall'Aglio *et al.,* 1991). Interestingly Livingstone et al have demostrated a loss of GLUT4 expression in skeletal muscle , a major insulin-responsive glucose consuming tissue (Livingstone *et al.*, 1995). Similar reductions in GLUT4 content of skeletal muscle are rarely observed in the metabolic syndrome in humans, and this suggests that the altered cardiovascular and metabolic phenotype observed in the MHS differs from that observed in humans. Nevertheless, the demonstration of altered carbohydrate metabolism in a rodent model of hypertension further highlights the complex relationships that can exist between the haemodynamic and metabolic status.

The obese Zucker rat, described in Section 1.9.1, is another model that displays a phenotype similar to that observed in the human metabolic syndrome, although again there appears to be a different underlying abnormality. It appears that much of the defect observed in the Zucker is a direct result of obesity (Livingstone *et al.*, 1995). Although obesity undoubtedly contributes to the worsening of many of the defects seen in the metabolic syndrome, it is well documented that they occur independently of obesity.

Although the models described above have been beneficial in understanding the relationship between insulin resistance and hypertension, perhaps the most useful models are the spontaneously hypertensive rats (SHR's).

1.10.4 SHR

The spontaneously hypertensive rat (SHR) is a rodent model of genetic hypertension which has been employed as a tool to study the development of hypertension and metabolic syndromes in humans. Originally developed by Okamoto's group in Japan, the SHR was derived through extensive inbreeding of Wistar rats, specifically selecting for off-spring with elevated blood pressures (Okamoto *et al.*, 1974). These animals were then subsequently brother x sister mated for several generations, in order to generate the SHR colony (Okamoto *et al.*, 1974).

Of particular advantage in using the SHR is that it displays many of the metabolic disturbances which often occur in conjunction with hypertension in humans, namely hyperinsulinaemia, hypertriglyceridaemia and resistance to the cellular effects of insulin (Iritani *et al.*, 1977; Reaven *et al.*, 1989; Aitman *et al.*, 1999). Reaven *et al* first showed in the late eighties that the SHR displayed resistance to the effects of insulin *in vitro*. Isolated epididymal adipocytes showed a diminished ability to mediate insulin-stimulated glucose transport (Reaven *et al.*, 1989). Data generated using oral glucose tolerance tests (OGTT) have generally produced concordant results (Aitman *et al.*, 1997).

In addition to defects in carbohydrate metabolism, the SHR exhibits further defects analogous to those seen in the human metabolic syndrome, namely abnormalities in lipid metabolism. Although the ability of insulin to modulate catecholamine-mediated lipolysis is not altered in the SHR, the maximum isoprenaline stimulated lipolysis is indeed reduced (Reaven *et al.*, 1989). The SHR also displays elevated serum triglyceride levels compared to WKY controls (Aitman *et al.*, 1999), again a feature common to the human metabolic syndrome.

Due to these apparent similarities it is therefore of interest to define the underlying abnormalities responsible for the phenotype observed in the

SHR. In order to do this Aitman *et al* employed an essentially genetic strategy (Aitman *et al.*, 1999).

1.10.4.1 Potential defects underlying the metabolic abnormalities in the SHR

In order to identify and characterise markers that segregate with the phenotypes of interest in the SHR, that is defective insulin action, increased basal triglycerides and reduced effect of isoprenaline, extensive genetic analyses were carried out. In order to facilitate detection of Quantitative trait Loci (QTL's), a large F_2 cohort was produced by back crossing two previously produced inbred WKY and SHR strains. Extensive phenotypic and genotypic analysis of these animals revealed the presence of several QTL's for SHR defects in glucose and fatty acid metabolism, hypertension and hypertrigyceridaemia, and that several of these QTL's map to a single locus on rat chromosome 4 (Aitman *et al.*, 1999).

Recently further analysis of these QTL's has yielded valuable information regarding the phenotype observed in the SHR. To further confirm the importance of this region on chromosome 4, a congenic rat strain was produced where the SHR QTL on chromosome 4 was replaced with the corresponding region of chromosome 4 from the normotensive and non-insulin resistant Brown-Norway (BN) rat. The resultant SHR.4 exhibited a **partial** correction in the defect in insulin-mediated glucose uptake, and a **complete** correction of the defect in isoprenaline mediated lipolysis (Aitman *et al.*, 1999). This provides very strong evidence therefore for the involvement of this area in regulating both carbohydrate and lipid metabolism.

To characterise further the QTL region on chromosome 4 this SHR.4 strain was then used in conjunction with normal control SHR and WKY. By using an elegant technique known as cDNA micro-array analysis, several clones were identified where the expression between SHR and SHR.4 was

significantly different. One of these clones mapped in the centre of the QTL using radiation hybrid mapping, and was functionally therefore a very likely candidate. This clone was revealed to encode the gene for the putative fatty acid transporter Cd36/Fatty Acid Translocase (FAT), which is known to map to chromosomes analogous to rat chromosome 4 in both mouse and human (Aitman *et al.*, 1999). The location of the *Cd36* gene therefore identified the gene as potentially central to the deficits in glucose and fatty acid metabolism mapping to chromosome 4 in SHR rats.

Since Aitman's group identified the apparent importance of the *Cd36* gene product, other groups have generated similar data that reinforces this. Pravenec *et al* were also able to partially correct the SHR defects in glucose and lipid metabolism by transferring a segment of BN chromosome 4 (including the *Cd36* region) into the SHR chromosome (Pravenec *et al.*, 1999). This work not only reinforces a role for the *Cd36* gene product, but also demonstrates that a single chromosome region can influence a broad spectrum of cardiovascular risk factors involved in the metabolic syndrome. Despite the convincing demonstration of the linkage between the Cd36 defect and the SHRSP phenotype however, the actual cellular mechanisms involved in this link remain elusive.

1.10.4.2 Cellular functions of the Cd36 protein

(i) Fatty Acid Transport

Although transport of long chain fatty acids (FA) across plasma membranes was once thought to involve simple diffusion, a strong body of evidence now indicates that long chain FA transporter proteins are involved, implicating a facilitated process (Febbraio *et al.*, 1999). Certainly expression of long chain FA transporters is prevalent in tissues such as adipose, mammary epithelia, small intestine and both cardiac and skeletal muscles (Febbraio *et al.*, 1999); where increased FA transport would be expected to occur.

The Cd36/FAT (Fatty Acid Translocase) protein, identified to be of importance in the SHR by Aitman's group, encodes such a Fatty Acid Receptor/Transporter protein (Aitman *et al.*, 1999). Growing evidence implicates this protein as central to long chain FA transport in tissues where there is strong demand for FA's. (Febbraio *et al.*, 1999). Much of the work carried out in order to elucidate a role for Cd36 has involved studies where the protein is either over-expressed or deleted completely, in mouse models (Febbraio *et al.*, 1999; Ibrahimi *et al.*, 1999).

Work from Ibrahimi's group demonstrated that muscle from mice over-expressing Cd36 displayed an increased capacity for FA oxidation, and increased FA uptake from the blood (Ibrahimi *et al.*, 1999). The mice also had lower total and Very Low Density Lipoprotein (VLDL)-TAG, and a general decrease in total body fat (Ibrahimi *et al.*, 1999).

In contrast to this, Febbraio's group by generating *Cd36* null mice, created the reverse phenotype. Studies using these animals have revealed a striking phenotype, which includes elevated fasting plasma (serum) cholesterol, non-esterified FA and triacylglycerol (TAG) levels (Febbraio *et al.*, 1999). This suggests a role for Cd36 in clearing these substances from the blood stream and promoting their uptake into the relevant tissues, functioning indeed as a fatty acid transporter. *Cd36* null mice also show a diminished ability to transport oleate (a typical long chain FA) at low FA:Bovine Serum Albumin (BSA) ratios, analogous to those present in physiological situations. Should Cd36 function in such a role suggested by these studies, then absence could play a role in the development of any conditions where there are abnormalities in lipid metabolism.

(i) Receptor for modified LDL species

More recent work however, again by Febbraio's group, has suggested that the reverse could also be true. Cd36 is a member of the Scavenger Receptor Family, a large group of trans-membrane proteins which all

possess the ability to bind large negatively charged molecules, such as oxidised or modified LDL (ox-LDL/m-LDL) (Febbraio *et al.*, 2000). Scavenger receptors are believed to play an important role in the development of atherosclerosis, where they function in atherosclerotic plaque formation. Although plaque formation is ultimately dependent on a variety of circulating and locally released factors, lipid deposition in the plaque is central to its development and progression. This lipid deposition is achieved by the insertion of foam cells, monocyte-derived macrophages that have taken up large amounts of m-LDL, into the intima and vessel walls. By allowing entry of m-LDL into the macrophages (therefore allowing them to turn into foam cells) the importance of Cd36 in this process is believed to be by functioning as a receptor for the m-LDL (Febbraio *et al.*, 2000). Indeed Febbraio showed that *Cd36* knockout mice, when crossed in to an *ApoE* gene (postulated to play a role in protection against development of atherosclerosis) negative environment, decreased the development of atherosclerosis. This is backed up by a reduction in foam cell production, measured in vitro (Febbraio *et al.*, 2000). Finally, the ability of Cd36 to bind and promote uptake of nitro-LDL species has also been observed (Podrez *et al.*, 2000). This is thought to be of relevance as monocytes themselves have systems responsible for generating reactive nitrogen species, which easily react with circulating-LDL.

1.10.5 SHRSP

Derived from the same lines as the stroke-resistant SHR (referred to through-out this thesis as the SHR), the stroke-prone SHR represents a sub-group of SHR which displays even higher blood pressures than the SHR and, as the name suggests, is more prone to cerebrovascular lesions resulting in stroke (Okamoto *et al.*, 1974). Although extensive studies have aided in characterising the vascular abnormalities in the SHRSP, there have been a lack of studies aimed at examining the metabolic status of this particular sub-strain. This issue is also addressed in Chapter 3 of

this thesis, where the metabolic phenotype of the SHRSP is examined with relevance to the SHR and potential importance of the CD36 deletion.

1.11 Insulin Resistance and other Metabolic disorders

Although insulin resistance is most often associated with conditions such as NIDDM and Syndrome X, the condition also forms a part of many other diseases. One of the most important of these is in disorders of the female endocrine system where insulin resistance forms an integral part of the disease phenotype, for example Polycystic Ovarian Syndrome (PCOS). This is a complex disease and although it is unclear whether the insulin resistance is a cause or consequence, its presence undoubtedly worsens the outcome. The links between PCOS, insulin resistance and intra-cellular signalling by insulin are extensively discussed in Chapter 4, with an aim to addressing how defective insulin resistance could contribute to the phenotype observed in PCOS.

1.12 Aims

As discussed in the introduction to this chapter, insulin action within mammalian tissues is a complex process, regulated at several different levels. Because of this complexity insulin action in mammalian cells can easily be perturbed. Perhaps the most obvious illustration of this is in Insulin Dependent Diabetes Mellitus (IDDM), where there is a primary defect in insulin secretion and patients require the addition of exogenous insulin to survive. More common however than IDDM is NIDDM. NIDDM occurs when there is a resistance to the actions of insulin in the peripheral target tissues, principally muscle and adipose tissue; and a failure of the pancreas to secrete adequate amounts of insulin to overcome this. Insulin resistance also occurs in other conditions, and it is found particularly associated with cardiovascular disease syndromes where hypertension, abnormal lipid metabolism and obesity also occur.

Animal models have been very useful in studying the links between insulin resistance. Ones of the aims of this thesis is to address the relationship between metabolic disorders and hypertension in a genetic model of hypertension, the SHRSP. This will involve characterization of the SHRSP phenotype with regard to carbohydrate and lipid metabolism, with an aim to determining how this relates to the SHR phenotype. Insulin signalling in isolated adipocytes will also be examined in a bid to determine if altered signalling may underlie any metabolic defects detected in SHRSP adipocytes.

As discussed in Section 1.11 (and also later in Chapter 4) insulin resistance is also commonly associated with disorders of the female endocrine system, of which PCOS is the best characterised example. A further aim of this thesis is to determine potential mechanisms by which abnormal levels of important female sex hormones can influence the development of insulin resistance in a cell culture model of a peripheral insulin-sensitive tissue. Again, the main aim of this work is to relate this

to the intracellular signalling pathways activated by insulin in adipose tissue.

2 Materials and Methods

2.1 Materials

All reagents used during this project were of the highest quality available and were obtained from the following suppliers:

2.1.1 General Reagents

Alpha Laboratories, Eastleigh, Hampshire, UK
WAKO NEFA-C Kit

Boehringer Mannheim, GmbH, Germany
Complete ™ Protease Inhibitor Tablets

Bio-Rad Laboratories, Hemel Hempstead, Hertfordshire, UK
N, N, N', N',-tetramethylethylenediamine (TEMED)

BDH, Loughborough, Leicestershire, UK
acrylamide
ammonium persulphate
Dow Corning silicone oil, 100/200CS
D-glucose
glycerol
glycine
Hepes
hydrochloric acid
methanol
potassium chloride
sodium dodecyl sulphate (SDS)
sodium chloride
sodium dihydrogen orthophosphate dihydrate
sodium diaminoethanetetra-acetic acid (EDTA)
sodium hydrogen carbonate

Gelman Sciences Ltd., Northampton, UK

sterile acrodisc 0.2μM filters

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

Lorne Laboratories, Twyford, Essex, UK

collagenase- type 1 for adipocyte isolation

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

calcium chloride hexahydrate

dimethyl sulphoxide (DMSO)

magnesium chloride hexahydrate

magnesium sulphate heptahydrate

NovoNordisk, DK-2880, Bagsvaerd, Denmark

Human Actrapid® Insulin solution- 100 IU/ml

Porcine Insulin

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

Ultima-Flo scintillation fluid

Premier Brands UK, Knighton Adbaston, Staffordshire, UK

Marvel powdered milk

Schliecher & Schuell

nitrocellulose membrane (0.45μM)

Sigma Chemical Company Ltd., Poole, Dorset, UK

adenosine

aprotinin

BSA (A-7030) used for protein estimations

BSA (A-7888) used for adipocyte buffers

bromophenol blue

cytochalasin B

2-deoxy-D-glucose

diisopropyl fluorophosphate (DFP)

DL-dithiothreitol

E64

estriol

estrone

Pepstatin A

Triton X-100

Whatmann International Ltd., Maidstone, UK

Whatmann 3mm filter paper

2.1.2 Antibodies

Amersham International Plc, Aylesbury, Buckinghamshire, UK

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

HRP-conjugated donkey anti mouse IgG antibody

Sigma Chemical Company Ltd., Poole, Dorset, UK

HRP-conjugated donkey anti sheep IgG antibody

HRP-conjugated mouse anti sheep/goat IgG antibody

TCS Biologicals, Botolph Claydon,Buckingham, UK

"immunoaffinity purified" rabbit IgG directed against the C-terminal 14 amino acids ([C]YASINFQKQPEDRQ) of rat liver IRS-1

"Protein A-purified" rabbit IgG directed against a GST Fusion protein containing amino acids 976-1094 of mouse IRS-2

"Protein A-purified" rabbit IgG directed against a GST fusion protein containing the full-length 85kDa sub-unit of PI3'Kinase

"immunoaffinity purified" sheep IgG directed against a GST fusion protein corresponding to residues 1-149 of human Akt-1 (PKBα) Pleckstrin Homology domain

"immunoaffinity purified" sheep IgG directed against a 15 residue synthetic peptide (RPHFPQFSYSASGTA) corresponding to the C-terminal (residues 466-480) of rat Akt-1 (PKBα)

"immunoaffinity purified" sheep IgG directed against a KLH conjugated synthetic peptide (RYDSLGSLELDQRTH) corresponding to amino acids 455-469 of rat Akt-2 (PKBβ)

sheep IgG directed against the C-terminal 14 amino acids of human GLUT4

2.1.3 Cells

American Type Culture Collection, Rockville, USA

3T3-L1 fibroblasts

2.1.4 Tissue Medium and Reagents

Gibco BRL, Paisley, Lanarkshire, UK

Foetal Calf Serum (FCS)

Newborn Calf Serum (NCS)

Dulbecco's Modified Eagles Medium (DMEM)

Sigma Chemical Company Ltd., Poole, Dorset, UK

Penicillin Streptomycin

Trypsin

2.1.5 Cell Culture Plastics

AS Nunc, DK Roskilde, Denmark

50ml centrifuge tubes

Bibby Sterilin, Ltd., Stone, Staffordshire, UK

sterile pipettes

Costar

75cm^2 tissue culture flasks

Fred Baker Ltd.

10cm tissue culture dishes

6 well tissue culture plates

2.1.6 Radioactive Materials

Amersham International Plc, Aylesbury, Buckinghamshire, UK

[γ^{32}P]-ATP

NEN DuPont (UK) Ltd., Stevenage, Herts, UK

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

2.2 Buffers and medium

2.2.1 Cell Culture medium

Serum Free DMEM

100U/mL Penicillin, 100U/mL Streptomycin in DMEM

NCS DMEM

100U/mL Penicillin, 100U/mL Streptomycin in DMEM, 10% (v/v) NCS

in DMEM

FCS DMEM

100U/mL Penicillin, 100U/mL Streptomycin in DMEM, 10% (v/v) FCS in DMEM

Sterile Trypsin solution for cell passage

25% (w/v) trypsin in PBS (see section) was syringe filtered through a 0.2μM membrane and stored in 50ml sterile centrifuge tubes at -20^{o}C.

2.2.2 Adipocyte Buffers

Collection Buffer

118mM NaCl, 5mM $NaHCO_3$, 4.7mM KCl, 1.2mM KH_2PO_4, 1.2mM $MgSO_4.7H_2O$, 25mM Hepes, 0.2μM adenosine, 2.5mM $CaCl_2$, 1% (w/v) BSA (Fraction V), 3.0mM glucose

Digestion Buffer

118mM NaCl, 5mM $NaHCO_3$, 4.7mM KCl, 1.2mM KH_2PO_4, 1.2mM $MgSO_4.7H_2O$, 25mM Hepes, 0.2μM adenosine, 2.5mM $CaCl_2$, 1% (w/v) BSA (Fraction V), 3.0mM glucose , Type 1 collagenase (for exact amounts see 2.7.8)

Wash Buffer

118mM NaCl, 5mM $NaHCO_3$, 4.7mM KCl, 1.2mM KH_2PO_4, 1.2mM $MgSO_4.7H_2O$, 25mM Hepes, 0.2μM adenosine, 2.5mM $CaCl_2$, 1% (w/v) BSA (Fraction V)

2.2.3 General Buffers

Hes Buffer

20mM Hepes, 1mM EDTA, 225mM sucrose, pH 7.4

KRP Buffer

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

Lysis Buffer

50mM Hepes, pH 6.5, 150mM NaCl, 100mM NaF, 10mM $NaPO_4$, 1mM Na_3VO_4, 1mM EGTA, 1.5mM $MgCl_2$, 10% (v/v) glycerol, 1% (v/v) Triton-X 100,10μg/ml leupeptin, 10μg/ml aprotinin, 10μg/ml PMSF.

Phosphate Buffered Saline (PBS)

150mM NaCl, 10mM $NaH_2PO_4.dH_20$ pH 7.4

2.2.4 SDS-PAGE Buffers

Electrode Buffer

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

Sample Buffer

93mM Tris-HCl pH 6.8, 20mM dithiothreitol, 1mM Na-EDTA, 10% (w/v) glycerol, 2% (w/v) SDS, 0.002% (w/v) bromophenol blue

2.2.5 Immunoblot Buffers

Towbin Buffer (for transfer of proteins onto nitrocellulose)

25mM Tris Base, 192mM glycine, 20% (v/v) methanol

Wash Buffer

20mM Tris base, 150mM NaCl, 0.03% (v/v)Tween-20, pH 7.4

2.3 Cell Culture

2.3.1 Growth and maintenance of 3T3-L1 Fibroblasts

3T3-L1 fibroblasts were seeded into 75cm^2 flasks and maintained at 37^{o}C/5% CO_2 in a humidified incubator. Cells were fed every two days with DMEM/10% (v/v) NCS/100U/ml Pen strep until they reached confluency.

2.3.2 Trypsinisation of 3T3-L1 Fibroblasts

Medium was aspirated from cells in a 75cm^2 flask and the cells then washed with 10mls pre-warmed sterile PBS. This PBS was then removed and replaced with Trypsin (in sterile PBS). Cells were returned to the incubator and left for around 5 minutes to allow cells to detach. After this the trypsin was neutralized by the addition of 10mls medium, and cells seeded as desired. On average the cells from one 75cm^2 flask were seeded onto ten 10 cm plates.

2.3.3 Preparation of 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 of dexamethasone was prepared by a 1:20 dilution of 2.5mM dexamethasone in ethanol with 10% (v/v) FCS/DMEM prior to use. A 500X sterile stock solution of methyl isobutylxanthine (IBMX) was prepared by dissolving 55.6mg IBMX in 1.0ml 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 of 3T3-L1 Fibroblasts

Cells were routinely differentiated 48 hours post-confluency. Medium was aspirated from cells and replaced with 10mls differentiation medium (outlined above) per 10cm plate. Cells were incubated in this medium for two days. After this time the medium was carefully aspirated and replaced with DMEM/10% (v/v) FCS/100U/ml Pen strep containing 1μg/ml Insulin. Cells were incubated in this medium for a further two days. Cells were then fed every two days with DMEM/10% (v/v) FCS/100U/ml Pen strep. Cells were used between days 8-13 after differentiation, the point at which insulin stimulated glucose transport is maximum.

2.3.5 Freezing and storage of 3T3-L1 Fibroblasts

Confluent cells from a $75cm^2$ flask were removed as described in 2.3.2. Cells were then centrifuged (1000 x g at room temperature for 5 minutes) in a 13.5ml centrifuge tube to pellet cells. Medium was then aspirated off and replaced with 1ml sterile 10% (v/v) NCS/DMEM containing 10% (v/v) glycerol which had been pre-equilibrated in 10% CO_2 for 1 hour. Cells were transferred to sterile cryovials (0.5ml per vial), wrapped in cotton wool and frozen at $^{-}80^{o}C$ overnight. The next day the cotton wool was removed and the vials were transferred to a liquid N_2 for long term storage.

2.3.6 Resurrection of 3T3-L1 Fibroblast stocks

10mls of sterile DMEM/NCS/100U/ml Pen strep was placed in a $25cm^2$ flask and left in the incubator for at least 30 minutes to allow medium to equilibrate with CO_2 and humidity. During this time a vial of cells was

removed from liquid nitrogen storage and placed in a flask containing liquid nitrogen for transfer to the culture hood. The vial was removed from the nitrogen and sprayed with ethanol, whereupon the cap was loosened to relieve pressure. The cap was slightly tightened and the vial swirled in a 37^{o}C water bath for approximately two minutes. The vial was then removed from the water bath whilst a small amount of frozen medium remained in the vial. The vial was sprayed with ethanol again, the pre-equilibrated flask returned to the hood, and the cells rapidly transferred to the flask using a sterile plastic pipette. Cells were left to attach and then medium was replaced the next day.

2.3.7 Measurement of 2-Deoxy-D-Glucose uptake in 3T3-L1 adipocytes

Uptake of 2-deoxy-D-glucose was measured using the method adapted from Campbell, PhD thesis, 1997.

Adipocytes in 6 or 12 well plates were quiesced in serum-free DMEM for at least two hours. Cells were then transferred to the hot plate, where the medium was aspirated and replaced with 1ml KRP (37^{o}C). This was then aspirated and replaced with 1ml KRP/1% (w/v) BSA (37^{o}C). For stimulation insulin was added where desired and plates swirled briefly to ensure adequate mixing throughout the plate. Following stimulations, to half of the wells at each condition 1µl of 1mM Cytochalasin B was added to give a final concentration of 10µM. After 1 minute uptake was initiated by the addition 50µl of 2-deoxy-D-glucose (deGlc) solution (50µM, 0.5µCi/well). Plates were gently mixed and after a further three minutes radioactivity was removed. Plates were then rapidly washed by immersing 3 times in a beaker containing ice-cold PBS. Plates were then allowed to dry and, after this time, 1ml of 1% (v/v) Triton X-100 was added to each well and the plates wrapped in Cling-film to avoid evaporation. Cells were then solubilised by shaking at room temperature for two hours. After this time the solution from each well was removed to

a scintillation vial, ensuring the volumes had not altered dramatically, scintillant was added, and the radioactivity was measured using a Beckman Scintillation counter. Data was quantified as picomoles 2-deoxy-D-glucose transported per minute per million cells, assuming 2.1 x 106 cells per well of a 6 well plate (Brant, thesis, University of Glasgow, 1994).

2.3.8 Hormonal treatment of 3T3-L1 adipocytes

3T3-L1 adipocytes in 10cm dishes or 6 well plates were treated for 12 (membrane/lysate preparation) or 48 hours (transport assay) with 100nM of the desired steroid, in FCS/DMEM. The next day the cells were quiesced in serum-free DMEM for at least two hours. After this time, insulin was added where desired and cells then subjected to transport assay (refer 2.3.7) or membranes (refer to 2.3.9) or lysates (refer to 2.3.10) prepared.

2.3.9 Preparation of total membranes from 3T3-L1 adipocytes

Adipocytes in 10cm dishes were quiesced in serum-free DMEM for at least two hours. Cells were then transferred to the hot plate. For stimulations insulin was added where desired and plates swirled briefly to ensure adequate mixing throughout the plate. After the desired time medium was aspirated and dishes washed 2 times in ice cold PBS (pH 7.4) (see section 2.2.3). This was then replaced with 1ml ice-cold Hes (see section 2.2.3) plus protease inhibitors. Cells were harvested by scraping with a plastic cell scraper and then homogenised in a hand-held Homogeniser (20 up/down strokes). Samples were then transferred to 13.5ml centrifuge tubes and centrifuged at 1000 x g (4^{o}C) for 10 minutes, to pellet nuclear material and any un-broken cells. The resulting supernatant was then centrifuged at 100,000 x g (4^{o}C) to yield soluble protein (supernatant) and membrane fractions (pellet). The pellet was usually re-suspended in 10 times volume of Hes plus protease inhibitors.

Samples were then subjected to protein assay (see section 2.6) and snap-frozen in aliquots.

2.3.10 Preparation of whole cell lysates from 3T3-L1 adipocytes

Adipocytes in 10cm dishes were quiesced in serum-free DMEM for at least two hours. Cells were then transferred to the hot plate. For stimulation insulin was added where desired and plates swirled briefly to ensure adequate mixing throughout the plate. After the desired time medium was aspirated and dishes washed 2 times in ice cold PBS (pH 7.4) (see section 2.2.3). This was then replaced with 1ml ice cold Lysis buffer (see section 2.2.3) plus protease inhibitors. Cells were harvested by scraping with a plastic cell scraper and then homogenised in a hand-held Homogeniser (20 up/down strokes). Samples were then transferred to 1.5 Eppendorf tubes and centrifuged at 30,000 x g (4^{o}C) for 10 minutes, to pellet nuclear material and any whole cells. The resulting lysate (supernatant) was then subjected to protein assay (see section 2.6) and snap-frozen in aliquots.

2.3.11 PKB Activity Assay in 3T3-L1 adipocytes

Eppendorf tubes were set up containing 50µl of a 50% Protein G Sepharose bead slurry. These beads were then washed three times by centrifugation in the lysis buffer outlined in 2.2.3. The washed Protein G beads were then incubated with 4µg anti-PKBα/PH (see section 2.1.2), with shaking at 4°C to allow the antibody to pre-couple to the beads. After two hours 300-500µg cell lysate prepared as outlined in 2.3.9 was added to each the eppendorf and left to incubate for at least 2 hours on a rotary wheel at 4°C. After this the Eppendorfs were briefly centrifuged to collect the immunoprecipitates. These were washed two times in lysis buffer minus Triton-X 100 and then washed and re-suspended in 25µl kinase buffer containing 50mM Tris, pH 7.6, 10mM $MgCl_2$, 1mM DTT. The kinase reaction was initiated by the addition of a mix containing

50μM ATP, 3μCi [γ^{32}P]ATP and 30μM PKB specific substrate peptide (RPRAATF, kindly donated by Dr Robin Plevin) in a 5μl volume. After 30 min at 15°C, the reaction was stopped by adding 10μl of 300mM orthophosphoric acid to give a final concentration of 75mM. The samples were then spotted onto 1cm^2 squares of p81 paper, placed in a covered sandwich tub and washed twice in 75mM orthophosphoric acid, twice in water and allowed to dry. They were then counted.

2.4 Animals

2.4.1 Animal upkeep and blood pressure measurement

WKY and SHRSP rats were housed within the Joint Animal Facility within the University of Glasgow. Animals were maintained on a 12 hour light/dark cycle, fed standard laboratory chow and allowed access to water *ad libitum.* Blood pressure measurements were made by insertion of telemetry probes and by tail cuff measurements, according to standardised techniques (Jeffs et al., 1997).

2.4.2 Adipocyte dissection and preparation

Epididymal fat pads were removed from rats killed by CO_2 overdose and placed immediumtely into a 50 ml centriufuge tube containing pre-warmed (37^{o}C) collection solution in a Dewar flask (see 2.2.6) for transfer to the laboratory. Cells were then weighed and rapidly transferred to a fresh pre-warmed tube containing digestion solution, to which collagenase was added (4mls of a 2mg/ml solution per gram of fat). Fat pads were finely minced using scissors and then placed into a 37^{o}C water bath. Digestion was then carried out with rapid shaking for around 30 minutes, whereupon it was terminated by the addition of a large (15-20ml) volume of wash buffer. The solution was then passed through a plastic tea strainer to allow digested cells and buffer to pass through but keep any

undigested fat or other material away from the cells. The cells were then allowed to float to the top of the tube and the medium from below removed and replaced with 15mls fresh buffer. This washing stage was repeated 3-4 times over a 30-minute period, ensuring that the tube was always immersed in the water bath so that the cells were kept at 37^{o}C constantly. Finally, washed cells were aliquoted at 40% (v/v) cytocrit for storage (not normally longer than 30 minutes).

2.4.3 Measurement of 2-Deoxy-D-Glucose uptake in rat primary epididymal adipocytes

Adipocytes were aliquoted into the desired number of pre-warmed 50ml centrifuge tubes at 10% (v/v) cytocrit (100µl cells/900µl buffer per tube). For stimulations insulin was added where desired, then both treated and basal tubes were shaken gently for the desired time. Following stimulations, to one tube at each condition 10µl of 1mM Cytochalasin B was added to give a final concentration of 10µM. After 1 minute uptake was initiated by the addition 25µl of 2-deoxy-D-glucose (deGlc) solution (10µM, 2.5µCi/ml). Tubes were gently mixed during uptake and after a further three minutes three 200µl aliquots were removed to 0.5ml eppendorf tubes containing 200µl Dow Corning Oil. Tubes were then rapidly centrifuged (500 x g/30-secs) and the adipocytes (which float on the oil above the aqueous layer) were then removed to scintillation vials using glass pasteur pipettes. The cells were then solubilised in 1ml Triton X solution (1% v/v) for at least 30 minutes. Scintillant was added and samples then counted using a Beckman Scintillation Counter. Data was quantified as picomoles 2-deoxy-D-glucose transported per minute.

2.4.4 Measurement of fatty acid release from rat primary epididymal adipocytes

Adipocytes were aliquoted into the desired number of pre-warmed 50ml centrifuge tubes at 10 %(v/v) cytocrit (100µl cells/900µl buffer per tube).

For treatment, insulin or isoproterenol was added as indicated in the legends to the figure legends in chapter 3. Following stimulations, 50µl of the buffer was removed to a glass test-tube. Fatty acid release was then measured using the WAKO NEFA-C kit exactly as described in the protocol provided, using an oleic acid internal standard. Fatty acid concentration was quantified as mmoles per litre.

2.4.5 Preparation of total membranes from rat primary epididymal adipocytes

Adipocytes were prepared and stimulated as for transport assay (see section 2.4.3). To terminate stimulation medium was aspirated from below and the cells were washed 2 times in warmed KRH (pH 7.4). Next this was replaced with 500µl ice-cold Hes plus protease inhibitors per 2mls of packed cells, and cells were then rapidly homogenised by repeated passage through a 23G needle attached to a 1 or 2ml syringe. Membranes and soluble proteins were then prepared as described in Section 2.3.8. The membrane pellet produced from a 2ml cell volume was then typically suspended in 100µl Hes plus protease inhibitors.

2.4.6 Preparation of whole cell Lysates from rat primary epididymal adipocytes

Adipocytes were prepared and stimulated as for transport assay (see section 2.4.3). To terminate stimulation medium was aspirated from below and cells washed 2 times in warmed KRH (pH 7.4). This was replaced with 1ml ice cold Lysis buffer protease inhibitors and cells were then rapidly homogenised by repeated passage through a 23G needle attached to a 1 or 2ml syringe. Samples were then transferred to 1.5 eppendorf tubes and centrifuged at 10,000 x g (4^{o}C) for 10 minutes to pellet nuclear material, matrix proteins and any remaining whole cells. The resulting lysate (supernatant) was then subjected to protein assay (see section 2.6) and snap-frozen in aliquots

2.5 Protein Assay

2.5.1 Bichiconnic Acid Assay

The assay was carried out essentially as per manufacturers protocol, with a few minor modifications.

A working stock of assay reagent was prepared by diluting concentrated reagent A 1 in 50 with reagent B. Eight standard BSA solutions were prepared (ranging from 0 to 5mg/ml in 2 fold increments) and these solutions were prepared in the same buffer as unknown sample wherever possible. Duplicate 10µl lots of each sample were then added to a 96 well plate, followed by 200µl of diluted reagent. The plate was incubated at 37C for 30-minutes, and after this time the absorbance of the samples was read on an automated plate counter. The standard curve was plotted according to a 2^{nd} order polynomial fit using Cricket Graph II ™, and unknown protein concentrations were calculated according to the corresponding equation.

2.6 SDS-PAGE

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 (v/v) in 125mM Tris-HCl (pH 6.8), 0.1% (w/v) SDS, polymerised with 0.1% (w/v) ammonium persulphate and 0.05% (v/v) TEMED.

The resolving gel consisted of 6.5-10% acrylamide/0.18-0.28% bisacrylamide in 0.383mM Tris-HCl (pH 8.8), 0.1% (w/v) SDS,

polymerised with 0.1% (w/v) ammonium persulphate and 0.01% (v/v) TEMED. The protein samples were solubilised in sample buffer (see section 2.2.3) and loaded into 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 gel had reached the desired position. For Bio-Rad mini-PROTEAN gel a constant voltage of 100-120 volts for 1-2 hours (depending on percent acrylamide in resolving gel) was used. Large gels were electrophoresed for around 5 hours at 150 volts.

2.7 Immunoblotting of proteins

After separation of the proteins as described in 2.5, the gels were removed from the plates and equilibrated in Towbins buffer (see 2.2.5) for 10 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 Towbins buffer. This was then sandwiched between two layers of Whatmann 3MM filter paper, which had also been pre-soaked in Towbin 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 255mA for 2 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.

2.7.1 Immunodetection of GLUT1, GLUT4, IRS-1, IRS-2, PKBα/β, PI3'K p85 sub-unit, phospho-tyrosine containing proteins and CD36 on nitrocellulose membranes

To block non-specific binding sites on the nitrocellulose the membrane was incubated was shaken in 5% (w/v) non-fat milk ('Marvel')/Wash buffer for at least 1 hour. The nitrocellulose was then placed into 1%

(w/v)(non-fat milk/Wash buffer, containing either GLUT1 (serum 1:200), GLUT4 (serum 1:500), IRS-1 (1μg/ml), IRS-2 (1μg/ml), PKBα (1μg/ml), PI3'K p85 sub-unit (1μg/ml), 4G10 (phospho-tyrosine residues) (1μg/ml), or CD36 (serum 1:2000), (see section 2.1.2 for more detail). The nitrocellulose was then incubated overnight at 4°C on a shaking platform. The next day the nitrocellulose was washed five times at ten-minute intervals with Wash buffer. The nitrocellulose was then incubated with either HRP linked-donkey anti-rabbit (GLUT1, GLUT4, IRS-1, IRS-2, PI3'K p85 sub-unit), HRP linked-donkey anti-sheep (PKBα/β), or HRP linked-anti-mouse (phospho-tyrosine, CD36) all at a concentration of 1μg/ml. The nitrocellulose was then washed as before. Finally the nitrocellulose was subjected to Enhanced Chemi-Luminescence (ECL) using an Amersham kit according to the manufacturers protocol. The blot was then wrapped in cling-film, exposed to film for 30 seconds - 10 minutes, and developed using an X-Omat processor.

2.8 Statistical analysis

Unless otherwise indicated, results are expressed as means ± standard error. Statistically significant differences were determined using a Students t-test.

3.1 Insulin Resistance in the SHRSP is not linked to aberrant Cd36 expression

3.1 Introduction

3.1.1 SHR and CD36

As discussed in Section 1.10, aberrant expression of Cd36 protein shows strong linkage some aspects of the SHR phenotype, although it's precise role remains elusive. We saw how one hand Cd36 absence in mouse models appear to lead to elevated circulating lipids, a well known cardiovascular risk factor. The SHR strain (with aberrant Cd36 expression), also has elevated triglycerides. These data strongly suggest a role for Cd36 in promoting FA uptake, since uptake is reduced in its absence. Interestingly, in spite of the cardiovascular risk factors present in the SHR, it does not however develop atherosclerosis. This is concordant with the view that Cd36 (and other proteins of the scavenger receptor family) play a role in development of foam cells and subsequent atherosclerotic lesions, and hence absence will reduce potential to develop atherosclerotic lesions.

Nevertheless, in spite of the apparent links between Cd36 and lipid abnormalities in the SHR it is unlikely to completely explain the observed phenotype. Indeed, as discussed in Section 1.10, only 40% of the defect in glucose metabolism is linked to Cd36 deletion, and nothing is known regarding the effect at the cellular level. This strongly suggests that some other factor or locus contributes to the phenotype seen in the SHR. Interesting in this regard is the demonstration that there exists an SHR strain which although phenotypically similar to Aitman's Cd36 deletion strain, has normal Cd36 expression. Indeed Gotoda *et al.*, have demonstrated lack of the Cd36 deletion in an SHR strain maintained independently of Aitman's NIH derived SHR's. Interestingly the SHR deletion variant of Cd36 is also not observed in strain of the stroke-prone SHR (SHRSP) maintained by Pravenec *et al.* This group showed, by

Southern blotting, that their SHRSP strain possessed a *Cd36* cDNA band pattern identical to that observed in the non-insulin resistant normotensive BN rat (Pravenec *et al.*, 1999). Although this further discounts the role of Cd36 in the development of spontaneous hypertension, no metabolic parameters were included in this study. There is, therefore, a need to address the role of Cd36 deletion within syndromes where spontaneous hypertension and altered carbohydrate metabolism are demonstrated to exist in parallel.

Clearly, by addressing this issue we will begin to further understanding of the importance of Cd36 variants in the development of spontaneous hypertension and metabolic syndromes.

3.1.2 Aims

The work in this chapter aims to address this issue by measuring whether any defects in glucose and fatty acid metabolism are present in adipocytes from our SHRSP strain, and whether these defects associate with a deletion variant of Cd36.

3.2 Results

3.2.1 Establishment of linearity of 2-Deoxy-D-glucose (DeGlc) uptake in rat primary epididymal adipocytes

As early as the 1960's Rodbell demonstrated that it was possible to measure glucose uptake into isolated primary adipocytes, and that this glucose uptake was markedly enhanced by the addition of insulin (an effect now known to be primarily mediated by the GLUT4 glucose transporter isoform) (Robdell, 1964). Based on the work of Rodbell others, DeGlc uptake into primary adipocytes is generally studied within a 3 or 5 minute time period (Rodbell, 1964; Olefsky, 1976; and Ciaraldi, 1988). Figure 3.1 shows, using an adaptation of the method of Rodbell, that glucose uptake in our system is also in the linear range within the chosen 3 minute uptake time and beyond, both in the presence or absence of insulin. For this reason a 3 minute uptake has been used routinely in these experiments.

3.2.2 Typical dose-response curves to insulin in WKY and SHRSP adipocytes

Figure 3.2 illustrates a typical dose-response curve to insulin established using the 3 minute DeGlc uptake outlined above. Insulin stimulation was carried out for 15 minutes, although maximal responses were reached at around 10 minutes and the effect persisted for up to 30 minutes.

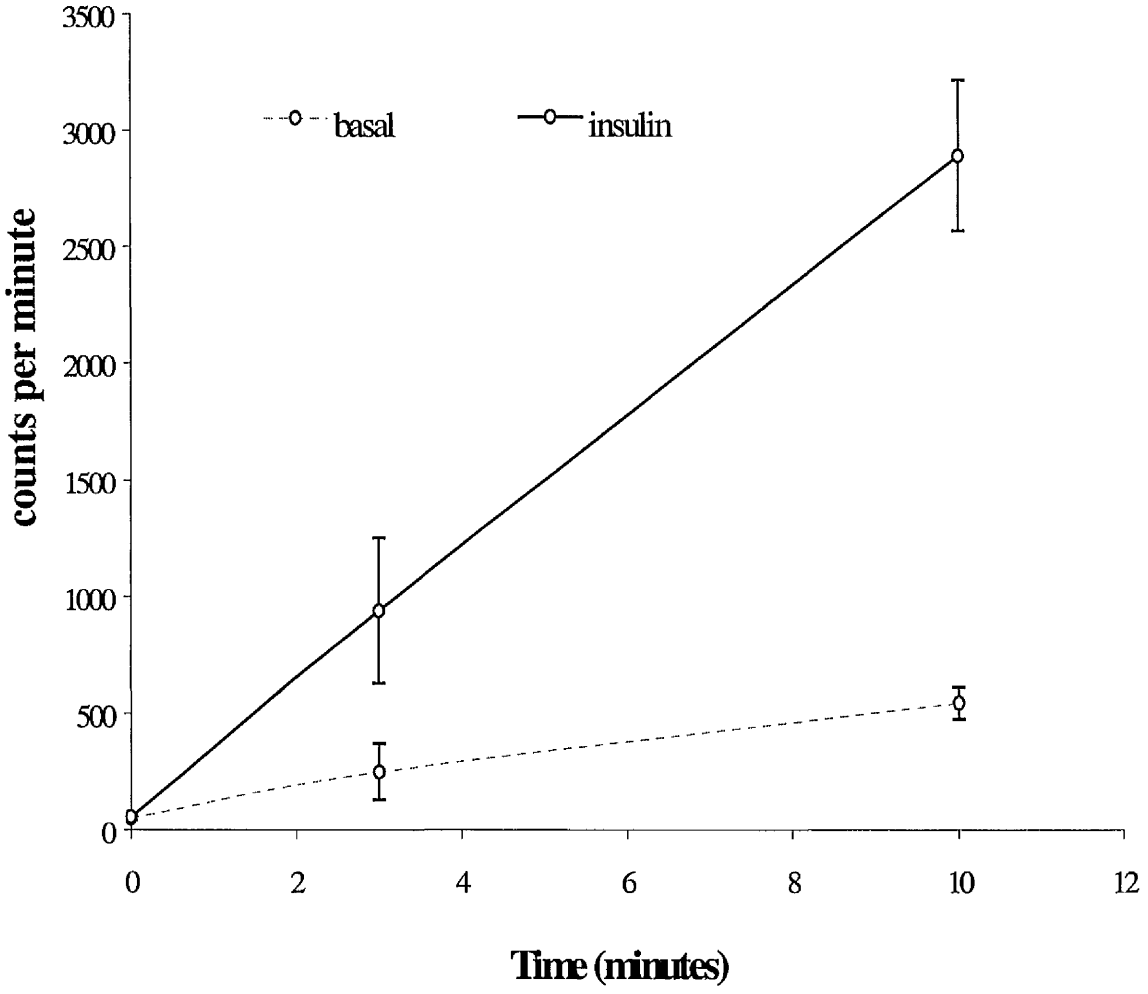


Figure 3.1 2-Deoxy-D-glucose uptake falls within the linear range when uptake is studied over a 3 minute time period

Epididymal adipocytes were prepared as described in Section 2.2.4. DeGlc uptake was performed essentially as described in Section 2.4.3, although both 3 and 10 minute uptake times were included.

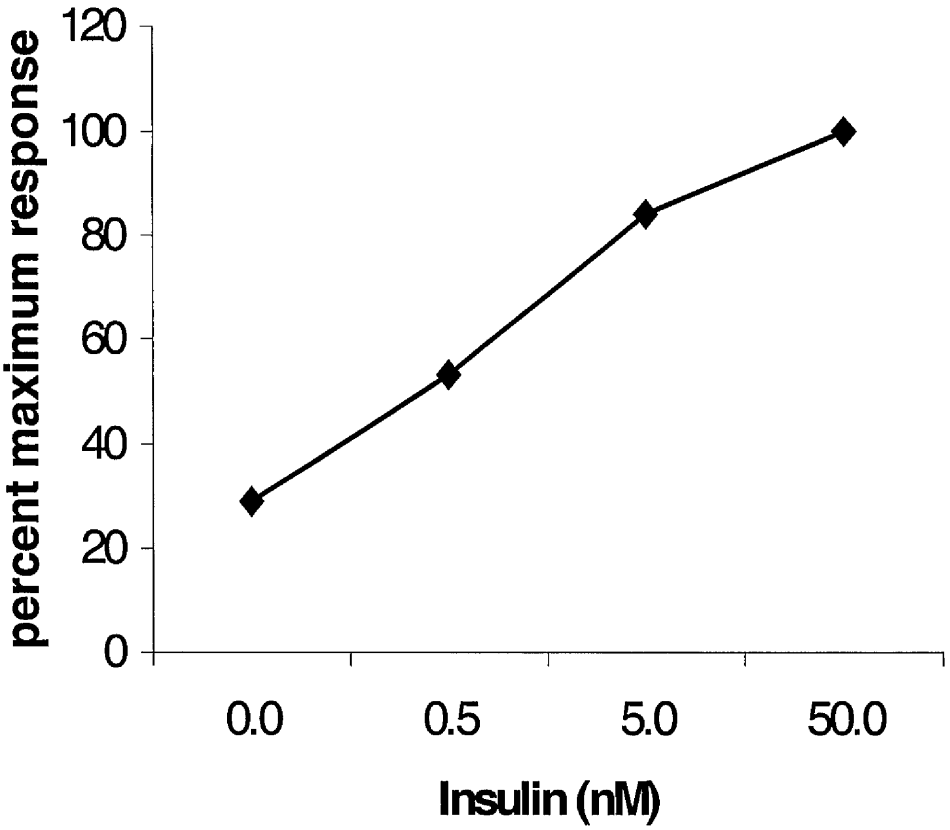


Figure 3.2 Typical Dose Response Curve to insulin in WKY adipocytes

Epididymal adipocytes were prepared and DeGlc uptake assayed as described in Sections 2.4.2 and 2.4.3. Data is taken from one experiment, and values are an average of three triplicate 200µl aliquots from each stimulation. Standard Errors (SE) within the assay are minimal (typically less than 1%) and are therefore not shown.

3.2.3 Glucose transport in WKY and SHRSP adipocytes as a measure of in vitro insulin sensitivity

Although there is variability in the response to insulin observed in both the WKY and SHRSP sub-sets, Figure 3.3 shows how there is clearly an overall strain-related difference in the ability of insulin to promote glucose transport. As there is no significant difference between the size of the cells (1.60 ± 0.06 arbitary units WKY, vs 1.71 ± 0.05 arbitary units SHRSP), data are expressed as picomoles DeGlc transported per minute per 20µl packed cells. When expressed in this way, the maximal response observed in SHRSP adipocytes is clearly diminished compared to WKY (3.2 ± 1.7 versus 20.1 ± 7.8 fold respectively; $p<0.05$). There is however no significant difference between basal rates of glucose transport between WKY and SHRSP.

3.2.4 NEFA release in WKY and SHRSP adipocytes- the effects of isoprenaline and insulin

Resistance to the effects of insulin in the SHR is not only restricted to glucose disposal but also present at the level of fatty acid release. There are no such data regarding this action of insulin in the SHRSP. In Figure 3.4, it can be seen that basal and isoprenaline-stimulated fatty acid (NEFA) release do not differ between WKY and SHRSP adipocytes. In contrast, however, the ability of insulin to inhibit lipolysis is markedly reduced in SHRSP adipocytes. When data are expressed as a percentage of maximum WKY response, the ability of insulin to inhibit isporenaline stimulated NEFA accumulation is reduced in the SHRSP compared to the WKY. For example, in Figure 3.5 it can be seen that, at 0.05nM insulin, there is a 72 ± 3% reduction in WKY compared to 15 ± 2% in SHRSP.

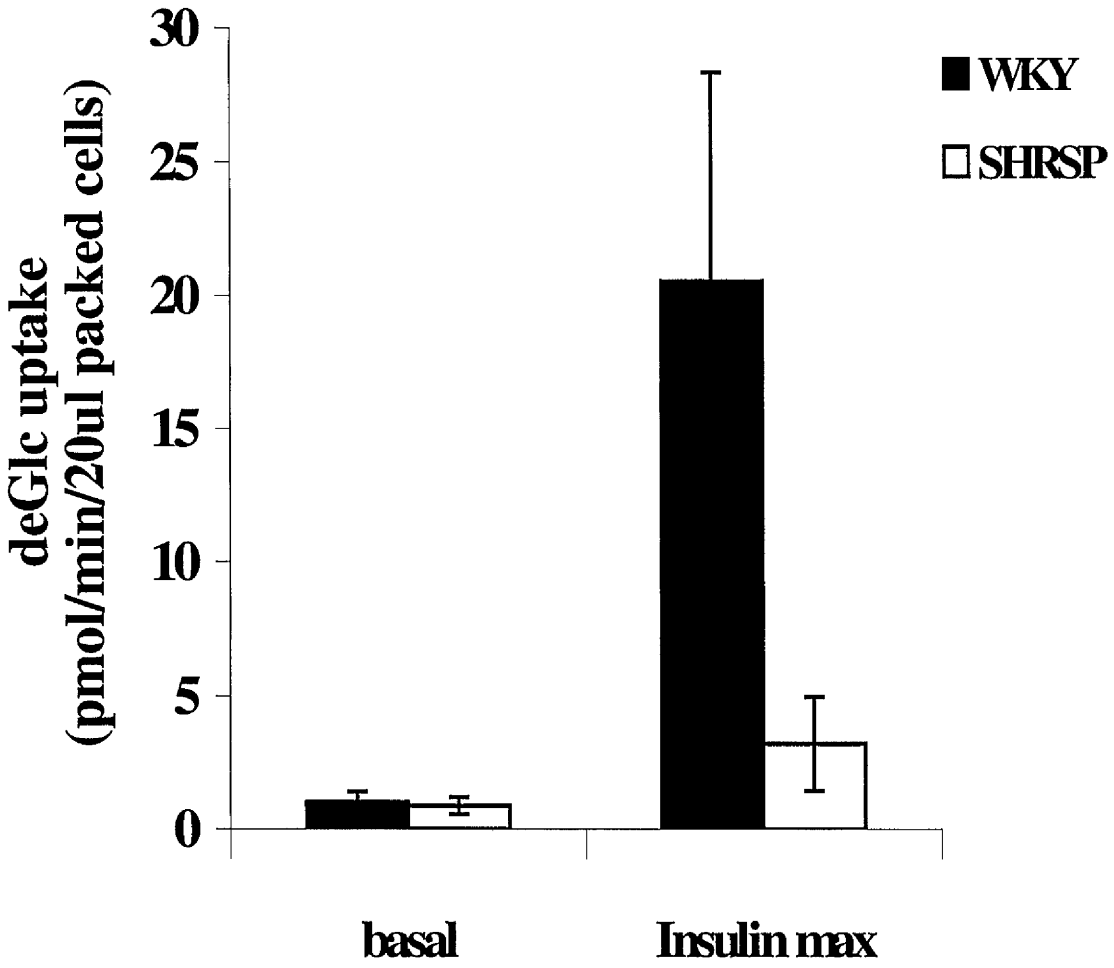


Figure 3.3 Glucose transport in WKY and SHRSP adipocytes as a measure of in vitro insulin Sensitivity

Epidiymal adipocytes were prepared and DeGlc uptake was measured according to the methods outlined in Section 2.4.2 and 2.4.3. Data from three individual experiments is presented, and error bars represent the standard error of the mean. Statistical significance was determined using the Students t-test. Using this test, there was found to be no significant difference between the basal rates in SHRSP and WKY adipocytes, whereas the insulin-stimulated response (50nM insulin/15 minutes) was significantly reduced in SHRSP adipocytes compared to WKY ($p=0.01$).

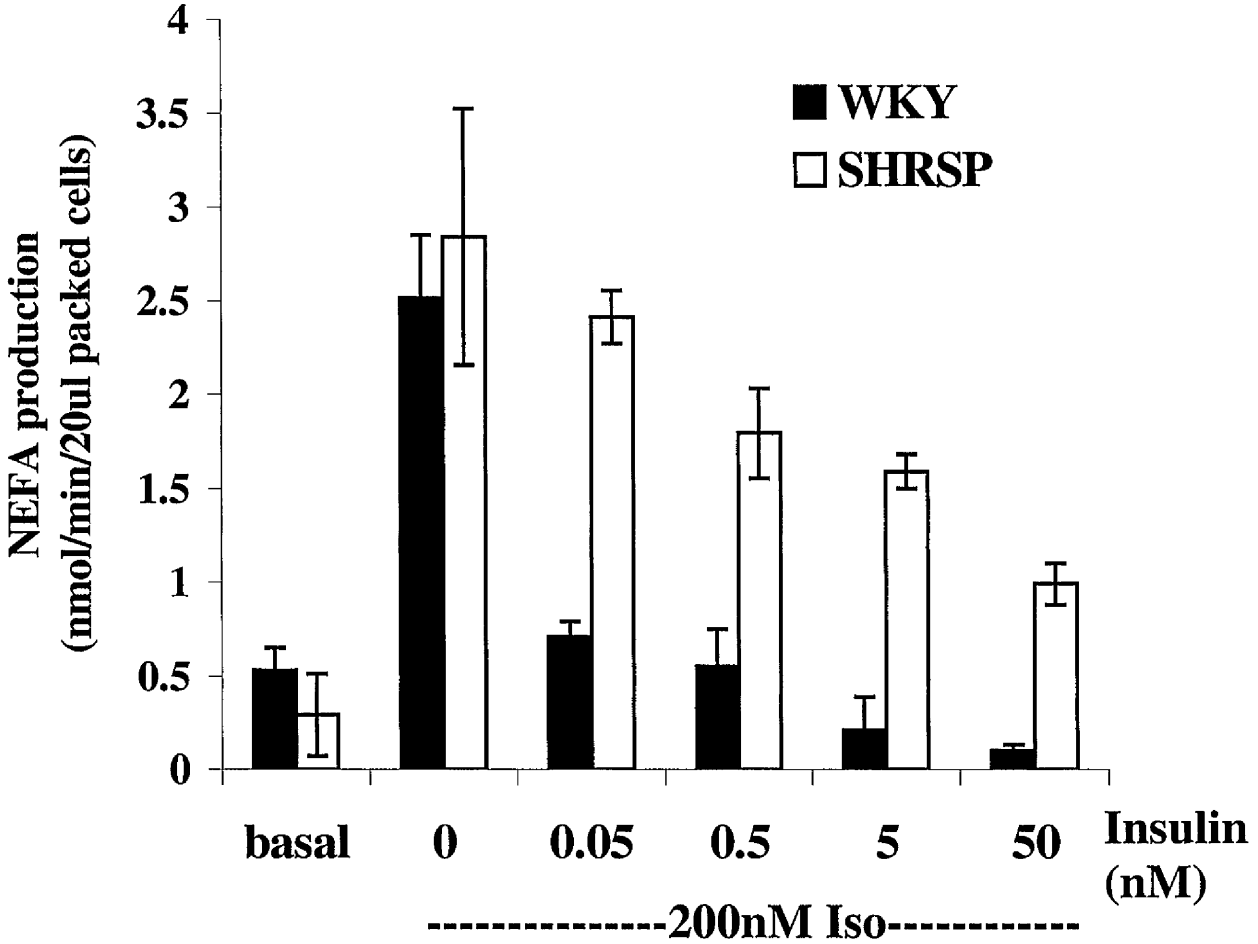


Figure 3.4 NEFA release in WKY and SHRSP adipocytes- the effects of isoprenaline and insulin

Epidiymal adipocytes were prepared according to the method outlined in Section 2.4. Cells were incubated in the presence of isoprenaline (200nM), isoprenaline plus insulin (0-50nM) or neither for 30 minutes, and NEFA release then measured according to the methods outlined in Section 2.4.4. Data from four individual experiments is presented, and error bars represent the standard error of the mean. Using the Students t-test, there was found to be no significant difference between the basal rates of lipolysis in SHRSP and WKY, whereas the effect of insulin to reduce lipolysis was dramatically reduced in SHRSP adipocytes ($p<0.05$).

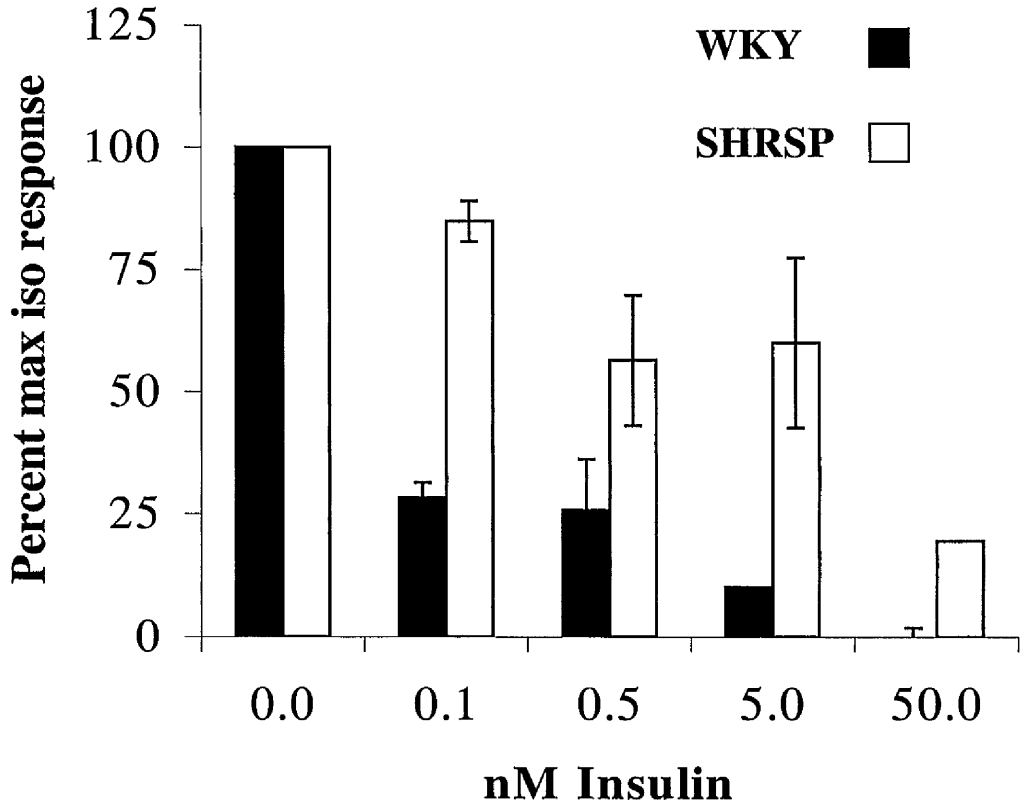


Figure 3.5 Impaired ability of insulin to inhibit isopreanline-stimulated NEFA release in SHRSP adipocytes

This figure illustrates the impaired ability of insulin to inhibit lipolysis in SHRSP adipocytes. Using the data presented in Figure 3.4, every response was expressed as a percentage of the maximum lipolysis stimulated by isoprenaline in WKY or SHRSP adipocytes (although these values did not differ significantly). It is clear in WKY adipocytes that insulin is able to effectively reduce lipolysis even at relatively low (0.05nM) insulin concentrations, and that it reduces it to basal levels or below (data not shown). In contrast, in SHRSP adipocytes, much higher doses of insulin are needed and even then the response is much poorer than that seen in WKY. Data is expressed as percentage maximum WKY response, and error bars represent S.E.M. $P< 0.05$ at all data points for SHRSP versus WKY, using the students t-test.

3.2.5 Serum levels of Insulin, Glucose, NEFA's and Triglycerides in WKY and SHRSP

Table 3.1 highlights the major differences in serum levels of several metabolic and cardiovascular markers in the SHRSP and WKY. As has been observed in the SHR (and indeed in human hypertension), serum triglycerides are markedly elevated in SHRSP compared to WKY animals (0.8 ± 0.1mM in WKY versus 1.8 ± 0.3mM in SHRSP; $p<0.001$, using the students t-test). Despite the impairment in the ability of insulin to inhibit lipolysis in adipocytes, the overall serum NEFA levels are not significantly different between the two strains. All other parameters, aside from blood pressure, did not differ significantly between SHRSP and WKY.

3.2.6 Levels of *Cd36* mRNA in WKY and SHRSP adipocytes assessed by Northern blotting

To determine expression level and transcript size cDNA Northern analysis of Cd36 expression was carried out in adipose tissue from SHR, SHRSP, WKY and BN rats. Using a probe from the 5' end of the *Cd36* cDNA, the overall level of expression was found to be similar in all four strains. In Figure 3.6 it can be seen that the transcript size in SHRSP was identical to the major 2.8kb transcript seen in WKY and BN. The two major transcripts (3.8kb and 5.4kb) in SHR were not observed in the SHRSP or other controls. (This experiment was performed on our behalf by Anne M. Glazier, Hammersmith Hospital, Middlesex, UK).

	WKY	SHRSP
Systolic blood pressure *(telemetry)(mm Hg)*	**131 ± 12**	**180 ± 14*** **
Diastolic blood pressure *(telemetry)(mm Hg)*	**94 ± 10**	**128 ± 10*** **
Serum TG *(mmol/L)*	**0.8 ± 0.1**	**1.8 ± 0.3***
Serum NEFA *(mmol/L)*	**0.39 ± 0.07**	**0.30 ± 0.06**
Serum glucose *(mmol/L)*	**8.3 ± 0.5**	**7.9 ± 0.4**

Table 3.1 Blood Pressures and Serum levels of Glucose, NEFA's and Triglycerides in WKY and SHRSP

All blood pressure measurements were made according to the methods outlined in Section 2.4.1. Glucose was measured using the glucose oxidase technique, and triglycerides were measured using standard enzymatic techniques. NEFA's were measured according to the methods outlined in Section 2.4.4. 6 animals were used for each measurement, and the errors respresent the standard error of the mean. *** denotes $p<0.05$, * denotes $p<0.001$, using the students t-test.

3.2.7 Sequence analysis of *Cd36* cDNA from WKY and SHRSP adipocytes

Based on the experiment outlined on page 92, it is apparent that the SHRSP cDNA is identical to the WKY cDNA, except for a Glutamine to Arginine substitution at position 262, as shown in Table 3.2. Although this substitution does introduce a charge difference, it is also present in the BN rat, indicating that it is unlikely to be related to hypertension or the other observed defects since the BN rat is defined as normal in these respects. Further evidence against a selection role for this mutation is that, in this region both the WKY and the SHR express a glutamine residue. (This experiment was performed by Anne M. Glazier, Hammersmith Hospital, Middlesex, UK).

3.2.8 Expression of Cd36 protein in WKY and SHRSP total adipocyte membrane fractions assessed by immunoblotting

In order to determine whether the *Cd36* gene encoded a synthesised protein, we performed immunoblotting. Figure 3.7 illustrates that the amount of Cd36 protein in adipocyte total membranes does not differ between SHRSP and WKY. Clearly this does not rule out the presence of any functional mutations in SHRSP. However it does distinguish the SHRSP from the SHR, since Cd36 is completely missing from SHR adipocyte membranes.

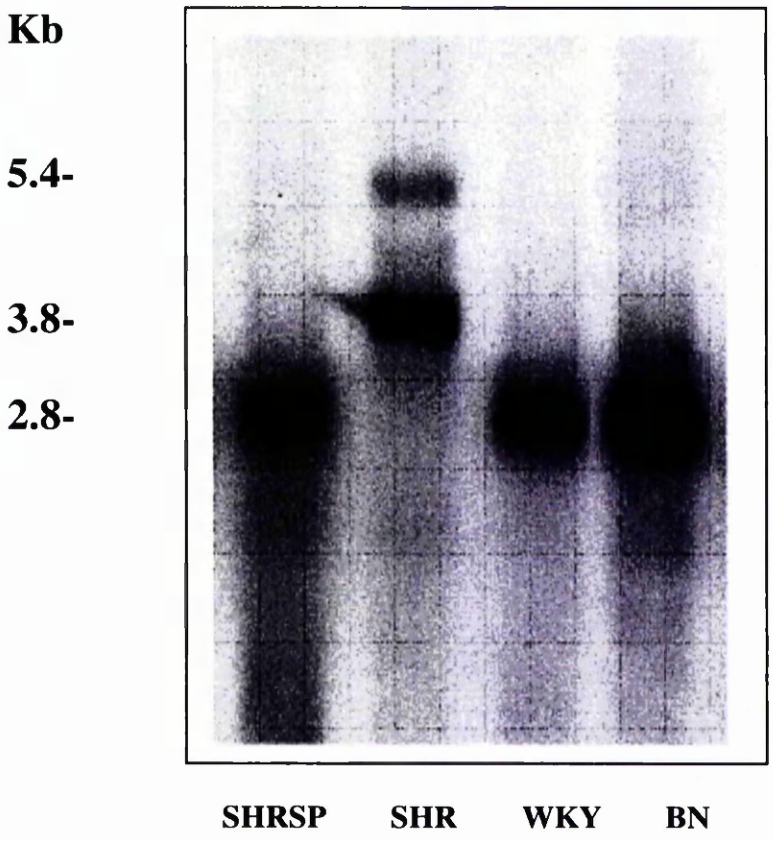


Figure 3.6 Levels of CD36 mRNA in WKY and SHRSP adipocytes assessed by Northern blotting

Total RNA, prepared from epididymal fat by acid phenol extraction, was separated by electrophoresis on formaldehyde gels. The RNA was transferred onto Hybond-N (Amersham). A cDNA probe was then PCR amplified from the 5' end of the WKY cDNA, extending from nucleotides 462 to 1052 of the published sequence (Abumrad *et al*, 1993), and the probes labelled and purified using the RediPrime random prime labelling system and a G50 Nick Spin Column (Pharmacia).

Strain	Codon 262	
	Amino acid Sequence	Nucleotide Sequence
SHRSP	CGA	Arg
SHR/NCr1Br	CAA	Gln
WKY	CAA	Gln
BN	CGA	Arg

Table 3.2 Sequence analysis of CD36 cDNA from WKY and SHRSP adipocytes

PCR amplification of cDNA with AmpliTaq Gold (Perkin Elmer) was carried out with Dnase treated total RNA extracted from epididymal adipose tissue with the DNA/RNA extraction kit (Qiagen). cDNA was prepared with the 1st Strand Synthesis Kit (Boehringer), and purified through a G50 Spin Column (Pharmacia). PCR products were purified with Centricon 100 columns (Millipore) and directly sequenced with the BigDye Sequencing Kit (Perkin Elmer) on an BI Prism 377 DNA sequencer.

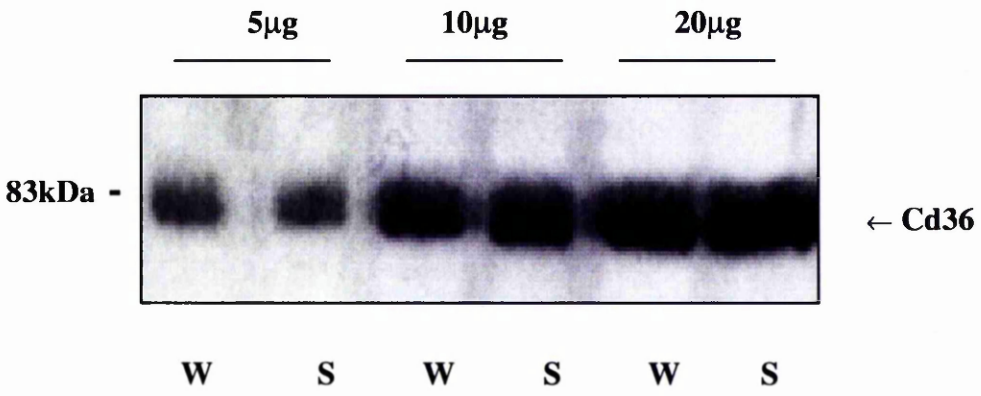


Figure 3.7 Level of Cd36 protein in WKY and SHRSP total adipocyte membranes assessed by Immunoblotting

Total membranes were prepared from WKY and SHRSP adipocytes as outlined in section 2.4.5. Samples were separated by SDS-PAGE, as outlined in section 2.6, and then immunoblotted for Cd36 protein using a mouse monoclonal antibody as described in section 2.7. Shown is a representative immuno-blot, which was typical of a result obtained on three separate occassions.

The position of the Molecular Weight marker is shown, and Cd36 identified by the arrow at the right.

3.3 Discussion

3.3.1 Glucose metabolism in epididymal adipocytes

Although adipose tissue only accounts for up to around 10% of whole body glucose uptake from the blood, the other main sites being muscle and brain (Chapter 24, Zubay, 1996), the primary adipocyte has become widely accepted as a model system for studying the mechanisms of glucose transport. This is due in part to problems faced when using muscle, particularly in manipulation of whole muscle and difficulties in maintaining muscle cell cultures. Although the adipocytes can be obtained from several different fat depots within the rat, it is generally accepted that epididymal adipocytes represent one of the best model systems (Gliemann, 1968; Gliemann, 1971; Ciaraldi, 1988),as these cells are easy to dissect and generally retain good responses to insulin.

Within the adipocyte, basal glucose requirements are maintained largely by glucose entry via the GLUT1 glucose transporter which, as discussed in Chapter 1, resides predominantly at the plasma membrane in the basal state. Upon insulin stimulation, the large amount of glucose entering the cell can be explained by the movement of more glucose transporter proteins to the plasma membrane. Although a small amount of these transporters are likely to be GLUT1, the predominant transporter in this phase is the GLUT4 isoform (discussed in more detail in Chapter 1). GLUT4 translocates from intracellular stores to the plasma membrane in order to facilitate the entry of such large amounts of glucose (see Section 1.4 and 1.5).

When attempting to quantify movement of glucose transporters such as this, several factors have to be considered with regard to the primary adipocyte. Of relevance is the fact that the cells themselves are relatively fragile, meaning that the manipultions need to be carried out rapidly and under strictly controlled conditions of temperature, pH, etc. Care must also be taken to ensure that the cells do not become unnecessarily stressed, as

this could ultimately activate pathways that may interfere with the experimental measurements. Fortunately the inclusion of stabilising compounds, such as BSA, into the media surrounding the cells overcomes the majority of these problems.

It is well documented that adipocytes in vivo are exposed to a variety of paracrine and endocrine factors that can have positive and negative effects on adipocyte metabolism. Perhaps one of the best studied is adenosine, and addition of adenosine to the media has proved to be most useful when manipulating these cells in vitro (Ciaraldi, 1988; Bush, 1988). Endogenous adenosine is released from the cells as they are maintained in suspension, and it has been observed that fluctuations in this adenosine release can dramatically alter the basal activity and viability of the adipocyte. To overcome this, large amounts of adenosine are included in the media, thus in a sense, desensitising the cell to the effects of any locally released adenosine.

Apart from problems regarding adipocyte health, similar challenges are faced regarding expression of the data in a meaningful way. Unlike the majority of cells maintained in culture, which generally grow uniformly on dishes, primary adipocytes are maintained in suspension. This in itself creates difficulty when attempting to standardise between different incubations. Although there are several potential ways of doing this none are completely satisfactory. Ideally data should be expressed per cell number but this has drawbacks. As previously mentioned, adipocytes are fragile and obtaining reliable and accurate numbers is very difficult. Even though there is no significant difference in the cell size between these two strains, inevitable handler error in measurements such as this may skew data. Similar problems are encountered with other methods, for example fluid volume of sample, which are again dependent on a variety of factors which may differ between cells from different strains. Measurements of protein or dry weight are commonly used, although these encounter similar problems and also do not take in to account the percentage of dead cells at the time of assay. Thus, data presented here is simply expressed as a factor

of packed cell volume. Although this may have drawbacks it does allow consistency of approach between experiments. When data are expressed in this manner, it is apparent in Figure 3.3 that the ability of SHRSP adipocytes to transport DeGlc in the basal state is not altered, when compared to WKY controls. It is reasonable therefore to conclude that the machinery involved in mediating basal glucose transport (discussed in detail in Section 1.4.1) appears to be similarly functional in SHRSP and WKY.

In contrast to this, however, the ability of SHRSP adipocytes to mediate insulin-stimulated DeGlc uptake is clearly reduced. This data argues strongly that insulin-stimulated glucose transport is impaired in the SHRSP compared to the WKY, and Figure 3.3 illustrates for the first time that the SHRSP is indeed insulin-resistant at the level of glucose uptake. The potential mechanisms behind this are extensively discussed in Chapter 4.

3.3.2 Lipid metabolism in the SHRSP

Human subjects with hypertension not only commonly have defects in carbohydrate metabolism, but also an altered state of lipid metabolism characterised by elevated triglycerides and LDL-cholesterol. (Iritani *et al.*, 1977; Reaven *et al.*, 1989; and Aitman *et al.*, 1999).

The effect of insulin on fatty acid metabolism can be most easily studied by measuring its ability to modulate catecholamine-mediated NEFA release. Stimulation of cells with a synthetic catecholamine such as isoprenaline will result in activation of Gsα which will, by presently poorly defined mechanisms involving cAMP, activate the Hormone Sensitive Lipase (HSL) (Clifford *et al.*, 2000). Once activated the HSL will cleave intracellular fatty acids to generate and release NEFA's into the extracellular environment. Since insulin is an anabolic hormone, it functions to inhibit the action of the HSL, again by poorly defined mechanisms. The SHR displays abnormal catecholamine-mediated fatty

acid release, a phenomenon also observed in human hypertension (Aitman *et al.*, 1999). Obviously an important biological process, lipolysis is subject to tight regulation within the cell, and a large part in this process is played by negative-feedback regulation. Indeed after a period of stimulated release NEFA's will eventually cause a negative feedback on further release from the adipocyte. Nevertheless, over a shorter period of time (up to around half an hour), it is possible to gain a measure of the activity of the HSL in response to exogenous agents simply by measuring NEFA release.

In the absence of any lipolytic stimulus, basal NEFA secretion from adipocytes is generally low (Aitman *et al.*, 1997) and, as can be seen from Figure 3.4, this does not differ in SHRSP and WKY adipocytes. Thus the basic machinery involved in maintaining basal adipocyte NEFA production appears not to be altered in these cells. It appears that the lipolytic effect of isoprenaline is also not significantly different, Figure 3.4 indicating that SHRSP adipocytes possess an equal capacity to activate the HSL over the time period measured. Importantly however, the ability of insulin to inhibit NEFA release is impaired in the SHRSP, as demonstrated in Figures 3.4 and 3.5. This is of particular relevance, in that the SHRSP adipocytes are able to activate lipolysis normally but are unable to exert at least one important physiological control on this. Whether normal negative feedback responses to NEFA (initiated in response to elevated NEFA's over a long time period) are also altered in SHRSP adipocytes remains to be addressed, although again this would be of relevance since any impairment would also result in elevated NEFA release for a longer time period.

Although this impairment would be expected to alter the overall levels of NEFA within the blood, Figure 3.6 shows that there is no overall difference in NEFA levels in serum of SHRSP and WKY rats. This is possibly because the rats are not specifically fasted and given access to standard chow *ad libitum*. In addition there are other factors which could contribute. It is possible that the epididymal adipocyte is not the major regulator of NEFA levels, and it is likely that many of the effects seen in

this particular fat depot are at the local level. Obviously it would therefore be of interest to measure anti-lipolysis in other fat depots which may play a larger role. Secondly, although adipocytes are clearly important in fat metabolism, other tissues like muscle are also involved (Febbraio *et al.*, 1999; and Ibrahimi *et al.*, 1999). Again therefore, it would be of interest to examine NEFA metabolism in other tissue types such as muscle. Finally, it is possible that changes will be observed in serum NEFA levels in older animals, as they are exposed to continually altering fat metabolism over a longer time period and any compensatory mechanisms could be reduced with age.

In contrast to the NEFA levels, the SHRSP clearly has elevated levels of triglycerides in serum compared to the WKY controls. This is similar to the hypertriglyceridaemia observed in human hypertension, thought to be implicated in the development of atherosclerosis (Donnelly *et al.*, 1992; Connell *et al.*, 1994).

Clearly it is thus emerging that the phenotype observed the SHR, and now the SHRSP, is similar to that seen in humans and therefore places them central to understanding the development of human hypertension and related conditions such as Syndrome X. In summary, the SHR displays elevated blood pressure, reduced insulin-stimulated glucose uptake and a reduced insulin-mediated inhibition of lipolysis. Although the SHR also has elevated blood pressure and defective insulin-mediated glucose disposal, there are subtle differences. Interestingly, the basal rate of glucose transport in the SHR appears elevated compared to WKY controls (Reaven *et al.*, 1989) in addition to the reduced maximal stimulation. Only the maximal response is altered in the SHRSP. The ability of isoprenaline to stimulate lipolysis is also impaired in SHR adipocytes, in contrast to the apparent normal stimulation in SHRSP adipocytes. Thus although the SHRSP and SHR phenotypes are clearly similar, there are important differences. Subsequently this suggests the presence of different underlying abnormalities.

3.3.3 Potential defects behind the SHRSP phenotype

The nature of the defect in the SHRSP suggests the involvement of some locus which, either directly or indirectly has global effects on carbohydrate and lipid metabolism and blood pressure regulation. This is discussed below.

3.3.4 Cd36

As discussed in the introduction to this chapter and in Chapter 1, the emerging role of Cd36 in the SHR has placed it central to understanding how hypertension and metabolic defects develop in spontaneous hypertension in general (Aitman *et al.*, 1999; Pravenec *et al.*, 1999; de Winther *et al.*, 2000). It has emerged however, that although it certainly plays a role in the SHR, its significance in other cases is less clear. For example, although some hypertensive patients who also exhibit metabolic disorders have missing or dysfunctional Cd36, this is not a feature in the majority of patients with elevated blood pressure (de Winther *et al.*, 2000).

Indeed, this also seems to be the case with other animal model systems. For example, Gotoda *et al* were also able to observe the presence of a *Cd36* deletion mutation in three SHR sublines derived from the NIH but not in SHR/Izm and SHRSP/Izm strains (one of which exhibited insulin resistance) that had been maintained in Japan (Gotoda *et al.*, 1999). Thus, these findings led to the conclusion that the SHR *Cd36* deletion has somehow arisen *de novo* in the NIH colony. Our studies show that the SHRSP strain maintained in Glasgow (derived from NIH stocks) similarly does not display the *Cd36* deletion, indicating that the *Cd36* deletion identified by Aitman's group probably arose after the Glasgow SHRSP strain was derived. Nevertheless, the data from Aitman and Pravenec are very convincing that in their SHR strain Cd36 is, or is close to, the major locus accounting for the abnormalities in lipid and glucose metabolism.

Taken in context, these studies indicate that there are clearly several factors other than the *Cd36* locus involved in the pathogenesis of spontaneous hypertension. Also of relevance is that only up to 40% of the defect in glucose metabolism observed in the SHR is explained by the *Cd36* deletion (Aitman *et al.*, 1999) and other factors of potential importance in the SHRSP could also be involved in this aspect of the SHR phenotype.

Interestingly however, it must be considered that Cd36 itself could be one of these other factors, in that the gene expression is normal but the function is somehow altered. In the introduction to this chapter (Section 3.1.3), the involvement of Cd36 in mediating the effects of ox-LDL was discussed. It has emerged recently that one of these effects is to cause the movement of eNOS from caveolae in endothelial cells. This effect was found to be related to the localisation of Cd36 to caveolae, illustrated both by immunoblotting and co-immuno-precipitation of Cd36 with a caveolin-1 antibody (Uittenbogaard *et al.*, 2000). Although this study has undoubtedly given insight into the cellular functions of Cd36, it also highlights the apparent importance of cellular localisation in Cd36 function.

This consequently raises the question of whether Cd36 localisation (and hence potentially some functions) are different in the SHRSP and WKY. In the results section of this Chapter (Figure 3.7) we observed that Cd36 protein expression in total membrane preparations was not altered. Recent observations in our laboratory have revealed that Cd36 is distributed similarly between WKY and SHRSP adipocytes, in that Cd36 is present in both the plasma membrane (PM) and the Low Density Membrane (LDM) fractions (Ian Salt, personal communication, data not shown). This is similar to the distribution observed by Bonen *et al,* where they found Cd36 to be located primarily in the intracellular membrane (IM) and PM fractions in skeletal muscle cell study (Bonen *et al*, 2000). Interestingly they observed that Cd36 moved from the IM to the PM in response to insulin in these cells, in a manner analogous to GLUT4. In contrast Cd36 did not appear to move significantly in response to insulin (1μM for 15

minutes) in our WKY or SHRSP adipocytes. This is quite possibly however a reflection both of differences in the conditions studied and the sub-cellular fractionation procedure, and hence further studies are needed. This will involve further sub-cellular fractionation under different conditions, and also eventual separation and characterisation of caveolae from both WKY and SHRSP adipocytes.

3.4 Conclusions

Although the SHRSP has been well characterised previously with regard to hypertension, the results in this chapter represent the first demonstration that this strain also has impairments in carbohydrate and lipid metabolism. This is therefore of considerable importance as it allows the more effective use of the strain as a model for the development of spontaneous hypertension in conjunction with metabolic disorders.

Of further importance is the discovery that the Cd36 putative fatty acid transporter is normally expressed in adipocytes from SHRSP, in contrast to the deletion observed in the related SHR (Aitman *et al.*, 1999). This reinforces the growing theory that, although altered Cd36 expression certainly plays a key role in the SHR and a limited role in human hypertension, it clearly does not explain the metabolic changes in all cases of spontaneous hypertension.

Clearly there exist other underlying deficits which must explain the SHRSP phenotype. This is of interest at the level of the SHRSP phenotype and could quite possibly impinge on other syndromes of spontaneous hypertension. Indeed in the SHR, Cd36 deletion does not account for all of the observed phenotype and so it is quite possible that some similar deficit functions here and in the SHRSP. A similar scenario could be envisaged in cases of spontaneous hypertension in humans; Cd36 deletion has been shown to play a limited role and it is quite possible that defects in the SHRSP play a role here.

Further analysis of Cd36, concentrating specifically on sub-cellular localisation studies and analyses of Cd36 transporter activity, may reveal other potential mechanisms underlying the SHRSP phenotype. Also of relevance is a cellular analysis of insulin action, including the role of signalling and trafficking (discussed in Chapter 4), processes which are key to all of the biological effects of insulin. Finally, genetic profiling strategies are undoubtedly central to studies such as this and will begin to

provide answers to what genes and molecules may be significant in the phenotype of these animal models, and ultimately humans. This is discussed in more detail in the general discussion (Section 5.2.3).

4 Sex Hormones and Insulin Resistance

4.1 Introduction

4.1.1 Insulin Resistance and Polycystic Ovarian Syndrome

As outlined in the previous chapters, resistance to the effects of insulin is a phenomenon present in, and central to a wide variety of disease states. Polycystic Ovarian Syndrome (PCOS) is yet another example of such a disorder, where reduced insulin sensitivity appears to be an integral characteristic of the disease (Dunaif *et al.*, 1992; Rosenbaum *et al.*, 1993; Taylor and Marsden., 2000; Lyall and Gould., 2000).

In order to understand how defective insulin action can impinge on a condition such as PCOS it is necessary to define the underlying endocrine abnormalities present within the disease state. This, in turn, calls for an understanding of the normal regulatory steps involved in ovarian hormone action. This is described below.

4.1.2 Oestrogen Function

Oestrogens are responsible for the development and maintenance of the female reproductive cycle. In pre-pubertal females, the level of circulating oestrogens is low, however in response to an increase in hormone secretion from the anterior pituitary and the hypothalamus there is a rapid increase in sex steroid secretion at the onset of puberty. These oestrogens are synthesised mainly by the ovary and throughout puberty the hormones are directly responsible for the maintenance of the reproductive organs, and the development of secondary sexual characteristics. There is also an accelerated growth phase and closure of the epiphyses of the long bones, representing the point at which adult height is reached. From this point on, and throughout the reproductive lifetime, the oestrogens are then responsible for maintenance of the menstrual cycle and the hormone

changes associated with pregnancy. This function is however not performed alone, but in conjunction with progesterones and other gonadotrophic hormones (extensively reviewed in Speroff, 1994).

4.1.3 Ovulation and Annovulation

Just before the onset and during ovulation, escape from the negative feedback effects of oestrogen, progesterone and inhibin allows for a rapid increase in the secretion of follicle stimulating hormone (FSH) from the anterior pituitary. This surge in FSH levels acts to promote growth of the emerging follicles within the ovary, and enhances the ability of these developing follicles to carry out steroidigenesis (Speroff, 1994). Continued growth of the follicles, promoted by both autocrine and paracrine factors maintains the high sensitivity to FSH, thus allowing the conversion from a microenvironment dominated by androgens to one largely influenced by oestrogens. One important aspect of this is the FSH-mediated stimulation of the appearance of luteininzing hormone (LH) receptors, a prerequisite for ovulation and luteinization. The rapidly increased levels of circulating oestradiol are then responsible for triggering ovulation, that is release of one mature follicle from the ovary (Speroff, 1994). This follicle is the one which has acquired the highest level of LH receptors and ability to carry out steroidigenesis (defined by highest levels of aromatase activity) in response to FSH. This successful follicle is also characterised by high oestrogen (for central feedback action) and greatest inhibin production (for local and central effects) (Speroff, 1994). Following release of this follicle there is a 2nd surge of oestrogen, this time accompanied also by a surge in progesterone levels. This point is known as the luteal phase, where FSH and LH levels begin to fall, and eventual demise of the corpus luteum occurs. As a result of degeneration of the corpus luteum, the local ovarian environment if freed from the action of oestrogen and progesterone, allowing FSH levels to once again rise and hence initiate the next cycle (Speroff, 1994).

From events described above it is apparent that normal ovarian function is regulated by the complex interaction of several hormones, the levels of which change dramatically at different phases. This complex regulation does not occur in annovulatory subjects suffering from PCOS. Indeed PCOS patients do not display the fluctuations in hormone levels seen in normal subjects, instead many of the ovarian hormones can be described as being in a 'steady-state' (Speroff, 1994). This does not necessarily mean that the levels are always low, simply that they are secreted in a manner that is unable to promote normal follicular growth, development and release. Phenotypes within the disease are diverse and patients will general display several other characteristics, including the presence of polycystic ovaries as defined by ultra-sound, oligo- or ammenorrhoea, raised testosterone, androstenedione, dehydroepiandrosterione (DHA), DHA sulphate, luitenizing Hormone (LH), 17-hydroxyprogesterone and oestrone concentrations, and abdominal obesity. Current lines of evidence indicate that the testosterone, androstendione and DHA are predominantly derived from the ovary, whereas the DHA suphate is predominatly adrenal. The increased circulating levels of oestrogens are thought to be less due to a direct increase in secretion, but more due to the peripheral conversion of the excess andostenedione to oestrone (Speroff, 1994).

4.1.4 Annovulation, PCOS and insulin action

Central also to a large majority of PCOS sufferers is the presence of a marked reduction in sensitivity to insulin, existing with the other endocrine disorders in an apparently integrated fashion (Dunaif *et al.*, 1992; Rosenbaum *et al.*, 1993).

Perhaps the first documented observations of a relationship between ovarian function and insulin sensitivity was the observation of 'Diabetes des femmes a barbe' or 'Bearded-diabetic women', in France in the early part of the last century, the beard resulting from the hyper-androgenism and the diabetes reflecting the impaired insulin sensitivity (Archard and

Thiers, 1921). Subsequently further evidence that excess of insulin could influence ovarian function came from observations on six PCOS sufferers who showed extreme syndromes of insulin resistance. Although the insulin resistance could be attributed to Insulin Receptor antibodies in three of these cases, other insulin resistant PCOS subjects without antibodies were soon identified (Taylor and Marsden., 2000). Subsequently Burghen *et al*, using an Oral Glucose Tolerance Test (OGTT), demonstrated that a cohort of PCOS patients displayed a low sensitivity to insulin compared to non-PCOS sufferers (Burghen *et al.*, 1980)

Perhaps one major caveat regarding early studies on insulin sensitivity in PCOS subjects concerns the obesity often seen in these patients. Indeed there are clearly relationships between obesity and insulin sensitivity independent of PCOS, in that increased adiposity usually reflects a reduced sensitivity to insulin. Thus the insulin resistance itself may not be so much related to the endocrine disorders but rather the obesity. Nevertheless this has actually proved not to be the case and further studies (utilising both OGTT techniques and clamps) have demonstrated that the insulin insensitivity of PCOS subjects exists independently of obesity, in large well-characterised and matched groups of patients (Taylor and Marsden., 2000)

4.1.5 Characteristics of the Insulin Resistance of PCOS

In order to understand how insulin resistance affects or is affected by the abnormalities observed in PCOS subjects it is necessary to define this insulin resistance at the cellular level. The insulin resistance of PCOS, as described by Dunaif in 1989, is due to peripheral and not hepatic insensitivity to insulin (Dunaif *et al.*, 1989). Indeed Marsden *et al* studied isolated adipocytes from PCOS patients and revealed that both insulin receptor binding and insulin-stimulated glucose uptake were markedly impaired when compared to age, sex and weight matched normal controls

studied during the follicular phase of the normal menstrual cycle (Marsden *et al.*, 1999). An important feature of this study is that patients who had polycystic ovaries but regular menses were, thus affording a greater differentiation between normal and the characteristic PCOS group. These studies therefore suggest that some association exists between impairment in the ability to mediate normal ovulation and reduced insulin sensitivity.

4.1.6 Insulin Resistance of PCOS- cause or consequence?

Clearly the studies described above indicate that insulin resistance and annovulation are associated. Nevertheless, whether insulin resistance is indeed a cause or consequence of the other factors remains to be addressed. It is well established that improvements in insulin sensitivity can be obtained in Type 2 Diabetics by treatment with insulin sensitising agents such as metformin or the thiazolidinediones, troglitazone or roziglitazone (Fonseca *et al.*, 2000; Raskin *et al.*, 2000). If the insulin resistance in PCOS is responsible for the other defects then potentially correction of insulin insensitivity with such drugs could also ameliorate the other symptoms of PCOS. Treatment of PCOS subjects with metformin revealed indeed that fasting and glucose-stimulated insulin levels could be reduced. Interestingly, metformin treatment also reduced the activity of p450c17α, an enzyme important in steroid metabolism and known to be elevated in PCOS (Lyall and Gould., 2000). Nevertheless other studies have produced conflicting results, in some studies insulin-sensitisers causing normalisation of menstrual cycles and hormone levels but having little effect in other studies (Taylor and Marsden., 2000). This probably reflects the complexity of PCOS as a disease and careful control and monitoring of phenotypes studied may reduce some of this conflict in responses.

In agreement with a role for insulin in causing the other endocrine abnormalities observed in PCOS is the fact that insulin is able to influence circulating androgen levels in different ways. Human ovaries possess

insulin receptors and insulin is able to directly stimulate androgen release from the ovary in PCOS sufferers (Lyall and Gould., 2000). This agrees with the view that ovarian and not adrenal androgens are the main source of excess androgens in PCOS. The apparent inability of insulin to mediate normal biological effects in peripheral tissues but still retain a stimulatory effect on the ovary could be explained in several ways. This could include either activation of distinct or slightly different signalling pathways or receptors within the ovary and peripheral cells, maintenance of some other interacting pathway or sensitising mechanism within the ovary but not within the periphery (or some inhibitory pathway in periphery but not ovary), or even an indirect effect on the ovary via insulin action in the pituitary. Indeed insulin receptors are present in the pituitary and the ability of insulin to alter anterior pituitary function has been shown *in vitro* (Lyall and Gould., 2000).

Within the body the levels of free sex hormones are also influenced by their binding to sex hormone binding globulins (SHBG's). PCOS subjects will generally display around a 50% reduction in SHBG levels, hence contributing to the elevated levels of free and available steroids in this condition. Interestingly, insulin is able to cause reductions in SHBG levels and hence indirectly causes elevations in free sex hormone levels (Lyall and Gould., 2000).

Thus clear evidence exists which implicates insulin as being responsible, at least in PCOS and related disorders, for modulating sex hormone levels and hence ovarian function.

Nevertheless there is also evidence to suggest the reverse, that excess circulating ovarian hormones themselves can indeed promote the development of insulin resistance. Perhaps one of the best illustrations of this is in pregnancy where marked increases in circulating oestrogens and progestogens occur early on and, as a result carbohydrate, lipid and intermediary metabolism become less sensitive to the effects of insulin (Taylor and Marsden., 2000). Further evidence is that women on the

combined oral contraceptive pill (containing an oestrogen and a progestogen) will often develop impaired glucose tolerance and a degree of insulin resistance with long term usage (Kasdorf and Kalkoff., 1988). Similar problematic reductions in insulin sensitivity are also observed in post-menopausal women on hormone replacement therapy (HRT), where HRT is often advised to maintain the cardio-protective effects of oestrogens (Taylor and Marsden., 2000).

Thus there are clearly a plethora of diseases where oestrogen reduction of peripheral insulin sensitivity may exacerbate, or even initiate disease development. For example in PCOS, although oestrogen effects on peripheral insulin responsive tissues are unlikely to explain the whole disease phenotype, undoubtedly effects such as these will make the condition worse. It is therefore important to understand how oestrogen treatment is able to influence insulin responsiveness in target tissues.

4.1.7 Insulin Resistance in other chronic annovulatory states?

If the insulin resistance of PCOS is reflective simply of the lack of ovulation in these patients, it follows that other chronic annovulatory states may also be associated with insulin insensitivity even under a completely different hormone profile. To try to answer this question patients suffering from hypogonadotrophic hypogonadism (HH) were studied with regard to measuring insulin sensitive responses in primary adipocytes. Interestingly this revealed that insulin receptor binding was reduced by around 40%, as was the maximum glucose uptake in these patients (Taylor and Marsden., 2000). This clearly demonstrates a further link between insulin resistance and annovulation at least in part independent of hormone profile, since PCOS and HH differ in this regard.

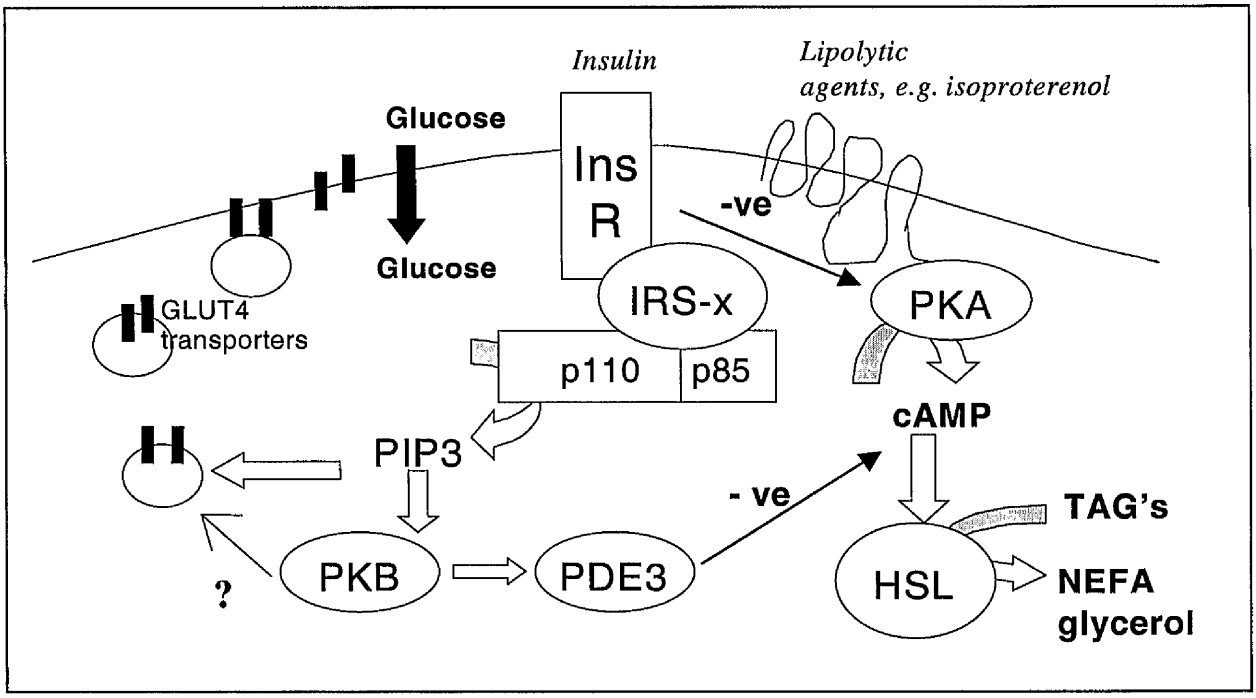
4.1.8 Potential mechanisms of oestrogen-induced insulin resistance in target cells

The signalling pathways activated by insulin in fat and muscle are outlined in detail in Chapter 1. Essentially, oestrogens could modulate any of the important steps in insulin signalling, shown below in Figure 4.1.

The most likely explanation however, given the known mechanisms of sex steroid action, is that the oestrogens are acting at the level of DNA transcription and influencing the production of proteins important in insulin action.

Sex steroid receptors are members of a super-gene family that includes receptors for steroids, thyroid hormones, vitamin D3 and Retinoic acids (Hyder *et al.*, 1999). All of these receptors are intracellular. Binding of the ligand to the receptor results movement of the complex to the nucleus, where it the acts as a transcription factor to influence DNA directed RNA and protein synthesis.

There are actually two different oestrogen receptor forms, derived from two different gene products- oestrogen receptor (ER) α and β, both of which bind to oestrogen response elements (ERE's) present within distinct DNA sequences (Hyder *et al.*, 1999). It is generally believed that the ERα and ERβ bind to different ERE's with different affinities, and this is likely to be one mechanism of selectivity (Hyder *et al.*, 1999). ER action is also putatively influenced by a 160kDa associated protein, Oestrogen Receptor Associated Protein (ERAP 160). Indeed mutational analysis of this protein has revealed that the ability of ER to activate transcription parallels the ability to bind ERAP 160 (Halachmi *et al.*, 1994).



InsR	insulin receptor	**PDE3**	Phosphodiesterase 3B
IRS-x	insulin receptor substrates	**PKA**	Protein Kinase A
p110	PI3'K catalytic sub-unit	**HSL**	Hormone Sensitive Lipase
p85	PI3'K regulatory sub-unit	**cAMP**	Cyclic Adenosine Monophosphate
PI3'K	phosphatidylinositol 3'-kinase	**TAG's**	Triglycerides
PKB	Protein Kinase B	**NEFA**	Non-esterified Fatty Acids

Figure 4.1 Putative signalling pathways mediating the metabolic effects of insulin in 3T3-L1 adipocytes

4.1.9 Aims

This study was designed to answer the question of whether sex steroids are able to exert a direct effect on insulin responsive tissues. To avoid the problems associated with biopsy tissue, such as availability and difficulties in manipulation, a model cell line was used, the 3T3-L1 adipocyte. The 3T3-L1 adipocyte is derived from the 3T3-L1 fibroblast by selective induction into an insulin-responsive phenotype according to the methods outlined in 2.3.3 and 2.3.4. Once differentiated, the 3T3-L1 adipocyte responds well to insulin and many of the insulin-responsive signalling pathways are well characterised. Unfortunately, it is not possible to culture the 3T3-L1 adipocytes in an environment exactly mimicking that seen in conditions such as PCOS, where the cells are exposed to consistantly elevated levels of hormone over long-periods of time, since these cells only retain the insulin-responsive phenotype for around 5 days. As an alternative these cells have been incubated with physiological or pharmacological doses of sex steroid for up to 24 hours. Although this treatment is not ideal, it is thought to be a broadly representative adaptation using this model.

Previous work by Ian Campbell has shown that treatment of 3T3-L1 adipocytes with either oestradiol (E1), oestrone (E2) or oestriol (E3) causes a marked reduction in the ability of these cells to mediate insulin-stimulated glucose transport (Campbell, Ph.D Thesis, University of Glasgow, 1997). The aim of this chapter is to determine whether this effect is due to a direct action on insulin signalling in these cells.

4.2 Results

4.2.1 The effect of E1, E2 and E3 on insulin-stimulated glucose transport in 3T3-L1 adipocytes

Previous work by Ian Campbell has established that treatment of 3T3-L1 adipocytes with either E1, E2 or E3 was able to markedly reduce the ability of these cells to mediate insulin-stimulated glucose transport, the effect being significant after 8 hours and maximal at 24 hours (data not shown). Maximal effects of each sex steroid were seen at a dose of 100nM. Figure 4.2 shows that 12 hours of treatment with 100nM E1, E2 or E3 was similarly able to reduce the maximum insulin-stimulated glucose transport in 3T3-L1 adipocytes in my hands, with an order of potency equal to that seen in previous studies (E2 ≥ E3 > E1).

4.2.2 The effect of E1, E2 and E3 on total levels of GLUT4 in 3T3-L1 adipocytes

As described in detail in Chapter 1, the main insulin-responsive glucose transporter is the GLUT4 isoform, which translocates from intracellular stores to the plasma membrane in response to insulin. Since GLUT4 is so central to this process, it follows that any alterations in expression of this protein could explain diminished glucose transport. This appears not to be the case in this system. Figure 4.3 shows how GLUT4 expression remains unaltered after 12 hours of steroid treatment. This is also illustrated in Figure 4.8, where data from three experiments is quantified. In contrast plasma membrane lawn assays (carried out by Gwyn Gould, University of Glasgow) revealed that the translocation to the plasma membrane in response to insulin was reduced by 65 + 8% in E1-treated cells, 54 + 8% in E2-treated cells and 51 + 9% in E3-treated cells ($p<0.05$ using the students t-test in each case) (data not shown).

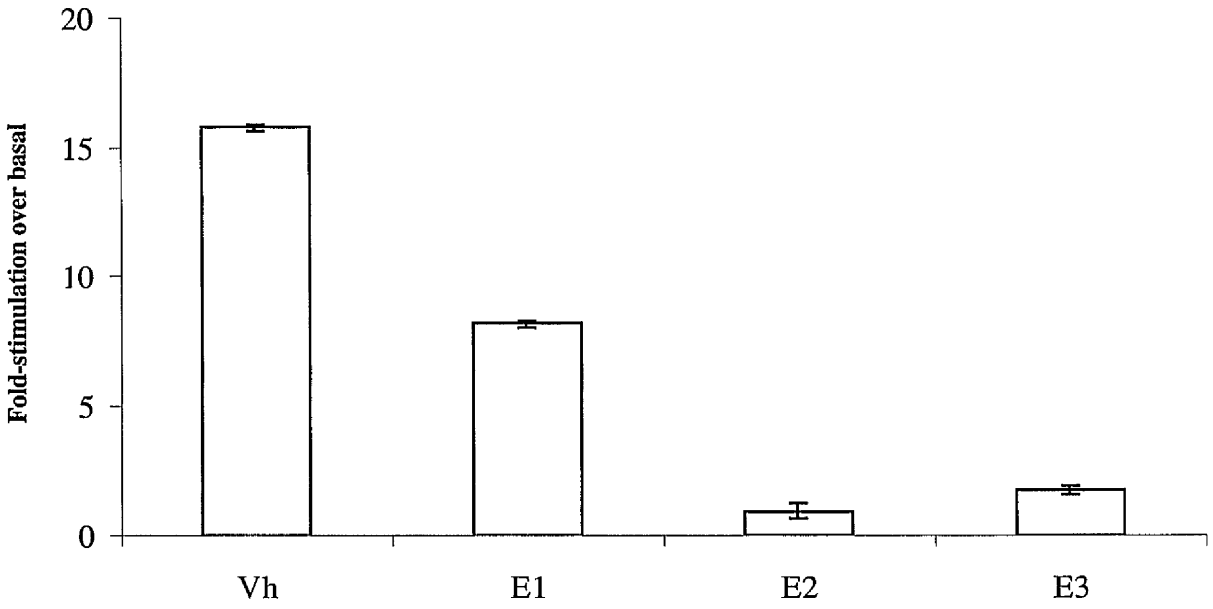


Figure 4.2 The effect of E1, E2 and E3 on insulin-stimulated glucose transport in 3T3-L1 adipocytes

3T3-L1 adipocytes, 4 days post-differentiation, were incubated over-night in the presence of vehicle or 100nM E1, E2 or E3. The next day cells were washed and then incubated in serum-free DMEM for a minimum of 2 hours. After this time 2-Deoxy-D-Glucose uptake assays were carried out according to the method outlined in 2.3.7, using 100nM insulin for 15 minutes to stimulate uptake.

Data shown is representative of 3 such experiments, and the error bars represent the standard error of the mean.

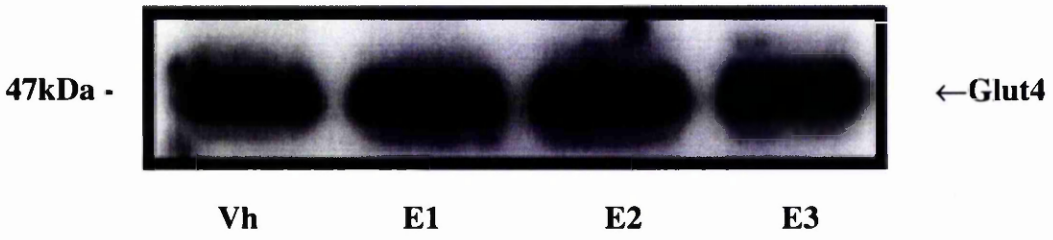


Figure 4.3 The effect of E1, E2 and E3 on total levels of GLUT4 in 3T3-L1 adipocytes

Steroid treatments were carried out on 10cm dishes of 3T3-L1 adipocytes in the same way as outlined in Figure 4.2, and whole cell lysates prepared as outlined in Section 2.3.10. Samples were resolved by SDS-PAGE, as outlined in section 2.6, and then immuno-blotted for GLUT4 protein using a rabbit polyclonal antibody as described in section 2.7.

The blot shown is typical of a representative set of data obtained on three separate occasions. The position of the molecular weight marker is shown, and GLUT4 identified by the arrow at the right.

Quantification of three experiments of this type revealed no significant difference in GLUT4 levels between the 4 treatment groups (see Figure 4.8).

4.2.3 The effect of E1, E2 and E3 on total levels of IRS-1 and IRS-2 in 3T3-L1 adipocytes

Since GLUT4 levels are normal but both insulin-stimulated glucose transport and GLUT4 translocation are reduced within these cells, it follows that levels or functionality of some other protein leading to these steps must be altered. As outlined in Chapter 1 there are potentially several signalling molecules, alterations in the activity or levels of which could reduce insulin signalling. IRS-1 and IRS-2 are the two of first effector molecules activated in response to insulin (see Section 1.4.3), and they have been illustrated to be of vital importance. Figure 4.4 illustrates that E1, E2 and E3 are indeed able to alter the total cellular expression of IRS-1 and although E2 and E3 appear more potent than E1, all steroids are able to significantly reduce the levels of the protein. Also seen in Figure 4.4 is the ability of the steroids to reduce total cellular levels of IRS-2, with an almost complete abolition by all 3 steroids. This is demonstrated graphically in Figure 4.8, where data from three experiments is quantified.

4.2.4 The effect of E1, E2 and E3 on total levels of PI3'Kinase in 3T3-L1 adipocytes

It is well established that the main immediate downstream target of the IRS proteins is PI3'Kinase, and so the effect of E1, E2 and E3 on cellular levels of this protein was examined. In common with the IRS proteins, steroid treatment caused a dramatic reduction (> 50% reduction compared to vehicle) in the levels of the p85 sub-unit of PI3'K as can be seen in Figure 4.5. Data from three such experiments is quantified in Figure 4.8.

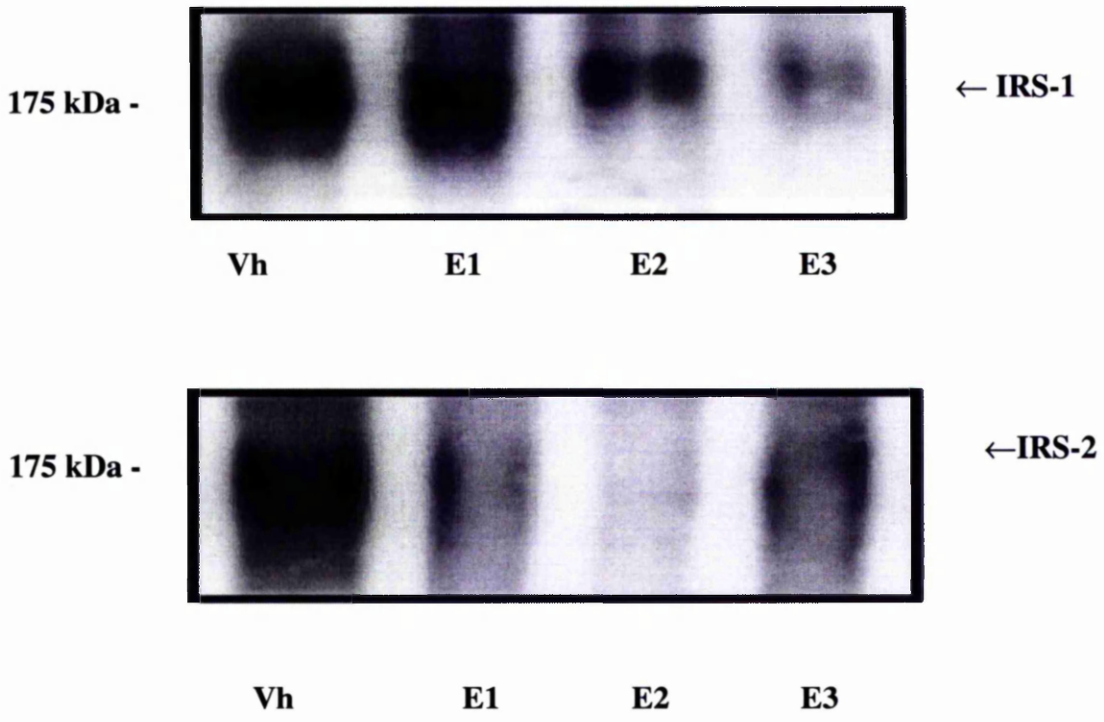


Figure 4.4 The effect of E1, E2 and E3 on total levels of IRS-1 and IRS-2 in 3T3-L1 adipocytes

Whole cell lysates were prepared as outlined in Figure 4.3. Samples were then resolved by SDS-PAGE, as outlined in section 2.6, and immuno-blotted for IRS-1 or IRS-2 protein using a rabbit polyclonal antibody as described in section 2.7.

The blot shown is typical of a representative set of data obtained on three separate occasions. The position of the molecular weight marker is shown, and IRS-1 and IRS-2 are identified by the arrows at the right.

Quantification of three experiments of this type revealed that all three steroids were able to significantly reduce the levels of IRS-1 and IRS-2 in whole cell lysates (see Figure 4.8).

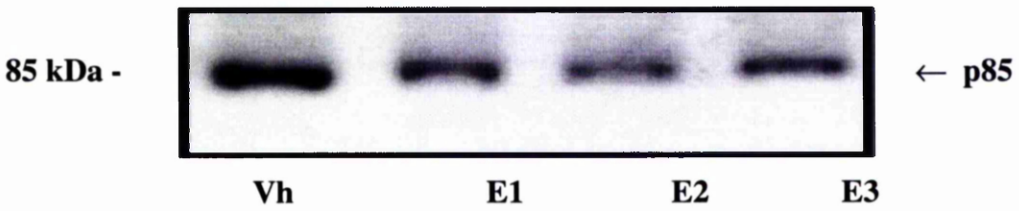


Figure 4.5 The effect of E1, E2 and E3 on total levels of PI3'Kinase p85 sub-unit in 3T3-L1 adipocytes

Whole cell lysates were prepared as outlined in Figure 4.3. Samples were then resolved by SDS-PAGE, as outlined in section 2.6, and immuno-blotted for p85 protein using a rabbit polyclonal antibody as described in section 2.7.

The blot shown is typical of a representative set of data obtained on three separate occasions. The position of the molecular weight marker is shown, and p85 is identified by the arrow at the right.

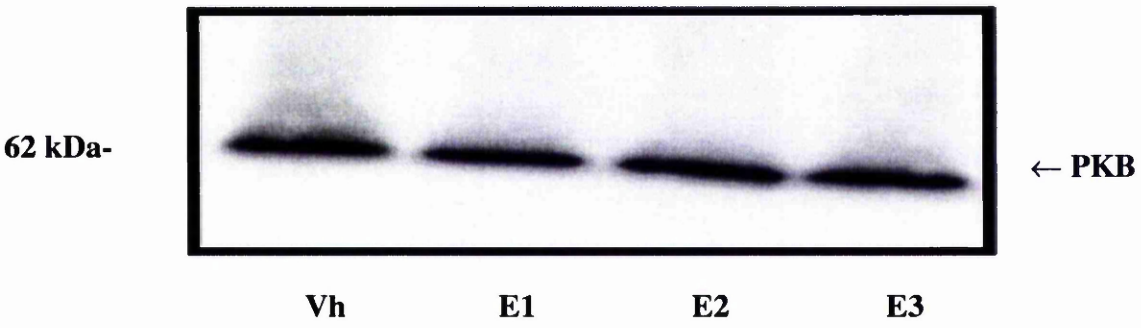
Quantification of three experiments of this type revealed that all three steroids were able to significantly reduce the level of p85 PI3'K subunit in whole cell lysates (see Figure 4.8).

4.2.5 The effect of E1, E2 and E3 on total levels and activity of PKB in 3T3-L1 adipocytes

The precise role of PKB in mediating glucose transport is still controversial. Nevertheless many studies support a role for this protein. Certainly insulin-stimulation will cause a rapid increase in PKB activity within 3-5 minutes. In contrast to the effects of the sex steroids to reduce cellular levels of the IRS proteins and PI3'K however, Figure 4.8 and Figure 4.6A illustrate that total PKB levels remain unchanged in response to steroid treatment. In spite of this however, steroid treatment is able to dramatically reduce the extent of PKB activation in response to insulin stimulation (E1, E2 and E3; 34.5 ± 9.6, 25.5 ± 8.1 and 82.5 ± 13.1 percent maximum insulin response respectively, n=3, $p<0.05$ using students t-test).

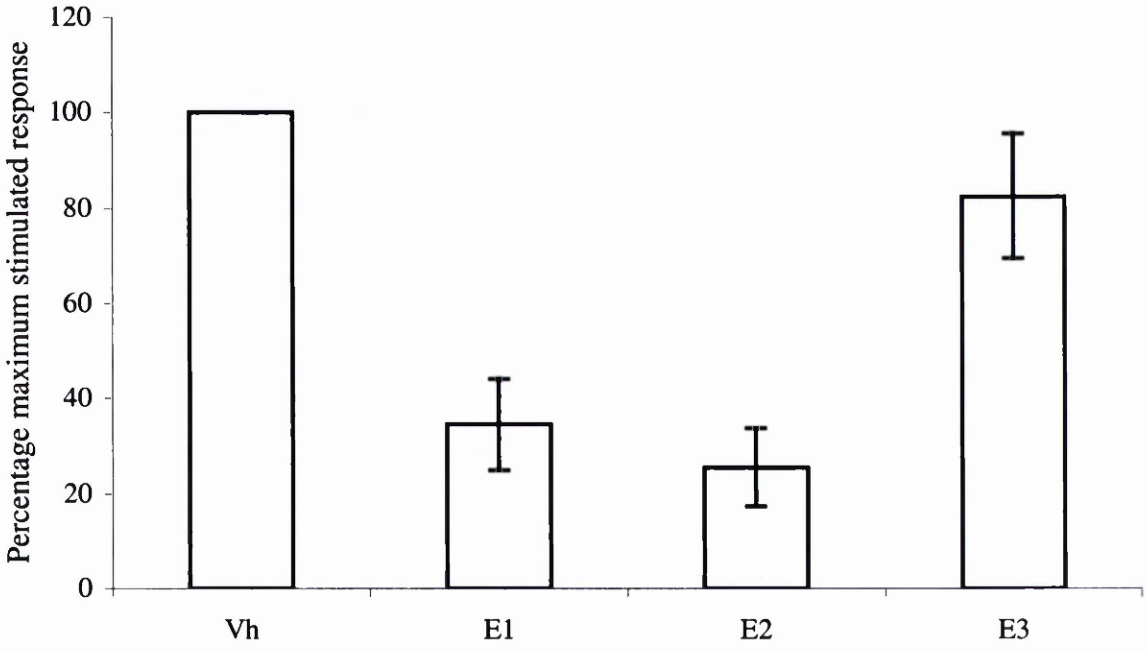
This reduction in activity in spite of normal expression suggests that the reduction in PKB activity is a consequence of impaired up-stream IRS-PI3'K interaction, and presumably activation.

The rank order of potency of E1, E2 and E3 for this effect on PKB is different than that seen for the effects on glucose transport and the up-stream proteins. This agrees with the belief that other intermediary proteins (which may or may not be regulated by the steroids) are involved in the steps between PI3'K and PKB.



Panel A

Levels of PKB protein in control (vehicle-treated) and steroid-treated 3T3-L1 adipocytes



Panel B

PKB activity in control (vehicle-treated) and steroid treated 3T3-L1 adipocytes

Figure 4.6 The effect of E1, E2 and E3 on levels and activity of PKB in 3T3-L1 adipocytes

For legends see next page

Figure 4.6 The effect of E1, E2 and E3 on levels and activity of PKB in 3T3-L1 adipocytes

Panel A

Whole cell lysates were prepared as outlined in Figure 4.3. Samples were then resolved by SDS-PAGE, as outlined in section 2.6, and immuno-blotted for PKB protein using a rabbit polyclonal antibody as described in section 2.7.

The blot shown is typical of a representative set of data obtained on three separate occasions. The position of the molecular weight marker is shown, and PKB is identified by the arrow at the right.

Quantification of 3 experiments of this type revealed no significant difference in PKB levels between the 4 treatment groups (see Figure 4.8).

Panel B

3T3-L1 adipocytes, 4 days post-differentiation, were incubated over-night in the presence of vehicle or 100nM E1, E2 or E3. The next day cells were washed and then incubated in serum free DMEM for a minimum of 2 hours. After this time, half of the cells from each treatment were stimulated with 100nM Insulin for 5 minutes. Lysates were then prepared as above, and then subjected to a PKB activity assay exactly as outlined in Section 2.3.11.

Data shown is representative of 3 such experiments, and is expressed relative to the maximum stimulation obtained in control (vehicle-treated) cells, with the error bars representing the standard error of the mean

4.2.6 The effect of E1, E2 and E3 on sub-cellular distribution of IRS-1 and IRS-2 in 3T3-L1 adipocytes

Although the effect of the steroids to reduce the total levels of IRS proteins and PI3'K could easily explain the inhibition of insulin action observed in these cells, it is also of interest to determine whether the steroids are able to influence the sub-cellular distribution of the IRS proteins. This is largely due to the emerging importance of sub-cellular localisation in insulin-induced activation of the IRS proteins, and possibly in the development of insulin resistance. As outlined in Chapter 1 activation of IRS-1 and IRS-2 occurs while engaged in a 'scaffold network' situated within the plasma membrane environment. Stimulation with insulin is believed to release this protein into the cytosol. This is believed to be of great importance as aberrant movement is believed to play a role in the development of insulin resistance. Indeed in Figure 4.7 it can be seen that treatment of 3T3-L1's with E1, E2 or E3 results in a dramatic reduction in the levels of IRS-1 in the membrane fraction and a concomitant increase in the cytosolic fraction. Steroid treatment also appears to reduce the amount of membrane-associated IRS-2 but, in contrast to the effects observed with IRS-1, Figure 4.7 shows that this is accompanied by a similar decrease in the soluble fraction.

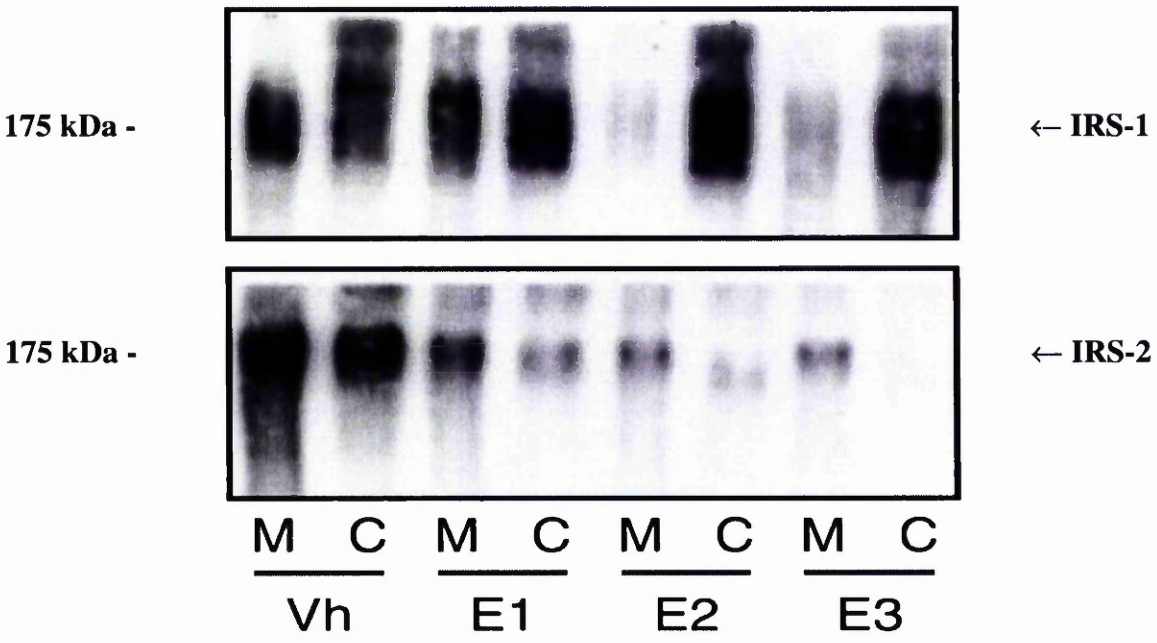


Figure 4.7 The effect of E1, E2 and E3 on sub-cellular distribution of IRS-1 and IRS-2 in 3T3-L1 adipocytes

In order to determine the effects of E1, E2 and E3 on the intracellular distribution pattern of IRS-1 and IRS-2, membrane and cytosolic fractions were prepared after steroid treatment as outlined in Section 2.3.9. Samples were resolved by SDS-PAGE, as outlined in section 2.6, and then immuno-blotted for IRS-1 or IRS-1 protein using a rabbit polyclonal antibody as described in section 2.7.

The blot shown is typical of a representative set of data obtained on three separate occasions. Note that approximately 10% of the membrane and 5% of the cytosolic protein obtained from one 10cm dish was loaded per lane.

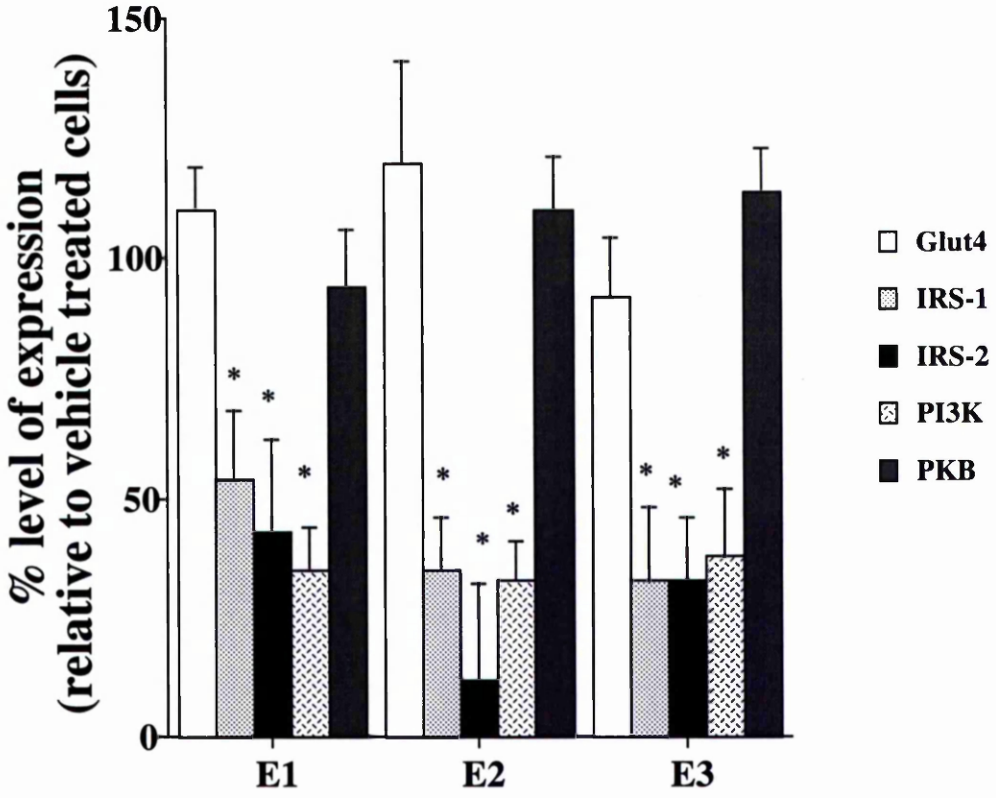


Figure 4.8 Quantification of the levels of GLUT4, IRS-1, IRS-2, p85 and PKB in steroid treated lysates

The bar graph illustrates a quantification of blots typical of those obtained in Figures 4.4 to 4.6. Expression levels in steroid treated cells are expressed as a percentage of expression in control (vehicle)-treated cells. Error bars represent the standard error of the mean. $P<0.05$, using the students t-test.

4.3 Discussion

4.3.1 Effects of E1, E2 and E3 to inhibit insulin-stimulated glucose transport

Although the association between circulating oestrogen levels and impaired insulin action has long been appreciated there has up until now been a failure to demonstrate a direct effect of sex steroids on peripheral insulin-sensitive tissues. The data presented in Figure 4.2 however are a direct demonstration of such an effect, where E1, E2 and E3 are able to effectively inhibit insulin-stimulated glucose transport in the 3T3-L1 adipocyte, a suitable cell-culture model of an insulin-sensitive peripheral tissue. Although the 3T3-L1 adipocyte is clearly far removed from the adipocytes of human PCOS subjects (discussed in more detail in Section 4.1.6) the results presented clearly have implications for the development of such disorders.

Current lines of evidence do not favour a role for oestrogen action on peripheral tissues in initiating the onset of PCOS (Taylor and Marsden., 2000), nevertheless it is thought that such an action does likely have a role in propagating the disease once it has began to develop. Indeed, some current lines of evidence suggest that the persistent hyper-insulinaemia of PCOS is able to influence the elevated oestrogen secretion and that the reverse is also true (refer to Section 4.1.4).

With regard to the insulin resistance present in PCOS, Dunaif *et al* illustrated that this is a peripheral and not a hepatic insulin resistance (Dunaif *et al.*, 1989). In other words, the insulin resistance is not related to any impairment at the level of the liver to exert normal control over glucose homeostasis but more a reduced ability of peripheral tissues to respond normally to insulin. This therefore suggests impairment in some mechanism within the peripheral tissues, which is responsible for mediating the steps between insulin binding and the biological effects. This fits well will the data presented in Figure 4.2, since oestrogen

treatment is also affecting some machinery within the 3T3-L1 adipocyte, resulting in development of cellular insulin resistance.

The data presented also has implications for other disorders where insulin resistance is associated with an altered sex hormone profile. Pregnancy, long-term use of the combined-oral contraceptive pill and the use of HRT can all result in a state of cellular insulin resistance. Although it is perhaps not ideal to group these three conditions together, they do all develop into an insulin resistant state by apparently similar mechanisms- the presence of elevated circulating levels of oestrogens. Therefore it is quite possible that the development of insulin resistance in these conditions follows a similar pattern to that observed in the 3T3-L1 adipocyte cell system.

Due therefore to the potential similarity between mechanisms observed in the 3T3-L1 and potential mechanisms of peripheral insulin resistance in the conditions discussed above it is of interest to further define the mechanisms of insulin resistance in the 3T3-L1.

4.3.2 Effects of E1, E2 and E3 on GLUT4 Function and Expression

In common with the ability of the sex steroids to inhibit insulin-stimulated glucose transport, reductions in movement of GLUT4 to the plasma membrane have also been observed (Campbell, PhD thesis, University of Glasgow, 1997). Thus therefore suggests that defects are present either at the level of GLUT4 itself, or in the upstream signalling proteins.

Rosenbaum *et al* have shown similar reduction in sensitivity to insulin in PCOS patients, which is apparently due to an obesity-independent reduction in GLUT4 expression in adipocytes (Rosenbaum *et al.*, 1993). This is not the case in our study where the expression of GLUT4 is not altered after a 12 hour incubation with sex steroids. It is however quite likely that the differences observed in our system may relate to how the parameters are actually measured. Indeed PCOS patients observed at the

early stages of this disease may show a lesser reduction in GLUT4 content and conversely, were it possible to treat 3T3-L1's with steroid for much longer periods, reductions in GLUT4 expression may be observed.

Despite the lack of effect of the sex steroids on GLUT4 expression, they are clearly able to reduce insulin-stimulated GLUT4 translocation and transport in the 3T3-L1 adipocyte. It therefore follows that sex steroids are able somehow to modulate some of the other key steps leading to glucose transport.

4.3.3 Effect of E1, E2 and E3 to reduce levels of key insulin signalling molecules

The early signalling pathways activated by insulin binding to the receptor are well appreciated. This is discussed in more detain in Section 1.4.2. Due to the key role of many of these proteins they are key targets for oestrogen-induced inhibition of glucose transport.

(i) IRS-1 and IRS-2

Although the actions of IRS-1 and IRS-2 overlap, and can be partly replaced by other IRS proteins such as IRS-3 (see Section 1.4.3), numerous studies have high-lighted the vital roles of both proteins in mediating insulin-stimulated glucose transport (Holman and Kasuga, 1997). The observation in Figure 4.4 that total levels of IRS-1 and IRS-2 are dramatically reduced suggests that this effect is least partly responsible for the impairment in insulin-stimulated glucose transport in observed response to oestrogen treatment. Obviously a reduction such as this has implications for all metabolic actions of insulin, since these proteins are vital adaptor molecules, linking the upstream binding of insulin to its receptor to activation of all downstream responses. One of these downstream responses, activation of PI3'K, is discussed below.

(ii) PI3'K

Undoubtedly, the reduction in IRS protein levels will have dramatic effects on the activation of PI3'K simply as a consequence of there being a reduced capacity for activation. Nevertheless there is little doubt that any alterations in expression of PI3'K itself will also fundamentally impair the ability of insulin to signal effectively. Therefore it is of interest that the IRS proteins appear not unique in being targets of the sex steroids. Indeed it can be seen in Figure 4.3.4 that the levels of the p85 sub-unit of PI3'K are significantly reduced also. Since many studies using inhibitors or dominant negative enzyme sub-units (see Section 1.4.4) have demonstrated how reduced p85 expression can dramatically impair insulin-stimulated glucose transport it is very likely that this will also contribute to the development of cellular insulin resistance in these cells.

Thus it appears that the oestrogen induced-insulin resistance in 3T3-L1 adipocytes develops as a result of reduced levels of three important signalling molecules, IRS-1, IRS-2 and the p85 sub-unit of PI3'K. This will ultimately reduce the capacity of these cells to transduce the signals leading to the normal insulin-activated responses. The links between an altered biological response in spite of normal expression of key mediators of that response- glucose transport/GLUT4, and impaired upstream signalling has therefore been well illustrated. However it is also of interest to determine whether other downstream responses are similarly affected.

(i) PKB

It does appears that the reduction in IRS-1/2 and p85 levels is able to affect downstream biological responses other than activation of glucose transport. Insulin causes a rapid and transient activation of PKB in adipocytes and although PKB activation is believed to play some role in glucose transport the exact mechanisms are unclear. Certainly studies using over expression of specific PKB isoforms have illustrated how insulin-stimulated glucose transport is enhanced (Foran *et al.*, 1999; Hill *et*

al., 1999). In contrast however studies where PKB expression is reduced by various means have often failed to show any effect on glucose transport (still to find refs). PKB is also thought to be involved in mediating the anti-lipolytic effects of insulin, another important physiological effect induced by insulin in these cells. This is discussed in more detail in Section 1.4.5.1. Nevertheless, regardless of the specific role(s) of PKB it is undoubtedly intrinsically linked to insulin signalling and biological effects. As Figure 4.6 illustrates, although the expression of PKB remains unaltered, the insulin-stimulated activity is significantly reduced in response to sex steroid treatment indicating that the reduced levels of IRS proteins and PI3'K contribute to reduced activation of downstream targets other than those classically involved in glucose transport. Importantly the basal activity of PKB is unaltered, further suggesting that the defect is not at the level of the downstream response but with the up-stream proteins, IRS-1, IRS-2 and PI3'K.

(ii) Mitogenic signalling

From the results presented in this chaper it appears that sex steroid induced changes in the levels of upstream signalling molecules are able to effectively reduce metabolic signalling events such as glucose transport in 3T3-L1 adipocytes. It remains to be addressed whether this inhibition also impinges on the mitogenic effects of insulin, for example MAPK or p70s6K activation in unclear. It is likely that IRS-1/2 and PI3'K activated events may be altered in some way, simply as a consequence of the reduced levels of upstream proteins. Nevertheless, several mitogenic actions of insulin can be activated independently of the IRS proteins and PI3'K, including activation of MAPK, and it is feasible that the activity and levels of these proteins will be unaltered. This would be analogous to the work recently reported by Cusi *et al*, where a group of NIDDM patients were seen to have impaired IRS-1/2 PI3'K coupling but normal MAPK activation (Cusi *et al.*, 2000). It remains to be addressed whether such as situation exists in the 3T3-L1 adipocyte and indeed the functional implication of such a situation in a condition like PCOS.

4.3.4 Impaired IRS-1/2 function in sex steroid-induced insulin resistance- potential mechanisms

Although the reduced expression of IRS-1/2 and PI3'K can easily explain the observed reduction in insulin-stimulated glucose transport, it has recently emerged that many factors other than protein expression are important in the activation of signalling cascades (White and Kahn., 1994; Inoue *et al.*, 1998; Clark *et al.*, 2000).

In the field of IRS-1/2 signalling, the importance of tyrosine phosphorylation has long been appreciated, the phosphorylation of both proteins on distinct tyrosine residues being essential for their interaction with downstream signalling molecules (refer to Section 1.4). Recently however it has also emerged that the cellular localisation of the IRS proteins is crucial to their functioning (Inoue *et al.*, 1998; Clark *et al.*, 2000; and also refer to section 1.4.3). Indeed, recent work by Clark *et al* has illustrated that the IRS proteins appear to associate with a cytoskeletal fraction that is insoluble in non-ionic detergents and associates with the particulate fraction of cell membranes. It is believed that this complex may perform a unique function, allowing the IRS proteins to interact with the IR and also providing a location for IRS interaction with downstream target molecules. IRS proteins are subsequently released from this scaffold after insulin stimulation occurs. Interestingly, release from this platform is also associated with development of insulin resistance, the IRS proteins failing to interact with or being abnormally released from the scaffold, hence preventing the normal interactions (Clark *et al.*, 2000).

It appears that sex steroid treatment of 3T3-L1 adipocytes also induces resistance to the effects of insulin in a mechanism similar to this. It can be seen from Figure 4.7, that both IRS-1 and IRS-2 are located largely in the membrane fraction of basal 3T3-L1 adipocytes, but steroid treatment dramatically alters this. In the case of IRS-1, there is an apparent movement of the protein from the membrane to the soluble protein fraction, presumably reflecting disassembly of IRS-1 from its normal site

inside the cell. Assuming a mechanism similar to that outlined by James *et al*, this movement of IRS-1 will alter the subsequent ability of IRS-1 to interact with the IR and its ability to interact with downstream effector molecules. Importantly, however, the IRS-1 increase in the soluble fraction is much less than the amount of IRS-1 released from the membrane complex. This is in agreement with the view that a large percentage of the released protein does not remain within the cell and is actually degraded (Clark *et al.*, 2000).

Similar reductions, shown also in Figure 4.7, are observed in the IRS-2 content of membranes after sex steroid treatment. This presumably reflects a similar disassociation of IRS-2 with a key membrane component to which it localises to function properly. Little IRS-2 was found in the soluble fraction, again reinforcing the thought that the released protein is indeed degraded.

There are potentially several reasons why the levels of IRS-1 in the membrane and the cytosol appear higher than those of IRS-2. Firstly, it may simply be a reflection of there being higher total levels of IRS-1 within the cell. Another possibility is that IRS-2 is simply degraded more rapidly than IRS-1, and hence is less easy to detect. It is also possible that the IRS-1 antibody has a higher affinity than the IRS-2 antibody, and as a result more easily detects a signal. Indeed a combination of both these, and other, factors is likely to be involved. In this regard, it can be seen that the levels of IRS-1 and IRS-2 in whole cell lysates (Figure 4.4) appear to be less than the total amount detected in the membrane and cytosol fractionation (Figure 4.7). Although this might be due to differential protein loading this is unlikely as similar amounts of protein were loaded per lane for each blot. Another more likely explanation is simply differences in processing of the blots, in that although the western blots were carried using the same protocol, there could possibly be subtle differences between experiments.

Nevertheless, the data presented in this Chapter clearly illustrates the ability of sex steroids to modulate insulin-mediated biological responses, by inhibiting the function of key upstream signalling molecules.

4.3.5 Potential Mechanisms of sex steroid action in 3T3-L1 adipocytes

The effect of the sex steroids to reduce the intracellular levels of insulin-sensitive signalling components could quite easily be explained by an action of the steroids at the gene level (discussed in Section 4.1.8). Although the genes expressed in the mature 3T3-L1 adipocyte will undoubtedly differ significantly form the genes expressed in the cells of the ovary, the general mechanism of oestrogen action will be similar. Although there is a lack of data regarding characterisation of sex steroid receptors in 3T3-L1 adipocytes, several studies have revealed the presence of oestrogen receptors in mature human adipose tissue (Pedersen *et al.*, 1992; Pedersen *et al.*, 1996). In our study, it was observed that steroid treatment didn't reduce the total recovery of protein (data not shown) from the cells and so it is unlikely that the steroids are having a general effect to suppress protein production.

More likely the steroids are acting at specific EREs located within the genes, to selectively modulate RNA and protein synthesis. EREs within the IRS or p85 genes have not been extensively studied but it is highly likely that such sites will exist. The apparent differing potencies of the steroids is likely to be due to a combination of several factors, including potency of ligand-binding to receptor or expression of different ER isoforms which may favour binding of one steroid over another.

The ability of the sex steroids to influence intracellular location of the IRS proteins (discussed in Section 4.3.4) is perhaps more difficult to explain. One possible explanation is that the sex steroids are influencing expression of other, as yet unidentified, accessory proteins which are important in

targeting of the IRS proteins. Should the expression of specific proteins be up- or down-regulated, then mis-targeting (and hence destruction) would occur. Another possible, but perhaps less likely, explanation is that perhaps the ligand-bound ER complex has some other biological function and is able to directly influence the formation of insulin signalling components.

4.4 Conclusions

Many studies have demonstrated associations between endocrine disorders such as PCOS and impaired insulin sensitivity but the molecular mechanisms behind these are poorly understood. It is generally perceived such conditions are multi-factorial and that sex steroid induced changes in peripheral insulin-sensitive tissues are not sufficient to fully explain the observed phenotype (Taylor and Marsden., 2000). Nevertheless it is feasible that a direct action of oestrogens to supress insulin action could influence already activated disease states (Taylor and Marsden., 2000). To date, however, there have been a lack of studies looking from this aspect. This study aimed to address this by studying the effect of oestrogens on insulin sensitivity in 3T3-L1 adipocytes.

Oestrogen action on 3T3-L1 adipocytes was indeed found to significantly inhibit insulin-stimulated glucose transport. Interestingly this was not due to any change in GLUT4 expression but in fact due to aberrant expression and localisation of the up-stream signalling intermediates IRS-1 and IRS-2. This effect is undoubtedly in part mediated by direct effects of oestrogen-ER complexes to influence the new synthesis of the IRS proteins, and other accessory proteins. A direct action of ligand-ER complexes on formation of insulin-signalling complexes must also be considered.

These observations are of interest not only with regard to insulin signalling and the development of insulin resistance in culture models, but also of clinical relevance. As already illustrated, there are many conditions where there is an excess of sex steroids and impaired insulin-sensitivity. Although it is unlikely that the affects seen will adequately explain all such conditions, it is likely that similar effects could contribute to the development and worsening of the disease state.

Further work would be aimed at looking more closely at different aspects of the effects of the sex steroids on 3T3-L1 adipocytes, for example effects

on levels and activity of mitogenic signalling intermediates in order to determine whether this pathway is similarly affected. It would also be of interest to look more closely at the IRS genes, and determine the presence and location of specific oestrogen responsive areas. Finally the ultimate aim would be to take this forward into human adipocytes, and determine the whether similar defects could be observed in adipocytes obtained from PCOS subjects or similar compared to controls.

5 General Discussion and Further Work

5.1 Insulin action and inaction

As discussed throughout this thesis, insulin resistance is a phenomenon present in a variety of disease states. Perhaps the most obvious of these is NIDDM, where the profound insulin resistance in target tissues is coupled with an inadequate pancreatic β cell compensatory response (Dale *et al.*, 1996; Gould, 1997; Brady *et al.*, 1999). NIDDM is not unique in this regard however, and insulin resistance is also associated with a variety of cardiovascular disorders. Indeed, as discussed in more detail in Section 1.10, there is a well established interaction between primary hypertension and insulin resistance (Mitchell *et al.*, 1990; Morris *et al.*, 1994)., and this frequently couples with abnormal lipid metabolism in a condition referred to as the Metabolic Syndrome or Syndrome X (Morris *et al.*, 1994). Insulin resistance is also a common feature of disorders of the female endocrine system, and is particularly associated with PCOS and HRT users (Dunaif *et al.*, 1992; Rosenbaum *et al.*, 1993; Taylor and Marsden., 2000; Lyall and Gould., 2000).

Since the insulin resistance present in all of these conditions exists at the level of peripheral target tissues (Morris *et al.*, 1994; Taylor and Marsden., 1994), it is reasonable to assume that there may be a defect at the level of insulin signalling within these cells. As discussed in Section 1.4 to 1.6, the early pathways involved in propagating the insulin signal to the inside of the cell are fairly well characterised (reviewed in Holman and Kasuga., 1997), although the precise mechanisms of GLUT4 translocation are less clear. Changes in any of these signalling events could potentially indicate a primary defect, or alternatively may reflect an adaptive response to a change within some other tissue such as muscle.

5.2 Insulin action in the SHRSP

5.2.1 Potential roles of Cd36

Although the SHR was first demonstrated to be insulin resistant over ten years ago, defective expression of the putative fatty acid transporter Cd36 was the first abnormality observed at the molecular level (Aitman *et al.*, 1999). With the demonstration that around 40% of the defect in carbohydrate metabolism and almost **all** of the defect in lipid metabolism could be accounted for by absence of this protein (Aitman *et al.*, 1999; Pravenec *et al.*, 1999), a suggestive role in the development of spontaneous hypertension in general arose. Interestingly however, although this protein may be linked to an enhanced risk of developing abnormal lipid metabolism (Ginsberg, 2000), its links with spontaneous hypertension in humans is less clear. Only a small percentage of cases of spontaneous hypertension are associated with abnormal Cd36 expression (de Winther *et al.*, 2000). Interestingly Gotoda and colleagues have studied an SHR strain, the SHR/Izm, that displays **normal** Cd36 expression, despite being similar to Aitman's SHR in other ways (Gotoda *et al.*, 1999). These lines of evidence are therefore suggestive that although Cd36 undoubtedly plays a role in some cases of spontaneous hypertension, it is unlikely to have such a universal role as once anticipated.

In agreement with this the data presented in Chapter 3 illustrates how the similarly hypertensive relative of the SHR, the SHRSP, exhibits metabolic abnormalities similar to those seen in the SHR, but in common with the SHR/Izm has **normal** Cd36 expression.

5.2.2 Further SHRSP studies

5.2.2.1 Cd36 and other molecules

Although *Cd36* gene expression and protein levels are normal in SHRSP adipocytes, the question remains as to whether it could contribute in other ways. As discussed in Section 3.3.4, localisation to specific cellular compartments appears essential for at least some of the functions of Cd36, particularly its role in mediating uptake of ox-LDL. It is therefore feasible that Cd36 could contribute to the SHRSP phenotype, not through reduced expression but by a somehow altered cellular distribution that impinges on normal function. Current ongoing studies are aimed at determining whether Cd36 protein is similarly distributed between different cellular compartments in SHRSP and WKY adipocytes, and whether this distribution can be differentially affected by insulin stimulation.

5.2.2.2 Insulin Signalling

It is highly likely however that there are other molecules which are important in the SHRSP phenotype, and which could also contribute to aspects of the SHR phenotype not explained by the Cd36 deletion, and in cases of spontaneous hypertension in humans. The observation that the SHRSP is indeed insulin resistant at the level of glucose uptake into adipocytes (discussed in Chapter 3) and skeletal muscle (personal communication from Declan James) suggests that the abnormality may lie at the level of insulin signalling in these cells.

The molecules known to be important in transducing the signalling steps activated by insulin include the IR, IRS-1 and IRS-2 and PI3'K (Holman *et al.*, 1997). Future work in this area will concentrate firstly on determining whether the key molecules in insulin-stimulated GLUT4 translocation are not only expressed normally, but also activated normally. With regard to IRS-1 and IRS-2 this will involve the specific immuno-precipitation of

these proteins, followed by immunoblotting with specific phospho-tyrosine antibodies. In this regard it would also be of interest to determine the serine phosphorylation of either of the proteins, as serine phosphorylation of IRS-1 and IRS-2 is associated with the development of insulin resistance (DeFea *et al.*, 1997; Li *et al.*, 1999; Wang *et al.*, 1998). It will also be necessary to determine the specific sub-cellular localisation of each of the proteins, as removal from specific sites is believed to be important in the development of insulin resistance in 3T3-L1 cells chronically treated with insulin (Clark *et al.*, 2000). Assessment of the activity of important signalling enzymes such as PI3'K, PKC ι, ζ and λ, and PKBβ will also be carried out in order to establish whether defects at this level could contribute to the SHRSP phenotype.

In addition to the important intracellular signalling molecules involved in mediating the effects of insulin, there are multiple proteins involved in the GLUT4 translocation (see Section 1.5). Analysis of the levels and expression of these proteins in SHRSP and WKY cells will reveal whether aberrant expression of these proteins could contribute the phenotype of the SHRSP.

5.2.2.3 Genetic studies

With the aim of further characterising the SHRSP phenotype at the genetic level, and identifying important loci and marker molecules other than Cd36, extensive genetic and phenotypic studies will be carried out. The studies presented in this thesis have revealed a significant impairment in insulin-stimulated glucose uptake and inhibition of lipolysis in SHRSP adipocytes compared to WKY. The p-values are however within the range of 0.01, a value considered inadequate for QTL mapping studies, and thus strategies to enhance the differences between SHRSP and WKY phenotype will be therefore employed. Pravenec and colleagues have described a method where rats fed a high (60%) fructose diet display an exaggerated insulin resistance (Pravenec *et al.*, 1999). Phenotypic

analyses of SHRSP and WKY fed such a diet can then be carried out, including measurement of serum markers, insulin-stimulated glucose uptake and anti-lipolysis, and of systolic and diastolic blood pressures. Such an approach will allow for selection of animals with a robust phenotype, and these animals can then be used to generate an F_1 generation via SHRSP x WKY crosses (male SHRSP x female WKY, and *vice versa*). The mode of inheritance of a given phenotype in these F_1 hybrids will be established, and results from this will dictate the approach for further breeding strategies. The approach outlined above should result in the identification of one or more QTL's for insulin resistance in the SHRSP. These QTL's are however likely to represent large chromosomal regions (spanning around 20-30cM), and the ultimate aim is to identify progressively smaller regions. This will be achieved using congenic strains, where regions containing potential QTL's of interest in one strain (the recipient) are replaced with the homologous region from another strain (the donor). Should transfer of a particular area of DNA confer a phenotype different from the control recipient strain, then it can be concluded that this area is important in a specific trait. In the long term work would be aimed at advancing towards very small areas (1cM), using techniques such as positional cloning and cDNA microarray analyses.

5.3 Insulin resistance in PCOS

5.3.1 Characteristics of PCOS

As discussed in Chapter 4, insulin resistance is a feature present in a variety of syndromes associated with an abnormal balance of hormones in the female reproductive system. This includes users of the combined oral contraceptive pill, HRT, and PCOS subjects (Taylor and Marsden., 2000). With regard to PCOS it is though that the peripheral insulin resistance is unlikely to be the primary defect, although its presence is thought to contribute to worsening of the disease phenotype (Taylor and Marsden., 2000). For this reason it is of value to determine how an altered sex

hormone profile may lead to the development of insulin resistance in insulin sensitive tissues, in an aim to understanding how it could be prevented or reduced.

5.3.2 Potential contribution of insulin resistance in PCOS

Interestingly it was observed in a cell culture model of a peripheral insulin sensitive tissue, the 3T3-L1 adipocyte, that treatment with three different sex steroids was able to reduce the insulin sensitivity of these cells as indicated by a reduced insulin-stimulated glucose uptake. This effect involved a steroid-induced down-regulation of several proteins known to be important in insulin signalling processes. Protein levels of IRS-1, IRS-2, and the p85 regulatory subunit of PI3'K were dramatically reduced in lysates from steroid treated cells. In addition, steroid treatment caused a redistribution of IRS-1 and IRS-2 from the membrane to the cytosolic fraction. Clark and colleagues described a similar scenario in 3T3-L1 adipocytes, where they demonstrated a movement of IRS-1 and IRS-2 away from a membrane associated compartment to the cytosol, resulting in development of a cellular insulin resistance (Clark *et al.*, 2000). Interestingly the altered signalling in the steroid treated 3T3-L1 cells, resulted in impairment of insulin-mediated responses other than glucose transport. Indeed, although expression of PKB was not dramatically altered by steroid treatment, the insulin-induced stimulation was dramatically reduced. This is similar to the scenario observed with GLUT4, where expression of the protein was unaltered but the response to insulin was dramatically impaired.

5.3.3 Further studies regarding the relationship between PCOS and insulin resistance

Although many studies have illustrated the links between these two conditions, little has been established regarding links at the molecular

level. Studies focusing on GLUT4 levels in PCOS subjects have produced conflicting results, although it is generally thought that many PCOS subjects do have a significantly reduced level of GLUT4 in adipocytes (Rosenbaum *et al.*, 1993). Obviously this does not agree with the results presented in Chapter 5, where the levels of GLUT4 remain unchanged by steroid treatment. This suggests that the reduced level of GLUT4 observed in PCOS subjects is an adaptive change, and occurs as a result of continuing abnormal hormone balance. As discussed in Chapter 5, treatment of 3T3-L1 adipocytes with high doses of sex steroids does broadly mimic the environment to which adipocytes from PCOS subjects will be exposed. Because it is not possible to expose these cells to steroids for long periods of time however, the long-term adaptive changes are unlikely to develop. In contrast if adipocytes could be obtained from very early stage PCOS subjects, as well as advanced cases, it may be possible to observe how GLUT4 levels change within these cells throughout the disease progression. Obviously it would also be of interest to establish how the insulin signalling pathways are modulated in these primary adipocytes from PCOS subjects, and to determine whether the defects observed in the 3T3-L1 adipocytes may also be of significance in PCOS adipocytes.

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