The role of protein kinase C ε in insulin receptor trafficking and insulin action

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The Role of Protein Kinase C ε in Insulin Receptor Trafficking and Insulin Action

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ABSTRACT

The development of type 2 diabetes is reaching epidemic proportions and identifying ways to modulate insulin levels in order to maintain euglycaemia is important in understanding how to better treat this disease. It is now accepted that lipid oversupply can detrimentally affect insulin action and the lipid activated kinase, protein kinase C epsilon (PKCε), has been implicated in the development of insulin resistance and progression to type 2 diabetes. We have previously shown that global PKCε knockout (PKCε KO) mice are protected from high fat diet induced glucose intolerance, in part through reduced hepatic insulin clearance.

We have shown using wild type (WT) and PKCε KO mouse embryonic fibroblasts (MEFs) as a model that ablation of PKCε perturbs insulin uptake and this was associated with a reduction in insulin-stimulated insulin receptor (InsR) redistribution within the cell, by subcellular fractionation. This was associated with a differential localisation of the InsR in the basal state in PKCε KO MEFs, with a greater proportion of the IR localised to cellular lipid microdomains. Insulin-stimulated InsR tyrosine phosphorylation within the kinase domain at sites 1162/1163 was reduced, however this defect in InsR phosphorylation did not translate to defective downstream signalling, with insulin-stimulated PKCε KO MEFs having normal or enhanced Akt/PKB, Erk1/2 and IRS-1 phosphorylation. Potential mechanisms for these differences were investigated by examining key candidates such as expression of the InsR substrate, Ceacam1 and the docking protein Grb14, to deduce the effect upon InsR internalisation and signalling, with Ceacam1 expression found to be greatly reduced in PKCε KO MEFs. Insulin signalling was further investigated in primary hepatocytes and *in vivo* following stimulation of de novo insulin secretion to examine these findings in more physiological settings.

The role of PKCε in lipid-induced insulin resistance was also investigated as PKCε has been shown to affect lipid metabolism. The incorporation of the fatty acid palmitate into distinct lipid classes was examined, as alterations in cellular lipid composition could contribute to alterations in insulin action, however no major changes were observed. However, following palmitate treatment of MEFs, PKCε KO MEFs displayed a greater preservation of downstream insulin signalling compared to WT MEFs. The data is consistent with a role for PKCε in the generation of insulin resistance through the modulation of lipid metabolism, rather than merely acting downstream of specific lipid intermediates.

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Publications

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Raddatz K, Turner N, Frangioudakis G, Liao BM , **Pedersen DJ**, Cantley J, Wilks D, Preston E, Hegarty BD, Leitges M, Raftery MJ, Biden TJ, Schmitz-Peiffer C. "Time-dependent effects of Prkce deletion on glucose homeostasis and hepatic lipid metabolism on dietary lipid oversupply in mice." (2011) Diabetologia. 54(6):1447-56.

Directly due to this thesis

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Poster Presentations

Pedersen DJ and Schmitz-Peiffer C. "The roles of Protein Kinase C epsilon (PKCε) and the Myristoylated Alanine-Rich C Kinase Substrate (MARCKS) in the regulation of Insulin Receptor trafficking." (2008) Australian Diabetes Society Conference, Melbourne, Australia.

Pedersen DJ and Schmitz-Peiffer C. "The role of Protein Kinase C ε in the Regulation of Insulin Receptor Trafficking and Insulin Action." (2010) American Diabetes Association 70th Scientific Sessions Conference, Orlando, USA.

Pedersen DJ and Schmitz-Peiffer C. "Protein kinase C epsilon Ablation Impairs Insulin Receptor Trafficking but Not Signalling." (2010) XI International Symposium on Insulin Receptor and Insulin Action, Naples, Italy.

Oral Presentations

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Awards

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ABBREVIATIONS

2-DG	2-deoxyglucose
A/A	Antibiotic/Antimycotic
aPKC	Atypical PKC
BCA	Bicotinic Acid Protein Assay
BSA	Bovine Serum Albumin
Ceacam1	Carcinoembryonic Antigen-Related Cell Adhesion Molecule
DAG	Diacylglycerol
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulphoxide
EGFR	Epidermal Growth Factor Receptor
EM	Electron Microscope
EtOH	Ethanol
FCS	Foetal Calf Serum
FA	Fatty Acid
GABA	Gamma Amino Butyric Acid
Grb	Growth Factor Receptor Bound Protein
GTT	Glucose Tolerance Test
HEK	Human Embryonic Kidney
HFD	High Fat Diet

IGF	Insulin-like Growth Factor
IKK	Inhibitor of κB Kinase
InsR	Insulin Receptor
IR	Insulin Resistance
IRS	Insulin Receptor Substrate
JNK	c-Jun N-terminal Kinase
KO	Knockout
LDL	Low-Density Lipoprotein
LPL	Lipoprotein Lipase
MAPK	Mitogen Activated Protein Kinase
MEF	Mouse Embryonic Fibroblast
NFκB	Nuclear Factor κB
PAGE	Polyacrylamide Gel Electrophoresis
PDK1	Phophoinositide Dependent Kinase
PI3K	Phosphatidyl-Inositol 3-Kinase
PIP_2	Phosphatidyl-Inositol-4,5-bisphosphate
PIP_3	Phosphatidyl-Inositol-3,4,5-triphosphate
PKC	Protein Kinase C
PM	Plasma Membrane
PP2A	Protein Phosphatase 2A
PS	Phosphatidylserine

PTB	Phosphotyrosine Binding Domain
RACK	Receptor of Activated C Kinase
SDS	Sodium Dodecyl Sulphate
Ser	Serine
SOCS	Suppressor Of Cytokine Signalling
STAT	Signal Transducer and Activator of Transcription
T2D	Type 2 Diabetes
TG	Triglyceride
Thr	Threonine
TRFR	Transferrin Receptor
TTBS	Tris-buffered Saline with Tween
Tyr	Tyrosine
WT	Wild type

Table of Contents

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1

General Introduction

1.1 Overview of Glucose Homeostasis and Type 2 Diabetes

Type 2 diabetes (T2D) is a multifactorial disease of glucose homeostasis afflicting a growing proportion of the world's population and is now starting to reach epidemic levels. This increase in prevalence is the result of a number of factors including genetic susceptibility and the increased availability of nutrient poor, energy dense foods (Harris, Pomeranz et al. 2009). It has been linked to the onset of related disorders such as cardiovascular disease and hepatosteatosis.

Blood glucose homeostasis is tightly controlled through a complex process of hormonal regulation and cross talk between various tissues of the body, in order to regulate the production, use and storage of glucose. The hormone insulin is crucial to maintaining euglycaemia. The development of insulin resistance (IR) and its progression to T2D is characterised by a reduced sensitivity of the peripheral tissues to insulin, resulting in impaired metabolism of glucose primarily by muscle and fat, whilst the liver fails to suppress hepatic glucose production (HGP). Initially the pancreatic β-cells compensate for this by secreting more insulin to overcome the reduced response to the hormone by the target tissues, in order to maintain the metabolic response to clear glucose from the blood. Over time IR can develop into overt T2D as the β-cells become dysfunctional and lose responsiveness to glucose levels, later undergoing apoptosis which ultimately results in defective insulin secretion and uncontrolled blood glucose levels (Saltiel and Kahn 2001).

It is now accepted that lipid oversupply leading to obesity plays a causative role in the induction of IR and the progression to T2D (Boden 1997). Increased availability of lipid in the circulation and subsequent storage in non-adipose tissues can lead to impaired glucose clearance from the blood (Boden, Chen et al. 1994; Dresner, Laurent et al. 1999), particularly by skeletal muscle, together with a reduction in the inhibition of hepatic glucose output. Insulin elicits its action through a cascade of phosphorylation events following binding and subsequent activation of its cell-surface receptor. The exact mechanisms leading to IR are still not fully understood, particularly the causation of defects at certain nodes in the canonical insulin signal transduction pathway resulting in the metabolic defects observed. The role of various lipid intermediates in this process is being further studied, in particular metabolites utilising fatty acid (FA) in their formation, such as diacylglycerol (DAG) and ceramide (Holland, Bikman et al. 2011a). These may diminish activation of the insulin

signalling cascade via negative signalling intermediates such as novel PKC isozymes (Schmitz-Peiffer, Browne et al. 1997; Samuel, Liu et al. 2007) and protein phosphatases (Cazzolli, Carpenter et al. 2001).

1.2 Glucose Homeostasis, Insulin Resistance and Obesity

1.2.1 Regulation of blood glucose levels

In the healthy state, the body is able to maintain glucose levels within a stringent range, through the ability of insulin to act on insulin target tissues to promote glucose clearance from the circulation. During the fasted state, as blood glucose levels decline, the pancreatic α-cells secrete glucagon which acts on the liver and to a lesser extent other organs such as the kidneys to stimulate endogenous glucose production. Following a meal, however, the spike in glucose levels stimulates the pancreatic β-cells to secrete insulin, to regulate fuel storage and maintain glucose homeostasis. This is achieved through promotion of glucose uptake and storage as glycogen primarily in skeletal muscle, which is the major site of glucose disposal in the body, and to a lesser extent in fat; whilst also shutting off endogenous glucose production by the liver (Figure 1.1). The ability of the body to maintain control of energy and glucose metabolism is essential as chronically elevated blood glucose levels lead to many health complications including blindness, renal failure, cardiovascular disease and non-alcoholic fatty liver disease (Biddinger and Kahn 2006).

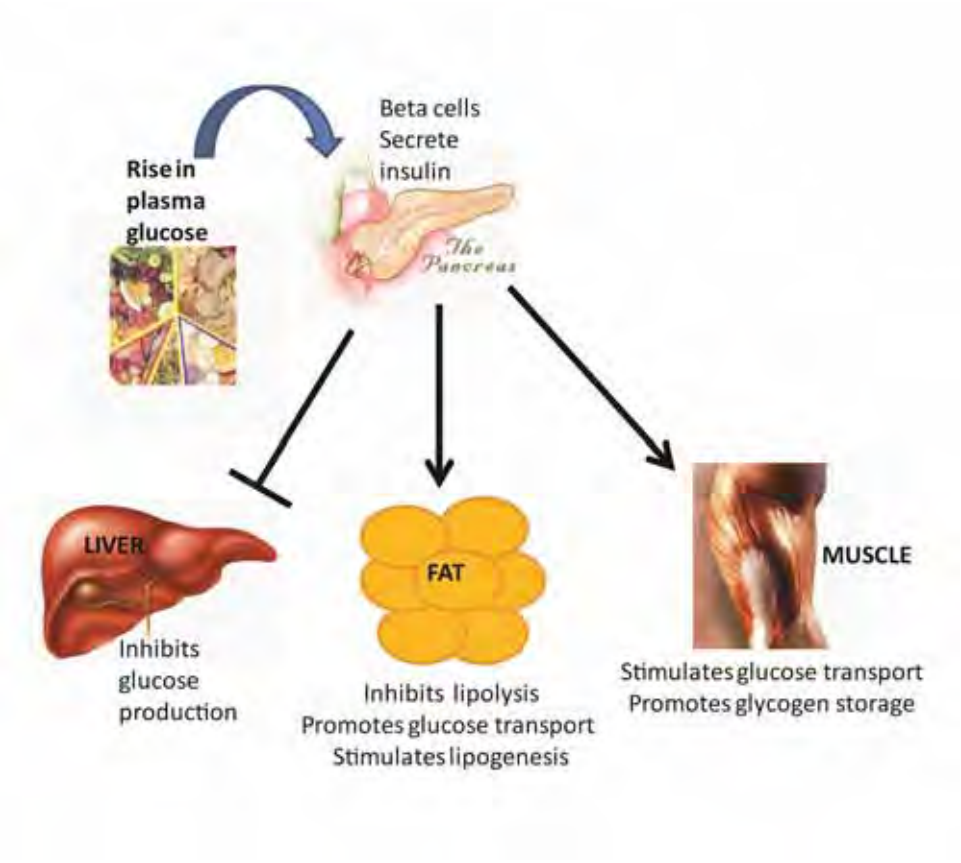

FIGURE 1.1 Basic Overview of insulin control of glucose homeostasis
Blood glucose levels are tightly controlled in the healthy state. Following a meal, blood glucose levels rise and stimulate the β-cells of the pancreas to secrete insulin. Insulin promotes the uptake of glucose primarily into muscle as well as fat, while inhibiting glucose production by the liver.

1.2.2 Progression from insulin resistance to Type 2 Diabetes

The diminished ability of the insulin-target tissues of the body to respond to insulin, resulting in a reduced capacity of the body to regulate fuel utilisation, primarily involving impaired glucose disposal, can be defined as IR. Initially, IR is counteracted as the pancreas compensates through hyper-secretion of insulin in order to maintain euglycaemia (DeFronzo 2009). Over time, however, this increased demand placed upon the β-cells can lead to cellular exhaustion, resulting in a defective response to increased circulating glucose levels, a loss of β-cell mass, and the onset of overt T2D. A distinguishing feature between T2D and Type-1 diabetes is observed in the early stages of disease progression: it is aberrant insulin action rather than the complete inability to produce insulin that defines T2D, whereas type-1 diabetes ensues upon auto-immune attack on the β-cells.

1.2.3 The regulation of glucose metabolism by insulin and defects occurring upon insulin resistance

The development of IR and T2DM arises from a milieu of factors and progression of the disease may involve a synergy of the insulin target tissues (liver, adipose tissue, skeletal muscle, pancreas and brain), which become less sensitive to insulin, most often associated with altered lipid metabolism and increased delivery and therefore abnormal deposition of lipid to insulin sensitive tissues (McGarry 2002). The manifestation of IR can lead to a constellation of metabolically detrimental characteristics referred to as the metabolic syndrome, including weight gain, diabetes, heart disease, non-alcoholic fatty liver disease and hypertension. The wide ranging effects of these diseases arising from aberrant fuel utilisation drives the research to define a potential unifying cause of the disease.

Insulin acts on the different tissues of the body to elicit tissue-specific actions. It has previously been shown using dietary models in rodents that peripheral tissues develop IR over different time courses, with the liver becoming insulin resistant before muscle and adipose tissue (Kraegen EW, Clark PW et al. 1991). In order to better understand the specific actions of insulin in each tissue, global as well as tissue specific insulin receptor (InsR) knockout (KO) mice have been engineered to abolish insulin signalling and downstream action. Muscle is responsible for the greatest utilisation of glucose (Abdul-Ghani and DeFronzo 2010) and as such muscle IR is defined as the inability for insulin to stimulate glucose uptake and storage into glycogen. In adipose tissue, insulin is responsible for promoting glucose clearance, although its contribution to whole body glucose clearance is minimal compared to muscle, whereas stimulating lipogenesis and inhibiting lipolysis are more important. The liver is the primary site for clearing insulin from the circulation as well as functioning as a central regulator of glucose metabolism, through HGP and to a minor degree glucose uptake. Further to this, the liver is responsible for storing glycogen, and plays a key role in FA and amino acid metabolism.

Initially the β-cells compensate for the reduced actions of insulin through increasing insulin secretion (Kubota, Tobe et al. 2000). While this is able to compensate in the short-term, with time this progresses to hyperinsulinemia and the onset of T2D, as the target tissues become less sensitive to insulin through and the β-cells are no longer able to cope with the increased demand (DeFronzo 2009). The insulin resistant liver fails to efficiently clear insulin secreted

from the pancreas at first-pass (Duckworth, Bennett et al. 1998), while glycogen, gluconeogenesis and glycogenolysis are insufficiently regulated, yet lipogenesis is maintained (Consoli and Nurjhan 1990; Brown and Goldstein 2008). Insulin stimulates the translocation of the glucose transporter, GLUT4, to the plasma membrane (PM) to facilitate the process of glucose uptake, and promote the storage of glucose as glycogen (Shulman, Rothman et al. 1990). The defect in glucose transport is likely to result from a defect in insulin signal transduction as insulin independent glucose transport, such as during exercise, is not impaired in insulin resistant and diabetic patients (Kahn 1996; Hawley 2004). In adipose tissue, insulin acts to promote the uptake of glucose, storage of lipid and to block lipolysis, thereby reducing the delivery of lipids to peripheral tissues. In the insulin resistant state, insulin-stimulated lipogenesis is maintained, while lipolysis is poorly regulated, resulting in expanded adipose tissue mass and increased FA release into the circulation which can act upon other tissues such as muscle and liver to further exacerbate the insulin resistant state (Groop, Saloranta et al. 1991; Olefsky and Glass 2010). The brain relies upon glucose production from the liver and has more recently been identified as a key regulator of insulin action within adipose tissue and liver as well as its control over β-cell function (Obici, Zhang et al. 2002; Pocai, Lam et al. 2005; Lin, Plum et al. 2010; Stanley, Pinto et al. 2010; Calegari, Torsoni et al. 2011). It has been demonstrated by lipid infusions and fat-feeding that IR can develop in the brain, leading to an impaired response to insulin and adipokines such as leptin which can cause aberrant neural firing and dysfunctional control over functions such as pancreatic secretion, HGP, adipose tissue lipogenesis and skeletal muscle glucose transport (Bamshad, Aoki et al. 1998; Könner, Janoschek et al. 2007; Nogueiras, Wiedmer et al. 2007; Stanley, Pinto et al. 2010; Calegari, Torsoni et al. 2011; Coomans, Biermasz et al. 2011; Marino, Xu et al. 2011).

Different tissue specific InsR KO mice displayed distinct phenotypes, with liver specific KO developing glucose intolerance due to an inability to suppress HGP as well as exhibiting reduced insulin clearance and β-cell secretion, whereas the muscle and fat specific InsR KO mice displayed normal glucose tolerance (Brüning, Michael et al. 1998; Michael, Kulkarni et al. 2000; Blüher, Michael et al. 2002). More recently, work from the Accili lab has demonstrated that reconstitution of InsR into brain, liver and β-cells of global InsR KO mice is sufficient to rescue diabetes (Okamoto, Nakae et al. 2004). However, as Glut4 is required for insulin-stimulated glucose uptake, ablating the InsR and therefore insulin signalling only

in Glut4-expressing cells of the brain, muscle and adipose tissue in mice induced diabetes, whilst reconstitution of InsR specifically in these tissues of global InsR KO mice failed to rescue the onset of diabetes (Lin and Accili 2011; Lin, Ren et al. 2011). Furthermore, reconstituting the InsR into the liver of global InsR KO mice failed to rescue the ability of insulin to inhibit HGP (Okamoto, Obici et al. 2005). Taken together, these findings indicate that the pathogenesis from IR to T2D is complex and most likely the result of cross talk between insulin resistant tissues rather than the result of a single defect in a single tissue.

1.3 Components of The Insulin Signalling Pathway

1.3.1 Initiation of insulin signalling

Insulin elicits its actions upon its target tissues through binding to its cell surface receptor (Freychet, Roth et al. 1971). Binding of insulin to the extracellular region of the InsR induces autophosphorylation and activation of the receptor, which promotes redistribution of the InsR towards clathrin-coated pits of the PM for cellular internalisation (Fehlmann, Carpentier et al. 1982). Upon reaching the acidic endosomal compartment, insulin dissociates from the receptor and is targeted for degradation, whilst the free receptor recycles back to the PM to continue the process (Gorden, Carpentier et al. 1978; Carpentier 1994). With increased circulating insulin levels, the InsR can be targeted for degradation as well in order to modulate the signal (Okabayashi, Maddux et al. 1989). In some instances, however, insulin may be released back into the circulation by receptors prior to and after internalisation (Duckworth, Bennett et al. 1998). The rate of insulin internalisation is rapid, with a half maximal rate of internalisation of 2-3 min, reaching steady state with recycling rate of receptors after 6-8 min (Wang, Sonne et al. 1983; Marshall 1985; Kublaoui, Lee et al. 1995). The activated receptor is able to recruit various substrates and docking proteins in order to promote specific insulin-induced effects as described below.

1.3.1.1 Metabolic and mitogenic actions of insulin

Insulin exerts its anabolic effects on a wide variety of cellular processes and as such promotes the storage of fuels. Although central to maintaining glucose homeostasis by promoting glucose uptake and intracellular storage as glycogen, insulin also participates in many other metabolic processes. These include promoting the synthesis and storage of lipid (lipogenesis and FA esterification), while inhibiting the processes of lipolysis and proteolysis. Insulin also acts to prevent glycolysis and gluconeogenesis in the liver, which are induced in times of fasting. In addition to these metabolic activities, insulin is a key regulator of lifespan (Narasimhan, Yen et al. 2009) and supporting mitogenic pathways. The hormone induces cell growth and differentiation by promoting gene expression and protein transcription and translation. These processes are regulated by a complex network of signalling interactions, modulated through spatial organisation, phosphorylation and other post-transcriptional modifications in the various target tissues of the body.

1.3.2 The insulin signalling cascade

1.3.2.1 The insulin receptor

The InsR belongs to a family of receptors including insulin-like growth factor receptor 1 (IGF-1R) and the insulin receptor-related receptor. It is a tyrosine kinase transmembrane receptor, responsible for eliciting the actions of the hormone insulin. The InsR is a hetero tetrameric protein, consisting of 2 extracellular α-helix chains, to which insulin binds, and 2 β-subunit chains, which traverse the membrane and on the intracellular portion contains a kinase domain and multiple sites for docking of substrates. The α and β glycoproteins are linked together by disulphide bonds (Massague, Pilch et al. 1981) and noncovalent interactions (Figure 1.2). Binding of insulin to the InsR causes a conformational change in the receptor, which increases the kinase activity of the β-subunit. The change in conformation results in bringing the 2 β-subunit chains into close proximity and exposes the catalytic ATP-binding pocket, enabling the InsR to bind ATP (Kasuga, Karlsson et al. 1982). This in turn results in autophosphorylation within the kinase domain (Kasuga, Karlsson et al. 1982; Van Obberghen, Rossi et al. 1983). The tyrosine (Tyr) sites within the kinase domain

(Tyr 1158/1162/1163) are *trans*-phosphorylated by the opposing β-subunit and this is followed by *cis*-phosphorylation at Tyr 965 and 972 within the juxtamembrane domain (Cann and Kohanski 1997). This autophosphorylation stabilises the activated kinase and appears essential for receptor internalisation and signal transduction. While some studies have shown internalisation to be independent of autophosphorylation (Carpentier, Van Obberghen et al. 1981; Trischitta, Wong et al. 1989; Backer, Kahn et al. 1989a), most studies involving mutations of the Tyr kinase domain of the InsR, have shown a much slower rate of internalisation and diminished metabolic signalling when InsR autophosphorylation is inhibited (McClain, Maegawa et al. 1987; Smith, Seely et al. 1991; Burgess, Wada et al. 1992; Carpentier, Paccaud et al. 1992). Phosphorylation of the Tyr 972 site has been shown to be essential in transmitting the metabolic arm of insulin action, as it primarily serves as a docking site for InsR substrates, but also in mediating the mitogenic actions, through Shc binding (Paz, Boura-Halfon et al. 2000). The cytoplasmic C-terminus tail of the InsR β-subunit contains further Tyr autophosphorylation sites, 1328/1334, which have been shown to be redundant for internalisation and kinase activation of the InsR; however these sites are thought to play an important role in propagating the mitogenic actions of insulin (Paz, Voliovitch et al. 1996).

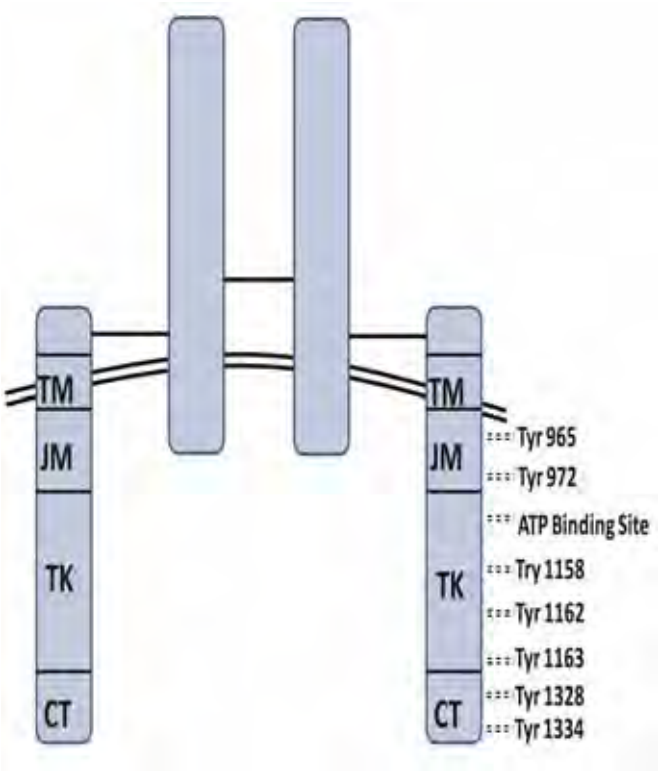

FIGURE 1.2 – Structure of the insulin receptor.
The InsR β-subunit contains two Tyr phosphorylation sites in the juxtamembrane domain, and the Tyr 972 site serves as a docking site for IRS-1 and Shc. The Tyr kinase domain contains an ATP binding site and three autophosphorylation sites that are thought to be important in promoting full kinase activation of the InsR. More distal in the C-terminal domain, two further phosphorylation sites exist which are not involved in InsR activation.

While insulin remains bound to the InsR, the receptor remains active for signalling. This enables the receptor to propagate the cellular effects of insulin from both the PM and also intracellularly from the endosomes, where the InsR maintains a high level of activation until insulin dissociates (Backer, Kahn et al. 1989b; Burgess, Wada et al. 1992; Bevan, Burgess et al. 1995; Kublaoui, Lee et al. 1995). It has been demonstrated that InsR dephosphorylation is required before it can be recycled back to the plasma membrane and that this requires dissociation of insulin from the InsR (Carpentier, Hamer et al. 1996). In studies covalently linking insulin to the receptor, however, the InsR along with the bound insulin molecule is mainly targeted for degradation, with the recycled complex returning to the PM at a significantly slower rate (Carpentier, Gazzano et al. 1986).

Due to differential splicing of the receptor encoded by exon 11, there are two isoforms of the InsR, with isoform A lacking 12 amino acids proximal to the C-terminus of the α-chain (Seino and Bell 1989; De Meyts and Whittaker 2002) and being more predominant during foetal development. The InsR-A isoform has a greater binding affinity for insulin but also IGF-II and therefore plays a greater role in mitogenic growth than InsR-B which is more abundant in adult organisms and more important for transmitting the metabolic actions of insulin. Whether these two isoforms exhibit differential rates of internalisation or biological activity has been debated, (Yamaguchi, Flier et al. 1991; Calzi, Choice et al. 1997; Uhles, Moede et al. 2003; Giudice, Leskow et al. 2011), with it now becoming more accepted that despite no difference in β-subunit structure the A isoform is endocytosed at a slower rate (Giudice, Leskow et al. 2011).

1.3.2.2 Insulin receptor adaptors and substrates

Autophosphorylation on Tyr residues of the InsR within the activation loop, and subsequent kinase stabilisation, results in further phosphorylation of Tyr residues which recruit substrates to activate several signalling pathways. The diverse biological actions that insulin can induce result from the recruitment of several substrates to the activated InsR, including the insulin receptor substrate (IRS) family, p66src homology 2 domain containing protein (Shc), adaptor protein containing pleckstrin homology (PH) and Src homology-2 (SH2) domains (APS), Carcinoembryonic antigen-related cell adhesion molecule 1 (Ceacam1), Grb2 associated binder-1 (Gab-1), Casitas B-lineage Lymphoma proto-oncogene (c-Cbl), signal transducer and activator of transcription (STAT) 5B and growth factor receptor bound-7 (Grb7) family members Grb14 and Grb10 (Kahn and White 1988; White 1998; Pessin and Saltiel 2000; Nishida and Hirano 2003). There are in fact more than 100 molecules either directly or indirectly involved in insulin signal transduction (Taniguchi, Emanuelli et al. 2006). Much research has focussed on the role of the IRS family, due to their high specificity for the InsR and the predominant role they play in the tissue response to insulin. Each substrate can act as an adaptor however, and is able to activate distinct pathways to produce specific effects, including alterations in glucose and lipid metabolism, protein synthesis, mitogenesis and gene expression (Rosen 1987). The different pathways initiated by the various substrates are summarised below (Figure 1.3).

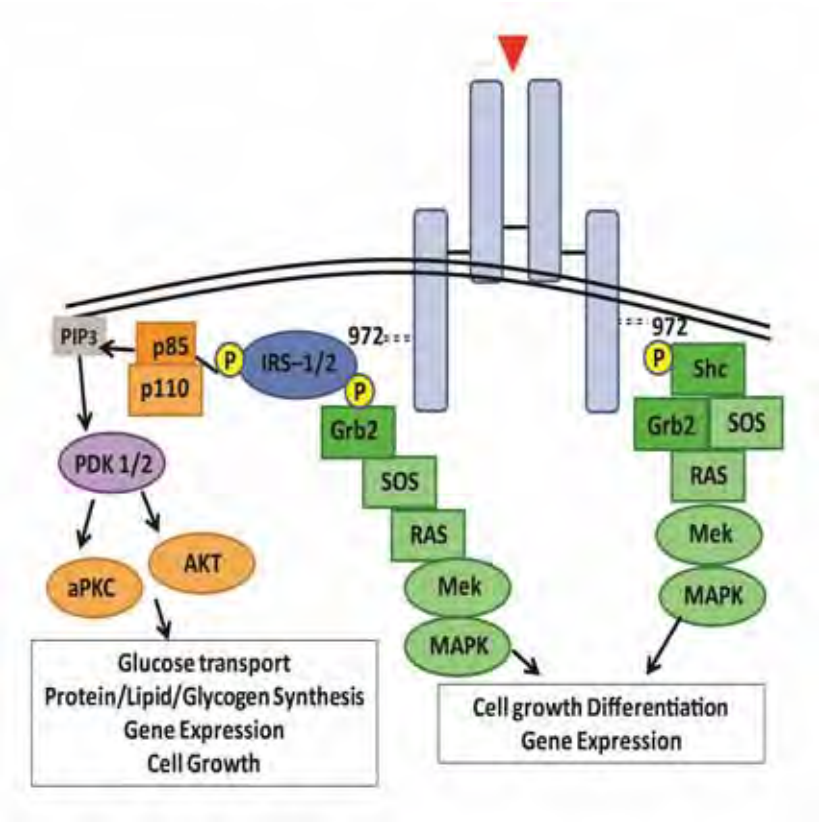

Figure 1.3 – Schematic of the insulin signalling cascade
Activation of the InsR results in signalling through multiple pathways. This occurs through Tyr phosphorylation of IRS and other Shc. PI3K docks with Tyr phosphorylated IRS residues, leading to PIP_3 generation, PDK-1 activation and subsequent Akt activation. This pathway is involved in metabolic actions such as glucose transport, glycogen synthesis and lipid storage. The Ras/MAPK pathway can be activated via SOS and GRB2, to stimulate the actions of insulin on growth and proliferation. InsR, insulin receptor; IRS, insulin receptor substrate; PI3K, phosphoinositol-3-kinase; PDK-1, phosphoinositide-dependent kinase-1; SOS, son-of-sevenless; Grb2, Growth factor receptor-bound protein 2; MAPK, mitogen-activated protein kinase.

The ability of the activated InsR to recruit a variety of substrates and adaptors is important to amplify the insulin signal, as a means by which to overcome receptor stoichiometric constraint. In this way, multiple substrates can be engaged and activated by a single receptor to transmit the cellular signal, and the effectiveness of this can be influenced by the spatial localisation of the InsR, which can determine the substrates it can attract and interact with (Di Guglielmo, Baass et al. 1994; Heller-Harrison, Morin et al. 1995; Kublaoui, Lee et al. 1995; Ceresa, Kao et al. 1998; Sorkin, McClure et al. 2000).

The IRS family is comprised of six members, of which two (IRS-1 and IRS-2) have been shown to be involved in the regulation of insulin action, whilst the other isoforms have roles in different signalling pathways (Brummer, Schmitz-Peiffer et al. 2010). As such, the

remainder of this review will mainly focus on IRS-1 and to a lesser extent IRS-2, given their requirement in eliciting almost all of insulin's downstream, actions. This was highlighted by the double KO of IRS-1 and 2 in liver of mice, which abolished insulin signalling in the face of normal InsR activation, leading to uncontrolled hyperglycaemia and hyperinsulinemia (Dong, Park et al. 2006). Upon activation of the InsR, IRS-1 is recruited to bind to the NPxY sequence at Tyr 972 (where x is any amino acid) within the juxtamembrane region of the InsR containing the phosphotyrosine binding (PTB) domain (O'Neill, Craparo et al. 1994; Wolf, Trüb et al. 1995). This results in phosphorylation of IRS-1, at Tyr residues which serve as docking sites for adaptor proteins containing Src-homology 2 (SH2) domains which further transmit the insulin signal. IRS-1 was first identified by White and Colleagues in insulin-stimulated FAO cells (White, Maron et al. 1985) and was later found to be Tyr phosphorylated by the InsR in vitro (Sun, Rothenberg et al. 1991; Sun, Miralpeix et al. 1992). IRS-2 was later discovered after immunoprecipitating IRS-1 from insulin stimulated FAO cells (Miralpeix, Sun et al. 1992). Although IRS-1/2 share only ~43% amino acid homology overall, their phosphotyrosine-binding (PTB) and pleckstrin homology (PH) domains have much higher homology (Sun, Wang et al. 1995). It is these domains that are crucial for the recruitment of IRS proteins to the activated InsR and their subsequent Tyr phosphorylation and activation, as IRS-1 lacking the PTB domain is unable to be Tyr-phosphorylated by the InsR (O'Neill, Craparo et al. 1994). Furthermore, the PH domains can promote protein-protein interactions and are also crucial in localising IRS-1 to the InsR, as they direct proteins to bind phosphoinositides in biological membranes, where in this case the InsR is activated at the PM. Phosphorylation of Tyr residues on IRS-1 serves in turn to generate docking sites for SH2 domain containing proteins, to further propagate the signal (Pawson and Gish 1992; Zhou, Shoelson et al. 1993). IRS-1 also contains numerous Tyr and serine (Ser) residues, which can be phosphorylated to modulate the insulin signal (White 1998) and some of these will be discussed later in the chapter.

Although IRS-1/2 are similar in their activation and recruitment to the InsR, IRS-2 has been found to interact with the InsR at Tyr residues within the InsR kinase domain (Wu, Tseng et al. 2008). Furthermore, the adapters recruited by each isoform are similar with few being isoforms-specific (Hanke and Mann 2009). It has, however, been hypothesised that the two isoforms may regulate metabolism differently in liver and skeletal muscle, with IRS-1 proposed to have a greater influence over glucose metabolism and IRS-2 more so lipid

metabolism (Taniguchi, Ueki et al. 2005; Bouzakri, Zachrisson et al. 2006). Other members of the IRS family appear to exert little control over metabolism and growth (Brummer, Schmitz-Peiffer et al. 2010).

Several other substrates of the InsR have been identified, the actions of which are not as specific to the InsR and IGF-1R as those of the IRS family of proteins;

Ceacam1 is a transmembrane glycoprotein which acts as a specific InsR but not IGF-1R substrate (Najjar, Blakesley et al. 1997). Ceacam1 upregulates insulin induced internalisation of the InsR through its role as an InsR substrate in liver, but not muscle or fat (Accili, Perrotti et al. 1986). Ceacam1 is constitutively phosphorylated at Ser 503 and this phosphorylation is required for interaction of Ceacam1 and the InsR (Najjar, Phillippe et al. 1995), and in turn leads to Ceacam1 phosphorylation on Tyr 488. This interaction with the InsR induces receptor endocytosis and therefore Ceacam1 can act to modulate the insulin signal. Through its actions upon hepatic insulin clearance, Ceacam1 has been associated with not only glucose homeostasis but also modulation of lipid metabolism, particularly in the liver but also in adipose tissue (Dai, Abou-Rjaily et al. 2004; DeAngelis, Heinrich et al. 2008).

Gab1 shares a similar amino acid homology and structural features with IRS-1 but acts as a substrate for both InsR and epidermal growth factor receptor (EGFR) (Holgado-Madruga, Emlet et al. 1996). It lacks a PTB domain which may reduce its affinity for the InsR specifically; however it does possess a PH domain and sites for SH2 and SH3 domain containing proteins to bind (Nishida and Hirano 2003).

Shc contains an N-terminal PTB domain and a C-terminal SH2 domain, allowing Shc to be recruited to the activated InsR at Tyr 972 but also the EGFR (Luzi, Confalonieri et al. 2000). Binding results in Tyr phosphorylation, enabling Shc to interact with the adaptor protein Grb2 and Son-of-Sevenless (SOS) resulting in downstream activation of the Ras-Raf-MEK-Erk1/2 pathway (Saltiel and Kahn 2001).

APS binds the InsR at phosphotyrosine sites within the activation loop, where it in turn is Tyr phosphorylated and has been proposed to link the InsR and c-Cbl (Moodie, Alleman-Sposeto et al. 1999; Ahmed, Smith et al. 2000).

Grb14 binds to the InsR to protect is kinase domain from Tyr dephosphorylation as it competes with the Tyr phosphatase PTP1B (Hemming, Agatep et al. 2001; Holt and Siddle 2005). Binding of Grb14 to the InsR, although enhancing InsR Tyr phosphorylation, competes with IRS-1 and reduces IRS-1 binding and activation (Kasus-Jacobi, Perdereau et al. 1998; Cooney, Lyons et al. 2004; Nouaille, Blanquart et al. 2006). Expression of Grb14 has been found to be increased in peri-epididymal adipose tissue of Gotokakizaki rats and ob/ob mice as well as subcutaneous adipose tissue of human diabetic subjects (Cariou, Capitaine et al. 2004). The major effect of Grb14 on insulin action has been reported to occur in liver and muscle of rodents, both *in vivo* and *ex vivo* (Cooney, Lyons et al. 2004).

The CAP/Cbl pathway involves Tyr phosphorylation of APS and c-Cbl, which forms a complex with CAP and upon phosphorylation moves to lipid rafts of the PM, where it binds flotillin (Hu and Hubbard 2005). This pathway has been implicated in promoting glucose uptake independent of phosphatidylinositol 3-kinase (PI3K)/Akt (Chiang, Baumann et al. 2001). However, its importance in glucose transport has been brought into question as this pathway appears to be lacking in skeletal muscle (JeBailey, Rudich et al. 2004) and inhibition of the pathway in culture and *in vivo* has no effect upon glucose transport (Mitra, Zheng et al. 2004; Molero, Jensen et al. 2004).

1.3.2.3 Canonical insulin receptor signalling

The major pathways initiated from activated IRS are PI3K and mitogen-activated (MAP). The PI3K pathway is responsible for insulin's metabolic actions, whereas mitogenic actions can also be stimulated through Shc activation of the MAP kinase pathway. Activation of IRS proteins and therefore the metabolic actions of insulin occur rapidly following InsR activation at the PM, prior to internalisation of the InsR (Heller-Harrison, Morin et al. 1995; Kublaoui, Lee et al. 1995). The spatial localisation of signalling molecules can be crucial in how well the cellular signal is distributed. In the case of IRS-1, in the basal state it is mostly found at intracellular membranes with a smaller portion in the cytosol (Heller-Harrison, Morin et al. 1995; Inoue, Cheatham et al. 1998; Clark, Molero et al. 2000). Upon insulin stimulation, the greater proportion of Tyr phosphorylated IRS-1 is found located at intracellular membranes and this is independent of InsR endocytosis (Heller-Harrison, Morin et al. 1995). However

with time, IRS-1 redistributes to the cytosol of the cell where it is less phosphorylated, thereby reducing the signal (Heller-Harrison, Morin et al. 1995; Clark, Molero et al. 2000). These results have been confounded by some fluorescence microscopy studies, however, where it has been reported that upon insulin stimulation, IRS-1 translocated from the cytosol to membrane ruffles (Jacobs, LeRoith et al. 2001). What is evident, however following InsR activation is that IRS-1 is required in proximity to the PM for activation, and with time IRS-1 moves toward the cytosol. Interestingly either InsR or IGF-1R endocytosis is required for activation of Shc and its dependent pathways, but not for IRS-1 phosphorylation (Ceresa, Kao et al. 1998; Chow, Condorelli et al. 1998). In fact blocking InsR endocytosis reduced activation of the mitogenic pathway without altering the metabolic actions (Ceresa, Kao et al. 1998). This is due to Shc, Grb2 and Erk/MAPK, key components of the mitogenic pathway, being localised to endosomal membranes, where internalised receptors remain active until dissociated from their ligand (Di Guglielmo, Baass et al. 1994; Pol, Calvo et al. 1998; Rizzo, Shome et al. 2000). Internalisation of the InsR, other than dampening the signal by reducing the PM-bound InsR number, can also act to promote the activation of target proteins not located at the PM or internal membrane region of the cell. Therefore membrane activation of the InsR is responsible for eliciting the metabolic actions of insulin, but internalisation of the activated InsR is required for the mitogenic actions to be fully activated.

Upon Tyr phosphorylation of IRS-1, class Ia PI3K association is induced, as the regulatory p85α translocates from the cytosol towards the PM where IRS-1 is phosphorylated (Inoue, Cheatham et al. 1998). This association is due to the SH2 domain of PI3K interacting with the phosphorylated YxxM motifs on IRS (Myers, Backer et al. 1992) and leads to the metabolic actions of insulin including cellular glucose uptake, glycogen and lipid synthesis. There are multiple isoforms of both the regulatory (p85α, p55α, p50α, p85β, p55γ) and catalytic (p110α, p110β, p110δ) subunits of PI3K, due to alternative splicing (Shepherd, Withers et al. 1998). The p85- and p110-kDa proteins are cross-linked and therefore form a complex (Carpenter, Duckworth et al. 1990). The regulatory subunits compete to bind active IRS proteins (Taniguchi, Emanuelli et al. 2006); however it is thought that the regulatory subunit p85α is the predominant regulatory isoform involved. PI3K is able to exert both lipid and Ser kinase activity but its dominant role, is the generation of phosphatidylinositol-3,4,5-triphosphate (PI-3,4,5-P_3) to stimulate GLUT4 translocation to the PM (Fruman, Meyers et al. 1998). The generation of 3'-phosphoinositides recruits PH domain-containing Ser kinases

to the PM, such as 3'- phosphoinositide-dependent protein kinase 1 (PDK1), Akt and atypical Protein kinase C (aPKC) isoforms ζ and λ (Alessi, Deak et al. 1997; Mora, Komander et al. 2004). Activated PDK1 phosphorylates Akt within its kinase regulatory loop at threonine (Thr) 308, although full activation of Akt requires Ser 473 phosphorylation (Hill, Feng et al. 2002; Pearce, Komander et al. 2010) by PDK2 activity. Moreover PDK1 is also able to phosphorylate aPKC within its activation loop (Pearce, Komander et al. 2010) giving rise to full activation independent of PDK2.

There are 3 mammalian isoforms of Akt, sharing high amino acid identity. The Akt1 isoform is ubiquitously expressed and thought to be involved mainly in growth effects; Akt2 is highly expressed in the insulin-sensitive tissues liver, muscle and fat and thought to be predominantly involved in the metabolic actions induced by Akt activation and Akt3 displays limited expression except in brain and testes (Cho, Mu et al. 2001a; Cho, Thorvaldsen et al. 2001b; Bouzakri, Zachrisson et al. 2006; Cleasby, Reinten et al. 2007; Schultze, Jensen et al. 2011). The major action of Akt in response to insulin is to promote GLUT4 translocation to the PM and increase glucose uptake in muscle and adipocytes, whereas in liver it regulates gluconeogenesis through its action on FOXO (Li, Monks et al. 2007). There are a number of cellular substrates Akt is able to phosphorylate, and therefore it mediates a variety of responses to insulin. Some of its targets include glycogen synthase kinase 3 (GSK3 – glycogen synthesis), Akt substrate of 160kDa (AS160/TBC1D4 – glucose transport), FOXO transcription factors (gene expression – gluconeogenic), TSC-1/2 (protein synthesis via mTOR) and Bcl-2-associated death promoter (BAD - apoptosis) (Manning and Cantley 2007; Vasudevan and Garraway 2010). Although Akt activation in muscle and adipose tissue mediates GLUT4 translocation, other mechanisms are also likely to be required to facilitate the process such as actin remodelling, vesicle release and membrane fusion (Lopez, Burchfield et al. 2009; Rowland, Fazakerley et al. 2011).

The mitogenic actions of insulin are regulated through the Ras/MAP kinase pathway (Avruch 2007), involving Shc or IRS interaction with Grb2 and the recruitment of son of sevenless (SOS) to the PM. The extent to which Shc and IRS are involved, or whether one dominates over the other is unclear (Myers, Wang et al. 1994), but localisation experiments indicate that full mitogenic activity is achieved with InsR internalisation which results in increased Shc activation (Pruett, Yuan et al. 1995; Ceresa, Kao et al. 1998). The conformational change of

SOS further activates Ras and the cascade of events activating Raf, MEK and ERK produce many of the growth and survival actions of insulin (Ramos 2008).

1.3.2.4 Regulation of insulin signalling

Cellular insulin signalling involves a cascade of events that are modulated by reversible phosphorylation through the actions of protein kinases and phosphatases. This dynamic control can alter protein conformation as well as localisation and therefore the ability of particular proteins to interact.

Protein tyrosine phosphatases (PTPs) are one family of phosphatases capable of regulating insulin signalling through their actions on Tyr-phosphorylated residues of target proteins. Key PTPs that regulate insulin signalling include: leukocyte antigen related (LAR) PTP, Src homology region 2-containing protein tyrosine phosphatase-2 (SHP-2) and PTP-1B.

LAR is a trans-membrane PTP targeting the InsR (Ahmad and Goldstein 1997) and overexpression inhibits insulin action (Zhang, Li et al. 1996). Its actual role is not clear however, since LAR KO mice display impaired insulin stimulated glucose uptake and hepatic glucose production regulation (Ren, Li et al. 1998), despite improved basal blood glucose levels.

SHP-2 was initially thought to improve insulin signalling, with deletion of SHP-2 in muscle inducing whole body insulin resistance, although its precise role remains unresolved. Conflicting results from studies suggest that SHP-2 interacts with the InsR, IRS-1 and IRS-2 (Rocchi, Tartare-Deckert et al. 1996; Hayashi, Shibata et al. 2004). It has been proposed that both SH2 domains of SHP-2 are required to interact with IRS-1 for full activation, however other studies have suggested it could in fact act as a negative regulator of insulin action and to date this has not been clarified (Myers, Mendez et al. 1998; Maegawa, Hasegawa et al. 1999).

PTP1B is a negative regulator of insulin signalling (Asante-Appiah and Kennedy 2003; Galic, Hauser et al. 2005) that targets Tyr residues of the InsR kinase domain and may also target IRS-1 following insulin stimulation (Seely, Staubs et al. 1996; Bandyopadhyay, Kusari

et al. 1997; Goldstein, Bittner-Kowalczyk et al. 2000). PTP1B KO mice remain insulin sensitive on a high-fat diet (HFD), with most of its actions associated with the liver and muscle (Elchebly, Payette et al. 1999; Klaman, Boss et al. 2000; Egawa, Maegawa et al. 2001). Due to resistance of the mice to HFD-induced obesity it has also been linked to effects in the brain (Saltiel and Kahn 2001). It has been identified as localising to the cytosolic face of the ER, however it is released into the cytosol following cleavage (Frangioni, Beahm et al. 1992; Dubé and Tremblay 2005). Despite this localisation, it remains unclear how and where it associates with the InsR.

PTPs can themselves be regulated by a number of factors which affect the assembly of signalling complexes (Neel, Gu et al. 2003). Such modifications include Tyr phosphorylation of the PTPs to generate docking sites for SH2 domain containing proteins, proteolytic cleavage to facilitate cellular translocation (Gil-Henn, Volohonsky et al. 2001), dimerisation (Wallace, Fladd et al. 1998) and oxidation (Xu, Rovira et al. 2002).

Other phosphatases that have been implicated in negatively regulating insulin action at more downstream sites include:

Phosphatase and tensin homologue (PTEN) contains a catalytic domain similar to that of PTPs, however it targets the lipid PI-3,4,5-P_3 through binding to the PM via its C2 domain and dephosphorylating PI-3,4,5-P_3 to PI-4,5-P_2, thus inhibiting the activation of Akt (Maehama and Dixon 1998).

Protein phosphatase 2 (PP2A) is a Ser/Thr phosphatase that reduces the activation of Akt by preferential dephosphorylation of the Thr 308 site but also can reduce Ser 473 phosphorylation (Pim, Massimi et al. 2005; Andrabi, Gjoerup et al. 2007; Padmanabhan, Mukhopadhyay et al. 2009). Ceramide accumulation has been documented to inhibit Akt activation through increased PP2A activity (Cazzolli, Carpenter et al. 2001; Ruvolo 2001; Stratford, Hoehn et al. 2004).

Pleckstrin homology domain leucine-rich repeat protein phosphatase (PHLPP) acts to specifically reduce Akt activity through dephosphorylating Akt at ser-473 (Gao, Furnari et al. 2005; Brognard, Sierecki et al. 2007).

T-cell protein tyrosine phosphatase (TCPTP) is involved in the attenuation of STAT3 activation and insulin signalling in the liver, to regulate gluconeogenesis (Fukushima, Loh et al. 2010).

Apart from the number of Tyr phosphorylation sites of the IRS proteins targeted by the InsR (Sun, Miralpeix et al. 1992), they also contain a number of Ser sites (Sun, Miralpeix et al. 1992). Insulin signalling can be modulated through Ser phosphorylation of effector molecules such as the InsR and IRS-1, to reduce their interaction with substrates and binding partners. In the basal state, IRS-1 is heavily phosphorylated on Ser residues, causing it to migrate higher than its predicted molecular weight. Phosphorylation of IRS-1 on ser residues can occur through a number of kinases including PKCs, MAP kinases and cyclic-AMP dependent protein kinase (White 1998; Zick 2005). It is established now that increased Ser phosphorylation of IRS proteins can inhibit its activation and downstream insulin action (White 2002; Gual, Le Marchand-Brustel et al. 2005). However due to the numerous sites as well as kinases that can perform this modification, the relative importance of the sites described are not fully clear.

The lack of clarity as to the initial negative regulation of insulin signalling, particularly emanating from negative regulation of IRS-1 is in part due to the fact that the same residue has been reported to be phosphorylated by different kinases and in some cases with opposing effects. A critical site of negative Ser phosphorylation on IRS-1 is the Ser 307 site in mice. This site is associated with reduced IRS-1 activation and IR (Aguirre, Werner et al. 2002). Phosphorylation of the mouse Ser 307 site of IRS-1 is increased by insults such as TNFα, chronic (high) insulin, increased FA and amino acids (Aguirre, Uchida et al. 2000; Rui, Aguirre et al. 2001; Carlson, White et al. 2004). These metabolic insults are thought to activate JNK, which targets this site to attenuate insulin signalling and action (Aguirre, Uchida et al. 2000; Hirosumi, Tuncman et al. 2002; Werner, Lee et al. 2004). However, more recently the White laboratory has proposed through mutation studies *in vivo* that this site correlates with enhanced insulin action as opposed to the negative regulation of signalling observed in cultured cells (Copps, Hancer et al. 2010). The rodent IRS-1 Ser 632/635 sites have also been associated with negative regulation of insulin action through reducing the interaction between IRS-1 and PI3K (Ozes, Akca et al. 2001; Bouzakri, Roques et al. 2003) by mTOR and MAP kinase pathways. More recently, activation of the Ser 632/635 site by Rho-kinase has been shown to enhance insulin signalling and action

(Furukawa, Ongusaha et al. 2005). Such opposing observations have also been shown to be the case at other Ser sites (Gual, Le Marchand-Brustel et al. 2005). These examples show the complexity involved in the regulation of signalling pathways and differences that may exist in cell culture models are not always reflective of the *in vivo* situation where there are many more factors involved in regulating the cellular response.

Many proteins involved in the negative regulation of insulin signalling and action are activated by a variety of stimuli associated with IR and diabetes, including lipid intermediates, cytokines, reactive oxygen species (ROS) and excess nutrient supply. These include activation of inflammatory pathway components such as inhibitor of κB kinase (Iκκ) (NFκB) and members of the MAP kinase family; p38 MAPK, JNK 1/2 (Shoelson, Lee et al. 2003; Ozcan, Cao et al. 2004; Gual, Le Marchand-Brustel et al. 2005; Vallerie and Hotamisligil 2010); PKCs (Schmitz-Peiffer 2000; Kim, Fillmore et al. 2004; Samuel, Petersen et al. 2010) and SOCS proteins (Yasukawa, Sasaki et al. 2000; Ueki, Kondo et al. 2004) to name a few. Once activated these proteins target proximal insulin signalling components such as the InsR and IRS-1 to downregulate their activation through Ser phosphorylation or in the case of the SOCS proteins, sterically block the interaction with substrates and adaptor proteins. Adding to this complicated nature of signal modulation is the number of other mechanisms postulated, such as O-linked glycosylation of proteins (D'Alessandris, Andreozzi et al. 2004; Sparrow, Gorman et al. 2007); adipose secreted cytokines (Greenberg and McDaniel 2002); hexosamine pathway activation (Davidson, Hunt et al. 1994); excess nutrient supply (Hoy, Bruce et al. 2007; Hoy, Brandon et al. 2009); and oxidative stress (Hoehn, Salmon et al. 2009; Chang and Chuang 2010) although some study results have confounded this view (Loh, Deng et al. 2009). Furthermore these mechanisms are thought to interact with each other, such as DAG inhibiting insulin signalling through PKC activation which has also been shown to activate JNK itself (Comalada, Xaus et al. 2003).

The fine balancing act required to maintain appropriate cellular responses is further highlighted with many of the proposed insults in fact being generated in the activation of insulin signalling to further promote the actions of insulin, however, when produced in excess can have detrimental effects leading to IR and the pathogenesis of diabetes. This has been shown with regard to the production of ROS and oxidative stress. Upon InsR activation ROS are produced and these can act to inhibit phosphatases such as PTP1B and PTEN (Goldstein,

Mahadev et al. 2005; Vardatsikos, Sahu et al. 2009) and enhance insulin sensitivity *in vivo* (Loh, Deng et al. 2009). In excess, however, ROS can impair signalling through mislocalisation of proteins (Rudich, Tirosh et al. 1998; Tirosh, Potashnik et al. 1999; Ogihara, Asano et al. 2004) whereby although total cellular activation of IRS-1 and PI3K was not impaired, its activation at intracellular membranes where it exerts its effects on substrates was found to be reduced. The link between excessive oxidative stress and reduced insulin sensitivity is now gaining more interest (Loh, Deng et al. 2009; Giacco and Brownlee 2010). It has also been shown that the localised production of ROS can have differing effects on insulin sensitivity (Veal, Day et al. 2007; Hoehn, Salmon et al. 2009; Shibata, Hakuno et al. 2010). This applies to many other stimuli including ER stress and excess availability of nutrients such as glucose and amino acids.

Overall, modulation of the insulin signalling pathway is regulated by many factors at different levels of the signalling cascade. Studies have implicated reduced IRS-1 Tyr phosphorylation in the pathogenesis of IR and diabetes, however it is now becoming more evident that it is not this simple and that regulation can occur further downstream for example at Akt. A conclusive mechanism has not yet been defined, due to the interplay of spatial localisation of signalling molecules and the role that storage of lipid metabolites and excess nutrient toxicity can influence these pathways, resulting in metabolic derangements.

1.4 Lipids and Insulin Resistance

1.4.1 Generation of lipids

The alarming rise in T2D in recent decades has also coincided with increased lipid consumption in our diets (Harris, Pomeranz et al. 2009). Although there is clearly a genetic component that can predispose people to the development of the disease (O'Rahilly 2009), the dramatic global rise in the prevalence of the disease indicates there is an interaction between genes and environment. Increased fat supply may result in an overwhelming of the ability of cells to suitably deal with the lipid through β-oxidation and triacylglycerol (TG) storage, so that detrimental lipid metabolites build-up intracellularly.

Within the body, delivery of non-esterified FA to the peripheral tissues results predominantly from hydrolysis of TG in adipose tissue (Gordon and Cherkes 1956). Following a meal, lipoprotein lipase hydrolyses dietary TG for uptake by adipose tissue (Karpe, Dickmann et al. 2011). However, in excess not all FAs are able to be absorbed, resulting in increased FAs in the circulation which may then accumulate in other tissues. Various methods of cellular uptake of FA have been proposed, including simple diffusion (Hamilton and Kamp 1999) and, what is now more accepted, facilitated diffusion (Berk and Stump 1999). The discovery of FA transporters such as fatty acid binding proteins and CD36, and the effects of mutations to disrupt FA cellular transport, have given the facilitated transport theory strong support (Kiens 2006; Binas and Erol 2007; Holloway, Jain et al. 2009).

Tissues can utilise FA in different ways, involving 3 major pathways; 1) Storage as TG through the glycerolipid synthesis pathway, 2) conversion into sphingolipids or 3) oxidation for energy.

1.4.1.1 Cellular fate of free fatty acids

The enzymes involved in the storage and metabolism of lipids have been identified and modulation of these has helped to clarify roles that particular lipid species play in the development of metabolic disease.

The Kennedy pathway is the major route of TG synthesis, which predominantly occurs in the endoplasmic reticulum (ER). This pathway involves esterification of acyl-CoAs with a glycerol backbone, starting with glycerol-3-phosphate (Prentki and Madiraju 2008). Sequential enzyme-catalysed reactions produce lysophosphatidic acid, phosphatidic acid, DAG and TG. Phosphatidic acid and DAG themselves can be used to generate membrane phospholipids or be further modified towards TG synthesis. The TG is stored primarily within lipid droplets in adipose tissue, however in the disease state excess availability can lead to ectopic deposition within other peripheral tissues such as liver, muscle and heart (Goodpaster, He et al. 2001; Nelson, Prasad et al. 2007; Yamaguchi, Yang et al. 2007). The liver also plays an important role in lipid metabolism to modulate the profile in the circulation, as it is able to utilise synthesised TG to form lipoproteins such as very low density lipoproteins (VLDL) for secretion through incorporation with apolipoprotein B.

Furthermore, palmitoyl-CoA is able to enter the sphingolipid pathway which ultimately leads to the synthesis of the bioactive signalling metabolite, ceramide from its inactive precursor dihydroceramide following a series of reactions primarily taking place within the ER (Bartke and Hannun 2009; Hanada, Kumagai et al. 2009). A small proportion of the pathway may also take place in the mitochondria.

The third pathway that lipids entering the cell can take involves oxidation upon transport into the mitochondria by carnitine palmitoyltransferase 1 (CPT-1). The action of CPT-1 can be inhibited by build-up of malonyl-CoA which is generated by acetyl-CoA carboxylase (ACC) (Choi, Savage et al. 2007; Hoehn, Turner et al. 2010). Fatty acyl-CoA species transported into the mitochondria undergo β-oxidation, with the acetyl-CoA generated entering the tricarboxylic acid (TCA) cycle, where it is oxidised for energy production. The build-up of acetyl-CoA favours its conversion to malonyl-CoA by ACC, which inhibits the actions of CPT-1 and therefore reduces FA oxidation.

1.4.2 Tissue-specific regulation of lipid metabolism

With increased FA in the circulation, lipid uptake and deposition increases in insulin-sensitive tissues and in time this ultimately reduces the response of the tissues to insulin. This development of lipid-induced IR arises from exceeding the capability of the cells to oxidise or appropriately store the lipid. This is in part overcome through limiting cellular lipid transport, as has been demonstrated in mice lacking lipoprotein lipase (LPL) or CD36 (Hajri, Han et al. 2002; Goudriaan, Dahlmans et al. 2003). These proteins are crucial for tissue lipid uptake as lipoprotein lipase hydrolyses TG in lipoproteins to generate FA and monoacylgycerol, which can be further broken down to produce a FA and glycerol. The FA generated are transported into the cell by facilitated diffusion through receptors. Adipose tissue is a key site of lipid storage, with insulin stimulating glucose uptake, which is relatively minor compared to that of skeletal muscle; as well as lipogenesis and importantly, inhibition of lipolysis (Stumvoll and Jacob 1999). The importance of adipose tissue in modulating lipid availability to other peripheral tissues and the detrimental effects this can have in terms of IR have been demonstrated in extreme cases such as lipodystrophic conditions. In such cases, where adipose tissue is minimal, severe IR ensues due to

hypertriglyceridemia and increased ectopic lipid deposition in non-adipose tissue such as liver or muscle (Moitra, Mason et al. 1998; Garg 2004). Increased myocellular lipid storage has been associated with IR, especially defects in glucose transport (Goodpaster, He et al. 2001; Dube, Amati et al. 2008). Insulin action in the liver reduces HGP, while inhibiting the secretion of lipoproteins (Julius 2003; Browning and Horton 2004). Impaired insulin sensitivity of the liver can act to increase HGP while lipogenesis remains insulin sensitive, resulting in promotion of intrahepatic lipid storage and lipoprotein secretion. As insulin levels rise through β-cell compensation, hyperinsulinemia worsens the pathogenesis of IR to T2D (Wiegman, Bandsma et al. 2003).

1.4.3 Cellular lipid metabolism

1.4.3.1 The Randle cycle hypothesis

In 1963, Randle and colleagues showed that FFA compete with glucose for substrate oxidation in isolated rat heart (Randle, Garland et al. 1963). In this study, it was observed that intracellular glucose-6-phosphate (G6P) levels were increased following lipid exposure. This led to the hypothesis that increased fat oxidation augmented acetyl-CoA and citrate levels, resulting in inhibition of phosphofructokinase and a concomitant rise in G6P levels. These increased levels of G6P were proposed to inhibit hexokinase, and the resulting increased unphosphorylated glucose levels to reduce glucose transport.

This theory was later contradicted by other studies. In one study, infusions of lipid into healthy subjects found that plasma levels of 500 μM FA reduced intracellular G6P levels, probably through inhibition of muscle glucose transport after 4-6 h and led to a block in insulin-suppression of hepatic glucose production. At a higher level of FA infusion, glycogen synthesis was also inhibited (Boden, Chen et al. 1994). These findings were supported in later studies by Roden *et al*. involving lipid infusions during a hyperinsulinaemic-euglycaemic clamp for 6 h. It was found that G6P levels declined after 1.5 h of lipid infusion and after 3 h this was associated with reduced whole body glucose disposal and reduced glycogen synthesis (Roden, Price et al. 1996). The conclusion that increased circulating FA reduce glucose uptake was strengthened by the finding that the

defect in intracellular glucose content was associated with reduced IRS-1 association with PI3-kinase (Dresner, Laurent et al. 1999).

It has been demonstrated that obese individuals actually have reduced LPL activity, despite FA predominantly arising from adipose tissue lipolysis (Gordon, Cherkes et al. 1957), and furthermore, FA do not increase proportionately with fat mass (Karpe, Dickmann et al. 2011). It is now becoming clearer that rather than the circulating FA themselves, it is the increased intracellular lipid metabolites which can act as signalling molecules to interfere with insulin action (Schmitz-Peiffer, Craig et al. 1999; Cazzolli, Mitchell et al. 2007; Samuel, Petersen et al. 2010; Holland, Miller et al. 2011b).

1.4.3.2 The effect of lipid metabolites on insulin action

In recent times, lipid metabolites have emerged as signalling entities capable of interfering with insulin signalling through the activation of Ser/Thr kinases and phosphatases. Some species that have been implicated in impaired insulin action include the ganglioside GM3, DAG and ceramide (Schmitz-Peiffer, Craig et al. 1999; Tagami, Inokuchi et al. 2002; Aerts, Ottenhoff et al. 2007; Kumashiro, Erion et al. 2011; Holland, Miller et al. 2011b).

The storage product, TG is thought to be a relatively inert species itself, as a number of studies have now shown that increasing TG storage can overcome the lipotoxic effects of lipid oversupply (Listenberger, Han et al. 2003; Liu, Zhang et al. 2007; Peter, Weigert et al. 2009). The fact that highly insulin-sensitive, endurance-trained athletes exhibit increased intramuscular TG levels also suggest that TG itself is not a negative regulator of insulin action (Goodpaster, He et al. 2001).

Another species that has gained much research attention regarding negative regulation of insulin action is DAG. The hypothesis being that increased intracellular DAG activates PKC isoforms (especially θ and ε) which reduce IRS-1 association with PI3K (Yu, Chen et al. 2002; Samuel, Liu et al. 2007). A recent study by Shulman and colleagues showed that rather than DAG as a whole, “cytosolic” DAG localised to lipid droplets correlated with IR (Kumashiro, Erion et al. 2011). Further to the idea that a subpopulation of DAG may be the mediator, as opposed to the whole pool, is the fact that unsaturated DAG species serve as

more potent PKC activators (Wakelam 1998). Although activation of PKC isoforms have been associated with enhanced DAG accumulation and the development of lipid induced IR (Schmitz-Peiffer, Browne et al. 1997; Samuel, Liu et al. 2007), some studies have also correlated DAG with insulin sensitivity (Monetti, Levin et al. 2007; Holland, Brozinick et al. 2007b; Amati, Dubé et al. 2011). In a study by Amati *et al.*, although absolute DAG content was increased in the muscle of insulin sensitive subjects, there were particular sub-species of DAG that were increased in the muscle of IR subjects (Amati, Dubé et al. 2011) and as such the effects may be more associated with particular species and localisation than with the overall pool. Furthermore, overexpression of the enzyme DAG kinase ε increased phosphatidic acid levels concomitant with reduced DAG and reduced PKC activation, despite inducing IR through reduced IRS-1 Tyr phosphorylation (Cazzolli, Mitchell et al. 2007).

Ceramide, a product of the sphingolipid pathway has also gained credence as a metabolite capable of inducing negative effects upon glucose metabolism (Schmitz-Peiffer, Craig et al. 1999; Cazzolli, Carpenter et al. 2001). The sphingolipid pathway has been investigated for its detrimental effects on insulin action as inhibition of the pathway at the most proximal steps with chemical agents such as myriocin and cycloserine have shown to induce beneficial metabolic effects (Holland, Brozinick et al. 2007b; Yang, Badeanlou et al. 2009). Ceramide has been linked to impaired insulin signalling through inhibition of Akt via activation of protein phosphatase 2A (Cazzolli, Carpenter et al. 2001; Stratford, Hoehn et al. 2004) as well as PKCζ (Powell, Hajduch et al. 2003; Fox, Houck et al. 2007) and therefore correlated with IR (Amati, Dubé et al. 2011). Furthermore, a recent study by Holland and co-workers showed that the insulin sensitising lipokine, adiponectin, can induce ceramidase activity through its receptor (Holland, Miller et al. 2011b). Despite the strong evidence correlating ceramide to IR, other studies have failed to implicate ceramide as a primary candidate. Studies have shown increased hepatic ceramide levels are not associated with induction of IR (Monetti, Levin et al. 2007; Boden 2008).

The approaches taken thus far have not identified any clear targets as instigators of metabolic decline, with continued debate over DAG, ceramide or other species as being the most toxic. However, the advancement in mass spectrometry technology, for studying lipid species, together with more target-specific approaches, promise to paint a clearer picture in the future. Furthermore, location of the lipid metabolites may be as important, with the biology of the

system *in vivo* complicated by a constellation of tissue effects rather than cell-autonomous roles inducing the observed phenotype.

1.4.3.3 The athlete's paradox

The confounding nature of many experiments in rodents and cell culture models, searching for one particular lipotoxic metabolite has been corroborated by the so-called athletes paradox. With studies showing that obese and T2D subjects had high intramyocellular lipid levels (Pan, Lillioja et al. 1997; Goodpaster, Thaete et al. 2000) it was thought that this may in fact explain the reduced insulin sensitivity in these patients. However, it was later observed that highly trained endurance athletes, who are extremely insulin sensitive, were found to have similarly elevated intramyocellular lipid content (Goodpaster, He et al. 2001; van Loon, Koopman et al. 2004) as well as enhanced oxidative capacity. This paradoxical finding has led to recent research utilising the advancement in lipidomic technology to analyse samples for particular species that may correlate with insulin sensitivity in endurance athletes or not in sedentary subjects (Dube, Amati et al. 2008; Amati, Dubé et al. 2011). The anomaly with the endurance trained athletes compared to sedentary insulin resistant subjects is that despite higher intramyocellular TG levels they also exhibit increased oxidative capacity, possibly leading to reduced lipid intermediates.

The recent study by Amati and co-workers showed that although overall intramuscular DAG content correlated with insulin sensitivity, it was only the DAGs saturated at both acyl chains that tended to correlate with insulin sensitivity rather than at just one position (Amati, Dubé et al. 2011). This somewhat contrasts with another recent study by Bergman *et al.* which showed that saturated DAG species were lower in the endurance trained subjects (Bergman, Perreault et al. 2010), however the way in which these lipid species were measured also differed. Moreover, in the same recent study by Goodpaster and colleagues, ceramide content correlated with IR, both saturated and unsaturated forms (Amati, Dubé et al. 2011).

It is now becoming accepted that it is not lipid content *per se*, but particular species of lipid metabolites and their compartmentalisation that play a key role in bringing about the detrimental metabolic defects observed following increased lipid supply. In order to clarify the apparent discrepancies, it may be appropriate to better utilise the advanced lipidomic technology available for looking at specific species rather than examining total levels.

1.4.4 Fatty liver – A benign condition or instigator of the pathogenesis of metabolic disease?

It has been hypothesised that accumulation of fat in non-adipose tissue, particularly the liver contributes to the development of IR and its associated metabolic disorders. The development of hepatic steatosis arises due to a number of factors that together bring about the condition. These include IR in adipose tissue, leading to increased lipolysis and delivery of FA to the liver, reduced FA oxidation capacity of the liver in concert with increased hepatic lipogenesis and altered lipoprotein secretion (Tessari, Coracina et al. 2009).

Evidence has accumulated over the years to suggest that a steatotic liver is involved in the genesis of IR and the pathogenesis of cirrhosis and cardiovascular disease (Angulo 2002; Roden 2006; Larson-Meyer, Newcomer et al. 2011). Identifying whether steatosis instigates the progression toward further metabolic disease in patients afflicted with the condition is difficult, as elevated insulin and glucose levels can themselves drive hepatic lipogenesis. It is hypothesised that hepatic steatosis is associated with increased levels of lipid metabolites, including acyl-CoA, ceramide and DAG, with DAG hypothesised to activate PKC isoforms to cause Ser phosphorylation of IRS-1/2 to perturb insulin action (Samuel, Petersen et al. 2010). It is the accumulation of metabolites that is postulated to impair insulin action, as increasing TG storage in the absence of intermediary metabolites does not result in IR (Monetti, Levin et al. 2007; Jou, Choi et al. 2008). The liver-specific transgenic diacylglycerol O-acyltransferase (DGAT2) mouse in fact exhibits significantly increased hepatic TG levels, yet is immune from IR (Monetti, Levin et al. 2007). Furthermore, deletion of adipose triglyceride lipase in mice results in enhanced liver lipid deposition on a high fat diet (HFD), yet the mice are protected from IR (Haemmerle, Lass et al. 2006; Hoy, Bruce et al. 2011). It has also been demonstrated that elongation of long chain fatty acids-6 (Elovl6)-null mice are protected from IR and hyperinsulinemia on a HFD, despite developing obesity and hepatic steatosis (Matsuzaka, Shimano et al. 2007).

The progression of hepatic steatosis to diabetes is often associated with altered adipokine and cytokine profiles and the activation of inflammatory pathways (Cai, Yuan et al. 2005; Sabio, Cavanagh-Kyros et al. 2009). Yet, it has been demonstrated that the supposedly pro-

inflammatory cytokine interleukin-6 (IL-6) can have protective effects upon hepatic insulin sensitivity (Wunderlich, Luedde et al. 2008; Matthews, Allen et al. 2010; Awazawa, Ueki et al. 2011). Inhibiting NFκB activation during the development of liver steatosis can result in a protection from IR (Cai, Yuan et al. 2005). Moreover, the way in which the lipid is stored within adipocytes can affect insulin sensitivity (Nishino, Tamori et al. 2008; Puri, Ranjit et al. 2008).

Therefore it seems plausible that it is not the total fat content of the liver that drives the pathogenesis of a steatotic liver to a more harmful condition, but the type of fat and how it is stored, together with interactions from the peripheral tissues and their cytokine profiles that promote the pathogenesis of metabolic disease.

1.4.5 Lipid induced insulin resistance

As discussed previously, IR and T2D are associated with increased intracellular lipid content, particularly lipid metabolites that can themselves act as signalling molecules to modulate insulin signalling. Although there has not been a single causative factor delineated as yet, the altered lipid moieties of peripheral tissues during increased lipid supply have all been shown to affect glucose homeostasis. Lipid infusions have shown that increased FA supply can increase DAG, ceramide and acetyl-CoA to negatively regulate insulin signalling components at various points of the cascade in muscle of rats and humans (Griffin, Marcucci et al. 1999; Itani, Ruderman et al. 2002; Reynoso, Salgado et al. 2003).

Rodents subjected to a HFD display distinct tissue timelines of IR development. Kraegen and colleagues demonstrated that hepatic IR develops after as little as 3 days of a HFD, whereas skeletal muscle IR develops later, at around three weeks (Kraegen EW, Clark PW et al. 1991). At the same time it was seen that adipose tissue also became IR after just three days as assessed by labelled glucose disposal. This highlights the fact that tissues become less sensitive to insulin at different stages in the pathogenesis of the disease rather than all at once.

It can also be noted that in the development of hepatic IR, insulin is unable to suppress HGP, apolipoprotein B synthesis and VLDL secretion, yet the liver remains sensitive to insulin's

stimulatory effect upon lipogenesis through SREBP-1 (Kraegen EW, Clark PW et al. 1991; Tessari, Coracina et al. 2009). It also appears that in the progression of metabolic disease, the hepatokine profile secreted by the liver alters, becoming more antagonistic of insulin signalling in other tissues, such as with increased Fetuin-A secretion (Auberger, Falquerho et al. 1989; Hennige, Staiger et al. 2008; Dasgupta, Bhattacharya et al. 2010). A feature of adipose tissue IR is a reduction in insulin stimulated glucose uptake and a block in the suppression of lipolysis. This leads to increased FA levels in the circulation that are taken up by other tissues, while the adipokine profile is also altered, increasing inflammatory secretions and reducing those involved in insulin sensitising actions such as adiponectin (Holland, Bikman et al. 2011a; Holland, Miller et al. 2011b; Kalupahana, Massiera et al. 2012). Skeletal muscle primarily shows a defect in insulin-stimulated glucose transport, however insulin-independent glucose transport remains intact, indicating the defect is associated with the insulin signal transduction pathway (Zierath 2002). As these key insulin target tissues develop IR, the β-cells of the pancreas compensate through increased mass to enhance insulin secretion (Terauchi, Takamoto et al. 2007). In more recent times, central control over the peripheral tissue response to insulin has been shown to exist. Studies from various groups have shown central control over HGP, adipocyte and β-cell function (Obici, Zhang et al. 2002; Könner, Janoschek et al. 2007; Stanley, Pinto et al. 2010; Calegari, Torsoni et al. 2011; Lam, Chari et al. 2011; Marino, Xu et al. 2011). More recently insulin-stimulated glucose transport into skeletal muscle has been linked to central mechanisms (Coomans, Biermasz et al. 2011).

IR has largely been associated with defects in insulin signalling (Petersen and Shulman 2006) as a consequence of activation of various serine/threonine kinases such as PKCs, JNK, NFκB as well as phosphatases that downregulate activation of insulin signalling components (Schmitz-Peiffer 2000; Benoit, Kemp et al. 2009; Samuel, Petersen et al. 2010; Vallerie and Hotamisligil 2010; Holland, Bikman et al. 2011a). Reduced IRS-1 Tyr phosphorylation has long been implicated in the reduced insulin action (Dresner, Laurent et al. 1999; Aguirre, Uchida et al. 2000; Yu, Chen et al. 2002; Zick 2005), however several studies now suggest the primary defect is downstream of IRS-1 (Nadler, Stoehr et al. 2001; Hoehn, Hohnen-Behrens et al. 2008). Despite these discrepancies, it is clear that defects in the signal transduction pathway of insulin are likely to be a primary cause as insulin-independent

glucose metabolism is unimpeded. A number of mechanisms have been proposed including lipotoxicity, oxidative stress, inflammation and ER stress (Hotamisligil 2006).

The MAPK family member, JNK is activated by a number of stressors that are present in obesity and IR (Hotamisligil 2006) including ER stress, saturated FA and toll-like receptor 2 and 4 activation. It has also been demonstrated that activation of JNK can increase phosphorylation of Ser and Thr residues on IRS-1/2 (Aguirre, Werner et al. 2002; Solinas, Naugler et al. 2006). The three members of the JNK family show high homology and the ubiquitously expressed isoforms JNK 1 and JNK 2 show some redundancy, with JNK 1 compensating for JNK 2 deletion in tissues such as liver and adipose (Tuncman, Hirosumi et al. 2006). Tissue-specific inactivation and deletion studies of JNK isoforms have led to conflicting findings. Initial studies inhibiting both JNK 1 and 2, as well as JNK 1 specifically, by RNA interference in the whole liver showed improved insulin sensitivity and glucose homeostasis (Yang, Wilcox et al. 2007; Singh, Wang et al. 2009). Recent work, however, where JNK 1 was genetically deleted specifically in hepatocytes of mice, resulted in impaired insulin sensitivity and glucose homeostasis on a normal diet. Somewhat surprisingly, however, the phenotype was overcome when fed a HFD (Das, Sabio et al. 2009; Sabio, Cavanagh-Kyros et al. 2009). Deletion of JNK isoforms, particularly JNK 1, in other specific tissues has led to different effects, suggesting that the overall physiological phenotype due to JNK activation involves tissue cross-talk rather than cell-autonomous roles (Kaneto, Xu et al. 2002; Sabio, Das et al. 2008; Sabio, Cavanagh-Kyros et al. 2009; Sabio, Kennedy et al. 2010).

The lipid metabolite, ceramide has been shown to interfere with insulin signalling by inhibition of Akt activation, through two different mechanisms. Firstly, ceramide is able to enhance the activity of protein phosphatase 2A (Cazzolli, Carpenter et al. 2001), which acts to dephosphorylate Akt at Ser 473. Secondly, ceramide can activate PKC ζ, which also acts to inhibit Akt activation (Powell, Hajduch et al. 2003; Fox, Houck et al. 2007). Another lipid metabolite, implicated in impaired insulin action is DAG. It has been demonstrated that DAG elicits its negative regulation through activation of PKC θ (primarily in muscle) and ε (in liver), which Ser phosphorylate IRS-1/2 and therefore impair insulin-stimulated glucose metabolism (Samuel, Liu et al. 2007; Samuel, Petersen et al. 2010).

Adding further complexity to this is that these different processes can also interact with each other to induce inhibitory actions. An example of this is PKCε activation by lipopolysaccharide treatment, which induces JNK activation which in turn mediates secretion of the pro-inflammatory cytokine TNFα by macrophages to induce apoptosis (Comalada, Xaus et al. 2003). Furthermore, saturated FA can activate JNK and promote the IR through effects on β-cell and hepatocyte function (Malhi, Bronk et al. 2006; Solinas, Naugler et al. 2006).

Apart from these well documented metabolites, membrane phospholipid content can also be remodelled. It has also been demonstrated that FA, particularly saturated FA, can alter membrane composition and therefore affect fluidity of many cell types including lymphocytes, adipocytes and hepatocytes (Pjura, Kleinfeld et al. 1982; Luo, Rizkalla et al. 1996; Clamp, Ladha et al. 1997; Stulnig, Huber et al. 2001). Intriguingly the insulin sensitising drug, metformin has been shown to increase adipocyte membrane fluidity (Muller, Denet et al. 1997). Furthermore, it has been demonstrated that increasing membrane fluidity alone can increase basal glucose transport (Pilch, Thompson et al. 1980; Whitesell, Regen et al. 1989). It has also been shown that hyperinsulinemia, a hallmark of IR, can alter the membrane phospholipid content of obese non-diabetic patients (Younsi, Quilliot et al. 2002). Moreover, many of the signalling defects associated with the pathogenesis of IR from increased lipid supply are associated with activation at the PM and furthermore, reduced membrane fluidity has been associated with impaired glucose transport in adipocytes as well as in erythrocytes of insulin resistant patients (Pilch, Thompson et al. 1980; Younsi, Quilliot et al. 2002).

Although much literature has focussed on the role that inflammation plays in the development of IR (Shoelson, Lee et al. 2003; Olefsky and Glass 2010), it has recently been questioned as to whether this mediates the condition only in the longer term rather than being an underlying mechanism for the initial development of the disease. This was recently suggested by Lee *et al.* who demonstrated that macrophage-induced inflammation was not involved in early IR, but manifests over time (Lee, Li et al. 2011). Furthermore, a recent study involving short term over-feeding in humans induced weight gain and reduced insulin sensitivity without any increase in inflammatory markers in the circulation or subcutaneous adipose tissue (Tam, Viardot et al. 2010). Given the many metabolic derangements occurring within the first one to three weeks of a HFD, inflammation is unlikely to be the primary

mechanism, but instead the lipid entities accumulating in tissues could underlie the pathogenesis, due to their ability to affect a wide range of kinases and phosphatases independent of inflammation.

1.5 Protein Kinase C

The PKC family of proteins belong to the AGC group of Ser/Thr kinases. They are ubiquitously expressed and act as important lipid sensitive signalling molecules through activation by specific lipids especially DAG (and the co-factor phosphatidylserine) (Kishimoto, Takai et al. 1980). The PKC isozymes are involved in numerous cellular events including gene expression, metabolism, cytoskeletal reorganisation, proliferation, differentiation, survival and trafficking, which affect a number of outcomes such as neural processes, immunity, inflammation, tumor progression, ischemia, and diabetes (Considine, Nyce et al. 1995; Balciunaite, Jones et al. 2000; Racke, Wang et al. 2001; Chen and Tian 2011; Son, Hong da et al. 2011). The PKC family is encoded by nine genes, divided into three groups based upon their mechanism of activation: cPKCs (conventional PKCs: α, β and γ) are activated by Ca^{2+} and DAG; the activation of nPKCs (novel PKCs: δ, ε, η and θ) requires DAG but is independent of Ca^{2+}; and aPKCs (atypical PKCs: ζ and ι/λ), activated by increased phosphatidylinositol-3,4,5-bisphosphate (PIP_3) leading to PDK1 activation while also being insensitive to Ca^{2+}. The effects modulated by PKC signal transmission can be cell and isozyme specific and are highly dependent upon cellular localisation. The remainder of this literature review will discuss regulation of the PKC family, with a focus on the novel PKCε isozymes, which has been implicated in liver IR and InsR trafficking (Samuel, Liu et al. 2004; Samuel, Liu et al. 2007; Schmitz-Peiffer, Laybutt et al. 2007).

1.5.1 PKC structure

The PKCs are classed into their categories based upon structural similarity, together with their activation profile. In general PKCs consist of an N-terminal regulatory domain linked by a hinge region to a C terminal catalytic domain. Despite the homology amongst PKC members, they do serve a variety of non-redundant functions. The regulatory domain, responsible for activation and localisation of the kinase, is where the greatest discrepancy

exists between the PKC classes, with the kinase domain relatively conserved between all isozymes (Figure 1.4). Within the regulatory domain, the C1 domain binds DAG and phorbol esters (Sharkey, Leach et al. 1984) while phospholipids such as phosphatidylserine bind the C2 domain of cPKCs in a Ca^{2+} dependent manner (Newton and Johnson 1998; Johnson, Giorgione et al. 2000). Work by Blumberg and colleagues, demonstrated the DAG/PMA interaction with PKC occurs in a stoichiometry of 1 mol ligand/mol PKC (Konig, DiNitto et al. 1985). The C2 domain of conventional PKC isozymes binds anionic phospholipids with a selectivity for phosphatidylserine (Conesa-Zamora, Lopez-Andreo et al. 2001) but also phosphatidylinositol-4,5-bisphosphate (PIP_2), which selectively targets these PKC isozymes to the PM (Evans, Murray et al. 2006; Corbalan-Garcia, Guerrero-Valero et al. 2007).

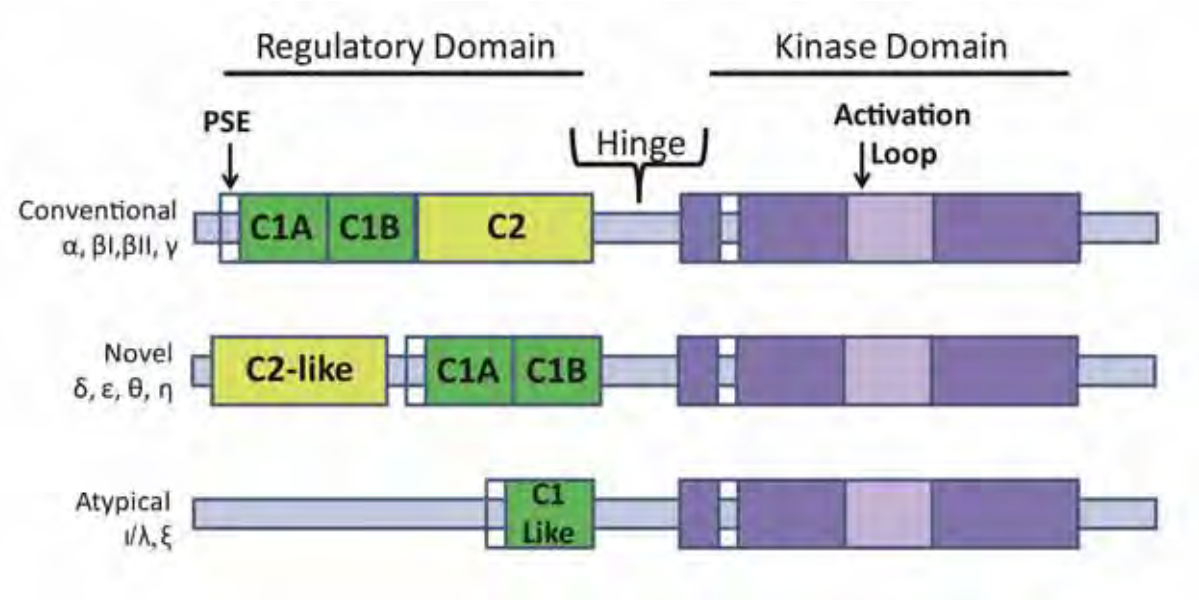

Figure 1.4 Structure of the PKC family
PKCs are a family of Ser/Thr kinases, sub-divided into subclasses based on their domain composition and activation requirements. All isozymes have a conserved kinase domain. The conventional isozymes contain a C1 domain that binds DAG or phorbol esters, and a C2 domain that binds phosphatidylserine (PS) in a Ca^{2+} dependent manner. The novel subclass contain a C1 domain and C2-like domain that does not bind Ca^{2+}. The atypical PKCs do not contain functional C1 or C2 domains rendering them DAG and Ca^{2+} independent.

The nPKCs, like the cPKCs also contain tandem C1 domains that bind DAG but in this case the C2-like domain is missing key residues required for Ca^{2+} binding (Ochoa, Garcia-Garcia et al. 2001). The nPKCs only require DAG for regulation, and the affinity for DAG is much greater than that of cPKCs (Giorgione, Lin et al. 2006) which has been credited to one

residue within the C1b domain (Dries, Gallegos et al. 2007). This increased affinity allows nPKCs to respond more efficiently to DAG as they do not require Ca^{2+} to sense alterations in DAG content. Despite this, the nPKCs translocate to the membrane at a slower rate than cPKCs (Newton 2003).

The C1 domain of aPKCs has an altered ligand-binding pocket that is unable to bind DAG or phorbol esters (Kazanietz, Bustelo et al. 1994; Pu, Peach et al. 2006). As they also lack the requirement for regulation by Ca^{2+}, it is thought that their PB1 domain promotes these protein-protein interactions along with the c-terminal PDZ ligand (Lamark, Perander et al. 2003). It has been demonstrated that the lipids PIP_3 via PDK1 and ceramide can activate atypical PKC ζ (Nakanishi, Brewer et al. 1993; Muller, Ayoub et al. 1995).

All PKCs share a conserved C-terminal kinase domain that serves as a phosphorylation-dependent docking site for substrates. Moreover, they also contain an N-terminal autoinhibitory pseudosubstrate sequence to keep the isozymes in an inactive conformation until specifically stimulated (Poole, Pula et al. 2004). This has been postulated to occur through the pseudosubstrate domain binding the active site and masking the Thr phosphorylation site within the kinase activation loop, which can be relieved by ligands such as DAG (Newton 1995).

1.5.2 PKC activation and modulation

PKC isozymes undergo a series of constitutive phosphorylations that are essential for the stabilisation of the enzymes and promotion of catalytic competence (Parekh, Ziegler et al. 2000). This is known as maturation, with the non-phosphorylated form referred to as immature. Recent studies indicate heat shock protein-90 (HSP90) and the mammalian target of rapamycin (mTOR) complex 2 (mTORC2) as playing key roles in this process to facilitate further phosphorylation events (Guertin, Stevens et al. 2006; Ikenoue, Inoki et al. 2008; Gould, Kannan et al. 2009). Phosphorylation by PDK1 is accepted to be the first phosphorylation event in stabilising PKCs which is demonstrated in studies by Alessi and colleagues where cells lacking PDK1 exhibit low PKC expression (Balendran, Hare et al. 2000). This PDK1 dependent phosphorylation occurs at a conserved Thr site within the

activation loop, as demonstrated by mutation of this site to alanine or valine, and this further inhibits subsequent phosphorylation at two C-terminal sites proposed to be autophosphorylation sites (Cazaubon, Bornancin et al. 1994; Orr and Newton 1994). Mature PKC is held in the inactive conformation by a number of intra-molecular interactions.

In the inactive state, PKCs localise primarily to the cytosol engaged in scaffolding interactions (Schechtman and Mochly-Rosen 2001). The hydrolysis of PIP_2 generates the key cPKC second messengers; Ca^{2+} and DAG. cPKCs translocate to the PM following Ca^{2+} binding where they interact with anionic phospholipids such as PIP_2 and the C1 domain binds PM-localised DAG. Following this membrane localisation and interaction, the autoinhibitory pseudo-substrate sequence is released to permit substrate phosphorylation. On the other hand nPKCs respond directly to DAG alterations for which they have an approximate two-orders-of-magnitude higher affinity (Giorgione, Lin et al. 2006). This less specific requirement for targeting to the PM by Ca^{2+} leads to somewhat increased basal localization to DAG enriched membranes of nPKCs (Carrasco and Merida 2004). This has led to the use of phorbol esters such as phorbol 12-myrystate 13-acetate (PMA), which are natural DAG mimetic compounds, to activate PKCs and examine their biological effects in various systems (Kraft and Anderson 1983). The cytosol to PM redistribution is used as a measure of PKC activation.

Sustained activation of PKCs induces their degradation and chronic phorbol ester treatment has been used to take advantage of this and deplete cell systems of phorbol ester-responsive isozymes (Rodriguez-Pena and Rozengurt 1984; Huang, Yoshida et al. 1989). This lack of specificity for a particular isozyme together with the ability of phorbol esters to activate other C1 domain containing proteins requires careful consideration. The actual mechanism behind the degradation is not exactly clear, however active PM localised PKCs take on an unstable conformation that makes them significantly more susceptible to dephosphorylation (Dutil, Keranen et al. 1994). Work by Newton and colleagues identified the PH domain leucine-rich repeat protein phosphatase (PHLPP) as a candidate to dephosphorylate the hydrophobic motif of cPKCs and nPKCs, causing them to move into a detergent-insoluble cell fraction, where they are further dephosphorylated by an okadaic acid sensitive phosphatase (Brognard, Sierecki et al. 2007; Gao, Brognard et al. 2008), making them more susceptible to degradation.

It was identified by Mochly-Rosen and colleagues that protein scaffolds are critical in modulating PKC activity (Mochly-Rosen, Khaner et al. 1991; Mochly-Rosen and Gordon 1998). A number of scaffold proteins have since been identified with the C2 domain implicated in many of these interactions forming (Brandman, Disatnik et al. 2007). Such scaffold proteins have been termed receptors for activated C kinase (RACKs) or receptors for inactive C kinase (RICKs), depending upon the conformation of the PKCs, however their interaction alleviates the autoinhibition of the isozyme involved (Ron and Mochly-Rosen 1995a). These proteins form interactions that maintain PKCs in an active conformation and can lead to prolonged signalling through activation and spatial localisation of the PKC. Due to the interactions being based around sequence identity, peptide inhibitors can interfere with these complexes and modulate signal transduction and actions of specific PKC isozymes (Tsunoda, Sierralta et al. 1997; Churchill, Qvit et al. 2009). Another group of scaffold proteins, the A-kinase anchor proteins (AKAPs) primarily act to bind and sequester Protein Kinase A to distinct cellular locations, but also bind phosphatases such as protein phosphatases a, 2A and 2B which can act to dephosphorylate downstream PKA targets and also PKCs (Perkins, Wang et al. 2001).

The importance of localisation has been shown in a number of studies, where altering the localisation of particular PKC isozymes through sequence mutations or binding with different scaffold proteins can significantly alter their cellular function (Quittau-Prevostel, Delaunay et al. 2004; Diouf, Collazos et al. 2009; Saurin, Brownlow et al. 2009). Such events have been identified with PKCα and PKCε and could represent significant consequences for the pathogenesis of various disease states in which the isoforms participate.

1.5.3 PKCε

PKCε is a member of the nPKC family which as described above are activated by DAG in a Ca^{2+} independent manner. PKCε is ubiquitously expressed and involved in regulating numerous cell functions including cell survival, proliferation, differentiation, gene expression, metabolism, trafficking and ischemia (Aksoy, Goldman et al. 2004; Churchill and Mochly-Rosen 2007; Samuel, Liu et al. 2007; Schmitz-Peiffer, Laybutt et al. 2007; Newton, Kim et al. 2007a; Van Kolen, Pullan et al. 2008). PKCε has been implicated in pathways of

the nervous system, immune system, cardiovascular system and endocrine systems, indicating PKCε activation could play a role in the development of a number of diseases.

1.5.3.1 PKCε structure, activation and modulation

PKCε shares structural features with other PKC isoforms in that it contains a C1 domain containing two cysteine-rich motifs that bind DAG, a C2-like domain that binds phospholipids, a pseudosubstrate domain, C3 and C4 catalytic domains with an ATP binding site and an activation loop (Poole, Pula et al. 2004; Newton and Ron 2007b). A unique feature of PKCε is a six-amino-acid actin-binding motif between the C1a and C1b subdomains (Prekeris, Mayhew et al. 1996) (Figure 1.5). As is the case with other PKCs, PKCε requires phosphorylation to prime the kinase for full enzymatic activity by allosteric regulators including DAG, PIP3 and FFA (Moriya, Kazlauskas et al. 1996; Graness, Adomeit et al. 1998). This involves phosphorylation at three conserved priming sites in the catalytic domain: Thr 566 in the activation loop, Thr 710 within the Thr-proline turn motif and Ser 729 of the hydrophobic motif. Phosphorylation of Thr 566 is mediated by PDK1, whereas Thr 710 and Ser 729 are proposed to be autophosphorylated in PKCε, as in cPKCs (Cenni, Doppler et al. 2002), although recent evidence suggests mTORC2 could transphosphorylate these sites (Facchinetti, Ouyang et al. 2008; Ikenoue, Inoki et al. 2008). These phosphorylation events appear to be regulated rather than constitutive in PKCε, which is supported by the findings that unphosphorylated PKCε can be recruited for signalling by activated receptors involved in PI3K activation by which PDK1 is activated (Cenni, Doppler et al. 2002; Olive, McGeehan et al. 2005). Studies have shown that unphosphorylated PKCε associates with the anchoring protein centrosome- and Golgi-localized PKN (protein kinase N)-associated protein (CG-NAP), via the catalytic domain of PKCε and therefore phosphorylation of Thr 566 is proposed to occur at the Golgi apparatus (Takahashi, Mukai et al. 2000). More recently, additional PKCε sites have been proposed to be autophosphorylated in vitro: Ser 234, Ser 316 and Ser 368 (Durgan, Cameron et al. 2008), but this does not appear to occur in intact cells, but in fact involve transphosphorylation by cPKCs.

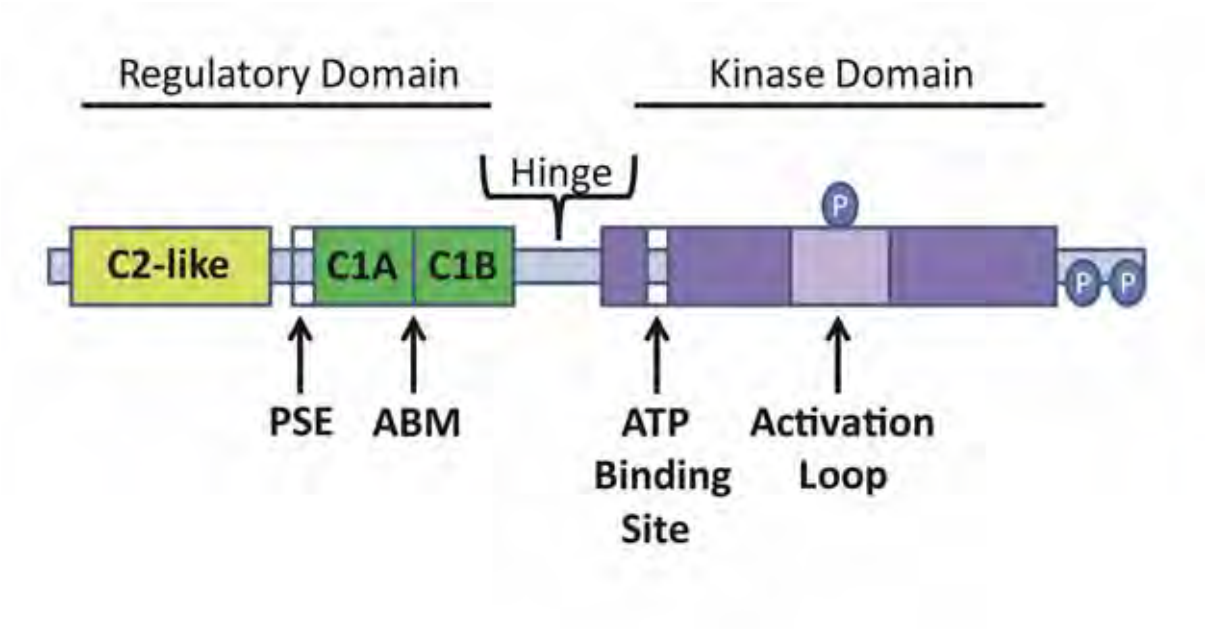

Figure 1.5 Structure of PKCε
The C2-like domain promotes membrane translocation of PKCε. DAG and phorbol esters bind the C1 domains, with CIB thought to be important for FA-induced targeting of the PKCε. The Thr 566 site in the activation loop is essential for enzymatic activity, with the Thr 710 and Ser 729 sites autophosphorylation sites, required to maintain PKCε stability for second messengers. The actin-binding motif is located between the C1A and C1B domains.
PSE = pseudosubstrate domain, ABM = Actin Binding Motif

1.5.3.2 PKCε binding partners and substrates

PKCε is involved in a wide array of cellular events, which is reflected in the number of binding partners and substrates to which it has been linked. Such pathways include regulation of activity of other kinases, cytoskeletal remodelling and ion channel regulation whose functions have also been associated with disease pathogenesis, including IR.

Although it is beyond the scope of this review to name all the proteins that have been described as interacting with PKCε, some of the major binding partners are described below in more detail, the biological function of which has been demonstrated to be modulated directly by PKCε. In addition PKCε can form signalling complexes with c-Src and its family member Lck, TRAM, β-actin, histone H1, 14-3-3, Akt and PKD (Csukai, Chen et al. 1997; Prekeris, Hernandez et al. 1998; Besson, Wilson et al. 2002; Ping, Song et al. 2002; Xu and Rumsby 2004; McGettrick, Brint et al. 2006; Aziz, Manoharan et al. 2007). Despite the diverse signalling processes PKCε is involved with, it tends mainly to be associated with promoting survival and growth (Saurin, Durgan et al. 2008).

RACKs (receptors for activated C-kinase)

PKCε can be targeted to certain subcellular compartments through its interactions with binding partners such as RACKs, to promote its association with substrates. There have been two RACKs identified; RACK1 (Ron, Luo et al. 1995b) and the coatomer protein *β*-COP' also known as RACK2, which binds activated PKCε with much greater affinity (Csukai, Chen et al. 1997). RACK1 has been associated with regulating a number of functions including translation, transcription, focal adhesions and JAK-STAT signalling (Usacheva, Smith et al. 2001; Besson, Wilson et al. 2002; He, Vagts et al. 2002). RACK1 has other binding partners and therefore not all of these actions may be attributed to its association with activated PKCs and more specifically PKCε, for which it has a significantly lower affinity compared to other PKCs (Mochly-Rosen 1995; Besson, Wilson et al. 2002). RACK2 is proposed to mediate ER-Golgi retrograde transport, and inhibition of the interaction with PKCε using a specific peptide inhibitor has been used to examine PKCε signal modulation. The interaction of activated PKCε with RACK2 requires Ser 729 phosphorylation and targets PKCε to the Golgi (Xu, He et al. 2007). Finally, PKCε has been associated with the regulation of expression of RACK 1 and 2 (Pass, Gao et al. 2001).

STAT3

The transcription factor STAT3 has recently been identified as a substrate of PKCε. The activation of STAT3 has been shown to be important in many models of tumorigenesis. STAT3 contains two sites for activation: Tyr 705 and Ser 727, which can be phosphorylated by PKCε (Aziz, Manoharan et al. 2007; Aziz, Hafeez et al. 2010). Knockdown of PKCε with RNA interference inhibits STAT3 Ser 727 phosphorylation and reduces the activity of STAT3, measured by DNA binding and activation of transcription, which implicates STAT3 in tumorigenesis. The functional consequences of STAT3 interaction with PKCε has been demonstrated to functionally enhance prostate cancer progression in humans and mice. Furthermore, STAT3 activation could play a role in negative regulation of insulin action through its control over the SOCS proteins, which as discussed earlier (Section 1.3.2.4) can negatively regulate insulin signal transduction through direct binding of the InsR or IRS proteins.

Actin

PKCε regulates the function of the major cytoskeletal structural proteins (Akita 2008a) and with its unique actin binding motif, located at amino acid residues 223-228 between the C1 domains, activation of PKCε by phorbol esters, DAG or arachidonic acid, promotes PKCε binding to F-actin (Prekeris, Mayhew et al. 1996). This interaction also acts to stabilise PKCε in an active conformation (Prekeris, Hernandez et al. 1998). The function of the PKCε –F-actin interaction may be important in the cytoskeletal reorganisation involved in glutamate exocytosis from the dentate gyrus and protein trafficking.

1.5.3.3 PKCε physiological functions

Determining the exact role of particular PKC isozymes in a signalling pathway has been difficult due to a number of factors including the overlap in function of isozymes, the variety of substrates involved and particularly the non-specificity of PKC activators and inhibitors. As such direct substrates are often unknown. The phorbol ester, DAG-mimetic PMA is widely used to activate PKCs, however to elucidate specific roles, often it is needed to be used in combination with other non-specific inhibitors. These difficulties are being reduced with the development of more specific peptides and the use of PKC isozymes KO mouse models. Deletion of PKCε in mice and through targeted inhibition in rats has supported conclusions from cell systems; however compensation by other isoforms can confound the interpretation of phenotypes observed in KO mice. Such studies have implicated PKCε actions in a number of tissues and physiological processes including lipid metabolism, anxiety, nociception, ethanol sensitivity, renal function, immunity, cardiovascular protection and tumour growth (Akita 2008b). Some of the functions of PKCε are discussed below.

<u>Neural system</u>

PKCε is most abundantly expressed in the central nervous system, mediating such processes as neurite outgrowth, possibly through its actin binding domain (Hundle, McMahon et al. 1995; Zeidman, Troller et al. 2002). PKCε has also been shown by Messing and colleagues to be important in the brain for alcohol dependency through regulation of GABA signalling (Proctor, Poelchen et al. 2003; Olive, McGeehan et al. 2005; Qi, Song et al. 2007) as well as

for cocaine addiction (Olive, Mehmert et al. 2000). Furthermore, with its role in synaptic function, PKCε has been implicated in the regulation of nociceptor function in pain (Khasar, Lin et al. 1999).

Immune system

The importance of PKCε in the immune response of the body to inflammatory signals has been demonstrated in a number of studies. The Parker laboratory has demonstrated that PKCε is required for macrophage activation through recruitment to toll-like receptor (TLR) 4 via MyD88 and that this is critical for downstream TLR signalling through NFκB (Faisal, Saurin et al. 2008). Moreover, MAPK pathways have been reported to be negatively regulated by PKCε in lipopolysaccharide-activated macrophages (Valledor, Xaus et al. 2000). It has also previously been demonstrated that PKCε KO mice are hypersensitive to gram-negative and gram-positive bacterial infections with reduced survival (Castrillo, Pennington et al. 2001). The absence of PKCε has also been associated with increased IKK phosphorylation and protection against TNFα-induced apoptosis (Mayne and Murray 1998; Tojima, Fujimoto et al. 2000). Furthermore, PKCε has been demonstrated to be important for activation and gene expression in various immune cells, especially T-cells (Krappmann, Patke et al. 2001; Maulon, Mari et al. 2001; Denys, Hichami et al. 2005). This makes PKCε activation a potential candidate for mediating the inflammatory response seen in the pathogenesis of metabolic disease, however recent data has shown inflammation is not involved in the early development of IR, when fat-fed WT animals are already glucose intolerant. Therefore a role for PKCε in inflammation-induced IR is probably not a primary effect.

Carcinogenesis

PKCε has been shown in a number of studies to be important in cell signalling pathways mediating apoptosis and metastasis (Kermorgant and Parker 2005; Okhrimenko, Lu et al. 2005; Gundimeda, Schiffman et al. 2009). The involvement of PKCε in regulating ras-signaling through Raf-1 activation has been demonstrated in various cancer cells (Hamilton, Liao et al. 2001) as well as its involvement in tumorigenic HGF/c-Met signalling pathway (Kermorgant and Parker 2005). Recently, Saurin and co-workers demonstrated that PKCε

controls the final step of cytokinesis and through a RhoA-dependent mechanism, indicating a key role for PKCε in carcinogenesis (Saurin, Durgan et al. 2008).

Cardiovascular disease

It is well established that PKCε plays a crucial role in cardioprotection from ischemic reperfusion injury (Lawrence, Kabir et al. 2005; Rathore, Zheng et al. 2006). PKCε has also been demonstrated to play a role in eliciting the beneficial effects of ethanol induced ischemia and its cardioprotective actions through formation of a PKCε, Akt and eNOS complex (Zhou, Karliner et al. 2002). The signalling pathways modulated by PKCε to induce cardioprotection are thought to involve PI3K, (Tong, Chen et al. 2000) and the activation of the anti-apoptotic transcriptional factor NFκB (Li, Liu et al. 2000). Furthermore, PKCε activation of the MAPK pathway members ERK, JNK, and p38MAPK have been implicated in mediating the cardioprotective signalling events from a mitochondrial location (Baines, Zhang et al. 2002). It has also been postulated that PKCε plays a role in cardiac hypertrophy, and this appears to be tightly controlled as inhibition of PKCε by peptides has resulted in hypercardiomyopathy, leading to death, whilst enhanced activation in transgenic mice has also resulted in hypertrophy and this was associated with reduced cardiac function (Takeishi, Ping et al. 2000; Wu, Toyokawa et al. 2000).

Metabolic disease

Studies in both humans and rodents have also shown that in PKCε expression and activation is elevated in diabetic and insulin resistant subjects (Considine, Nyce et al. 1995; Schmitz-Peiffer, Browne et al. 1997; Qu, Seale et al. 1999; Samuel, Liu et al. 2007; Kumashiro, Erion et al. 2011). This will be discussed in greater detail below. Increased PKCε activation in glucose intolerant patients has also been reported to be associated with cardiomyopathy, nephropathy and increased risk of vascular disease (Malhotra, Reich et al. 1997; Littler, Wehling et al. 2005; Della-Morte, Raval et al. 2011).

1.5.3.4 PKCε, Insulin Resistance and Lipid Metabolism

Increased expression and activation of PKCε has been associated with IR and diabetes in humans as well as various rodent models of lipid oversupply (Considine, Nyce et al. 1995; Samuel, Liu et al. 2004; Schmitz-Peiffer, Laybutt et al. 2007). This has bought about a focus of research into the mechanisms involved in PKCε signalling and its modulation of insulin action through regulation of the insulin signal transduction pathway. The negative modulation of insulin action by PKCε has been demonstrated by various methods of PKCε inhibition. Antisense oligonucleotide knockdown of PKCε in liver and WAT of rats fed a HFD for 3 days elicited protection against diet induced hepatic IR (Samuel, Liu et al. 2007). In this study it was suggested that the improved insulin action was due to the abolition of PKCε inhibition of InsR kinase activity (Samuel, Liu et al. 2007). Furthermore, global PKCε KO animals also show protection from HFD-induced glucose intolerance, both in the short term (1 week) and long term (16 weeks) (Raddatz, Turner et al. 2011). The early protection was associated with improved insulin action, primarily at the level of the liver (Raddatz, Turner et al. 2011). In the longer term, enhanced insulin availability was proposed to promote glucose tolerance following the onset of peripheral IR, associated with reduced insulin-stimulated glucose uptake (Schmitz-Peiffer, Laybutt et al. 2007; Raddatz, Turner et al. 2011). As such, many of the beneficial effects of PKCε inhibition on metabolism appear to occur through improved hepatic insulin action, despite not lowering hepatic lipid content (Samuel, Liu et al. 2007), and in fact PKCε deletion has been associated with increased hepatic lipid deposition (Raddatz, Turner et al. 2011). As well as PKCε being a negative regulator of hepatic insulin action, studies have shown that it also plays a role in adipocytes. Samuel and co-workers showed that although the greatest protection observed was hepatic insulin action, antisense oligonucleotide knockdown of PKCε also resulted in improved adipose insulin sensitivity as measured by insulin-stimulated glucose uptake (Samuel, Liu et al. 2007). In this study, due to the nature of the hyperinsulinaemic-euglycaemic clamp, adipose lipolysis could not be accurately determined due to the high insulin levels effectively abolishing lipolysis. Moreover, in a mouse adipocyte 3T3-L1 cell line, PKCε overexpression was found to enhance IL-6 secretion through an ERK-stimulated pathway, which may lead to systemic IR (Ohashi, Kanazawa et al. 2005). However the negative role of IL-6 on insulin sensitivity (Mohamed-Ali, Goodrick et al. 1997; Bastard, Jardel et al. 2000; Vozarova, Weyer et al. 2001) has recently been brought into question with many studies showing it actually

plays a positive role on hepatic insulin action (Carey, Steinberg et al. 2006; Pedersen and Fischer 2007; Holmes, Mesa et al. 2008; Matthews, Allen et al. 2010; Awazawa, Ueki et al. 2011). Although PKCε activation has been associated with the development of IR in some models (Laybutt, Schmitz-Peiffer et al. 1999), the specific actions of PKCε may also be masked somewhat by activation of other nPKC isozymes, such as PKCδ or θ with lipid-induced IR (Schmitz-Peiffer, Browne et al. 1997; Griffin, Marcucci et al. 1999; Frangioudakis, Burchfield et al. 2009; Frangioudakis, Garrard et al. 2010). It has previously been hypothesised that the spatial regulation of PKCs during activation is crucial. Furthermore Collazos and colleagues demonstrated that PKCs may act in a co-ordinated cascade leading to further activation of isoforms and therefore eliminating a particular isozyme could have impacts on the function of other isozymes that are not necessarily in the same PKC class (Collazos, Diouf et al. 2006). In contrast, an early study showed that reduced PKCε activity was associated with adipocyte dysfunction in a genetic mouse model of IR (Frevert and Kahn 1996).

Models of IR that have been associated with increased PKCε activation have been also exhibited increased levels of the lipid metabolite DAG (Samuel, Liu et al. 2004; Kumashiro, Erion et al. 2011). While PKCε has been associated with muscle IR in these cases, it also appears to play a major role in hepatic IR (Samuel, Liu et al. 2004; Samuel, Liu et al. 2007; Raddatz, Turner et al. 2011).

The activation of PKCs has been implicated in downregulation of key components of the insulin signalling pathway. The mechanism of PKCε-mediated IR has been postulated to result from increased IRS-1 Ser phosphorylation, resulting in reduced Tyr phosphorylation and a defect in the activation of the PI3K-Akt pathway, as well as the promotion of IRS-1 degradation (Zick 2005; Erion and Shulman 2010). Whether this negative modulation of insulin signalling involves direct phosphorylation by PKCε of proximal insulin signalling components such as the InsR and IRS-1 or indirectly through activation of stress signalling serine/threonine kinases including NFκB, Erk and JNK (De Fea and Roth 1997a; De Fea and Roth 1997b; Aguirre, Werner et al. 2002; Samuel, Liu et al. 2007) has not yet been clarified. A direct interaction with the InsR may impair trafficking and sorting of the InsR, without affecting endocytosis (Hribal, D'Alfonso et al. 2001), however in contrast to this it has been reported that PKCε ablation in mice reduces insulin clearance on a chow diet (Schmitz-Peiffer, Laybutt et al. 2007), which depends upon InsR-mediated endocytosis.

As has been discussed previously, activation of PKCs is highly dependent upon DAG which could arise from a number of stimuli other than lipids, including growth-factor signalling pathways (Standaert, Avignon et al. 1996a; Standaert, Avignon et al. 1996b) or high glucose levels (Ishizuka, Hoffman et al. 1989). Furthermore, saturated and unsaturated lipids induce IR by distinct mechanisms (Schmitz-Peiffer 2000). These are hypothesised to involve unsaturated species of DAGs strongly activating PKCs (Hodgkin, Pettitt et al. 1998; Cazzolli, Craig et al. 2002; Cazzolli, Mitchell et al. 2007). A recent study however demonstrated that PKCε activation and downregulation of InsR expression was associated with an increase in the circulation of the FAs palmitate and myristate in Ob/Ob mice (Dasgupta, Bhattacharya et al. 2011). Furthermore, this impairment on the InsR as a consequence of FA activation of PKCε was supported in cell culture models using the saturated FA palmitate (Dasgupta, Bhattacharya et al. 2011). As described earlier saturated lipid species are thought to have a greater impact upon ceramide levels through *de novo* synthesis, rather than DAG-associated PKC activation and are proposed to inhibit insulin action through negative regulation of Akt by activation of PP2A and atypical PKCζ (Schmitz-Peiffer, Craig et al. 1999; Holland, Miller et al. 2011b). This may be an oversimplification though and would require further investigation, as ceramide can incorporate any type of FA onto its backbone through re-modelling pathways, which has been shown to occur *in vivo* with high-fat feeding of predominately saturated or polyunsaturated diets in mice (Frangioudakis, Garrard et al. 2010). The tissue specific effects of PKCε in the pathogenesis of T2D may be opposing in nature, but with tissue cross-talk *in vivo*, these messages lead to the overall physiological phenotype. An example of this is the protective effect of PKCε in the heart following exposure to lipid and hyperglycaemia, where it acts to protect against oxidative stress damage (Sparagna, Jones et al. 2004; Malhotra, Kang et al. 2005), while PKCε activation has been reported to be increased in the kidney of diabetic subjects, whereas ablation of PKCε is associated with renal fibrosis, again indicating a protective role of PKCε in this tissue (Meier, Menne et al. 2007).

1.6 SUMMARY AND AIMS

IR is a common defect in the metabolic syndrome and a key indicator for T2D. The rise in sedentary lifestyle and consumption of an energy dense diet are major factors implicated in the disease pathogenesis. Further understanding the mechanisms involved in inducing IR through nutrient oversupply, in particular lipids, may aid in developing better therapies.

The exact mechanisms involved in the link between increased lipid availability and IR have not been fully elucidated. It is now understood the importance that lipid species play in cellular signalling cascades and therefore increased lipid availability could activate pathways modulating insulin action. The lipid-activated PKC family has been proposed to be involved in the pathogenesis of IR, particularly activation of the novel isozyme, PKCε in liver. This is hypothesised to result from accumulation of lipid intermediates, such as DAG, and its associated activation of PKCε to impair insulin signalling either through direct means or indirectly by further activation of stress signalling kinases. The liver plays a key role in glucose homeostasis through its regulation of glucose levels by gluconeogenesis, and also synthesises and secretes lipids into the circulation for use by other peripheral tissues of the body. Furthermore hepatic IR has been shown to occur first in the face of lipid oversupply.

Many studies have demonstrated reduced IRS-1 Tyr phosphorylation and subsequent PI3K activation as being involved in the reduced actions of tissues to insulin. Tyr phosphorylation of IRS-1 is inhibited by Ser/Thr phosphorylation, in which PKCε has been implicated. We have demonstrated that PKCε KO mice are protected from glucose intolerance induced by a HFD (Schmitz-Peiffer et al. 2007). These mice also exhibit reduced hepatic insulin clearance even on a chow diet which may improve insulin availability for insulin-stimulated glucose disposal without overworking the β-cell in the early development of IR.

The major aims of this thesis were:

- To determine the role of PKCε in regulating hepatic InsR trafficking
- To determine whether altered trafficking impacts insulin signal transduction
- To investigate the mechanisms involved in the development of IR in multiple cellular models of PKCε ablation, using mouse embryonic fibroblasts (MEFs), and primary hepatocytes, following lipid oversupply

To this end studies focussed on proximal components of the insulin signalling pathway such as the InsR and IRS-1, which PKCε has been reported to negatively regulate.

CHAPTER 2

Materials and Methods

2.1 Materials

General reagents of analytical grade were obtained from Sigma (St Louis, USA), Calbiochem (Alexandria, NSW, Australia) and Invitrogen (Victoria, Australia) unless otherwise stated. Labelled 2-deoxy-D-[2,6-^{3}H]glucose and [^{32}P]-orthophosphate were from Amersham (Buckinghamshire, UK). The human insulin analogue Actrapid™ from Novo Nordisk (Copenhagen, Denmark) or fluorescein isothiocyanate labelled insulin from Molecular Probes (Oregon, USA) were used for all insulin stimulation experiments. PKC α, PKCδ, PKCε, caveolin 1, flotillin-1, Rab 11, STAT3 (phospho Tyr 705, Ser 727 and total), SH2B, PTP1B and insulin receptor (β-subunit) antibodies were obtained from BD Transduction Labs (New South Wales, Australia). PKCδ, MARCKS, SH2B and Grb14 antibodies were from Santa Cruz (California, USA). Insulin Receptor (β-subunit), Akt/PKB (phospho Ser473, Thr308 and total), MARCKS (phospho Ser 152/156), p44/42 MAPK (phospho Thr202/Tyr204 and total), Src (phospho Tyr 416), IRS-1 (phospho Ser636/639), JAK2 (phospho Tyr 1107/1108 and total), STAT3 (Ser 727 and total), Early Endosome Antigen 1 (EEA1), SOCS3 and pan-cadherin antibodies were from Cell Signalling Technologies (California, USA). IRS-1 (total) and Grb14 were also purchased from Millipore (California, USA). LAMP-1 antibody was from Developmental Studies Hybridoma Bank (Iowa, USA). The p66Shc (phospho Tyr 239/240) antibody was from Sapphire Bioscience (New South Wales, Australia). IGF-1/ insulin receptor (phospho Tyr1162/1163), IRS-1 (phospho Tyr612) and β-actin antibodies were from Sigma (Missouri, USA). Horseradish peroxidise-linked (HRP-linked) donkey anti-rabbit IgG was from Jackson Labs (Pennsylvania, USA), sheep anti-mouse IgG was obtained from GE Healthcare (Buckinghamshire, UK) and donkey anti-goat HRP linked secondary antibody from Santa Cruz (California, USA). The CEACAM-1 antibody was a kind gift from Professor Andre Marette (Montreal Diabetes Research Centre, Canada). TEMED, 40 % Acrylamide solution and 2 % Bis Solution and 26-well Criterion Gel cassettes were from BIO-RAD (California, USA). Pre-cast 10 and 15 well BIS-Tris (10 % and 4-12 %) and 3-8 % Tris-Acetate gels were from Invitrogen (California, USA). One Shot Top10 Chemically Competent E.coli was also purchased from Invitrogen (California, USA). Complete Protease Inhibitor Tablets and PhosSTOP (containing calf alkaline phosphatase, potato acidic phosphatase, human acidic phosphatase, rabbit PP1, human PP2A and human PTP) tablets were from Roche (New South Wales, Australia). Non-esterified fatty acid (NEFA) assay kits were purchased from Wako Pure Chemical Industries (Osaka, Japan),

insulin ELISA kits were from Linco Research (Missouri, USA) and Adeno-X Rapid Titer Kit was from Clontech (California, USA). Ketamine under the trade name Ketamav 100 was purchased from Mavlab Pty Ltd (Brisbane, Australia). PKCε KO mice, generated by deletion of Exon 1, were a kind gift from Professor Michael Leitges (Biotechnology centre of Oslo University).

All tissue culture plasticware was purchased from Becton-Dickinson (New Jersey, USA). Cell culture media and trypsin was purchased from Invitrogen (Victoria, Australia); a solution containing penicillin, streptomycin and amphotericin B as fungizone (A/A) was from Gibco BRL Life Technologies (Nebraska, USA); foetal calf serum (FCS) was from Thermo Fisher Scientific Australia (Victoria, Australia) except dialysed FCS for primary hepatocytes which was from Roche (New South Wales, Australia). Coverslips were mounted in Immuno-Fluore from MP Biomedicals (Illkirch, France). Polyvinylidene fluoride (PVDF) transfer membranes were purchased from Millipore (New Hampshire, USA) and enhanced chemiluminescence (ECL) reagents were obtained from PerkinElmer Life Sciences (Massachusetts, USA).

2.2 Cell Culture

Isolation of Primary Mouse Embryonic Fibroblasts (MEFs)

Pure C57Bl/6 mice of heterozygous genotype for PKCε were mated, and checked for a vaginal plug, which was observed as day 0.5. On day 10.5, the pregnant female was sacrificed by cervical dislocation, the abdomen wiped with 70 % (v/v) ethanol (EtOH) and the abdominal cavity was opened using scissors. The gut and intestines were pushed aside to expose the embryos which were dissected and placed into 60 mm petri dishes containing 3 mL of saline on ice. Embryos were then removed from the placenta and yolk sac, with each individual embryo placed into a single 60 mm petri dish containing ~2 mL of ice-cold phosphate buffered saline (PBS). Visible visceral organs such as liver and heart were dissected out using a microscope, with the head removed for genotyping. The embryo was then rinsed with PBS before sectioning into approximately 2 mm pieces with forceps and the tissue transferred into a 50 mL Falcon tube containing 5 mL of ice cold 0.05 % (w/v)

trypsin/EDTA solution. This was incubated overnight at 4 °C to diffuse into the tissues. The following day, excess trypsin/EDTA solution was removed without disturbing the embryonic tissue pellet and samples incubated at 37 °C for 30 min. DMEM with 5 mM glucose (low glucose DMEM) containing 10 % (v/v) FCS and 1 % (v/v) A/A was added to the tissue, which was disaggregated by vigorous pipetting with a 5 mL pipette. Undigested tissue was allowed to sediment briefly and the supernatant, containing disaggregated cells, seeded into a 100 mm culture dish and incubated at 37 °C in a 5 % (v/v) CO_2 incubator until cells grew confluent. The cells were then passaged into two T75 Falcon flasks, and termed passage 1, with one flask used in experiments, while the other was grown to confluence and divided into 1mL aliquots and frozen at -80 °C for subsequent use. Three each of wild type and PKCε knockout pools of MEFs were generated and selected after genotyping as described below in section 2.15.1.

WT MEFs		**KO MEFs**	
MEF Pool Nomenclature	**Thesis Nomenclature**	**MEF Pool Nomenclature**	**Thesis Nomenclature**
ε10 WT	WT1	ε3 KO	KO1
ε31 WT	WT2	ε33 KO	KO2
ε30 WT	WT3	ε34 KO	KO3

Table 2.1. Nomenclature of MEF pools used.

MEFs were maintained at 37 °C and 5 % (v/v) CO_2 in low glucose DMEM supplemented with 10 % (v/v) FCS and 1 % (v/v) A/A.

Isolation of Primary Mouse Hepatocytes

Primary hepatocytes were isolated from ~12-17 week old adult male wild type (WT) or PKCε knockout (KO) mice. Mice were anaesthetised by intraperitoneal injection of ketamine (175µg/g.bw) and xylazine (35µg/g.bw) and once withdrawal reflex no longer persisted, the mouse was pinned to a dissection board. The abdomen was wetted with 70 % (v/v) ETOH, cut open and intestines were moved to one side with a sterile cotton tip. A peristaltic pump was used to perfuse the liver with Hanks Buffered Saline Solution (HBSS) (50mM Hepes at pH 7.4 containing 138 mM NaCl, 5.6 mM Glucose, 5.4 mM KCl, 0.34 mM Na_2HPO_4, 0.44

mM $NaHCO_3$) supplemented with 0.5 mM EGTA, warmed to 37 °C, at a rate of 4-5 mL/min, using a 21*g* butterfly needle (Becton Dickinson). The needle was inserted into the inferior vena cava, clamped, and the hepatic portal vein cut. The liver was perfused for 10-15 min, before warmed HBSS supplemented with 2 mM $CaCl_2$ (HBSS-$CaCl_2$) and 1 mg/mL collagenase was then perfused through the liver until digested, as identified by an inflated liver with gaps visible between patches of cells when gently pressed with a sterile cotton tip. The gall bladder was then removed and the liver isolated into ~5-10 mL of HBSS-$CaCl_2$, where the outer membrane of the liver lobes was gently broken with fine forceps and agitated to release the cells, which were then filtered through a 100 µm cell strainer with ice cold HBSS-$CaCl_2$ into a fresh 50 mL Falcon tube, made up to 50 mL and placed on ice. Gentle centrifugation (3 min at 50 *g*) was used to pellet intact, viable cells, while the supernatant was discarded. Cells were resuspended in HBSS-$CaCl_2$ buffer using a sterile plastic pasteur pipette and centrifuged again for 3 min at 50 *g*. This wash was repeated, after which the cells were resuspended in ~5 mL of cold M199 media and filtered through a 100 µm cell strainer into a fresh 50 mL tube. Cells were counted using a haemocytometer, viability was assessed and cells seeded at 3.2×10^5 cells/mL in 6-well tissue culture plates.

To promote adherence, primary hepatocytes were cultured for 4 h in M199 medium supplemented with 1 % (w/v) penicillin G sodium/streptomycin sulphate (P/S), 0.1 % (w/v) BSA, 2 % (v/v) dialysed FCS, 100 nM dexamethasone and 100 nM insulin. Hepatocytes were then rinsed once with warm PBS and medium changed to M199 medium supplemented with 1 % (w/v) P/S, 100 nM dexamethasone and 1 nM insulin if not being used the following day, otherwise insulin was not included in the overnight culturing medium.

2.3 Fatty Acid And Insulin Treatments

Preparation of fatty acid:BSA conjugates

Lipid-containing media stocks of 20x fatty acid (FA):BSA were prepared by dissolving 27.6 g of BSA in 150 mL of low glucose DMEM (for treatment of MEFs) or M199 medium (for treatment of primary hepatocytes) to yield an 18.4 % (w/v) BSA solution. Sodium palmitate was dissolved into 50 mL of the BSA/DMEM solution to yield a final concentration of 8

mM. The resulting FA:BSA conjugate in DMEM and control BSA/DMEM solution were sterilised through a 0.2 μm filter, aliquoted into sterile tubes and stored at -20 °C until used. FA concentration of the couplings was checked regularly by NEFA assay (Wako Pure Chemical Industries Ltd.) as per manufacturer instructions.

Cell Treatments

For FA treatment of MEFs, palmitate:BSA/DMEM and control:BSA/DMEM stocks were diluted 1:20 into low glucose DMEM and the resulting 0.4 mM palmitate 0.92 % (w/v) BSA solution, or control 0.92 % (w/v) BSA solution, was added to the MEFs which were cultured for 24 h. Prior to insulin treatment of MEFs, the media was aspirated, wells washed with warm PBS to remove residual serum, and cells incubated in control or palmitate-containing serum-free media for 2 h. Insulin (100 nM) was then added to the cells for the indicated time at the indicated dose.

For FA treatment of primary hepatocytes, palmitate:BSA/DMEM and control:BSA/DMEM stocks were diluted 1:20 into insulin free M199 culturing media to yield a 0.4 mM palmitate or BSA control as described for MEFs above. Following the 4 h adherence period after isolation, hepatocytes were washed with warm PBS to remove residual serum and insulin before media containing 0.4 mM palmitate or control were added to the primary hepatocytes, which were cultured in an incubator for 24 h, and the final 16 h at 37 °C with 5 % CO_2 prior to 10 nM insulin stimulation for the indicated time.

Following insulin treatment, cells were washed three times with ice-cold PBS on ice and harvested in RIPA lysis buffer (65mM Tris-Cl pH 7.4 containing 150 mM NaCl, 5 mM EDTA, 10 mM NaF, 10 mM $Na_4P_2O_7$, 10 % (v/v) glycerol, 1 % (v/v) NP40, 0.1 % (w/v) SDS, 2 mM phenylmethylsulfonyl fluoride, 100 μM leupeptin, 2 mM benzamidine, 2 mM sodium orthovanadate and one Complete Protease Inhibitor tablet per 50 mL of lysis buffer) unless otherwise indicated.

2.4 Protein Quantitation

2.4.1 Bicinchoninic Acid (BCA) Assay

According to manufacturer instructions, briefly reagent B was diluted 1:50 with reagent A (Pierce). All samples, together with 7 standard BSA solutions (0, 31.3, 62.5, 125, 250, 500, 1000 and 2000 μg/mL) were made up in 100 μL of milliQ water. A 25 μL aliquot of each sample was made up to a concentration between 0.1 and 1000 μg/mL to fit the standard curve and added to a well of a 96-well plate. The diluted reagent (300 μL) was added to each standard and sample in duplicate and incubated for 30 min at room temperature. The absorbances were read at 562 nm (Beckman DU 640B, Beckman Coulter Inc) and sample protein concentrations were determined from the linear curve derived from the standard concentrations.

2.4.2 Bradford Protein Assay

A stock solution of Bio-Rad Assay dye reagent (Bio-Rad Laboratories, Inc.) diluted 1:4 with milliQ water was initially prepared. All samples, together with 7 standard BSA solutions (0, 100, 200, 400, 600, 800, 1000 and 2000 μg/mL) were made up in 100 μL of milliQ water. A 25 μL aliquot of each was added to a well of a 96-well plate and diluted dye reagent (300 μL) was added to each standard and sample in duplicate and incubated for 5 min at room temperature. The absorbances were read at 595 nm (Beckman DU 640B, Beckman Coulter Inc) and sample protein concentrations were determined from the linear curve derived from the standard concentrations.

2.5 Immunoblot Analysis

Cell lysates were sonicated (10 pulses using a Branson Sonifier 250 and microtip at power setting 2 and 20 % duty cycle) and incubated on ice for 15 min before centrifugation at 16,200 *g* for 15 min at 4 °C. The supernatant was retained in a fresh tube and total protein determined by BCA assay (Pierce) as described below. Samples were equalised for protein in the same final volume of RIPA buffer, followed by addition of 4X NuPAGE sample buffer. The samples, typically 10 μg protein per lane were then subjected to SDS-PAGE using either

a 7 % or 10 % pre-cast gel (Invitrogen) or inhouse-cast 26-well gel. Gels were transferred at 0.4 mA for 2 h onto low fluorescence 0.45 µm PVDF membrane (Millipore), which had been pre-activated in methanol for ~30 s, then blocked after transfer by washing for 1 h in Tris-buffered saline containing 0.05 % (v/v) Tween-20 (TTBS), and 5 % (w/v) skim milk powder. Membranes were incubated in primary antibody diluted into TTBS containing 5 % (w/v) BSA, 0.05 % (v/v) phenol red and 0.02 % (w/v) sodium azide) overnight on rollers at 4 °C. The membranes were then washed in TTBS and incubated for one hour at room temperature with secondary antibody diluted in TTBS and 1 % (w/v) milk powder. All secondary antibodies were diluted 1:5000. The membranes were then washed in TTBS before ECL reagent was added to the membranes and bands visualised on X-ray film developed by a 100plus automatic x-ray film processor (All-Pro Imaging) and quantified using the computer program Image J (National Institutes of Health, USA).

Antibody dilutions used are tabled below:

Antibody	Dilution Used	Supplier
pInsR (Tyr 1162/1163)	1:500	Sigma
pIRS-1 (Tyr 612)	1:1000	Invitrogen
pIRS-1 (Ser 636/639)	1:1000	Cell Signaling Technology
pAkt (Ser 473)	1:1000	Cell Signaling Technology
pErk 1/2 (Thr 202/Tyr 204)	1:1000	Cell Signaling Technology
p66Shc (Tyr 239/240)	1:1000	Sapphire Bioscience
PKC epsilon	1:500	BD Transduction Labs
Total InsR (Beta subunit)	1:1000	BD Transduction Labs & Cell Signaling Technology
Total IRS-1	1:1000	Millipore
Total Akt	1:1000	Cell Signaling Technology
Total Erk 1/2	1:1000	Cell Signaling Technology
Ceacam1	1:500	Professor Andre Marette
Beta Actin	1:5000	Sigma
Grb14	1:250	Santa Cruz & Millipore
pSrc family Tyr kinase (Tyr 416)	1:1000	Cell Signaling Technology
SOCS3	1:1000	Cell Signaling Technology
Flotillin-1	1:1000	Cell Signaling Technology
Pan Cadherin	1:1000	Cell Signaling Technology
Rab 11	1:1000	Cell Signaling Technology
EEA1	1:1000	Cell Signaling Technology
Donkey ant-rabbit	1:5000	Jackson Labs
Sheep anti-mouse	1:5000	GE Healthcare

Table 2.2 Antibody dilutions used for immunoblotting.

2.6 Subcellular Fractionation

Cells were grown to confluence in a 15 cm plate. After serum starvation for 2 h, cells were treated with or without 100 nM insulin for the indicated time at 37 °C. Media was then aspirated and plates placed on ice and washed three times with ice cold PBS and harvested on ice in 1 mL of fractionation buffer (20mM Hepes at pH 7.4 containing 250 mM Sucrose, 1mM EDTA, 10 mM NaF, 2 mM phenylmethylsulfonyl fluoride, 100 μM leupeptin, 2 mM benzamidine, 2 mM sodium orthovanadate and one Complete Protease Inhibitor tablet per 50 mL of lysis buffer). Cells were disrupted by Nitrogen cavitation (450 psi nitrogen gas for 15 min in a 50mL nitrogen chamber on ice). Lysates were collected and unbroken cells removed by centrifugation at 500 *g* for 10 min at 4 °C. Supernatants were transferred to a fresh tube and protein content measured by Bradford assay (Biorad). Equal amounts of protein (500 μg) were loaded onto a 15 % iodixanol Opti-Prep gradient (Axis-Shield) which was made up to 11 mL with fractionation buffer in an Optiseal™ centrifuge tube (5/8’’; Beckman) and centrifuged in an NVT 65.1 rotor at 100,000 *g* for 4 h at 4 °C to form a continuous gradient. Fractions were removed from the top of the gradient and from each fraction, 65 μL of sample lysate was made up with 4X NuPAGE sample buffer, incubated for 10 min at 70 °C and run on a 4-12 % or 7 % pre-cast gel for analysis by immunoblotting for membrane markers and insulin signalling components.

2.7 Insulin Uptake Assays

2.7.1 Insulin Uptake by Fluorescence Microscopy

MEFs were seeded in poly-L-lysine coated (5 *μ*g/mL) 96-well glass bottom plates and cultured in DMEM supplemented with 10 % (v/v) FCS and 1 % (v/v) A/A at 37 °C and in 5 % (v/v) CO_2 in a humidified incubator. MEFs were grown to around 90-95 % confluency before being serum-starved for 2 h in DMEM without FCS. Cells were then incubated with 100 nM fluorescein isothiocyanate (FITC) labelled insulin (Molecular Probes) for 10 min at 37 °C. To stop ligand internalisation, medium was removed by rapid inversion of cell culture plates and plates placed on ice. Cell surface-bound insulin was removed by incubating the cells in an ice-cold acid wash solution (0.2 M acetic acid + 0.5 M NaCl, pH 4.5) with gentle

shaking for two 5 min washes followed by two washes with ice-cold PBS for 5 min each. Cells were immediately fixed with 4 % (w/v) paraformaldehyde for 15 min at 37 °C and nuclei stained with 4'-6-Diamidino-2-phenylindole (DAPI). The cells were left at 4 °C in PBS overnight, with quantitative analysis of the FITC-insulin uptake was performed on ~3 x 10^3 cells by an automated acquisition and analysis system (Image Xpress Micro (IXM), Molecular Devices, Sunnyvale, CA). Nine images were collected from each well, averaging 20-30 cells per image. The average integrated intensity of the FITC-insulin signal per cell was calculated for each well using the IXM system and the data expressed as a percentage of uptake relative to WT cells.

2.7.2 Insulin Uptake by FACS

MEFs were incubated in serum free medium for two hours prior to insulin stimulation. For the last 20 min, MEFs were incubated with 50 μM monensin in order to block receptor recycling and therefore enable measurement of insulin uptake only. Insulin receptor internalisation was stimulated with the addition of 100 nM FITC-insulin for 10 min at 37 °C. To stop receptor internalisation, medium was removed by rapid inversion of cell culture plates and cells placed on ice, followed by two washes in ice-cold PBS for 5 min each at 4 °C with gentle shaking. Non-internalised FITC insulin was stripped from the cell surface with an acid wash (DMEM, 0.2 % (w/v) BSA adjusted to pH 4.5 with HCl) three times for 5 min each while gently shaking. This was followed by three lots of 5 min ice-cold PBS washes before cells were trypsinised (0.25 % (w/v) trypsin in 1 mM EDTA solution) on ice for 15 min in 1 mL. Cells were harvested and centrifuged at 1,200 *g* for 5 min at 4 °C. Cells were then washed twice in ice-cold FACS buffer (PBS, 0.1 % (w/v) BSA, 0.1 % (w/v) sodium azide) and resuspended in FACS buffer containing 2 % (w/v) paraformaldehyde to fix the cells, in the absence of light at 4 °C for 4-16 h. Cells were centrifuged at 1,200 *g* for 5 min at 4 °C and washed three times in ice-cold FACS buffer, before being passed through a 70 μm filter and analysed using a FACSCanto™ instrument. Data was analysed using FlowJO software (Treestar, Oregon, USA).

2.8 DNA Assay and Insulin Receptor Half-Life Determination

MEFs seeded into a 6-well plate were treated with a supramaximal dose of insulin (1 μM) to stimulate insulin receptor activation and recycling in the presence of 50 μg/mL cycloheximide for 24 h, in order to assess the rate of receptor degradation over time. Cells were harvested on ice in 250 μL RIPA buffer following two washes with ice cold PBS, sonicated and DNA assay performed. DNA in MEF lysates following sonication was assayed with a microplate fluorescence reader (BMG Labtech, Victoria, Australia) using Sybr-green (Molecular Probes). Sybr-green was diluted 1:10,000 in PBS and 200 μl added to 10 μl samples in a 96-well plate. Fluorescence in each well was measured using a fluorescence plate reader fitted with a 485/518 nm excitation/emission filter set. A standard curve was generated by plotting fluorescence units against DNA concentration for a series of Salmon Sperm DNA standards in RIPA buffer. MEF lysate volumes were adjusted to normalise DNA content. Samples were subjected to SDS-PAGE and immunoblotting, after addition of 4x NuPAGE sample buffer.

2.9 Membrane Fluidity Analysis by 2-Photon Microscopy with Laurdan Staining

Cells were seeded onto glass coverslips and grown to around 70-80 % confluence. MEFs were then incubated for 40 min at 37 °C with a final concentration of 10 μM Laurdan (Molecular Probes) added to the growth medium. Following this incubation, the medium was aspirated off, the cells washed three times with warm PBS and fixed for 20 min at room temperature with 4 % (w/v) paraformaldehyde. The cells were then imaged with a 2-Photon microscope (Leica DM IRE2 microscope). Laurdan was excited at 800 nm and emission intensities were simultaneously recorded in the range of channel 1: 400-460 nm and channel 2: 470-530 nm. Intensity images were converted into Generalised Polarisation (GP) images (WiT software). The fluorescent lipid probe, Laurdan has improved the ability to measure membrane fluidity through its lipophilic properties enabling it to incorporate into membrane phospholipid tails. Laurdan exhibits a blue shift in emission when more water molecules are included in the membrane lipid bilayer. As such the degree of membrane order or fluidity can be expressed as a normalisation of the ratio of intensity from the two emission channels

used in 2-photon microscopy. This is referred to as the GP value. Values are in the range of -1 to +1, with more ordered domains closer to +1 (Gaus, Zech et al. 2006). The outer membrane area of the cell was defined as the region of interest and the mean GP of this region was determined using ImageJ. GP values were then corrected using the sensitivity correction factor (G-factor) which is the ratio of emission intensity of Laurdan in DMSO between the two channels for each experiment.

2.10 Adenoviral Methods

2.10.1 Adenoviral Amplification

Recombinant adenovirus for the expression of wild type PKCε (WT PKCε) was generated as described previously (Cazzolli, Craig et al. 2002) . Briefly, a histidine-tagged WT PKCε construct was linearised and subcloned into the pAdEasy system, enabling co-expression of GFP and PKCε, and a pAdEasy-derived virus expressing GFP only was used in control infections as previously described (He, Zhou et al. 1998). All recombinant viruses were amplified from concentrated stocks using the human embryonic kidney (HEK)-293 cell line. HEK cells were maintained in high glucose DMEM, supplemented with 10 % FCS (v/v), and 1 % A/A (w/v). One 150 cm^2 flask of HEK cells was infected with 10 μL of concentrated virus stock (~10^5 infectious units (ifu)/μl), at approximately 75-80 % confluency. After five days at least 90 % of cells had detached and lysed. The media and remaining cells were collected from the flask, sonicated for 20 pulses (20 % duty cycle and output control level 4), and centrifuged for 10 min at 1,000 *g*. The supernatant, containing the virus, was diluted into fresh DMEM and used to infect ten 150 cm^2 flasks of HEK cells at around 75-80 % confluency. This second round of infection was left for 48 h, until most cells were easily detached (but had not lysed), indicating they had been successfully infected with the virus. The cells were dislodged from the surface of the flasks by gentle agitation, and transferred into 50 mL tubes. The suspension was centrifuged (250 *g* for 10 min at room temperature) and the supernatant was discarded. The cell pellets were pooled and resuspended in 5 mL of sterile PBS. The cells were stored at -80 °C until purification.

2.10.2 Purification by Caesium Chloride Density Gradient Centrifugation

Adenovirus-containing cells were thawed and sonicated (20-25 pulses – 30 % duty cycle and output level 4). The supernatant was centrifuged for 10 min at 3,000 *rpm*, supernatant collected, made up to 6.8 mL with sterile PBS and transferred into a Beckman Optiseal™ centrifuge tube. The tube was filled by addition of 4 mL of saturated CsCl:PBS, mixed by gentle pipetting and the tube sealed. Centrifuge was carried out at 40,000 *rpm* for 4 h at 10 °C in an NVT 65 rotor. The formation of a CsCl gradient resulted in a thin, white band of virus. A 25 g needle was inserted into the top of the tube, while a 2 mL syringe with a 21 g needle was used to puncture the side of the tube just below the virus band to be harvested. The harvested virus was dialysed in a 0.5-3 mL 10,000 MW dialysis cassette (Pierce) at 4 °C for 2.5 h with three changes of dialysis buffer (10mM Tris-Cl at pH 7.4 containing 400 mM NaCl and 10 mM $MgCl_2$) after 1 h and then each subsequent 30 min. The virus was removed from the dialysis cassette with a needle and transferred to a sterile 1.5 mL eppendorf tube. Sterile glycerol was added to a final concentration of 10 % (v/v) and the virus aliquoted and stored at -80 °C.

2.10.3 Estimation of Viral Titre

Adenovirus titre was estimated using an Adeno-X Rapid Titre kit (Clontech). HEK cells were seeded in a 12-well plate at 5 x 10^5 cells per well in high glucose DMEM as described above. Concentrated viral stock was serially diluted in DMEM to yield dilutions between 10^{-2} and 10^{-6} fold, and 100 µl of diluted virus added drop wise per well in duplicate. Cells were then incubated for 48 h in a 5 % (v/v) CO_2 humidified incubator at 37 °C. Media was aspirated and cells were dried, fixed with 100 % methanol and incubated at -20 °C for 10 min. Methanol was aspirated and wells were washed three times with PBS containing 1 % (w/v) BSA (PBS/BSA). Following the final wash, 0.5 ml mouse anti-hexon (a viral coat protein) antibody, diluted 1:1000 in PBS/BSA, was added to wells and incubated for 1 h at 37 °C with occasional mixing. Cells were then washed three times with PBS/BSA and rat anti-mouse antibody conjugated to HRP, diluted 1:500 in 0.5 mL PBS/BSA, was added followed by incubation for 1 h at 37 °C. The secondary antibody solution was then aspirated, cells washed three times with PBS/BSA and 500 µl of 1 x Diaminobenzidine (DAB) working solution (10 x DAB substrate diluted 1:10 with peroxidise buffer) added followed by incubation at room temperature for 10 min before aspiration of DAB solution and addition of

1 mL PBS. Wells were then examined under a microscope with a 20x objective. Three fields of view were examined per well. Virus-positive cells were identified as those stained brown. The mean number of positive cells were obtained and used to calculate viral titre as ifu/ml.

2.10.4 Adenoviral Infection of MEF Cells

MEFs (3 x 10^4 cells per well of a 6-well plate) and incubated for 24 h when they had grown to approximately 60 % confluency. Media was then changed to low glucose DMEM containing 10 % (v/v) FCS only and the amount of virus required to give 80–100 % infection efficiency was individually determined by visualizing GFP expression using fluorescence microscopy after 48 h. This was found to be 5 x 10^7 ifu/well of a 6 well plate and 28.2 x 10^7 ifu/dish for a 10 cm dish for both WT PKCε and control pAdEasy-derived virus expressing GFP only. At 5-6 h post infection, media was changed to DMEM with 10 % (v/v) FCS only and incubated for a total of 48 h in a 37 °C, 5 % (v/v) CO_2 humidified incubator. Experiments for infected cells were then carried out as described for uninfected cells.

2.11 Cloning Of Retrovirus and Generation of Stable MEF Cell Lines Re-Expressing WT PKCε

2.11.1 PKCε Construct Preparation for Making Retrovirus

Rat PKCε cDNA, previously cloned into the Gateway entry vector, pDonR by Sakura Narasimhan (The Garvan Institute of Medical Research, Sydney) was subcloned into the Gateway retroviral vector pQXCIP by standard Gateway techniques (Invitrogen). This construct was used to transform Top10 competent cells, which were then streaked on agar plates containing kanamycin and incubated overnight at 37 °C. Several colonies were selected and shaken at 37 °C in 2 mL of LB media with kanamycin for 8 h. Subsequently 1 mL was diluted with 1 mL of 100 % glycerol and stored at -80 °C as a glycerol stock, while the remainder was used for DNA miniprep (Promega) for confirmation of PKCε presence by restriction enzyme analysis. The glycerol stocks of confirmed constructs were grown up for a maxiprep (Promega) to yield sufficient stocks of DNA to be used for transfecting PlatE cells for retroviral production.

2.11.2 Generation of PKCε Retrovirus with PlatE Cells

PlatE cells were seeded at a density of 1 x 10^5 into a 10 cm dish one day prior to transfection, in high glucose DMEM with 10 % (v/v) FCS and no antibiotics. The following day, WT PKCε subcloned into the retroviral vector pQXCIP (pQXCIP/WT PKCε) or as a control, empty pQXCIP vector, were transfected into PlatE cells with lipofectamine 2000 (Invitrogen): PlatE cells were washed twice with PBS and 5 mL of Opti-MEM was added to each 10 cm dish. Dishes were transfected with 15 µg DNA, diluted in Opti-MEM and complexed 1:1 µg/mL with lipofectamine 2000. The complexes were added dropwise to the cells and incubated for 3 h at 37 °C before media was removed and replaced with 8 mL high glucose DMEM with 10 % (v/v) FCS and no antibiotics. After a further 24 h, media was removed from the cells and replaced with 6 mL of DMEM (10 % (v/v) FCS, no antibiotics) and incubated for 48 h. The media was harvested from the cells and centrifuged at 3,000 *rpm* for 5 min to remove any cells. The supernatant was filtered through a 0.45 µm filter (Amicon) before concentration using an Amicon 100 kDa tube and centrifuged at 4,000 *g* for 15 min to concentrate the virus. The concentrated virus was resuspended to half the original volume in low glucose DMEM with 10 % (v/v) FCS and no antibiotic. Retroviral stocks were then aliquoted and stored at -80 °C until use. The PlatE cells remaining on each dish were harvested and analysed for PKCε expression by immunoblotting.

2.11.3 Infecting MEFs with Retrovirus

MEFs were seeded into 10 cm dishes and grown to 50 % confluency. Media was aspirated and cells washed twice with PBS. A 2 mL retroviral aliquot was diluted to 6 mL in low glucose DMEM (10 % (v/v) FCS, no antibiotics) and added to the dishes. Polybrene was added to a final concentration of 8 µg/mL and cells incubated for 36 h at 37 °C. Media was then removed, cells washed twice with PBS and 8 mL of low glucose DMEM selection media containing 10 % (v/v) FCS, 1 % (v/v) A/A) and 2 µg/mL puromycin was added to the cells. MEFs were cultured in this medium until untransfected MEFs had detached, as determined by comparison with a non-infected dish. Selection of cells expressing the retrovirus were used as a stable MEF cell line re-expressing WT PKCε. Cells selected after infection with empty vector served as control. All experiments were carried out on these MEFs as described for uninfected MEFs.

2.12 Incorporation of Palmitate into Lipid Pools

MEFs were seeded in a 12 well-plate the day prior to palmitate exposure. Cells were treated with 0.4 mmol/l palmitate coupled to 0.9 % BSA (w/v) and 0.74 MBq/ml [^{14}C] palmitate (1.76 TBq/mol) in ethanol for 24 h. To measure [^{14}C] palmitate incorporation, media was aspirated and cells washed three times with ice-cold PBS before harvest in 1 mL lysis buffer of milliQ water with 0.1 % (v/v) SDS, containing one Complete Protease Inhibitor tablet per 50 mL. Lipids were extracted from the cells according to the Folch method (Folch, Lees et al. 1957) for analysis by TLC as described previously (Magnani, Brockhaus et al. 1981). Briefly, 10 µL of the cell lysate was kept to assay total protein content, while the lipids were extracted with the addition of 1 mL of the Folch extraction solution chloroform:methanol (2:1, v/v) to each sample, which was then vortexed briefly and incubated for 10 min at room temperature, vortexed again and incubated for another 10 min. Samples were then centrifuged at 16,200 *g* for 10 min at room temperature. The upper aqueous phase was transferred to a new eppendorf tube, while 125 µL of water was added to the remaining, lower organic phase. This was vortexed, incubated for 10 min, vortexed again and incubated for a further 10 min before being centrifuged at 16,200 *g* for 10 min at room temperature. The upper aqueous wash was removed and pooled with the previous aqueous phase. The remaining lipid-containing organic phase was dried under nitrogen, redissolved in 25 µL of 100 % ethanol and 2 µL transferred to a vial containing 4 mL of scintillation fluid for counting to determine the total radioactivity in the extracted lipids. A 20 µL aliquot of the remainder of the samples, as well as lipid standards, were spotted onto TLC plates, pre-activated with methanol, and plates run in a pre-equilibrated TLC tank with 100 mL of hexane:diethylether:acetic acid (80:18:2) for 30-45 min when the solvent system had ascended to approximately 1 cm from the top of the plate. The plate was air dried for 5 min and exposed to Kodak MR film for 24 h at -80 °C. After developing the film, the plate was exposed to iodine for 24 h to visualise migration of lipid standards. The ^{14}C-labelled lipid spots comigrating with diacylglycerol, triglyceride and cholesterol ester standards were individually scraped, placed in 4 mL of scintillation fluid, vortexed briefly and counted by liquid scintillation spectrometry.

2.13 Electron Microscopy (EM)

To analyse MEF cells by electron microscopy, cells were fixed with a solution of 4 % (w/v) paraformaldehyde (PFA), made fresh from a stock 20 % (w/v) solution, 0.1 % (w/v) glutaraldehyde (EM grade) in 0.12 M phosphate buffer, pH 7.4. Fixed cells were then prepared for electron microscopy by Katanyu Pongstaporn and David Ryugo (Neuroscience program, Garvan Institute of Medical Research, Sydney, Australia). In the case of liver tissue, mice aged 16-18 weeks were anaesthetised by intraperitoneal injection of ketamine (175 µg/g.bw) and xylazine (35 µg/g.bw) and once withdrawal reflex no longer persisted, the mouse was pinned to a dissection board. The abdomen was wet with 70 % (v/v) ETOH, cut open and intestines moved to one side with a sterile cotton tip before cutting up through the diaphragm on either side of the sternum and the flap of skin was pinned down. The peristaltic pump was primed with warm PBS and turned up to a rate of 10-12 mL/min. The heart was supported with forceps and while still beating a 25 *g* needle was inserted into the lateral left ventricle. The pump was then turned on, the right atrium cut so blood flowed out and PBS pumped around the systemic circulation until the blood cleared. PBS was then replaced with PFA-phosphate buffer for 3-5 min until the mouse became rigid. Following fixation, the liver was removed from the mouse and placed in fixative solution for approximately 2 h. Liver tissue was then cut into thin sections (<1 mm) with a razor blade and placed in 1 % (v/v) osmium tetroxide solution for 15-30 min before washing six times for 5 min each in maleate buffer (pH 5). Liver tissue was then placed in 1 % (v/v) uranyl acetate overnight at 4 °C and the following day, the liver was dehydrated with resin (polybed 812), which was polymerized at 60 °C and the liver placed into a BEEM capsule-polymerize resin and ultrathin sections were collected for examination by electron microscope.

MEF cells used for electron microscopy were grown to approximately 70 % confluence in a 10 cm dish in a 37 °C incubator with 5 % (v/v) CO_2. Medium was aspirated from the dish, cells washed three times with ice-cold PBS and fixed with a solution of 4 % (w/v) paraformaldehyde (PFA), 0.1 % (w/v) glutaraldehyde (EM grade) in 0.12 M phosphate buffer, pH 7.4. Following fixation for 20 min, cells were prepared for EM as described above for the liver once it had been fixed and sectioned.

2.14 Glucose Transport in Primary MEFs

Primary MEFs, seeded in a 12-well plate were washed with 1 mL pre-warmed KRH buffer (136 mM NaCl, 4.7 mM KCl, 1.25 mM $MgS0_4$, 1.2 mM $CaCl_2$, 20 mM Hepes, pH 7.4) per well, and then incubated at 37 °C in KRH Buffer + 0.2 % BSA in the presence or absence of 100 nM insulin for 30 min. Non-specific uptake was measured by the inclusion of 10 μM cytochalasin B. Ten minutes before the end of the incubation, 50 μL of START solution (2 mM 2-deoxyglucose, 10 μCi/mL 2-deoxy-D-[2,6-3H] glucose in KRH) was added to the wells. The plates were rinsed rapidly with ice-cold PBS, and the cells lysed by incubating in 1 mL 10 M KOH at 37 °C for approximately 30 min. The samples were homogenised by pipetting up and down, and 800 μL of the sample was mixed with 10 mL scintillation fluid, and counted for radioactivity. The remainder of the lysate was used for protein determination by BCA assay.

2.15 Animal Studies

The generation and maintenance of wild-type (WT) and PKCε knockout mice was described previously (Schmitz-Peiffer, Laybutt et al. 2007). Ethical approval for mouse studies was granted by the Garvan/St Vincent's Hospital Animal Ethics Committee. Male mice at 10-14 weeks of age were fed either a standard chow diet (10.88 kJ/g; 8 % fat, 21 % protein and 71 % carbohydrate; Gordon's Specialty Stock Feeds, Yanderra, NSW, Australia) or with a lard-based high-fat diet prepared in-house (19.67 kJ/g, 45 % fat, 20 % protein and 35 % carbohydrate, based on rodent diet D12451; Research Diets, New Brunswick, NJ, USA) for one week. For glucose tolerance tests, mice were fasted for 6h prior to intraperitoneal injection of 2 g/kg glucose (ipGTT) and blood glucose was measured at the indicated times using an Accu-Chek II (Roche Nutley, NJ, USA) blood glucose monitor.

2.15.1 Mouse Genotyping

Mice were maintained on a hybrid 129/Sv x C57BL/6 background using PKCε heterozygous breeding pairs; age-matched wild-type and PKCε KO littermates were used for experiments.

Genotyping of progeny was performed by PCR. Primer sets to differentiate between PKCε wild type, heterozygous, and homozygous knockout were:

KO forward primer TACCCGTGATATTGCTGAAGA;

wild type forward primer CCCCCACTCCCCGCCCCGAC;

and knockout reverse primer GCTGTTGGTCTTTTGCTTGGTG.

These 3 primers were all used in the same PCR reaction and generated products of 200 bp for WT, 400 bp for KO, and both 200 bp and 400 bp for heterozygous. Mice were maintained on a mixed C57BL/6 and 129 background.

2.15.2 L-Arginine Stimulated *In Vivo* Insulin Secretion

Mice at 9-12 weeks of age were fed either a standard chow diet or a lard-based high-fat diet prepared in-house as described previously (Raddatz, Turner *et al.* 2011) for one week. Mice were fasted for 6 h prior to intraperitoneal injection of 1.5 g/kg L-arginine. Blood glucose levels were measured at 0, and 9 min using an Accu-Chek II (Roche Nutley, NJ, USA) blood glucose monitor, while blood samples were also taken at these time points into 18 mM EDTA/saline and centrifuged at 13,000 *rpm* for 1 min. Supernatant was stored at -80ºC for analysis of insulin levels by ELISA (Chrystal Chem Inc, USA). After 10 min, mice were culled by cervical dislocation, tissues harvested and snap frozen in liquid nitrogen until analysed for signalling by immunoblotting.

2.16 Statistical Analysis

Differences among groups were assessed by unpaired Student's t test or ANOVA using Tukey-Kramer post hoc test. These analyses were performed using GraphPad Prism 5 (Graph-Pad Software, La Jolla, CA, USA) software. Data are presented as mean ± standard error of the mean (SEM) unless otherwise stated. Deviations were deemed significant at $P < 0.05$.

CHAPTER 3

The Effect of PKCε on Insulin Receptor Trafficking

3.1 Introduction

In the insulin resistant state, the peripheral tissues of the body exhibit reduced sensitivity to the actions of insulin. To compensate for this, the body increases circulating insulin levels to overcome the reduced clearance of glucose from the plasma into insulin sensitive tissues, such as muscle and adipose tissue. The two major mechanisms which drive this insulin increase are (1) enhanced insulin secretion from the β-cells of the pancreas and (2) reduced insulin clearance, due to reduced InsR internalisation in the liver, which is responsible for the greatest removal of insulin from the circulation. This reduction in the ability of the liver to clear insulin efficiently, thereby increasing circulating levels of insulin, has been documented in previous studies of both insulin resistant and diabetic humans and rodents (Duckworth, Bennett et al. 1998).

It is critical that the InsR is able to bind and respond to the presence of ligand by internalising the ligand that subsequently gets degraded in lysosomes, in order to prevent chronic hyperinsulinemia. However the resulting internalisation is also important for dampening the insulin response, by reducing the number of PM-localised InsRs. Studies in 3T3-L1 adipocytes as well as primary rodent and HepG2 liver cell lines where InsR activity was measured in different cellular compartments following insulin treatment, illustrated that the InsR is most highly activated upon reaching endosomes, providing a platform from which to signal (Burgess, Wada et al. 1992; Kublaoui, Lee et al. 1995). Trafficking of the InsR and therefore insulin clearance can be perturbed by a variety of factors including InsR cellular localisation, PM integrity, activation of negative regulators of the InsR and alterations in complexes required to stimulate receptor internalisation, such as the presence of the adapter protein Ceacam1 in liver. Ceacam1 has been identified as a specific InsR substrate in liver but not in muscle or adipose tissue (Poy, Yang et al. 2002) and is thought to be part of a complex of proteins required for internalisation of the occupied InsR. Ceacam1 can therefore participate in modulation of circulating insulin levels, InsR localisation and trafficking to regulate InsR signalling through promoting its internalisation, and by extension, de-activation.

Alterations in cellular lipid content and composition may detrimentally impact InsR trafficking, with membrane lipid species, such as the ganglioside GM3 implicated in controlling membrane movement of the InsR (Kabayama, Sato et al. 2007). Membrane

fluidity, which can alter depending on membrane lipid composition, has also been identified to affect insulin action and activity of the InsR (Pilch, Thompson et al. 1980; Whitesell, Regen et al. 1989). Various PKC isoforms have been reported to interact with the InsR and regulate its intracellular fate and localisation (Formisano, Oriente et al. 1998). The novel isozyme, nPKCε, has been associated with development of IR in humans and various rodent models (Considine, Nyce et al. 1995; Schmitz-Peiffer, Browne et al. 1997; Samuel, Liu et al. 2004). Furthermore, nPKCε has been linked to diminished InsR activity and insulin action in insulin target tissues, either through direct association or indirect, undefined mechanisms (Schmitz-Peiffer, Browne et al. 1997; Samuel, Liu et al. 2007; Schmitz-Peiffer, Laybutt et al. 2007).

Previously it has been shown that antisense oligonucleotides against PKCε can reverse short-term diet-induced glucose intolerance in rats. This was reported to be achieved in part through improved hepatic insulin sensitivity and enhanced InsR kinase activity, as PKCε may directly associate with the InsR (Samuel, Liu et al. 2007). Consistent with this, recent research has shown that global PKCε KO mice are protected from short and long-term HFD-induced glucose intolerance (Schmitz-Peiffer, Laybutt et al. 2007; Raddatz, Turner et al. 2011). However, after 16 weeks of both high fat and chow feeding, hepatic insulin clearance was reduced in PKCε KO animals during a glucose tolerance test (GTT) (Schmitz-Peiffer, Laybutt et al. 2007). If the ablation of PKCε increases InsR kinase activity, as reported by Shulman and colleagues (Samuel, Liu et al. 2007), it would be expected that this would promote InsR internalisation, but the opposite was observed in global PKCε KO mice. These apparent differences may be explained by a number of differences in experimental design such as animal model, length of intervention and deletion strategy, despite the similar overall metabolic protection observed in both studies.

In the insulin resistant state the loss of InsR activity and the associated decrement in insulin internalisation can over time exacerbate the condition by leading to the development of chronic hyperinsulinemia. However, enabling the hepatic InsR to modulate insulin levels through clearance only during times of demand, such as ingestion of a meal, could acutely increase the circulating levels of insulin without over-stimulating the β-cell. This would help overcome the reduced insulin sensitivity displayed by the peripheral target tissues and thus restore glucose disposal. In this chapter, the mechanism leading to reduced insulin clearance previously observed in PKCε KO mice was investigated using MEFs as a cell model.

3.2 METHODS

3.2.1 Subcellular Fractionation

Cells were grown to confluence in a 15 cm plate. After serum starvation for 2 h, cells were treated with or without 100 nM insulin for the indicated time at 37 °C and media was aspirated, plates washed with ice cold PBS. Cellular fractionation (section 2.6) and analysis by western blot (section 2.5) was performed as described previously.

3.2.2 Insulin Uptake by Fluorescence Microscopy

MEFs were seeded and grown to around 90-95 % confluence in poly-L-lysine coated (5 μg/mL) 96-well glass bottom plates, serum-starved for 2 h in DMEM without FCS. Cells were then incubated with 100 nM FITC-labelled insulin for 10 min at 37 °C. Quantitative analysis of the FITC-insulin uptake was performed as described in section 2.7.1.

3.2.3 Insulin Uptake by FACS

MEFs were incubated in serum free medium for two hours prior to insulin stimulation. For the last 20 min, MEFs were incubated with 50 μM monensin in order to block receptor recycling and therefore enable measurement of insulin uptake only. Insulin receptor internalisation was stimulated with the addition of 100 nM FITC-insulin for 10 min at 37 °C. Cells were prepared for FACS analysis of FITC-insulin uptake as described in section 2.7.2.

3.2.4 DNA Assay and Insulin Receptor Half-Life Determination

MEFs seeded into a 6-well plate were treated with 1 μM insulin in the presence of 50 μg/mL cycloheximide for 24 h, in order to assess the rate of receptor degradation over time. Cells were harvested on ice in 250 μL RIPA buffer following two washes with ice cold PBS, sonicated and DNA assay performed. MEF lysate were harvested and adjusted to normalise

DNA content as described in section 2.8. Samples were subjected to SDS-PAGE and immunoblotting, after addition of 4x NuPAGE sample buffer.

3.2.5 Adenoviral Infection of MEF Cells

MEFs were seeded and allowed to grow to approximately 60 % confluency. At 5-6 h post infection, media was changed and MEFs were infected with virus as described in section 2.10.

3.2.6 Retroviral Infection of MEF Cells

Cells selected for expressing the retrovirus, due to puromycin resistance were used as a stable MEF cell line re-expressing WT PKCε, as described in section 2.11. Cells selected after infection with empty vector served as control. All experiments were carried out on retroviral expressing MEFs as described for uninfected MEFs.

3.2.7 Membrane Fluidity Analysis by 2-Photon Microscopy with Laurdan Staining

Cells were seeded onto glass coverslips and grown to around 70-80 % confluence. MEFs were then incubated for 40 min at 37 °C with a final concentration of 10 μM Laurdan (Molecular Probes) added to the growth medium. Following this incubation, cells were prepared to be imaged with a 2-Photon microscope (Leica DM IRE2 microscope) as described in section 2.9. Laurdan was excited at 800 nm and emission intensities were simultaneously recorded in the range of channel 1: 400-460 nm and channel 2: 470-530 nm. Intensity images were converted into Generalised Polarisation (GP) images (WiT software). The outer membrane area of the cell was defined as the region of interest and the mean GP of this region was determined using ImageJ. GP values were then corrected using the G-factor, which is described in section 2.9.

3.2.8 Electron Microscopy (EM)

MEF cells and fresh liver were fixed and prepared for electron microscopy as described in 2.13. Images were taken by the Garvan EM technician Katanyu Pongstaporn.

3.2.9 Statistical Analysis

Student's t test was performed using GraphPad Prism 5 (Graph-Pad Software, La Jolla, CA, USA) software. Data are presented as mean ± standard error of the mean (SEM). Deviations were deemed significant at $P < 0.05$.

3.3 Results

3.3.1 PKCε null MEFs display reduced insulin internalisation as determined by fluorescence microscopy

In order to investigate the effect of PKCε ablation on insulin uptake, MEFs were generated from embryos of PKCε heterozygous mice bred on a pure C57BL/6 background as previously described in section 2.2. Presence or absence of PKCε expression was confirmed by PCR and western blot analysis (Figure 3.3.1). Three independent paired PKCε KO and WT MEF cell lines were generated.

Figure 3.3.1 PKCε expression in three separate WT and KO pools of MEFs.
Embryos were harvested from the womb of the pregnant female on embryonic day 12 and grown to confluence. Cells were harvested in RIPA buffer and PKCε expression was assessed by immunoblotting following PCR confirmation of PKCε deletion. Shown are triplicates of each cell line.

Prior research (Schmitz-Peiffer, Laybutt et al. 2007) had shown that PKCε KO animals exhibited reduced insulin clearance on a normal chow diet *in vivo* and this was supported in experiments examining [I^{125}] insulin uptake into primary hepatocytes. In order to determine if PKCε KO MEFs recapitulate this phenotype, FITC-insulin was used to analyse cellular insulin uptake. Initial insulin uptake experiments were performed on MEFs seeded into a 96-well glass bottom plate and analysed using an ImageExpress microscope, based on internal cellular fluorescence intensity. The IXM analysis software corrected fluorescence intensity on a per cell basis, according to proximity to the nucleus. For these experiments WT1 and KO1 MEFs were used.

Consistent with previous [I^{125}] uptake data from primary hepatocytes, the PKCε KO MEFs displayed a large reduction in insulin uptake compared to WT (50 % ± 2 vs 100 % ± 7, n=2) (Figure 3.3.2). This suggests that the absence of PKCε can interfere with InsR internalisation. To test whether this is due to a general endocytosis defect, internalisation of other receptors was analysed. Uptake of Texas-Red Transferrin uptake (7 min) was in fact increased in PKCε KO MEFs (Figure 3.3.3A), while FITC-EGF (15 min) was unchanged in PKCε KO MEFs compared to WT MEFs (Figure 3.3.3B). These experiments suggested that the defect in ligand uptake is specific for the InsR rather than a general trafficking impairment.

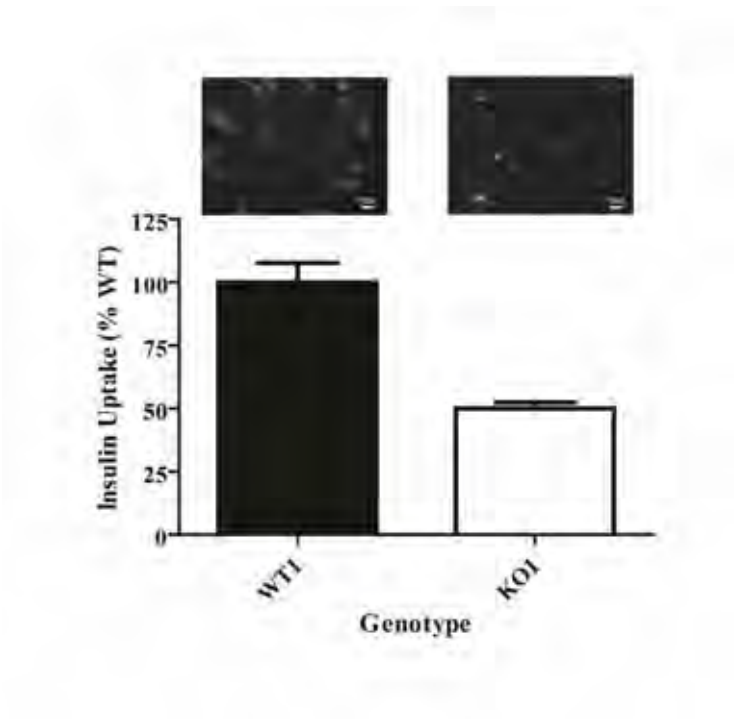

Figure 3.3.2 FITC-Insulin uptake in MEFs.
WT (■) and PKCε KO MEFs (□) were serum starved for 2 h and stimulated with 100 nM FITC-Insulin for 10 min at 37 °C. Cells were fixed and insulin uptake analysed using an ImageExpress microscope, on a per cell basis, based on internal cellular fluorescence intensity. Data are means ± Range; n = 2 independent experiments.

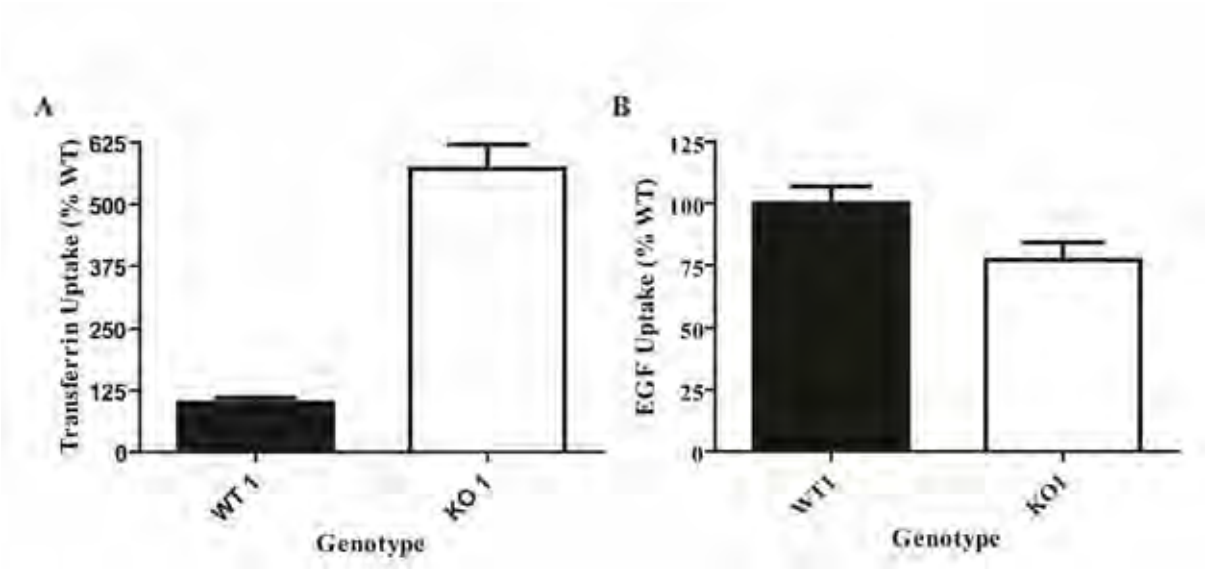

Figure 3.3.3 Uptake of ligands preferentially endocytosed by clathrin-mediated internalisation in MEFs. WT1 (■) and KO1 MEFs (□) were serum starved for 2 h and stimulated with **A:** Texas-Red Transferrin and **B:** FITC-EGF for 15 min at 37 °C. Cells were fixed and uptake analysed using an ImageExpress microscope, on a per cell basis, based on internal cellular fluorescence intensity. Data are means ± SEM; n = 2 independent experiments.

3.3.2 PKCε null MEFs display reduced insulin internalisation by FACS analysis

To extend these results using two different pools of WT and PKCε KO MEFS, cellular FITC-insulin uptake was measured by FACS. Cells were pre-treated with 30 μM monensin in order to trap internalised insulin in the endosomal compartment where it would not be degraded. This allows the rate of InsR endocytosis to be measured independently rather than the net effect of internalisation and recycling. The use of monensin has also been shown to increase FITC fluorescence intensity (Maxfield 1982; Ogris, Carlisle et al. 2001; Betts and Koup 2004) and therefore improve the sensitivity for measuring uptake of labelled insulin. Cells were treated in the same way as described for fluorescence microscopy, with addition of 100 nM FITC-insulin to the MEFs at 37 °C for 10 min. Media was then removed, cells washed and fixed with 4 % paraformaldehyde prior to analysis on a FACSCanto I^{TM}. Unstimulated cells were used as a control, in order to account for cellular autofluorescence. The experiments were performed with two separate lines of KO and WT MEFs and the results support the findings of the previous experiment, with a significant reduction in insulin uptake observed in KO MEFs compared to WT. Overall the KO MEFs exhibited ~21 % reduction in insulin uptake (79 % ± 7 vs 100 % ± 2, n=4-5 $p < 0.05$) (Figure 3.3.4).

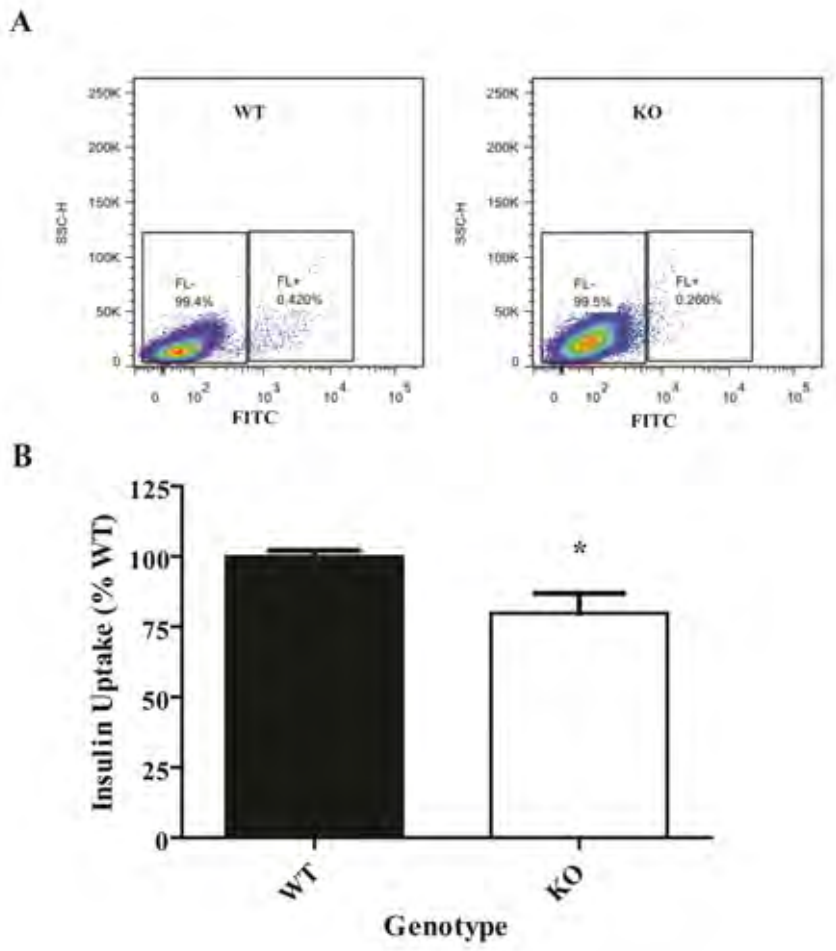

Figure 3.3.4 FITC-Insulin uptake in MEFs by FACS analysis.
WT (■) and PKCε KO MEFs (□) were serum starved for 2 h and stimulated with 100 nM FITC-Insulin for 10 min at 37 °C. Cells were fixed and insulin uptake analysed on a FACSCanto I^{TM}. **A:** Representative FACS plot of WT and KO MEFs. **B:** Graphical analysis of insulin uptake. Data are means ± SEM; n = 4-5 independent experiments in two separate MEF pools. (* = $p < 0.05$).

In these experiments, a low percentage of cells exhibited uptake of the FITC-labelled insulin. As a consequence the stimulation of the insulin signalling components, IRS-1, InsR and Akt/PKB with unlabelled and FITC-labelled insulin were compared. It was seen that the FITC-labelled insulin induced a similar activation of these proteins compared to unlabelled insulin (Figure 3.3.5). This ruled out the possibility that the FITC-insulin was not stimulating the insulin signalling pathway and the low cell numbers positive for FITC-insulin is likely due to other factors. These could include sensitivity of the FACSCanto I^{TM} compared to that of fluorescence microscopy and greater cell death in the FACS experiments from washing and filtering the cells prior to analysis.

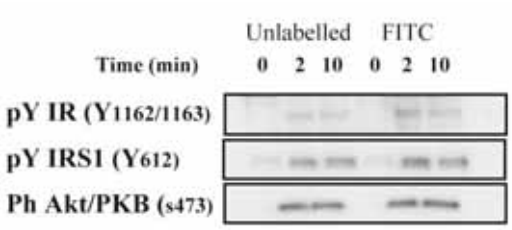

Figure 3.3.5 Insulin signalling cascade activation by unlabelled insulin and FITC-insulin.
WT MEFs were serum starved for 2 h and stimulated with 100 nM unlabelled or FITC-Insulin for the indicated times at 37 °C. Cells were harvested and analysed by western blot. n = 1.

Taken together, these experiments confirm that PKCε KO MEFs exhibit reduced insulin uptake, most likely due to defective endocytosis of the InsR as InsR content is not different between genotypes (Figure 3.3.6). These findings are consistent with enhanced insulin levels observed in PKCε KO mice, even in the absence of any increase in insulin secretion, such as observed in chow-fed mice and show that the MEFs could be a good model for the liver and therefore to examine InsR trafficking.

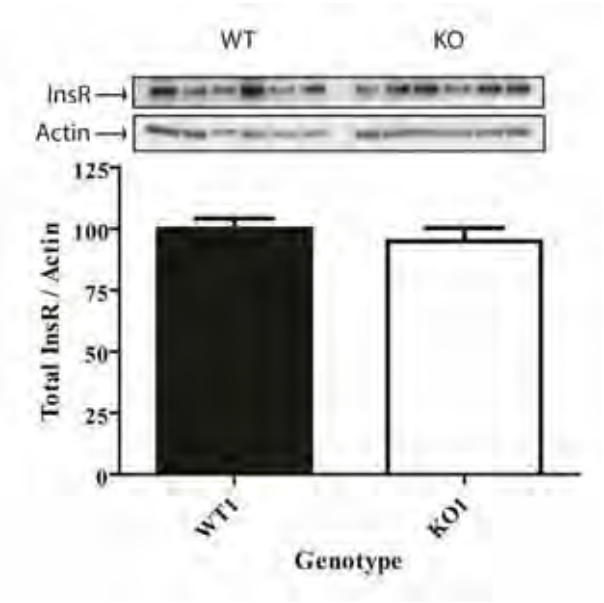

Figure 3.3.6 Insulin receptor expression of WT1 and KO1 MEFs.
MEFs were harvested in RIPA buffer and InsR expression examined by immunoblotting. Data are means ± SEM; n = 3 independent experiments

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3.3.3 PKCε KO MEFs have altered insulin receptor localisation

Having ascertained that PKCε KO MEFs display reduced insulin internalisation, it was next explored whether the localisation of the InsR was altered. The InsR is usually internalised by a process of clathrin-mediated endocytosis (Carpentier 1994), however in some instances, the InsR may be localised to caveolae or membrane microdomains (Foti, Porcheron et al. 2007), which can be important for the insulin signal cascade and therefore insulin action. To investigate whether the reduced rate of InsR internalisation in PKCε KO MEFS is associated with altered localisation of the InsR, subcellular fractionation was performed by Optiprep density gradient centrifugation and localisation of proteins were determined by immunoblotting.

Initial experiments characterised the different subcellular compartments. Flotillin1-enriched fractions (fractions 3-4) were taken as regions of membrane microdomains (peak 1) and early endosomal antigen 1 (EEA1), a marker for early endosomes, was most highly enriched within fractions 5-6 (peak 2). Pan-cadherin was used as a general PM marker and had a relatively broad distribution, with major peaks in fraction 11 as well as fractions 17-19 (peak 3) (Figure 3.3.7). The distribution of these markers was similar in both WT and KO cells and therefore combined, as shown in Figure 3.3.7 below.

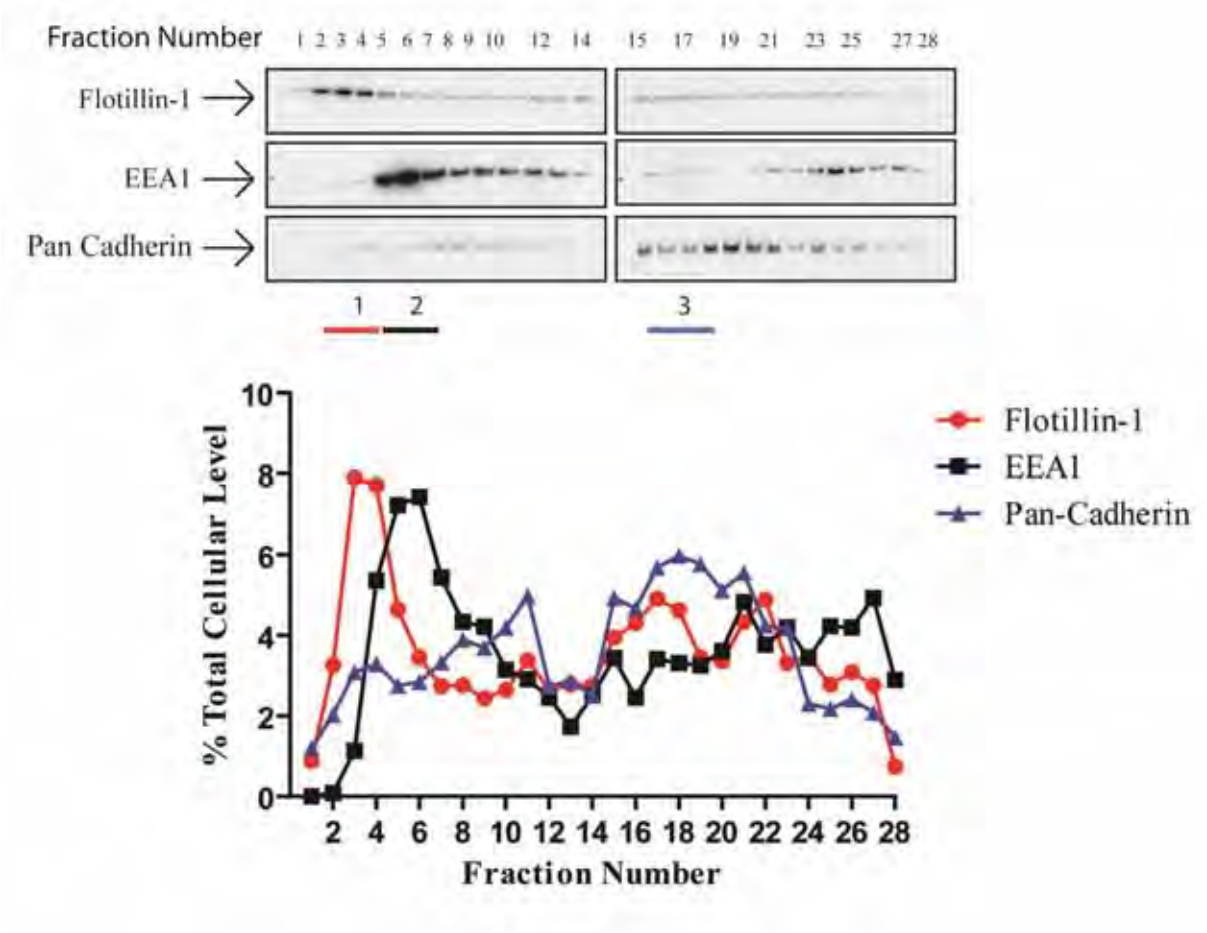

Figure 3.3.7 Subcellular fractionation compartment characterisation.
WT1 and KO1 MEFs were serum starved for 2 h and stimulated with 100 nM insulin for 2-10 min at 37 °C. Cells were harvested in lysis buffer and cells disrupted by nitrogen cavitation, before being separated over a 15 % Opti-Prep gradient centrifuged to form a continuous gradient. Flotillin1 was used as a lipid raft marker, EEA1 for early endosomes and pan-Cadherin as a plasma membrane marker. Data are means; n = 2 independent experiments.

There was a striking difference in localisation of the InsR between the two genotypes, with the InsR of the KO1 MEFs found to be highly enriched in peak 1, coinciding with the flotillin1-enriched fractions of the gradient, while in the WT1 MEFs there was a more even cellular distribution of the InsR in the basal state. It was also apparent that the insulin-induced redistribution of the InsR was perturbed in the KO1 MEFs compared to the WT1 cells. Following insulin treatment for 2 and 10 min, the InsR of WT1 cells redistributed to other fractions of the gradient, likely to be recycling endosomes (fractions 11-14) (Figure 3.3.8). This is consistent with reduced internalisation of the InsR in the absence of PKCε.

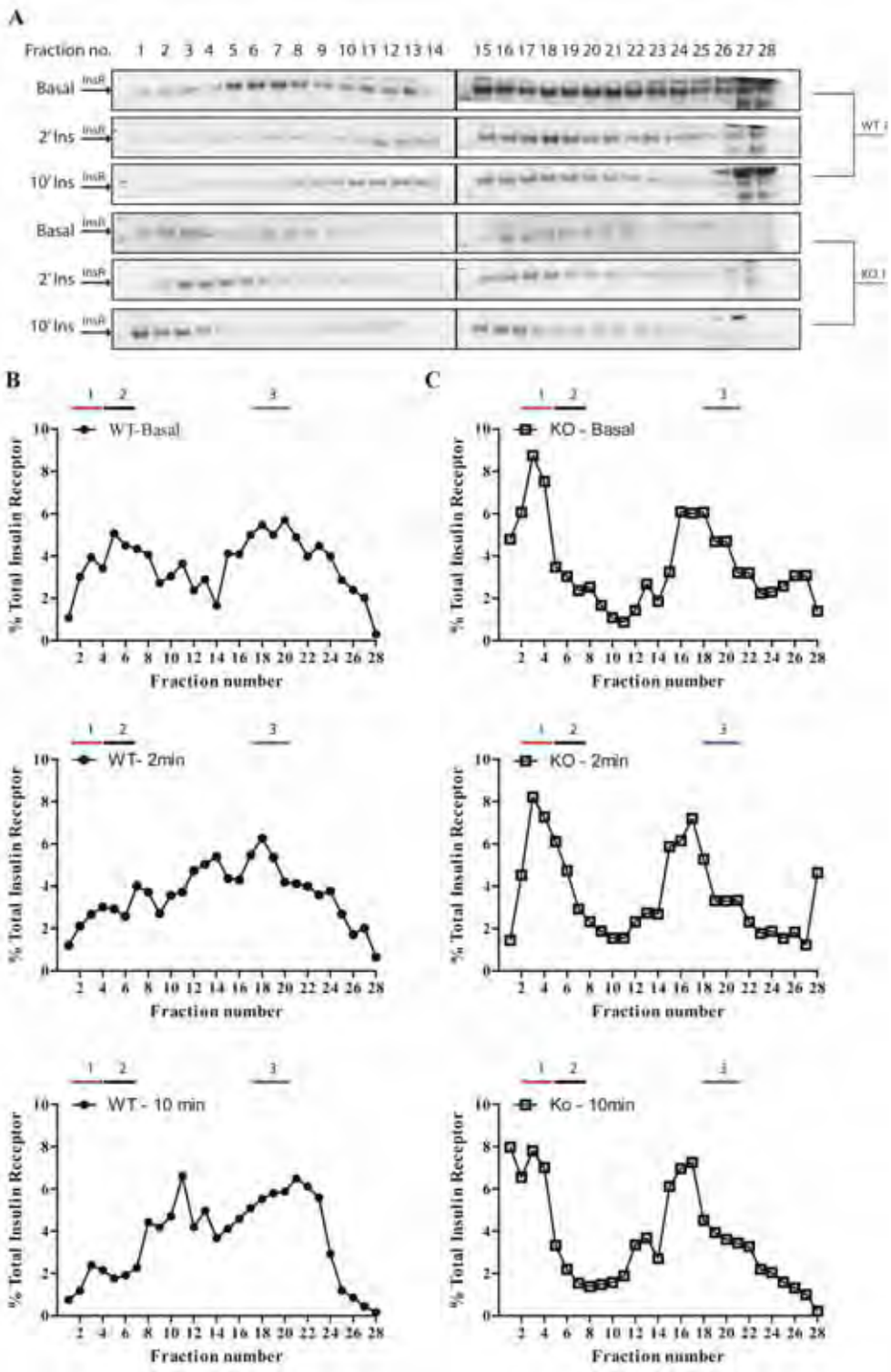

Figure 3.3.8 Insulin receptor trafficking and localisation in WT and PKCε KO MEFs, following subcellular fractionation.
WT1 and KO1 MEFs were serum starved for 2 h and stimulated with 100 nM insulin for 2-10 min at 37 °C. Cells were harvested in lysis buffer and subject to pressurised disruption under N_2 gas, before being separated over a 15 % Opti-Prep gradient and centrifuged to form a continuous gradient. **A:** Insulin receptor localisation was analysed by western blot. The profile was quantified for **B:** WT1 and **C:** KO1 MEFs using ImageJ. Data are means; n = 2 independent experiments.

3.3.4 Altered insulin receptor trafficking does not affect its half-life

It has been shown that chronic insulin stimulation can reduce the half-life of the InsR in various cell lines such as Rat 1 Fibroblasts, HEK cells and lymphocytes as well as in human and rodent models with hyperinsulinemia from around 9-12 h to 4-6 h (Kasuga, Kahn et al. 1981; Grako, Olefsky et al. 1992). Given that KO1 MEFs have reduced insulin uptake and display perturbed InsR trafficking upon acute insulin stimulation, we next examined whether the absence of PKCε affects InsR half-life. This was carried out in MEFs treated with a high dose of insulin (1 μM) to stimulate InsR trafficking, in conjunction with 50 μg/mL of cycloheximide, which prevents the synthesis of new proteins. This was carried out over a 24 h time course and analysed by immunoblotting lysates that were equalised for DNA content. It was observed that with insulin treatment in the presence of cycloheximide, the apparent InsR half-life was ~5-6 h (Figure 3.3.9), which is in agreement with previous reports (Grako, Olefsky et al. 1992). The rate of InsR degradation was unchanged in WT1 and KO1 MEFs.

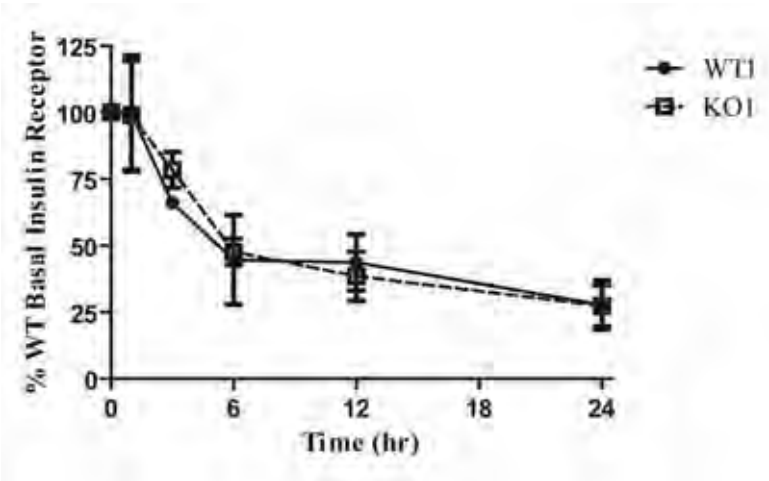

Figure 3.3.9 Insulin receptor half-life.
WT1 and KO1 MEFs were treated with 1μM insulin in the presence of 50 μg/mL cycloheximide for the indicated times at 37 °C, to detect the rate of insulin receptor degradation and therefore its half life with insulin. Data are means ± SEM; n = 3 independent experiments per time point.

These results suggest that upon chronic insulin stimulation, the altered trafficking of the InsR in the absence of PKCε does not significantly affect the rate of InsR degradation.

3.3.5 Ablation of PKCε does not alter gross membrane morphology or membrane fluid dynamics

The experiments described above demonstrated that PKCε KO MEFs have altered cellular InsR distribution that may affect its internalisation. Consequently it was investigated if this could be due to differences in gross membrane morphology or lipid order, which have been shown to perturb lateral movement of the InsR in the membrane and thus likely its rate of endocytosis (Kabayama, Sato et al. 2007). These questions were addressed through examination of MEFs by electron microscope (EM) for morphological differences and by incorporation of the lipid Laurdan into the PM to analyse the lipid order. Changes in either may modify InsR trafficking and hence endocytosis.

The EM experiments were carried out on freshly fixed liver sections, as well as WT and KO MEFs, which were grown to confluence and fixed in phosphate buffer containing 4 % paraformaldehyde and 1 % gluteraldehyde. The liver tissue and cells were then prepared for EM as described in the methods. Specimens were sliced and micrographs were obtained by the Garvan EM technical assistant Katanyu Pongstaporn. There was no observable difference in gross physical morphology around the PM in the MEFs (Figure 3.3.10A) or liver (Figure 3.3.10B) between the WT and PKCε KO conditions.

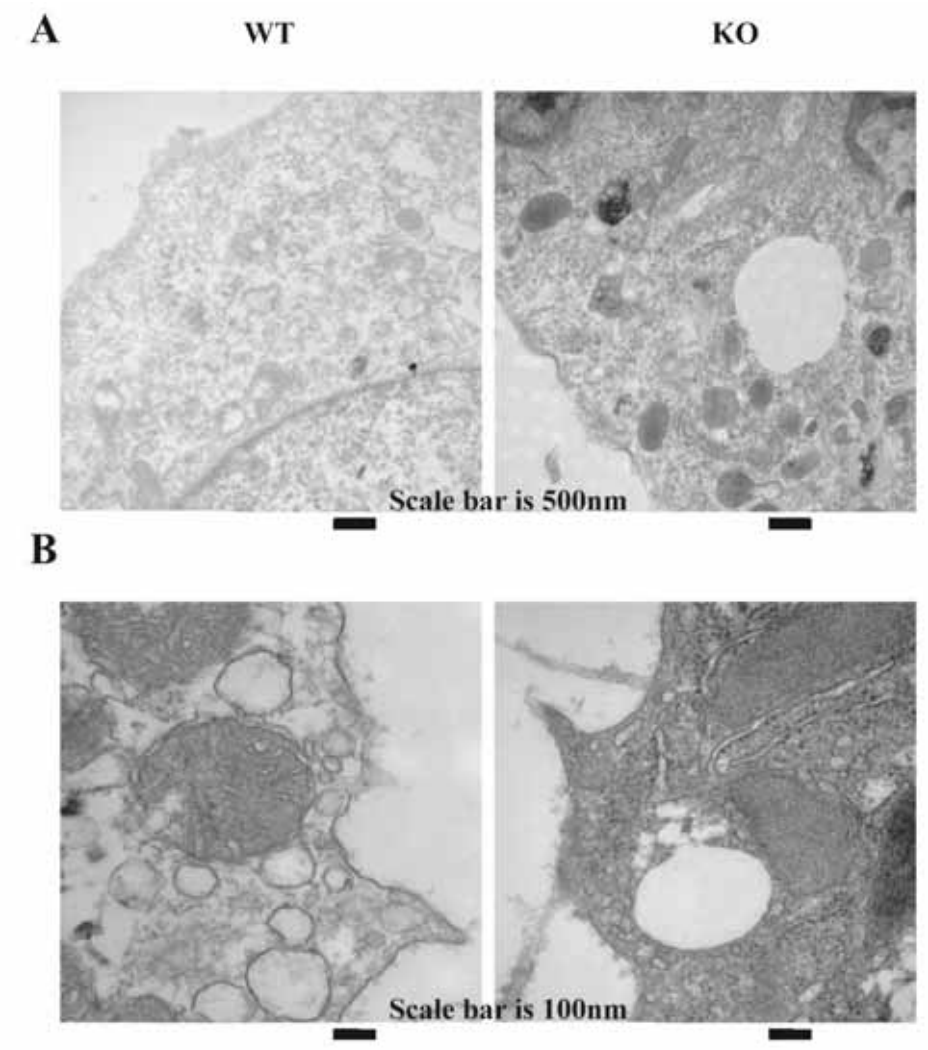

Figure 3.3.10 Electron microscope images of WT and PKCε KO MEFs and liver.
A: WT and KO MEFs as well as **B:** liver samples from WT and PKCε KO animals were fixed and prepared for imaging by electron microscopy. Representative images are shown above.

Although we saw no difference in the physical properties of the PM, it may be that the lipid content of the PM is modified in the absence of PKCε. This possibility was studied, using the lipid stain Laurdan, as described in Chapter 1. Laurdan was included in the media of the cells for 40 min at a concentration of 10 μM, to enable its incorporation into the membranes of live cells as a measure of membrane lipid order (Gaus, Zech et al. 2006). The cells were then fixed followed by visualisation using two-photon microscopy and quantification as described in the methods. Quantification of 20-40 cells per genotype showed no significant difference in membrane lipid order between WT and KO MEFs (Figure 3.3.11). As described in section 2.9, a GP value of -1 indicates a more fluid membrane and +1 a more

ordered membrane, based upon the ratio of emission intensities measured at the two different wavelengths. The higher the value, the more ordered the PM, which correlates to the red/purple colour and the more fluid area of the cell is indicated by the blue/green region.

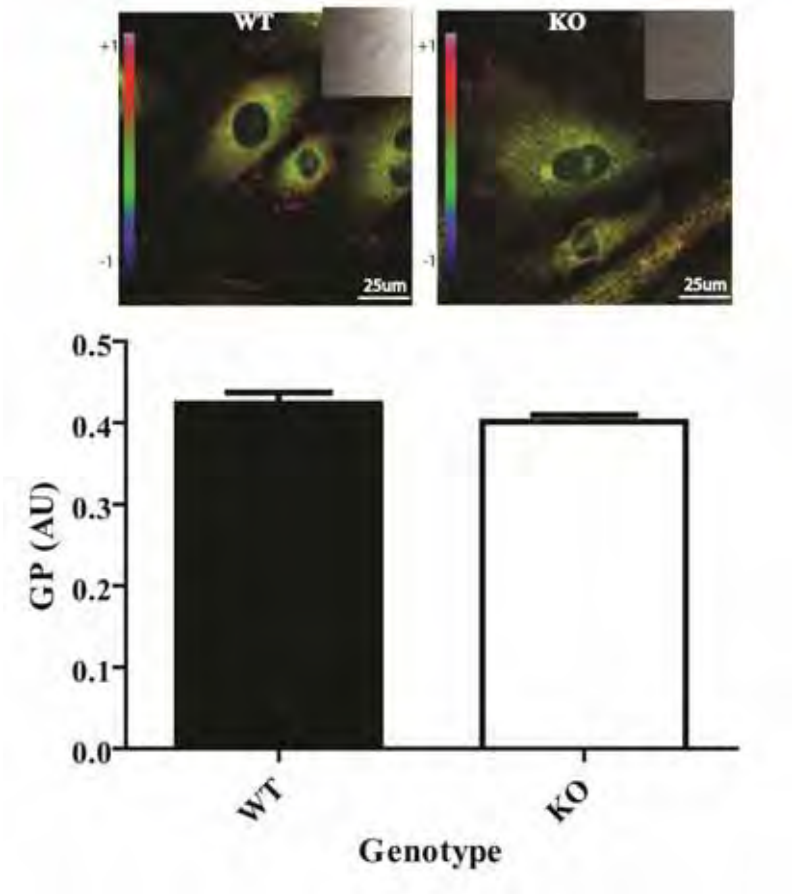

Figure 3.3.11 2-photon microscopy of Laurdan as a measure of membrane fluidity in WT and PKCε KO MEFs.
WT and PKCε KO MEFs were treated with 10 μM Laurdan for 45 min. Media was aspirated from the cells, washed with warm PBS and fixed with 4 % paraformaldehyde to be imaged by 2-photon microscopy. Representative images are shown above of all cells quantified. Data are means ± SEM; n = 20-40 cells from two separate MEF pools.

Altogether, these EM and Laurdan findings suggest that the altered InsR distribution and trafficking in the PKCε KO MEFs is not due to either morphological or biochemical differences of the PM.

3.3.6 Reconstitution of PKCε alters insulin receptor localisation

It was next investigated whether alterations in InsR trafficking were directly related to the presence or absence of PKCε. The cellular fractionation experiments were repeated after re-expression of an adenoviral PKCε construct in PKCε KO cells, or GFP as a control in both KO and WT MEFs. After 48 h of adenovirus treatment, immunoblotting demonstrated PKCε was overexpressed compared to endogenous WT cells; however the cellular distribution of PKCε was the same for the overexpressed and endogenous protein. Overexpressed PKCε localised to the same fractions of the gradient as the endogenous PKCε (Figure 3.3.12).

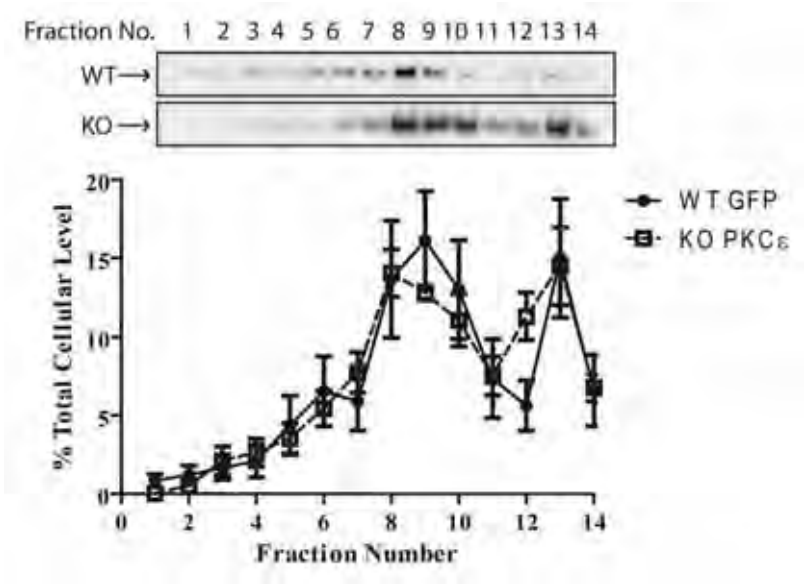

Figure 3.3.12 PKCε localisation in WT and PKCε KO MEFs, following subcellular fractionation.
WT and PKCε KO MEFs were serum starved for 2 h and stimulated with 100 nM insulin for 10 min at 37 °C. Cells were harvested in lysis buffer and subject to high pressure nitrogen for disruption, before being separated over a 15 % Opti-Prep gradient and centrifuged to form a continuous gradient. PKCε localisation was analysed by immunoblotting. Data are means ± Range; n = 2 independent experiments each performed in two separate MEF cell lines.

In these fractionation experiments, the precise distribution of the cell compartment markers was slightly different to that observed previously. Importantly, however, they followed a similar pattern, with the flotillin-1 enriched fractions identified early in the gradient (peaking in fractions 5-6) followed by the markers for early endosomes and recycling organelles, EEA1 and rab11 respectively, which peaked in fractions 7-9 along with a smaller peak in fractions 12-13 (Figure 3.3.13). The overlap of EEA1 and Rab11 is not unexpected as they

represent organelles on a continuum. This may also be due to the middle part of the gradient not being separated as greatly as anticipated, because fewer fractions harvested in these experiments compared to earlier in order to compare the entire gradient on a single polyacrylamide gel. For the purposes of explaining the trafficking, region 1 refers to the PM region of the gradient, region 2 the flotillin-enriched fractions, region 3 the EEA1 and Rab11 enriched fractions and region 4 the most dense organelle compartments of the gradient.

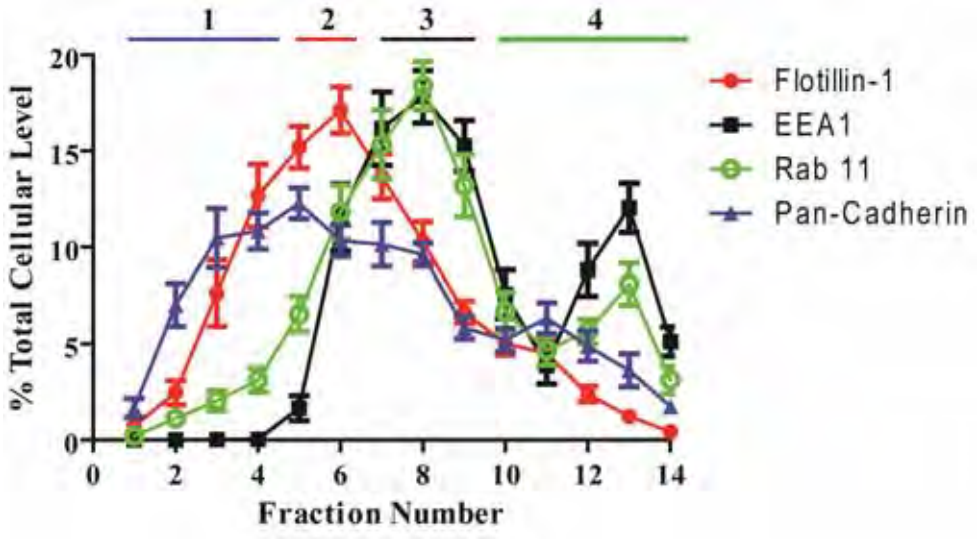

Figure 3.3.13 Subcellular fractionation compartment characterisation.
WT and PKCε KO MEFs were treated with adenovirus for 48 h prior to gradient fractionation. Cells were serum starved for 2 h and stimulated with 100 nM insulin for 10 min at 37 °C. Cells were harvested in lysis buffer and subject to high pressure nitrogen for disruption, before being separated over a 15 % self-forming continuous Opti-Prep gradient. Flotillin1 was used as a lipid raft marker, EEA1 early endosomes, Rab11 for recycling endosomes, Pan-Cadherin as a PM marker. Data are means ± SEM; n = 2 independent experiments performed each in two separate MEF cell lines.

The aim of these fractionation experiments was to examine the effect of PKCε reconstitution on InsR localisation in PKCε KO MEFs. These experiments were performed independently using two pools of WT and PKCε KO cells and it was found that the organelle markers were localised to the same regions in all MEF pools. Insulin treatment for 10 min induced InsR re-distribution in the WT MEFs compared to the basal state (Figure 3.3.14A), with a higher proportion of the InsR localised within regions 2 and 3 of the gradient (Figure 3.3.14D). In contrast, the PKCε KO MEFs, expressing GFP adenovirus as a control, did not exhibit alterations in cellular InsR redistribution with insulin stimulation compared to the basal state (Figure 3.3.14B), indicating a trafficking defect, which is consistent with the previous findings in the KO1 MEFs as well as the reduced insulin uptake data. Furthermore, in the

absence of PKCε, the InsR is much more highly localised within the lightest density portion of the gradient (region 1 and 2), which contrasts that seen in the WT cells (Figure 3.3.14D).

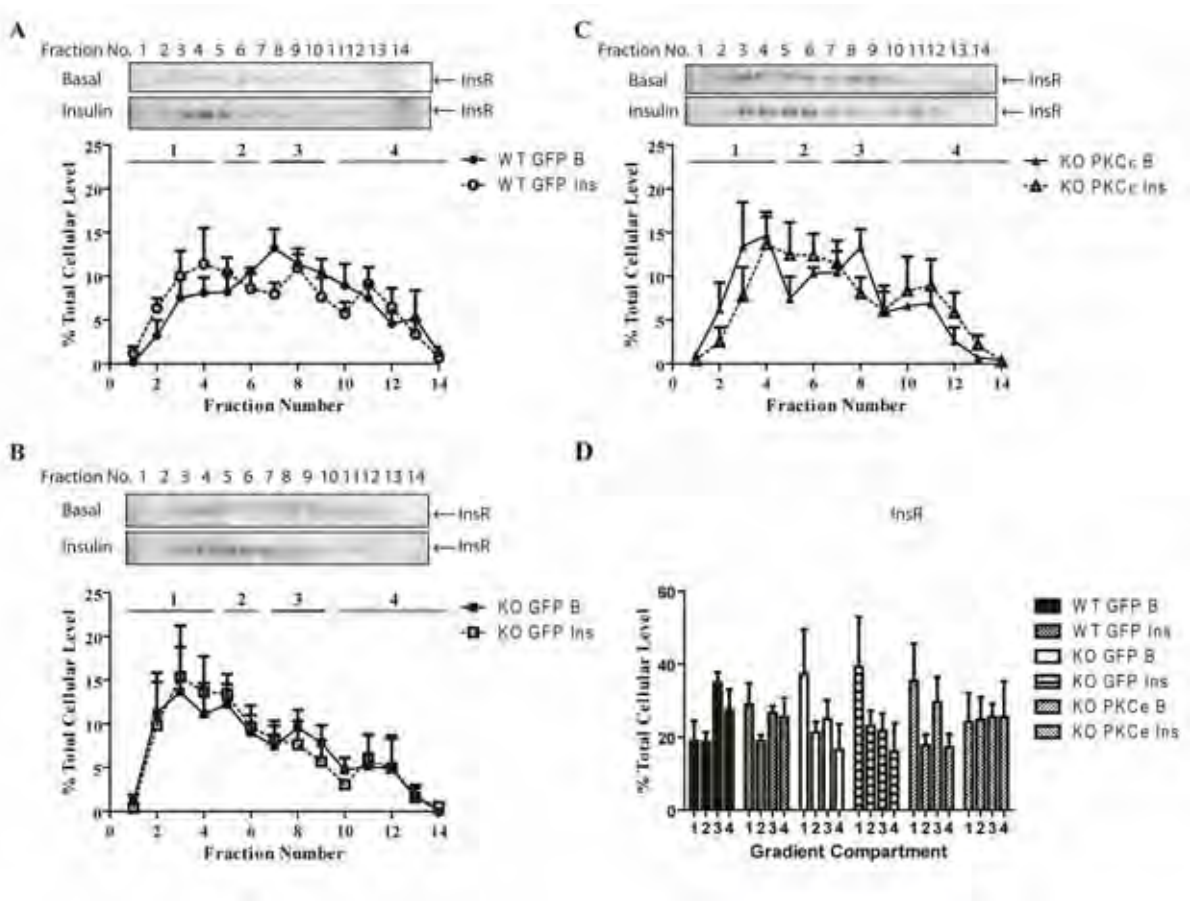

Figure 3.3.14 Insulin receptor trafficking and localisation in WT and PKCε KO MEFs, following reconstitution of PKCε into KO MEFs.
WT and PKCε KO MEFs were treated with GFP control or PKCε adenovirus for 48 h. Cells were serum starved for 2 h and stimulated with 100 nM insulin for 10 min at 37 °C. Cells were harvested in lysis buffer and subject to high pressure cellular disruption, before being separated over a 15 % Opti-Prep gradient and centrifuged to form a continuous gradient. InsR localisation was analysed by immunoblotting in **A:** WT MEF control, **B:** KO MEF control and **C:** KO overexpressing PKCε cells. **D:** Localisation of the InsR within each nominated region of the gradient was also compared between the conditions. Data are means ± Range; n = 2 independent experiments performed in 2 independent MEF cell lines.

Overexpression of PKCε in the KO MEFs resulted in a slight redistribution of the InsR in the basal state compared to control KO cells, with a tendency for a higher proportion of cellular InsR located within the denser region 3 of the gradient (Figure 3.3.14D). This reflects more closely that seen in the WT MEFs. Following insulin treatment, the InsR showed a slight redistribution compared to the basal state, and this redistribution was more prominent compared to KO control cells, indicating that the reconstitution of PKCε was able to

influence InsR trafficking (Figure 3.3.14C). This altered distribution resulted in a greater proportion of the InsR being localised to regions of the gradient associated with more dense organelles.

Although not as evident a redistribution as in previous fractionation experiments, with a more diffuse InsR expression across the gradient, this may be explained by the reduced resolution in the middle fractions of the gradient, where the early endosomes and recycling organelles are present (Figure 3.3.13). Despite the insulin-stimulated redistribution of the InsR being less clear than in the initial fractionation experiments, the fact that there was an alteration with the re-expression of PKCε into the KO MEFs, does suggest that the absence of PKCε can alter InsR localisation and trafficking.

In summary, reconstitution of PKCε into KO MEFs resulted in a different cellular localisation of the InsR and was associated with a more pronounced redistribution following insulin stimulation. This pattern of cellular redistribution observed is more consistent with that seen in WT cells with a higher percentage of InsR localising to the more dense organelle compartments of the gradient. Preliminary data suggests there is no difference in PM-localised InsR expression (data not shown), therefore these experiments suggest that the absence of PKCε can alter InsR localisation and redistribution, and its deletion could potentially impair the rate of internalisation.

3.3.7 Overexpression of PKCε in PKCε KO MEFs reverses the loss of Ceacam1

As Ceacam1 expression has been shown to be influenced by PKC activation, together with the ability of Ceacam1 to promote InsR internalisation, its expression was examined in the MEFs, as a potential mechanism responsible for the reduced insulin uptake.

Ceacam1 expression was analysed by immunoblotting in three separate pools of WT and PKCε KO MEFs. All PKCε KO MEF pools were found to have a major reduction in Ceacam1 expression compared to their WT control (Figure 3.3.15). All pools of PKCε KO MEFs displayed a similar reduction in Ceacam1 levels, with an overall reduction of approximately 94 % (6 % ± 1 vs 100 % ± 9 of WT basal, $p < 0.0001$, n = 3-6 independent experiments performed in each MEF pool).

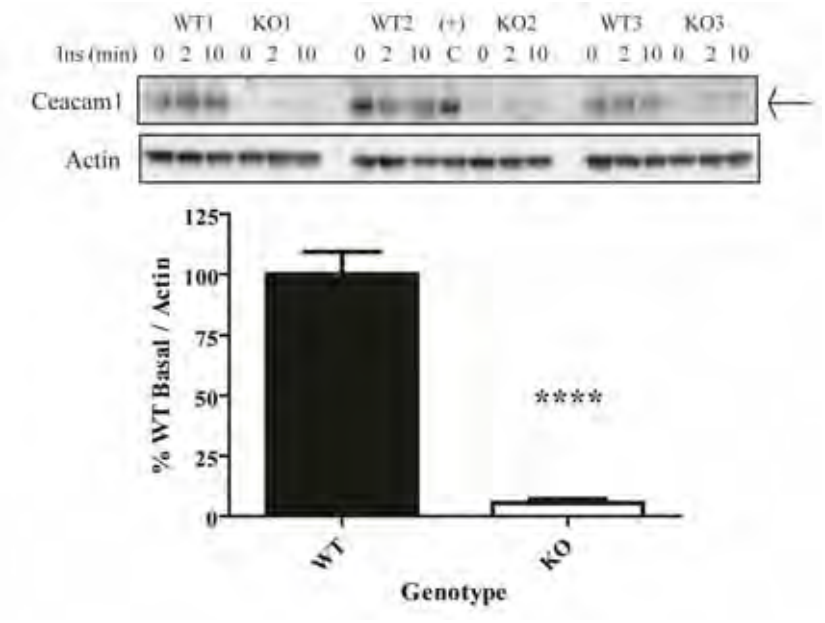

Figure 3.3.15 Ceacam1 expression in WT and PKCε KO MEFs
WT and PKCε KO MEFs were harvested in RIPA buffer and expression was analysed by immunoblotting. Ceacam1 was expressed as a percentage of the corresponding WT MEF pool. Data are means ± SEM; n = 3-6 independent experiments in three separate MEF pools. (**** = $p < 0.0001$ vs WT).

The diminution in Ceacam1 appears to be closely associated with a lack of PKCε expression because when PKCε was reconstituted with adenovirus for 48 h, Ceacam1 expression was partially restored in reconstituted PKCε KO MEFs compared to PKCε KO MEFs infected with GFP expressing adenovirus ($p < 0.001$, n = 4-5 independent experiments performed in two separates MEF pools). This led to an increase in Ceacam1 expression to ~38 % of WT control levels (Figure 3.3.16).

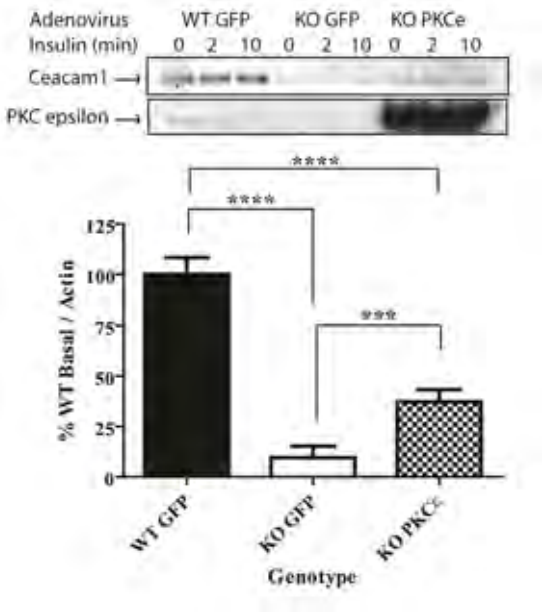

Figure 3.3.16 Effect of adenovirus-mediated PKCε reconstitution on Ceacam1 expression in PKCε KO MEFs.
WT MEFs were infected with GFP control adenovirus and PKCε KO MEFs with GFP control or PKCε adenovirus for 48 h. MEFs were treated with 100 nM insulin for up to 10 min, harvested and Ceacam1 expression analysed by immunoblotting. For all data analysis, CEACAM1 expression was expressed as a percentage of WT basal. Data are means ± SEM; n = 3-6 independent experiments performed in two separate MEF pools. (**** = $p < 0.0001$ vs WT control; *** = $p < 0.001$ vs KO control).

Such changes in protein levels may take longer than 48 h to restore, possibly due to indirect effects on either transcription or the rate of proteosomal degradation. Therefore, a PKCε re-expressing stable cell line was generated from the KO1 MEFs by infection with a retrovirus and this cell line allows the analysis if re-expressing PKCε for longer time at a more physiological level could have a greater impact. This was indeed the case, with PKCε expression only ~1.7-fold that of endogenous and a restoration of Ceacam1 to higher levels (75 % ± 14 vs 100 % ± 22 of WT1 basal, $P > 0.05$; n = 3 independent experiments performed in triplicate) than was observed with adenovirus treatment (Figure 3.3.17A). These experiments were performed in cells selected for virus-mediated PKCε expression by 5-10 passages with puromycin. In fact, in experiments performed when PKCε had been re-expressed for the longest time (10 passages), Ceacam1 levels returned to levels most similar to that seen in the WT1 (88 % ± 4, n = 1 experiment in triplicate) (Figure 3.3.17B).

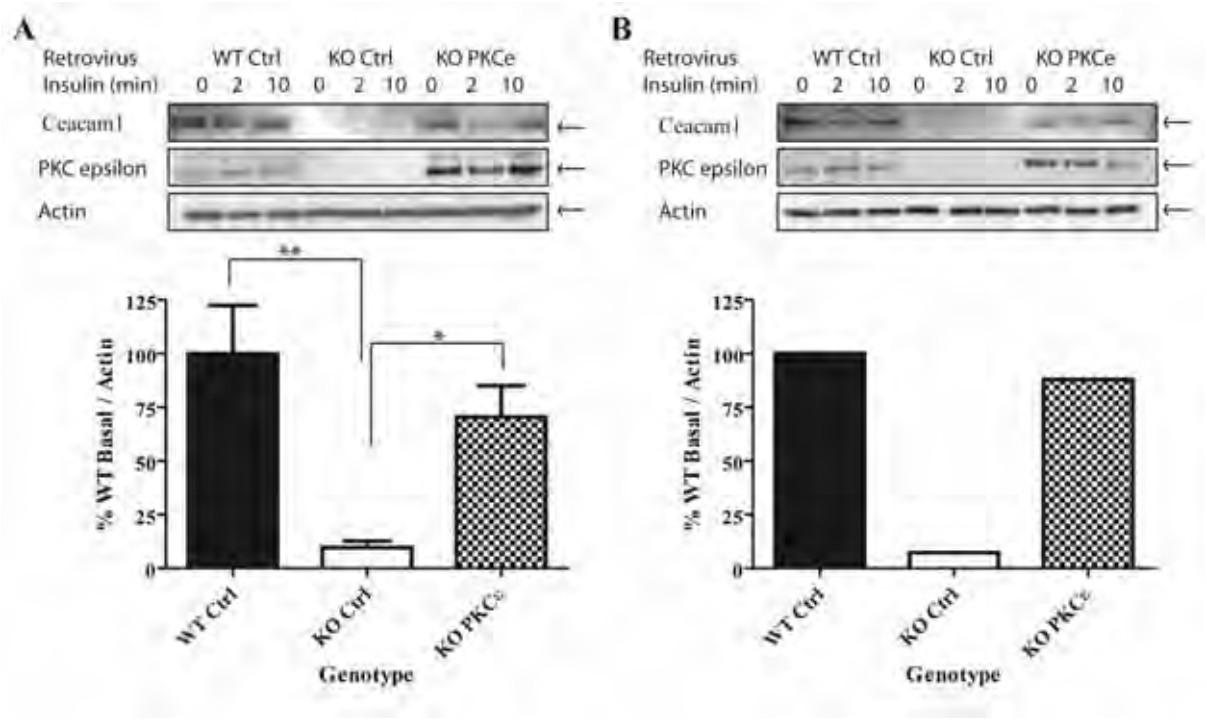

Figure 3.3.17 Effect of reconstitution of PKCε with retrovirus on Ceacam1 expression in MEFs
WT1 MEFs were infected with empty control retrovirus and PKCε KO1 MEFs with empty control or PKCε retrovirus for 5-10 passages. MEFs were harvested and Ceacam1 expression analysed by immunoblotting. **A:** The 3 individual retrovirus re-expression experiments combined and **B:** the third independent retrovirus re-expression experiment alone were analysed for Ceacam1 expression. For all data analysis, Ceacam1 expression was expressed as a percentage of WT basal. Data are means ± SEM; n = 1-3 independent experiments. (* = $P < 0.05$ vs KO control, ** = $P < 0.01$ vs WT Ctrl).

These retrovirus experiments demonstrate that PKCε can directly affect Ceacam1 expression levels. Furthermore this could be a candidate for the altered InsR trafficking observed in the PKCε KO MEFs, through modulation of Ceacam1, which is known to act as an InsR substrate and form a complex to promote InsR endocytosis. Taken together, the adenoviral reconstitution of PKCε into PKCε KO MEFs modestly altered InsR localisation in a manner reflective of the enhanced Ceacam1 levels, which were also restored with retrovirus. These findings support the hypothesis that the absence of PKCε reduces insulin uptake by impairing Ceacam1 expression or activation, which has been shown to directly influence InsR internalisation.

3.4 Discussion

Previously published work has demonstrated that PKCε ablation in mice reduces hepatic insulin clearance (Schmitz-Peiffer, Laybutt et al. 2007) in chow fed animals. Reduced hepatic insulin clearance has been reported to be associated with the development of IR in both rodents and humans (Duckworth, Bennett et al. 1998). The aim of the experiments performed in this chapter were to elucidate the mechanisms involved in the reduced hepatic insulin clearance of PKCε KO mice, which were hypothesised to be due to perturbed rates of InsR trafficking. The key findings of the experiments described here were that in the absence of PKCε, InsR trafficking is perturbed following an acute dose of insulin in primary MEFs and this altered InsR trafficking is not due to cellular morphological differences or membrane fluidity, but correlates with the expression of the InsR substrate Ceacam1, known to be required for InsR internalisation.

Impaired hepatic insulin clearance has been shown to be associated with IR and the development of T2D (Duckworth, Bennett et al. 1998). The results from this chapter showing that PKCε null MEFs display reduced insulin uptake following an acute stimulation for 10 min agrees with previous reports from global PKCε KO animals (Schmitz-Peiffer, Laybutt et al. 2007). Often in studies eliciting reduced insulin clearance, IR has already manifested itself and as such the defective clearance of insulin from the periphery is a chronic phenomenon, resulting in continually higher circulating insulin levels and therefore promotes the onset of further metabolic deterioration. In PKCε KO animals this however appears to contribute to improved glucose tolerance rather than resulting in progressive hyperglycemia.

Acute insulin stimulation led to a reduction in insulin uptake in PKCε KO MEFs. This reduction in insulin internalisation was not observed with transferrin uptake, a measure of general clathrin-mediated endocytosis. The insulin clearance reduction is consistent with previous data from isolated primary hepatocytes of global PKCε null animals, which showed reduced uptake of I^{125} insulin over 15 min (Schmitz-Peiffer, Laybutt et al. 2007). The decrement in insulin uptake by the MEFs shown in this chapter is likely due to a perturbation in InsR trafficking in the short term. Chronic exposure to high doses of insulin can act to downregulate InsR numbers, by promoting internalisation and degradation of the InsR rather than recycling in order to dampen the signal. The defect in InsR internalisation in KO cells did not affect the half-life of the InsR. No difference was observed in the rate of InsR

degradation between genotypes and this finding supports the idea that the InsR trafficking defect is acute and does not alter degradation rate or the level of InsR expression between genotypes. This may act to enhance metabolic insulin signalling in the short term, as components of the metabolic arm of insulin signalling are activated at the PM. This is examined in Chapter 4. Furthermore the insulin signal can be terminated through internalisation of its cell surface receptor, as the InsR is deactivated following dissociation of the ligand-receptor complex in endosomes (Carpentier 1994).

Transferrin uptake was also investigated by fluorescence microscopy and surprisingly this was found to be increased in KO compared to WT cells. This suggests that the defective trafficking in the absence of PKCε is not a general trafficking deficiency but specific to the InsR. Earlier work (Lakadamyali, Rust et al. 2006) showed that the transferrin receptor (TrfR) is readily internalised through clathrin-coated pits. On the other hand, internalisation of the low-density lipoprotein receptor (LDLR) and EGFR was internalised through only ~15 % of clathrin-coated pits, during 15 min incubation at 37 °C. These pits also contained transferrin, and this selective pit association was observed following acute stimulation at 4 °C. In contrast, following 2 h of stimulation at 4 °C, the LDL receptor tended to have less spatial specificity and had in fact moved into ~50 % of pits, similar to that of TrfR. If this phenomenon translates to the InsR, it can be inferred that a block in InsR trafficking may not be reflected in general cellular trafficking, but in fact remain relatively isolated to the internalisation of the InsR itself, possibly due to a specific signal from the InsR or PM/pit regions responsible for internalisation of the InsR not being recognised to initiate endocytosis. An early paper by Carpentier et al using primary hepatocytes and HEK cells (Carpentier, Fehlmann et al. 1985) supported this hypothesis to a degree. Using electron microscopy, Carpentier et al were able to show that insulin became internalised through clathrin coated pits, and that there are concentrated regions of the cell, which may be more highly specific to internalisation of the InsR and its bound ligand.

In the initial cell fractionation experiments performed in this chapter on the KO1 and WT1 MEFs, there was a reduced redistribution of the InsR in the PKCε KO MEFs compared to that of the WT over 10 min of insulin treatment. This was also associated with a different localisation of the InsR, with it being more highly localised to the membrane microdomain fractions of the gradient, as denoted by enriched flotillin-1 expression. A disparate cellular distribution of the InsR was also observed between the genotypes in the basal state. This

could be due to a variety of factors including an altered endocytotic signal or different PM structure, in such a way that in the PKCε KO MEFs the InsR may have more affinity for membrane microdomains and/or caveolae due to their number, size or associated adaptor molecules or even altered PM lipid composition. It was investigated whether PKCε affected the gross physical structure of the PM and this turned out not to be the case, with EM experiments showing no distinct differences between the PKCε KO and WT livers or MEFs.

The effects of membrane composition and fluidity on insulin action are currently the focus of much research (Muller, Denet et al. 1997; Hulbert, Turner et al. 2005). The ganglioside GM3 is present in microdomains of the PM (Kabayama, Sato et al. 2007), particularly in adipocytes and changes in levels of lipids such as GM3 could impact upon membrane fluidity and by extension perturb InsR trafficking. It has been reported recently that lateral movement of the InsR along the PM can in fact be changed through alterations in the expression of GM3 (Kabayama, Sato et al. 2007). The study by Inokuchi and colleagues demonstrated that the InsR-caveolin1 complex maintains the InsR in a more static state, in more ordered domains and this was proposed to promote insulin signalling from the caveolae. The current studies, in which we observed no discernable difference in membrane fluidity between the KO and WT MEFs, suggest that that there is no difference in PM microdomains or membrane composition/fluidity to explain the impaired InsR trafficking of the PKCε KO MEFs.

The PKCε re-expression experiments with adenovirus led to a subcellular redistribution of the InsR with insulin stimulation. This redistribution was similar to the one observed in the WT MEFs, while GFP expressing control PKCε KO MEFs showed no InsR redistribution with insulin stimulation. In the initial fractionation experiments, where more fractions were taken, the redistribution differences may have been more obvious. Furthermore, it has been reported that calnexin can be a marker of non-microdomain PM and use of this antibody in conjunction with pan-Cadherin could potentially identify PM regions with more clarity (Lingwood and Simons 2007).

Previously, Kahn and colleagues specifically targeted deletion of the InsR in hepatocytes (Michael, Kulkarni et al. 2000). In these animals, complete loss of hepatic insulin action was observed with a major primary defect being reduced hepatic insulin clearance, leading to marked hyperinsulinemia. Although demonstrating that high circulating insulin levels,

primarily through reduced hepatic clearance, can produce further complications, this is an extreme case where insulin signalling in the liver was a primary contributor to the severe defect seen with glucose homeostasis. A more recent study showed that overexpression of a defective mutant of Ceacam1 in the liver of mice leads to altered hepatic insulin clearance, while the InsR remains functional and insulin signalling is intact (Poy, Yang et al. 2002). In that study, the Ser 503 site on Ceacam1 was mutated to alanine, resulting in Ceacam1 inactivation and absence of Tyr phosphorylation by the activated InsR. As such, the propensity for Ceacam1 to act as an InsR substrate in promoting InsR internalisation and by extension insulin clearance was significantly reduced. The secondary metabolic derangements that occurred as a result of this were less severe than those observed in the liver specific InsR KO mice.

Interestingly, the effect upon hepatic lipogenesis and insulin clearance in the Ceacam1-mutant expressing mice, were similar but more detrimental than those reported in global PKCε KO mice (Schmitz-Peiffer, Laybutt et al. 2007; Raddatz, Turner et al. 2011). Furthermore, the defects observed in the Ceacam1-mutant transgenic mice were also the major ones reported in global Ceacam1 KO mice, suggesting that the liver is the tissue where Ceacam1 is most prominent in its actions, in agreement with earlier work demonstrating it to be an InsR substrate in liver but not fat or muscle (Accili, Perrotti et al. 1986). Ceacam1 defective mice develop a fatty liver, hyperinsulinemia through a continual reduction in hepatic insulin clearance as well as slightly elevated insulin secretion (Poy, Yang et al. 2002; Dai, Abou-Rjaily et al. 2004; DeAngelis, Heinrich et al. 2008). It has been shown that activation of PKC isoforms with 12-O-tetradecanoylphorbol-13-acetate (TPA) can enhance Ceacam1 expression and that this PKC-dependent enhancement of Ceacam1 expression is transcriptionally controlled (Bamberger, Briese et al. 2006). Furthermore, Ceacam1 contains multiple predicted PKC phosphorylation sites according to the Scansite database including for PKCε. It is therefore not implausible to hypothesise that PKCε can alter Ceacam1 activity or expression which could alter InsR trafficking. Whether there is a specific isoform of PKC responsible for activation of Ceacam1 has not been elucidated, but the PKCε re-expression studies in this chapter strongly implicate PKCε as a regulator of Ceacam1 expression.

In addition to increased expression of Ceacam1 in the PKCε KO MEFs was a restoration of InsR redistribution by insulin with PKCε reconstitution. This indicated that Ceacam1 is

regulated by PKCε and its lack of expression could impair InsR redistribution/trafficking in the PKCε KO MEFs. However PKCε reconstitution did not localise the InsR of PKCε KO MEFs to precisely the same portion of the gradient as that of the WT. These results were from adenovirus reconstituted cells, however, where a lesser restoration of Ceacam1 was observed compared to retrovirus. Therefore whether Ceacam1 alone is able to rescue InsR localisation is not clear at present.

In summary, the data from this chapter support the hypothesis that ablation of PKCε alters InsR trafficking acutely. This could be due to PKCε directly affecting the association and therefore activity of the InsR substrate, Ceacam1, which could result in reduced insulin uptake due to perturbed InsR internalisation in the short term. This alteration in InsR trafficking could also affect signalling, because impaired InsR endocytosis was linked to enhanced activation of metabolic signalling and reduced mitogenic signalling (Ceresa, Kao et al. 1998).

CHAPTER 4

Effect of PKCε Ablation on Insulin Signalling and Action

4.1 Introduction

There are a large number of studies showing that insulin action is mediated by a series of translocation and phosphorylation-based events (Saltiel and Kahn 2001). In the healthy state, binding of insulin induces the intrinsic activity of the InsR, which in turn recruits substrates such as IRS proteins that become phosphorylated and activated. Phosphorylation on Tyr residues of these substrates serve as a platform for SH2-domain containing proteins to dock including PI3K and Grb2. Through further activation of these docking proteins, the insulin signal is amplified, and the metabolic and mitogenic actions of insulin propagated.

Most of the actions of insulin are transmitted through IRS-1 and 2. The importance of IRS mediated signalling has been demonstrated in mice deficient in both IRS-1 and IRS-2 in the liver, which display defective insulin signalling, despite normal InsR activity (Dong, Park et al. 2006). It is thought that the activated InsR at the PM and internal membranes of the cell is important for activation of IRS (Heller-Harrison, Morin et al. 1995; Kublaoui, Lee et al. 1995), whereas activation of the InsR intracellularly is required for activation of the MAPK arm to stimulate the mitogenic actions of insulin (Ceresa, Kao et al. 1998).

There are a variety of factors that can modulate the signalling process to dampen and terminate the insulin signalling cascade, and its associated metabolic effects. These include endocytosis of the InsR and sequestration from the PM, insulin degradation following receptor binding and the activation of phosphatases and Ser/Thr kinases. In Chapter 3, PKCε KO MEFs were shown to exhibit altered InsR trafficking. This change in localisation of the InsR could affect signalling downstream from the receptor (Ceresa, Kao et al. 1998). Prior research has demonstrated that the InsR is most active at the endosomal compartment of the cell, the initial destination following binding of insulin (Kublaoui, Lee et al. 1995). At this site, the InsR remains active for signalling until insulin is dissociated from the receptor in a lower pH environment and insulin degraded by the insulin-degrading enzyme (Affholter, Fried et al. 1988; Duckworth, Hamel et al. 1990). That PKCε KO MEFs show a perturbed InsR distribution upon insulin stimulation compared to WT cells could result in a dysregulation of the insulin signalling cascade and/or downstream action.

The Ser/Thr kinase, PKCε has been shown to negatively regulate insulin signalling through Ser phosphorylation of IRS-1 and possibly the InsR itself (Samuel, Liu et al. 2007).

Therefore, ablation of PKCε may result in reduced serine phosphorylation of the most proximal components of the insulin signalling cascade and an enhanced signal generation. Enhanced activation of the insulin signalling pathway may lead to modified expression of the proteins therein, as an adaptive mechanism to counter-regulate and dampen the signal (Carpentier 1994).

The altered InsR localisation in PKCε KO MEFs may also result in a reduced ability of other InsR substrates to access the activated receptor. This could include negative regulators of insulin signalling, such as SOCS3 (Emanuelli, Peraldi et al. 2000) and the Grb family member, Grb14, which acts to downregulate insulin signalling emanating from the InsR (Cooney, Lyons et al. 2004; Depetris, Hu et al. 2005). In addition, Ceacam1, an InsR substrate which in Chapter 3 was shown to be altered in PKCε KO MEFs, has been shown to bind Shc. Ceacam1 activation by the InsR acts to sequester Shc, thereby reducing signalling through the ras-mitogen activated mitogenic pathway.

Therefore the aim of this chapter was to investigate potential alterations in the proximal insulin signalling cascade in MEFs deficient in PKCε, given the alterations in InsR trafficking described in Chapter 3.

4.2 METHODS

4.2.1 Glucose Transport in Primary MEFs

Primary MEFs, seeded in a 12-well plate were washed with 1 mL pre-warmed KRH buffer per well, and then incubated at 37°C in KRH Buffer + 0.2% BSA in the presence or absence of 100 nM insulin for 30 min. Non-specific uptake was measured by the inclusion of 10 μM cytochalasin B. Ten minutes before the end of the incubation, 50 μL of START solution was added to the wells. The samples were harvested as described in section 2.14. The sample (800 μL) was mixed with 10 mL scintillation fluid, and counted for radioactivity. The remainder of the lysate was used for protein determination by BCA assay.

4.2.2 Retroviral Reconstitution of Grb14 Into MEFs

The Grb14 WT and control retroviral plasmid constructs were kindly donated by Professor Roger Daly (Garvan Institute, Darlinghurst, NSW). PlatE cells were seeded at a density of 1 x 10^5 in 10 cm dishes one day prior to transfection, in high glucose DMEM with 10 % (v/v) FCS and no antibiotics and the retrovirus infection was performed as described in section 2.11. The concentrated media-containing virus was resuspended to half the original volume in low glucose DMEM with 10 % (v/v) FCS and no antibiotic. Retroviral stocks were aliquoted and stored at -80 °C until use. The PlatE cells remaining on each dish were harvested and analysed for Grb14 expression by immunoblotting as described in section 2.5. MEFs were infected with the retrovirus as described in section 2.11.3.

4.2.3 Statistical Analysis

Differences among groups were assessed by unpaired Student's t test or ANOVA using Tukey-Kramer post hoc test. These analyses were performed using GraphPad Prism 5 (Graph-Pad Software, La Jolla, CA, USA) software. Data are presented as mean ± standard error of the mean (SEM). Deviations were deemed significant at $P < 0.05$.

4.3 Results

4.3.1 PKCε null MEFs exhibit reduced insulin receptor phosphorylation but normal or enhanced downstream signalling

To investigate the effect that ablation of PKCε exerts upon the insulin signalling cascade, particularly the metabolic arm through IRS-1 and Akt, three separate primary MEF cell lines were stimulated with 100 nM insulin over a 30 min time course. The PKCε KO MEFs displayed significantly reduced InsR tyrosine phosphorylation at the autophosphorylation sites Tyr 1162 and Tyr 1163, compared to WT ($P < 0.001$, AUC). The greatest difference was observed 2 min after insulin stimulation, with the KO MEFs exhibiting approximately 86% reduction in InsR phosphorylation (Figure 4.3.1A). In stark contrast to this, downstream insulin signalling was not defective. Phosphorylation of IRS-1 at the Tyr 612 site showed a trend to be increased above WT (Figure 4.3.1B), while Akt phosphorylation at the Ser 473 site was also found not to be different to the WT MEFs over the 30 min insulin time course (Figure 4.3.1C). It was further investigated if IRS-1 Ser phosphorylation was altered given Ser phosphorylation has been documented to negatively regulate IRS-1 activation and PKCε also being a Ser/Thr kinase that has been reported to target IRS-1. Inhibitory phosphorylation of IRS-1 at the Ser 636/639 site, which has been shown to impair PI3K activation and therefore Akt signalling. This site was examined and found not to be different between the KO and WT MEFs (Figure 4.3.1D).

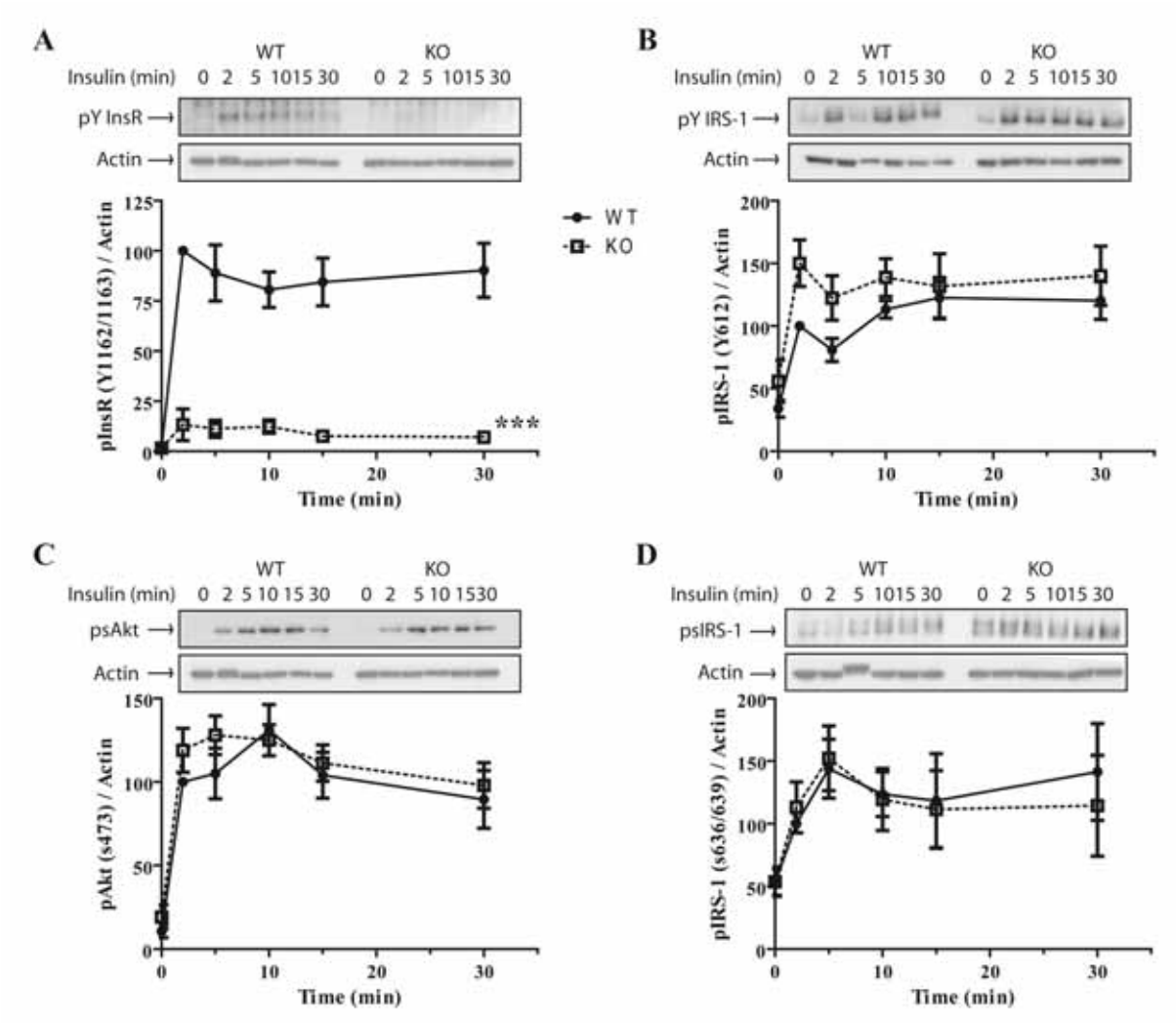

Figure 4.3.1 Phosphorylation of components of the proximal metabolic arm of insulin signalling in WT and PKCε KO MEFs.
WT (●) and PKCε KO MEFs (□) were serum starved for 2 h and stimulated with 100 nM insulin for the indicated times at 37 °C. Cells were washed three times with ice cold PBS, harvested in RIPA buffer and phosphorylation of components of the insulin signalling pathway were analysed by immunoblotting; **A:** InsR Tyr 1162/1163(AUC $P < 0.0001$), **B:** IRS-1 Tyr 612, **C:** Akt Ser 473 and **D:** IRS-1 Ser 636/639. Data are means ± SEM; n = 3-10 independent experiments in three separate MEF pools. (*** = $P < 0.001$ vs WT, AUC).

Importantly, as previously demonstrated in Chapter 3 in the WT1 and KO1 MEFs, InsR expression was not different between the genotypes over the three separate pools examined (Figure 4.3.2A); therefore the reduction in InsR phosphorylation is not simply due to reduced protein levels. Moreover, total IRS-1 (Figure 4.3.2B) and further downstream, Akt expression levels (Figure 4.3.2C) were not seen to be altered between the genotypes.

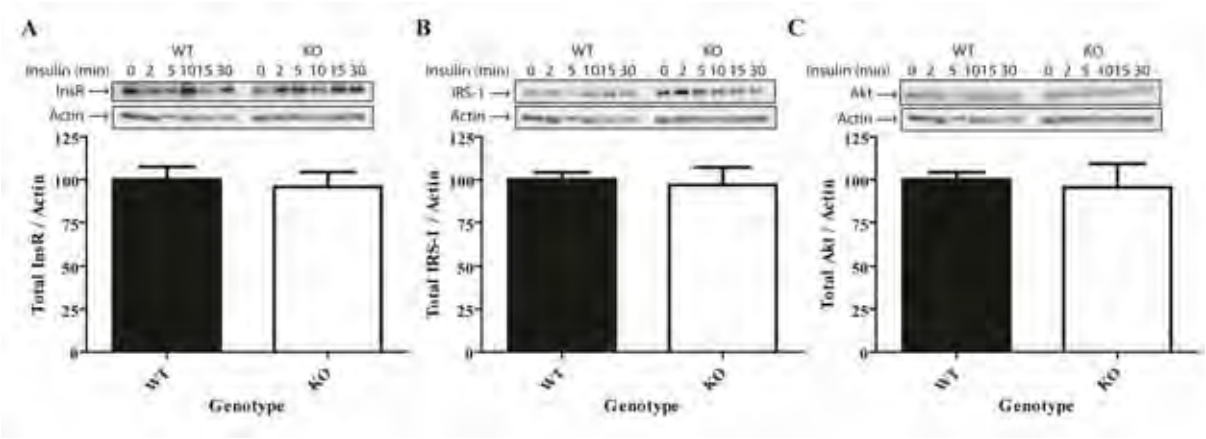

Figure 4.3.2 Expression of proximal components of the metabolic arm of insulin signalling in WT and PKCε KO MEFs.
WT (■) and PKCε KO MEFs (□) were serum starved for 2 h and stimulated with 100 nM insulin for the indicated times at 37 °C. Cells were washed three times with ice cold PBS, harvested in RIPA buffer and expression of **A:** InsR, **B:** IRS-1 and **C:** Akt were analysed by immunoblotting. Data are means ± SEM; n = 3-10 independent experiments in three separate pools of MEFs.

Because inhibition of InsR internalisation has been demonstrated to impair activation of mitogenic components of the insulin signalling cascade, phosphorylation of p44/42 MAPK (Erk 1/2) and p66 Shc were examined. It was found that Erk 1/2 phosphorylation at Thr202/Tyr204 was upregulated in PKCε KO MEFs ($P < 0.05$, AUC) over the 30 min time course (Figure 4.3.3A). However, this enhancement was not due to enhanced upstream Shc Tyr 239/240 phosphorylation (Figure 4.3.3B), or altered expression of Erk 1/2 protein (Figure 4.3.3C).

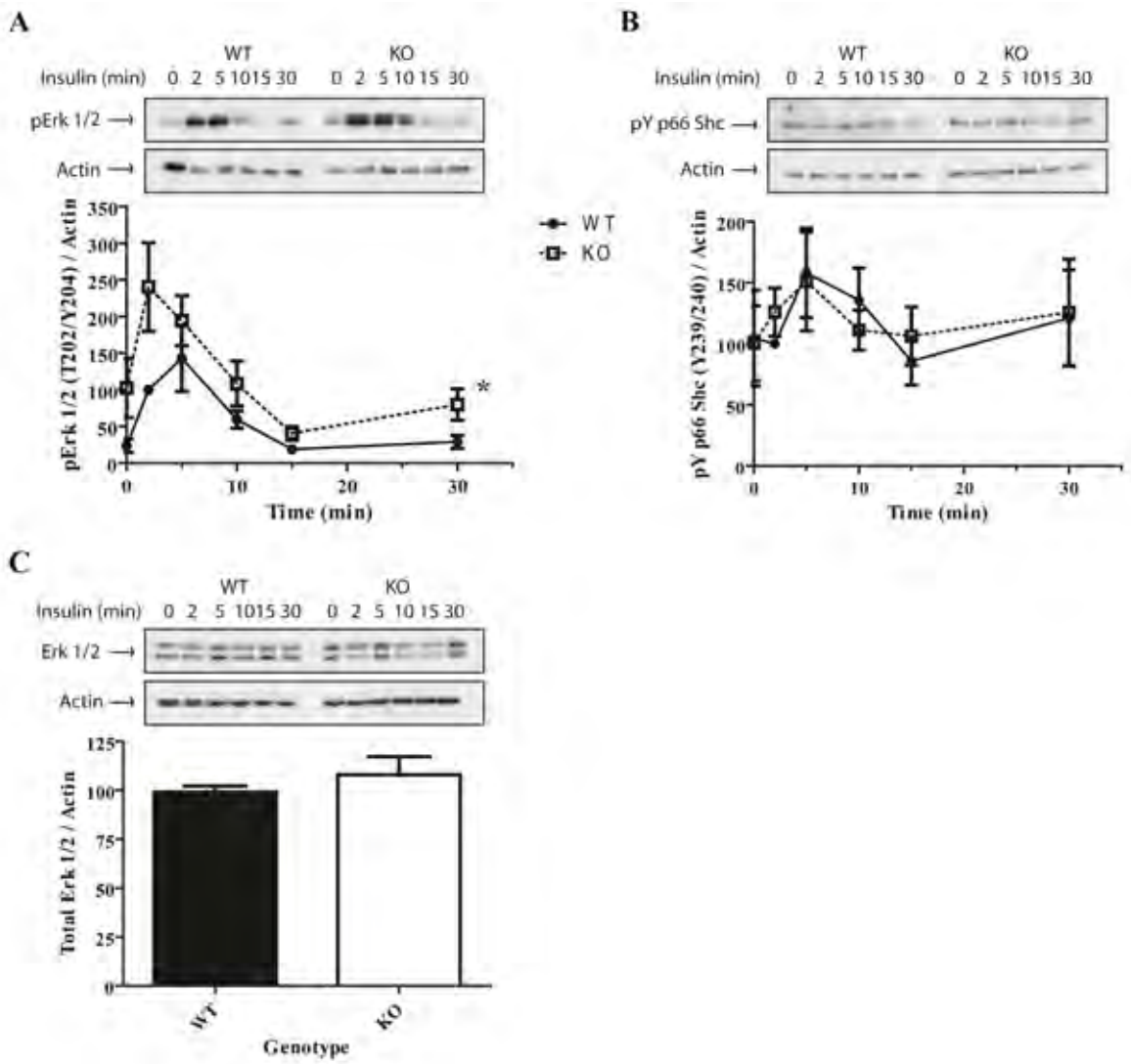

Figure 4.3.3 Phosphorylation of mitogenic components of insulin signalling in WT and KO MEFs. WT (■) and PKCε KO MEFs (□) were serum starved for 2 h and stimulated with 100 nM insulin for the indicated times at 37 °C. Cells were washed three times with ice cold PBS, harvested in RIPA buffer. **A:** Erk 1/2 phosphorylation; **B:** p66Shc phosphorylation; and **C:** Erk 1/2 expression were analysed by immunoblotting. Data are means ± SEM; n = 3-10 independent experiments in three separate pools of MEFs (* = $P < 0.05$ vs WT AUC).

These data show that the defect in InsR autophosphorylation is seen consistently with PKCε deletion. Despite this defect, the KO MEFs exhibit at least normal downstream insulin signalling. These alterations are not due to changes in expression of the signalling components studied, namely InsR, IRS-1, Akt and Erk 1/2.

To determine whether the unaltered or enhanced insulin signalling profile observed in the KO MEFs despite reduced InsR phosphorylation was due to altered negative regulation of signalling from the InsR, SOCS3 protein expression was examined. Using the three separate MEF pools, there was no significant change in SOCS3 expression (Figure 4.3.4) and therefore SOCS3 is unlikely play a role in the signalling profile observed.

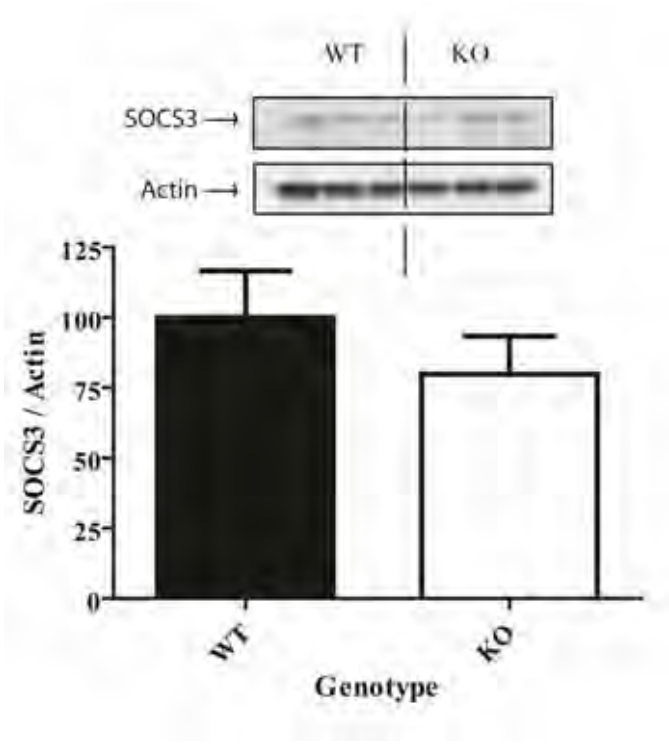

Figure 4.3.4 SOCS3 expression in WT and KO MEFs.
WT and PKCε KO MEFs were harvested in RIPA buffer and SOCS3 expression was analysed by immunoblotting. Data are means ± SEM; n = 3 independent experiments in 3 separate MEF pools.

4.3.2 General phosphatase inhibition can enhance insulin receptor phosphorylation in PKCε KO MEFs

To determine whether the InsR in PKCε KO MEFs was able to be phosphorylated, a broad range phosphatase inhibitor cocktail, PhosStop, was used in the culture medium of KO1 and WT1 MEFs. In these experiments, cells were incubated with the phosphatase inhibitor for 20 min prior to insulin stimulation. It was observed that inhibition of phosphatases was able to enhance phosphorylation of the InsR in both KO1 and WT1 cells (Figure 4.3.5).

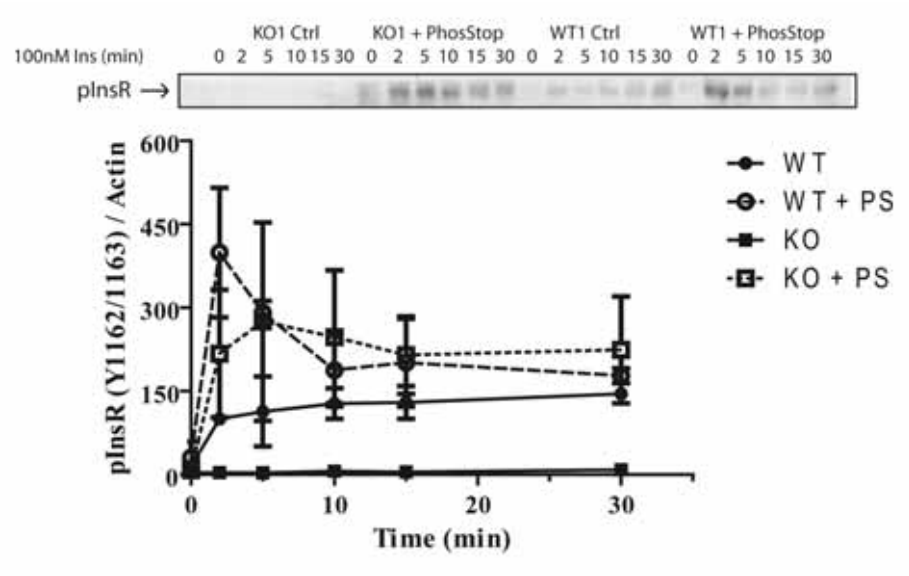

Figure 4.3.5 Insulin receptor phosphorylation in WT1 and KO1 MEFs treated with or without a general phosphatase inhibitor.
WT and PKCε KO MEFs were serum starved for 2 h and stimulated with 100 nM insulin for the indicated times at 37 °C. Cells were washed three times with ice cold PBS, harvested in RIPA buffer and InsR Tyr 1162/1163 phosphorylation was analysed by immunoblotting. Data are means ± Range; n = 2 independent experiments.

This was not unexpected, as it has previously been shown that treatment of cells with the Tyr phosphatase inhibitor pervanadate can increase InsR phosphorylation (Solow, Harada et al. 1999). A preliminary experiment looking at FITC-insulin uptake in the presence of PhosStop, showed no alteration in insulin uptake in KO1 MEFs compared to untreated KO1 MEFs (data not shown). This indicates that despite the phosphatase inhibitor increasing phosphorylation of the InsR, the reduced InsR phosphorylation does not alter InsR internalisation. This data suggests that the InsR in WT MEFs may be protected against phosphatase activity by some means.

4.3.3 Grb14 expression does not affect insulin receptor phosphorylation in MEFs

A potential explanation for the altered signalling profile displayed by the PKCε KO MEFs is a change in Grb14 level or function. Grb14 KO mice have been shown to have reduced InsR Tyr phosphorylation yet enhanced downstream metabolic actions, similar to that observed in the PKCε KO MEFs (Cooney, Lyons et al. 2004). Therefore, Grb14 protein expression was examined in three separate pools of KO and WT MEFS. The KO1 MEFs had a significant

reduction in Grb14 protein expression (100 ± 10 % in WT1 vs 33 ± 11 % in KO1, $p < 0.01$ n= 7) (Figure 4.3.6A).

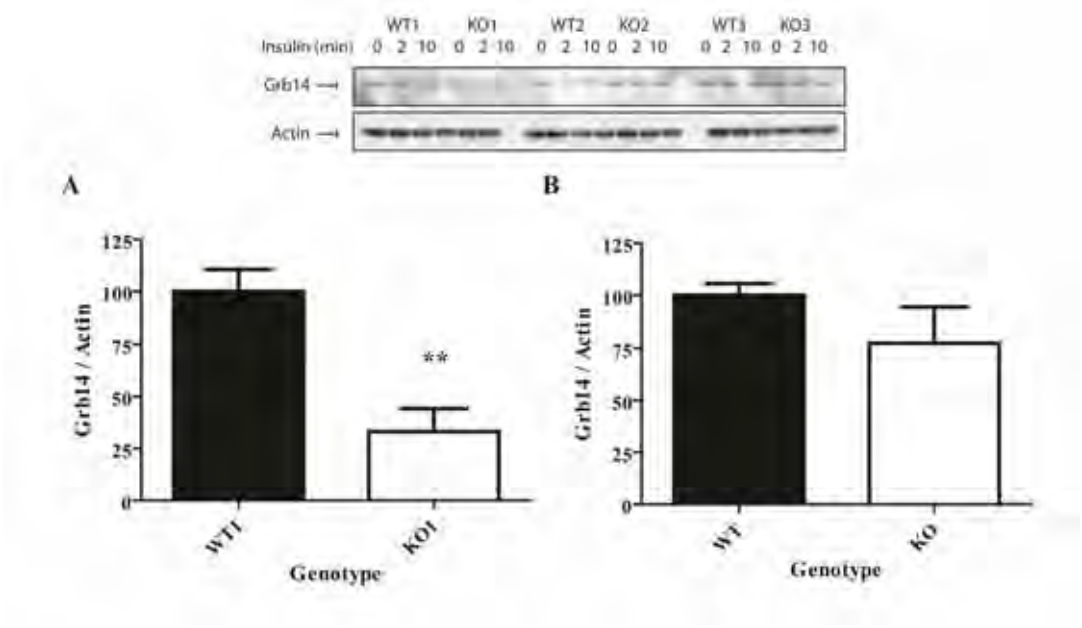

Figure 4.3.6 Grb14 expression in WT and PKCε KO MEFs.
WT (■) and PKCε KO MEFs (□) were serum starved for 2 h and stimulated with 100 nM insulin for up to 10 min at 37 °C. Cells were washed three times with ice cold PBS, harvested in RIPA buffer and Grb14 protein expression was analysed in **A:** WT1 and KO1 MEFs and **B:** All three MEF pools combined Grb14 by immunoblotting. Data are means ± SEM; n = 3-7 independent experiments (** = $P < 0.05$ vs WT).

This however was not observed in the KO2 or KO3 pools and therefore overall there is no difference in Grb14 expression (Figure 4.3.6B). This implies that the signalling profile seen in the KO MEFs compared to the WT is not simply due to lower Grb14 expression.

To further exclude a role for Grb14, the protein was re-expressed in the KO1 pool of MEFs using a retroviral vector, to examine whether restoration of the Grb14 level resulted in the normalisation of InsR phosphorylation and downstream signalling. This reconstitution resulted in expression above that seen in the KO1 control cells, but still less than that seen in the WT1 cells (Figure 4.3.7).

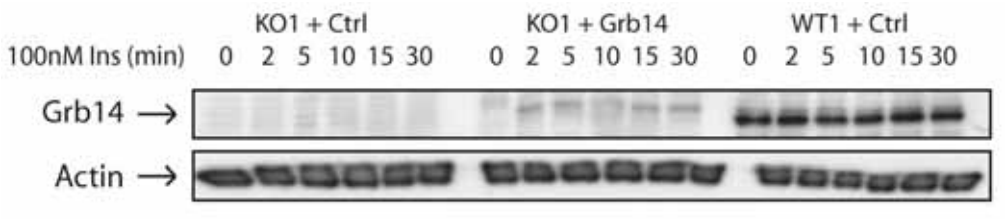

Figure 4.3.7 Grb14 expression with and without retrovirus in WT1 and PKCε KO1 MEFs.
WT and PKCε KO MEFs were serum starved for 2 h and stimulated with 100 nM insulin for the indicated times at 37 °C. Cells were washed three times with ice cold PBS, harvested in RIPA buffer and Grb14 expression was analysed by immunoblotting.

The KO1 MEFs re-expressing Grb14, showed no significant difference in their signalling profile compared to those expressing the control retrovirus at the level of InsR, Tyr and Ser phosphorylated IRS-1 and Akt (Figure 4.3.8). This finding confirms further that Grb14 is not responsible for the observed alterations seen in the insulin signalling cascade between the PKCε KO and WT MEFs.

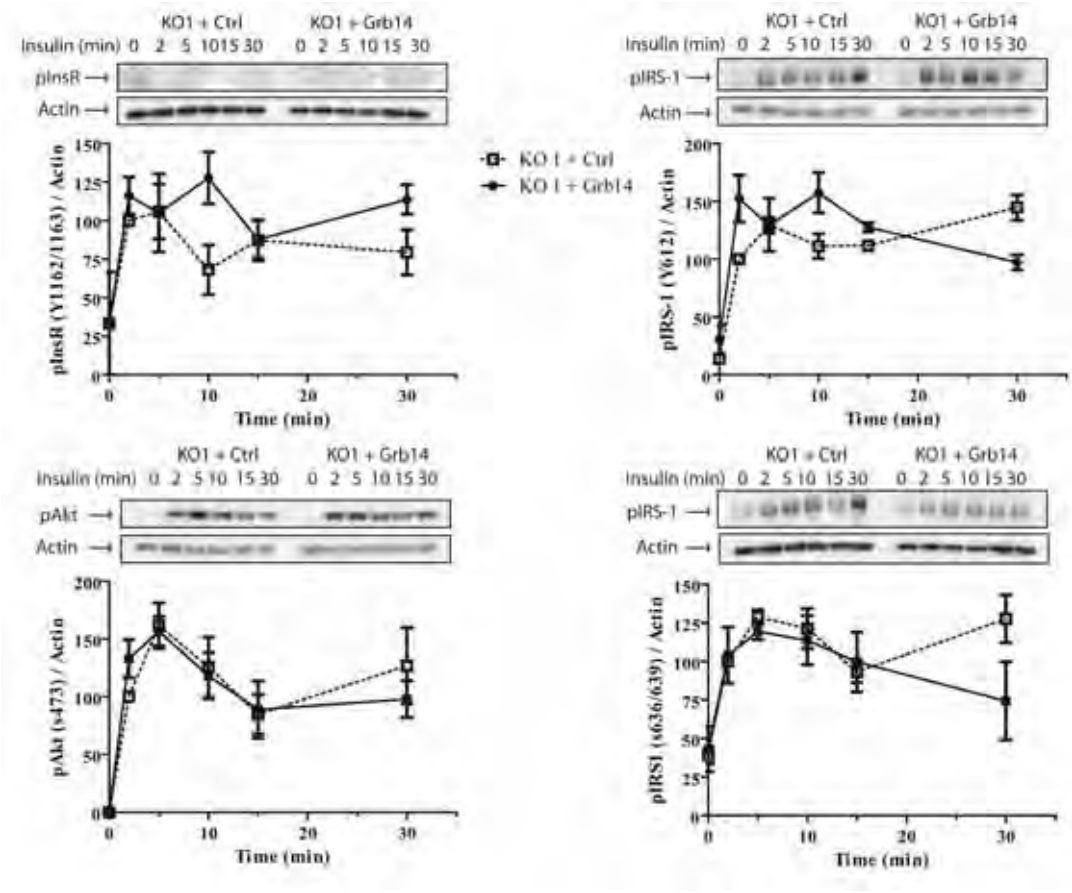

Figure 4.3.8 Insulin signalling in KO1 MEFs with or without Grb14 retroviral re-expression.
WT (■) and PKCε KO MEFs (□) were serum starved for 2 h and stimulated with 100 nM insulin for the indicated times at 37 °C. Cells were washed three times with ice cold PBS, harvested in RIPA buffer and proximal components of the insulin signalling pathway were analysed by immunoblotting. Data are means ± SEM; n = 3 independent experiments.

4.3.4 Reconstitution of PKCε into KO MEFs does not alter insulin receptor phosphorylation

To determine if the observed signalling changes, particularly the reduced InsR phosphorylation, could be reversed by reconstitution of PKCε, adenovirally-mediated expression of PKCε was employed in two separate pools of WT and KO MEFs. As previously shown in Chapter 3, PKCε was greatly overexpressed above endogenous WT levels, following 48 h of infection with the adenovirus (see Figure 3.3.15). Cells were subsequently stimulated with 100 nM insulin for up to 10 min. The overexpression of PKCε did not alter InsR phosphorylation in the PKCε KO MEFs (Figure 4.3.9A). While the reconstitution of PKCε shifted IRS-1 Tyr 612 phosphorylation toward WT levels (Figure

4.3.9B), without an alteration in IRS-1 Ser 636/639 phosphorylation (Figure 4.3.9D). While Akt Ser 473 phosphorylation remained similar between control and reconstituted KO MEFs compared to WT MEFs (Figure 4.3.9C).

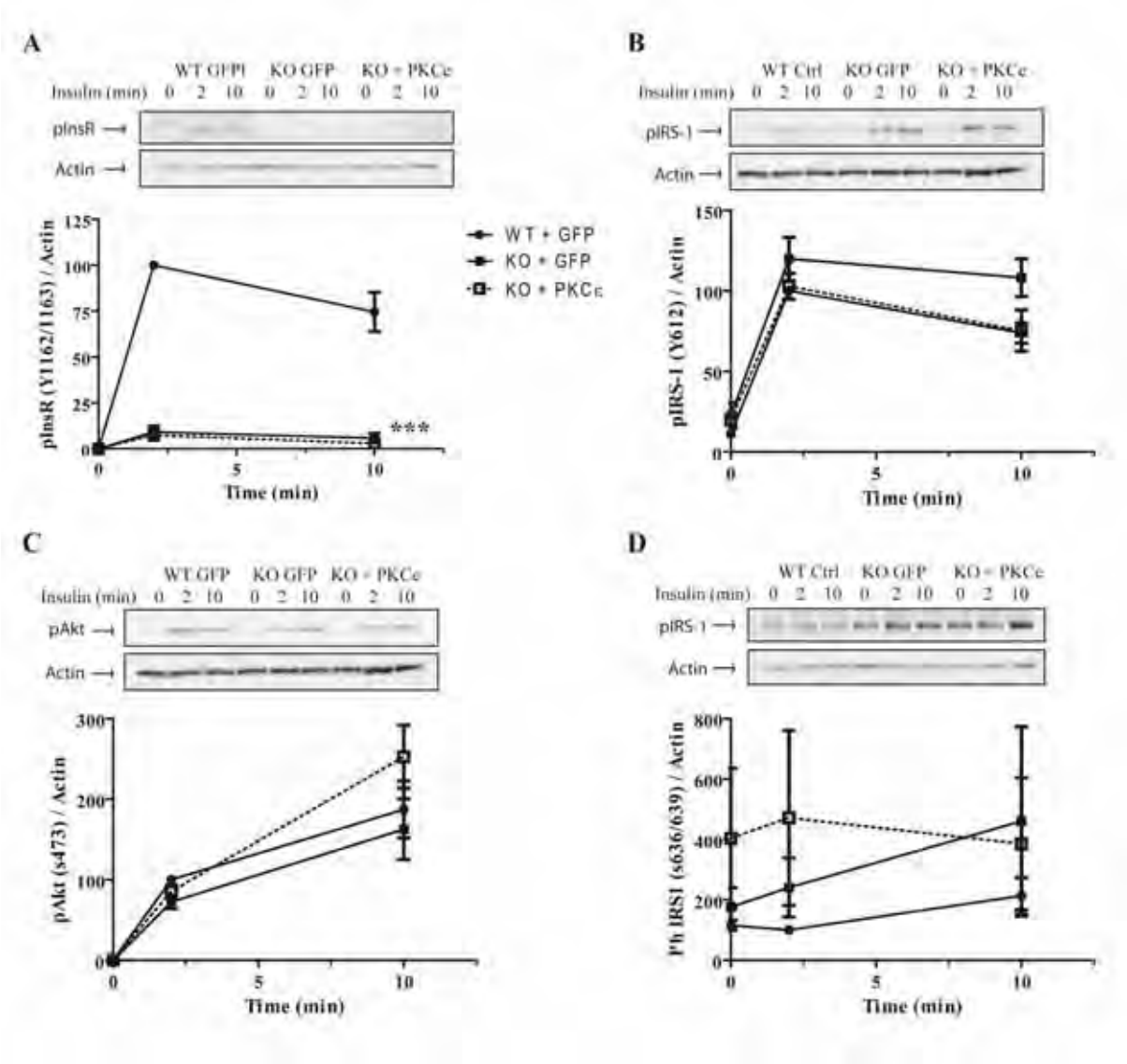

Figure 4.3.9 Insulin signalling in WT and KO MEFs reconstituted with a PKCε adenoviral construct. WT and PKCε KO MEFs were serum starved for 2 h and stimulated with 100 nM insulin for the indicated times at 37 °C. Cells were washed three times with ice cold PBS, harvested in RIPA buffer and insulin signalling analysed by immunoblotting. The proximal components of the insulin signalling pathway; **A:** InsR Tyr 1162/1163 phosphorylation, **B:** IRS-1 Tyr 612 phosphorylation **C:** Akt Ser 473 phosphorylation and **D:** IRS-1 Ser 636/639 phosphorylation were examined by immunoblotting. Data are means ± SEM; n = 3-5 independent experiments in two separate MEF lines. (*** = P < 0.001 vs WT GFP, AUC).

PKCε overexpression did not alter Grb14 protein expression in the KO1 MEFs (Figure 4.3.10), indicating that the low Grb14 expression seen specifically in the KO1 MEFs, but not in KO2 or KO3, was not due to PKCε ablation alone.

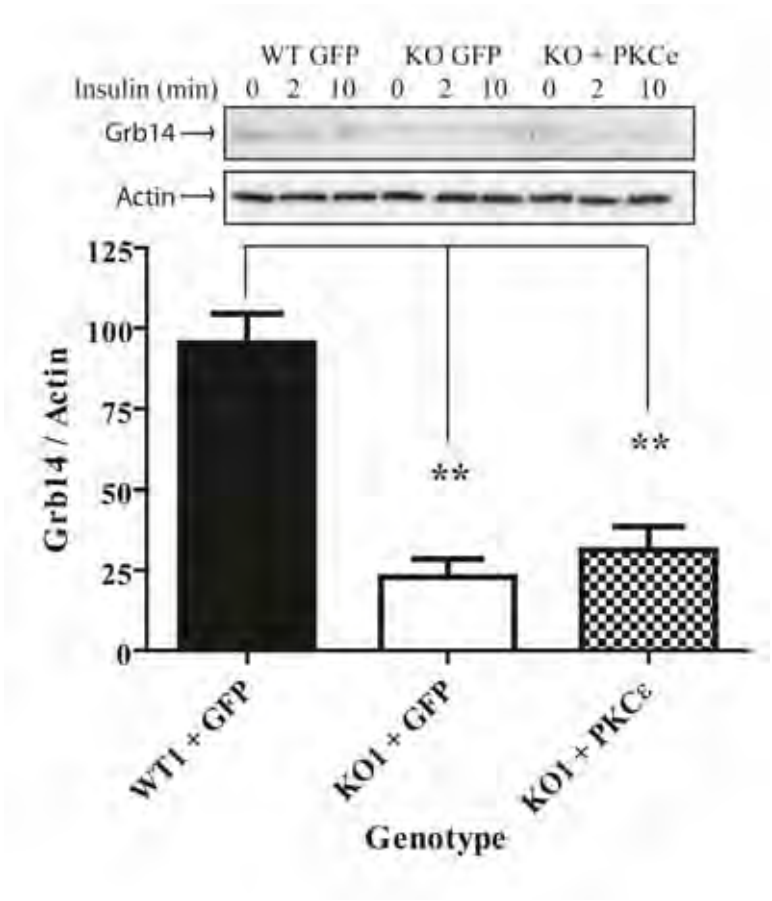

Figure 4.3.10 Grb14 protein expression KO1 MEFs reconstituted with a PKCε adenoviral construct. WT (■) and PKCε KO MEFs (□) were serum starved for 2 h and stimulated with 100 nM insulin for the indicated times at 37 °C. Cells were washed three times with ice cold PBS, harvested in RIPA buffer and Grb14 expression analysed by immunoblotting. Data are normalised to Grb14 expression in the basal state. Data are means ± SEM; n = 3 independent experiments (** $P < 0.01$ vs WT).

Taken together, these experiments suggest that the signalling alterations observed are an adaptation of PKCε KO MEFs, enabling them to survive in culture, rather than being due more directly to the absence of PKCε.

PKCε was also re-expressed in the KO1 MEF pool with a retrovirus construct to determine if reconstituting PKCε at more physiological levels for longer is required to overcome the changes in InsR phosphorylation. Consistent with the results from the reconstitution of PKCε using adenovirus, when PKCε was re-expressed in KO1 MEFS by retroviral techniques, the

decreased InsR phosphorylation was not reversed (Figure 4.3.11A). Similarly, IRS-1 Tyr 612 (Figure 4.3.11B) and Akt Ser 473 phosphorylation levels were not altered (Figure 4.3.11C).

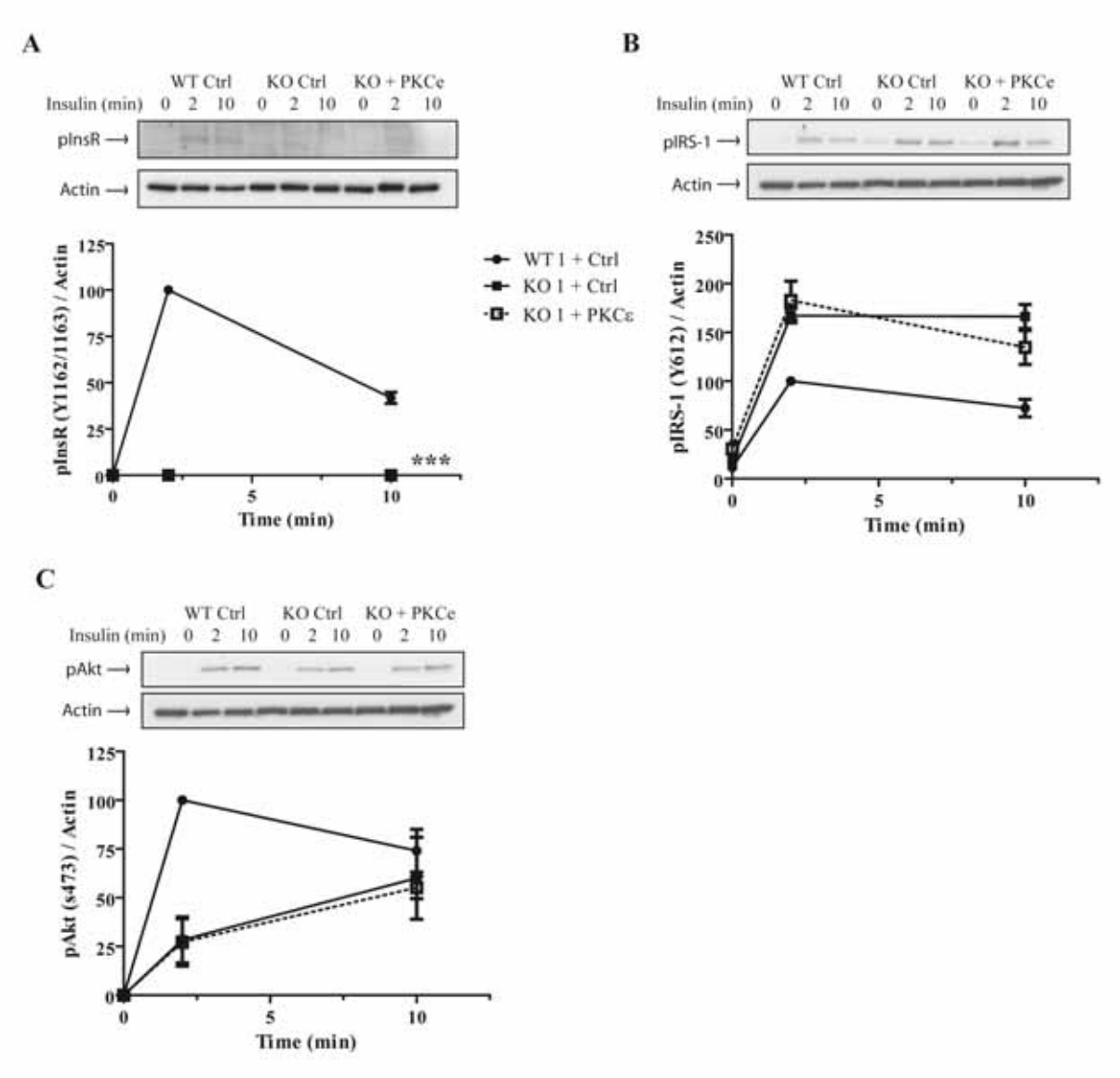

Figure 4.3.11 Insulin signalling in WT1 and KO1 MEFs reconstituted with a PKCε retroviral construct. WT and PKCε KO MEFs were serum starved for 2 h and stimulated with 100 nM insulin for the indicated times at 37 °C. Cells were washed three times with ice cold PBS, harvested in RIPA buffer. **A:** InsR Tyr 1162/1163; **B:** IRS-1 Tyr 612 and **C:** Akt Ser 473 phosphorylation were analysed by immunoblotting. Data are means ± SEM; n = 3 independent experiments (*** = P < 0.001 vs WT Control, AUC).

4.3.5 Glucose uptake is not different between WT and PKCε KO MEFs

It was next examined if the absence of PKCε resulted in any functional differences in terms of glucose metabolism. The KO1 and WT1 MEFs showed no difference in insulin stimulated glucose uptake (Figure 4.3.12A). In these experiments, the absolute level of glucose uptake was relatively small (< 0.1 pmol/min/mg) compared to that seen in other cell systems such as myotubes (Cazzolli, Craig et al. 2002; Taylor, Ye et al. 2006).

Given that only a small percentage of Akt phosphorylation is required for near maximal glucose transport (Hoehn, Hohnen-Behrens et al. 2008), the lack of differences between genotypes was not an unexpected finding. Nevertheless, there was an insulin dependent increase in glucose transport ($P < 0.05$) into both the KO1 and WT1 MEFs (Figure 4.3.12B).

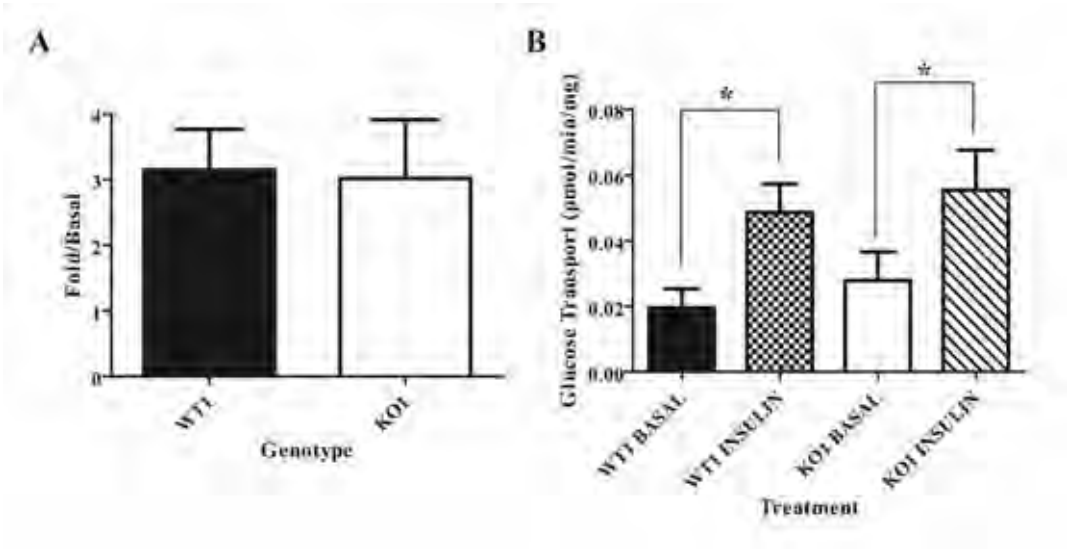

Figure 4.3.12 Glucose uptake into WT1 and KO1 MEFs.
WT (■) and PKCε KO MEFs (□) were serum starved for 2 h prior to stimulation with 100 nM insulin for 30 min at 37 °C. Over the last 10 min, 2 mM 2-deoxyglucose and 10 µCi/mL 2-deoxy-D-[2,6-3H]glucose in KRH was added to the media. Cells were washed three times with ice cold PBS, harvested in 1 M KOH and radioactivity counted on a β-counter. Data are means ± SEM; n = 3 independent experiments (* = $P < 0.05$ vs Basal).

Taken together with the results of Chapter 1 where it was shown general trafficking was not perturbed, these findings indicate general transport is not defective in the PKCε KO MEFs.

4.4 Discussion

The InsR Tyr kinase is activated upon ligand binding, resulting in autophosphorylation within the kinase domain, followed by further trans- and cis- phosphorylation (Kido, Nakae et al. 2001). It is thought that this kinase activation and subsequent Tyr phosphorylation is required to maintain the receptor in its active conformation, promoting endocytosis of the InsR while also providing a platform to which its substrates, IRS-1 and 2, can dock and propagate the signal (Carpentier 1994; Heller-Harrison, Morin et al. 1995). Furthermore, the spatial localisation of the activated InsR has been demonstrated to affect substrate activation and therefore the metabolic and mitogenic actions of insulin (Kublaoui, Lee et al. 1995; Ceresa, Kao et al. 1998). The Ser/Thr protein kinase, PKCε, has been shown to negatively regulate insulin signalling, through Ser phosphorylation of IRS-1 and some groups have reported this to be the case with the InsR itself (Samuel, Liu et al. 2007). Having demonstrated in Chapter 3 that the PKCε KO MEFs exhibit reduced insulin internalisation and InsR redistribution with insulin stimulation, it was investigated if this could affect activation of particular components of the insulin signalling cascade.

In these signalling experiments, it was found that the PKCε KO MEFs exhibited reduced InsR phosphorylation within the kinase domain compared to the WT MEFs. Despite this, the phosphorylation of proximal downstream insulin signalling components examined, IRS-1 (Tyr 612), Akt (ser 473) and Erk 1/2 (T202/Tyr204), were not altered but in fact tended to be enhanced. It was also observed that inhibitory IRS-1 phosphorylation at Ser 636/639, a key site for modulating PI3K binding and activation, and SOCS3 expression were not affected with PKCε deletion. Furthermore, using a general phosphatase inhibitor, the InsR in PKCε KO MEFs could be phosphorylated to a similar degree as that seen in the WT, suggesting that in the PKCε KO MEFs, the InsR may be subject to greater phosphatase activity. A key regulator of this is the InsR adaptor protein, Grb14. In the KO1 MEFs, Grb14 was indeed found to be downregulated, yet when further examined in two other KO and WT cell lines there was no significant difference overall between the genotypes. Moreover, re-expression of Grb14 into the KO1 MEFs failed to rescue the defect in insulin-stimulated InsR phosphorylation. Finally, when PKCε was reconstituted in KO MEFs, the InsR phosphorylation failed to be restored. To investigate the consequences of insulin signalling

in the MEFs with a physiological readout, insulin-stimulated glucose transport was examined and found not to be different between the genotypes.

The metabolic arm of the insulin signalling pathway through IRS-PI3K-Akt has been proposed to be activated at the PM (Ceresa, Kao et al. 1998), whilst the mitogenic arm through ras-MAPK is thought to be initiated from endosomal compartments, therefore requiring InsR internalisation (Ceresa, Kao et al. 1998). This was highlighted by Ceresa *et al*. using a dynamin mutant, K44A/dynamin, which inhibited InsR endocytosis. In this case, IRS-1 Tyr phosphorylation was not affected and neither was Akt, despite a modest reduction in PI3K activity. On the other hand, this block in InsR internalisation led to a marked reduction in MAPK signalling. Many studies have also shown the requirement for InsR autophosphorylation in promoting its internalisation (McClain, Maegawa et al. 1987; Carpentier, Paccaud et al. 1992; Kublaoui, Lee et al. 1995). Given these aforementioned studies and the results from the trafficking experiments in Chapter 3 as well as those examining InsR phosphorylation in this chapter, it may be expected that downstream signalling of the mitogenic signalling pathway in particular, which requires InsR endocytosis to be activated, could be impaired. It was, however observed that both proximal metabolic signalling as well as mitogenic signalling components were not impaired and in fact tended to be enhanced in the PKCε KO MEFs. In the current studies, Tyr phosphorylation of the InsR within the kinase domain was examined. The activation of this site leads to further autophosphorylation at Tyr 972, which is important for IRS-1 and Shc binding to mediate their effects. However, it was difficult to obtain a signal from the Tyr 972 antibody used and as such this important site was not examined in these studies. The enhanced phosphorylation of IRS-1, however suggests that activation at this site is not impaired in KO MEFs. Furthermore, although in Chapter 3 a reduction in InsR internalisation was observed, it was not a complete block. Therefore the InsR that is reaching the endosomal compartment in the KO MEFs may be sufficient for activating the mitogenic signalling of Shc and Erk 1/2.

To examine the InsR phosphorylation defect in PKCε KO MEFs, cells were treated with a broad range phosphatase inhibitor and InsR phosphorylation could be rescued to similar levels seen in WT MEFs. This phosphatase treatment also induced a slight increase in InsR phosphorylation in the WT MEFs, which has also previously been reported in cells treated with the Tyr phosphatase inhibitor, pervanadate (Solow, Harada et al. 1999). The reduction in InsR phosphorylation observed in PKCε KO MEFs is not likely to explain the reduced InsR

internalisation as in preliminary experiments, when examined in the presence of the phosphatase inhibitor cocktail, insulin uptake was not increased in KO MEFs compared to untreated insulin-stimulated cells (data not shown). The results of the phosphatase treatment described in this chapter indicate the possibility that phosphatases, such as the InsR specific phosphatase, PTP1B may target the InsR to a greater degree in the PKCε KO MEFs. A primary candidate mechanism for this possibility is an alteration in the adaptor protein Grb14.

Grb14 competes with IRS-1 to bind to the activated InsR and therefore can act to negatively regulate insulin signalling and action, while preserving InsR signalling (Holt and Siddle 2005). Grb14 KO mice have been shown to have reduced InsR phosphorylation, yet enhanced downstream signalling through IRS-1 and Akt (Cooney, Lyons et al. 2004). Grb14 expression was examined and in the KO1 pool of MEFs, which exhibited a large reduction in Grb14 protein expression compared to WT1. Reconstituting Grb14 to levels more similar to those of WT MEFs with retrovirus failed to rescue InsR phosphorylation or affect the overall signalling profile seen in the KO1 MEFs. Together with the fact that the two other pools of PKCε KO MEFs did not show changes in Grb14 expression and the fact that reconstitution of PKCε did not alter Grb14 expression in the KO1 MEFs, altered InsR phosphorylation observed in the PKCε KO MEFs is not likely to be due to altered Grb14 expression or function. Moreover, due to the non-specificity of the phosphates inhibitor used, these experiments were unable to identify a candidate phosphatase that may be more active against the InsR in the KO MEFs. Although the altered InsR localisation shown in Chapter 3 may make the InsR of KO MEFs more susceptible to phosphatase activity, this was not examined in these experiments.

To further examine whether the reduced InsR phosphorylation could be due to deletion of PKCε, adenoviral and retroviral mediated techniques were used to reconstitute KO MEFs with PKCε. Following 48 h infection with adenovirus, there was no change observed in the signalling profile of PKCε KO MEFs. It was hypothesised that changes may require longer to take place and re-expressing PKCε at more physiological levels may be required if such changes were dependent upon a tight stoichiometry of the proteins inducing the changes. However, re-expressing PKCε at more endogenous levels for a longer period of time with a retroviral PKCε construct also did not reverse the signalling alterations seen in the KO MEFs. These results suggest that the perturbed InsR phosphorylation in the PKCε KO MEFs is an

indirect effect of PKCε ablation and may have arisen as an important adaptation for growth and survival in culture in the absence of PKCε.

Further to this, to determine whether the metabolic actions of insulin, as well as upstream signalling were not altered with the loss of PKCε, glucose uptake experiments were performed in the KO1 and WT1 MEF pools. As expected with the signalling profiles observed, there was no difference in uptake in the basal or insulin stimulated state, suggesting that the metabolic action of insulin is not altered and the altered trafficking does not impact on this process. Although an insulin induced increase was observed, this may not be due to the stimulation of GLUT4 trafficking, as studies have reported this insulin-sensitive glucose transporter is not always expressed in mouse embryonic cells (Hogan, Heyner et al. 1991; Aghayan, Rao et al. 1992; Tonack, Fischer et al. 2004). Furthermore, glucose uptake in embryonic cells is thought to occur through passive diffusion rather than stimulated uptake. The low rate of glucose transport observed in these experiments suggests that the machinery for insulin-stimulated glucose transport, as occurs in fat or muscle cells, may not be fully expressed in MEFs. However, given the increase seen with insulin and the lack of an effect of PKCε deletion, there does not appear to be a defect at this downstream level of insulin action, but glucose transport studies in primary skeletal muscle or fat is needed to confirm this.

Overall the results of this chapter indicate that ablation of the Ser/Thr kinase PKCε does not affect insulin signalling or action in normal culture conditions, in the absence of deleterious metabolic insults. In preliminary experiments, when MEFs were stimulated at a lower dose of insulin (1 nM), that is unlikely to activate the IGF-1R, the insulin signalling profiles were not different to those observed in this chapter (see Appendix I). To confirm that the signalling is being propagated from the InsR and not other receptors with affinities for insulin such as the IGF1 receptor, it would be necessary to perform an InsR kinase assay to confirm that it is indeed active, despite having reduced phosphorylation. In order to reveal differences between the WT and KO MEFs that may exist in the insulin signal transduction pathway and its associated actions, it may be necessary to treat the cells with insults that activate PKCε to allow disparities to be revealed. Lipid oversupply is one such insult, and the effect of this intervention on insulin signalling in PKCε KO MEFs will be described in Chapter 5.

CHAPTER 5

Effect of PKCε Ablation on Insulin Signal Transduction in MEFs under conditions of Lipid Oversupply

5.1 Introduction

The link between increased lipid availability and IR is well established, however the exact mechanisms remain unclear. This association has been shown in various models, including increased dietary supply of lipid to rats (Storlien, James et al. 1986; Kraegen EW, Clark PW et al. 1991), lipid infusion in rodents and humans (Kim and Youn 1997; Schmitz-Peiffer, Craig et al. 1999; Hevener, Reichart et al. 2001; Watt, Hevener et al. 2006; Frangioudakis and Cooney 2008; Hoy, Brandon et al. 2009), and lipid treatment in cell culture (Schmitz-Peiffer, Craig et al. 1999; Cazzolli, Craig et al. 2002; Hoehn, Hohnen-Behrens et al. 2008). *In vitro* studies have used saturated (palmitate C16:0), monounsaturated (oleate C18:1) and polyunsaturated (linoleate C18:2) FA oversupply to study the effects of lipids on inducing IR in the myotubes as assessed by glucose transport defects (Thompson, Lim-Fraser et al. 2000; Cazzolli, Carpenter et al. 2001; Malhi, Bronk et al. 2006; Solinas, Naugler et al. 2006; Cazzolli, Mitchell et al. 2007). Whilst these experiments have identified that both saturated and unsaturated FA induce IR, this occurs through two distinct signalling mechanisms (Schmitz-Peiffer, Craig et al. 1999; Cazzolli, Craig et al. 2002; Holland, Brozinick et al. 2007b). It is generally accepted that a build up of lipid metabolites, such as DAGs and ceramides, along with activation of inflammatory/stress signalling pathways are associated with impaired cellular glucose transport and metabolism, as well as the differential signalling defects that have been reported (Schmitz-Peiffer, Craig et al. 1999; Samuel, Liu et al. 2004; Stratford, Hoehn et al. 2004; Neschen, Morino et al. 2005; Hotamisligil 2006).

Defective insulin action can occur either via reduced activation of IRS-1/2 Tyr phosphorylation with or without increased inhibitory Ser phosphorylation, or through reduced activation of Akt (Schmitz-Peiffer, Craig et al. 1999; Samuel, Liu et al. 2004; Hoehn, Hohnen-Behrens et al. 2008). Inhibition of Akt by palmitate, for example, is proposed to result from enhanced activation of protein phosphatase 2A (Cazzolli, Carpenter et al. 2001; Stratford, Hoehn et al. 2004) or increased PKCζ activation (Powell, Hajduch et al. 2003; Powell, Turban et al. 2004) due to the increased ceramide accumulation that occurs under these experimental conditions. A number of other signalling intermediates have also been proposed to be involved in negative modulation of insulin signalling, including activation of JNK (Aguirre, Uchida et al. 2000; Hirosumi, Tuncman et al. 2002; Weston and Davis 2007), NFκB (Shoelson, Lee et al. 2003) and the lipid activated PKCs (Schmitz-Peiffer, Browne et al. 1997; Griffin, Marcucci et al. 1999; Yu, Chen et al. 2002).

In relation to inflammatory or stress signalling, it has been demonstrated that saturated but not unsaturated FA, can lead to increased inflammatory signalling through JNK activation (Solinas, Naugler et al. 2006); and elevated saturated FAs, such as palmitic and stearic acid (C18:0), have been reported in the plasma of obese subjects (Reaven, Hollenbeck et al. 1988). Furthermore, it was recently shown by Holzer *et al*. that the Tyr kinase, c-Src, can be recruited to membrane microdomains in the presence of saturated FA oversupply. Activation of c-Src within membrane microdomains led to activation of JNK and inhibition of insulin signalling (Holzer, Park et al. 2011). The novel PKC isozyme, PKCε, has also been linked to JNK activation (Comalada, Xaus et al. 2003) and it has recently been shown that PKCε deletion can protect mice from IR after one week of high fat feeding (Raddatz, Turner et al. 2011). This is consistent with the hypothesis that increased FFA can increase intracellular DAG levels and via PKC activation and subsequent IRS-1 Ser phosphorylation, lead to reduced IRS-1 Tyr phosphorylation.

Lipid oversupply has long been shown to affect membrane phospholipid composition and as such impact fluidity (Borkman, Storlien et al. 1993; Vessby, Tengblad et al. 1994; Pan, Lillioja et al. 1995). FA in particular, are able to incorporate into the PM phospholipids, and incorporation of the saturated FA palmitate, but not polyunsaturated FA, has been shown to reduce membrane fluidity (Pjura, Kleinfeld et al. 1982; Clamp, Ladha et al. 1997). This is now receiving increasing attention in the context of insulin signalling (Ibrahim, Natrajan et al. 2005; Chen, Liu et al. 2006; Dobrzyn, Jazurek et al. 2010), which is likely to be affected by changes in membrane composition, since many elements of signalling cascades emanate from membrane microdomains.

Overall, it is thus becoming increasingly evident that the mechanisms leading to inhibition of insulin signalling and action are complex. Additive effects of numerous lipid species and signalling intermediates, rather than of one particular entity are likely to play a role. For example, localisation of lipid metabolites, increases in particular sub-pools of lipid metabolites, as well as membrane alterations which may sequester signalling intermediates into specific cellular compartments are all important in the development of lipid-induced IR (Amati, Dubé et al. 2011; Holzer, Park et al. 2011; Kumashiro, Erion et al. 2011). In this chapter, it was examined whether inhibition of insulin signalling through increased saturated FA oversupply may be attenuated with the ablation of the lipid activated PKCε in MEFs.

5.2 METHODS

5.2.1 Fatty Acid Treatments

For FA treatment of MEFs, palmitate:BSA/DMEM and control:BSA/DMEM stocks were diluted 1:20 into low glucose DMEM and the resulting 0.4 mM palmitate 0.92 % (w/v) BSA solution, or control 0.92 % (w/v) BSA solution, was added to the MEFs and the cells cultured for 24 h. Prior to insulin treatment of MEFs, the media was aspirated, wells washed with warm PBS to remove residual serum, and cells incubated in control or palmitate-containing serum-free media for 2 h. Insulin (100 nM) was then added to the cells for the indicated times.

5.2.2 Thin Layer Chromatography

The chromatography tank was prepared by adding 150 mL of chloroform:methanol:0.25 % (v/v) KCI (5:4:1) to a glass chromatography tank (25 cm x 27 cm x 10 cm) lined with filter paper and sealed. Vapours in the tank were allowed to equilibrate for approximately 2 h. The silica gel plate was cut to size and a line drawn approximately 1.5 cm from the bottom of the plate. Samples or standards were spotted along the line and the plate was then placed into the equilibrated developing chamber. The tank was tightly covered with a lid and the plate was left until the ascending solvent line had reached approximately 1 cm from the top of the plate (30-45 min). The TLC plate was carefully removed from the tank and allowed to air dry thoroughly in a chemical fume hood, before being exposed to iodine.

5.2.3 Membrane Fluidity Analysis of Palmitate-Treated MEFs by 2-Photon Microscopy with Laurdan Staining

MEFs were seeded onto glass coverslips and grown to around 70-80 % confluence. Following 24 h of palmitate or control treatment, cells were incubated for 40 min at 37 °C with a final concentration of 10 μM Laurdan and harvested for analysis as described in section 2.9. GP values are in the range of -1 to +1, with more ordered domains closer to +1

as described in section 2.1. The outer membrane area of the cell was defined as the region of interest for analysis using ImageJ.

5.2.4 Statistical Analysis

Differences among groups were assessed by unpaired Student's t test or ANOVA using Tukey-Kramer post hoc test. These analyses were performed using GraphPad Prism 5 (Graph-Pad Software, La Jolla, CA, USA) software. Data are presented as mean ± standard error of the mean (SEM). Deviations were deemed significant at $P < 0.05$.

5.3 Results

5.3.1 PKCε null MEFs display protected insulin signalling following treatment with the saturated fatty acid palmitate

To study the effect of lipid oversupply on insulin signalling, WT and PKCε KO MEFs were incubated with 400 μM palmitate for 24 h, before being stimulated with 100 nM insulin for the indicated times. Despite MEFs having significantly reduced ($p < 0.01$, ANOVA) InsR phosphorylation under both control and palmitate treated conditions (Figure 5.3.1A), there was no change in the level of IRS-1 Tyr phosphorylation in the presence of palmitate in either genotype (Figure 5.3.1B). WT MEFs did however display impaired ($p < 0.01$, AUC) Akt Ser 473 phosphorylation in the presence of palmitate, while PKCε KO MEFs did not (figure 5.3.1C). This was observed in the absence of any alteration in IRS-1 Ser phosphorylation at Ser 636/639 (Figure 5.3.1D). In summary, following 24 h of palmitate exposure, PKCε KO MEFs exhibit preserved Akt signalling whereas WT MEFs did not, despite similar signalling profiles at the level of IRS-1.

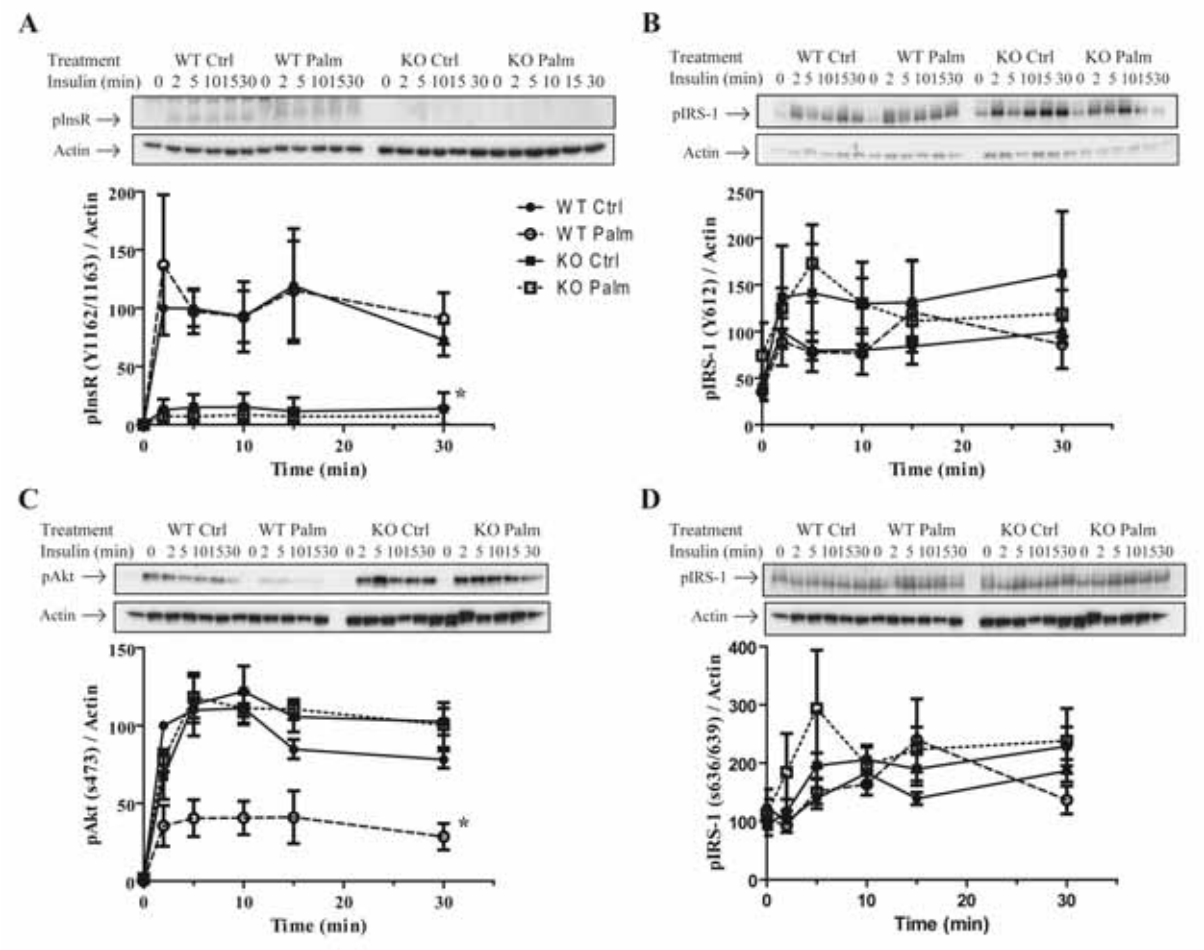

Figure 5.3.1 Phosphorylation of proximal components of the metabolic arm of insulin signalling in WT and KO MEFs.
WT and KO MEFs were incubated for 24 h in the presence or absence of 400 µM palmitate, serum starved for 2 h and stimulated with 100 nM insulin for the indicated times at 37 °C. Cells were washed three times with ice cold PBS, harvested in RIPA buffer. **A:** InsR Tyr 1162/1163, **B:** IRS-1 Tyr 612, **C:** Akt Ser 473 and **D:** IRS-1 Ser 636/639 phosphorylation were analysed by immunoblotting. Data are means ± SEM; n = 3-4 independent experiments in two separate MEF pools. (* = P < 0.05, AUC).

There was no difference in InsR protein expression between genotype or treatment (Figure 5.3.2A). IRS-1 protein expression was not different between genotypes under control conditions, but decreased in WT MEFs following palmitate treatment ($P < 0.05$); this was not observed in the KO MEFs (Figure 5.3.2B). A reduction ($P < 0.001$) in Akt expression was also observed with palmitate treatment in the WT MEFs only (Figure 5.3.2C). The magnitude of the reduction in Akt protein levels in palmitate-treated WT MEFs, however, is not sufficient to account for the entire defect observed in Ser 473 Akt phosphorylation (Figure 5.3.2D).

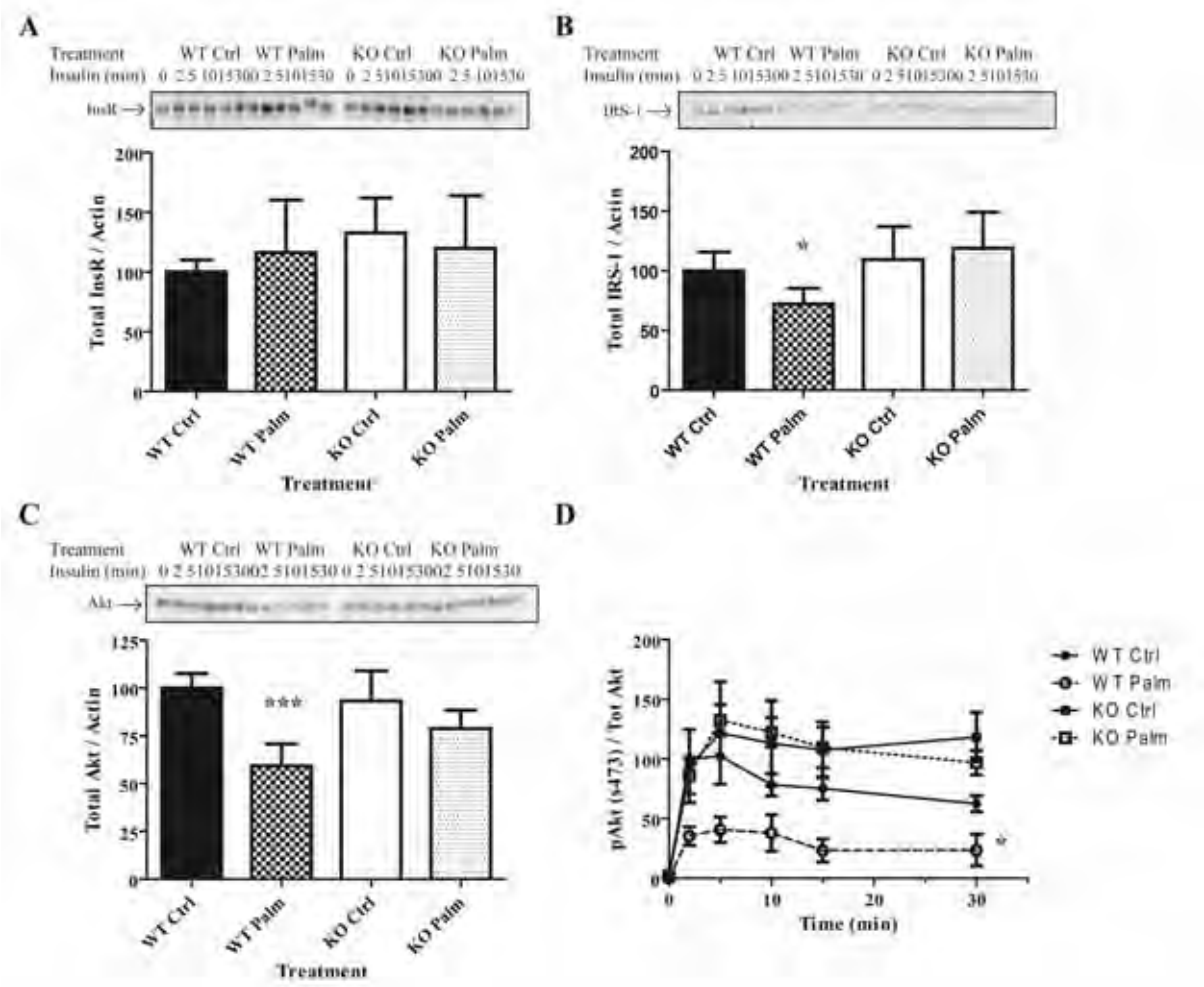

Figure 5.3.2 Expression of proximal components of the insulin signalling pathway.
WT and KO MEFs were incubated with or without 400 µM palmitate for 24 h, serum starved for 2 h and stimulated with 100 nM insulin for the indicated times at 37 °C. Cells were washed three times with ice cold PBS, harvested in RIPA buffer. **A:** InsR, **B:** IRS-1 and **C:** Akt expression was measured by immunoblotting. **D:** Akt Ser 473 phosphorylation was corrected for Akt expression. Data are means ± SEM; n = 3-4 independent experiments in two separate MEF pools. (* = P < 0.05; *** = P < 0.001).

5.3.2 The role of JNK in regulating insulin signalling in MEFs following incubation with palmitate.

Examination of phosphorylation of JNK (54kDa) as a marker of stress signalling revealed that it was elevated (P < 0.05, AUC) above control conditions in both KO and WT MEFs with palmitate, but more so in the latter (Figure 5.3.3A). In WT MEFs, JNK (46kDa) phosphorylation was significantly increased following palmitate exposure (P < 0.05, AUC), while in the KO MEFs there was a less pronounced trend for JNK (46kDa) phosphorylation to increase (P = 0.05, AUC) with palmitate (Figure 5.3.3A). Since it has recently been

hypothesised that palmitate exposure can sequester c-Src in membrane microdomains, leading to increased c-Src activation and enhanced JNK activation (Holzer, Park et al. 2011), Tyr phosphorylation of Src family kinases was examined in the current study, however, no difference was observed between genotype or treatment conditions (Figure 5.3.3B).

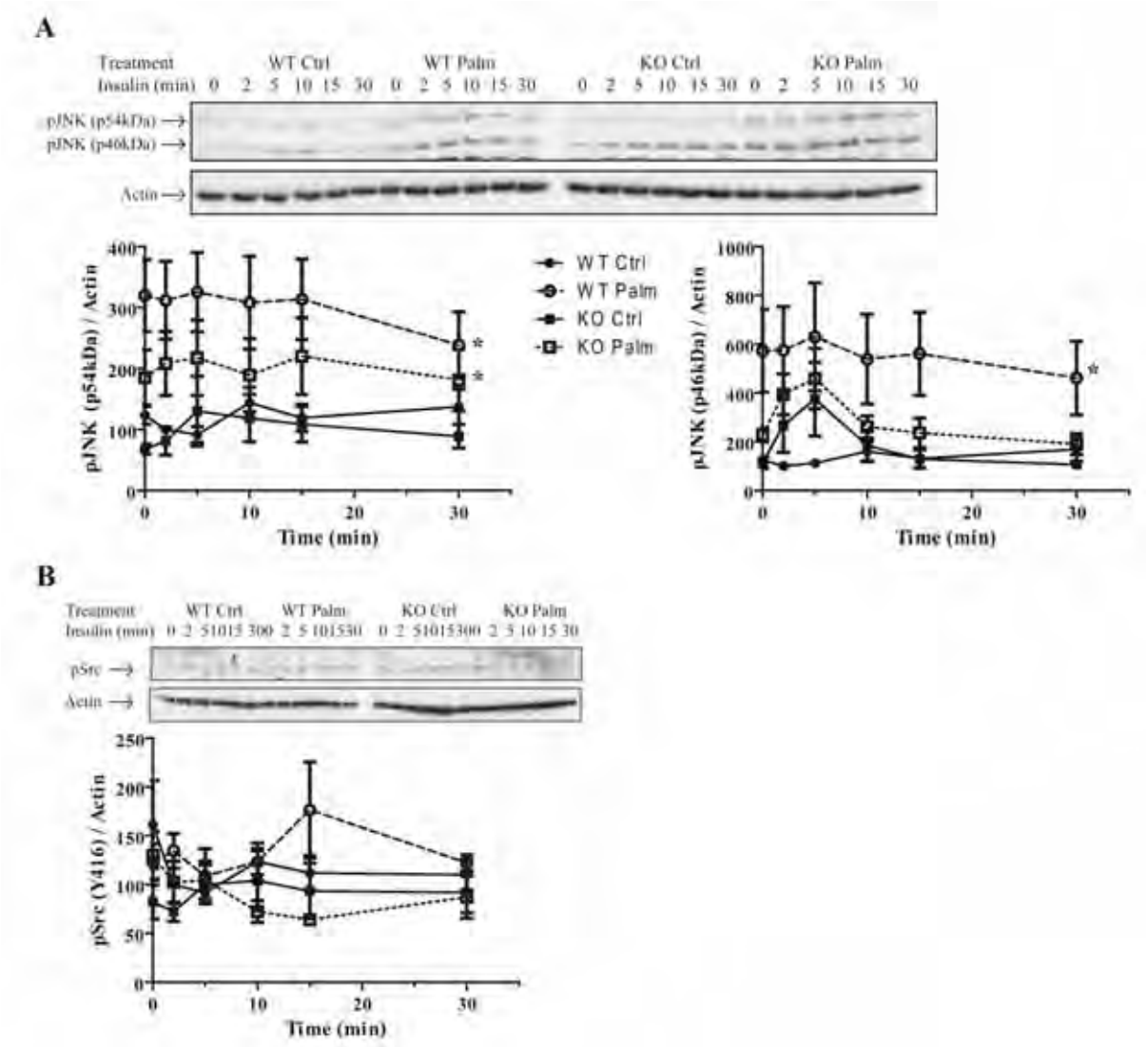

Figure 5.3.3 Activation of stress signalling components involved in the negative regulation of insulin signalling in WT and KO MEFs.
WT and KO MEFs were incubated in the presence or absence of 400 µM palmitate for 24 h, serum starved for 2 h and stimulated with 100 nM insulin for the indicated times at 37 °C. Cells were washed three times with ice cold PBS, harvested in RIPA buffer. **A:** JNK (p54 and 46 kDa); and **B:** Src family kinase phosphorylation were analysed by immunoblotting. Data are means ± SEM; n = 2-4 independent experiments in two separate pools of MEFs. (* = P < 0.05).

It has also been reported that JNK activation is responsible for increased phosphorylation of IRS-1 at Ser 636/639, which can impair PI3K and Akt activation. However, as we saw no difference at this Ser site (Figure 5.3.1D) and no difference in Src phosphorylation (Figure 5.3.3B), Src induced JNK activation is likely not the reason for the reduced Akt phosphorylation observed in palmitate-treated WT MEFs in this study.

Both JNK (54kDa) ($P < 0.01$) and JNK (46kDa) ($P < 0.001$) expression were reduced in the KO MEFs under control conditions, and a similar but not significant trend was observed following palmitate in this genotype (Figure 5.3.4).

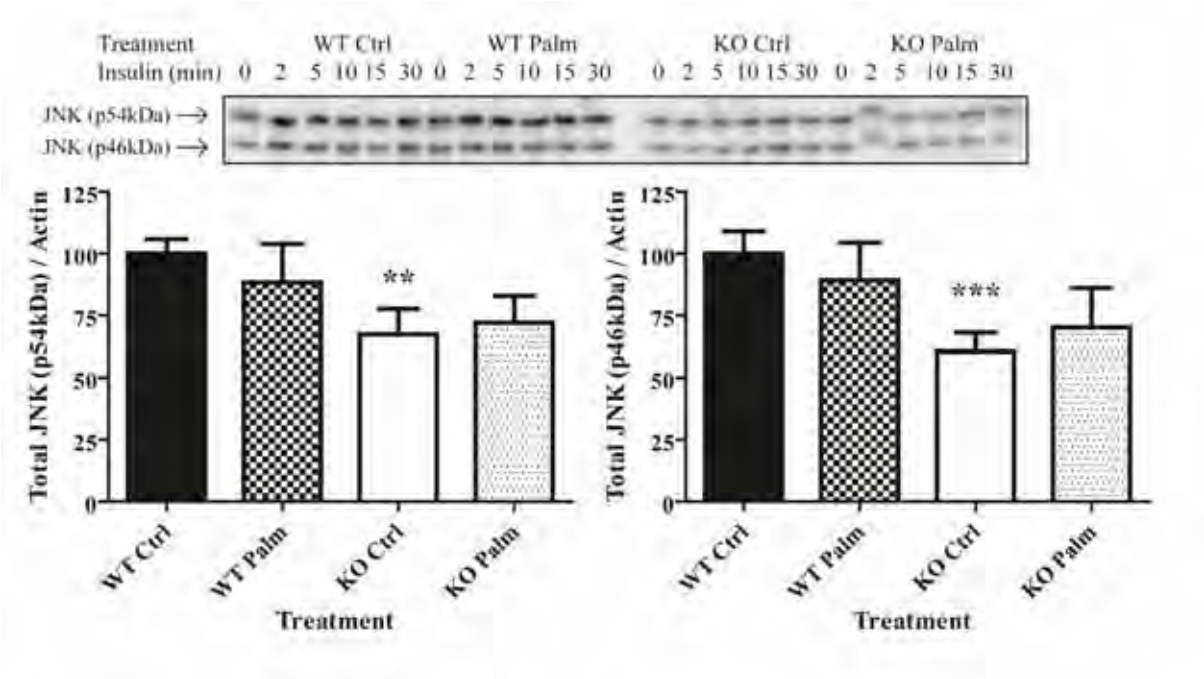

Figure 5.3.4 Expression of JNK (p54 and p46kDa) in WT and KO MEFs.
WT and KO MEFs were incubated for 24 h in the presence or absence of 400 μM palmitate, serum starved for 2 h and stimulated with 100 nM insulin for the indicated times at 37 °C. Cells were washed three times with ice cold PBS, harvested in RIPA buffer. JNK (p54 and p46kDa) expression was measured by immunoblotting. Data are means ± SEM; n = 3-4 independent experiments in two separate MEF pools (** = $P < 0.01$; *** = $P < 0.001$).

Given JNK expression is lower in the KO MEFs compared to WT, the palmitate-induced change in JNK phosphorylation when corrected for total protein, shows a trend to be lower in the KO MEFs compared to WT (Figure 5.3.5).

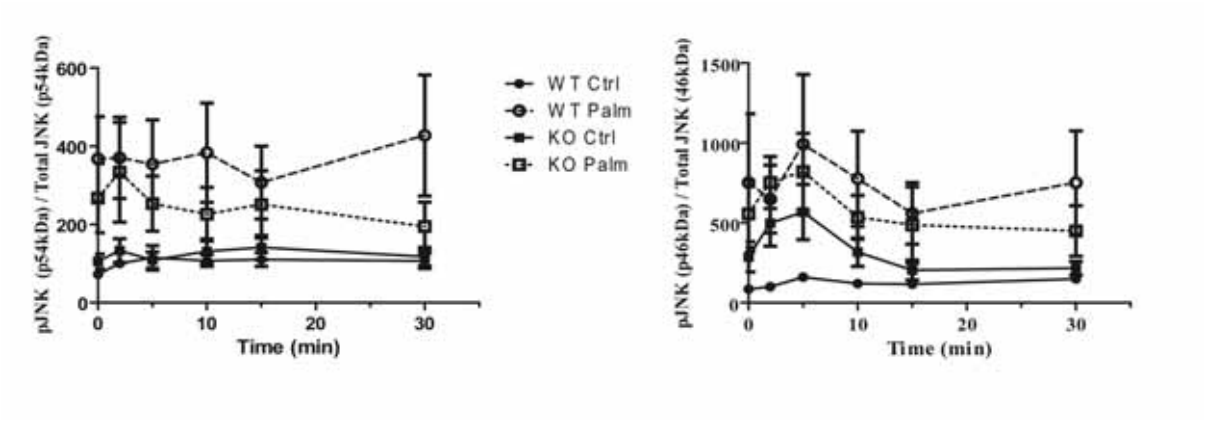

Figure 5.3.5 Phosphorylation of JNK corrected for total expression of JNK.
WT and KO MEFs were incubated for 24 h with or without 400 µM palmitate, serum starved for 2 h and stimulated with 100 nM insulin for the indicated times at 37 °C. Cells were washed three times with ice cold PBS, harvested in RIPA buffer. JNK (p54 and p46kDa) expression was measured by immunoblotting. Data are means ± SEM; n = 3-4 independent experiments in two separate MEF pools.

5.3.3 The role of palmitate incorporation into distinct lipid species may explain the protected insulin signalling observed in PKCε null MEFs.

It was next investigated whether ^{14}C-labelled palmitate became differently incorporated into distinct lipid species in the WT and KO genotypes. Overall, the KO MEFs displayed non-significant reduction in cellular DAG levels and a slight increase in TAG compared to WT MEFs (Figure 5.3.6). These small changes may be biologically significant, and follow a similar profile to that seen in previously published data from primary hepatocytes of the mice (Raddatz, Turner et al. 2011). Since DAGs activate PKCs, a potentially lower level of DAG together with ablation of PKCε may explain some of the preserved Akt activation seen following palmitate treatment in the KO MEFs. There were no differences observed in cholesterol or cholesterol ester (CE) (Figure 5.3.6), suggesting that if anything, alterations are specific to the glycerolipid synthesis pathway and may be associated with PKC activation status.

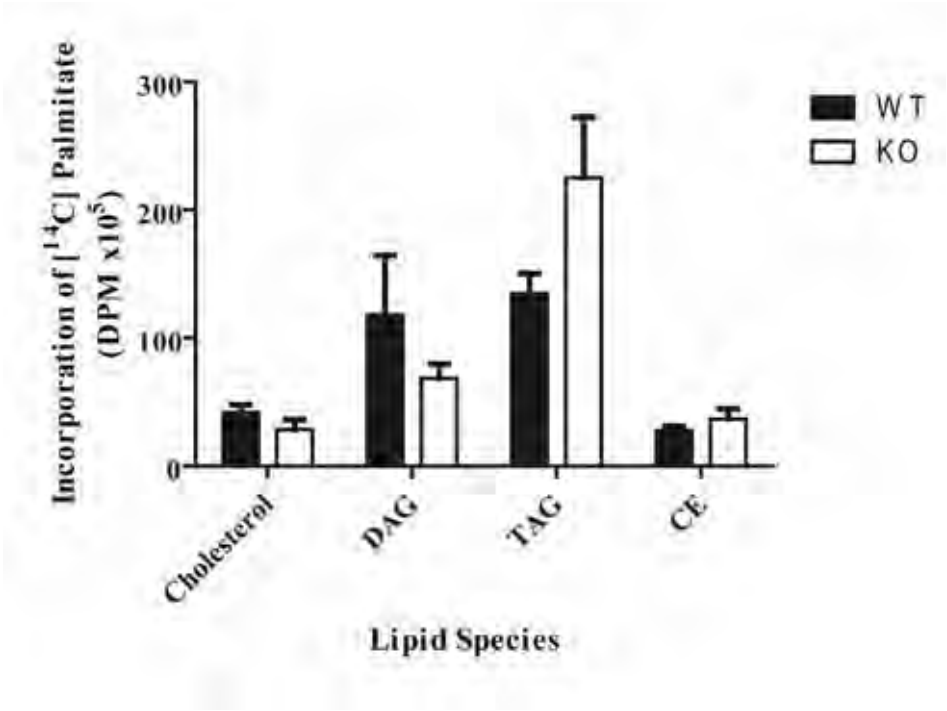

Figure 5.3.6 ^{14}C-Palmitate incorporation into various lipid species pools in WT and KO MEFs.
WT (■) and PKCε KO (□) MEFs were incubated with ^{14}C-Palmitate for 24 h at 37 °C. Cells were washed three times with ice cold PBS, harvested in 0.1 % PBS:DMSO and run on a TLC to measure lipid species. Data are means ± SEM; n = 3 independent experiments performed in duplicate.

5.3.4 Plasma membrane fluidity is not different between WT and PKCε KO MEFs following 24 h incubation with the saturated fatty acid palmitate.

Alterations in PM fluidity have been suggested to disrupt insulin action in times of increased lipid availability, as well as impact cellular trafficking which is important for insulin action and maintaining glucose homeostasis (Read and McElhaney 1976; Pilch, Thompson et al. 1980). It was therefore determined whether palmitate-treated WT and KO MEFs exhibited alterations in membrane fluidity. Using the lipid stain Laurdan, PM fluidity was not different between the KO and WT MEFs, with or without palmitate treatment (Figure 5.3.7), as depicted by no difference in GP value, a measure of membrane order based upon the degree of water penetration into the membrane.

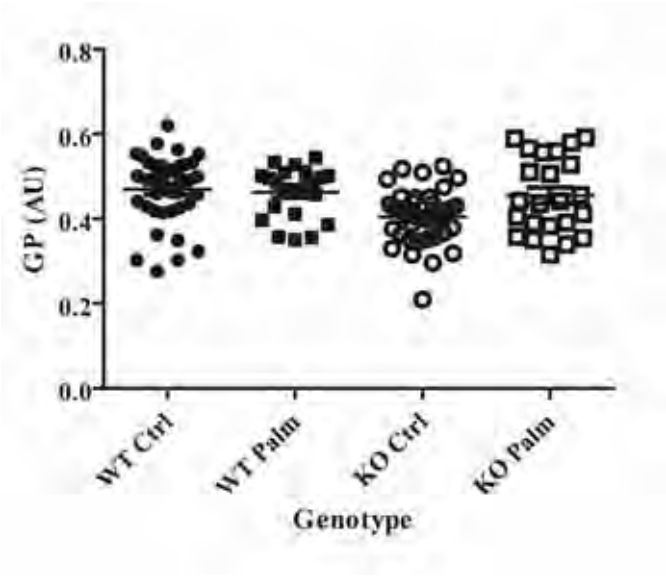

Figure 5.3.7 Membrane fluidity measured with Laurdan staining in WT and KO MEFs.
WT and PKCε KO MEFs were incubated for 24 h in the presence or absence of 400 µM palmitate. For the final 45 min, 10 µM Laurdan was added to the media at 37 °C. Cells were washed three times with warm PBS and fixed with 4 % paraformaldehyde. Cells were analysed by 2-photon microscopy. Data are means ± SEM; n = 23-40 individual cells.

Therefore membrane fluidity also does not likely explain the preservation of Akt signalling observed in PKCε KO MEFs. Interestingly however, the KO MEFs displayed more localised, punctate regions of ordered lipid domains (as indicated by the arrows in Figure 5.3.8), compared to the WT MEFs. In contrast, the WT MEFs appeared to exhibit a more general cellular increase in ordered lipid regions (as indicated by rectangles in Figure 5.3.8) than was seen in the control conditions without palmitate treatment (see Chapter 3 Figure 3.3.11).

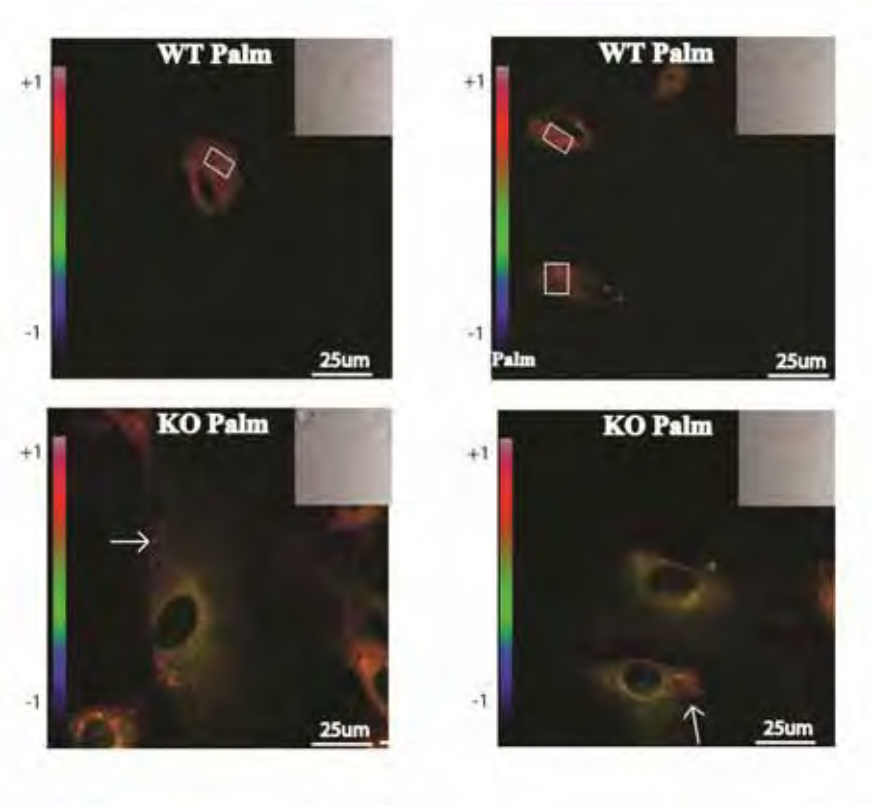

Figure 5.3.8 Lipid order measured with Laurdan staining in palmitate-treated WT and KO MEFs. WT and PKCε KO MEFs were incubated for 24 h in the presence of 400 μM palmitate. For the final 45 min, 10 μM Laurdan was added to the media at 37 °C. Cells were washed three times with warm PBS and fixed with 4 % paraformaldehyde. Cells were analysed by 2-photon microscopy. Data are means ± SEM; n = 23 individual cells.

Taken together with the results of section 5.3.3, despite no difference in PM fluidity, overall cellular lipid storage and metabolism may be altered between the KO and WT MEFs under conditions of lipid oversupply.

5.4 Discussion

In times of increased lipid availability, glucose disposal by peripheral tissues becomes impaired as FA can interfere with insulin action. The precise mechanism(s) inducing insulin resistance have not yet been defined, however the involvement of particular lipid species and the activation of PKC isozymes in the development of insulin resistance can be considered strong candidates (Schmitz-Peiffer 2000; Benoit, Kemp et al. 2009; Samuel, Petersen et al. 2010). Although there are reports of insulin resistance existing without defects in Akt activity (Kim, Nikoulina et al. 1999; Nadler, Stoehr et al. 2001), many of the aberrant metabolic actions of insulin have been linked with defects in the IRS-Akt pathway (Aguirre, Uchida et al. 2000; Zick 2005; Samuel, Petersen et al. 2010). In the experiments described in this chapter, it was assessed whether ablation of the lipid-activated PKCε could result in a protection against impaired insulin signalling observed in the face of the metabolic insult, i.e. with exposure to the saturated FA palmitate, as rodent models with specific deletion of PKCε had shown protection from HFD induced glucose intolerance and defining potential mechanisms was the aim of the current work.

The observation that in two separate lines of MEFs, PKCε deletion resulted in preserved insulin-stimulated Akt Ser phosphorylation following lipid treatment, indicated that PKCε may be involved in the downregulation of insulin signalling seen following increased lipid availability. In these experiments Akt but not IRS-1 phosphorylation was the common site of preserved insulin signalling and this is in agreement with recent work, whereby defects in insulin action arose from a variety of insults independent of IRS-1 mediated signalling (Hoehn, Hohnen-Behrens et al. 2008). Furthermore, those studies showed that very little Akt activation (~5%) was required in order to elicit near maximal GLUT4 translocation, whilst previous studies have demonstrated that activation of Akt and glucose transport are not linearly dependent (Whitehead, Molero et al. 2001; Hoehn, Hohnen-Behrens et al. 2008). Although in these studies IRS-2 phosphorylation was not examined, previous work in the laboratory has not observed any differences between the genotypes on liver IRS-2 activation. Furthermore the experiments performed in this chapter were performed in MEFs and IRS-2 is proposed to be more important in hepatic insulin action and as such was not reported here. The data shown in this chapter indicate that exposure of MEFs to the saturated FA palmitate for 24 h induced defects in Akt phosphorylation, which are prevented by ablation of PKCε (Figure 5.3.1C).

The lipid-activated PKCε has been implicated in the inhibition of the insulin signalling pathway. It has been proposed that its activation by lipids such as DAG results in translocation from the cytosol to the PM, where it can induce inhibitory Ser phosphorylation of IRS-1 (Samuel, Liu et al. 2007). Other studies however suggest that PKCε can interact with other signalling intermediates, forming signalling complexes to carry out its actions. Intermediates that PKCε has been shown to associate with include the Tyr kinase, c-Src, the MAPK family member JNK and the transcription factor STAT3 (Song, Vondriska et al. 2002; Aziz, Manoharan et al. 2007; Rybin, Guo et al. 2007). Previous work has shown that c-Src localised to lipid rafts is more catalytically active than that found in the cytosol (Mukherjee, Arnaud et al. 2003). Furthermore a recent paper by Holzer *et al.* demonstrated that palmitate can specifically induce c-Src recruitment to lipid microdomains in several cell types, including MEFs (Holzer, Park et al. 2011), and this sequestration of c-Src in the lipid microdomains promoted JNK activation. JNK has been identified as a key mediator in impairing insulin action following lipid oversupply (Hirosumi, Tuncman et al. 2002) and PKCε has been linked to activation of JNK (Comalada, Xaus et al. 2003). JNK has also been implicated in enhanced inhibitory Ser phosphorylation of IRS-1 at Ser 636/639, which is a site thought to impair PI3K association and therefore reduce Akt signalling. When palmitate-induced JNK phosphorylation was examined in WT and KO MEFs, its activation relative to control conditions was found to be enhanced in both KO and WT MEFs, but the palmitate-induced increase tended to be less pronounced in the KO MEFs. Moreover, there was no evidence of increased cellular Src activation with palmitate treatment in either genotype. In these experiments, however, localisation and activation of specific cellular compartment pools of Src was not investigated. With JNK activation being increased in both genotypes (albeit potentially more in WT than KO), this proposed pathway is probably not solely involved in the downregulation of Akt phosphorylation observed in the WT MEFs. Activation of the NFκB stress signalling pathway was not investigated as a complementary assessment to JNK. However, the finding that IRS-1 Ser phosphorylation was not different at the Ser 636/639 site examined suggests that similar to JNK, NFκB may not have a key role in this model, despite the fact PKCε has been linked to activation of this pathway. Furthermore, during the experiments for this thesis, NFκB activation was shown not to be altered in isolated liver of mice fed a HFD for 1 week ((Raddatz, Turner et al. 2011). It does not rule out the involvement of this pathway, but it is something that remains to be tested.

With lipid oversupply, a build up of lipid intermediates can occur within the cell. Many lipids are proposed to be signalling intermediates themselves, with DAGs activating PKCs for example (Zhang, Liu et al. 2007; Zhang, Christianson et al. 2010; Stout, Liu et al. 2011). Increased lipid availability results in an increase in the intracellular DAG pool, which in turn activates PKCε. Activated PKCε can then act to reduce insulin signalling through negative regulation of IRS-1 activation (Morino, Petersen et al. 2006). Additional complexity is likely, with FA composition and cellular localisation of lipids being important for the negative actions upon metabolism. Specifically, unsaturated DAG is a more potent activator of PKCε than saturated (Wakelam 1998) and more recently it was proposed that 'cytosolic' DAG, specifically associated with lipid droplets, correlates with PKCε activation and reduced insulin sensitivity in obese subjects (Kumashiro, Erion et al. 2011). In contrast DAGs involved in acute signalling events are primarily generated at the PM and therefore consistent with a distinct localisation of PKC activity. It has also been reported that DAG can in fact correlate with insulin sensitivity, with increased liver or muscle DAG not inducing negative metabolic actions through altered insulin sensitivity (Monetti, Levin et al. 2007; Amati, Dubé et al. 2011). There are multiple steps along the glycerolipid synthesis pathway, ultimately involving generation of TG from DAG and alterations at one step can affect levels of other lipid intermediates. In these experiments, cellular DAG and TG content changed relatively little with PKCε deletion. It is however difficult to conclude that changes in the overall DAG pool were causal to the improved downstream insulin signalling in KO MEFs.

As discussed previously, this further supports the theory that it is not the overall content of one particular lipid species that exacerbates the negative metabolic actions induced from lipid oversupply but rather a concerted action of a number of bioactive lipids. The sphingolipid ceramide, also found to be elevated in several models of FA oversupply, has been shown to negatively regulate Akt through enhanced activation of the protein phosphatase 2A or PKCζ, which act to dephosphorylate Akt or prevent its activation by inhibitory phosphorylation respectively (Cazzolli, Carpenter et al. 2001; Hajduch, Balendran et al. 2001; Powell, Hajduch et al. 2003; Holland, Miller et al. 2011b). Ceramide accumulation was not assessed in the current studies. Furthermore, and in contrast to some studies, a recent report from the Goodpaster laboratory showed, using trained athletes, sedentary and obese sedentary subjects that intramuscular DAGs correlated with insulin sensitivity, whereas ceramide levels were associated with insulin resistance (Amati, Dubé et al. 2011). Interestingly in this study, DAG

species with an unsaturated FA on both positions was more highly associated with IR than those with only one unsaturated or saturated FA species. This further highlights the complexity concerning the role of lipid intermediates in the induction of IR. In the studies performed in this chapter, only the saturated FA palmitate was used and a comparison of the effects of unsaturated species which are thought to be more potent activators of PKCs could yield further insights. Furthermore, the diet consumed by rodents and humans is generally composed of a mixture of saturated and unsaturated FA; so using a mixture of FA species could be more useful. Given the protection observed at the level of Akt in PKCε KO MEFs with the use of saturated FA only, it could be that alterations in sphingolipid species are also relevant in this model. It would therefore be of benefit to perform lipidomic analyses of the lipid treated MEFs to determine the levels of various lipid intermediates, not just DAG/TG, and their different species that may be associated with the preserved Akt phosphorylation in PKCε KO MEFs.

Studies have shown that the insulin resistant state induced by obesity is associated with alterations in membrane properties (Muller, Denet et al. 1997; Younsi, Quilliot et al. 2002). It has also been demonstrated that increasing membrane fluidity can increase glucose transport (Pilch, Thompson et al. 1980; Whitesell, Regen et al. 1989). Younsi *et al.* have shown in erythrocytes of obese non-diabetic women that membrane phospholipid content is correlated with hyperinsulinemia (Younsi, Quilliot et al. 2002). In that study, sphingomyelin and phosphatidylethanolamine proved to be the strongest predictors of insulin sensitivity in lean and obese subjects. Furthermore, FA can be incorporated into membrane phospholipids and the alteration in the integrity of the PM could influence cell signalling and physiology. Many studies have shown that the saturated FA palmitate but not unsaturated FA can reduce membrane fluidity (Luo, Rizkalla et al. 1996; Clamp, Ladha et al. 1997). However, it has also been seen that the omega-3 and 6 fatty acids most affect membrane lipid composition, as cells are unable to synthesise these species themselves and therefore require their dietary supply (Hulbert, Turner et al. 2005). In the experiments performed in this chapter, no difference in PM fluidity as assessed by Laurdan staining was found. However the KO MEFs did appear to display a more localised cellular region of increased lipid order than the WT which appeared to increase cellular lipid order more generally throughout the cell. Therefore it could be that the PKCε KO MEFs store lipids differently to the WT and the localisation of lipid storage has been observed to affect activation of signalling pathways (Granneman and

Moore 2008; Nishino, Tamori et al. 2008; Puri, Ranjit et al. 2008; Consitt, Bell et al. 2009) which could impact upon insulin sensitivity. This needs to be further investigated to determine whether this could be a mechanism for the improved insulin signalling seen at Akt in PKCε KO MEFs following palmitate treatment, as cellular compartmentalisation and size of lipid stores which could be important were not measured in these studies.

The results in this chapter indicate that PKCε null MEFs exhibit protected downstream insulin signalling compared to WT upon exposure to saturated FA. The mechanism for this is not clear, but localisation of signalling components upstream of insulin-stimulated metabolic pathways has been postulated to be important in their actions (Tirosh, Potashnik et al. 1999; Takano, Haruta et al. 2001). Taken together with the fact no common differences were observed in some of the known negative regulators of insulin signalling examined, this represents a valid hypothesis. The protection observed may also be due to a difference in lipid handling which is in agreement with a recent report from this laboratory using primary hepatocytes and liver from PKCε KO and WT mice fed a high fat diet (Raddatz, Turner et al. 2011). The lipid handling could be altered in terms of species of lipid intermediates or from the Laurdan results, the localisation of lipid storage, resulting in the improved insulin signalling. Small alterations in activation of signalling proteins as well as the lipid species generated and stored could together lead to the overall detrimental metabolic effects. Whether this results in a decrease in insulin action cannot be concluded without further physiological measurements such as glucose uptake and production, as well as examining the effects of lipid oversupply in more physiological settings such as primary hepatocytes and fat-fed animals.

CHAPTER 6

The Effect of PKCε Deletion on Insulin Signalling in Primary Hepatocytes and Liver

6.1 Introduction

It has previously been shown that global PKCε KO mice are protected from high-fat diet induced glucose intolerance, both in the short and long term (Schmitz-Peiffer, Laybutt et al. 2007; Raddatz, Turner et al. 2011). The early protection is primarily due to a beneficial liver phenotype, with no difference observed between genotypes in glucose uptake into quadriceps muscle or epididymal white adipose tissue. Specifically, the PKCε KO mice display improved glucose and pyruvate tolerance, despite increased hepatic TG accumulation (Raddatz, Turner et al. 2011). In long-term fat-feeding studies, circulating insulin is elevated in PKCε KO mice, which helps to overcome the IR that may otherwise exist, as seen in the WT fat-fed mice (Schmitz-Peiffer, Laybutt et al. 2007).

A number of other studies have also implicated PKCε in the development of fat-induced IR in both humans (Considine, Nyce et al. 1995; Kumashiro, Erion et al. 2011) and rodents (Schmitz-Peiffer, Browne et al. 1997; Samuel, Liu et al. 2004; Samuel, Liu et al. 2007; Schmitz-Peiffer, Laybutt et al. 2007). Furthermore, it has been demonstrated that PKCε is the dominant PKC isoform correlated to hepatic IR with increased membrane localisation in liver samples of insulin resistant patients as well as rodents (Samuel, Liu et al. 2007; Jornayvaz, Birkenfeld et al. 2011). A variety of methods have been used to inhibit PKCε including antisense oligonucleotides, specific PKCε inhibitors, as well as global genetic deletion of PKCε (Samuel, Liu et al. 2007; Schmitz-Peiffer, Laybutt et al. 2007) and in all studies, inhibition of PKCε activation results in a protection against diet-induced glucose intolerance. Some studies have reported improved insulin signalling following PKCε deletion (Samuel, Liu et al. 2007), whereas this occurred in the absence of signalling changes in other studies (Schmitz-Peiffer, Laybutt et al. 2007; Raddatz, Turner et al. 2011). This could be due to a number of reasons, including different model systems examined, different timelines studied and different methods of inhibition. Perhaps most importantly though, studies have assessed insulin signalling following hyperinsulinaemic clamps (Samuel, Liu et al. 2007) or acute supra maximal infusions of insulin (Raddatz, Turner et al. 2011) after short term HFD. The duration of insulin stimulation in clamp studies before tissue collection (approximately 2 h) is not ideal for capturing initial signalling events that may be more important for insulin action; and pharmacological insulin levels following bolus injections only examine maximal signalling activation, not that which occur in a physiological setting, and may even overcome

subtle signalling defects through 'spare' signalling components that can be activated when stimulated in excess (Kono and Barham 1971; Rice, Lienhard et al. 1992).

In the recent study by Raddatz *et al.*, following a short-term HFD, PKCε KO mice have increased liver lipid storage but a relatively better insulin action overall. The lipid accumulation phenotype was further confirmed in studies using primary hepatocytes isolated from the animals and cultured with the saturated FA palmitate (Raddatz, Turner et al. 2011). In the primary hepatocyte studies, insulin signalling was not studied and therefore whether the improved metabolic actions seen in the PKCε KO animals are associated with the preservation of insulin signalling in a cell-autonomous manner, as seen in the MEF studies of this thesis (Chapter 5), has not been elucidated. Furthermore, as described above, *in vivo* insulin signalling in the PKCε KO mice has only been studied following supraphysiological bolus injection of insulin into the inferior vena cava. Moreover, it has not previously been shown directly whether PKCε KO animals are more insulin sensitive, despite showing improved glucose tolerance with lower insulin profiles in the short term.

It is known that L-arginine is a potent insulin secretagogue, which when administered by intraperitoneal injection can stimulate insulin secretion from the β-cell independent of glucose sensing (Palmer, Benson et al. 1976; Krause, McClenaghan et al. 2011). A potential advantage of such an approach would be it induces insulin secretion directly into the hepatic portal vein in a physiological manner and dose. Moreover, this technique clamps the β-cell to continue to secrete as maximal levels, which has been shown not to be different between the WT and PKCε KO islets, however on a HFD, PKCε KO islets have been demonstrated to be more glucose responsive (Cantley, Burchfield et al. 2009). As the largest effects of PKCε inhibition do appear to occur at the level of the liver, certainly in the short term, L-arginine administration may have a greater effect upon hepatic insulin action when compared to intravenous or intraperitoneal insulin injection.

Therefore, in this chapter, primary hepatocytes isolated from WT and PKCε KO animals were used to investigate the insulin signalling pathway in the presence or absence of lipid treatment, and whether the preserved insulin signalling observed in PKCε KO MEFs translated to primary cells such as from the liver. Additionally, L-arginine administration was optimised as a means for investigating sensitivity to endogenously secreted insulin.

6.2 METHODS

6.2.1 Isolation of Primary Mouse Hepatocytes

To promote adherence of freshly isolated primary hepatocytes, cells were cultured for 4 h in M199 medium supplemented with 1 % (w/v) penicillin G sodium/streptomycin sulphate (P/S), 0.1 % (w/v) BSA, 2 % (v/v) dialysed FCS, 100 nM dexamethasone and 100 nM insulin. Fresh M199 medium supplemented with 1 % (w/v) P/S, 100 nM dexamethasone and 1 nM insulin was added to culture the cells in if not being used the following day, otherwise insulin was not included in the overnight culturing medium.

For FA treatment of primary hepatocytes, palmitate:BSA/DMEM and control:BSA/DMEM stocks were diluted 1:20 into insulin free M199 culturing media to yield a 0.4 mM palmitate or BSA control. Following the 4 h adherence period after isolation, hepatocytes were washed with warm PBS to remove residual serum and insulin before media containing 0.4 mM palmitate or control were added to the primary hepatocytes, which were cultured in an incubator for 24 h, and the final 16 h at 37 °C with 5 % CO_2 prior to 10 nM insulin stimulation for the indicated time.

6.2.2 Glucose Tolerance Test

Mice at 9-14 weeks of age were fed either a standard chow diet or a lard-based HFD prepared in-house for one week. For glucose tolerance tests, mice were fasted for 6 h prior to intraperitoneal injection of 2 g/kg glucose (ipGTT) and blood glucose was measured from the tail vein at the indicated times using an Accu-Chek II (Roche Nutley, NJ, USA) blood glucose monitor.

6.2.3 L-Arginine-Stimulated *In Vivo* Insulin Signalling

6.2.3.1 Optimisation of L-Arginine dose

Mice, aged 8-10 weeks, were fasted for 6 h prior to intraperitoneal injection of L-arginine (1 g/kg or 3 g/kg), insulin (0.1 U/kg, 0.25 U/kg, 0.5 U/kg) or saline as a control. Blood glucose levels were measured at 0, 5 min and 9 min using an Accu-Chek II (Roche Nutley, NJ, USA) blood glucose monitor, while blood samples were also taken at these time points into 18 mM EDTA/saline and centrifuged at 13,000 *rpm* for 1 min. Supernatant was stored at -80 ºC for analysis of plasma insulin levels by ELISA (Chrystal Chem Inc, USA). After 10 min, mice were culled by cervical dislocation.

6.2.3.2 Administration of L-Arginine to WT and PKCε KO mice

Mice at 9-14 weeks of age were fed either a standard chow diet or a lard-based HFD for one week. Mice were fasted for 6 h prior to intraperitoneal injection of 1.5 g/kg L-arginine or equivalent saline volume and blood glucose was measured at 0 and 10 min using an Accu-Chek II (Roche Nutley, NJ, USA) blood glucose monitor.

6.2.4 Statistical Analysis

Differences among groups were assessed by unpaired Student's t test or ANOVA using Tukey-Kramer post hoc test. These analyses were performed using GraphPad Prism 5 (Graph-Pad Software, La Jolla, CA, USA) software. Data are presented as mean ± standard error of the mean (SEM) unless otherwise stated. Deviations were deemed significant at $P < 0.05$.

6.3 Results

6.3.1 PKCε null primary hepatocytes do not show any differences in insulin signalling under control conditions

To further examine the effect of PKCε ablation on insulin signalling in a more physiological setting than in MEFs, primary hepatocytes were isolated from WT and PKCε KO mice, cultured overnight and stimulated with 10 nM insulin. It was found that phosphorylation of the InsR was not different between genotypes (Figure 6.3.1A). This was opposite to what was observed in the KO MEFs, further supporting the hypothesis that the defect in InsR phosphorylation in the KO MEFs was likely a survival adaptation rather than a more direct PKCε-mediated event. Furthermore, phosphorylation of downstream components in the insulin signalling pathway, IRS-1, Akt and Erk 1/2, were not different between the genotypes (Figure 6.3.1B-E). IRS-1 Tyr phosphorylation in KO hepatocytes showed a trend to be increased above WT. Other than the finding of reduced InsR phosphorylation in the KO MEFs, this insulin signalling profile in isolated hepatocytes is in agreement with what was observed in the MEFS.

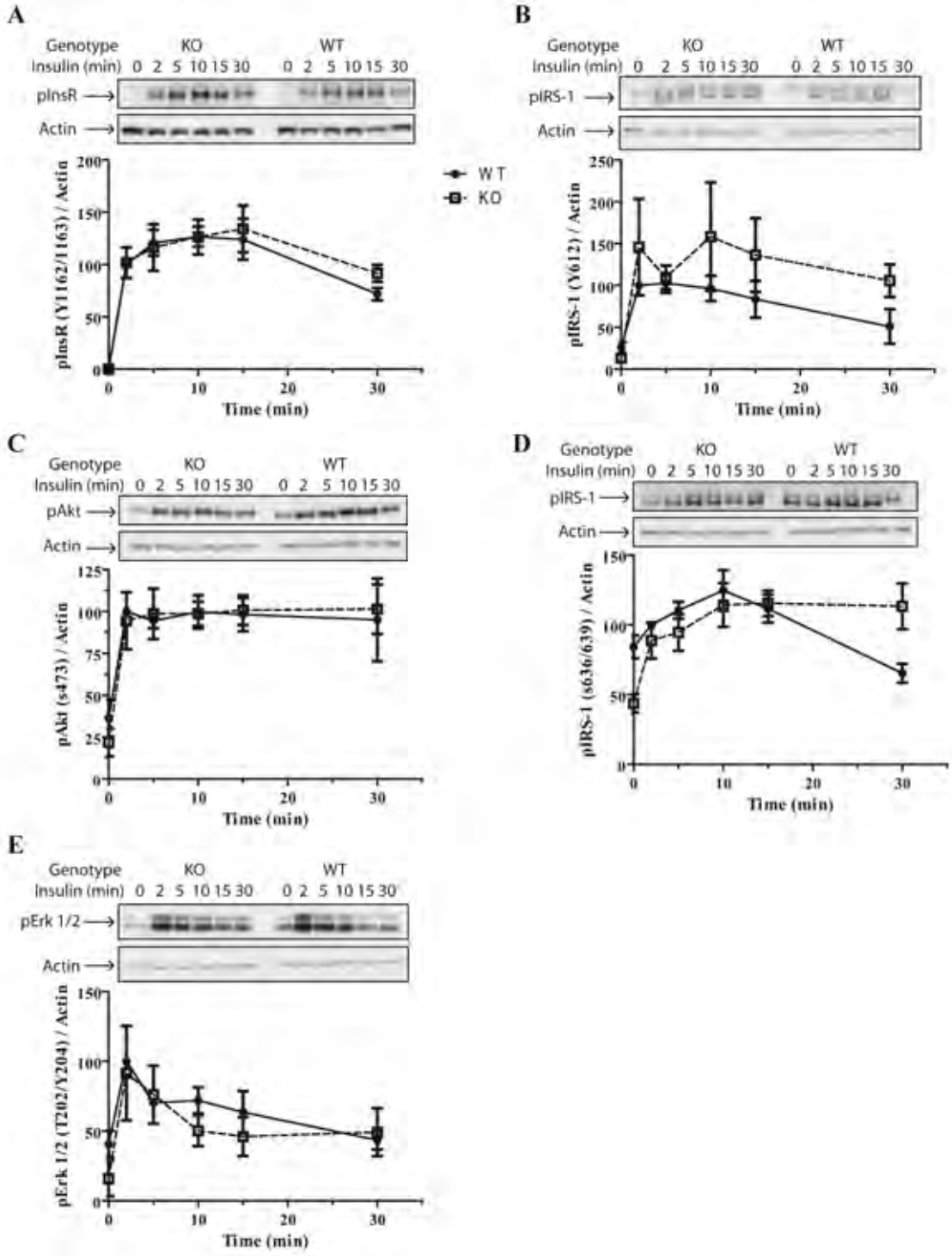

Figure 6.3.1 Insulin signalling pathway in primary hepatocytes isolated from WT and PKCε KO mice. WT and PKCε KO primary hepatocytes were serum starved for 16 h and stimulated with 10 nM insulin for the indicated times at 37 °C. Cells were washed three times with ice cold PBS, harvested in RIPA buffer and phosphorylation of insulin signalling components; **A:** InsR (Tyr 1162/1163), **B:** IRS-1 (Tyr 612), **C:** Akt (Ser 473), **D:** IRS-1 (Ser 636/639) and **E:** Erk 1/2 (Thr 202/Tyr 204) were analysed by immunoblotting. Values are normalised to WT 2 min insulin condition. Data are means ± SEM; n = 5 independent experiments.

Furthermore, the total protein expression of the signalling components measured (InsR, IRS-1, Akt and Erk 1/2) was not different between WT and KO primary hepatocytes (Figure 6.3.2A-D).

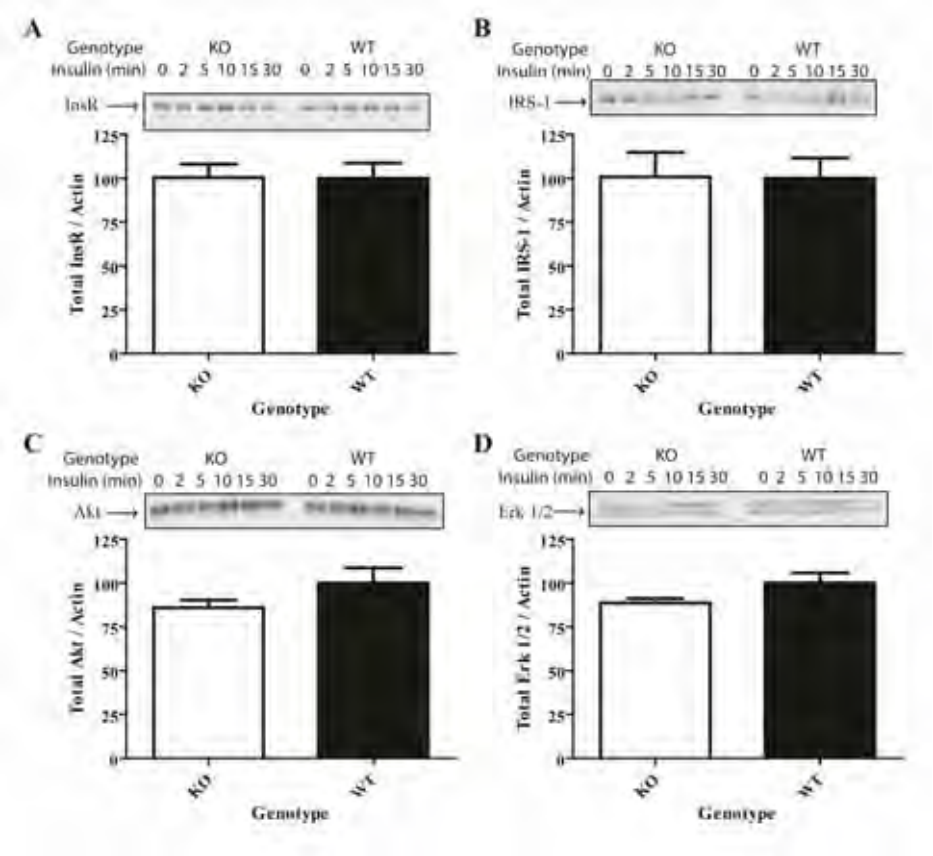

Figure 6.3.2 Expression of insulin signalling pathway components in primary hepatocytes isolated from WT and PKCε KO mice.
WT and PKCε KO primary hepatocytes were serum starved for 16 h and stimulated with 10 nM insulin for up to 30 min at 37 °C. Cells were washed three times with ice cold PBS, harvested in RIPA buffer and expression of; **A:** InsR, **B:** IRS-1, **C:** Akt and **D:** Erk 1/2 were analysed by immunoblotting. Expression was expressed as a percentage of WT after 2 min insulin treatment. Values are normalised to WT 2 min insulin condition. Data are means ± SEM; n = 5 independent experiments.

There was also no difference observed in Grb14 protein expression (Figure 6.3.3), supporting the conclusion drawn from experiments carried out in MEFs (Chapter 4), that altered Grb14 does not occur with PKCε deletion, nor does it influence any signalling alterations that may exist with this genotype.

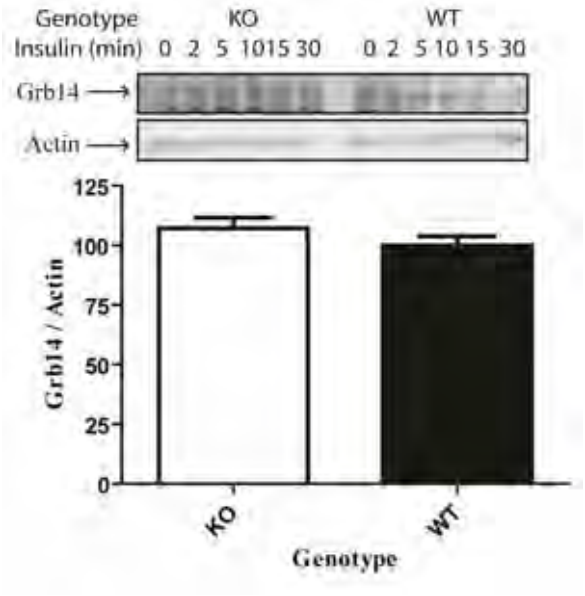

Figure 6.3.3 Grb14 protein expression in primary hepatocytes isolated from WT and PKCε KO mice. WT and PKCε KO primary hepatocytes were serum starved for 16 h and stimulated with 10 nM insulin for up to 30 min at 37 °C. Cells were washed three times with ice cold PBS, harvested in RIPA buffer and Grb14 expression analysed by immunoblotting. Values are expressed as a percentage of WT 2 min insulin treatment. Data are means ± SEM; n = 5 independent experiments.

6.3.2 Long-term palmitate treatment impairs insulin signalling in primary hepatocytes

Because the PKCε KO mice display protected glucose tolerance following high fat feeding, primary hepatocytes from WT and PKCε KO mice were incubated with the saturated FA palmitate to investigate any differences in insulin signalling due to lipid effects. Following 24 h incubation with palmitate, primary hepatocytes of both genotypes exhibited significantly reduced InsR Tyr phosphorylation ($P < 0.05$ KO control vs KO Palm, $P < 0.01$ WT control vs WT Palm), with the WT revealing a greater reduction compared to the KO ($P < 0.05$) (Figure 6.3.4A). This effect on signalling translated further downstream in that both IRS-1 Tyr ($P < 0.001$ vs genotype control) and Akt Ser phosphorylation ($P < 0.01$ vs genotype control) were significantly reduced in cells of both genotypes (Figure 6.3.4B-C). However, Akt exhibited a greater decrement in the WT hepatocytes ($P < 0.05$) compared to KO hepatocytes (Figure 6.3.4C), in agreement with what was seen in the MEFs (see Figure 5.3.1C).

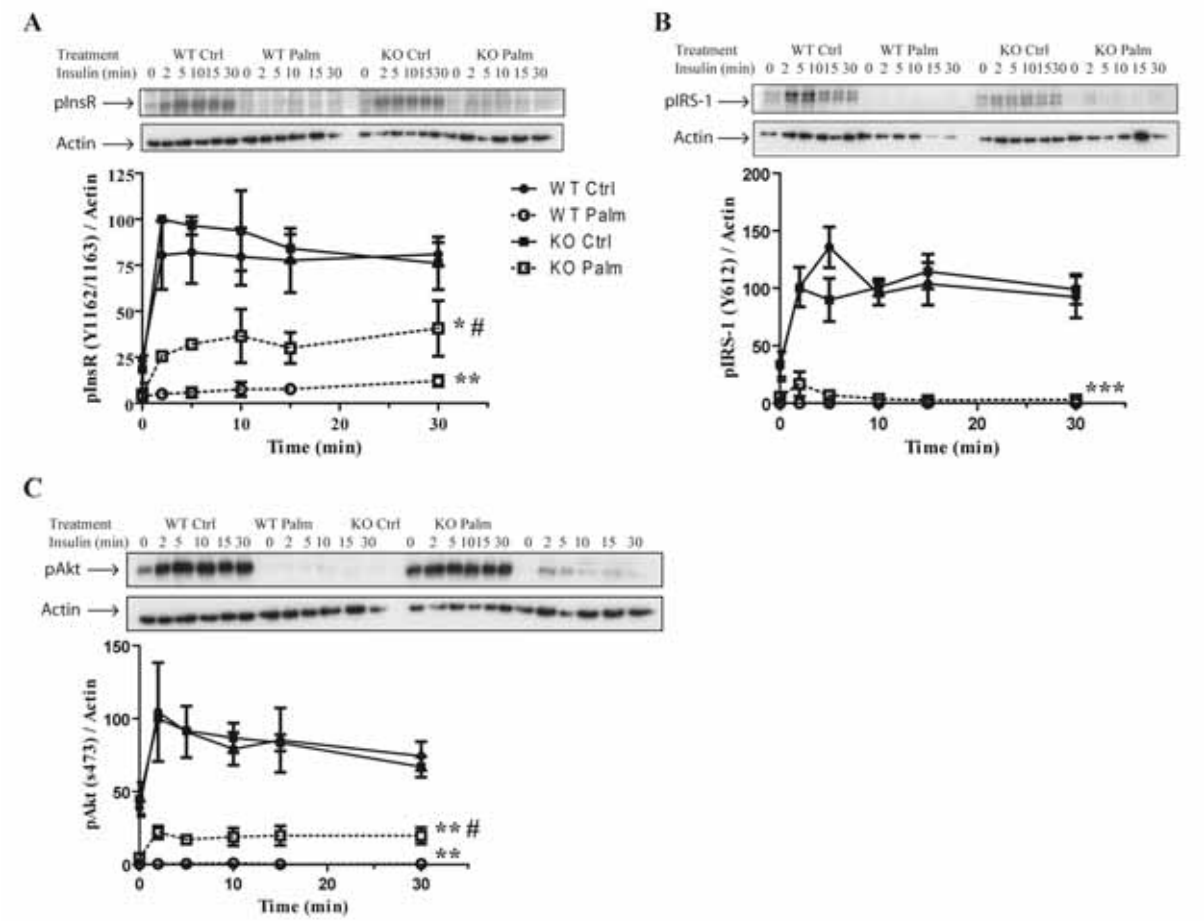

Figure 6.3.4 Phosphorylation of proximal components involved in the metabolic actions of insulin in primary hepatocytes.
Primary hepatocytes from WT and PKCε KO mice were incubated with 400 μM palmitate for 24 h. Over the final 16 h, hepatocytes were serum starved. Hepatocytes were stimulated with 10 nM insulin for the indicated times at 37 °C, washed three times with ice cold PBS and harvested in RIPA buffer. Phosphorylation of the insulin signalling components; **A:** InsR (Tyr 1162/1163), **B:** IRS-1 (Tyr 612) and **C:** Akt (Ser 473) were analysed by immunoblotting. Values are normalised to WT 2 min insulin condition. Data are means ± SEM; n = 3 independent experiments (* = P < 0.05 vs KO ctrl; ** = P < 0.05 vs genotype Ctrl; # = P < 0.05 vs WT Palm.).

Moreover, protein levels of all signalling components examined (InsR, IRS-1, Akt) were diminished following 24 h of palmitate exposure (Figure 6.3.5).

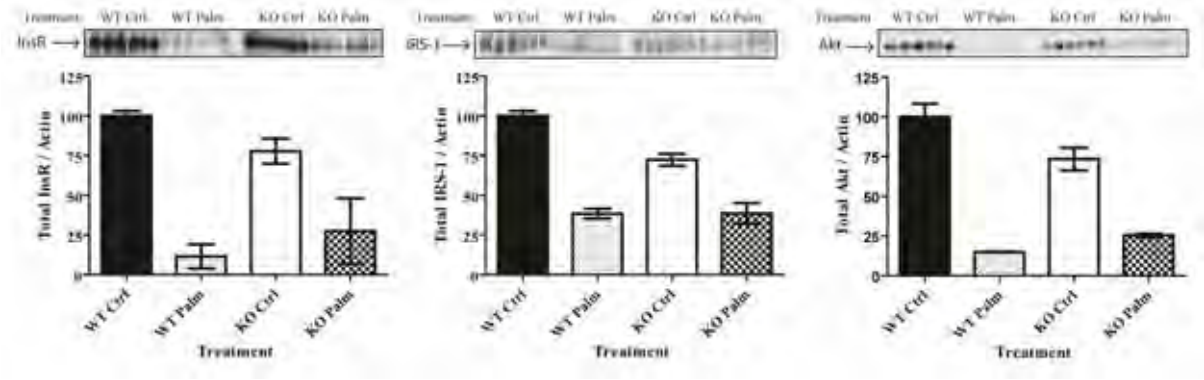

Figure 6.3.5 Expression levels of proximal signalling components involved in the metabolic actions of insulin in primary hepatocytes.
Primary hepatocytes from WT and PKCε KO mice were incubated with 400 μM palmitate for 24 h and stimulated with 10 nM insulin for up to 30 min at 37 °C. Cells were washed three times with ice cold PBS, harvested in RIPA buffer and InsR, IRS-1 and Akt analysed by immunoblotting. Values are expressed are normalised to expression of WT 2 min insulin condition. Data are means ± Range; n = 2 independent experiments.

The protective effect against downstream defects induced by palmitate in the KO hepatocytes still remains when the palmitate-induced changes in protein expression are accounted for, with the IRS-1 Tyr phosphorylation signalling profile still following a similar pattern to when corrected for β-actin (Figure 6.3.6A). Furthermore, when corrected for total Akt expression, Akt Ser phosphorylation is not different in KO hepatocytes treated with palmitate compared to the non-lipid treated condition, whereas the WT hepatocytes still exhibit a marked reduction (Figure 6.3.6B).

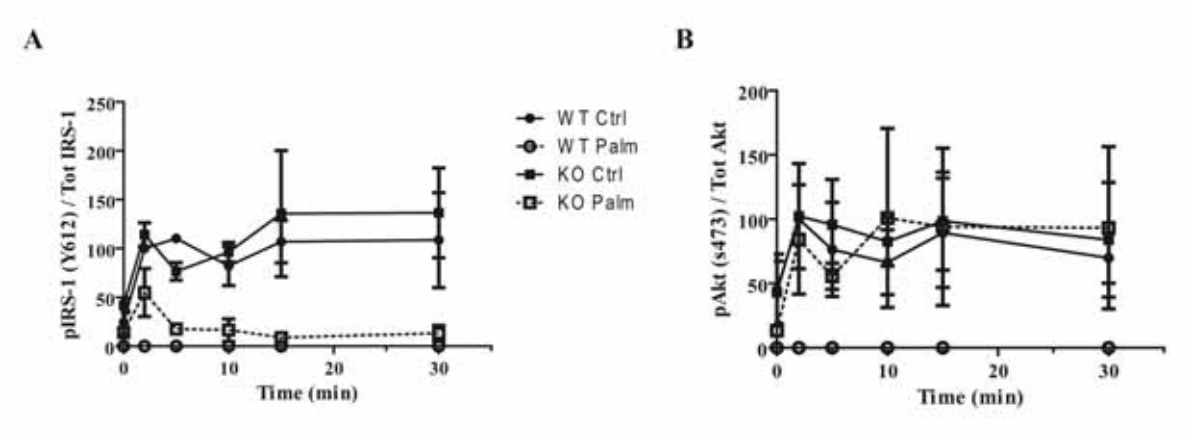

Figure 6.3.6 Phosphorylation of IRS-1 and Akt, corrected for total protein expression in primary hepatocytes.
Primary hepatocytes from WT and PKCε KO mice were incubated with or without 400 µM palmitate for 24 h and stimulated with 10 nM insulin for up to 30 min at 37 °C. Cells were washed three times with ice cold PBS, harvested in RIPA buffer. **A:** IRS-1 and **B:** Akt were analysed by immunoblotting. Values are expressed are normalised to expression of WT 2 min insulin condition. Data are means ± Range; n = 2 independent experiments.

It may be that a less prolonged exposure to lipid or a lower dose of lipid may more clearly elucidate signalling differences that can arise in primary hepatocytes under conditions of lipid oversupply, when expression of the signalling proteins examined are not reduced following lipid treatment, with and without PKCε deletion. As such further signalling components that could be involved in negatively regulating hepatic insulin sensitivity, such as JNK, IKK-β and STAT3 were not examined.

6.3.3 Optimisation of L-Arginine administration to stimulate endogenous secretion of insulin

PKCε KO mice are protected from glucose intolerance following short-term high fat feeding at the level of the liver. To examine insulin sensitivity upon physiological insulin release into the hepatic portal vein, insulin secretion was stimulated endogenously by intraperitoneal injection of L-arginine. To optimise this, pure C57/Bl6 mice were used. A dose-dependent response to L-arginine was observed on circulating insulin levels over a 9 min time course (Figure 6.3.7). Specifically, from a basal level of 0.5 ng/mL, 3 g/kg L-arginine raised circulating insulin levels to 3.4 ng/mL ($p < 0.01$), whereas 1 g/kg L-arginine only increased plasma insulin levels to 1.1 ng/mL. Saline injection as a control had no effect.

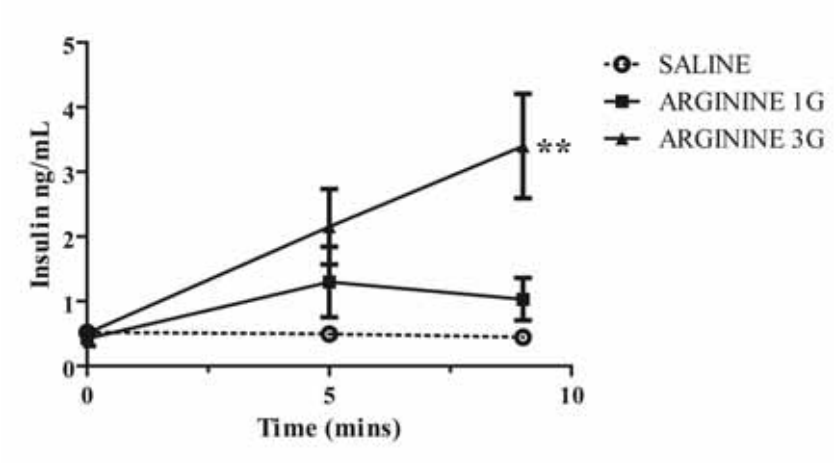

Figure 6.3.7 Plasma insulin levels following L-arginine administration by intraperitoneal injection.
Pure C57/Bl6 mice aged 8-10 weeks were injected with saline, 1 g/kg (■) or 3 g/kg (▲) L-arginine and plasma insulin levels were measured at 0, 5 and 9 min. Data are means ± SEM; n = 5 mice (** = P < 0.01 vs saline at 9 min).

The short time course was chosen as mice were to be culled for tissue harvest and examination of insulin signalling. Furthermore, the purpose of the study was to investigate insulin signalling *in vivo* in the presence of physiological insulin levels, which may be overridden with longer stimulation of insulin secretion by L-arginine.

Blood glucose levels increased over 9 min with saline injection, suggestive of a stress response of the mice to the injection; however this was blunted ($P < 0.05$, AUC) with both doses of L-arginine (Figure 6.3.8A). In addition, different doses of insulin administered by intraperitoneal injection were examined and a similar reduction in glucose levels compared to saline was observed with 0.25 U/kg and 0.5 U/kg ($P < 0.05$, AUC) (Figure 6.3.8B). When insulin was injected at a lower dose of 0.1 U/kg, however, glucose levels resembled those of saline injected animals, indicating a lack of an insulin response at this dose (Figure 6.3.8B).

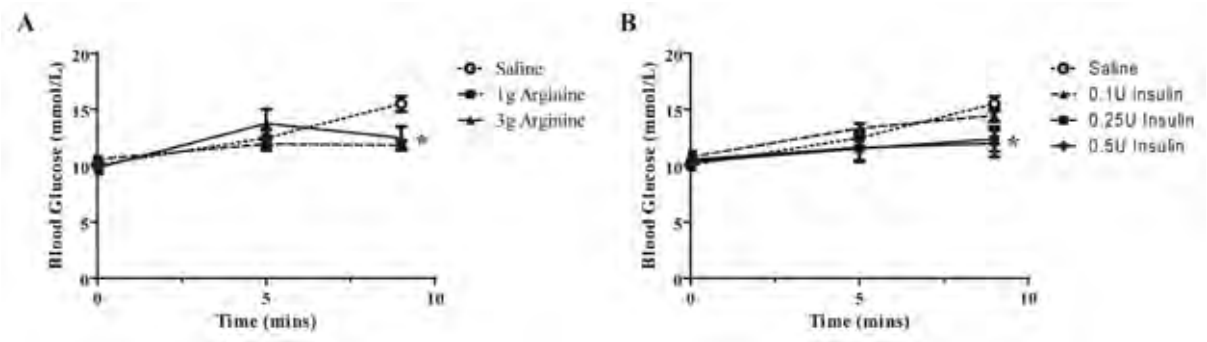

Figure 6.3.8 Blood glucose levels following L-arginine or insulin administration by intraperitoneal injection.
Pure C57/Bl6 mice aged 8-10 weeks were injected with **A:** saline or L-arginine (1 g/kg or 3 g/kg); and **B:** saline or insulin (0.1 U/kg, 0.25 U/kg or 0.5 U/kg). Blood glucose levels were measured at 0, 5 and 9 min. Data are means ± SEM; n = 5 mice. (* = P < 0.05 vs saline)

Insulin target tissues were harvested 10 min after injection of saline, L-arginine or insulin to analyse for insulin signalling activation. Phosphorylation of Akt was examined as the marker of insulin signal transduction activation, and was increased in all insulin-target tissues (liver, quadriceps and epididymal white adipose tissue) over saline (Figure 6.3.9A-C). In liver, L-arginine at 3 g/kg stimulated Akt phosphorylation to higher levels than the other treatments; these all increased Akt phosphorylation only moderately above saline, with the exception of the 0.1 U/kg insulin dose, where Akt phosphorylation remained at similar levels to saline treatment (Figure 6.3.9A). Insulin and L-arginine administration invoked a stronger fold-change activation effect above saline in quadriceps muscle, compared to the liver (Figure 6.3.9B). In epididymal white adipose tissue (eWAT), whilst there was a strong stimulation with insulin injection, the effect was not dose dependent; yet a clear dose-response was observed with L-arginine (Figure 6.3.9C). Together these results indicate that physiologically secreted insulin induced by L-arginine administration is able to activate insulin signalling, as indicated by Akt phosphorylation, in a dose-dependent manner in all insulin-target tissues examined.

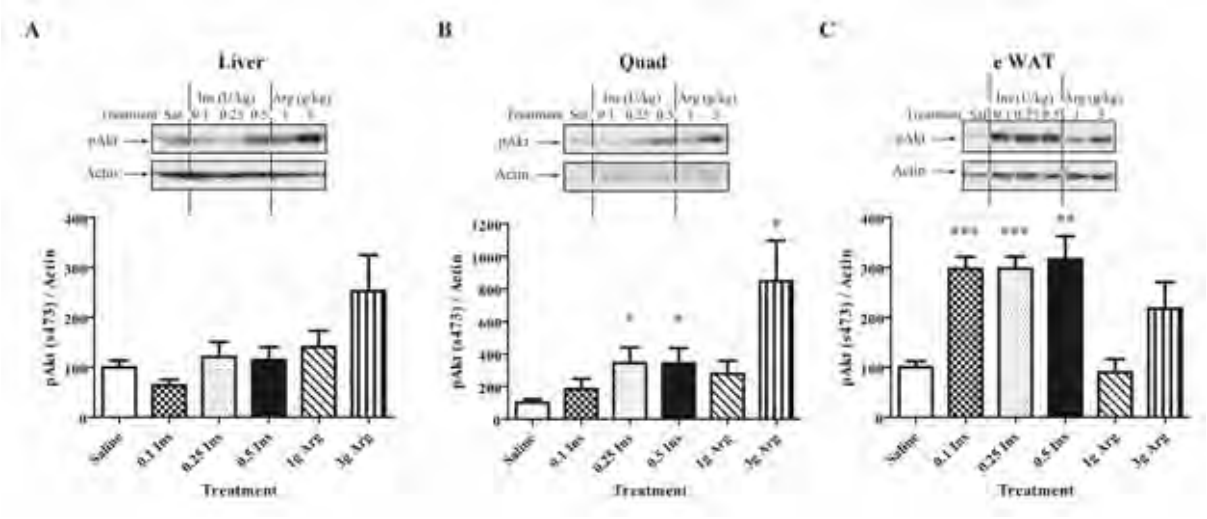

Figure 6.3.9 *In vivo* insulin signalling with insulin or L-arginine administration by intraperitoneal injection.
Pure C57/Bl6 mice aged 8-10 weeks were injected with insulin (0.1 U/kg, 0.25 U/kg, 0.5 U/kg) or L-arginine (1 g/kg or 3 g/kg). Insulin-target tissues; **A:** liver, **B:** quadriceps and **C:** eWAT were harvested 10 min post injection and signalling components analysed by immunoblotting. Data are means ± SEM; n = 4 mice per treatment. (* = P < 0.05 vs saline, ** = P < 0.05 vs saline, *** = P < 0.05 vs saline,)

To examine the effect of L-arginine stimulated insulin secretion in WT and PKCε KO mice, the methodology was optimised in chow-fed animals initially, to assess the degree that insulin levels would rise to in both the WT and KO mice. With L-arginine administration at a dose of 1.5 g/kg by intraperitoneal injection, plasma insulin levels increased similarly in chow-fed PKCε KO and WT animals by ~3.7-fold and ~3-fold to 1.4 ng/mL and 1.1 ng/mL respectively (Figure 6.3.10). These levels are similar to those reported in mice during re-feeding experiments (Agouni, Owen et al. 2010), supporting the use of this methodology for investigating physiological insulin signalling and action *in vivo*.

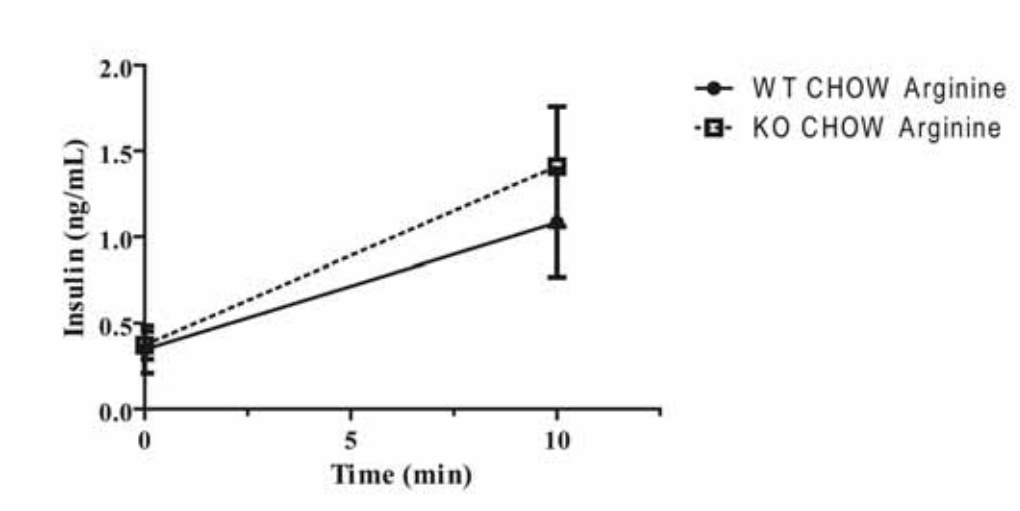

Figure 6.3.10 Circulating insulin levels following L-arginine administration.
Mice aged 10-12 weeks were injected with 1.5 g/kg L-arginine by intraperitoneal injection. Blood samples were collected at 0 and 10 min and insulin levels measured by ELISA. Data are means ± SEM; n = 5-6 mice.

In response to the similar levels of circulating insulin levels observed between the genotypes on a chow diet, glucose levels 10 min after L-arginine administration were not different between the PKCε KO and WT animals (Figure 6.3.11).

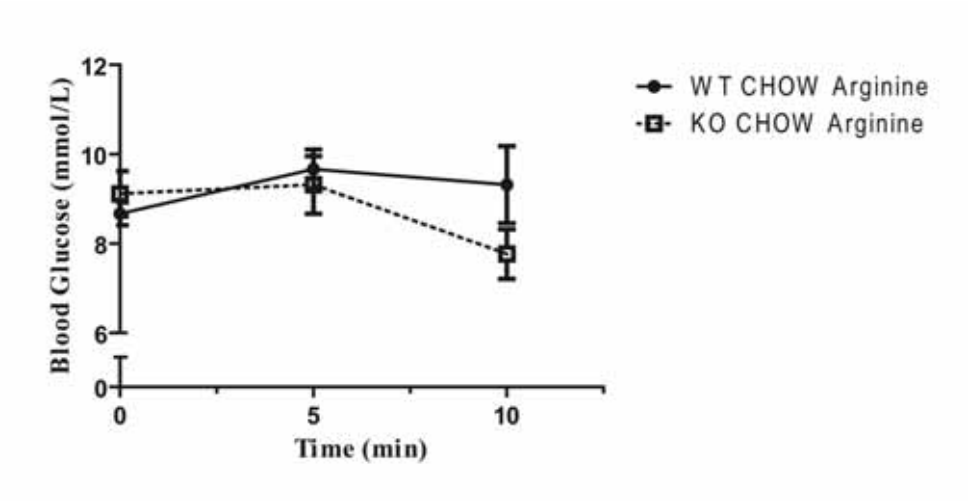

Figure 6.3.11 Blood glucose levels of chow-fed mice during an L-arginine tolerance test.
Mice aged 10-12 weeks were injected with 1.5 g/kg L-arginine by intraperitoneal injection and blood glucose measured at the indicated timepoints. Data are means ± SEM; n = 5-6 mice.

This was associated with a significantly enhanced InsR phosphorylation in the liver of the KO mice treated with L-arginine (P < 0.05 vs WT Arg), as well as a trend towards enhanced insulin signalling at the level of Akt phosphorylation in the liver of these mice (Figure 6.3.12).

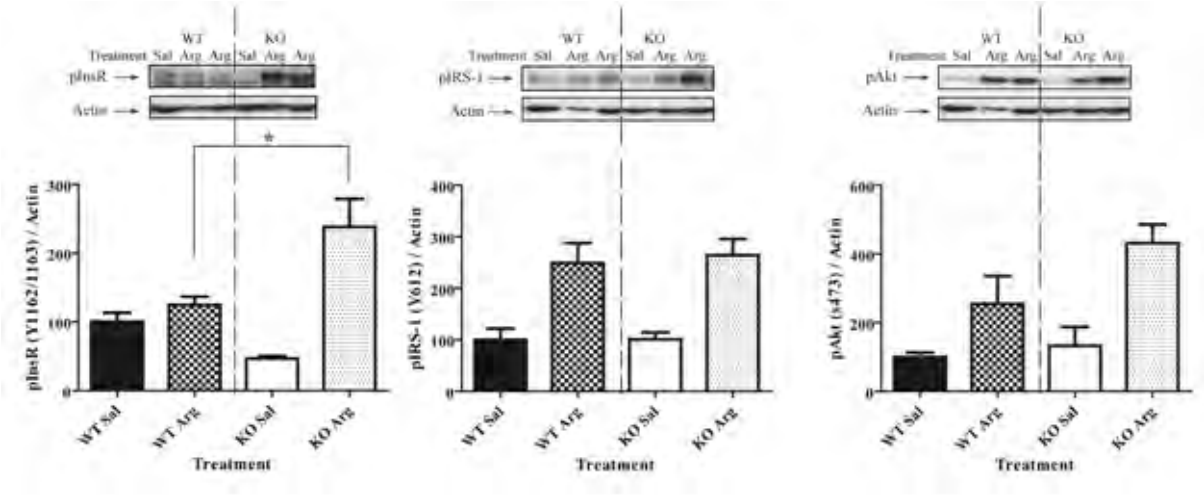

Figure 6.3.12 *In vivo* liver insulin signalling with L-arginine stimulated insulin secretion.
WT and PKCε KO mice aged 8-10 weeks were injected with 1.5 g/kg L-arginine. Tissues were harvested 10 min post injection and phosphorylation of proximal insulin signalling components; **A:** InsR (Tyr 1162/1163), **B:** IRS-1 (Tyr 612) and **C:** Akt (Ser 473) were analysed by immunoblotting. Data are means ± SEM; n = 4-5 mice per treatment (* = P < 0.05 vs WT Arginine).

High fat feeding has been shown to increase β-cell mass and therefore the capacity for the β-cell to secrete insulin (Terauchi, Takamoto et al. 2007). To ascertain whether 1 week of a HFD affected insulin secretion in response to L-arginine, fat-fed WT mice were investigated. Mice fed a lard-based HFD for 1 week were shown to be glucose intolerant (P < 0.001, AUC) (Figure 6.3.13A). Intraperitoneal injection of 1.5 g/kg L-arginine, as was used on the chow-fed animals, led to a marked increase in circulating insulin levels in the fat-fed mice after 10 min, rising to 9 ng/mL (P < 0.01), compared to only 1.1 ng/mL in the chow-fed mice (Figure 6.3.13B).

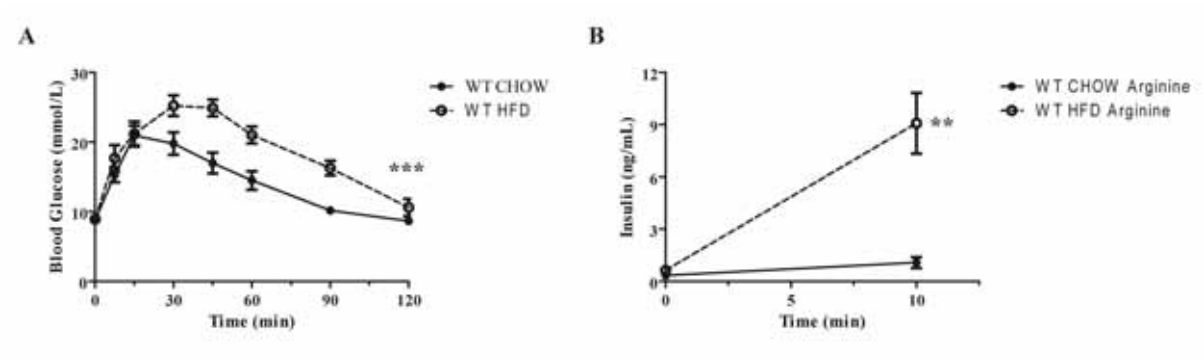

Figure 6.3.13 Blood glucose and insulin profiles of fat-fed WT mice during a GTT and L-arginine tolerance test.
Mice aged 10-12 weeks were fed an HFD for 1 week and were **A:** injected with 2 g/kg glucose by intraperitoneal injection to examine glucose tolerance and blood glucose measured at the indicated timepoints. In a subsequent study, fat-fed mice were **B:** administered 1.5 g/kg L-arginine by intraperitoneal injection and plasma insulin measured at the indicated timepoints. Data are means ± SEM; n = 5-6 mice (** = $P < 0.01$ vs chow 10 min, student's t-test, *** = $P < 0.001$ vs WT chow AUC).

Given the higher circulating insulin levels in WT HFD mice compared to WT chow mice, evaluation of signalling in fat-fed KO and WT mice was not pursued, as the higher insulin concentration would not allow a direct comparison to be made with chow-fed animals. In addition, although the circulating level of insulin was achieved via stimulation of a physiological process, it was not within a range that would be relevant in physiological insulin action, therefore negating the principle of the experiment.

6.4 Discussion

Results of the experiments in this chapter performed in primary hepatocytes were generally in agreement with previous results obtained using MEFs. Although IRS-1 Tyr phosphorylation tended to be increased in the PKCε KO hepatocytes, similar to the MEFs, this occurred without a concomitant reduction in Ser phosphorylation, as measured at Ser 636/639, a key site for PI3K binding. Also, that InsR phosphorylation was not affected in primary hepatocytes supports the hypothesis that the lack of InsR phosphorylation in PKCε KO MEFs was more due to an adaptation of the cells to survival in culture than a specific effect of PKCε deletion. Furthermore, Grb14 levels were not altered indicating PKCε deletion does not affect its expression in liver. The insulin signalling data presented herein is also in agreement with previous data obtained from primary hepatocytes of these animals (Schmitz-Peiffer, Laybutt et al. 2007), which did not exhibit pronounced metabolic differences on a chow diet and where there were no signalling differences detected in mice following insulin administration *in vivo*. Therefore it is not surprising that primary cells isolated from adult mice on a chow diet, such as hepatocytes in the present experiment, did not display alterations in the insulin signalling cascade as a result of PKCε deletion.

As previously mentioned, PKCε KO animals are protected from diet-induced glucose intolerance both in the short and long term (Raddatz, Turner et al. 2011), and the protection seen after 1 week of HFD was associated with improved hepatic glucose metabolism, as highlighted by improved glucose levels during glucose and pyruvate tolerance tests. This protection was associated with increased liver lipid deposition, however, which is in agreement with the TG accumulation observed in primary hepatocytes incubated with palmitate (Raddatz, Turner et al. 2011). In the experiments described in this chapter, incubation of primary hepatocytes with palmitate for 24 h resulted in diminished activation and expression of key insulin signalling components: InsR, IRS-1, Akt in both KO and WT hepatocytes. PKCε KO hepatocytes did exhibit a better preservation of Akt phosphorylation though, following 24 h of palmitate treatment compared to WT. It has been reported that the saturated FA palmitate can inhibit insulin signalling in hepatocytes through promoting degradation of the InsR in a time and dose-dependent manner for up to 48 h, with a 35% reduction seen after just 12 h of incubation (Ruddock, Stein et al. 2008). Other studies have shown that acute incubation of palmitate for as little as 30 min and up to 6 h (Malhi, Bronk et

al. 2006; Solinas, Naugler et al. 2006) can impair insulin signalling, with an associated increase in JNK activation. In the study by Malhi *et al.*, it was seen that lipid treatment for 24 h induced a less sustained activation of JNK in primary hepatocytes compared to the HepG2 hepatocyte cell line (Malhi, Bronk et al. 2006). In the experiments described in this chapter, preliminary data showed JNK expression and therefore cellular activation to be significantly reduced in both WT and PKCε KO primary hepatocytes following 24 h palmitate treatment (data not shown). As such, examining candidates whose activation may alter hepatic insulin sensitivity such as JNK and STAT3; the latter having been implicated in promoting hepatic insulin sensitivity through suppression of hepatic gluconeogenic gene expression (Inoue, Ogawa et al. 2004; Ramadoss, Unger-Smith et al. 2009; Fukushima, Loh et al. 2010); should ideally be examined under more refined conditions where palmitate does not induce protein degradation, as was observed in the experiments described in Section 6.3.2 of this chapter. However the limited number of mice available for use did not allow further studies on primary hepatocytes to be conducted. Therefore, to elucidate the subtle signalling differences in KO versus WT that appear to exist with lipid exposure in primary hepatocytes, an acute or lower dose palmitate regimen may be required. Under the current study conditions though, a greater decrement in insulin signalling at the level of Akt in WT hepatocytes was seen at 24 h compared to the PKCε KO hepatocytes. These results agree qualitatively with those seen in MEFs incubated for 24 h with palmitate, as detailed in Chapter 5.

The lack of a clear protection of insulin signalling observed in primary hepatocytes of PKCε KO mice may suggest that PKCε does not play a completely liver-intrinsic role in the protection of glucose tolerance observed *in vivo*, but requires synergistic effects from ablation in other tissues. It has been previously shown by the Shulman group that *in vivo* knockdown of PKCε in liver and WAT by antisense oligonucleotide results in enhanced InsR kinase activity, IRS-2 Tyr phosphorylation and Akt2 activation in isolated liver tissue from rats following a 20 min euglycaemic-hyperinsulinaemic clamp, despite no alteration in hepatic lipid profile (Samuel, Liu et al. 2007). This may indicate that deletion of PKCε in tissues other than the liver contribute to the protection seen in the animals, with regard to hepatic glucose production; this could result from an indirect effect that acts to improve liver insulin action by alleviating other metabolic perturbations. This question of whether PKCε plays a hepatocyte-specific role in modulating HGP could be answered through measuring glucose production into the media of cultured primary hepatocytes, an experiment that has yet to be

reported in mice with targeted deletion of PKCε. Preliminary data for these experiments did not produce consistent insulin-induced suppression of glucose production, and no obvious difference between WT and PKCε KO hepatocytes was observed (data not shown).

The increased lipid deposition observed in liver of global PKCε KO mice appears to be an intrinsic effect however, given it also occurs in isolated primary hepatocytes from the mice (Raddatz, Turner et al. 2011). Results acquired from PKCε KO MEFs in Chapter 5 indicated a more pronounced protection in insulin signalling following lipid treatment compared to WT than was observed in the primary hepatocytes in this chapter. Yet, this may be due to MEFs not being representative of a tissue-specific cell line such as the liver, but having the ability to differentiate into a variety of tissues under specific conditions (Vierbuchen, Ostermeier et al. 2010; Sekiya and Suzuki 2011). It has recently been shown that MEFs can differentiate into hepatocyte-like cells (Sekiya and Suzuki 2011) and this has also been demonstrated for neuron differentiation (Vierbuchen, Ostermeier et al. 2010). Therefore the results obtained from MEFs in these studies must be interpreted with care as they may be reflective of a combination of cell types, thus potentially contributing to differences compared to primary cells as described in this chapter.

In the L-arginine experiments described in this chapter, performed to stimulate endogenous insulin secretion, tissues were harvested to examine physiological insulin signalling. In these experiments, during the L-arginine tolerance test, blood glucose levels of chow-fed PKCε KO mice were reduced further than in WT mice, despite no difference in circulating insulin levels, possibly indicating enhanced insulin sensitivity. This increased insulin sensitivity is likely to be at the level of the liver, as data from our laboratory has shown no difference in glucose transport into muscle or adipose tissue (Raddatz, Turner et al. 2011). However, the observation that PKCε KO mice are more sensitive to the endogenously secreted insulin is a novel finding, since no differences were seen following an intraperitoneal insulin tolerance test (Raddatz and Schmitz-Peiffer unpublished data). This raises the possibility that secretion of insulin by physiological means may represent a more sensitive way of elucidating differences in insulin sensitivity.

Recently it was shown that intraperitoneal injection of insulin leads to different kinetics of insulin signalling in different insulin sensitive tissues (Agouni, Owen et al. 2010). In the

study by Agouni *et al.* it was determined that tissue harvest 5-10 min post injection, particularly of liver and muscle, was suitable to measure phosphorylation of insulin signalling components, at which time signalling tended to peak. For the experiments performed in this chapter it was hypothesised that stimulating endogenous secretion of insulin from the pancreas may be more appropriate to examine liver signalling, as the insulin is secreted directly into the hepatic-portal vein thereby reaching the liver first. Furthermore, endogenous insulin secretion would occur in an anatomically compatible manner, as occurs in the animal following a meal. Intraperitoneal injection of L-arginine acts as a potent endogenous insulin secretagogue (Palmer, Benson et al. 1976; Krause, McClenaghan et al. 2011), probably through membrane depolarisation of the β-cell via its actions on blocking potassium channels as well as stimulating calcium influx to promote exocytosis transport machinery (Thams and Capito 1999). Over a 10 min time period following L-arginine administration, mice on a chow diet exhibited insulin levels similar to those observed during a glucose tolerance test after 1 week of chow or HFD (Raddatz, Turner et al. 2011). However, WT mice fed an HFD for 1 week exhibited significantly elevated circulating insulin levels 10 min after L-arginine administration and this increase was greater than physiological levels expected to be secreted by a mouse following ingestion of a meal. Although it is established that β-cell mass increases with high fat feeding and this aids in overcoming the peripheral IR, the increase in circulating insulin expected after 1 week of lipid oversupply due to β-cell hyperplasia would be approximately 2-fold (Terauchi, Takamoto et al. 2007). Assessment of β-cell mass was beyond the scope of the experimental aims of the present studies. The approximate 5-8 fold increase in circulating insulin levels seen in this case is probably greater than the increase that would be expected purely due to an increase in β-cell mass or high fat feeding for 1 week, which is further supported by the 1.5 to 2-fold increase in insulin levels observed during a GTT after 1 week of high fat feeding in both WT and PKCε KO mice (Raddatz, Turner et al. 2011). Since insulin secretion stimulated by L-arginine is independent of glucose, it suggests that within 1 week of an HFD, this aspect of the β-cell secretory response is greatly upregulated. The mechanism behind this could be interesting to investigate further, however was beyond the scope of the current work.

Due to the differences in circulating insulin levels induced by L-arginine, insulin signalling was not compared between the chow and HFD-fed animals. However whether diet-induced signalling defects are to be expected after 1 week of fat feeding is unclear, with some studies

showing differences (Samuel, Liu et al. 2007) but many others reporting no difference, despite protection from IR or glucose intolerance in each case (Hoy, Bruce et al. 2007; Hoy, Brandon et al. 2009; Raddatz, Turner et al. 2011). In order to better examine insulin signalling with a HFD, and determine its contribution to overall insulin action in the model used in these experiments, a lower L-arginine dose would be required to elicit a modest increase in insulin. This would be a reasonable future experiment, as our pilot studies showed L-arginine is able to increase insulin levels in a dose-dependent manner.

Overall, it appears that there may not be hepatocyte-intrinsic alterations at least in chow-fed mice in the insulin signalling cascade. Results from primary hepatocytes also suggest that ablation of PKCε does not exhibit a pronounced preservation of insulin signalling with long term lipid oversupply. However, the KO mice display a tendency toward increased insulin sensitivity following *in vivo* insulin secretion. This suggests examining insulin signalling and sensitivity to endogenously secreted insulin at physiological levels may emphasise differences that do exist between the WT and PKCε KO mice that are biologically relevant after a short-term HFD, which may be masked by supramaximal doses of insulin.

CHAPTER 7

Summary and Future Directions

The development of IR has been associated with excess consumption of an energy dense diet, rich in simple carbohydrates and fat, leading to obesity and T2DM. Models of lipid oversupply induce impairments in insulin action such as reduced glucose transport, glycogen synthesis and insulin signal transduction (Schmitz-Peiffer 2000; Cazzolli, Carpenter et al. 2001; Kraegen, Cooney et al. 2001; Hoehn, Hohnen-Behrens et al. 2008). The molecular mechanisms leading to these defects remain unclear especially in terms of precisely which particular lipid species or signalling proteins are responsible for the onset of IR.

The Ser/Thr PKC family are ubiquitously expressed and have been implicated in modulating a variety of signal transduction pathways. Lipid second messengers such as DAG have been postulated to increase in times of lipid oversupply and this may lead to activation of PKC isozymes that in turn lead to defective insulin action. The novel PKC isozyme, PKCε, does not require calcium for activation and as such, lipid second messengers, particularly DAG are primarily responsible for its activation. Furthermore, PKCε has been demonstrated to be activated in fat-fed rat skeletal muscle, lipid-treated muscle cell-lines (Schmitz-Peiffer, Browne et al. 1997) as well as in liver of insulin resistant and diabetic humans (Considine, Nyce et al. 1995). Furthermore, hepatic DAG accumulation has been linked to PKCε activation and hepatic IR (Samuel, Liu et al. 2004; Kumashiro, Erion et al. 2011). It has also been shown that PKCε can impair InsR kinase activity and insulin signalling in times of excess lipid availability in the liver of rodents (Samuel, Liu et al. 2007), while PKCε ablation reduced hepatic insulin clearance even in chow-fed mice (Schmitz-Peiffer, Laybutt et al. 2007). The fact that two models of PKCε deletion or knockdown found protection against HFD-induced glucose intolerance, despite different mechanisms, could be due to the length of diet (3 days vs 16 weeks), model used (rats vs mice) as well as experimental techniques used to gauge metabolic parameters (euglycaemic clamp vs GTT).

The studies described in this thesis investigated the mechanisms affecting InsR endocytosis in the absence of PKCε and its role on insulin signal transduction in either the absence or presence of lipid oversupply. MEFs were used as a model to determine whether PKCε alters InsR trafficking and possibly the associated signal transduction pathway. As PKCε has been implicated in the development of hepatic IR, primary hepatocytes were further used as a model for examining its role on insulin signalling upon lipid treatment.

It has been demonstrated that PKCε null mice are protected from HFD-induced glucose intolerance, partly through enhanced insulin secretion in the long-term in order to better cope with the worsening peripheral IR, but also through reduced hepatic insulin clearance which was also present in chow-fed KO animals compared to WT. The reduced hepatic insulin clearance has been shown in PKCε KO primary hepatocytes previously (Schmitz-Peiffer, Laybutt et al. 2007) and the mechanism behind this was examined using primary MEFs. In support of the findings *in vivo* and from primary hepatocytes, the experiments described in Chapter 3 demonstrated that PKCε KO MEFs exhibited reduced insulin uptake and this was associated with perturbed InsR trafficking. This altered trafficking of the InsR was associated with a differential localisation of the InsR in the basal state, with a much higher proportion of the receptor localised to less dense membrane fractions compared to the WT. It has been proposed that altering membrane lipid composition can impair cellular trafficking and furthermore changes in the ganglioside GM3 have been associated with altered InsR movement and localisation with caveolae (Pilch, Thompson et al. 1980; Kabayama, Sato et al. 2007). The altered InsR localisation and trafficking in this case, was not however due to alterations in membrane morphology, as assessed by EM, or membrane lipid order, measured by Laurdan staining. In addition, reconstitution of PKCε resulted in increased insulin-stimulated InsR redistribution towards the denser cellular organelles as was seen in the WT MEFs, however basal InsR localisation remained more similar to that seen in PKCε KO MEFs. Associated with the increased redistribution of the InsR with PKCε reconstitution was an increase in expression of Ceacam1, closer to that observed in WT MEFs. The liver-specific InsR substrate Ceacam1, has been demonstrated to promote InsR endocytosis (Poy, Yang et al. 2002) and previous experiments using TPA have shown PKCs to regulate Ceacam1 expression and phosphorylation (Edlund, Wikstrom et al. 1998; Bamberger, Briese et al. 2006). The results from experiments involving MEFs in Chapter 3 demonstrate for the first time that a particular PKC isozyme, PKCε, can directly modulate Ceacam1 expression and this finding provides a potential mechanism for the reduced insulin uptake seen in the KO MEFs and hepatocytes.

To test these hypotheses, however, future work would involve examining insulin uptake in KO MEFs reconstituted with PKCε retrovirus where the nearest return to the WT phenotype was observed with regard to Ceacam1 expression. Importantly, the retroviral construct is not GFP-linked, enabling FITC-insulin to be used. This proposed experiment was unable to be

done due to Molecular Probes, who supply the FITC-insulin that has been characterised to be biologically active, no longer distributing to Australia. Furthermore, tracking the InsR following insulin-stimulation by immunofluorescence or EM studies could provide more detailed information regarding the compartmentalisation of the InsR in the absence of PKCε. Additionally, examining Ceacam1 association with the InsR in MEFs and primary hepatocytes or liver is needed to confirm this as a mechanism for the reduced insulin clearance. It has been reported that reduced phosphorylation of Ceacam1 at Ser 503 can impair its ability to associate with and therefore promote InsR endocytosis (Najjar, Phillippe et al. 1995; Estrera, Chen et al. 2001). Whether Ser 503 phosphorylation is reduced by PKCε deletion or if other Ceacam1 Ser sites are directly phosphorylated by PKCε and are required to promote the association of PKCε and the InsR, leading to Ceacam1 Tyr phosphorylation, need to be examined.

Interestingly, PKCε contains an actin-binding motif, enabling it to bind actin and potentially alter actin conformation. Preliminary experiments have shown that PKCε KO MEFs exhibit a reduced ability to reorganise actin upon insulin stimulation compared to WT MEFs. However due to the importance actin plays in multiple cellular trafficking events (Tong, Khayat et al. 2001; Lopez, Burchfield et al. 2009), together with the fact that the trafficking defect appears specific to the InsR, as shown by transferrin and EGF uptake not being significantly reduced in the KO MEFs (Chapter 3) and glucose transport unaffected (Chapter 4), it is difficult to understand how actin might specifically affect InsR trafficking in the absence of PKCε. Reconstituting PKCε with and without a mutation in its actin-binding domain and investigating actin reorganisation, InsR trafficking and insulin uptake could elucidate if either actin or Ceacam1 are key components of the mechanism by which PKCε impairs InsR trafficking.

It is known that impaired InsR internalisation can affect mitogenic signalling, while metabolic signalling remains intact (Ceresa, Kao et al. 1998). Because the experiments from Chapter 3 demonstrated that PKCε KO MEFs exhibit reduced InsR trafficking and therefore insulin uptake, activation of critical components of the insulin signalling cascade were examined. The fact that the MEFs tended to show an increase in IRS-1 Tyr phosphorylation and Akt Ser phosphorylation is in agreement with the hypothesis that reduced InsR internalisation could promote metabolic signalling. This signalling profile however, occurred

despite PKCε KO MEFs exhibiting significantly reduced InsR Tyr phosphorylation. In contrast to when InsR internalisation is completely blocked by inhibition of dynamin (Ceresa, Kao et al. 1998), PKCε KO MEFs also showed an increase in ERK 1/2 phosphorylation compared to WT. The fact that ERK 1/2 phosphorylation was not impaired is not irreconcilable with prior research, as the KO MEFs do not display a complete block in InsR internalisation, but instead a reduced rate of internalisation. Moreover, the trend for increased insulin signalling in the PKCε KO MEFs is not associated with reduced IRS-1 Ser phosphorylation. Inhibition of phosphatases increased InsR Tyr phosphorylation towards WT levels, and it may be that altered localisation of the InsR in KO MEFs results in enhanced phosphatase activity towards the InsR. Furthermore, overexpression of PKCε by adenoviral techniques failed to localise the InsR to that seen in the WT cells in the basal state and moreover did not overcome the reduced InsR Tyr phosphorylation. It did however, affect insulin-stimulated InsR redistribution. Additionally, treatment of PKCε KO MEFs with a phosphatase inhibitor failed to rescue the defect in insulin uptake, suggesting phosphorylation does not alter internalisation of the InsR directly in PKCε KO MEFs, however localisation of the InsR upon phosphatase inhibition was not investigated in these experiments. Because reconstitution of PKCε failed to alter InsR phosphorylation, but did affect trafficking, it could be that localisation of the InsR is independent of phosphorylation.

The fact that reconstitution of PKCε both acutely (by adenovirus) and chronically (by retrovirus), together with the signalling profile observed in primary hepatocytes showing no difference in InsR phosphorylation or downstream signalling, indicates that this phosphorylation defect of the InsR in MEFs may be an adaptation to survive in culture in the absence of PKCε rather than a direct effect of PKCε ablation. On the other hand, it has been reported that PKCs may activate their signalling networks in a cascade, requiring each other for further activation (Kewalramani, Fink et al. 2011) and therefore knocking out a single isozyme may result in non-isozyme specific effects, due to a perturbation in the PKC signalling network. This has recently been demonstrated by Collazos *et al.*, who showed that PKCε is downstream of PKCα and therefore their individual effects may be masked by signalling aberrations of the other isozymes (Collazos, Diouf et al. 2006). Importantly there was no alteration in the expression of other PKCs examined (see Appendix II), suggesting there was no compensation by other PKCs for the deletion of PKCε.

As such it needs to be confirmed that the signalling profile observed is propagated from the activated InsR, by examining InsR kinase activity between the WT and KO MEFs. It is hypothesised that this would not be different between genotypes as in experiments where cells were stimulated with as little as 1nM insulin (see Appendix I), a concentration at which IGF receptors should not be activated due to their lower affinity for insulin, the same signalling profile was observed. This indicates that IGF receptors are not compensating for the reduced InsR autophosphorylation, which could impact signalling.

It has been postulated that spatial localisation of signalling components can affect signal transduction, because of the requirement for interaction between components to maintain the cascade of events ultimately leading to propagation of the signal and its actions. Given PKCε is a lipid activated protein kinase that has been implicated in the development of IR, increased lipid supply could be expected to increase PKCε activation and highlight its actions. Furthermore, PKCε ablation elicits the largest metabolic benefit during high fat feeding. It has been shown that knockdown of PKCε with antisense oligonucleotides improves insulin sensitivity, particularly at the level of the liver with increased IRS-2 Tyr phosphorylation, Akt2 activity and InsR kinase activity (Samuel, Liu et al. 2007). It has been hypothesised that high fat feeding leads to accumulation of DAG which activates PKCε, resulting in reduced InsR activation and therefore impaired insulin action (Samuel, Liu et al. 2007). Although results from global deletion of PKCε in mice have not indicated improved activation of proximal insulin signalling components *in vivo*, as was seen by Samuel *et al*., this may have been due to methodological factors as discussed previously, and so signalling was re-examined first in MEFs to determine whether PKCε deletion could protect against lipid induced defects. In the experiments described in Chapter 5, PKCε KO MEFs exhibited a protection in insulin signalling at the level of Akt following 24 h palmitate treatment. Furthermore, although more subtle, experiments described in Chapter 6 demonstrated PKCε KO primary hepatocytes have improved Akt phosphorylation compared to lipid-treated WT hepatocytes. This effect after 24 h palmitate treatment was less pronounced than in the MEFs and was associated with significant downregulation of signalling components examined. Therefore more acute lipid treatment may be required to better investigate signalling differences, compared to that used for lipid partitioning experiments. In fact it has been shown that acute treatment of primary hepatocytes is able to induce activation of JNK and reduce insulin signalling and action (Malhi, Bronk et al. 2006; Solinas, Naugler et al. 2006)

and therefore potential alterations in JNK signalling between the genotypes may be observed following a shorter incubation with lipid

It has previously been shown that in primary hepatocytes from PKCε KO animals, 24 h palmitate treatment results in increased TG synthesis, however in MEFs, palmitate treatment was not associated with altered accumulation of lipid species of the Kennedy pathway. The MEFs do not represent a particular tissue, which could be a reason for the discrepancy, although there was a trend for the KO MEFs to exhibit enhanced TG synthesis. On the other hand, the protection in signalling observed at the level of Akt may be due to differences between the genotypes and other detrimental factors such as the sphingolipid metabolite ceramide, activation of phosphatases or possibly altered localisation of signalling components (Schmitz-Peiffer, Browne et al. 1997; Tirosh, Potashnik et al. 1999; Cazzolli, Carpenter et al. 2001; Holland, Miller et al. 2011b). As such lipidomic analysis of the lipid-treated cells would give greater insight into whether a particular lipid species or sub-pool of a particular lipid species correlates with the improved insulin signalling. In the lipid-treatment experiments described in Chapters 5 and 6, only the saturated FA palmitate was used, whereas it has been shown that unsaturated FA species can be more potent activators of PKCs. Therefore lipid-treating the MEFs and hepatocytes with unsaturated FAs or a mixture of saturated and unsaturated FA may provide additional mechanistic insight into the role of PKCε in lipid-induced IR and more reflect the dietary situation *in vivo*. Furthermore, optimising and examining HGP in isolated primary hepatocytes is necessary to investigate the lipid effect upon a more physiological function than signalling.

Previous studies have demonstrated that insulin action can be impaired in the absence of any observed insulin signalling defects in whole cell lysates or tissue extracts (Hoy, Bruce et al. 2007; Hoehn, Hohnen-Behrens et al. 2008) and it has been hypothesised that this may be the result of altered cellular localisation of signalling components causing reduced interaction among downstream cellular targets (Tirosh, Potashnik et al. 1999; Ogihara, Asano et al. 2004). The results from Chapter 3, together with the Laurdan results described in Chapter 5, whereby the KO MEFs tended to display more localised cellular regions of ordered lipid compared to the WT following 24 h palmitate treatment, suggest that the PKCε KO MEFs store lipids differentially to the WT. This compartmentalisation as well as a potential alteration in the lipid metabolites stored may impact upon the ability of a sub-pool of signalling molecules to be sufficiently activated. Furthermore, the increased lipid deposition

in primary hepatocytes as well as liver suggests that the lipid storage effect is a liver-intrinsic mechanism of PKCε. This increased hepatic lipid deposition is in contrast to the findings of Samuel *et al*., however this discrepancy may be explained by the study of Samuel *et al.* employing a more acute diet treatment over 3 days (Samuel, Liu et al. 2007).

Previously it has been shown that supramaximal administration of insulin into the inferior vena cava of mice did not reveal any defect in insulin signalling (Raddatz, Turner et al. 2011). The 'spare' component theory proposes that not all signalling molecules are activated under low or physiological levels of insulin, but with supramaximal doses, the 'reserve' components can become activated (Kono and Barham 1971; Rice, Lienhard et al. 1992) which could compensate for lipid-induced defects. It was recently reported that following intraperitoneal administration of supraphysiological levels of insulin, that insulin-target tissues elicited altered kinetics for insulin signalling activation (Agouni, Owen et al. 2010). It was therefore hypothesised that to determine whether the signalling protection observed in PKCε KO MEFs and isolated hepatocytes also occurred *in vivo*, it may be best to induce physiological levels of insulin secretion in the mice. In the experiments described in Chapter 6, examining insulin signalling under physiological doses of insulin that are secreted into the circulation could reveal subtle differences. To achieve this, the fact that L-arginine is able to stimulate insulin secretion *in vivo* in a more physiological manner was utilised. L-arginine stimulates insulin secretion independent of glucose sensing, through depolarisation of the β-cell, due to its actions upon potassium channels and intracellular calcium influx (Thams and Capito 1999). These experiments demonstrated that chow-fed PKCε KO animals tended to be slightly more insulin sensitive, based on their lower glucose profile in response to physiological insulin secretion. Furthermore, this was associated with slightly increased proximal insulin signal transduction in the KO mice. However, when WT mice were fat-fed for 1 week, they exhibited significantly increased L-arginine stimulated insulin secretion. It has been reported previously that after four weeks of fat-feeding, β-cell hyperplasia results in a 1.2-fold increase in β-cell mass (Terauchi, Takamoto et al. 2007). Surprisingly, the increase in circulating insulin levels observed in 1 week fat-fed WT mice, following L-arginine administration was approximately 9-fold. This is much higher than would be expected due to β-cell hyperplasia and as such this suggests that after as little as 1 week of fat-feeding, the β-cell secretory response due to L-arginine is markedly upregulated, although the mechanism for this was not addressed here.

In order to examine insulin signalling differences between the WT and KO mice on a HFD, it would be interesting to see if subtle differences are revealed by inducing physiological insulin secretion with a lower dose of L-arginine, which during optimisation experiments (Figure 6.3.7) was shown to increase insulin secretion in a dose-dependent manner. This method in chow-fed animals revealed that the KO animals have a tendency to be more insulin sensitive, which had not previously been observed upon supraphysiological insulin intraperitoneal injection by our lab, and it could be that minor, yet physiologically relevant differences, particularly within the liver, are more easily revealed in mice on a HFD.

The question remains as to whether PKCε deletion exerts its effects on improved hepatic insulin sensitivity directly or through synergistic effects of other tissues. The improved hepatic insulin action of rodent models lacking PKCε are supported by the results of Shulman and colleagues (Samuel, Liu et al. 2007) and more recently Raddatz *et al.*, who showed PKCε KO mice exhibit an improved glucose profile during a pyruvate tolerance test, indicative of reduced gluconeogenesis, which predominantly occurs at the level of the liver (Raddatz, Turner et al. 2011). It therefore raises the question of whether these phenotypes would be replicated in a liver-specific KO model. However, fat-fed liver-specific PKCε KO mice do not show any improvement during a GTT (Raddatz and Schmitz-Peiffer unpublished data). In previously published studies that have targeted PKCε expression in rodents, the common sites of PKCε deletion or knock down were the liver and adipose tissue. Given the lipogenic phenotype of the global PKCε KO mice and improved insulin action in rats with knockdown of PKCε by antisense oligonucleotide, the effect of the adipose tissue in the phenotype of the rodents could be critical to the hepatic improvements seen. It has been demonstrated that PKCε can induce differentiation of 3T3L-1 cells into adipocytes (Ohashi, Kanazawa et al. 2005), however its role in adipose tissue is yet to be carefully examined and targeting PKCε in adipose tissue alone or in combination with the liver could elucidate as yet unknown mechanisms of action of PKCε. Whether PKCε could enhance insulin action and therefore prevent uncontrolled lipolysis or improve the lipokine profile are two such ways by which it could exert its effects on liver through an adipose dependent manner. Furthermore, PKCε is highly expressed in neural tissue and the link between HGP and the brain is well established in mice (Obici, Zhang et al. 2002; Könner, Janoschek et al. 2007; Lin, Plum et al. 2010). It has been shown that HGP is controlled by agouti-related peptide (AgRP) expressing neurons of the hypothalamus, independent of feeding alterations (Könner, Janoschek et al. 2007).

PKCε has been demonstrated to be involved in many neural processes including pain, anxiety and alcohol withdrawal (Olive, Mehmert et al. 2001) and its actions in these non-liver tissues may synergistically result in the overall phenotype.

Many of the experiments described in this thesis have used MEFs as a model in which to investigate the effects of PKCε ablation. The MEFs are unique in that they represent a PKCε null cell line and can be cultured easily to examine the effects of PKCε ablation. This enables examination of PKCε dependent processes, such as Ceacam1 expression, which was modulated by the presence of PKCε. Although the MEFs did exhibit properties associated with liver and primary hepatocytes from the mice, as demonstrated by impaired insulin uptake, they are not truly representative of a specific tissue. Moreover as revealed in some experiments of this thesis, with regard to Grb14 expression, MEFs do exhibit clone-specific effects which may arise as the cells adapt to survive in culture after isolation. It is therefore imperative, as has been done in this thesis, to test findings in multiple MEF pools in order to confidently conclude that differences are due to ablation of a particular protein, in this case PKCε, and not due to clone-specific divergence in culture. In fact many published studies using MEFs do not state if experiments performed in MEFs have been characterised in a number of clones as was done in these experiments (Flinn, Yan et al. 2010; Holzer, Park et al. 2011; Peterson, Sengupta et al. 2011). Furthermore, the ability to differentiate MEFs with specific factors into specific tissue-like cells such as hepatocytes and neurons, make them a unique cell line in which to examine potential tissue-specific effects in a KO cell line. Therefore MEFs can be utilised to investigate mechanisms that a particular protein is involved in, however cross-referencing findings in multiple MEF pools with primary tissues is vital to delineate cell culture adaptations from protein-specific and tissue-autonomous effects.

In conclusion, the current experiments have demonstrated PKCε modulates InsR trafficking and this is associated with increased expression of the InsR substrate, Ceacam1 (Chapter 3). This is the first time a specific PKC, in this case PKCε, has been implicated in regulating Ceacam1. These experiments also demonstrated that deletion of PKCε improved insulin signalling under conditions of lipid oversupply at the level of Akt in MEFs and primary hepatocytes (Chapters 5 and 6). Furthermore the findings from the Laurdan experiments suggest that the localisation of lipid storage is altered between WT and PKCε KO MEFs

(Chapter 5). The *in vivo* experiments (Chapter 6) have also revealed the PKCε KO mice to be slightly more insulin sensitive to physiologically secreted insulin on a chow diet and this has not been observed previously in our lab (Chapter 6). Extending this method of physiological insulin secretion to short-term fat-fed mice could help ascertain differences associated with the improved hepatic insulin action and overall metabolic profile of the mice. Together these results indicate targeting PKCε at the level of the liver could alter insulin clearance and modulate insulin levels without overworking the β-cell. Furthermore, ablation of PKCε improved insulin signalling, with preserved Akt phosphorylation in cells under conditions of lipid oversupply, however the primary hepatocyte experiments suggest that signalling perturbation is a consequence rather than a cause of metabolic derangement, potentially involving differential lipid localisation or a build up of particular lipid metabolites that are unlikely to be in the glycerolipid/Kennedy pathway, and this needs to be further examined by lipidomic analysis. How these cellular effects induced by lipid treatment of primary hepatocytes and MEFs translate *in* vivo, requires further investigation in short-term fat-fed mice.

Appendix I

Low Dose Insulin Elicits The Same Signalling Profile In MEFs

A1.1 Aim

To ensure that the insulin signalling profile observed in the MEFs emanated from the activated InsR, MEFs were stimulated with 1 nM insulin to avoid potential activation of the IGF-1R, which insulin can activate at high doses, however the affinity for insulin by IGF receptors is much less than that of the InsR.

A1.2 Methods

MEFs were maintained at 37 °C and 5 % (v/v) CO_2 in low glucose DMEM supplemented with 10 % (v/v) FCS and 1 % (v/v) A/A. MEFs were seeded into 6-well plates and grown to around 90% confluent, before being serum starved for 2 h and stimulated with 1 nM insulin for the indicated times. Media was aspirated and MEFs washed three times with ice-cold PBS and harvested in RIPA buffer. Lysates were aliquoted with equal protein content and analysed by immunoblotting.

A1.3 Results

Stimulation with 1 nM insulin elicited a similar signalling profile (Figure A3.1) in MEFs as that observed using 100 nM insulin (see Figure 4.3.1).

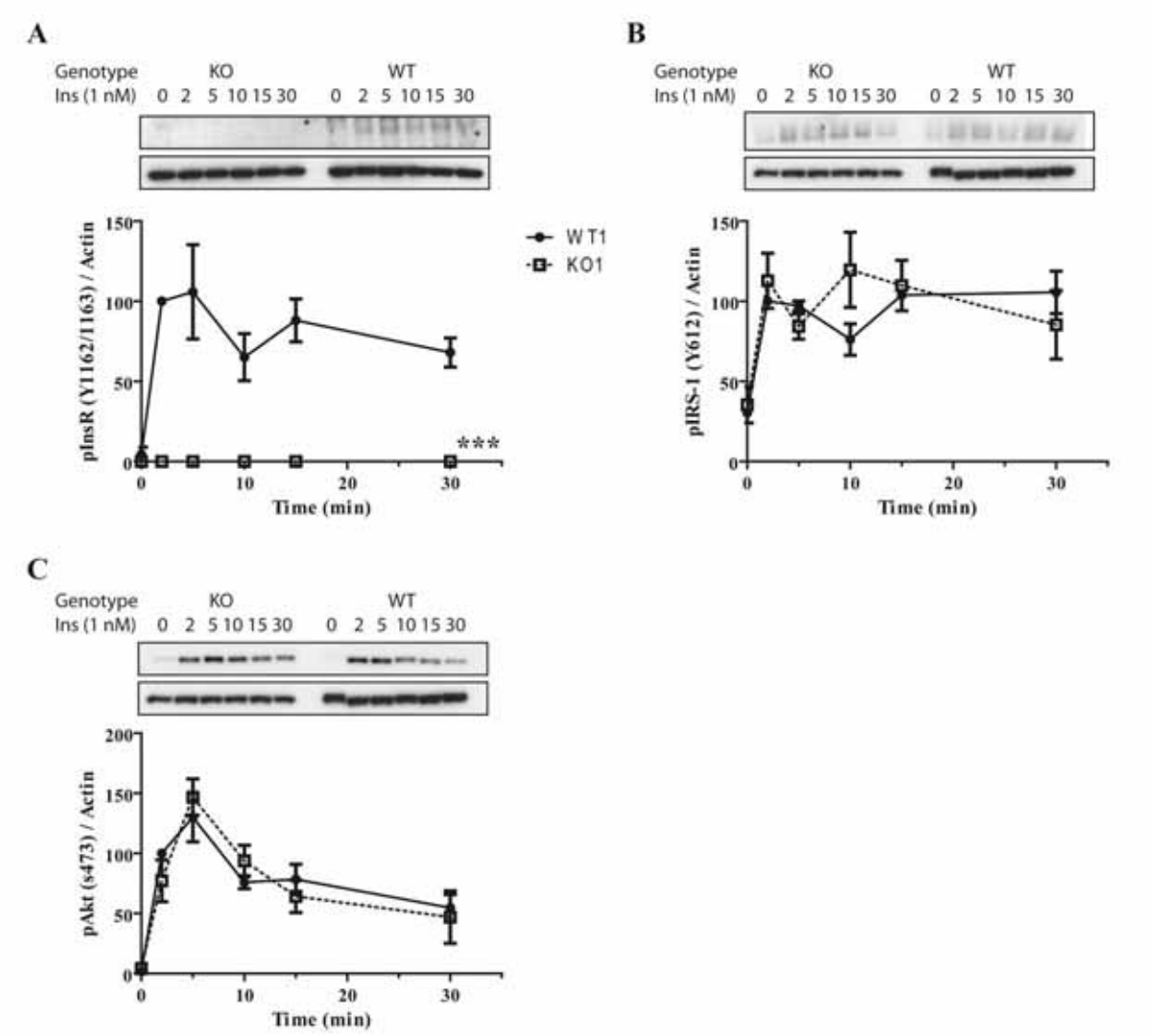

Figure A1.3.1 Insulin signalling in MEFs with 1 nM insulin.
WT (●) and PKCε KO MEFs (□) were serum starved for 2 h and stimulated with 1 nM insulin for the indicated times at 37 °C. Cells were washed three times with ice cold PBS, harvested in RIPA buffer and phosphorylation of components of the insulin signalling pathway were analysed by immunoblotting; **A:** InsR Tyr 1162/1163, **B:** IRS-1 Tyr 612 and **C:** Akt Ser 473. Data are means ± SEM; n = 3 independent experiments (*** $P < 0.001$).

These results indicate that the signalling profile observed in MEFs stimulated with 100 nM insulin, as was the case with the experiments described in this thesis, is most likely due to activation of the InsR.

Appendix II

PKC Isozyme Expression Is Not Altered by PKCε Ablation

A2.1 Aim

To examine whether other PKC isozymes altered their expression to compensate for PKCε ablation, expression of various isozymes in MEF lysates were examined by immunoblotting.

A2.2 Methods

MEFs were maintained at 37 °C and 5 % (v/v) CO_2 in low glucose DMEM supplemented with 10 % (v/v) FCS and 1 % (v/v) A/A. MEFs were grown to around 90% confluence, medium aspirated and cells washed three times with ice-cold PBS. Cells were harvested in RIPA buffer and lysates equalised for protein content and analysed by immunoblotting.

A2.3 Results

It was observed that PKC isozyme expression was not altered between WT and PKCε KO MEFs (Figure A2.3.1).

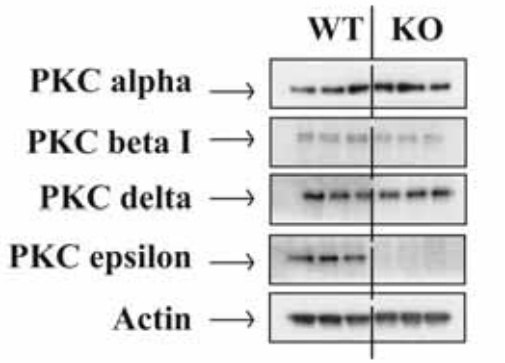

Figure A2.3.1 PKC isozyme expression in MEFs.
WT and PKCε KO MEFs were harvested in RIPA buffer and PKC certain isozymes were analysed by immunoblotting. A representative blot is shown of experiments.

This suggests that the phenotype observed due to PKCε deletion in MEFs is not masked by upregulation by other PKC isozymes to compensate for the absence of PKCε.

Chapter 8

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