The role of PtdIns(4,5)P_2 and its regulatory proteins in the development of insulin resistance in cell culture models.

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Contents

Abstract

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Insulin resistance, a key risk factor for type 2 diabetes, can be defined as when cells fail to respond effectively to insulin. In striated muscle and fat, this manifests as impaired insulin-stimulated glucose uptake due to reduced plasma membrane insertion of the glucose transporter GLUT4. In cell culture models, insulin resistance induced by chronic exposure to insulin, endothelin-1 or glucosamine, is correlated with reduced immunoreactivity of the lipid phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2) in plasma membrane sheets. However, the reason for this decrease, and whether other factors that induce insulin resistance affect PtdIns(4,5)P_2 levels, is unknown. Using L6 skeletal muscle myotubes and 3T3-L1 adipocytes, this project has investigated whether PtdIns(4,5)P_2 levels are perturbed in insulin resistance induced by several factors, including exposure to insulin, oxidative stress, and treatment with tumour necrosis factor α, endothelin-1 or angiotensin II (Ang II).

All these pre-treatments were found to abolish insulin-stimulated ^{3}H 2-deoxy-glucose uptake, and significantly decrease PtdIns(4,5)P_2 levels, measured in cell extracts by quantitative blotting using a PtdIns(4,5)P_2-specific probe, developed from the PH domain of phospholipase C (PLC) δ. Importantly the ability of insulin to stimulate glucose uptake can be restored by replenishing PtdIns(4,5)P_2 in L6 myotubes treated with insulin and Ang II. PtdIns(4,5)P_2 levels are regulated by three families of proteins; PIP kinases, which synthesise it, phosphatases, which remove phosphate groups from the inositol headgroup, and PLCs, which hydrolyse it. Membrane preparations from Ang II- and insulin-induced insulin resistant L6 myotubes showed no differences in PtdIns(4,5)P_2 production or dephosphorylation. However a significant increase in PLC activity was detected in membranes from insulin resistant cells and membrane localisation of PLCβ family members was increased in insulin resistant cells. Furthermore, studies using PLC inhibitors show a restoration of PtdIns(4,5)P_2 levels in insulin resistant cells, leading to partial reversal of insulin resistance.

This study therefore shows a causal link between decreased PtdIns(4,5)P_2 levels and insulin resistance in L6 myotubes, and that PLCs are the reason for the PtdIns(4,5)P_2 decrease in Ang II- and insulin-induced insulin resistance. PLCs, or their activation pathways, may thus be a novel target for combating insulin resistance, and preventing type 2 diabetes.

Declaration

No portion of the work referred to in the thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.

Copyright Statement

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Abbreviations

αMEM	α-modification of Eagle medium
AEBSF	4-(2-Aminoethyl) benzenesulfonylflouride hydrochloride
Ang II	Angiotensin II
aPKC	Atypical Protein Kinase C
APS	Adaptor protein with pleckstrin homology and Src homology 2 domains
AS160	Akt substrate of 160 kDa
BSA	Bovine serum albumin
CAP	Cbl-associated protein
CCD	Charge-couple device
Ctrl	Control
CPH	Carboxypeptidase H
DAG	Diacylglycerol
DMEM	Dulbecco's modified eagle medium
DPI	Diphenylene iodonium
ECL	Enhanced chemiluminescence
EDTA	Ethylenediamine tetraacetic acid
EGTA	Ethylene glycol tetraacetic acid
Endo-1	Endothelin-1
FBS	Foetal bovine serum
F-actin	Filamentous actin
G-protein	GTP-binding protein
GAP	GTPase-activating protein
GEF	Guanine nucleotide exchange factor

GLUT	Glucose transporter
GPCR	GTP-binding protein coupled receptor
GST	Glutathione S-transferase
GSV	Glucose transporter 4 storage vesicle
H_2O_2	Hydrogen Peroxide
HBS	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffered saline
HBSS	Hank's buffered salt solution
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HRP	Horse radish peroxidase
IBMX	3-Isobutyl 1-methylxanthine
Ins	Insulin
IP_3	Inositol (1,4,5)-trisphosphate
IPTG	Isopropyl β-D-thiogalactoside
IR	Insulin receptor
IRS	Insulin receptor substrate
iSH2	Inter-Src homology 2
LB	Luria-Bertani
MAPK	Mitogen activated protein kinase
mTOR	Mammalian target of rapamycin
NAC	N-acetyl-cysteine
NGS	Normal goat serum
NOX	NADPH oxidase
PB1	Phox/Bem 1
PBP10	Phosphoinositide-binding peptide

PBS	Phosphate buffered saline
PC1	Prohormone convertase 1
PC2	Prohormone convertase 2
PCR	Polymerase chain reaction
PDK1	Phosphoinositide-dependent kinase-1
PEG	Polyethylene glycol
PI3K	Phosphoinositide-3-kinase
PIKK	PI3K-related protein kinase
PIP4kins	Phosphatidylinositol 5-phosphate 4-kinases
PIP5kins	Phosphatidylinositol 4-phosphate 5-kinases
PIPkins	Phosphatidylinositol phosphate kinases
PH	Pleckstrin homology
PKC	Protein kinase C
PLC	Phospholipase C
PTB	Phospho-tyrosine binding
PtdIns	Phosphatidylinositol
PtdIns3*P*	Phosphatidylinositol 3-phosphate
PtdIns(3,4)P_2	Phosphatidylinositol (3,4)-bisphosphate
PtdIns(3,4,5)P_3	Phosphatidylinositol (3,4,5)-trisphosphate
PtdIns(3,5)P_2	Phosphatidylinositol (3,5)-bisphosphate
PtdIns4*P*	Phosphatidylinositol 4-phosphate
PtdIns(4,5)P_2	Phosphatidylinositol (4,5)-bisphosphate
PtdIns5*P*	Phosphatidylinositol 5-phosphate
PTEN	Phosphatase and tensin homolog
PTP	Protein tyrosine phosphatases
PVDF	Polyvinylidene difluoride
ROS	Reactive oxygen species

RTK	Receptor tyrosine kinase
RT-PCR	Reverse transcriptase-polymerase chain reaction
SDS	Sodium dodecyl sulphate
SER	Smooth endoplasmic reticulum
SH2	Src Homology 2
SHIP	Src homology 2 domain containing inositol polyphosphate 5-phosphatase
SKIP	Skeletal muscle- and kidney-enriched inositol phosphatase
SM	Sec61/Munc18
SNAP-23	Synaptosome-associated 23-kDa protein
SNAP-25	Synaptosome-associated 25-kDa protein
SNARE	Soluble *N*-ethylmaleimide sensitive factor attachment protein receptor
SOS	Son-of-sevenless
TBE	Tris/Boric Acid/ Ethylenediaminetetraacetic acid
TBS	Tris buffered saline
TCA	Trichloroacetic Acid
TEAB	Triethylamine bicarbonate
TLC	Thin layer chromatography
TLP	Theoretical lower phase
TNFα	Tumour necrosis factor α
TUP	Theoretical upper phase
VAMP	Vesicle-associated membrane protein

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1. Introduction

1.1 Insulin resistance and type 2 diabetes

1.1.1 Diabetes mellitus

Diabetes mellitus is a condition characterised by defects in glucose metabolism, and the hormone insulin, which lowers blood glucose levels. There are two types of diabetes, type 1 and type 2 (*1*). Type 1, or insulin-dependent, diabetes arises when the body is unable to produce insulin (*2*). Type 2, or non-insulin-dependent, diabetes occurs when the body fails to respond correctly to insulin, a phenomenon called insulin resistance (*3*). Type 2 diabetes is the more prevalent, affecting over 350 million people worldwide, and this number is predicted to increase drastically over the next 20 years (*1*, *4*). Insulin resistance and type 2 diabetes have been heavily linked with obesity and a sedentary lifestyle (*5*–*7*).

1.1.2 Insulin resistance

Insulin resistance can be defined as the inability of cells to respond appropriately to insulin. When skeletal muscle and adipose tissue begin to exhibit insulin resistance, they fail to increase glucose uptake as a response to insulin (*8*). In mammals this leads to hyperglycaemia, as levels of glucose in the blood fail to decrease (*3*, *9*). In an attempt to decrease the excess glucose insulin is secreted from the pancreas, possibly leading to a hyperinsulinaemic state (*9*). Hyperinsulinamia can lead to an increase in glucose uptake, but it is also known to exacerbate insulin resistance (*3*). Also, prolonged hyperinsulinaemia can lead to pancreatic β-cell death, with mammals being unable to produce sufficient insulin (*2*, *9*).

In the US alone it is estimated that over 79 million adults suffer from insulin resistance (*5*). Although insulin resistance is usually a precursor to type 2 diabetes, it is worth noting that patients whom exhibit insulin resistance do not always develop diabetes, but their chances of doing so are greatly increased (*5*, *7*, *10*, *11*). It is possible to improve insulin sensitivity through medication such as glucocorticoids (*12*, *13*), or lifestyle choices such as exercise and/or weight loss (*7*, *12*, *13*).

However, these treatments usually only delay the onset of type 2 diabetes (*12*), and a significant proportion of patients still develop type 2 diabetes (*12*, *14*).

A condition known as the metabolic syndrome is characterised by insulin resistance, abdominal obesity, hypertension and dyslipidaemia (*15*–*18*). The metabolic syndrome and insulin resistance are risk factors for cardiovascular disorders (*3*, *19*), various cancers (*3*, *20*–*22*) and Alzheimer's disease (*23*). In the UK it has been claimed that 10% of all patients admitted to hospital are suffering from diabetes and complications associated with it (*4*). Consequently the worldwide cost of treating diabetes is becoming astronomical (*4*, *13*).

It is important that the mechanism behind insulin resistance is thoroughly investigated, as reversing insulin resistance or preventing it from occurring can potentially stop the onset of type 2 diabetes, and other conditions associated it with it (*1*, *10*, *19*–*23*). To understand insulin resistance, it is first necessary to understand how insulin causes glucose uptake in healthy individuals.

1.2 Physiological insulin signalling

1.2.1 Insulin

Insulin is a hormone, secreted from the pancreas in response to increased plasma glucose. It stimulates many responses, but arguably the most important is to lower blood glucose levels, by increasing glucose uptake into skeletal muscle and adipose tissue (*24*). It does this by increasing the amount of glucose transporter (GLUT) 4 active at the plasma membrane (*25*, *26*). Insulin binds to a receptor tyrosine kinase (RTK) called the insulin receptor (IR) (*27*), causing receptor autophosphorylation (*28*) and recruitment of various proteins including the IR substrate (IRS) family of proteins (*29*–*31*), Shc (*32*–*34*), phosphoinositide 3-kinases (PI3Ks) (*35*, *36*) and Cbl (*37*–*39*). As well as increasing glucose uptake via the PI3K arm of signalling, insulin stimulates a Ras-Mitogen activated protein kinase (MAPK) pathway in insulin responsive cells (*40*, *41*).

Insulin is a 5.8kDa peptide hormone, secreted from pancreatic β cells, located in the Islets of Langerhans, as a response to increased blood plasma glucose levels (*42*).

When plasma levels of glucose are high, glucose enters the β cells, and glycolysis occurs. This leads to a rise in the ATP/ADP ratio, leading to the secretion of insulin (*43*). As well as glucose there are other stimuli which can increase insulin secretion, including nutrients such as Arginine, Leucine and fatty acids (*43*). Insulin is originally secreted as a pro-hormone, called proinsulin and must be cleaved by three enzymes; prohormone convertase 1 and 2 (PC1 and PC2) and carboxypeptidase H (CPH), also known as carboxypeptidase E (*44*). PC1 and PC2 cleave two separate positions, to remove a central portion from proinsulin, and generate a 31 amino acid residue known as the C-peptide (*45*, *46*). CPH then cleaves a further four amino acids to form the mature insulin hormone.

Insulin circulates in the bloodstream to sites of action. At basal conditions the plasma insulin concentration is very low, generally less than 25 pmol/L, but this increases to between 100 and 600 pmol/L after a meal when blood glucose levels are increased (*47*).

1.2.2 The insulin receptor

Insulin binds to a specific cell-surface receptor called the IR, which consists of four subunits; two transmembrane β chains and two extracellular α chains (*48*, *49*). The α chain has two splice variants which results in two isoforms of the IR; IR-A and IR-B, where the IR-B isoform contains an additional 12 amino acids in the extracellular chain (*50*). Each receptor has a specific expression pattern, with IR-B predominantly expressed in adipose tissue, liver and muscle (*51*). Therefore, it is thought that IR-B plays a major role in stimulating glucose uptake (*50*, *52*, *53*). In fact it has been suggested that defects in the expression of the particular isoforms might contribute to insulin resistance (*52*, *54*).

As mentioned above, the IR is a member of the RTK family (*27*), which possess intrinsic tyrosine kinase activity (*28*). RTKs are activated by the binding of a specific ligand, and in the case of IR-A and IR-B this is insulin (*51*). Insulin binds to the extracellular α chains, leading to a conformational change which activates the kinase activity of the β chains (*28*). The β chain undergoes autophosphorylation on several tyrosine residues (*55*). This has a two-fold effect; it increases the kinase activity and

recruits several proteins to the receptor, which in turn can be phosphorylated (*28*, *48*). Tyrosine residues 1162 and 1163 are important for increasing the kinase activity (*48*). Proteins including IRS (*30*, *31*), Shc (*32–34*) Phospholipase C (PLC) γ1 (*56*) and Cbl (*37–39*) are amongst the proteins recruited to the IR. These proteins interact with receptor via their Src Homology 2 (SH2) or phospho-tyrosine binding (PTB) domains which recognise phosphorylated tyrosine residues (*28*, *48*).

As mentioned above a key role for insulin is to increase glucose uptake in skeletal muscle and adipose tissue, by increasing GLUT4 translocation to the plasma membrane (*25*, *26*, *57*). Despite the huge amount of research into GLUT4 translocation and plasma membrane insertion the complete pathway is not completely known (see 1.5). It is thought that two pathways must be activated; a PI3K-dependent pathway, involving PI3K and Akt, and a PI3K-independent pathway involving Cbl and TC10 (*35*, *37*, *58*). Several families of proteins are known to play key roles in insulin-stimulated glucose uptake, including PI3Ks (*59–62*), Akt family members (also known as Protein Kinase B) (*62–64*) and the atypical protein kinases C (aPKCs) ζ or λ/ι (*58*, *65*, *66*) (see 1.3).

1.2.3 The IRS family

The IRS family contains six proteins, which display little homology in general (*67*). However, all IRS family members contain three distinct homologous regions; a PTB domain, a pleckstrin homology (PH) domain and an IRS-homology-3 domain (*67*, *68*), as well as several phosphorylation sites (*31*).

IRS-1 and -2 are the most closely related in sequence (*68*). IRS-1 is necessary for development, and IRS-1 deficient animals and cells exhibit growth retardation and slight insulin resistance (*69–71*). IRS-1-deficient animals also express diminished MAPK signalling in response to insulin (*72*) as IRS-1 can activate MAPK signalling via an interaction with Grb2 (*34*). IRS-2-deficient mice suffer more severely and develop type 2 diabetes (*70*, *71*, *73*). This suggests that both IRS family members carry out important roles in insulin signalling. IRS-1 is more important for the MAPK signalling pathway and IRS-2 is more important for insulin-stimulated glucose uptake (*72*). Decreased levels of IRS-1 and IRS-2 correlate with insulin resistance in human

subjects (*74*). Experiments on knockout mice lacking IRS-3 and -4 lead to mild if any defects in growth, development and glucose homeostasis (*75*, *76*). IRS-5 and -6 are poor substrates for the IR, and are only included in the family due to sequence homology (*77*).

The PTB domain from the IRS interacts with a phosphorylated tyrosine on the IR, Tyr960. By mutating this residue to an alanine, decreased IRS phosphorylation, PI3K activation and glucose uptake all occur (*74*). IRS-1 and -2 have at least 20 conserved tyrosine residues, which can be targeted for phosphorylation by the IR (*31*). These phosphorylated tyrosine residues can lead to the recruitment of other proteins including PI3K, PLCγ1, SHP2, and Grb2 (*34*, *71*, *74*, *78*). PI3K and PLCγ1 play roles in lipid turnover, and SHP2 and Grb2 have mainly mitogenic effects. PI3Ks also play a direct role in glucose uptake, discussed below (see 1.3.1).

1.2.4 MAPK signalling

As well as stimulating IRS activation, insulin stimulation also leads to the activation of ERK MAPK family. This pathway is mostly independent of the IRS-PI3K pathway described below (see 1.3.1) (*33*). As mentioned above Grb2 can be recruited to IRS-1 (*34*), but it can also be recruited to the IR via a different adaptor protein, Shc (*32*, *79*). Shc is recruited to the IR after stimulation (*32*–*34*), and is phosphorylated in a similar manner to IRS family members (*80*). Grb2 associates with son-of-sevenless (SOS), a guanine nucleotide exchange factor (GEF) which can cause the activation of Ras (*41*, *80*, *81*). Ras is a GTP-binding protein (G-protein), and in its active form (when bound to GTP) it can activate Raf (*82*). Raf in turn can activate the MEK family of protein kinases (*41*), leading to ERK phosphorylation and activation (*82*).

1.3 Kinases necessary for insulin-stimulated glucose uptake

1.3.1 Activation of PI3Ks

Insulin stimulation causes an increase in levels of several phosphoinositides, phosphorylated of the D3 position of the inositol headgroup: phosphatidylinositol (3,4,5)-trisphosphate (PtdIns(3,4,5)P_3), phosphatidylinositol 3-phosphate (PtdIns3*P*),

phosphatidylinositol (3,4)-bisphosphate (PtdIns(3,4)P_2), and phosphatidylinositol (3,5)-bisphosphate (PtdIns(3,5)P_2) (*35*, *36*, *83–87*). It does this by primarily activating PI3K family membranes (*35*, *36*, *74*). After insulin stimulation Class I PI3Ks are recruited to the plasma membrane via interactions with the IR or IRS (*35*, *36*, *74*, *88*, *89*). Lipid turnover is a necessary part of the insulin signalling pathway, particularly with respect to GLUT4 translocation and glucose uptake (*35*, *36*, *84*, *85*, *87*).

The PI3Ks superfamily are mostly kinases which phosphorylate phosphoinositides on the D3 position of the inositol ring (*90*, *91*). There are several classes within the PI3K superfamily, including 3 classes of lipid kinases (I, II and III) and class IV PI3K-related protein kinases (PIKKs). Each class of the lipid kinases possess different functions and structures (*89*, *92*) and classes I and II contain multiple isoforms (*93*). PIKKs include mammalian target of rapamycin (mTOR), ATM and ATR (*91*). These are considered to be members of the superfamily because of their sequence homology, but they are not true PI3Ks as they do not phosphorylate lipids.

Class I PI3Ks catalyse the phosphorylation of phosphatidylinositol (4,5)-bisphosphate (PtdIns(4,5)P_2) to PtdIns(3,4,5)P_3 (*94*, *95*). All class I PI3Ks contain two subunits, a 110 kDa catalytic subunit and a regulatory subunit (*93*). They can be split into 2 further groups, IA and IB, depending on the method of activation, and the subunits that make up the protein. Class IA PI3Ks tend to be activated by RTKs, whereas class IB are activated by G-protein coupled receptors (GPCRs) (*96*).

Class IA PI3Ks contain p110α, p110β or p110δ as the catalytic subunit, and there are five regulatory subunits; p50α, p55α, p55γ, p85α and p85β (*92*, *93*, *97*). It has been suggested that p110α and p110β both play a role in insulin signalling (*97*, *98*). Mice with a heterozygous loss of either p110α or p110β display slight impaired glucose uptake, whereas mice with a heterozygous loss of both display profound insulin resistance (*97*). This suggests some plasticity between the catalytic subunits. All of the regulatory subunits are expressed in skeletal muscle and adipose tissue, and can interact with p110α and p110β (*99*).

The catalytic subunit contains an adaptor binding domain which interacts with the regulatory subunit (*93*). Each regulatory subunit contains two conserved SH2 domains, flanking an inter-SH2 (iSH2) domain. These are called the N-SH2 and C-SH2 depending on their position either side of the iSH2 domain. The N-SH2 domain

plays a role in the dimerization of the protein by interacting with the catalytic domain (*100*), whereas the C-SH2 domain interacts with phosphorylated tyrosine residues, such as those found on the IR and IRS (*88*, *89*). When the C-SH2 domain interacts with a phosphorylated tyrosine this allows the structure to unfold, allowing the kinase domain to become active.

As mentioned earlier, Class IA PI3Ks generate PtdIns(3,4,5)P_3 after insulin stimulation (*35*). The PtdIns(3,4,5)P_3 generated has important roles in insulin signalling, and can act as a docking site for several specific PH domains, including those of Akt and its activator phosphoinositide-dependent kinase-1 (PDK1) (*101*, *102*). PtdIns(3,4,5)P_2 is necessary for insulin-stimulated glucose uptake as the inhibition of Class I PI3Ks, using wortmannin or LY294002, prevents insulin-stimulated glucose uptake (*60*, *103*, *104*). Wortmannin and LY294002 are non-specific inhibitors of Class I PI3Ks (*59*).

Class IB PI3Ks contain the catalytic subunit p110γ, and either p101, p87 or p84 as the regulatory subunit (*94*, *105*). The two subunits interact via the adaptor binding domain in the catalytic subunit, similar to class IA PI3Ks. When the regulatory subunits interact with the βγ subunits from heterotrimeric G-proteins, this allows the protein to unfold, allowing the catalytic domain to become active (*96*, *106*).

Insulin stimulation also causes the activation of Class II PI3Ks (*36*). Class II PI3Ks are large (approximately 170kDa) monomeric proteins (*36*, *107*). There are three class II PI3Ks in humans; PI3K-C2α, PI3K-C2β and PI3K-C2γ. *In vivo* these kinases phosphorylate phosphatidylinositol (PtdIns) and phosphatidylinositol 4-phosphate (PtdIns4*P*) on the D3 position of the inositol headgroup to generate PtdIns3*P* and PtdIns(3,4)P_2 respectively (*107*).

PI3K-C2α is the only class II PI3K that plays a known role in GLUT4 translocation (*36*). Working downstream of TC10, a small G-protein of the Rho family, PI3K-C2α generates PtdIns3*P* as a response to insulin (*84*). This lipid is necessary for GLUT4 translocation and activation (*87*) (see 1.5.3), although the mechanism is still not completely understood.

There is only one known class III PI3K, which consists of a catalytic subunit, PIK3C3, and a single regulatory subunit p150 (*89*). PIK3C3 produces PtdIns3*P* (*108*), but it does not play a role in insulin signalling (*109*).

1.3.2 Akt phosphorylation and activation

The generation of PtdIns(3,4,5)P_3 is necessary for the activation for Akt family members (*101*). Akt is a 56kDa serine/threonine kinase that has roles in cell proliferation, growth and metabolism, as well as a vital role in insulin-stimulated glucose uptake (*62*, *110*). There are three mammalian Akt isoforms, 1, 2 and 3, each of which is encoded by a separate gene, despite a highly conserved amino acid sequence. Obese mice display diminished Akt activation in response to insulin (*111*) but different isoforms are known to exhibit different functions and play different roles in insulin signalling (*110*, *112*, *113*). Akt1 and 2 are ubiquitous, although Akt2 is more prevalent in insulin-responsive tissues, and plays a bigger role in insulin signalling (*110*, *114*). Akt1-deficient mice show no difference in glucose- and insulin-tolerance tests (*112*), but Akt2-deficient mice are prone to insulin resistance, and type 2 diabetes (*110*). Akt3 is localized solely in the brain, and its functions are less clear, although it does not contribute to diabetes (*110*).

The protein kinase Akt is activated by insulin, via a PI3K-dependent pathway, shown in Fig. 1.1. Akt is able to phosphorylate many different proteins. Several targets include cell proliferation and anti-apoptotic proteins, including many Bcl-2 family members, eNOS, mdm2 and cyclin-dependent kinase 4 (*115*, *116*). Other targets include proteins involved in glucose uptake and metabolism, such as AS160 (Akt substrate of 160 kDa), PIKfyve and GSK3 (*24*, *117*). Akt2 also acts on GLUT4 storage vesicles (GSVs) directly, stimulating their translocation to the plasma membrane (*118*, *119*).

It is thought that Akt must be phosphorylated by PDK1 and TORC2 in order to become fully active (*120*), but an interaction with PtdIns(3,4)P_2 is also thought to play a role in the activation (*101*). After insulin stimulation, Akt is recruited to the plasma membrane via its PH domain, where levels of PtdIns(3,4,5)P_3 are increased. The Akt-PH domain recognises PtdIns(3,4,5)P_3 as well as preferentially binding to

PtdIns(3,4)P_2 (*101*, *121*). PtdIns(3,4)P_2 can be generated by Class II PI3Ks following the phosphorylation of PtdIns4*P* (*107*) or by the dephosphorylation of PtdIns(3,4,5)P_3 by a phosphoinositide 5-phosphatase (*101*, *122–125*).

PDK1 also contains a PH domain which interacts with PtdIns(3,4,5)P_3, and PI3K activity leads to translocation of this protein to the plasma membrane (*102*). PDK1 phosphorylates Akt on a threonine residue (Thr308 in Akt1, 309 in Akt2 and 305 in Akt3) leading to a conformational change, and partial activation of Akt (*102*). For full activation Akt must also be phosphorylated on a serine residue (Ser473 in Akt1, 474 in Akt2 and 470 in Akt3). This phosphorylation is carried out by TORC2 (*126*). However this phosphorylation may be dependent on a phosphoinositide 5-phosphatase (*101*, *122–125*). As mentioned earlier, the PH-domain of Akt binds preferentially to PtdIns(3,4)P_2, and binding to this lipid allows TORC2 access to the serine residue, and phosphorylation to occur (*101*, *121*). However, overexpression of SHIP2 (SH2 domain containing inositol polyphosphate 5-phosphatase 2), a phosphoinositide 5-phosphatase, has been shown to inhibit Akt phosphorylation, suggesting there may be a further, more complicated, level of control (*124*). A similar 5-phosphatase SKIP (skeletal muscle- and kidney-enriched inositol phosphatase) plays a similar role in insulin signalling and its overexpression can also cause insulin resistance (*124*, *125*).

The inositol 3-phosphatase PTEN (phosphatase and tensin homolog) can inhibit Akt activation by removing the phosphate group at the D3 position of the inositol headgroup of PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 (*127*, *128*). This suggests that the D3 position of the inositol headgroup must be phosphorylated in order for Akt activation to occur, showing the importance of PI3K signalling in Akt activation.

1.3.3 Activation of the atypical PKCs

Another protein kinase believed to play a role in insulin-stimulated glucose uptake is aPKC. There are two aPKCs, PKCζ and PKCλ/ι, These proteins are structurally very

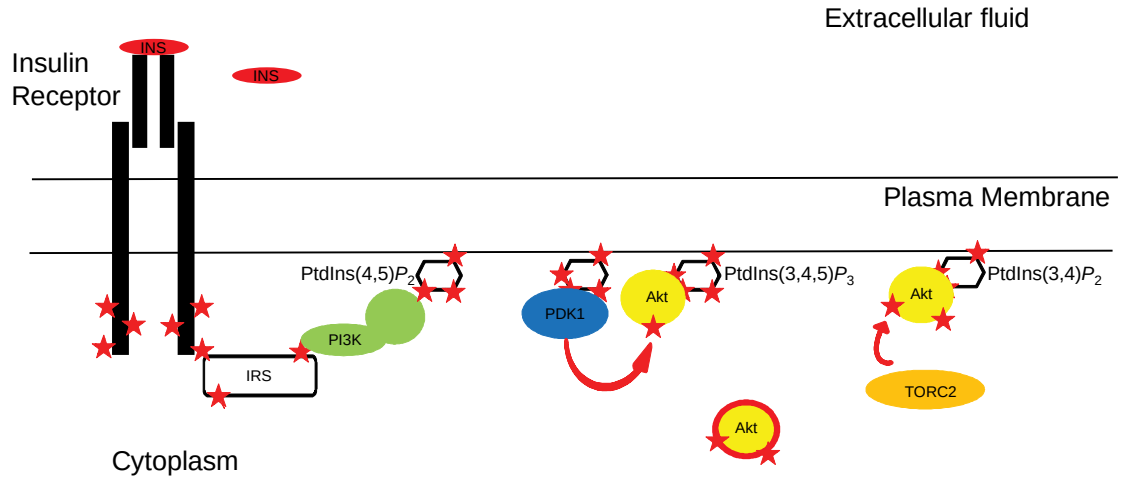

Figure 1.1 The activation of Akt by insulin stimulation. When insulin binds to the insulin receptor, the kinase activity of the receptor is activated, causing phosphorylation of the receptor and other proteins, including IRS. This leads to recruitment of PI3K and the generation of PtdIns(3,4,5)P_3. This in turn leads to recruitment of the kinase PDK1 and its substrate Akt. A second kinase TORC2 also phosphorylates Akt, leading to its full activation.

Key: Red stars show phosphate groups. Red arrows show the act of phosphorylation. Red border demonstrates activation.

similar, and like other PKCs contain a C1 and kinase domain, as well as a domain specific to the aPKCs called the Phox/Bem 1 (PB1) domain (*129–131*). This domain allows the aPKCs to interact with other proteins also featuring PB1 domains, which includes many scaffold proteins (*130*, *131*).

PKCζ and PKCλ/ι are activated by insulin stimulation, via a PI3K-dependent mechanism (*66*, *132*). PDK1 and PtdIns(3,4,5)P_3 have been shown to activate aPKCs *in vitro* (*133*). It is therefore suggested that a rise in PtdIns(3,4,5)P_3 at the plasma membrane leads to the recruitment of PKCζ and PDK1 (*65*). Once at the plasma membrane PKCζ can be phosphorylated on Thr410 in a similar manner to Akt (*134–136*). This threonine residue in conserved in PKCλ/ι, so it's suggested that the activation is similar between family members (*134*).

The involvement of aPKCs in glucose uptake is currently unclear, with various studies presenting contrary results. Several studies suggest that aPKCs play a crucial role in insulin-stimulated glucose uptake (*137*) and that inhibition of aPKCs diminishes insulin-stimulated glucose uptake (*138*, *139*). However, other studies were unable to replicate these results (*140*), and some have even shown that decreased aPKC levels actually increase glucose uptake (*141*, *142*).

The possible role of aPKCs in insulin-stimulated glucose uptake is unknown. Overexpressing aPKCs can cause actin reorganisation in a similar manner to insulin (*143*, *144*). This actin reorganisation is necessary for GSVs to undergo translocation to the plasma membrane (see 1.5.2) (*145*). Similarly the co-localisation of PKCζ with Rac-1, with GLUT4 on GSVs has been shown to be important for efficient GSV translocation in L6 myotubes (*143*, *145*).

PKCζ is thought to possibly carry out a regulatory role, phosphorylating several IRS family members to prevent further insulin signalling. However, interestingly it does not phosphorylate IRS-2, the family member primarily linked to insulin-stimulated glucose uptake, suggesting that PKCζ does not stop insulin-stimulated glucose uptake (*146*).

1.4 Phosphatidylinositol (4,5)-bisphosphate and the phosphoinositides

1.4.1 The phosphoinositides

Several of the phosphoinositides play crucial roles in insulin signalling and glucose uptake (*36*, *83–85*, *87*, *147–149*). PtdIns and its phosphorylated derivatives, the phosphoinositides, make up a small percentage of the total membrane lipids, roughly 6-8 % (*150–152*). PtdIns is a glycerophospholipid with two fatty acids and an inositol headgroup linked via a phosphodiester bond to the glycerol backbone. The fatty acids present are usually a saturated 18-carbon chain stearic acid in the first position, and a 20-carbon chain poly-unsaturated arachidonic acid, in the second position (*153*, *154*). A phosphodiester bond links the third position on the glycerol to the D1 position of the inositol ring.

As well as the phosphate at the D1 position, PtdIns can be phosphorylated on the inositol headgroup D3, D4 or D5 position (*152*). As any combination of these positions can be phosphorylated *in vivo* this gives rise to seven different phosphoinositides family members (see Fig. 1.2): the mono-phosphorylated PtdIns3*P*, PtdIns4*P* and phosphatidylinositol 5-phosphate (PtdIns5*P*), the bis-

phosphorylated PtdIns(3,4)P_2, PtdIns(3,5)P_2 and PtdIns(4,5)P_2, and the tris-phosphorylated PtdIns(3,4,5)P_3. PtdIns cannot be phosphorylated on the D2 or D6

positions due to stearic hindrances with the glycerol backbone (*155*). Fig. 1.2 shows the approximate cellular levels of phosphoinositides under basal conditions (*152*). The amounts of the lipids vary after stimulation, and they are often found at high concentrations at specific locations (*35*, *83–85*, *156*).

The specific location of the phosphoinositides, and the specificity of lipid-binding domains, means that the lipids play a role in ensuring the correct location of various binding proteins (*152*, *153*). The location of the lipids is generally linked to the localisation of the kinases and phosphatases which generate them (*157–159*).

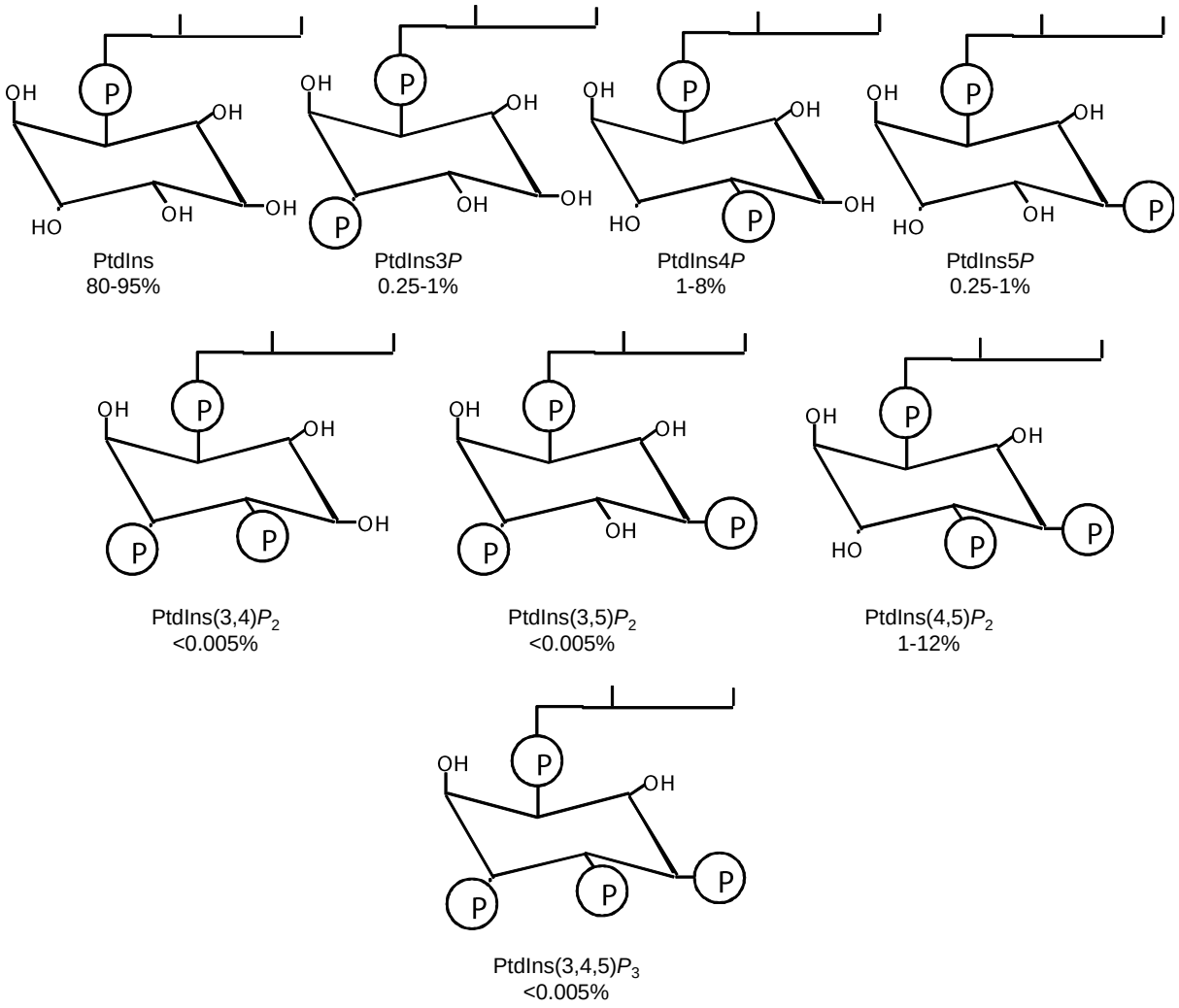

Figure 1.2. Headgroups of the phosphoinositides. The headgroup of phosphoinositides is an inositol ring. It can be phosphorylated in the D3, D4 or D5 position, or any combination of these. The individual headgroups of the various phosphoinositides are shown here, as well as their abundance under basal conditions, expressed as a percentage of total inositol lipids (152).

There is a larger amount of PtdIns4*P* than the other two mono-phosphorylated phosphoinositdes, PtdIns3*P* and PtdIns5*P* (*152*, *160*). PtdIns4*P* is found mostly at the plasma membrane, with distinct pools in the Golgi and endocytic compartments (*161*). PtdIns3*P* is also located in Golgi and endosomes, as well as in the smooth endoplasmic reticulum (SER) (*108*, *162–164*). Whilst there is a nuclear pool of PtdIns5*P* (*157*), the majority of the lipid is thought to be in plasma membrane, as well as also being present in the Golgi and SER (*164*, *165*).

PtdIns(4,5)P_2 is the most abundant of the bis-phosphorylated phosphoinositides, and it is found almost entirely at the plasma membrane (*159*, *161*, *163*, *166*), with a distinct nuclear pool (*167*, *168*). PtdIns(3,4)P_2 is present at very low concentrations at the plasma membrane, but is also present in the Golgi (*169–171*). The majority of PtdIns(3,4)P_2 is generated by the dephosphorylation of PtdIns(3,4,5)P_3 by the 5-phosphatases (*128*, *169–171*). After insulin stimulation, plasma membrane levels of PtdIns(3,4)P_2 increase (*35*, *36*). PtdIns(3,5)P_2 is located mostly on endosomes, where a kinase called PIKfyve generates it by phosphorylating PtdIns3*P* (*172*). PIKfyve is activated by the IR, causing an increase in PtdIns(3,5)P_2 as a result (*173*, *174*).

There are very low basal amounts of PtdIns(3,4,5)P_3 in cells, and these are mostly present at the plasma membrane. As mentioned earlier (see 1.3.1), stimulation of class I PI3Ks generates PtdIns(3,4,5)P_2 from PtdIns(4,5)P_2 (*94*, *95*).

1.4.2 PtdIns(4,5)P_2

Despite only making up less than 1% of total membrane lipids (*151*, *163*), PtdIns(4,5)P_2 has been implicated in many cellular processes, including endocytosis (*175*), exocytosis (*167*), the recruitment of proteins to the plasma membrane (*176–179*), modulation of channels and transporters (*180*, *181*), and cytoskeleton and actin reorganization (*182*, *183*).

PtdIns(4,5)P_2 is an important precursor for several secondary messengers. PLCs cleave the phosphodiester bond between the D1 position of the inositol headgroup ring and the glycerol backbone to form diacylglycerol (DAG) and inositol (1,4,5)-trisphosphate (IP_3) (*184*). IP_3 is important in calcium release and signalling (*185*),

and DAG is necessary for the activation of a C1 domain-containing proteins, in particular PKCs (*130*, *186*). As mentioned earlier (see 1.3.1), class I PI3Ks phosphorylate PtdIns(4,5)P_2 to PtdIns(3,4,5)P_3 (*94*, *95*), an important molecule in PDK1, Akt and PKCζ, activation (*131*, *136*, *187*).

1.4.3 Control of PtdIns(4,5)P_2 levels

Levels of PtdIns(4,5)P_2 are mainly controlled by three families of proteins, as can be seen in Fig. 1.3. PtdIns4*P* 5-kinases (PIP5kins) and PtdIns5*P* 4-kinases (PIP4kins), which are collectively known as the PIPkins, generate the majority of PtdIns(4,5)P_2 (*188*). PIP5kins are also known as type I PIPkins, and PIP4kins are known as type II PIPkins. PtdIns(4,5)P_2 specific phosphoinositide 4- and 5-phosphatases remove the phosphate group from the D4 or D5 position of PtdIns(4,5)P_2, respectively (*189*). PLCs, as mentioned earlier, hydrolyse the phosphodiester bond linking the inositol ring and the glycerol backbone (*184*).

A relatively small amount of PtdIns(4,5)P_2 is also removed by class I PI3Ks to form PtdIns(3,4,5)P_3 (*152*). PI3K activation occurs after stimulation with a ligand such as insulin (*35*, *36*, *74*, *88*, *89*), and the increase in PtdIns(3,4,5)P_3 and subsequent decrease in PtdIns(4,5)P_2 concentration is thought to be localised to a specific area, and does not contribute significantly to decreased global levels of PtdIns(4,5)P_2. However, a localised increase in PtdIns(3,4,5)P_3 could generate a low concentration of PtdIns(4,5)P_2 in a specific area. As some effects of PtdIns(4,5)P_2 are localised to clusters of the membrane (e.g. lipid rafts) decreased levels of PtdIns(4,5)P_2 could potentially prevent important lipid-protein interactions (*177*, *180*, *190*).

1.4.4 The PIPkins

A major difference between the PIPkins is the choice of substrate. PIP5kins phosphorylate PtdIns4*P* on the D5 position, and PIP4kins phosphorylate PtdIns5*P* on the D4 position (*188*). As mentioned earlier, the subcellular location of the phosphoinositides is very important, and similarly the PIPkins show different

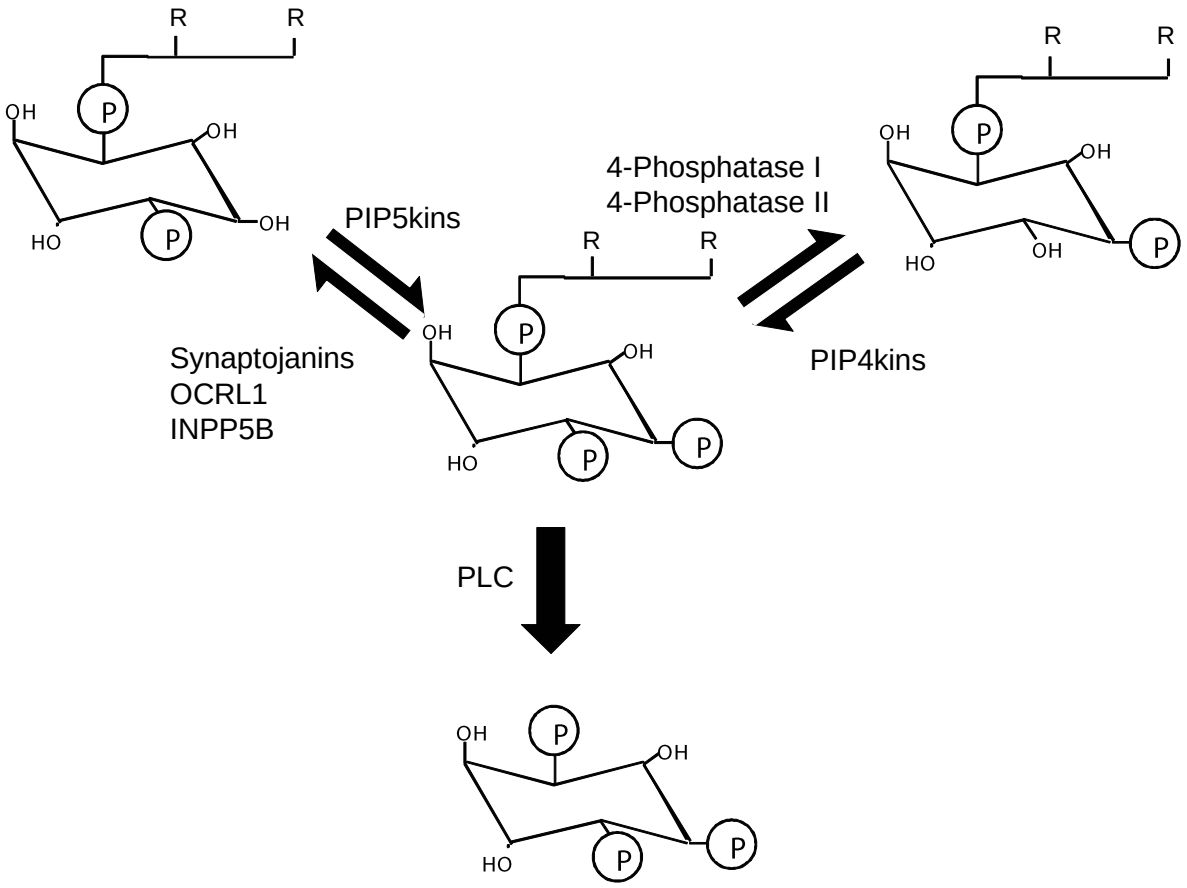

Figure 1.3 PtdIns(4,5)P_2 metabolism is controlled by various enzymes. This shows the interplay between PtdIns(4,5)P_2 and its pre-cursors PtdIns4*P* and PtdIns5*P*. The enzymes which control these reactions are shown above and below the arrows. In the case of the 5-phosphatases only PtdIns(4,5)P_2-specific 5-phosphatases are shown. The hydrolysis by PLCs and the generation of IP_3 is also shown. R represents fatty acids.

subcellular localisations, allowing for generation of PtdIns(4,5)P_2 at specific locations (*157*, *159*).

As the levels of PtdIns4*P* are higher than PtdIns5*P* (see Fig. 1.2) it is thought that the majority of PtdIns(4,5)P_2 is generated by type I PIP5kins (*89*, *191*). There are three isoforms, α, β and γ, and several splice variants, which share a similar kinase core (*191–193*). They are widely expressed, but each isoform is thought to have a specific localisation in the cell, allowing for the specific generation of PtdIns(4,5)P_2 in a particular location (*159*).

Type II PIPkins use PtdIns5*P* as a substrate. Again there are three isoforms of the PIP4kins, α, β and γ, with the α isoform being the most active (*194*). These isoforms can form heterodimers, and it is thought that the major role of the β and γ isoforms is

to cause specific localisation of the α isoform, depending on which heterodimers are formed (*194*, *195*). It is thought a major role of PIP4kin α is to function constantly to keep the levels of PtdIns5*P* low (*196*).

The other method of generating PtdIns(4,5)P_2 is by the dephosphorylation of PtdIns(3,4,5)P_3. However, as the level of PtdIns(3,4,5)P_3 is very low under basal conditions (see Fig. 1.2) it is unlikely to contribute to the comparatively high levels of PtdIns(4,5)P_2 under basal conditions (*152*).

1.4.5 PtdIns(4,5)P_2-specific phosphoinositide phosphatases

There are two types of phosphatases which remove PtdIns(4,5)P_2: phosphoinositide 4-phosphatases and phosphoinositide 5-phosphatases. Phosphoinositide 4-phosphatases remove the phosphate from the D4 position to form PtdIns5*P* (*197*), whereas phosphoinositide 5-phosphates remove the D5 phosphate to produce PtdIns4*P* (*198*).

PtdIns(4,5)P_2 4-phosphatases I and II are the two members of the 4-phosphatase family (*197*). Both of these phosphatases are located in late endosomes and lysosomes (*197*), although the type I isoform undergoes translocation to the nucleus under pro-apoptotic stress (*199*). Levels of PtdIns5*P* are increased in the nucleus of cells after UV irradiation (*157*), and the translocation of PtdIns(4,5)P_2 4-phosphatase I may play a role in increasing levels of the lipid (*199*).

The phosphoinositide 5-phosphatase family is much bigger, and contains 10 mammalian proteins including the synaptojanins, the SHIPs, OCRL1, INPP5A, INPP5B, INPP5E, INPP5J (also known as PIPP), and SKIP (*107*, *152*, *200*, *201*). These proteins contain a distinctive 5-phosphatase domain in their centre (*198*). *In vitro* many of these phosphatases appear to cleave PtdIns(4,5)P_2 but it is likely that *in vivo* the main regulators are the synaptojanins, OCRL1 and INPP5B (*107*). Other 5-phosphatases express specificity for other phosphoinositides or soluble inositol phosphates (*124*, *201*).

There are two synaptojanins, 1 and 2, which both specifically utilise PtdIns(4,5)P_2 (*201*). Synaptojanin 1-deficient mice exhibit increased plasma membrane

PtdIns(4,5)P_2 and vesicle accumulation, suggesting a key role for this phosphatase in exocytosis (*201*, *202*).

OCRL1 is one of only two 5-phosphatases with a Rab GTPase-activating protein (GAP) domain, the other being INPP5B (*200*). This domain binds to and activates the GTPase activity of Rab and this is thought to play a role in locating OCRL1 to clathrin-coated vesicles (*203*). OCRL1 shows a preference for PtdIns(4,5)P_2 but can also cleave the phosphate from PtdIns(3,4,5)P_3 and the soluble I(1,4,5)P_3 and I(1,3,4,5)P_4 (*198*). INPP5B is very similar to OCRL1 and contains a high sequence identity (*204*). It also contains a Rab GAP domain, and as such is able to interact with Rab proteins, but these are likely to be different Rabs to those with which OCRL1 interacts, as the subcellular location of INPP5B is different. INPP5B is found in the Golgi (*205*). It shows a very similar preference and specificity to OCRL1 with regards to phosphoinositides (*204*).

Many of the 5-phosphatases seem to have overlapping targets, but, in a similar manner to the kinases, specific phosphatases are expressed at specific locations in particular tissues, allowing for very specific generation and removal of phosphoinositides (*206*, *207*).

1.4.6 The PLCs

The majority of the PLC family are phosphoinositide-specific. PLCs cleave the sn-3 position, before the phosphodiester bond, removing the phosphate from the glycerol backbone and leaving it bound to the headgroup (*208*). There are thirteen mammalian phosphoinositide-specific PLCs, arranged into 6 subfamilies; β, γ, δ, ε, η and ζ .The different isoforms are separated based on structure, and all but the ε and ζ subfamilies contain multiple proteins (*184*, *209*), and most isoforms can be expressed as alternate splice variants. The different proteins show differential expression throughout organisms and tissues (*184*). All PLCs contain 4 EF hands, a C2 calcium-dependent lipid binding domain (*210*), and catalytic X and Y domains, which must interact to form a fully functional lipase (*184*, *211*). Calcium is therefore an important regulator of PLC activity (*212*, *213*). All but PLCζ contain a PH domain, and the PLCγ family members contain a second PH domain (*184*).

All of the phosphoinositide-specific PLCs play important signalling roles by generating IP_3 and DAG from PtdIns(4,5)P_2 (*186*), and are routinely recruited to RTK and GPCRs. As mentioned earlier, both IP_3 and DAG play important roles in calcium and PKC signalling (*214*). All of the PLCs are mainly cytosolic proteins, which are recruited to the plasma membrane upon stimulation and activation (*215*, *216*).

There are four PLCβ family members, 1 - 4. These lipases can be activated by GPCRs or by Rho GTPases (*209*, *217*). PLCβ family members can be stimulated by interactions with Gα, Gβγ or a combination of the two, and are able to interact with GPCRs via an elongated C-terminus (*218*).

The PLCγ family, containing PLCγ1 and γ2, is activated by RTKs (*56*, *78*, *218*, *219*) such as the IR. These lipases contain two SH2 domains, which can interact with phosphorylated tyrosine residues on the IR and IRS (*56*, *78*). Both members of the PLCγ family also contain a Src homology 3 (SH3) domain, and a split PH domain (*184*). The SH2 and SH3 domains allow PLCγ family members to interact with other proteins, whilst the PH domain allows for interaction with phosphoinositides (*220*, *221*).

The PLCδ family contains three proteins; PLCδ1, PLCδ3 and PLCδ4, which possess a high amount of similarity (*222*). The PLCδ family are the most sensitive to calcium, and it is thought that this is the main regulatory factor of these proteins (*212*). This suggests that these lipases are activated after the intracellular concentration of calcium has already risen by entry though calcium channels or release from intracellular stores (*223*).

There is only one member of the PLCε subfamily. PLCε is the largest PLC, and is the only one to contain a Ras family binding site (*224*). It is also known to function as a GEF for various Ras family G-proteins (*184*, *224*). It is activated after stimulation by GPCRs or RTKs, via protein-protein interactions with small G-proteins (*184*, *224*, *225*).

The PLCη family contains PLCη1 and the most recently discovered lipase, PLCη2. It is thought that the PLCη family are expressed highly in neurons, but there has been little work carried out on these proteins, and the full expression pattern has not been ascertained (*226*, *227*).

PLCζ, the smallest member of the PLC family (*184*), is localised to sperm cells, and plays a vital role in fertilisation; causing the oscillatory calcium waves necessary for activation of the egg and development (*228–230*).

1.5 GLUT4 translocation and activation

1.5.1 GLUT4

As mentioned above (1.2) insulin causes an increase in glucose uptake in skeletal muscle and adipose tissue by increasing plasma membrane levels of GLUT4. GLUT4 is mostly found in adipocytes and striated muscle (*231*). Insulin stimulates the translocation of GLUT4 to the plasma membrane to lower the blood glucose level (*57*).

A significant portion of GLUT4 is contained within intracellular vesicles, named GSVs (*26*, *232*, *233*). There is a small amount of GLUT4 present at the plasma membrane at all times (less than 10% of total GLUT4), and endocytosis and exocytosis occur constantly (*119*). This keeps the amount of GLUT4 at the plasma membrane fairly constant under basal conditions. Insulin alters the rate of endocytosis and exocytosis to increase the amount of GLUT4 at the plasma membrane (*26*, *233*, *234*). Insulin stimulation causes translocation of GSVs to the plasma membrane, meaning a greater amount of glucose can move into the cell (*231*, *235*). Insulin also causes the GSVs to get smaller, allowing for easier transport to the plasma membrane (*236*).

Insulin has different effects on GLUT4 cycling in skeletal muscle and adipose tissue (*237*). In skeletal muscle insulin inhibits endocytosis of GLUT4, leading to greater plasma membrane retention of the glucose transporter, and a 2-3 fold increase in plasma membrane levels of GLUT4 (*238*). However, in adipocytes insulin stimulates GLUT4 exocytosis to the plasma membrane, as well as increasing the amount of GLUT4 available for cycling (*237*, *239*). This leads to a 4-5 fold increase of GLUT4 at the plasma membrane.

1.5.2 GLUT4 translocation

The complete pathway of GLUT4 translocation and plasma membrane insertion is still unknown. However, it is known that the actin cytoskeleton (*240–242*), various small G-proteins (*243–245*), SNAREs (soluble *N*-ethylmaleimide sensitive factor attachment protein receptors) (*246*, *247*) and phosphoinositides (*83–85*, *87*, *148*, *149*, *240*, *248–250*) play crucial roles.

Insulin stimulates actin and microtubule reorganisation (*242*) allowing GSVs to move along the actin and microtubule cytoskeleton to fuse with the plasma membrane. After insulin stimulation, actin is remodelled from a compact meshwork to form filamentous actin (F-actin) (*149*, *240*). This F-actin allows GSVs and other proteins important in insulin signalling to undergo translocation to the plasma membrane (*248*, *251*, *252*). Preventing F-actin from forming by incubating cells with the cytoskeleton depolymerising agents cytochalasin B, Latrunculin A or nocodazole prevented glucose uptake from increasing after insulin stimulation (*235*, *251*). Similarly actin reorganisation, and the accumulation of important F-actin is lost in insulin resistant 3T3-L1 adipocytes (*148*, *149*, *248–250*, *253*), L6 myotubes (*240*, *242*) and primary cells (*240*, *242*).

Various small G-proteins direct the translocation of GLUT4 by directly interacting with GSVs and actin (*244*, *245*). These include members of the Rab and Rho families of proteins (*245*). As mentioned earlier, PI3K signalling leads to the activation of the protein kinase Akt (see 1.3.2) (*101*). One of the targets of Akt is AS160, a Rab GAP which regulates GLUT4 translocation (*243*). AS160 contains a Rab GAP domain, and two PTB domains, as well as six potential Akt phosphorylation sites, of which four are phosphorylated *in vivo* (*24*, *254*). Under basal conditions AS160 retains GSVs intracellularly, but after insulin stimulation Akt phosphorylates AS160, causing it to dissociate from GSVs. Constitutively active AS160, which cannot be phosphorylated on four of the six phosphorylation sites, prevented insulin-stimulated GLUT4 translocation and glucose uptake (*255*). The glucose uptake can be restored with a mutation causing inactivation of the GAP domain (*254*).

WASP family proteins regulate actin by recruiting the Arp2/3 complex, and forming actin nucleation sites (*252*, *256*). Arp2/3 nucleation sites protect the minus end of the

F-actin, stabilising an actin filament, and promoting growth. WASP family proteins are activated by a combination of Cdc42 and PtdIns(4,5)P_2 (*256*). Also, PtdIns(4,5)P_2 has been shown to regulate profilin and alpha-actinin (*257*, *258*), proteins which stabilise the plus end of actin filaments, by catalysing growth and causing cross linking respectively.

The full actin reorganisation pathway is not currently known, but it is thought that the mechanism differs between skeletal muscle and adipose tissue. Research carried out in L6 myotubes, a widely used model of skeletal muscle (*65*, *143*, *240*, *251*, *259*), suggests that Rac-1 is the major regulator of actin rearrangement in response to insulin (*143*), whereas in 3T3-L1 adipocytes, a commonly used model of adipose tissue (*248–250*, *253*, *260*), a more complicated pathway involving TC10 and Cbl seems more important (*145*).

In skeletal muscle PKCζ co-localises with GLUT4 and Rac-1 on GSVs after insulin stimulation (*143*, *145*). Rac-1 and Cdc42, two Rho family GTPases, can activate actin reorganisation and stabilisation (*261*, *262*). It is thought that the recruitment of PKCζ is an important regulator of Rac-1, and must be present for efficient actin reorganisation (*143*, *144*). However, the role for aPKCs in insulin-stimulated glucose uptake is still controversial (see 1.3.3).

TC10 phosphorylation leads to the activation of the class II PI3K, PI3K-C2α, in both L6 myotubes and 3T3-L1 adipocytes (*84*), which results in plasma membrane levels of PtdIns3*P* being increased. TC10 is a Rho family G-protein, which is anchored in lipid raft microdomains by two post translational modifications, farnesylation and palmitoylation (*244*, *263*).

In adipocytes TC10 activation is also needed for actin reorganisation (*145*). This requires a secondary, PI3K-independent pathway, known as the Cbl pathway, shown in Fig. 1.4 (*37*). Cbl associates with Cbl-associated protein (CAP) in the cytosol (*37*, *264*). After insulin stimulation APS (Adaptor protein with PH and SH2 domains) is rapidly phosphorylated by the IR (*264*), leading to the recruitment of Cbl-CAP which is also phosphorylated by the IR (*37*). The Cbl-CAP complex is then recruited to lipid rafts via the interaction of CAP with flotillin, a protein which is localised at lipid rafts (*265*). Cbl has many tyrosine residues, and can function as a scaffold protein when phosphorylated, recruiting many other proteins via their SH2 domains (*264*), and its

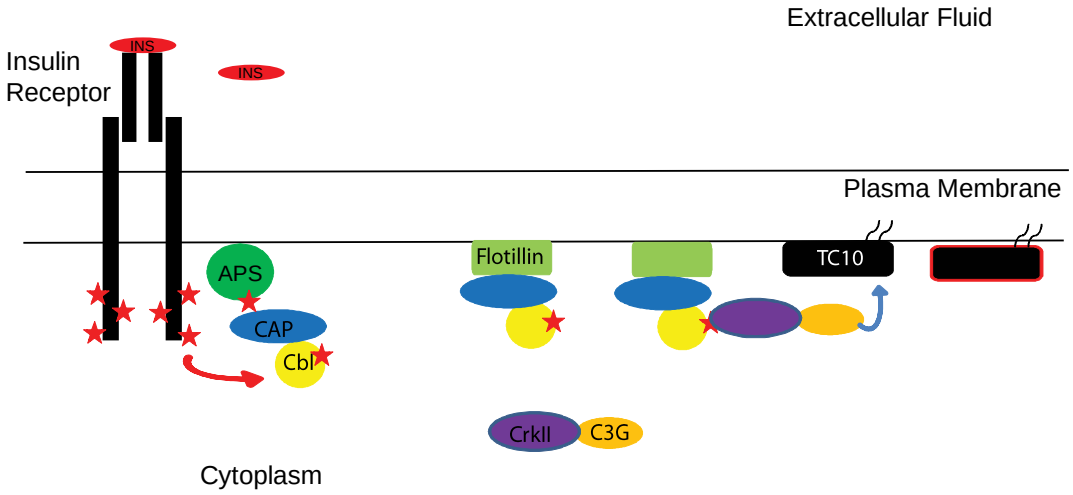

Figure 1.4 Insulin stimulation leads to the activation of TC10. Phosphorylation of APS by the IR leads to the recruitment of the protein oligomer Cbl-CAP. The IR then phosphorylates Cbl, and the Cbl-CAP dimer is recruited to lipid rafts , where CAP can interact with flotillin. This leads to the further recruitment of the CrkII-C3G oligomer, which can interact with the phosphorylated Cbl. The GAP activity of C3G activates TC10. TC10 plays roles in actin reorganisation, GSV translocation, and class II PI3K activation.

Key: Red stars show phosphate groups. Red arrows show the act of phosphorylation. Blue arrows show GAP activity. Red border represents activation.

phosphorylation results in a specific docking site for CrkII-C3G (*37*, *39*), leading to the recruitment of these proteins to the rafts also (*24*, *39*, *263–265*). This leads to a large complex of flotillin-CAP-Cbl-CrkII-C3G, localised at lipid rafts at the plasma membrane. Once at the plasma membrane C3G, a GEF, can activate TC10 (*36*, *263*, *265*). As mentioned earlier, TC10 can activate Class II PI3Ks leading to PtdIns3*P* production (*84*)

GLUT4 is known to localise to lipid rafts and it is believed that the flotillin-CAP-Cbl-CkrII-C3G-TC10 complex can act as a target for GSVs, and stimulate their translocation (*24*, *84*, *266*, *267*). However this pathway is still controversial as siRNA-mediated knockdown of several elements does not diminish glucose uptake (*114*).

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1.5.3 GLUT4 insertion into the plasma membrane

Once GSVs have undergone translocation to the plasma membrane, they must fuse with the membrane to insert GLUT4 into the membrane. SNARE proteins are involved in fusing vesicles during exocytosis; v-SNAREs are present on the vesicle, and t-SNAREs on the plasma membrane.

There are three main families of SNAREs involved with glucose uptake; the Vesicle-associated membrane proteins (VAMPs) on vesicles, syntaxins and the synaptosome-associated 25-kDa protein (SNAP-25) family of proteins on the plasma membrane (*246*, *247*, *268*). SNAREs all contain a small transmembrane domain, with almost the entirety of the protein exposed in the cytoplasm (*269*). The exposed sections of each SNARE can form a coiled coil with a second corresponding SNARE. This interaction provides the energy to allow the two membranes to fuse, an energetically costly procedure. This therefore allows the insertion of vesicle proteins to the plasma membrane (*269*).

VAMPs 2, 3 and 8 are known to play important trafficking roles, and are present in the GSVs (*247*). Depending on the cell type it is thought that different VAMPs are needed. VAMP2 is the most important family member in L6 myotubes, whereas 2, 3 and 8 appear equally important in 3T3-L1 adipocytes (*246*, *247*). In L6 myotubes perturbation to VAMP2 alone prevents GSV insertion and glucose uptake, whereas in 3T3-L1 adipocytes, all 3 VAMPs must be disturbed in order to abolish insulin-stimulated glucose uptake, and transfection with a single VAMP is enough to restore GLUT4 plasma membrane insertion and glucose uptake. (*247*)

Syntaxin4 and SNAP-23 (synaptosome-associated 23-kDa protein) are abundant at ruffled borders at the membrane and are necessary for GSV fusion (*270*). SNAP-23 is a member of the SNAP-25 family, and acts as a mediator between syntaxin4 and VAMP2 (*268*). Mutated SNAP-23, which cannot interact with either VAMP2 or syntaxin4, inhibits GLUT4 translocation. SNAP-23 has also been shown to interact with VAMPs 3 and 8 in non-insulin responsive cells, suggesting the mechanism is possibly similar for these SNAREs (*271–273*).

A family of proteins called Sec61/Munc18 (SM) proteins interact with syntaxins and regulate insulin-stimulated glucose uptake (*274*). In mammals there are three membrane localised SM family members, Munc18a (also known as Munc18-1), Munc18b and Munc18c (*275*). Only Munc18c specifically binds to syntaxin4, and is thought to play the biggest role in insulin signalling and GSV fusion (*275–277*). Munc18c is a negative regulator and forces syntaxin4 into a “closed” state, preventing the syntaxin4 interacting with SNAP-23 and the VAMPs.

Insulin stimulation causes dual phosphorylation of Munc18c by the IR on Tyr219 and 521, preventing the protein interacting with syntaxin4 (*276*, *278*). When Munc18c becomes phosphorylated, Syntaxin4 is no longer able to interact with it, and becomes "open" and is able to interact with SNAP-23. This complex can then interact with VAMPs present in GSVs (VAMP2 in L6 myotubes and VAMPs 2,3, and 8 in 3T3-L1 adipocytes), allowing incorporation of the GSVs into the plasma membrane (*268*). This pathway is shown in Fig. 1.5.

Overexpression of Munc18c inhibits GSVs from fusing with the plasma membrane (*276*) whereas adipocytes from Munc18c-deficient mice show increased insulin sensitivity (*277*). By mutating Munc18c tyrosine residues, using glutamic acid residues as phosphomimetics, the interaction between Munc18c and syntaxin4 can be completely abolished (*276*), increasing insulin sensitivity.

Syntaxins are a family of t-SNAREs necessary for the fusion of vesicles to the plasma membrane, which require PtdIns(4,5)P_2 binding for phosphorylation and

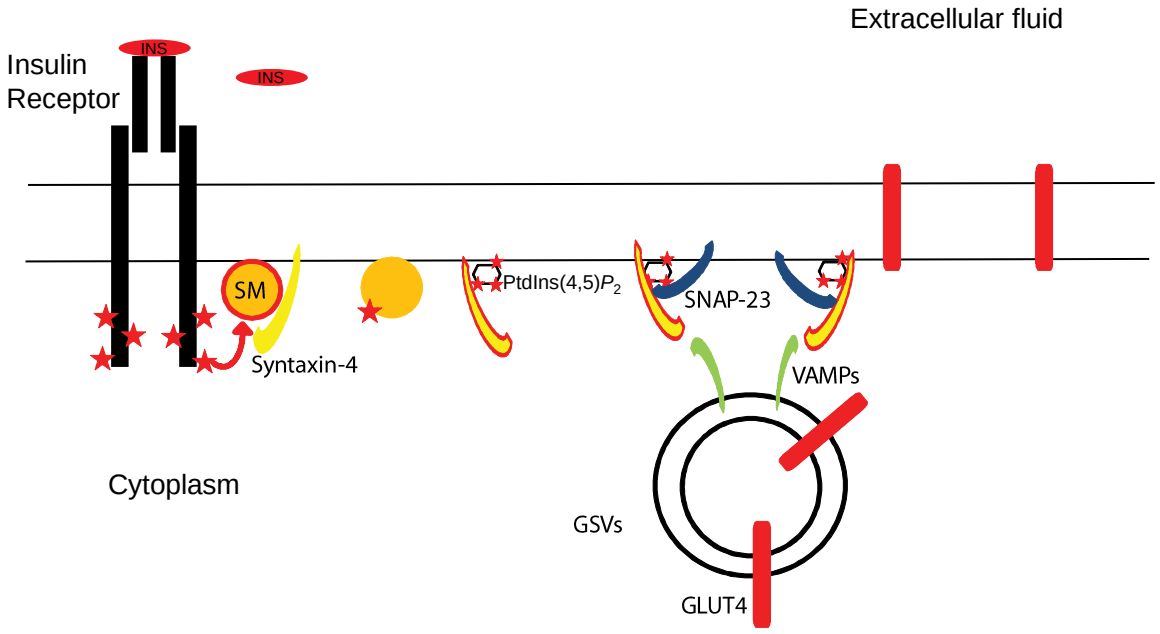

Figure 1.5 GSV fusion is reliant on SNARE interactions. The SM protein Munc18c inhibits syntaxin-4 until it is phosphorylated by the insulin receptor. Syntaxin-4 interacts with PtdIns(4,5)P_2 to become fully active. Once active, syntaxin-4 can interact with SNAP-23, these t-SNAREs can interact with VAMPs on GSVs, causing fusion with the plasma membrane and inserting GLUT4.

Key: Red stars show phosphate groups. Red arrows show the act of phosphorylation. Red borders represent activation.

activation (*177*, *190*). PtdIns(4,5)P_2 is important for syntaxin1 localisation and activation (*156*) and computational studies have suggested that high concentrations of PtdIns(4,5)P_2 and cholesterol are needed for the full activation of syntaxins (*190*). Both of these lipids are in relatively high concentration in lipid rafts (*279*), where GLUT4 is targeted (*24*, *84*, *266*, *267*).

1.5.4 Phosphoinositides in GLUT4 activation

Some evidence suggests that GLUT4 insertion into the plasma membrane is not enough to cause glucose uptake, and that the transporter must be activated, or "unmasked" (*87*, *148*, *149*, *280*). It has been suggested that an interaction with PtdIns3*P* and PtdIns(4,5)P_2 is needed in order for GLUT4 to carry out glucose transport (*87*, *148*).

Phosphoinositides can be delivered to cells using a carrier-mediated delivery system (*281*). Carrier-mediated delivery of PtdIns(3,4,5)P_3 causes GLUT4 translocation and plasma membrane insertion in both L6 myotubes and 3T3-L1 adipocytes, without increasing glucose uptake (*86*, *87*). Once GLUT4 is inserted into the membrane PtdIns3*P* is required to unmask the transporter and cause glucose uptake in L6 myotubes (*87*). The role of PtdIns3*P* in GLUT4 activation in 3T3-L1 adipocytes is not fully understood (*87*).

GLUT4 translocation can be induced using a cell-permeable phosphoinositide-binding peptide (PBP10) in a PI3K-independent manner, but glucose uptake did not occur until cells were further stimulated with insulin (*149*). Insulin stimulates PI3Ks (*35*, *36*, *74*), increasing levels of PtdIns(3,4,5)P_3 and PtdIns3*P* which are necessary for causing glucose uptake (*87*, *149*). PBP10 binds and sequesters PtdIns(4,5)P_2, preventing it from carrying out essential functions in insulin-stimulated glucose uptake (*148*). However, 3T3-L1 adipocytes overexpressing a PIP5kin, which can generate large amounts of PtdIns(4,5)P_2, can overcome this PBP10-induced inhibition, and can increase glucose uptake without insulin stimulation (*148*). It's thought that the PBP10 cannot sequester all of the PtdIns(4,5)P_2 generated, meaning that PtdIns(4,5)P_2 can interact with proteins important for GLUT4 activation (*148*).

PtdIns5*P* is also increased after insulin signalling (*83*, *85*). Carrier-mediated delivery of PtdIns5*P* alone leads to increased glucose uptake (*85*), whereas removal of PtdIns5*P* by expression of a PIP4kin or sequestering by transfection with a PtdIns5*P*-binding domain (the PHD domain from ING2 (*282*)) prevented insulin stimulated glucose uptake (*85*) and actin reorganisation (*83*). PIP4kin expression in L6 myotubes prevented the insulin-stimulated increase in PtdIns(3,4,5)P_3, and incubating cells with wortmannin, the Class I PI3K inhibitor, prevented the PtdIns5*P*-stimulated glucose uptake (*85*), suggesting a role for PtdIns5*P* in maintaining PtdIns(3,4,5)P_3 production in insulin signalling.

1.6 Mechanisms behind insulin resistance

1.6.1 Inducing insulin resistance

As mentioned earlier insulin resistance occurs when cells fail to respond correctly to insulin. In the case of skeletal muscle and adipose tissue this manifests as an inability to increase glucose uptake as a response to insulin. As type 2 diabetes affects over 350 million people worldwide (*1*, *4*), and insulin resistance is thought to affect millions more (*5*), there has been extensive research into the mechanism behind insulin resistance.

Insulin resistance is linked to obesity and the metabolic syndrome (*15–18*). In these conditions the plasma concentration of many molecules are abnormally raised, including angiotensin II (Ang II) (*283*, *284*), endothelin-1 (*285*), tumour necrosis factor α (TNFα) (*286*, *287*) insulin (*19*, *287–289*) and free fatty acids (FFA) (*290*). Organisms suffering from insulin resistance, obesity or the metabolic syndrome also exhibit increased oxidative stress (*17*, *291*). Lipid and glucose flux through cells is increased in obese organisms, leading to the accumulation of DAG (generated from FFAs) (*290*) and glucosamine-6-phosphate (*249*).

In organisms insulin resistance is multi-factorial, with many independent molecules contributing to the accumulation of the disease (*6*, *292*). It is not known which factors play preliminary roles, or arise later in the development of insulin resistance and type 2 diabetes. Using cell culture to investigate insulin resistance can be extremely

beneficial, as it allows a single factor to be investigated at a time. Treatment with Ang II (*293*), endothelin-1 (*250*, *253*), TNFα (*294*), insulin (*240*, *248*), hydrogen peroxide (*260*), glucosamine (*249*) or FFAs (*295*) can induce insulin resistance in cell culture models.

There are a few mechanisms which seem to be perturbed in multiple models of insulin resistance, suggesting an underlying theme. Some of these are discussed below.

1.6.2 PI3K/Akt signalling in insulin resistance

As multiple proteins are recruited to the IR, it is possible for a single pathway to be disturbed, whilst another pathway is unaffected. Commonly the MAPK arm of insulin signalling is unaffected in insulin resistant skeletal muscle and adipose tissue (*33*), whilst the IRS/PI3K/Akt pathway is often perturbed (*296*, *297*).

Using the Class I PI3K inhibitors wortmannin and LY290042 blocks GLUT4 translocation and insulin-stimulated glucose uptake (*60*, *103*, *104*). Reduced activity of PI3K has been linked to insulin resistance (*298*). The product of Class I PI3Ks is PtdIns(3,4,5)P_3 (*94*, *95*), which is known to play a crucial in GSV translocation (*86*, *87*). As such reduced PI3K activity also leads to decreased plasma membrane localisation of GLUT4 (*85*, *298*).

Reduced PI3K activity can also led to reduced Akt phosphorylation and activation, and insulin resistance is also known to correlate with reduced Akt phosphorylation (*240*, *299*). The Akt inhibitor AP12 has also been shown to diminish insulin-stimulated glucose uptake (*65*). As mentioned above (see 1.3.2) Akt2-deficient mice exhibit insulin resistance and diminished glucose uptake (*110*) suggesting the importance of PI3K/Akt signalling in insulin-stimulated glucose uptake. Akt deactivates AS160, and if Akt signalling is perturbed, AS160 is not phosphorylated, preventing GSV translocation (*243*).

TNFα pre-treatment has been shown to diminish the autophosphorylation of the IR, preventing full activation of the receptor's kinase activity, diminishing further phosphorylation of signalling proteins such as IRS, CAP and APS (*300*, *301*).

Signalling through the JNK pathway, TNFα and other cytokines can cause serine-phosphorylation of the IRS family of proteins (*302*–*304*). This prevents the IRS protein from interacting with the IR, greatly disrupting the PI3K pathway, and preventing Akt activation (*305*, *306*), causing decreased insulin-stimulated glucose uptake. PKCs are also known to phosphorylate IR and IRS, preventing insulin signalling (*290*, *307*, *308*).

1.6.3 Oxidative Stress

Oxidative stress has been implicated in insulin resistance and type 2 diabetes. Oxidative stress occurs when there is an imbalance between the production of reactive oxygen species (ROS) and the ability to detoxify the cell. This alters the redox state of the cell to a more oxidizing one. ROS and H_2O_2 production can be a normal result of many signalling pathways, and cells have mechanisms to control this increase, such as the antioxidant glutathione (*309*).

Several *in vitro* studies have shown that high levels of H_2O_2 and ROS may play a role in insulin resistance (*291*, *310*). Long term treatment with several of the molecules which cause insulin resistance leads to an increase in oxidative stress. Insulin, TNFα, Ang II and endothelin-1 treatments all increase levels of ROS (*291*, *293*, *311*–*314*). TNFα, Ang II and endothelin-1 activate NADPH oxidase (NOX) which generates superoxide species (*293*, *313*, *315*) and insulin stimulates ROS production (*316*). Although this is thought to play a physiological role in insulin signalling, it is possible that chronic exposure to insulin can cause oxidative stress and induce insulin resistance (*291*). It has also been suggested that hyperlipidaemia, another symptom of the metabolic syndrome, can cause excess H_2O_2 to build up in the mitochondria (*310*).

Antioxidants have been shown to restore insulin sensitivity in cell culture (*291*, *293*). Furthermore, studies using antioxidants have been able to reverse insulin resistance in animals models (*311*, *315*, *317*). This suggests that chronic oxidative stress induces insulin resistance and prevents glucose uptake, as opposed to the acute ROS production in insulin signalling (*316*).

1.6.4 PtdIns(4,5)P_2 in insulin resistance

Previous studies have shown that decreased plasma membrane immunofluorescence of PtdIns(4,5)P_2 correlates with insulin resistance, induced by exposure to insulin (*240*, *248*), endothelin-1 (*250*), or glucosamine (*249*). More importantly replenishing levels of PtdIns(4,5)P_2, using carrier-mediated delivery, can restore the insulin sensitivity, implying a causative relationship between decreased PtdIns(4,5)P_2 and insulin resistance (*240*, *248–250*). However, this technique has several drawbacks. A major one is that the mass levels of PtdIns(4,5)P_2 are not known, and the decreased immunofluorescence could be because of sequestration by binding proteins, rather than a decrease in actual lipid levels, as suggested by Bhonagiri *et al.* (*249*). Another problem is that the method of fixation can affect plasma membrane integrity and lead to misleading results (*161*, *167*, *318*).

Actin reorganisation is important in insulin-stimulated glucose uptake (*243*, *251*), and PtdIns(4,5)P_2 is known to recruit many proteins involved in regulation and formation of F-actin to the membrane (*258*, *319*). Via these interactions, PtdIns(4,5)P_2 is involved in the nucleation of actin, preventing capping of the filament plus end and stabilising acting filaments (*241*, *258*). As well as diminished plasma membrane insertion of GLUT4, decreased immunofluorescence of PtdIns(4,5)P_2 also correlates with decreased F-actin formation (*240*, *248–250*). Exogenous PtdIns(4,5)P_2, but not PtdIns(3,4,5)P_3, has been shown to reverse insulin resistance via actin related pathways (*240*, *248–250*).

1.6.5 DAG and Ca^{2+}

As mentioned earlier (see 1.2.2), organisms suffering from insulin resistance and the metabolic syndrome often express hyperlipidaemia (*18*, *320*), and high circulating levels of FFAs (*290*). This can lead to increased DAG accumulation, and increased intracellular levels of DAG is often found in the skeletal muscle of organisms suffering from insulin resistance (*290*, *307*). A major role for DAG is the activation of PKCs, and as such PKC activity is increased in the skeletal muscle of obese Zucker rats (*321*). Increased activity of PKCs is linked to reduced IR and IRS tyrosine

phosphorylation (*290*, *307*, *308*) preventing efficient PI3K activation and thereby preventing insulin-stimulated glucose uptake.

DAG can also be generated by the hydrolysis of PtdIns(4,5)P_2 by PLC activity (*184*). This also generates $IP_{3,}$ a second messenger which can release Ca^{2+} from intracellular stores (*185*). Raised intracellular levels of Ca^{2+} are also thought to play a role in insulin resistance (*322*, *323*).

1.6.6 Modeling insulin resistance in L6 myotubes and 3T3-L1 adipocytes

Two cell lines are commonly used to investigate insulin resistance and type 2 diabetes, the rat L6 myotubes and the mouse 3T3-L1 adipocytes. These cell lines have been extensively used to investigate GLUT4 mechanics and insulin resistance (*65*, *143*, *240*, *248–251*, *253*, *259*). L6 myotubes are differentiated from myoblasts (*240*), and 3T3-L1 adipocytes differentiate from fibroblasts (*324*). After differentiation the cells begin to express GLUT4, and increase their glucose uptake in response to insulin (*325–327*).

It has been shown that incubating L6 myotubes or 3T3-L1 adipocytes with Ang II (*293*), endothelin-1 (*250*, *253*), TNFα (*294*), insulin (*240*, *248*) hydrogen peroxide (*260*) or glucosamine (*249*) can induce insulin resistance. Plasma levels of Ang II, endothelin-1 and TNFα are increased in individuals suffering from type 2 diabetes (*283–287*). Hyperinsulinaemia is known to exacerbate insulin resistance (*3*) and insulin pre-treatment prevents GLUT4 translocation and insulin-stimulated glucose uptake (*240*, *248*). As mentioned above, individuals suffering from insulin resistance and type 2 diabetes exhibit increased oxidative stress (*17*, *291*), and hydrogen peroxide treatment can be used to mimic this oxidative stress, and this causes insulin resistance in cell culture (*260*).

1.7 Aims

The primary aim of this project was to investigate whether the mass levels of PtdIns(4,5)P_2 are decreased in insulin resistant L6 myotubes and 3T3-L1 adipocytes, as currently only plasma membrane immunofluorescence has been investigated

(*240*, *248–250*). Carrier-mediated delivery of exogenous PtdIns(4,5)P_2 was carried out to determine if the PtdIns(4,5)P_2 depletion plays a causal role in insulin resistance. It was then necessary to investigate the mechanism behind PtdIns(4,5)P_2 metabolism. Using membrane extracts from insulin sensitive and insulin resistant cells, the activity of PIPkins, phosphatases and PLCs were investigated. Further investigations were undertaken in an attempt to reverse the PtdIns(4,5)P_2 decrease, by directly manipulating the mechanism. The effects on PtdIns(4,5)P_2 levels and glucose uptake were investigated.

2. Materials and Methods

2.1 Materials

Cell lines used in the experiments were L6 myoblasts, obtained from ATCC, 3T3-L1 fibroblasts, a kind gift from Dr Melissa Westwood, University of Manchester, and COS-7 simian kidney cells, obtained from ECACC. Cell culture vessels were from Corning. Dulbecco's modified Eagle's medium (DMEM) and α-minimum essential medium (αMEM) were from PAA. Foetal bovine serum (FBS), penicillin-streptomycin, trypsin-ethylenediamine tetraacetic acid (EDTA), dexamethasone, 3-isobutyl-1-methylxanthine (IBMX), Ang II, endothelin-1, hydrogen peroxide, insulin, cytochalasin B and Isopropyl β-D-thiogalactoside (IPTG) were from Sigma Aldrich. 2-^{3}H-deoxyglucose, Hybond™-C extra reinforced nitrocellulose membrane used for protein-lipid overlay and Hybond™-P hydrophobic polyvinylidene difluoride (PVDF) membrane used for protein transfer were purchased from Amersham. Phosphate buffered saline (PBS) was from Gibco (Invitrogen). Ecoscint A was purchased from National Diagnostics. The Bradford assay reagent and BradfordUltra were purchased from Biorad. PtdIns(4,5)P_2 was purchased from Avanti. Chloroform and methanol were from Fisher. The expression vector pEGFP-C1 was purchased from Clontech. TNFα and the restriction endonucleases XhoI, SalI, HindIII and BamHI were purchased from New England Biolabs and oligonucleotide primers were custom synthesised by Eurogentec or Sigma Aldrich. Pfu Turbo was from Stratagene. The FastDigest® endonucleases were purchased from Fermentas. All reagents for DNA gel purification, and plasmid mini preps were provided in the QIAquick® Gel Extraction kit and the QIAprep® Spin Miniprep Kit from Qiagen. Silica-coated glass thin layer chromatography (TLC) plates, the rapid ligation kit and the buffers for the restriction endonuclease reactions were from Roche. The preparation of recombinant Glutathione S-transferase (GST)-tagged PLCδ PH domain was performed by Dr Sanaa Al-Ahdab and Dr David Jones. The primary antibodies, rabbit monoclonal anti-phospho-Akt Ser473 (9275), rabbit polyclonal anti-phospho-Akt Thr308 (4058) and rabbit polyclonal anti-Akt Antibody (9272) were from Cell Signaling Technology. The primary antibodies, rabbit polyclonal anti-PLCβ3 (51139-1-AP), rabbit polyclonal anti-PLCγ1 (18891-1-AP) and rabbit polyclonal anti-PLCδ1 (14228-1-AP) were kind gifts from Proteintech. The mouse monoclonal anti-

GST (sc-138) and monoclonal mouse PLCβ4 (sc-166131) antibodies were from Santa Cruz Biotechnology. The purified mouse monoclonal anti-PtdIns(4,5)P_2 (Z-P045) antibody, and the synthetic lipids, PtdIns4*P* and PtdIns5*P*, were from Echelon Biosciences. The Horse Radish Peroxidase (HRP)-conjugated secondary antibodies goat anti-mouse (PO447), goat anti-rabbit (PO448) and rabbit anti-goat (PO449) were obtained from Dako. Alexa Fluor® 488 Goat anti-mouse F(ab')2 fragment antibody and Prolong® Gold Antifade reagent were from Invitrogen. The EZ-Chemiluminescence chemicals and 30% polyacrylamide were from Geneflow. Glyceryl-glass, controlled pore (size 200-400 mesh, nominal diameter 3000Å) beads were from Sigma Aldrich. All other materials used were from Sigma Aldrich, unless specifically mentioned.

2.2 Cell Culture

2.2.1 Maintaining cells

All cells used in this project were incubated at 37 °C in 5% CO_2. 3T3-L1 fibroblasts, and COS-7 cells were maintained in high-glucose (4.5g/L) DMEM containing 10% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin. L6 myoblasts were maintained in αMEM with 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin and 2mM glutamine.

2.2.2 3T3-L1 fibroblast differentiation

The cells were grown to 70-80% confluence before splitting, roughly 3-4 days. Cells for differentiation were grown to 100% confluence and differentiated according to Elmendorf *et al.* (*324*). The medium was changed to DMEM containing 10% FBS, antibiotics, 0.17μM insulin, 1μM dexamethasone and 0.5mM IBMX. After four days the medium was changed to DMEM containing 10% FBS, antibiotics and 0.17μM insulin. This medium was replaced every two days. After 4 days the medium was changed to low-glucose (1g/L) DMEM containing 10% FBS and antibiotics only for at least two days. Experiments were performed on cells 10-12 days after initiation of differentiation, on cells from passage 4-9.

2.2.3 L6 myoblast differentiation

The cells were grown to 70-80% confluence before splitting. Cells for differentiation were grown to 100% confluence and differentiated according to McCarthy *et al*. (*240*) by changing the medium to αMEM containing 2% FBS and antibiotics. After 8-10 days cells were trypsinised and allowed to re-adhere in suitable dishes for experiments for two days. The cells used were from passage 4-10.

2.3 2-Deoxyglucose uptake

2.3.1 Prior treatments

24 hours prior to the experiment, 3T3-L1 adipocytes were incubated in serum-free low glucose DMEM, and L6 myotubes were incubated in serum-free αMEM. Non-specific glucose uptake was determined by incubating cells with the appropriate serum free media containing 10μM cytochalasin B 30 minutes prior to the experiment. Insulin resistance inducing factors were incubated with the cells for the appropriate time (see 2.6.1). All inducing factors were removed immediately before starting the experiment apart from insulin, which was removed 1 hour before starting the experiment.

2.3.2 Insulin stimulation and glucose uptake

Cells were incubated in either 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffered saline (HBS; 20mM Na-HEPES, pH 7.4, 140mM NaCl, 5mM KCl, 2.5mM $MgSO_4$, 1mM $CaCl_2$) or HBS containing 100nM insulin for 20 minutes at 37 °C. As described above, some cells were incubated with HBS containing 10μM cytochalasin B. A mixture of glucose and 2-^{3}H-deoxyglucose in HBS was added to give a final concentration of 100μM glucose, containing either 0.5 or 1 μCi/mL 2-^{3}H-deoxyglucose. The cells were then incubated at 37 °C for a further 10 minutes. The assay was stopped by the removal of all solutions, and the addition of ice cold HBS. After washing three times with ice cold HBS, ice cold HBS containing 0.1% sodium dodecyl sulphate (SDS) and 0.1% Triton X-100 was used to lyse the cells. The cells

were scraped and mixed with Ecoscint A scintillation fluid. The samples were left overnight, and then measured in a scintillation counter.

2.3.3 Bradford assay for protein concentration

Samples were normalised to protein concentration. Cells undergoing the same treatment of insulin resistance were lysed using 0.1% SDS, 0.1% Triton-X100 in HBS. 5 µL of this was added to 200 µL 4:1 water: Bradford reagent, or neat BradfordUltra reagent. The absorbance was read at 595 nm in a 96-well plate reader, and the results were normalized to a standard curve set up at the same time, using concentrations of bovine serum albumin (BSA) ranging from 0.05 mg/mL to 1 mg/mL.

2.4 PtdIns(4,5)P_2 measurements

2.4.1 Lipid extraction

Cells were taken from the incubator and placed on ice. The cells were washed with ice cold PBS, and 450 µL 1.2M HCl was used to lyse the cells. 600 µL CH_3OH and 500 µL $CHCl_3$ were added, and after vigorous mixing the samples were centrifuged at 16,000*g* for 1 minute. Two distinct phases were apparent; a lower organic phase and an upper aqueous phase. The lower organic phase was transferred to a fresh tube, and washed with 450 µL theoretical upper phase (TUP; 3:48:47 (v/v/v) $CHCl_3$: CH_3OH: 1M HCl). The lower phase was again transferred to a clean tube.

To ensure maximum lipid extraction, 450 µL theoretical lower phase (TLP; 86:14:1 (v/v/v) $CHCl_3$: CH_3OH: 1M HCl) was added to the original upper phase. This was thoroughly mixed and centrifuged at 16,000*g* for 1 minute, before the lower phase was removed and washed with TUP. This was pooled with the prewashed lower phase. The lipid samples were dried in a vacuum centrifuge, and stored under nitrogen at -80 °C.

2.4.2 Neomycin bead purification

Neomycin-coated beads were prepared as described by Schacht (*328*). 500 μL 5:10:12 (v/v/v) $CHCl_3$: CH_3OH: H_2O was added to clean glass vials. 50 μL neomycin beads were added to the glass vials, and were washed by centrifugation at 4,000*g* for 30 seconds. The supernatant was removed and the beads were washed with a high formate buffer (5:10:2 (v/v/v) $CHCl_3$: CH_3OH: ammonium formate (final concentration of formate was 500mM)). This was removed and the beads were washed with the first solvent again. This was removed with a Hamilton syringe to ensure maximal liquid removal. The beads were then resuspended in 50 μL low formate buffer (5:10:2 (v/v/v) $CHCl_3$: CH_3OH: ammonium formate (final concentration of formate was 50mM)). The dried lipid samples (from 2.4.1) were resuspended in 600 μL low formate buffer. Two 30 μL samples were taken for phosphate measurements (see 2.4.5). The rest was added to the beads, and this was incubated at room temperature for an hour. The samples were spun at 4,000*g* for 1 minute and the supernatant was removed. The beads were washed twice with 500 μL low formate buffer, before 500 μL freshly prepared triethylamine bicarbonate (TEAB) solution (2:6:3 (v/v/v) $CHCl_3$: CH_3OH: 2M TEAB) was added to the beads. 2M TEAB was prepared before each experiment by bubbling CO_2 through a 7:18 ratio of triethylamine and H_2O, on ice.

After an hour's incubation the supernatant was collected in a clean tube. To ensure maximal extraction, 500 μL TEAB solution was again added to the beads and the incubation and removal of the supernatant was repeated. The two supernatants were pooled together, and were dried in a vacuum centrifuge overnight.

2.4.3 Generation of GST-tagged PLCδ-PH domain

A vector driving the expression of GST-tagged PLCδ-PH domain was generated by Dr David Jones, The Paterson Institute for Cancer Research. Competent Rosetta *E. coli* cells were transformed using a heat shock transformation. Cells were incubated with 1 μL GST-tagged PLCδ-PH vector on ice for an hour. The cells were heated to 42 °C for 45 seconds before returning to ice for 2 minutes. 1 mL of Luria-Bertani (LB)

broth was added and this was incubated with shaking for an hour. This was spread on pre-warmed agar plates, and left to grow overnight.

Single colonies were incubated in 50 mL LB broth in the presence of 50µg/ml kanamycin at 37 °C. This culture was diluted into 450 ml of LB-kanamycin and incubated at 37 °C with shaking until an A_{600} of 0.6 was reached, at which time protein expression was induced by addition of IPTG to a final concentration of 0.1mM. The culture was incubated overnight at room temperature with vigorous shaking.

Cells were harvested by centrifugation at 6,000*g*, at 4 ˚C for 10 minutes. This was then washed in 250 mL ice cold wash buffer (50mM Tris, pH 8.0, 150mM NaCl, 5mM $MgCl_2$), before re-suspension in 20 mL wash buffer containing 1% (v/v) Triton X-100, 1% (v/v) Tween-20, 1mM 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF) and 5µM leupeptin. This suspension was lysed with a probe sonicator three times for 30 seconds, on ice. Centrifugation at 18,000*g*, at 4 ˚C for 10 minutes was carried out the remove insoluble material, and the soluble lysate was applied to a column of Glutathione agarose beads. The beads were pre-washed several times with ice cold wash buffer. The soluble lysate was passed through the column three times, before two washes with ice cold wash buffer. Proteins were then eluted by addition of 1mL of 20mM Glutathione in 50mM Tris, pH 8.0. The protein concentration was measured using a Bradford assay (2.3.3). Eluted proteins were diluted with glycerol (50% (v/v) final concentration) and stored at -20 °C until needed.

2.4.4 Protein lipid overlay

The PtdIns(4,5)P_2 mass assay was carried out as described by Divecha *et al*. (*158*) with slight modifications. A PtdIns(4,5)P_2 standard curve was prepared with amounts ranging from 50pmol to 0.195pmol. Lipids were spotted onto a reinforced nitrocellulose membrane in chloroform.

The nitrocellulose membrane was blocked with blocking solution (2% (w/v) BSA in Tris-buffered saline (TBS) (0.1M Tris, 0.3M NaCl pH 7.4) containing 0.05% (v/v) Tween-20) for an hour. The membrane was then incubated overnight at 4 °C with 0.5 or 1 µg/mL GST-PLCδ1-PH domain in blocking solution. The membrane was

washed with washing buffer (TBS containing 0.05% (v/v) Tween-20) and then incubated with a mouse anti-GST antibody. The membrane was washed again, and then incubated with an anti-mouse HRP-conjugated antibody.

After a final wash, visualization was carried out using enhanced chemiluminescence (ECL) with the EZ-Chemiluminescence detection system. A charge-couple device (CCD) camera (Image Reader LAS-1000; Fujifilm) was used for quantification. Densitometry was performed with AIDA software, with background subtraction.

2.4.5 Total phosphorus measurements

30 μL aliquots taken at the beginning of 2.4.2 were transferred to glass tubes and dried at 100 °C. Once dry, 220 μL 70% perchloric acid was added to the samples, and this was boiled at 170 °C for an hour. Once cool 1 mL H_2O, 600 μL 0.83% (w/v) ammonium molybdate (0.25% final concentration) and 200 μL freshly prepared 10% (w/v) ascorbic acid (1% final) were added with vortexing between each addition. This mixture was boiled for 7 minutes, rapidly cooled, and the absorbance at 820 nm was read. Reading were normalised to a standard curve, set up from the standards prepared from Na_2HPO_4 (0.1-0.5 μg).

2.5 Visualising PtdIns(4,5)P_2

2.5.1 Visualising PtdIns(4,5)P_2 using immunohistochemistry

3T3-L1 cells were seeded on glass coverslips before differentiation. Fully differentiated L6 myotubes were trypsinised, and then seeded on collagen-treated coverslips.

The cells were fixed according to Hammond *et al*. (*161*). Cells were quickly fixed by direct addition of formaldehyde and glutaraldehyde to the media, with a final concentration of 4% and 0.2% respectively. After 15 minutes, cells were washed with PBS containing 50mM NH_4Cl three times, and cooled for 2 minutes on a metal plate in an ice bath. All subsequent steps were carried out on ice, with chilled solutions. Cells were blocked and permeabilised using Buffer A (20mM PIPES, pH 6.8, 137mM

NaCl, 2.7mM KCl) containing 5% (v/v) normal goat serum (NGS), 50mM NH_4Cl and 0.5% (w/v) saponin for 45 minutes. 50 μg/mL GST-PLCδ1-PH was included in some samples at this stage, to allow the antibody to only bind PtdIns(3,4,5)P_3. Monoclonal anti-PtdIns(4,5)P_2 antibody was added at 2.5 μg/mL, in Buffer A containing 5% NGS and 0.1% saponin for 1 hour. After washing twice with Buffer A, the cells were incubated with the secondary antibody (in Buffer A with 5% NGS and 0.1% saponin) for 45 minutes. The slides were then washed 4 times with Buffer A, and post-fixed in 2% formaldehyde for 10 minutes on ice. The slides were then allowed to warm to room temperature for 5 minutes before the formaldehyde was removed, and rinsed with PBS containing 50mM NH_4Cl three times, before a final rinse with distilled water. Slides were allowed to dry, mounted using ProLong® Gold Antifade and left overnight to dry, before being sealed with nail varnish. Slides were visualised using a Nikon upright confocal microscope, and EZ-C1 software.

2.5.2 Visualising PtdIns(4,5)P_2 using the Tubby protein

The GFP-C1 Tubby vector was a kind gift from Professor John Challis, University of Leicester. Competent *E. coli* XL10-gold were transformed using a heat shock transformation using 1 μL GFP-C1 tubby vector, similar to 2.4.3.

A QIAprep spin mini prep was carried out, as per manufacturer's instructions, and a sample of the DNA extracted was sent to the DNA Sequencing Facility at the University of Manchester to confirm the presence of Tubby. The DNA sample was also cleaved with the restriction enzymes XhoI and SalI and run on a 0.8% (w/v) agarose, 0.5x Tris/Boric Acid/EDTA (TBE; 43mM Tris, 36mM Boric Acid, 1mM EDTA) gel to also test for the presence of a DNA sequence matching the size of GFP-Tubby.

Polymerase chain reaction (PCR) was carried out on the GFP-C1 Tubby vector using the following primers; upstream: 5'- ATC TGG TAC CGT CGA CCA CCA TGG GTA AAG GAG AAG -3' and downstream: 5'- GAA GCT TAG GCT CG CTA CTC GCA GGC CAG CTT GC -3'. The final reaction solution consisted of 200μM of each deoxynucleoside triphosphate, 100ng template DNA, 200nM of each primer and 2.5U Pfu Turbo DNA polymerase, in 50 μL 1x Pfu buffer. PCR was carried out by

first heating the samples to 95 °C for five minutes, followed by 35 cycles of heating to 95 °C for 30 secs, cooling to 60 °C for 30 secs, and then heating to 72 °C for 2.5 minutes. The reaction was finally heated to 72 °C for 10 minutes.

Fermantas FastDigest® BamHI and HindIII were used to cleave the pEGFP-C1 plasmid and the tubby-containing PCR product. This was incubated for 5 minutes at 37 °C. Following the manufacturer's instructions the Roche rapid ligation kit was used to ligate the plasmid and the vector.

The resulting DNA was transformed into competent *E.coli* cells using the heat shock transformation described earlier. A QIAprep mini prep was carried out, and the extracted DNA was digested with BamH1 and HindIII and run on a 0.8% (w/v) agarose, 0.5x TBE gel.

Calcium phosphate transfection was used to transiently transfect COS-7 cells. Cells were seeded onto coverslips, and incubated overnight. The next day the medium was replaced with fresh DMEM. 719ng of the GFP-C1 tubby vector was added to 60 μL 125mM $CaCl_2$. 2x Hank's buffered salt solution (HBSS; 42mM HEPES, pH 7.07, 274mM NaCl, 10mM KCl, 1.4mM Na_2HPO_4, 15mM glucose) was added dropwise whilst bubbling air through the mixture, and this was incubated at room temperature for 20 minutes. This was then added to the cells, which were then returned to the incubator. After 4-6 hours 15% (v/v) glycerol in PBS was added to the cells for no longer than 2 minutes. The cells were washed with PBS and then fresh medium was added. The cells were fixed with 4% (w/v) formaldehyde in 100mM $NaHPO_4$ pH 7.4, and mounted with Prolong® Gold Antifade reagent the next day. Slides were visualised on Axioplan 2 microscope.

When 3T3-L1 adipocytes were fully differentiated, they were transfected using an Amaxa Nucleofector® machine. Roughly 1 million cells were resuspended in 100 μL reaction buffer L. 2μg of DNA was added and this was mixed and transferred to a nucleofection cuvette, which was placed in the Nucleofector® machine. The programme A-033 was run, and the cells were removed and transferred to a dish containing a coverslip in 2 mL fresh medium.

The cells were fixed with 4% formaldehyde in 100mM $NaHPO_4$ as above. Slides were visualised using a Nikon upright confocal microscope, and EZ-C1 software.

2.6 Pre-treatments

2.6.1 Conditions for insulin resistance

Differentiated cells were incubated with, or without, one factor used to induce insulin resistance. This was added to serum-free αMEM or serum-free low glucose DMEM for L6 myotubes or 3T3-L1 adipocytes respectively. The conditions to induce insulin resistance are shown in table 2.1.

Table 2.1 Induction of insulin resistance

Method of Induction	Length of Induction	Concentration	Reference(s)
Ang II	10nM	24 hours	(*293*)
Endothelin-1	100nM	24 hours	(*250*)
Hydrogen Peroxide	100μM	3 hours	(*260*)
Insulin	5nM	12 hours	(*240*, *248*)
TNFα	40nM	24 hours	(*294*)

2.6.2 Carrier-mediated delivery of PtdIns(4,5)P_2

The protocol for the carrier-mediated delivery of PtdIns(4,5)P_2 was based on the one currently employed by the Elmendorf laboratory (*240*, *248*, *250*). A 2:1 mixture of PtdIns(4,5)P_2 and histone carrier, containing 12.5μM PtdIns(4,5)P_2, was mixed for 10 minutes in serum free αMEM. This was diluted in the appropriate medium and added to cells during the last hour of induction of insulin resistance, to make a final concentration 1.25μM PtdIns(4,5)P_2 and 0.625μM histone carrier. A carrier only control was also used.

2.6.3 Incubations with antioxidants

Differentiated L6 myotubes or 3T3-L1 adipocytes were incubated with either 5μM diphenylene iodonium (DPI), 10μM polyethylene glycol- (PEG) Catalase or 20mM N-acetyl Cysteine (NAC) for 24 hours, before carrying out glucose uptake assays.

2.7 Membrane activity assays

2.7.1 Membrane extraction

Cells were first washed with ice-cold PBS, and then scraped using a cell scraper in 1 mL PBS and transferred to an ice-cold falcon tube. Samples were then spun at 72*g* to pellet the cells, and the supernatant was removed. The pellet was resuspended in 1 mL ice-cold hypotonic swell buffer (5mM Tris, pH 7.4, 1.5mM KCl, 2.5mM $MgCl_2$, 5mM EDTA) and incubated for no more than 5 minutes on ice. This was passed through a needle 10 times, and inhibitors were added to the final concentration of 50mM NaF, 40mM β-glycerophosphate, 1mM Na_3VO_4, and 1mM AEBSF. This was then spun at 600*g* for 5 minutes at 4 °C, to pellet the nuclei. The supernatant was transferred to a Beckmann polycarbonate centrifuge tube and centrifuged at 100,000*g* for 1 hour at 4 °C. The supernatant was discarded and the pellet was resuspended in 1 mL ice-cold resuspension buffer (10mM Tris, pH 7.4, 1mM Ethylene glycol tetraacetic acid (EGTA), 1.5mM KCl, 5mM $MgCl_2$, 0.34M sucrose, 1mM Na_3VO_4, 40mM β-glycerophosphate, 50mM NaF, 1mM AEBSF). This was centrifuged at 100,000*g* for 1 hour at 4 °C. The supernatant was discarded and the pellet was resuspended in 100 µL resuspension buffer, and a Bradford assay was carried out as 2.3.3. 50 µL aliquots containing 100 µg/mL protein were snap-frozen in liquid nitrogen and stored at -80 °C until needed.

2.7.2 Kinase activity assay

Membrane preparations were thawed on ice. A mixture of ATP and γ-^{32}P ATP was made up in kinase buffer (10mM Tris, pH 7.4, 1mM EGTA, 1.5mM KCl, 5mM $MgCl_2$) and added to the membrane preparations making a final concentration of 5µM ATP, containing 10 µCi/mL γ-^{32}P ATP. In some samples micelles containing PtdIns4*P* or PtdIns5*P* (final concentration 200nM) were added at this stage. This was incubated at 30 °C for 5 minutes (for L6 myotube samples) or 15 minutes (for 3T3-L1 adipocyte samples). The reaction was stopped by adding 500 µL 1:1 $CHCl_3$:CH_3OH and the phases were split by the addition of 125 µL 2.4M HCl. This was vigorously mixed and spun at 16,000*g* for 1 minute, before being washed by removing 400 µL of the upper phase and replacing this with 400 µL theoretical upper phase. After washing a further

2 times, the lower phase was transferred to a clean tube, and dried down. This was kept at -20 °C until needed.

2.7.3. Generation of radioactively tagged PtdIns(4,5)P_2

For the phosphatase and PLC activity assay radioactively tagged PtdIns(4,5)P_2 had to be generated. Recombinant PIP4kinα was incubated overnight at 30°C with 0.1nmol PtdIns5*P* and 30μCi/mL γ-^{32}P-ATP in kinase buffer. The lipids were extracted as 2.7.2 with the lower phase being washed 4 times. The lower phase was collected and dried down, before being resuspended in 500 µL 1:1 $CHCl_3$:CH_3OH and kept at -20 °C until needed. Before use, 1 µL was counted using a scintillation counter under the tritium window to determine the Cherenkov count.

2.7.4. Phosphatase activity assay

Phosphatases and PLCs require different metal ions to function properly. Phosphatases require magnesium whereas PLCs require calcium (*198*, *208*, *213*) and by manipulating levels of these cations the specificity of the assay can be altered. Membrane samples were thawed on ice, and mixed with 60 µg alamethicin. Radioactively labelled PtdIns(4,5)P_2, sufficient to give 30,000 Cherenkov counts per sample, was dried down, along with unlabelled PtdIns(4,5)P_2 and resuspended in kinase buffer. The PtdIns(4,5)P_2 mixture was added to membrane samples to give a final concentration of 10µM PtdIns(4,5)P_2, and this was incubated at 37 °C for 30 minutes. The lipids were extracted as 2.7.2, before being washed once in a similar method to 2.7.2. The lower phase was transferred to a clean tube, dried down, and kept at -20 °C until needed.

2.7.5 PLC activity assay

Membrane samples were thawed on ice, and mixed with 60µg alamethicin. 100 µL 2.5x PLC buffer (25mM HEPES, pH 7.4, 2.5mM EGTA, 31.25mM LiCl, 220mM KCl, 62.5mM β-glycerophosphate, 2.76mM $CaCl_2$) was added and this was incubated at

30 °C for 10 minutes. Radioactively labelled PtdIns(4,5)P_2, sufficient to give 30,000 Cherenkov counts per sample, was dried down, along with unlabelled PtdIns(4,5)P_2, and resuspended in water. 100 µL was added to the samples to give a final concentration of 10 µM PtdIns(4,5)P_2, as well as a free Ca^{2+} concentration of 100µM. This was incubated for a further 30 minutes, before the reaction was stopped with 1250 µL 1:1 $CHCl_3$:CH_3OH and 312.5 µL 2.4M HCl. This was vigorously mixed and spun at 16,000*g* for 1 minute. 800 µL of the upper phase was removed and replaced with theoretical upper phase. Again, this was mixed and spun at 16,000*g* before the lower phase was transferred to a clean tube and dried down. Dried samples were kept at -20 °C until needed.

2.7.6 Thin layer chromatography

Silica-coated glass TLC plates were dipped in a solution containing 1% (w/v) potassium oxalate, 2mM EDTA, 50% (v/v) CH_3OH and then placed in an oven, at 120 °C, for at least 4 hours until dry. Samples extracted from the procedures outlined in sections 2.7.2, 2.7.4 and 2.7.5 were warmed to room temperature, dissolved in chloroform and spotted on the glass plate. These were resolved by TLC using a solvent system containing 40:28:10:6 (v/v) CH_3OH:$CHCl_3$:water:ammonia, and allowed to run for approximately 3 hours. Quantification was performed using a phosphoimager plate and AIDA densitometry software.

2.8 SDS-PAGE electrophoresis and western blot analysis

2.8.1 Preparation of protein extracts

Differentiated cells were treated as necessary, and washed twice with PBS. 2x Laemmli buffer (160mM Tris, pH 6.8, 20% (v/v) glycerol, 4% (w/v) SDS, 100 µg/mL bromophenol blue) with 10% (v/v) β-mercaptoethanol was added and the cells were scraped with a rubber scraper, and transferred to a 1.5 mL tube. Samples were boiled for 5 minutes and sonicated to reduce viscosity. The samples were stored at -20 °C until needed.

4x Laemmli buffer was added to thawed membrane extractions (from 2.8.1), and these were boiled for 5 minutes before running on an acrylamide gel immediately.

Samples were also extracted using a trichloroacetic acid (TCA) precipitation. First cells were lysed in ice cold RIPA buffer (50mM Tris, pH 7.4, 150mM NaCl, 1mM AEBSF, 1mM EDTA, 5 µg/mL leupeptin, 100µM Na_3VO_4, 40mM β-glycerophosphate, 50mM NaF, 1% (v/v) triton X-100, 1% (w/v) sodium deoxycholate, 0.1% (w/v) SDS). These were then centrifuged at 4,000*g*, and the supernatant was transferred to a fresh tube.

An equal volume of 20% (w/v) TCA was added, and this was incubated for 30 mins on ice. This was then centrifuged at 13,000*g* at 4 ˚C for 15 minutes. The supernatant was removed, and 300 µL ice cold acetone was added. This was centrifuged 13,000*g* at 4 ˚C for 5 minutes, and the supernatant was removed. The pellet was allowed to dry before being resuspended in 8M Urea with 1% (w/v) SDS under sonication, and a BradfordUltra protein assay was carried out. 4x Laemmli buffer was added to samples, and this was boiled for 5 minutes before running on an acrylamide gel.

2.8.2 SDS-PAGE electrophoresis and western blotting

Samples were run on either 8 or 10% acrylamide gels, and transferred onto a PVDF membrane using a liquid transfer system for two hours at 250mA, or overnight at 25mA in blotting buffer (189mM glycine, 25mM Tris, 20% (v/v) CH_3OH).

2.9 Reverse Transcriptase-PCR

2.9.1 RNA extraction

RNA extraction was carried out on differentiated L6 myotubes using an RNeasy Extraction kit from QIAgen. The RNA concentration was measured, and then stored at -80 °C until needed.

2.9.2 Reverse transcriptase-PCR

Reverse Transcriptase (RT)-PCR was carried out using a MyTaq One Step RT-PCR kit from BioLine, using a method based on the manufacturer's instructions. RT-PCR was carried out by first heating the samples to 45 °C for 20 minutes, and then heating to 95 °C for 1 minute. Then PCR was carried out using 40 cycles of 10 secs at 95 °C, 10 secs at 60 °C and then 30 secs at 72 °C. The resulting DNA was run on a 1.2% (w/v) agarose, 0.5X TBE gel.

Table 2.2 shows the primers used for RT-PCR.

2.10 Measuring PtdIns(4,5)P_2 in primary mice tissue

2.10.1 Animals

C57BL6 x 129SV male and female mice were kept on a normal chow diet (TestDiet, Richmond, IN, USA) for 8 weeks. After that they were placed on either a high fat (60%) or control diet (12% fat) for 7 weeks. The animals were killed by terminal anaesthesia with 3.5% isoflurane (in 3:7 O_2:N_2O), and perfused with 0.9% (v/w) NaCl in DEPC-treated water.

2.10.2 Lipid extraction

Muscle from the back legs was removed from mice after perfusion. The tissue was immediately transferred to $CHCl_3$ on ice. The tissue was sonicated, and kept on ice. Assuming muscle tissue to be 80% water 2:6:0.1 (v/v/v) $CHCl_3$:CH_3OH:concentrated HCl, was added for every 1.2g of tissue (*329*). This was centrifuged at 4,000*g* to remove non-soluble material, and the supernatant was transferred to a fresh tube.

1.25 mL $CHCl_3$ and 1.25 mL 1.6M HCl was added, with vigorous mixing after each addition. This was centrifuged at 10,000*g* and the lower phase was removed. This was dried in a vacuum centrifuge, and stored under nitrogen at -80 °C.

Neomycin bead purification, protein lipid overlay and ECL were carried out as 2.4.2-2.4.4.

Table 2.2 Primers used for RT-PCR against PLC isoforms.		
Target	Forward Primer	Reverse Primer
PLCβ1	GAA TGG TGC GCT TCC AGG CGG	CGC CCT TCT GGG GTC ACC TG
PLCβ2	CAA CCC TGT CCT GTT GCC CC	CTG GCC GAG GAC AGA GGC TCA
PLCβ3	ACC TGG TGA CCC TGC GAG TGG	GCG GTG GGT ACA GCA CCT CG
PLCβ4	CGC ATC CCC ATC ATC TAC TC	GAT CCG TGC CGC TGC AGA CA
PLCγ1	AGC CGC GGT GCG GAC AAA AT	GAT GTC CCC GCT GCG CTT GTT
PLCγ2	CTT GGT GCA CGC CAC CTC CC	ATC CCG GTT GGC TCC TCG CA
PLCδ1	GCT GAC GCT CAG CAC TGG GT	CAT CTG CCG CTG GGC CTT GG
PLCδ3	TCA AGG GCC GCC GCA AGA AC	CAG CCC CCT CAG GCG ACA AC
PLCδ4	TCC TAC CCA CGG TGA CTC TGG C	GTG AAG CCT TGC TCC AGG GGG
PLCε	CGA GGA GAG CGC CAG CGA	ACT TGC CAG GTT CGG CCA CG
PLCη1	GCA GTC CCT TGG TTG CGG GA	GCA GTC CCT TGG TTG CGG GA
PLCη2	GCA GTC CCT TGG TTG CGG GA	TGC CCA CAT GGG CAA GAT CA

2.10.3 Phosphorus measurements

As the phosphorus levels were very high in the tissues, the samples were diluted. After the perchloric acid digestion 22 µL was diluted 1 in 10 with fresh perchloric acid. The phosphate assay was then carried out as 2.4.6.

2.11 Statistical analyses

Unpaired T-Tests, One-way ANOVA with Bonferroni's multi comparison test, or Kruskal-Wallis One-way ANOVA with Dunn's multi comparison test were carried out using the GraphPad Prism programme.

3. Investigating a causal link between PtdIns(4,5)P_2 decrease and insulin resistance in cell culture models

3.1 Characterisation of cell types

Under physiological conditions in mammals, skeletal muscle and adipose tissue respond to insulin by increasing glucose uptake (*330*, *331*). When individuals begin to exhibit insulin resistance this response becomes diminished (*332*, *333*). As the induction of insulin resistance, and type II diabetes, in mammals is multi-factorial (*6*), it was decided to use cell culture models of skeletal muscle and adipocytes to investigate insulin resistance. Using cell culture models allows the effect of a single factor to be investigated, and is routinely used in the investigation of insulin-stimulated glucose uptake and type 2 diabetes (*65*, *143*, *240*, *248–251*, *253*, *259*).

The cells lines used were the rat L6 myoblast cell line as a model for skeletal muscle, and the mouse 3T3-L1 fibroblast cell line as a model for adipocytes. These cells undergo differentiation under the conditions stated in 2.2 (*240*, *324*).

When the L6 myoblasts begin to differentiate into myotubes, the cells fuse and become long, multi-nucleated cells. They begin to increase expression of GLUT4, the insulin-responsive glucose transporter (*57*), leading to increased insulin-stimulated glucose uptake (*325*). Glucose uptake assays were carried out to confirm that the cells had differentiated and that insulin would increase glucose uptake as reported (*240*). Upon insulin stimulation L6 myotubes increased their glucose uptake from 1.88 ± 0.3nmol glucose/mg protein to 3.13 ± 0.3nmol glucose/mg protein, and a 1.5-2 fold increase was seen consistently in untreated cells (Fig. 3.1).

When 3T3-L1 fibroblasts begin to differentiate they lose their characteristic shape and become rounded. The cells begin to generate lipid droplets, which are visible under a microscope. The cells also begin to increase their glucose uptake in response to insulin by expressing GLUT4 (*326*, *327*). Again, glucose uptake assays were carried out to confirm that the cells responded appropriately to insulin (*324*). 3T3-L1 adipocytes increased their glucose uptake from 0.41 ± 0.0nmol glucose per

mg protein to 1.13 ± 0.2nmol glucose per mg protein upon insulin stimulation (Fig. 3.2), and this 2.5-3 fold increase was consistently seen.

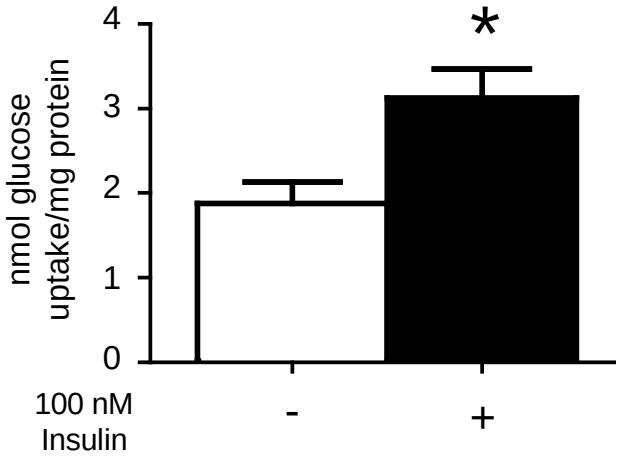

Figure 3.1 Insulin stimulation causes increased glucose uptake in L6 myotubes. Differentiated L6 myotubes were trypsinised and reseeded into 3.5 cm dishes. After two days incubation, the medium was changed to serum free αMEM for 24 hours. Cells were incubated with or without 100nM insulin as indicated and glucose uptake measured. Cells stimulated with insulin show increased glucose uptake. Results are mean±SEM (n=6; * P<0.005; Unpaired T-Test)

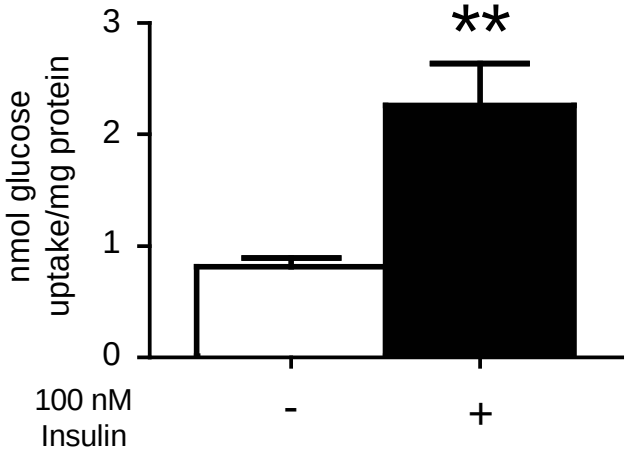

Figure 3.2 Insulin stimulation causes increased glucose uptake in 3T3-L1 adipocytes. Differentiated 3T3-L1 adipocytes were starved in serum free, low glucose DMEM for 24 hours. Cells were incubated with or without 100nM insulin and glucose uptake measured. Cells stimulated with insulin show increased glucose uptake. Results are mean±SEM (n=5; ** P<0.005; Unpaired T-Test)

3.2 Confirming insulin resistance in L6 myotubes and 3T3-L1 adipocytes

Insulin resistance is when cells lose the ability to respond appropriately to insulin (*3*). In the context of skeletal muscle and adipocytes this manifests as an inability to increase glucose uptake in response to insulin (*8*). Therefore, to define cells as insulin resistant, glucose uptake assays had to be carried out on cells incubated with factors known to induce insulin resistance.

Several factors which are known to be elevated in the blood of people suffering from type 2 diabetes, insulin resistance and obesity have previously been shown to induce insulin resistance in cell culture models. These include Ang II (*293*), endothelin-1 (*250*) and TNFα (*302*). Hyperinsulinaemia, indicative of insulin resistance in mammals, can also be modelled in cell culture, and insulin pre-treatment has been shown to cause insulin resistance (*240*, *248*). Individuals suffering from insulin resistance and obesity also exhibit increased oxidative stress in their skeletal muscle and adipose tissue (*111*). As a mimic for oxidative stress hydrogen peroxide has been used to induce insulin resistance in cell culture models (*260*).

Fig. 3.3 shows that exposing L6 myotubes to Ang II (10nM; 24hours), endothelin-1 (100nM; 24 hours), hydrogen peroxide (100μM; 3 hours), insulin (5nM; 12 hours) or TNFα (40nM; 24 hours) completely abolishes the insulin-stimulated glucose uptake seen in control cells, thereby rendering them insulin resistant. It is interesting to note that Ang II and insulin pre-treatments decrease basal glucose uptake, albeit not significantly. Basal glucose uptake in Ang II-induced insulin resistance cells is 71.1 ± 9.9% of the basal uptake in untreated L6 myotubes. The decrease in basal glucose uptake in insulin-induced in insulin resistant L6 myotubes was 60.9 ± 13.1% of untreated basal uptake.

Fig. 3.4 shows treatment with Ang II, endothelin-1, hydrogen peroxide, insulin or TNFα abolished insulin-stimulated glucose uptake in 3T3-L1 adipocytes. Unlike in L6 myotubes Ang II- and insulin-induced insulin resistant 3T3-L1 adipocytes did not exhibit decreased basal glucose uptake.

Under normal, physiological conditions stimulation by insulin leads to the phosphorylation, and activation, of Akt, (see 1.3) (*62*, *101*, *110*). Previous studies have shown that Akt phosphorylation is perturbed in insulin resistance (*240*, *299*). To investigate whether Akt phosphorylation, and therefore activation, was perturbed in the models of insulin resistance used in this study, cell extracts from insulin sensitive and insulin resistant L6 myotubes were prepared from cells before and after insulin stimulation. Western blotting was carried out, to look for changes in Akt phosphorylation.

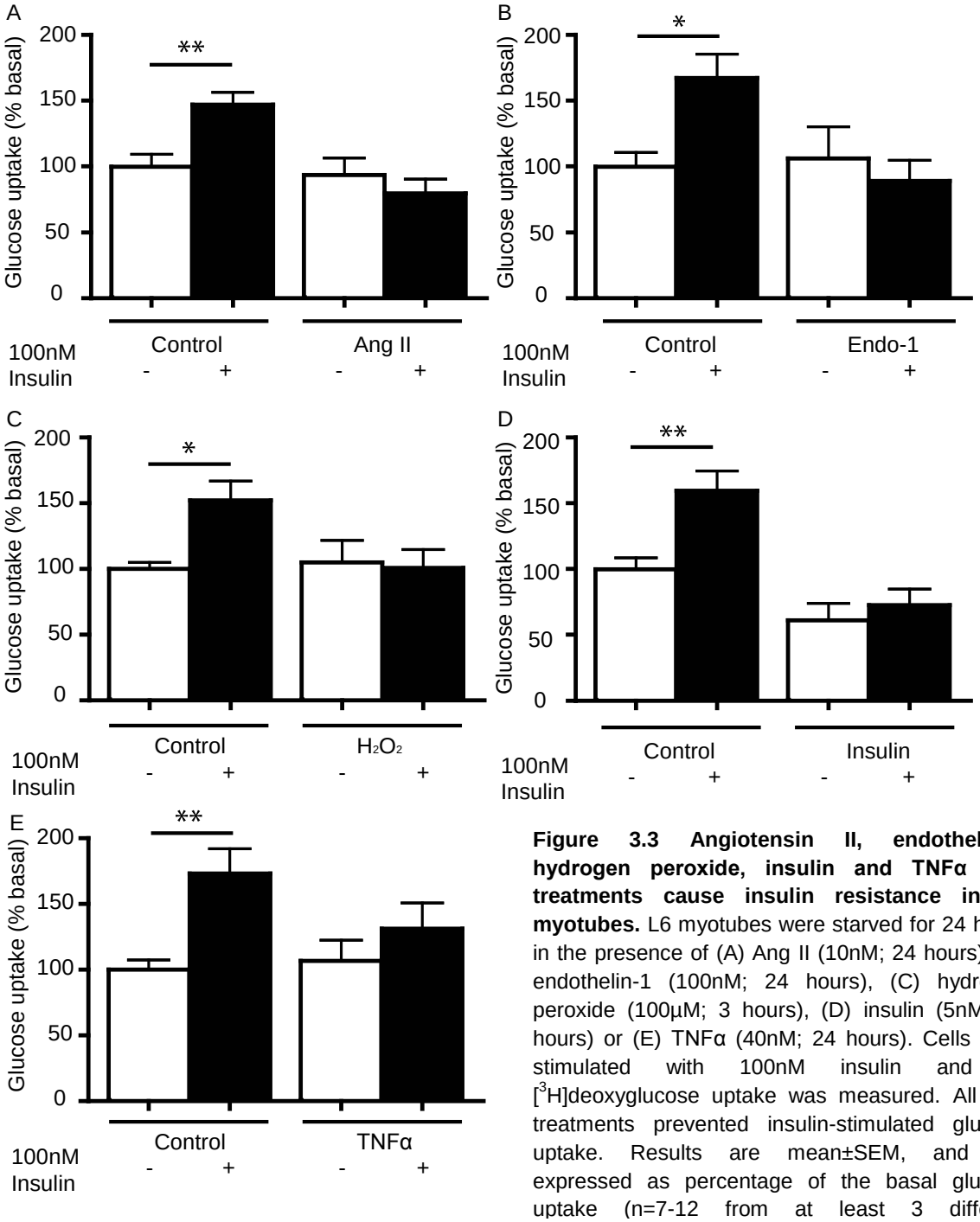

Figure 3.3 Angiotensin II, endothelin-1, hydrogen peroxide, insulin and TNFα pre-treatments cause insulin resistance in L6 myotubes. L6 myotubes were starved for 24 hours in the presence of (A) Ang II (10nM; 24 hours), (B) endothelin-1 (100nM; 24 hours), (C) hydrogen peroxide (100µM; 3 hours), (D) insulin (5nM; 12 hours) or (E) TNFα (40nM; 24 hours). Cells were stimulated with 100nM insulin and 2-[^{3}H]deoxyglucose uptake was measured. All pre-treatments prevented insulin-stimulated glucose uptake. Results are mean±SEM, and are expressed as percentage of the basal glucose uptake (n=7-12 from at least 3 different different independent experiments; * P<0.05; ** P<0.005; One-Way ANOVA with Bonferroni's Multi Comparison Test)

Despite cells showing no significant increase in glucose uptake upon insulin stimulation, Fig. 3.5 shows that Akt phosphorylation was not perturbed in cells rendered insulin resistant by exposure to Ang II, endothelin-1, hydrogen peroxide or insulin. This suggests that, under these conditions Akt activation still occurs correctly, and the deficiency associated with insulin signalling must occur later in the signalling cascade, or along a different pathway. However, the TNFα pre-treatment

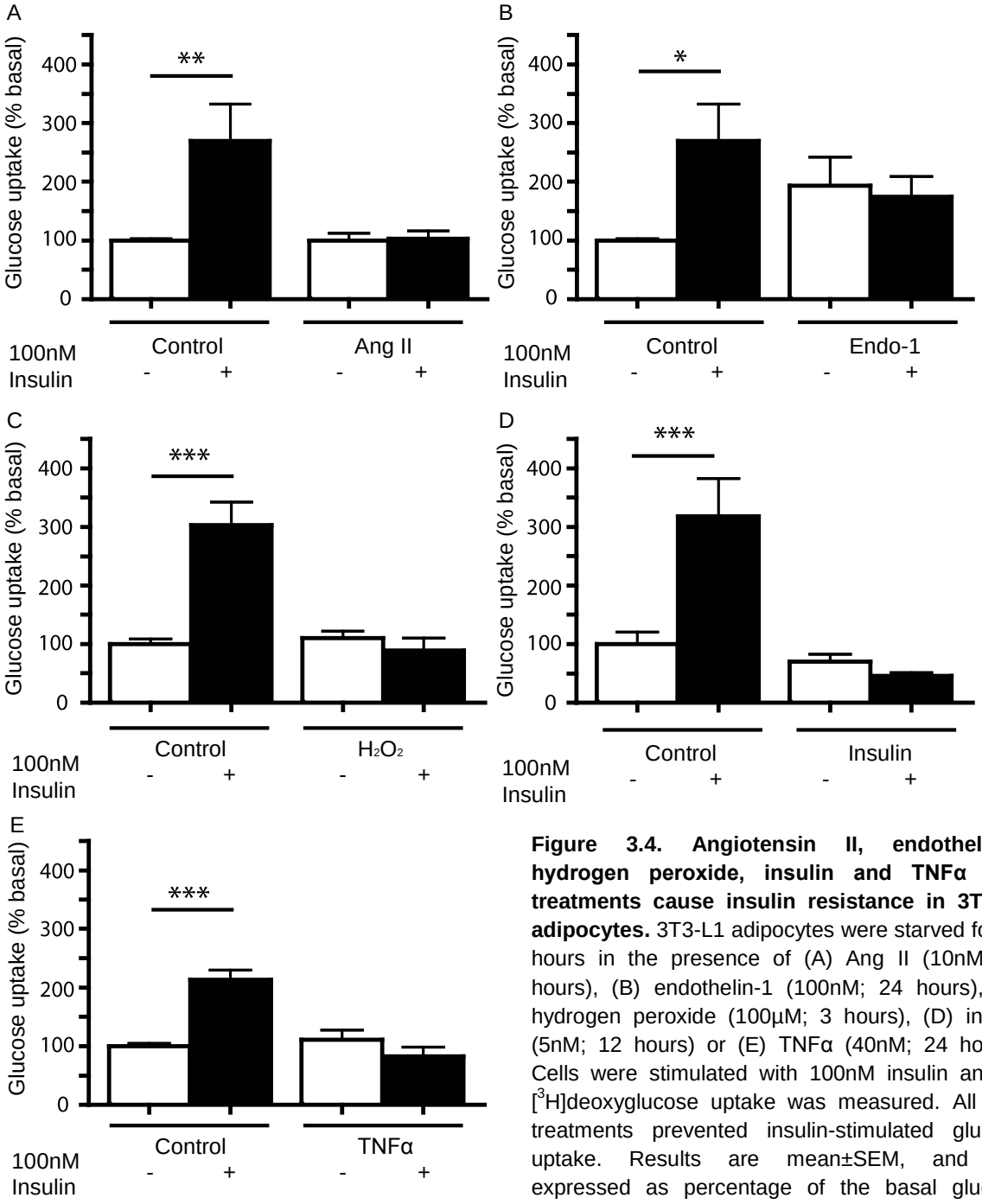

Figure 3.4. Angiotensin II, endothelin-1, hydrogen peroxide, insulin and TNFα pre-treatments cause insulin resistance in 3T3-L1 adipocytes. 3T3-L1 adipocytes were starved for 24 hours in the presence of (A) Ang II (10nM; 24 hours), (B) endothelin-1 (100nM; 24 hours), (C) hydrogen peroxide (100μM; 3 hours), (D) insulin (5nM; 12 hours) or (E) TNFα (40nM; 24 hours). Cells were stimulated with 100nM insulin and 2-[^{3}H]deoxyglucose uptake was measured. All pre-treatments prevented insulin-stimulated glucose uptake. Results are mean±SEM, and are expressed as percentage of the basal glucose uptake (n=8-15 from at least 3 different different independent experiments; * P<0.05; ** P<0.005; *** P< 0.001; One-Way ANOVA with Bonferroni's Multi Comparison Test)

diminishes serine phosphorylation, whilst not affecting the threonine phosphorylation, suggesting diminished TORC2 phosphorylation.

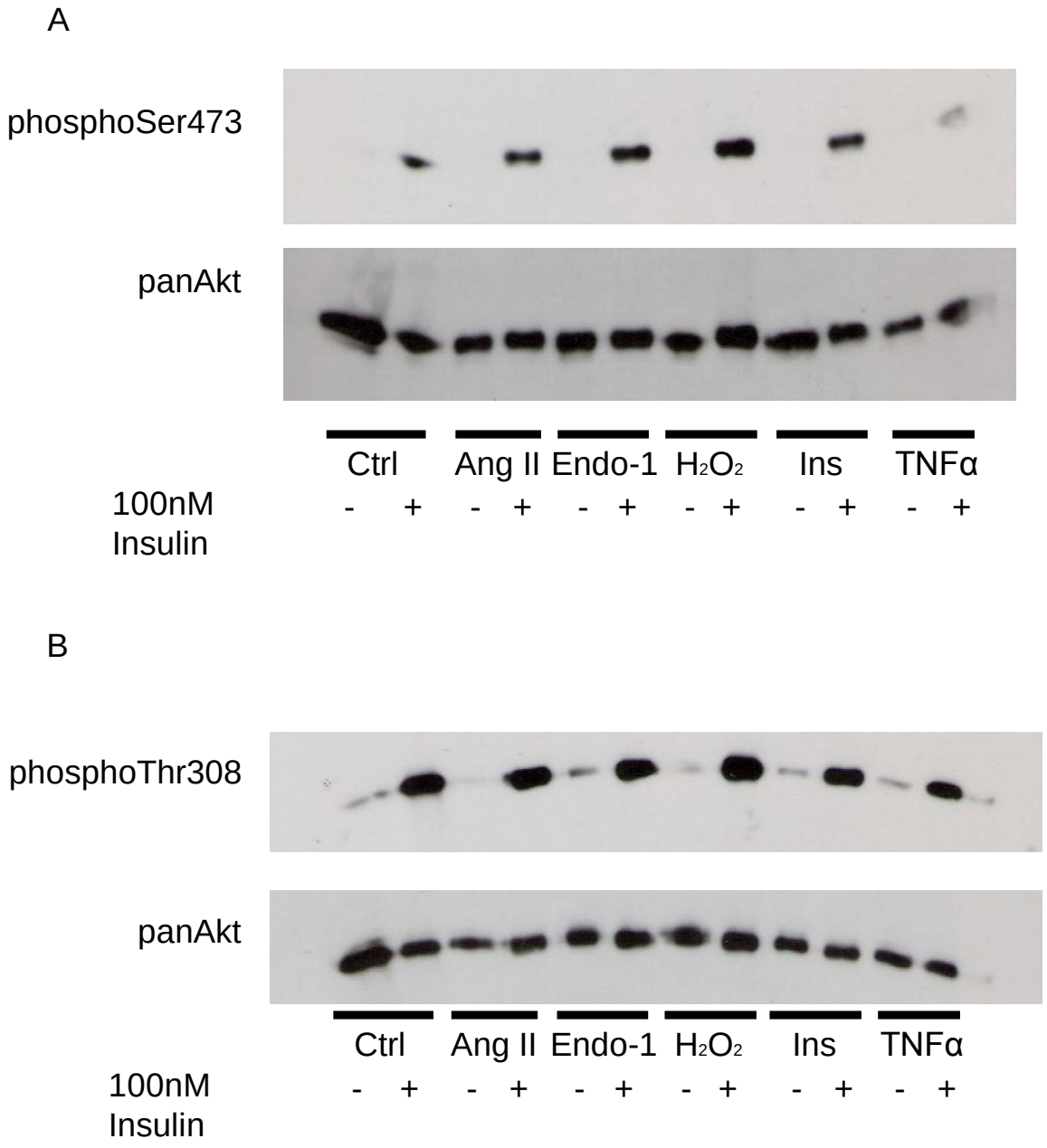

Figure 3.5 Ang II, endothelin-1, hydrogen peroxide and insulin pre-treatments do not affect Akt phosphorylation in L6 myotubes, whereas TNFα lowers Ser phosphorylation. Differentiated L6 myotubes were starved in the presence or absence of Ang II (10nM; 24 hours), endothelin-1 (Endo-1; 100nM; 24 hours), hydrogen peroxide (H2O2; 100µM; 3 hours), insulin (Ins; 5nM; 12 hours) or TNFα (40nM; 24 hours). Cells were then stimulated with 100nM insulin for 10 minutes. TCA precipitation was carried out to collect samples. 50µg total cell extract was separated by SDS-PAGE and western blotting was carried out. Membranes were incubated with (A) anti-phosphoAkt Ser473 or (B) anti-phosphoAkt Thr308, before being stripped and reprobed with anti-panAkt.

3.3 Mass levels of PtdIns(4,5)P_2 are decreased in insulin resistant L6 myotubes and 3T3-L1 adipocytes

It was decided to measure the mass levels of PtdIns(4,5)P_2 using a lipid protein overlay assay similar to that developed by Divecha *et al*. (*158*). Preliminary results showed that L6 myotubes contain 0.76 ± 0.1pmol PtdIns(4,5)P_2 per µg lipid phosphate, and 3T3-L1 adipocytes contain 0.53 ± 0.2pmol PtdIns(4,5)P_2 per µg lipid phosphate (data not shown).

Insulin resistant cells show strikingly decreased whole cells levels of PtdIns(4,5)P_2. The significantly decreased levels were found in all models of insulin resistance in both L6 myotubes (Fig. 3.6) and 3T3-L1 adipocytes (Fig. 3.7). It has been shown previously that PtdIns(4,5)P_2 plasma membrane immunofluorescence is decreased in membrane sheets prepared from 3T3-L1 adipocytes rendered insulin resistant by exposure to insulin (*248*), endothelin-1 (*250*) and glucosamine (*249*) and insulin-induced insulin resistant L6 myotubes (*240*). However, this is the first time the mass measurements of PtdIns(4,5)P_2 levels has been carried out in insulin resistant 3T3-L1 adipocytes and L6 myotubes induced by Ang II, endothelin-1, hydrogen peroxide, insulin and TNFα pre-treatments.

3.4 Using Tubby to investigate PtdIns(4,5)P_2 localisation

It is not known if the PtdIns(4,5)P_2 decrease is localised to specific areas, or if a total plasma membrane decrease occurs. To determine whether the observed PtdIns(4,5)P_2 decrease arises from the loss of a specific pool of lipid, or a more ubiquitous loss, the use of a marker for PtdIns(4,5)P_2 was attempted. The first marker used was the Tubby domain tagged with eGFP (*168*).

The Tubby family is a group of proteins which contain a carboxy-terminal domain known as the Tubby domain (*334*, *335*). The Tubby domain specifically binds to PtdIns(4,5)P_2, but not IP_3 (*151*, *168*, *336*), showing an advantage over the PH domain from PLCδ, a different PtdIns(4,5)P_2 marker which binds both (*337*). The

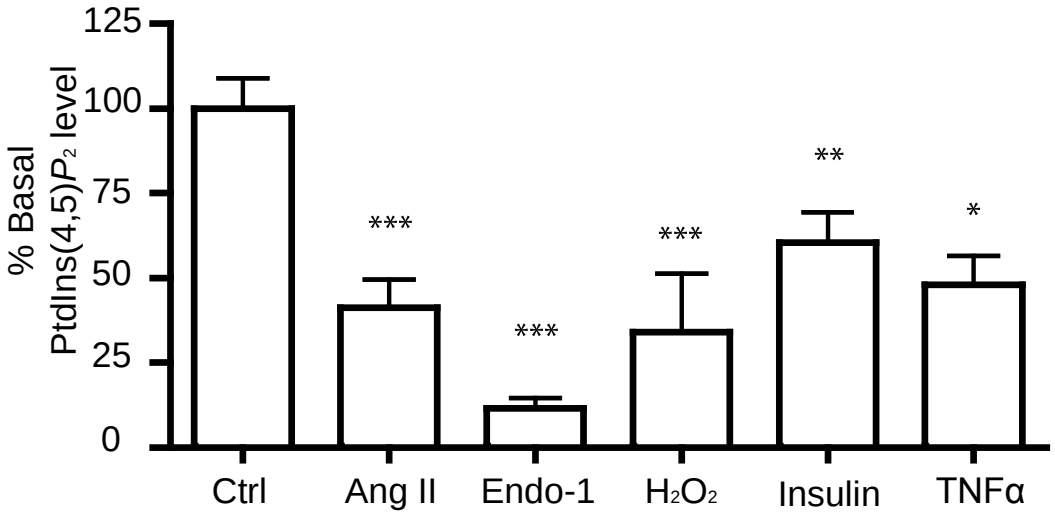

Figure 3.6 Mass levels of PtdIns(4,5)P_2 are decreased in insulin resistant L6 myotubes. Lipids were extracted from pre-treated myotubes, and measured for PtdIns(4,5)P_2 levels. Insulin resistant L6 myotubes showed decreased levels of PtdIns(4,5)P_2. Results are mean±SEM, and are expressed as percentage of basal L6 myotube PtdIns(4,5)P_2 levels. (n=7-28; All significant tests were against unstimulated control; *P<0.05 ; ** P<0.005;*** P<0.001; One-Way ANOVA with Bonferroni's Multi Comparison Test).

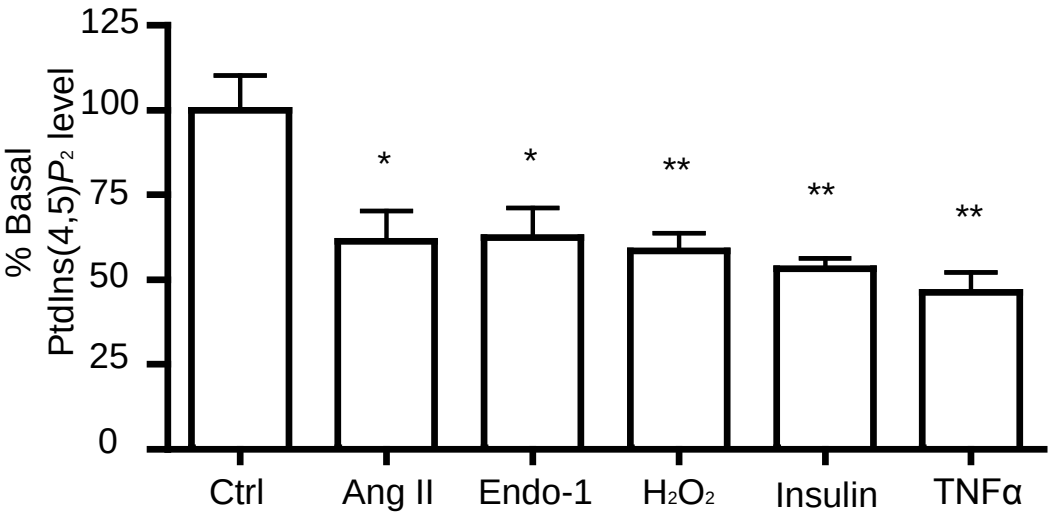

Figure 3.7 Mass levels of PtdIns(4,5)P_2 are decreased in insulin resistant 3T3-L1 adipocytes. Lipids were extracted from pre-treated adipocytes, and measured for PtdIns(4,5)P_2 levels. Results are mean±SEM, and are expressed as percentage of the basal 3T3-L1 adipocyte PtdIns(4,5)P_2 levels. (n=12-30; All significant tests were against control; *P<0.05 ; ** P<0.005; One-Way ANOVA with Bonferroni's Multi Comparison Test)

Tubby domain, tagged with a fluorescent moiety, has been used extensively as a specific marker for PtdIns(4,5)P_2 (*151*, *168*, *338*).

It was decided to subclone the Tubby sequence into the pEGFP-C1 vector to improve the fluorescence properties of the probe. PCR was carried out and the product underwent ligation into the pEGFP-C1 vector. DNA sequencing was also carried out which confirmed the presence of the eGFP-Tubby domain DNA sequence.

Preliminary studies showed plasma membrane localisation in eGFP-Tubby transfected COS-7 cells (Fig. 3.8). However, COS-7 cells transfected with only eGFP showed no such localisation, although they showed some nuclear localisation. It should also be noted that the fluorescence emitted from the eGFP-Tubby was lower than in cells transfected with eGFP only.

As 3T3-L1 adipocytes undergo terminal differentiation (*324*), they become difficult to transfect by traditional means. An Amaxa Nucleofector can be used, and this was attempted. When 3T3-L1 adipocytes were transfected with eGFP-Tubby, the plasma membrane localisation of PtdIns(4,5)P_2 was not observed in untreated cells starved for 24 hours (Fig. 3.9). The lack of clear plasma membrane localisation may be due to this length of time, and hence a long expression time. A long incubation time was chosen, as inducing insulin resistance with Ang II, endothelin-1 and TNFα takes 24 hours (*250*, *293*, *294*). Without clear plasma membrane localisation in the control cells it would be impossible to see any difference between insulin responsive and insulin resistant adipocytes. It was then decided to try a different approach to investigate the localisation of PtdIns(4,5)P_2, using an anti-PtdIns(4,5)P_2 antibody.

Like 3T3-L1 adipocytes, L6 myotubes cells are notoriously hard to transfect, by traditional means, as they do not undergo mitosis (*339*). Currently, the Amaxa Nucleofector cannot be used with L6 myotubes. However, it can be used for the undifferentiated myoblasts. Transfection using the appropriate buffer and programme for the myoblasts was carried out on differentiated L6 myotubes, but the transfection efficiency was very low (data not shown). Therefore transfection of L6 myotubes with eGFP-Tubby was not attempted in L6 myotubes.

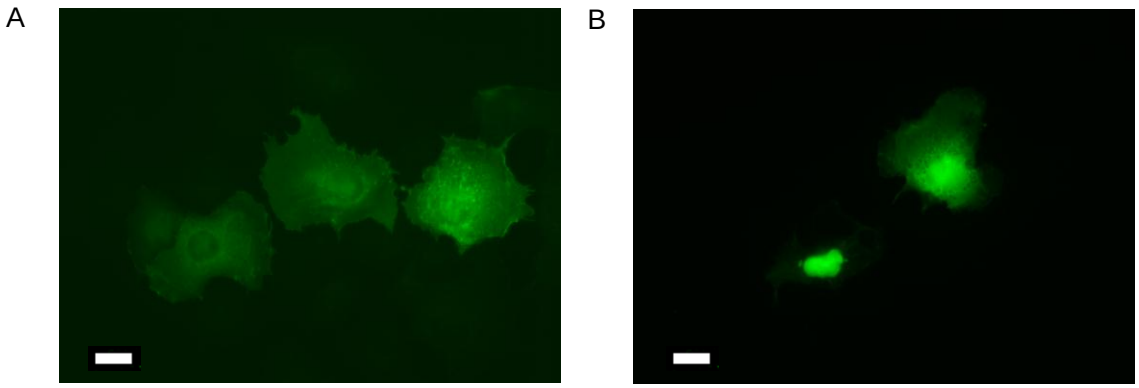

Figure 3.8. eGFP-Tubby shows plasma membrane localisation in COS-7 cells. COS-7 cells were transfected with eGFP-Tubby (A) or eGFP (B), and visualised on an Axioplan 2 microscope. Those cells transfected with eGFP-Tubby shows plasma membrane fluorescence, where cells transfected with GFP do not. Scale bar; 10µm.

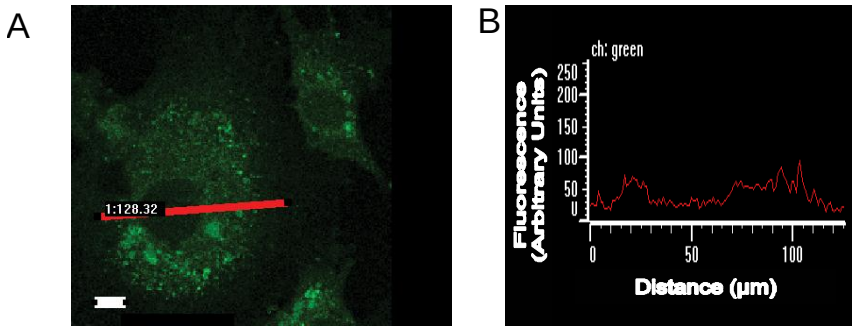

Figure 3.9 eGFP-Tubby does not show plasma membrane localisation in 3T3-L1 adipocytes. Differentiated 3T3-L1 adipocytes were transfected with eGFP-Tubby (A) and visualised on a Nikon upright confocal microscope. There was no plasma membrane localisation. Scale bar; 10µm.

3.5 Looking at PtdIns(4,5)P_2 localisation using immunohistochemistry

Using the method of fixation described by Hammond *et al*. (*161*), an anti-PtdIns(4,5)P_2 antibody was used to look at the location of the lipid. Using 3T3-L1 adipocytes again proved problematic, as there was no clear plasma membrane localisation seen in control cells (Fig. 3.10).

When using the L6 myotubes, however, a clear plasma membrane localisation can be seen in the insulin responsive cells (Fig. 3.11). This is lost in insulin resistant cells exposed to all treatments. These results did not show a localised decrease at

specific areas of the plasma membrane, and suggest that a global plasma membrane decrease occurs. A line profile can be used, which allows the respective fluorescence to be quantified. Fig. 3.11 G is a line profile which shows plasma membrane localisation in insulin-sensitive cells. Figs. 3.11 H-L show that this plasma membrane localisation is lost in insulin-resistant cells.

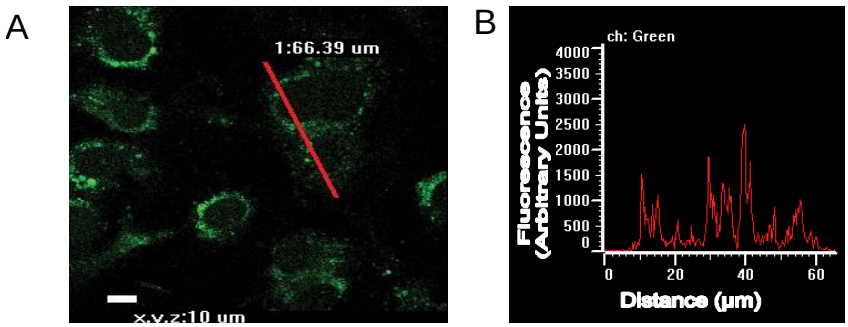

Figure 3.10 Immunohistochemistry showed no plasma membrane localisation in control 3T3 -L1 adipocytes. 3T3-L1 were starved for 24 hours were fixed following the instructions in Hammond *et al*., and incubated with anti- PtdIns(4,5)P_2 antibody, before washing and incubation with Alexa Fluor 488 Goat anti-mouse F(ab')2 fragment antibody. Afterwards cells were fixed again and visualised on a Nikon upright confocal microscope. A shows representative image of untreated 3T3-L1 adipocytes. B shows a line profile, across the red line in A. Line profiles measure the amount of fluorescence. Scale bar; 10µm

3.6 *Carrier-mediated delivery of PtdIns(4,5)*P_2 *restores insulin sensitivity in insulin resistant L6 myotubes*

Thus far, a correlation between insulin resistance and decreased PtdIns(4,5)P_2 levels in L6 myotubes and 3T3-L1 adipocytes has been identified. To determine whether the decreased levels of PtdIns(4,5)P_2 have a causative role in insulin resistance, or were simply a side effect, it is necessary to investigate whether restoring PtdIns(4,5)P_2 to basal levels, would also restore the insulin-stimulated glucose uptake seen in control insulin responsive cells. Carrier-mediated delivery of PtdIns(4,5)P_2 was therefore carried out.

Previous studies have shown that by using carrier mediated delivery of synthetic PtdIns(4,5)P_2, with two palmitoyl fatty acid chains, insulin-stimulated glucose uptake can be restored in insulin-induced insulin resistant L6 myotubes (*240*) and insulin- (*248*), endothelin-1- (*250*) and glucosamine- (*249*) induced insulin resistant 3T3-L1

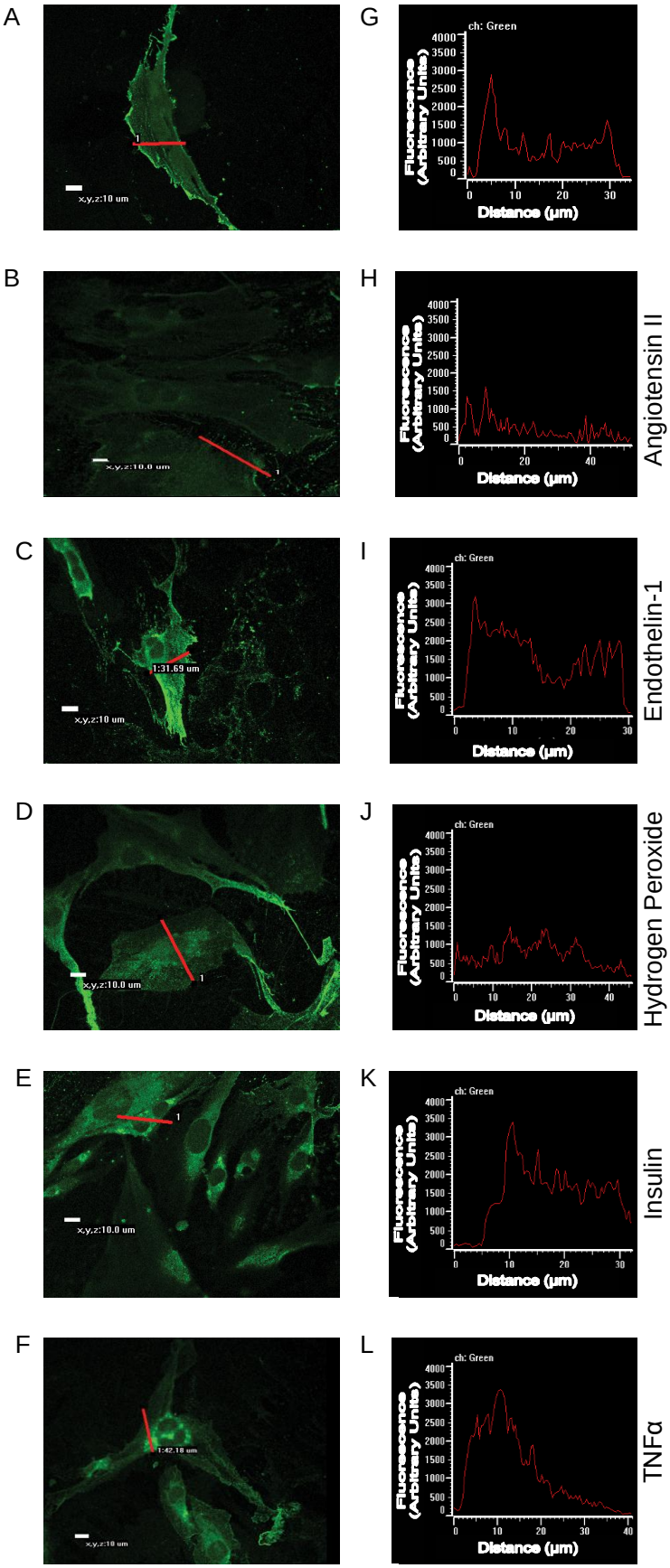

Figure 3.11 Plasma membrane localisation of PtdIns(4,5)P_2 is lost in insulin resistant L6 myotubes. Pre-treated L6 myotubes were fixed following the instructions in Hammond *et al*. (161), and incubated with anti- PtdIns(4,5)P_2 antibody, before washing and incubation with Alexa Fluor 488 Goat anti-mouse F(ab')2 fragment antibody. Afterwards they were fixed again and visualised on a Nikon upright confocal microscope. A-F show representative images. G-L show a line profile, across the red line. Line profiles measure the amount of fluorescence. Scale bar; 10µm

adipocytes. As di-palmitoylated PtdIns(4,5)P_2 occurs only rarely naturally (*340*), it is more physiologically relevant to use PtdIns(4,5)P_2 purified from porcine brain to

restore levels. This lipid is natural, and contained mostly 1-stearoyl-2-arachidonyl, which is the predominant form in mammals (*340*, *341*).

Due to time constraints, we chose to investigate further insulin resistance induced by insulin and angiotensin II in L6 myotubes. L6 myotubes were chosen as skeletal muscle is the site for the majority of insulin-stimulated glucose uptake in vivo (*8*). These two treatments were selected because they caused the largest decrease in PtdIns(4,5)P_2 levels, whilst inducing insulin resistance reliably (see Figs. 3.3 and 3.6).

Carrier-mediated delivery of PtdIns(4,5)P_2 restored the PtdIns(4,5)P_2 levels in insulin-induced insulin resistant cells (Fig. 3.12a). Using the carrier only did not affect PtdIns(4,5)P_2 levels. When the PtdIns(4,5)P_2 was restored to levels found in control cells, the insulin sensitivity was restored and insulin caused an increase glucose uptake similar to untreated cells (Fig. 3.12b).

It is interesting to note that carrier-mediated delivery of PtdIns(4,5)P_2 appears to increase the basal uptake of glucose in insulin-induced insulin resistant cells. When treated with carrier alone, insulin-induced insulin resistant cells take up 58.3 ± 12.4% of unstimulated control levels. However, when incubated with PtdIns(4,5)P_2 and carrier basal levels of glucose uptake increase to 100.8 ± 13.3% of unstimulated control levels. It has been previously suggested that PtdIns(4,5)P_2 is necessary to activate GLUT4 (*87*, *148*, *149*). As mentioned earlier (1.5) a proportion of GLUT4 is always present at the plasma membrane (*243*), and the fact that replenishing PtdIns(4,5)P_2 increases glucose uptake to control levels, without insulin stimulation, suggests that this lipid could be playing a role in activating GLUT family members.

In Ang II-induced insulin resistant L6 myotubes, carrier-mediated delivery of PtdIns(4,5)P_2 also restored PtdIns(4,5)P_2 levels (Fig. 3.13a). L6 myotubes with replenished PtdIns(4,5)P_2 exhibit partially restored insulin sensitivity (Fig. 3.13b). Ang II-induced insulin resistant cells treated with carrier only showed decreased basal glucose uptake, to 63.7 ± 12.1% of unstimulated control level. However, unlike in insulin-induced insulin resistant cells, PtdIns(4,5)P_2 replenishment does not restore this basal glucose uptake. Insulin stimulation causes a 2-fold increase in glucose uptake in cells treated with carrier-mediated delivery of PtdIns(4,5)P_2.

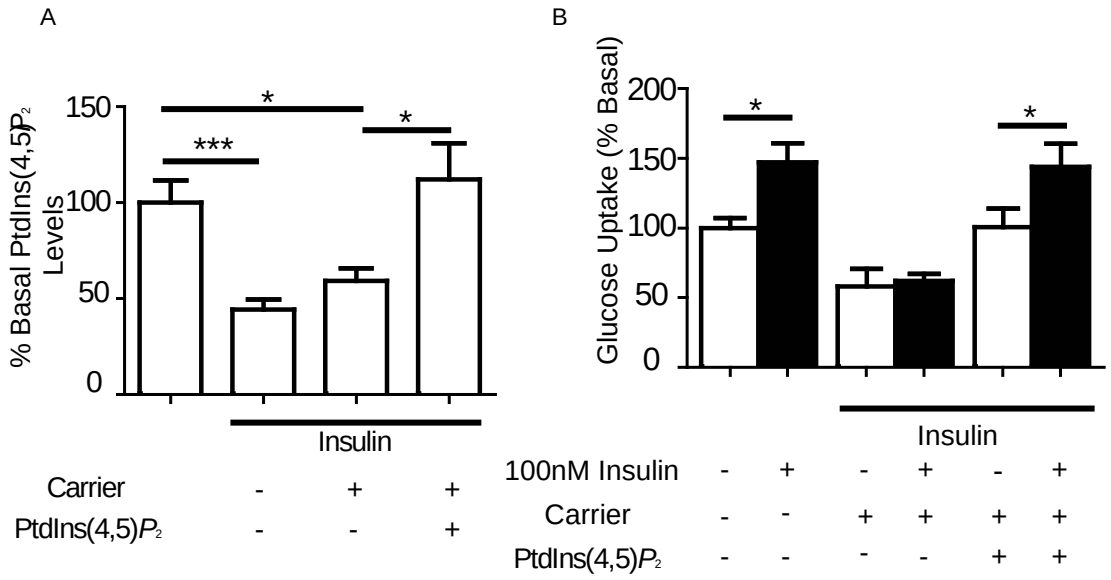

Figure 3.12 Carrier-mediated delivery of PtdIns(4,5)P_2 restores insulin sensitivity in insulin-induced insulin resistant L6 myotubes. Differentiated L6 myotubes were treated with insulin (5nM;12 hours) to induce insulin resistance. Cells were then treated with carrier only or carrier and 1.25µM PtdIns(4,5)P_2. (A) Lipids were extracted and PtdIns(4,5)P_2 levels were measured. Results are mean ± SEM , and are expressed as percentage of the control PtdIns(4,5)P_2. Treatment with carrier only did not alter PtdIns(4,5)P_2 levels in insulin resistant cells, but carrier-mediated delivery of PtdIns(4,5)P_2 increased PtdIns(4,5)P_2 levels. (B) Cells were stimulated with 100nM insulin and 2-[^{3}H]deoxyglucose uptake was measured. Results are mean ± SEM, and are expressed as percentage of the basal glucose uptake. Carrier-mediated delivery of PtdIns(4,5)P_2 restored insulin sensitivity, whereas carrier only treatment did not. (n=9-15 for the PtdIns(4,5)P_2 measurements; n=7-9 for glucose uptake measurements ; * P<0.05; *** P<0.001; One-way ANOVA with Bonferroni's Multi Comparison Test)

In untreated control cells insulin also produces a 2-fold increase in glucose uptake. This could suggest that Ang II is having a negative effect on other GLUT family members, as insulin-stimulated glucose uptake is a similar magnitude between untreated cells, and Ang II-treated cells treated with carrier-mediated delivery of PtdIns(4,5)P_2.

The fact that restoring PtdIns(4,5)P_2 levels reverses insulin resistance shows a causal link between decreased PtdIns(4,5)P_2 and insulin resistance. This is backed up by findings already reported in the literature, that PtdIns(4,5)P_2 is necessary for insulin-stimulated glucose uptake (*148*, *240*, *248–250*).

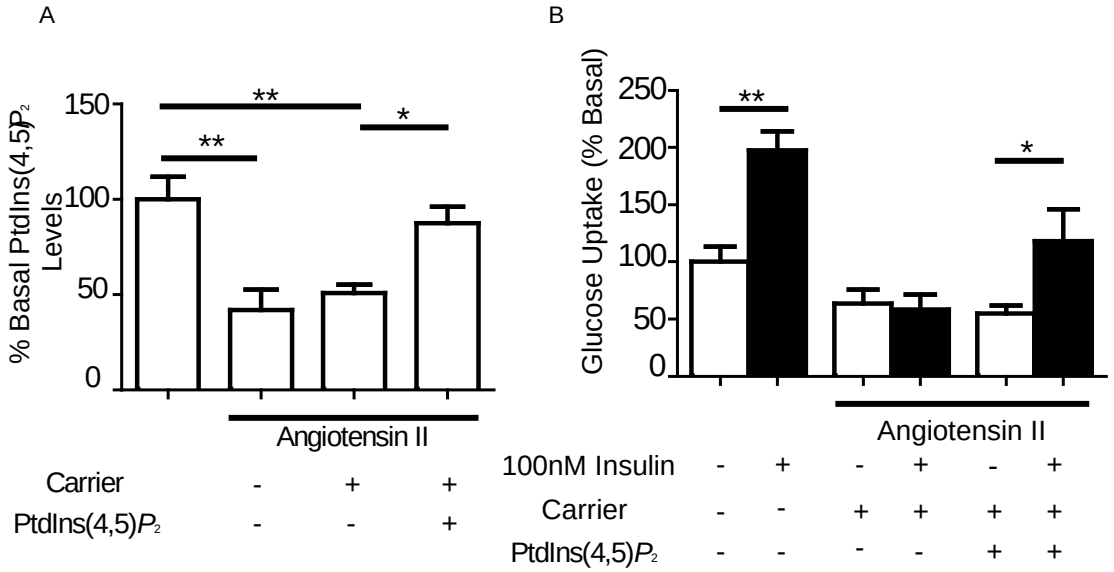

Figure 3.13 Carrier-mediated delivery of PtdIns(4,5)P_2 partially restores insulin sensitivity in Ang II-induced insulin resistant L6 myotubes. Differentiated L6 myotubes were treated with Ang II (10nM;24 hours) to induce insulin resistance. Cells were then treated with carrier only or carrier and 1.25µM PtdIns(4,5)P_2. (A) Lipids were extracted and PtdIns(4,5)P_2 levels were measured. Results are mean ± SEM, and are expressed as percentage of the control PtdIns(4,5)P_2. Treatment with carrier only did not alter PtdIns(4,5)P_2 levels in insulin resistant cells, but carrier-mediated delivery of PtdIns(4,5)P_2 increased PtdIns(4,5)P_2 levels. (B) Cells were stimulated with 100nM insulin and 2-[^{3}H]deoxyglucose uptake was measured. Results are mean ± SEM, and are expressed as percentage of the basal glucose uptake. Carrier-mediated delivery of PtdIns(4,5)P_2 partially restored insulin sensitivity, whereas carrier only treatment did not. (n=9-15 for the PtdIns(4,5)P_2 measurements; n=7-9 for glucose uptake measurements ; * P<0.05; ** P<0.005; One-way ANOVA with Bonferroni's Multi Comparison Test)

3.7 Oxidative stress

Oxidative stress is thought to play a role in inducing insulin resistance (see 1.6.3), and the use of antioxidants has been used to restore insulin sensitivity (*291*, *293*). Therefore it was decided to investigate the use of NAC, the NOX inhibitor DPI and PEG-catalase on insulin sensitivity.

Preliminary results showed that incubating L6 myotubes with DPI and PEG-catalase for 24 hours actually prevented insulin-stimulated glucose uptake, without any other factors being present (Fig. 3.14). Incubating L6 myotubes with NAC caused cell death. Similar results were seen in 3T3-L1 adipocytes (Fig. 3.15); 24 hour exposure to PEG-catalase and NAC induced insulin resistance, whereas DPI caused cell death. Therefore it was decided to abandon experiments using antioxidants to potentially reverse insulin resistance.

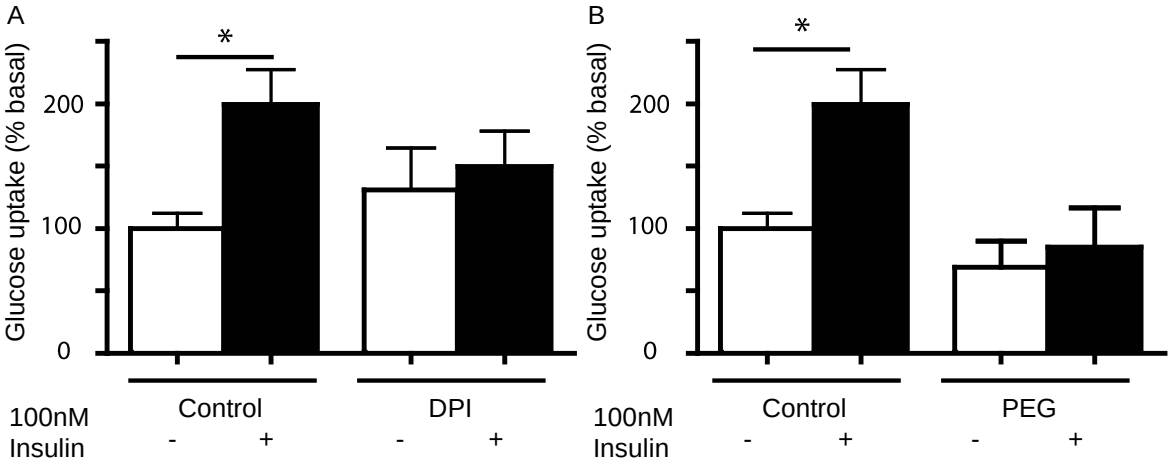

Figure 3.14 The antioxidants DPI and PEG-catalase induce insulin resistance in L6 myotubes. Differentiated L6 myotubes were starved for 24 hours in the presence of (A) DPI (5µM; 24 hours) or (B) PEG-Catalase (10µM; 24 hours). Cells were stimulated with 100nM insulin and 2-[^{3}H]deoxyglucose uptake was measured. All pre-treatments prevented insulin-stimulated glucose uptake. Results are mean ± SEM, and are expressed as percentage of the basal glucose uptake (n=4-6 from at least 3 different independent experiments; * P<0.05; One-way ANOVA with Bonferroni's Multi Comparison Test)

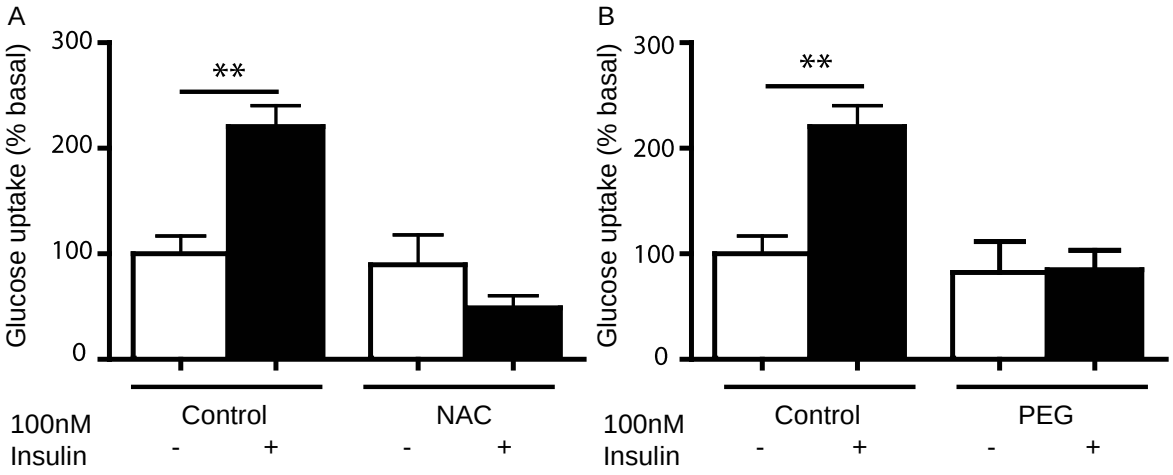

Figure 3.15 The antioxidants NAC and PEG-catalase induce insulin resistance in 3T3-L1 adipocytes. 3T3-L1 adipocytes were starved for 24 hours in the presence of (A) NAC (20mM; 24 hours) or (B) PEG-Catalase (10µM; 24 hours). Cells were stimulated with 100nM insulin and 2-[^{3}H]deoxyglucose uptake was measured. All pre-treatments prevented insulin-stimulated glucose uptake. Results are mean ± SEM, and are expressed as percentage of the basal glucose uptake (n=5-8 from at least 3 different independent experiments; ** P<0.005; One-way ANOVA with Bonferroni's Multi Comparison Test)

3.8 Discussion

These results presented here show that there is a clear correlation between insulin resistance, induced by Ang II, endothelin-1, H_2O_2, insulin and TNFα, and decreased mass PtdIns(4,5)P_2 levels in both L6 myotubes and 3T3-L1 adipocytes. Furthermore, restoring the PtdIns(4,5)P_2 levels in insulin- and Ang II-induced insulin resistant L6 myotubes can restore insulin sensitivity, and increase glucose uptake. This shows that PtdIns(4,5)P_2 plays a vital role in insulin-stimulated glucose uptake. These results confirm and extend previous studies which have shown that carrier-mediated delivery of synthetic PtdIns(4,5)P_2 can reverse insulin-, endothelin-1- and glucosamine-induced insulin resistance (*240*, *248–250*).

The first aim of this project was to investigate the levels of PtdIns(4,5)P_2 in insulin resistant cells, and as such it was necessary to induce insulin resistance in L6 myotubes and 3T3-L1 adipocytes. Previously it has been shown that treating cells with Ang II (*293*), endothelin-1 (*250*, *253*), hydrogen peroxide (*260*), insulin (*240*, *248*) or TNFα (*294*) can induce insulin resistance in cell culture models. This was replicated in this study in both cell types, as treatment with all factors abolished insulin-stimulated glucose uptake.

In L6 myotubes, Akt phosphorylation was also investigated. As mentioned earlier (1.3.2) insulin stimulation leads to the phosphorylation and activation of Akt which plays vital roles in GLUT4 translocation (*243*, *342*). With the exception of TNFα-induced insulin resistance there was no difference in Akt phosphorylation between insulin sensitive and insulin resistant L6 myotubes. This could suggest that the Akt pathway of insulin signalling is unperturbed in insulin resistance induced by Ang II, endothelin-1, hydrogen peroxide or insulin. As well as stimulating the phosphorylation of Akt, PI3Ks also cause the activation of other pathways, known to play a role in insulin signalling, notably Rac-1 (*145*) and aPKC activation (*133*), and it is possible that these pathways are perturbed and thus preventing insulin-stimulated glucose uptake. Rac-1 is important for insulin-stimulated actin reorganisation in L6 myotubes (*143*) and recent work has shown that activity of Rac-1 is diminished in ceramide-induced insulin resistance, in a PI3K-independent mechanism (*343*).

TNFα reduces serine phosphorylation of Akt. TNFα treatment has been shown to activate Akt (*344*, *345*) in the short term. Akt activity in HEK 293 cells was maximal after a 4 hour treatment with TNFα, whereas after 24 hours exposure to TNFα Akt activity was lower than in unstimulated cells (*344*). This suggests that long term TNFα exposure is inhibiting Akt activation, although the phosphorylation state of Akt was not investigated in this study.

24 hours exposure to endothelin-1, as used in this study, has been shown previously to diminish Akt phosphorylation in 3T3-L1 adipocytes (*299*). Even a short term (7 minutes) exposure to endothelin-1 has been shown to perturb PI3K activation and signalling (*346*), although Akt phosphorylation has been shown to be stimulated by a short term exposure to endothelin-1 (*347*). However, there have been no papers investigating Akt phosphorylation or PI3K signalling in endothelin-1-induced insulin resistant L6 myotubes, possibly suggesting different roles for endothelin-1 in the induction of insulin resistance in specific tissues.

The insulin treatment used in this study to induce insulin resistance (5nM; 12 hours) has already been shown not to affect Akt phosphorylation in 3T3-L1 adipocytes (*248*) and L6 myotubes (*240*). However, using a higher concentration of insulin (100nM) has been shown to prevent Akt phosphorylation (*240*).

A possible flaw could be the anti-phospho-Akt antibody itself, in that it does not recognise specific isoforms of Akt, and will bind to phosphorylated Akt1 or 2 equally. This could explain why the Akt phosphorylation does not appear to be altered in cells which fail to increase glucose uptake in response to insulin. As mentioned earlier (see 1.3.2), Akt2 is the most important family member in insulin-stimulated glucose uptake, but Akt1 is also phosphorylated by insulin signalling (*110*, *114*). As the antibody cannot distinguish between Akt family members it would be impossible to decipher if phosphorylation of a single family member was altered in insulin resistance. To investigate specific Akt isoforms it is possible to carry out an immunoprecipitation using an isoform-specific anti-Akt antibody. Then carry out western blotting using the anti-phospho-Akt antibodies.

Previously it has been shown that membrane sheets from 3T3-L1 adipocytes treated with insulin (*248*), endothelin-1 (*250*) or glucosamine (*249*), and L6 myotubes treated with insulin (*240*), exhibit decreased PtdIns(4,5)P_2 immunofluorescence . However, it

is important to look at the mass levels of the lipid, as this decreased immunofluorescence could be due to decreased levels of PtdIns(4,5)P_2 or because a binding protein is sequestering the lipid (*249*). This study has shown that the reduced immunofluorescence is due to decreased levels of PtdIns(4,5)P_2 in insulin resistant cells.

To look at the localisation of the lipid, it was decided to transfect cells with eGFP-Tubby. COS-7 transfected with eGFP-Tubby showed plasma membrane localisation, but this was not observed in 3T3-L1 adipocytes. As 3T3-L1 adipocytes do not undergo mitosis, they cannot be transfected by traditional means. However, they can be transfected using the Amaxa Nucleofector. This involves trypsinising the cells, and the amount of time needed for the cells to recover, as well as the length of time needed to induce insulin resistance (*250*, *293*, *294*) may have contributed to the lack of visible plasma membrane localisation. L6 myotubes cannot be transfected by the Nucleofector, so transfection with eGFP-Tubby was not attempted with these cells.

The immunohistochemistry approach using an anti-PtdIns(4,5)P_2 antibody was more successful, though only in L6 myotubes. Again, in 3T3-L1 adipocytes a clear plasma membrane localisation could not be seen in insulin sensitive cells. However, in insulin sensitive L6 myotubes a clear plasma membrane localisation can be seen, and this was completely lost in insulin resistant cells.

The fact that the 3T3-L1 adipocytes showed no distinguishable PtdIns(4,5)P_2 fluorescence was unexpected. The mass levels of PtdIns(4,5)P_2 in the adipocytes were lower than in L6 myotubes which could explain why immunofluorescence could only be seen in control L6 myotubes. However, recent work from the Elmendorf laboratory was able to use an antibody against PtdIns(4,5)P_2 to investigate immunofluorescence at the plasma membrane sheets in L6 myotubes (*240*) and 3T3-L1 adipocytes (*248*, *250*). Whilst immunofluorescence was decreased in insulin resistant cells, it was not below the level of detection, as this study has found. This may be due to the accessibility of PtdIns(4,5)P_2 in membrane sheets compared to that in whole cells. Another difference could be the actual antibody, as the antibody used by this study is different from that used by the Elmendorf laboratory (*240*, *248*–*250*).

It has been previously been shown that the actin dysregulation seen in insulin resistant skeletal muscle (*240*, *242*) and adipocytes (*348*) can be replicated in 3T3-L1 adipocytes (*248–250*, *253*) and L6 myotubes (*240*). As mentioned earlier (1.5), PtdIns(4,5)P_2 and many of its binding partners, play a role in actin regulation. By replenishing levels of PtdIns(4,5)P_2 with carrier-mediated delivery, not only is the insulin-stimulated glucose uptake restored, but the actin reorganisation is also (*240*, *248–250*). PtdIns(4,5)P_2 is known to interact with several actin binding proteins (*256–258*, *319*). It is therefore thought that PtdIns(4,5)P_2 regulates glucose uptake via its interactions with actin and actin binding proteins.

It is thought that syntaxins, which are necessary for GSV fusion, require PtdIns(4,5)P_2 for full activation (*190*). Decreased PtdIns(4,5)P_2 levels could prevent syntaxins from functioning, meaning that GSVs would be unable to fuse with the plasma membrane, and GLUT4 is not inserted into the plasma membrane.

PtdIns(4,5)P_2 is also thought to play a role in activation of GLUT4 (*87*, *148*). Carrier-mediated delivery of PtdIns(4,5)P_2 increases basal glucose uptake in insulin-induced insulin resistance (Fig. 3.12). This could suggest that the increase in PtdIns(4,5)P_2 is activating GLUT family members already present at the plasma membrane. However, this could not be seen in Ang II-treated cells (Fig. 3.13) suggesting that Ang II treatment may be preventing other members of the GLUT family from taking up glucose. GLUT1 and GLUT3 are both expressed in L6 myotubes (*349*) and could be inhibited by Ang II treatment. In fact Ang II has been shown to increase GLUT1 internalisation in a smooth muscle cell line (*350*), so it is possible that Ang II is carrying out a similar function in L6 myotubes. Currently the effect of Ang II on GLUT3 has not been investigated.

Oxidative stress is known to play an important role in insulin resistance (*317*), and antioxidants have been used previously to restore insulin sensitivity in 3T3-L1 adipocytes, L6 myotubes (*17*, *293*) and animals (*17*). However in this study these results could not be repeated. Instead, pre-treatments with the antioxidants NAC, PEG catalase, and the NOX inhibitor DPI lead to either insulin resistance or to cell death. In L6 myotubes 24 hour incubation with NAC lead to cell death, and treatment with DPI and PEG catalase lead to an abolition of insulin-stimulated glucose uptake,

whereas in 3T3-L1 adipocytes PEG catalase and NAC treatments prevented insulin-stimulated glucose uptake and exposure to DPI caused cell death.

It is known that insulin stimulation leads to ROS production, and it thought that this actually makes physiological contribution to insulin signalling and glucose uptake (*316*, *351*). The production of ROS has a role in regulating protein tyrosine phosphatases (PTPs) (*352*). PTPs play a crucial role in stopping RTK signalling, by dephosphorylating the tyrosine resides phosphorylated by the receptor. PTPs contain a cysteine residue, which must be reduced for dephosphorylation to occur (*353*). The reduced cysteine is necessary for forming a cysteinyl-phosphate intermediate with the relevant phosphorylated tyrosine (*351*). After insulin signalling levels of ROS transiently increase (*351*), leading to the oxidation of the necessary cysteine residue, which prevents PTPs from carrying out dephosphorylation of tyrosine residues (*316*, *354*). This means that RTKs, including the IR, and their targets stay phosphorylated, elongating signalling time (*316*, *353*, *354*).

PTP1B is thought to be the family member which negatively regulates insulin signalling (*351*, *354*), and shows a high specific activity towards the IR and IRS family members (*355*, *356*). Insulin stimulation prevents PTP1B enzyme activity by ~80%, but this can be reversed by treatment with PEG catalase (*354*). Blocking the inhibition of PTP1B leads to reduced phosphorylation of the IR and IRS family members (*354*).

Exposure to other antioxidants may well induce insulin resistance by preventing this ROS production, and allowing PTP1B to stay active. In keratinocytes TNFα stimulates Akt phosphorylation in a ROS-dependent mechanism (*345*, *357*). Treating cells with NAC has also been shown to decrease Akt phosphorylation caused by incubation with TNFα (*345*). This suggests that the long term treatment with antioxidants may be preventing glucose uptake by preventing the insulin stimulated ROS production, or by inducing cell death.

To summarise, this chapter has shown a clear link between decreased PtdIns(4,5)P_2 levels and insulin resistance in L6 myotubes and 3T3-L1 adipocytes. By restoring PtdIns(4,5)P_2 levels in insulin- and Ang II-induced insulin resistant cells, some insulin sensitivity was restored. In the case of insulin-induced insulin resistance this restoration of insulin sensitivity was almost identical to control cells. However, in Ang

II-induced insulin resistance this was a partial restoration, possibly due to the decreased basal glucose uptake.

The next aim for this project was to investigate the mechanism behind the PtdIns(4,5)P_2 decrease by investigating PIPkin, phosphatase and PLC activity. Once the mechanism was elucidated, attempts to reverse insulin resistance by manipulating this mechanism were carried out.

4. Identifying the mechanism behind the PtdIns(4,5)P_2 decrease in insulin resistant cells

As shown in the previous chapter there is a causative correlation between decreased PtdIns(4,5)P_2 levels and insulin resistance in L6 myotubes. Insulin sensitivity could be restored in L6 myotubes rendered insulin resistance by pre-treatments with Ang II (10nM; 24 hours) or insulin (5nM; 12 hours), by replenishing PtdIns(4,5)P_2 levels with carrier-mediated delivery. Therefore it was decided to investigate the mechanism behind the PtdIns(4,5)P_2 by looking at the 3 families of proteins which control levels of PtdIns(4,5)P_2; the PIPkins, phosphoinositide phosphatases and PLCs (*107*, *184*) (described in 1.4.3-1.4.6).

4.1 PIPkin activity is unaltered in membranes from insulin resistant L6 myotubes

PtdIns(4,5)P_2 is generated by a family of kinases known collectively as the PIPkins (see 1.4.4). PIP5kins use PtdIns4*P* and PIP4kins use PtdIns5*P* as their substrate (*188*). To investigate the possibility that in insulin resistance the activity of the PIPkins was perturbed membrane extracts were taken from insulin sensitive and insulin resistant L6 myotubes. It was decided to investigate cells rendered insulin resistant by exposure to insulin and Ang II pre-treatment. These conditions were chosen as they caused the greatest decrease in PtdIns(4,5)P_2 whilst reliably inducing insulin resistance (Figs. 3.3 and 3.6). Also the previous chapter has shown that carrier-mediated delivery of PtdIns(4,5)P_2 can partially restore insulin sensitivity in Ang II- and insulin-induced insulin resistant L6 myotubes (Figs. 3.12 and 3.13).

Membrane extracts were incubated with ^{32}P-γ-ATP, allowing the endogenous PIPkins present to create lipid products using the endogenous lipids. Lipids were then extracted, separated by TLC and levels of PtdIns(4,5)P_2 measured using a phosphoimager. This allowed the amount of ^{32}P-PtdIns(4,5)P_2 generated to be quantified, and the activity of the kinases to be investigated.

Figs. 4.1 and 4.2 show that there were no significant differences in kinase activity between insulin sensitive and insulin resistant L6 myotubes. The amount of ^{32}P-PtdIns(4,5)P_2 produced by the membranes was not altered between insulin sensitive and insulin resistant cells.

As the kinase activity assays utilised endogenous lipids, it is possible that the simple addition of γ-^{32}P-ATP was masking any differences in PIPkin activity due to differences in the amount of substrate. There are two families of PIPkins, with distinct substrates, and it was decided to investigate if the activity of a single family could be perturbed by using micelles of synthetic PtdIns4*P* or PtdIns5*P*. These micelles provided an excess of substrate, allowing for maximal kinase activity. Again, there was no significant difference in the level of ^{32}P-PtdIns(4,5)P_2 generated in membranes from insulin sensitive or insulin resistant cells (Figs. 4.1 and 4.2). This suggests that both the PIP5kins and PIP4kins are unperturbed in insulin resistance.

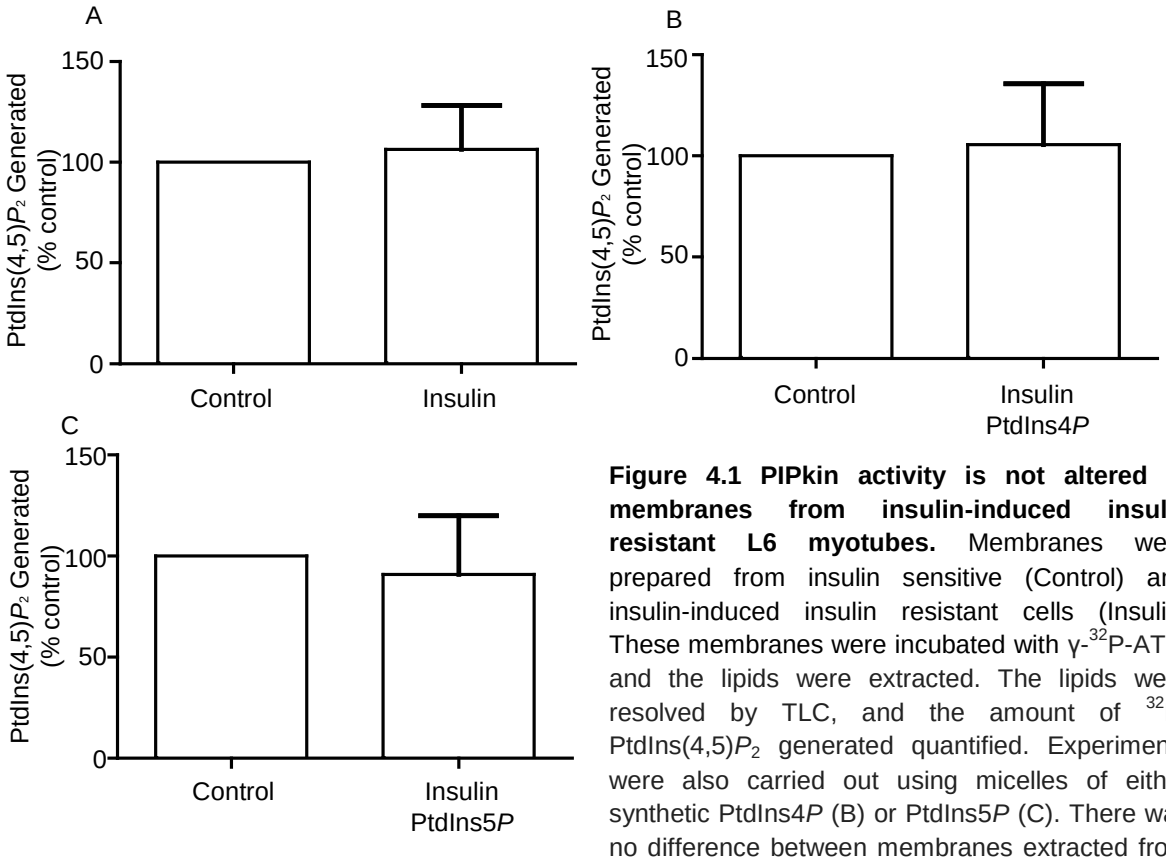

Figure 4.1 PIPkin activity is not altered in membranes from insulin-induced insulin resistant L6 myotubes. Membranes were prepared from insulin sensitive (Control) and insulin-induced insulin resistant cells (Insulin) These membranes were incubated with γ-^{32}P-ATP, and the lipids were extracted. The lipids were resolved by TLC, and the amount of ^{32}P-PtdIns(4,5)P_2 generated quantified. Experiments were also carried out using micelles of either synthetic PtdIns4*P* (B) or PtdIns5*P* (C). There was no difference between membranes extracted from insulin sensitive and insulin resistant cells in all cases. (n=4-8; Kruskal-Wallis ANOVA with Dunn's Multi Comparison Test)

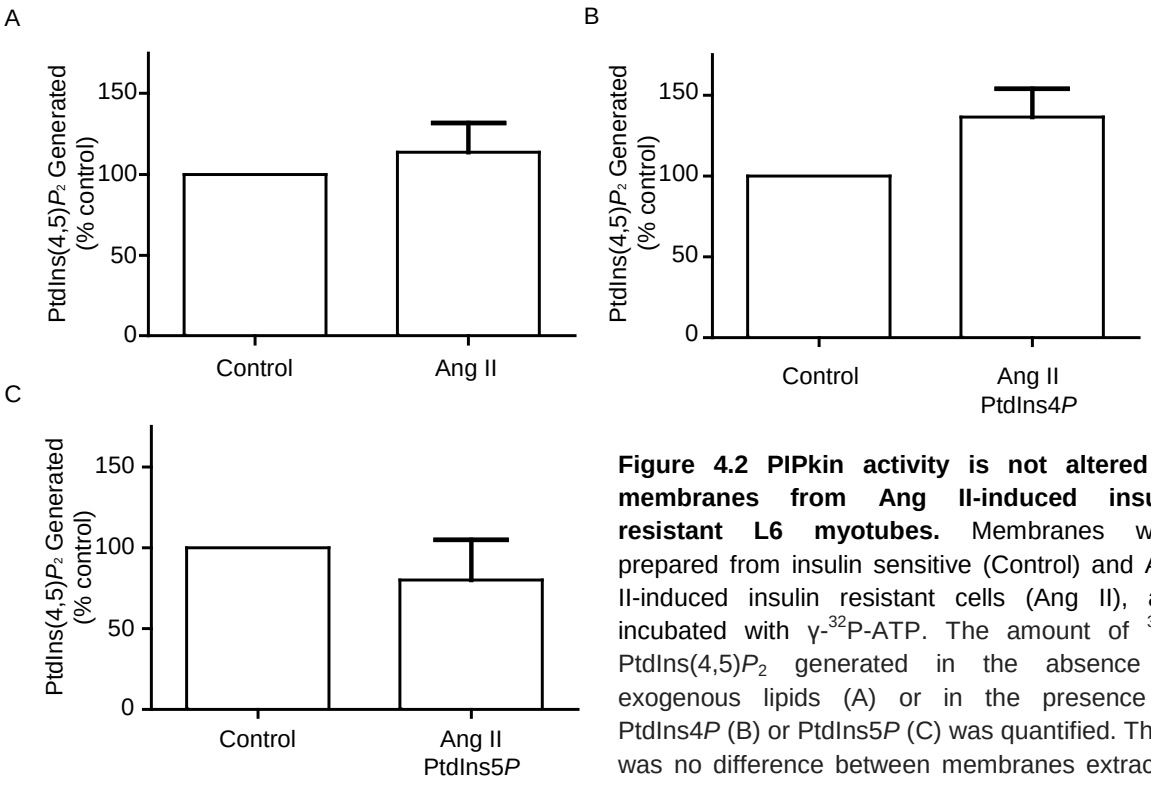

Figure 4.2 PIPkin activity is not altered in membranes from Ang II-induced insulin resistant L6 myotubes. Membranes were prepared from insulin sensitive (Control) and Ang II-induced insulin resistant cells (Ang II), and incubated with γ-^{32}P-ATP. The amount of ^{32}P-PtdIns(4,5)P_2 generated in the absence of exogenous lipids (A) or in the presence of PtdIns4*P* (B) or PtdIns5*P* (C) was quantified. There was no difference between membranes extracted from insulin sensitive and insulin resistant cells in all cases. (n=4-8; Kruskal-Wallis ANOVA with Dunn's Multi Comparison Test)

4.2 PtdIns(4,5)P$_2$-specific phosphoinositide phosphatase activity is not altered in membranes from insulin resistant L6 myotubes

As the kinase activity is not altered in insulin resistant cells, it was decided to investigate the activity of the phosphatases, which remove the phosphate from the D4 or D5 position of PtdIns(4,5)P_2 (see 1.4.5). The majority of PtdIns(4,5)P_2 in cells is de-phosphorylated by 5-phosphatases to PtdIns4*P*. To investigate the activity of the phosphatases PtdIns(4,5)P_2 labelled with a radioactive phosphate group on the D4 position was generated.

PtdIns(4,5)P_2, containing ^{32}P-PtdIns(4,5)P_2 with a reading of 30,000 Cherenkov counts, was incubated with the membrane extracts, under conditions allowing phosphatase activity, allowing the endogenous phosphatases to function. Again the lipids were extracted and separated by TLC, and levels of ^{32}P-PtdIns(4,5)P_2 and ^{32}P-PtdIns4*P* were quantified by phosphoimager.

After incubating the membrane extracts with ^{32}P-PtdIns(4,5)P_2 there was no significant difference between levels of ^{32}P-PtdIns(4,5)P_2 left in membrane extracts taken from insulin sensitive or insulin resistant cells (Figs. 4.3 and 4.4). A small amount of ^{32}P-PtdIns4*P* was generated but, again there was no difference between membrane extracts. Surprisingly there was no decrease in ^{32}P-PtdIns(4,5)P_2 between control cells and the original sample, suggesting that there was very little basal phosphoinositide phosphatase activity in membranes from insulin sensitive cells.

To investigate the low basal activity of phosphoinositide phosphatases further, alamethicin was used (*213*). Alamethicin is thought to aid interactions between lipids and proteins in the plasma membrane (*213*, *358*) allowing for better conditions for PLC activity (*213*). Using this compound led to a larger decrease in the amount of PtdIns(4,5)P_2, with 65.5 ± 8.4% of the original ^{32}P-PtdIns(4,5)P_2 remaining after incubation with membranes extracted from insulin sensitive L6 myotubes, compared to 100 ± 12.8% ^{32}P-PtdIns(4,5)P_2 remaining after incubation with membranes from insulin sensitive cells without alamethicin. There was, more importantly, no significant difference between insulin sensitive and insulin resistant cells in terms of

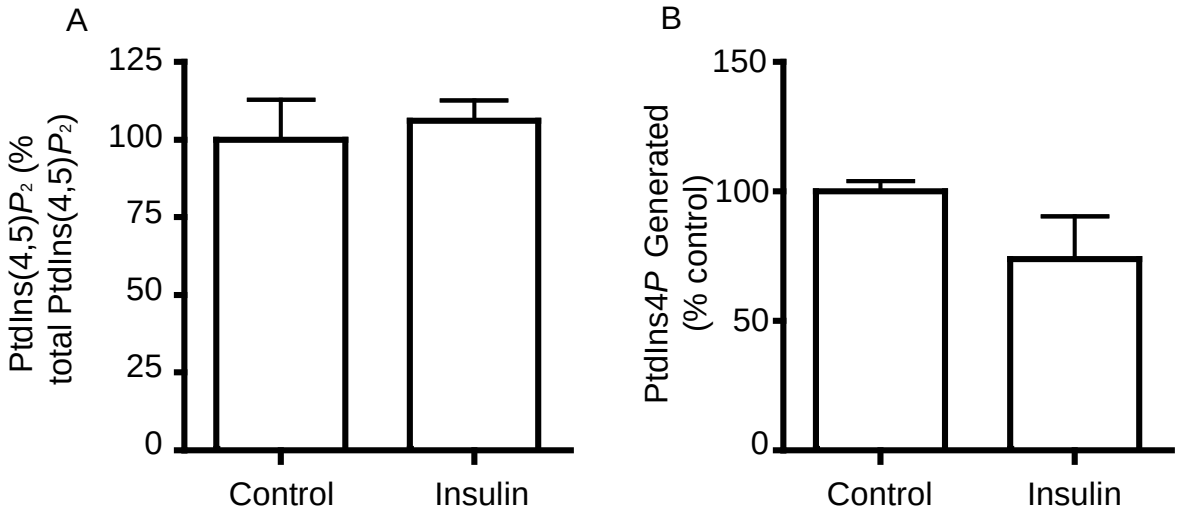

Figure 4.3 Phosphoinositide phosphatase activity is not altered in membranes from insulin-induced insulin resistant L6 myotubes. Membranes from insulin sensitive (Control) and insulin-induced insulin resistant cells (Insulin) were incubated with ^{32}P-PtdIns(4,5)P_2 under conditions supporting the activity of phosphatases. The amounts of ^{32}P-PtdIns(4,5)P_2 (A) and ^{32}P-PtdIns4*P* (B) were quantified. There was no difference in levels of these lipids between membranes extracted from insulin sensitive and insulin- induced insulin resistant cells (n=3-5; Kruskal-Wallis ANOVA with Dunn's Multi Comparison Test)

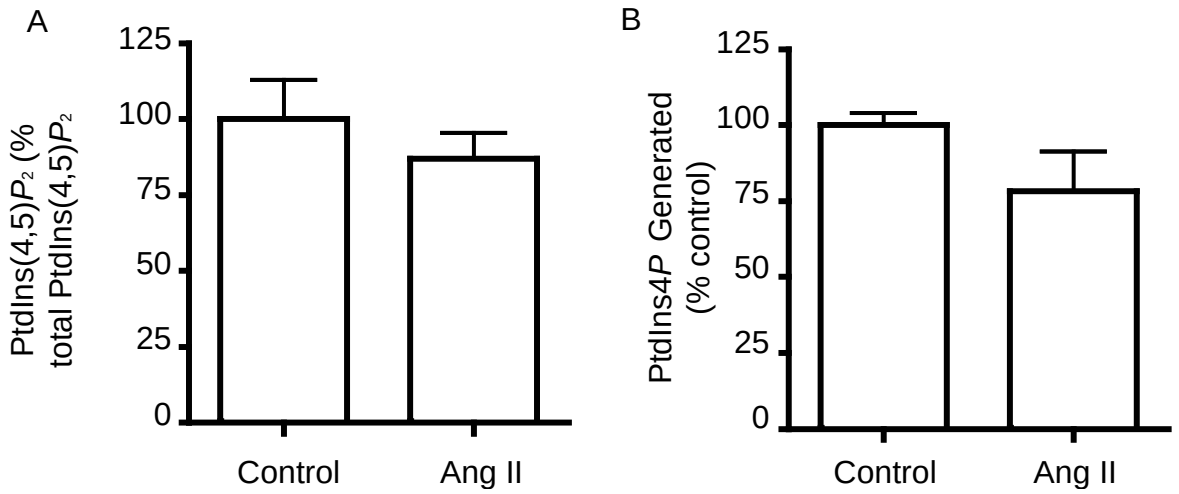

Figure 4.4 Phosphoinositide phosphatase activity is not altered in membranes from Ang II-induced insulin resistant L6 myotubes. Membranes from insulin sensitive (Control) and insulin resistant cells (Ang II) were incubated with ^{32}P-PtdIns(4,5)P_2 under conditions supporting the activity of phosphatases. The amounts of ^{32}P-PtdIns(4,5)P_2 (A) and ^{32}P-PtdIns4P (B) were quantified. There was no difference in levels of these lipids from membranes extracted from insulin sensitive and Ang II- induced insulin resistant cells. (n=3-6; Kruskal-Wallis ANOVA with Dunn's Multi Comparison Test)

^{32}P-PtdIns(4,5)P_2 or ^{32}P-PtdIns4P levels (Figs. 4.5 and 4.6). This suggests that the phosphoinositide phosphatases are also unaffected in insulin resistant L6 myotube membranes.

4.3 PLC activity is increased in membranes from insulin resistant L6 myotubes

The final family of proteins which control PtdIns(4,5)P_2 levels is the PLCs (see 1.4.6). These hydrolyse the phosphodiester bond of PtdIns(4,5)P_2, generating IP_3 and DAG (*184*). Radiolabelled PtdIns(4,5)P_2 was generated and incubated with membrane extracts in a buffer which allows PLC activity. The lipids were extracted, separated by TLC, and levels of ^{32}P-PtdIns(4,5)P_2 were quantified by phosphoimager.

Figs. 4.7 and 4.8 show that, under conditions which allow PLC activity, ^{32}P-PtdIns(4,5)P_2 levels are significantly decreased, when incubated with membranes from insulin resistant L6 myotubes. This suggests that the activity of PLCs is

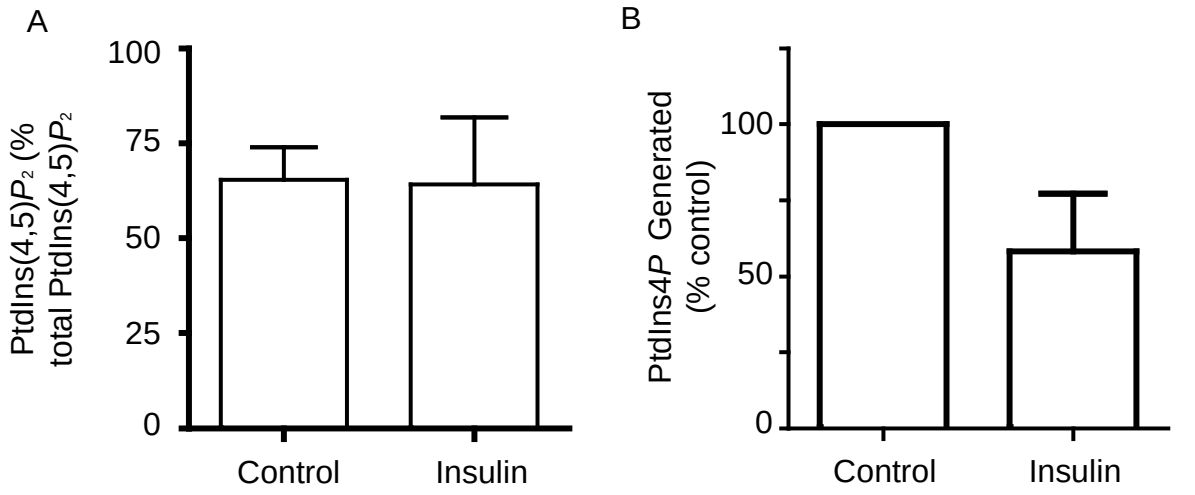

Figure 4.5 Alamethicin leads to increased phosphatase activity, but there is still no significant difference between insulin sensitive and insulin-induced insulin resistant membranes. Membranes from insulin sensitive (Control) and insulin-induced insulin resistant cells (Insulin) were incubated with alamethicin. ^{32}P-PtdIns(4,5)P_2 with a Cherenkov count of 30,000 was added, under conditions supporting the activity of phosphatases, but not of PLCs. The amounts of ^{32}P-PtdIns(4,5)P_2 (A) and ^{32}P-PtdIns4*P* (B) were quantified. There was no difference in levels of these lipids between membranes extracted from insulin sensitive and insulin- induced insulin resistant cells (n=3-5; Kruskal-Wallis ANOVA with Dunn's Multi Comparison Test)

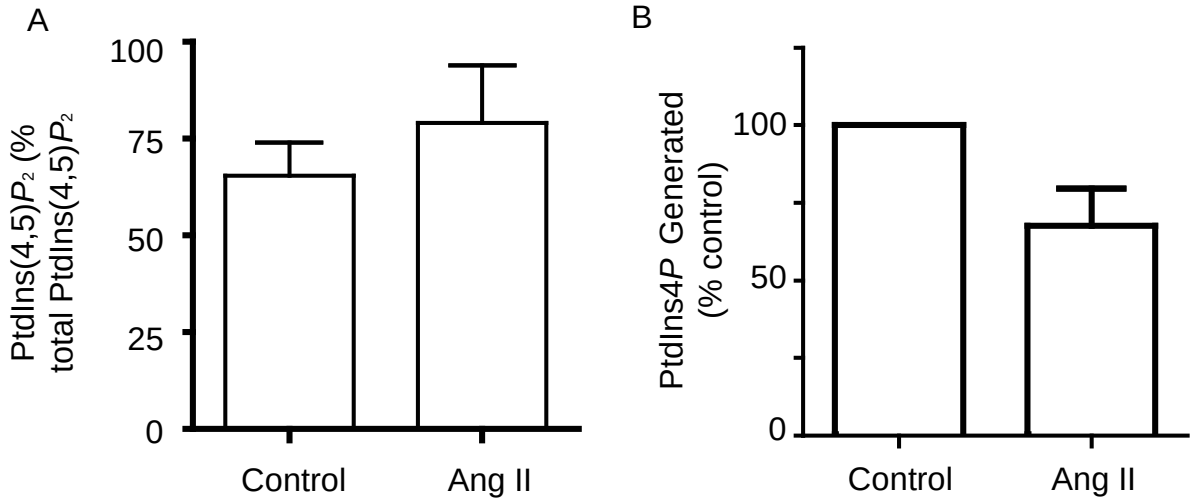

Figure 4.6 Alamethicin leads to increased phosphatase activity, but there is still no significant difference between insulin sensitive and Ang II-induced insulin resistant membranes. Membranes from insulin sensitive (Control) and Ang II-induced insulin resistant cells (Ang II) were incubated with alamethicin. ^{32}P-PtdIns(4,5)P_2 with a Cherenkov count of 30,000 was added, under conditions supporting the activity of phosphatases. The amounts of ^{32}P-PtdIns(4,5)P_2 (A) and ^{32}P-PtdIns4*P* (B) were quantified. There was no difference in levels of these lipids between membranes extracted from insulin sensitive and Ang II-induced insulin resistant cells (n=3-5; Kruskal-Wallis ANOVA with Dunn's Multi Comparison Test)

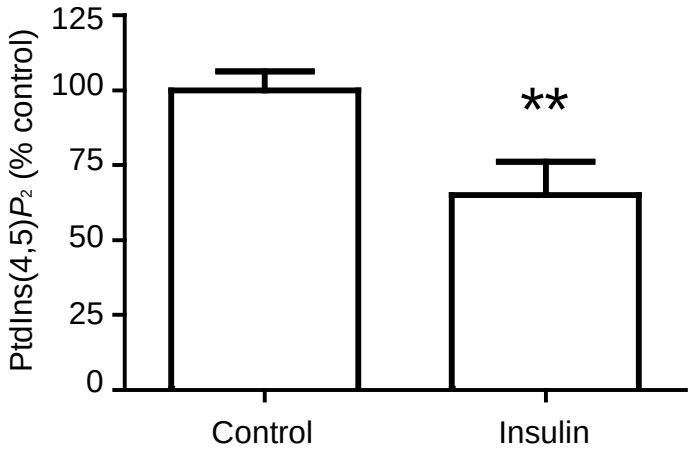

Figure 4.7 PLC activity is increased in membranes from insulin-induced insulin resistant L6 myotubes. Membranes were prepared from insulin sensitive (Control) and insulin-induced insulin resistant cells. These were incubated with ^{32}P-PtdIns(4,5)P_2 under conditions supporting the activity of PLCs, but not of phosphatases. After the incubation, the amount of ^{32}P-PtdIns(4,5)P_2 was quantified. Levels of ^{32}P-PtdIns(4,5)P_2 were significantly lower in membranes from insulin resistant cells. (n=7-10; ** P<0.01; Kruskal-Wallis ANOVA with Dunn's Multi Comparison Test)

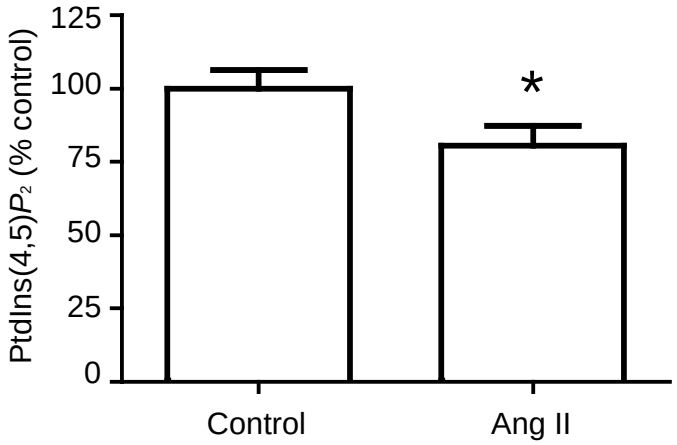

Figure 4.8 PLC activity is increased in membranes from Ang II induced-insulin resistant L6 myotubes. Membranes were prepared from insulin sensitive (Control) and Ang II-induced insulin resistant cells. These were incubated with ^{32}P-PtdIns(4,5)P_2 under conditions supporting the activity of PLCs. The amount of ^{32}P-PtdIns(4,5)P_2 was quantified and found to be significantly lower in membranes from insulin resistant cells. (n=10-12; * P<0.05; Kruskal-Wallis ANOVA with Dunn's Multi Comparison Test)

increased in membranes extracted from L6 myotubes insulin resistance induced by Ang II and insulin in L6 myotubes.

4.4 Chronic exposure to U73122 restores PtdIns(4,5)P$_2$ levels

After the findings above showed that PLC activity was increased in membranes from insulin resistant L6 myotubes, it was decided to investigate PLCs further. There are two pharmacological agents which inhibit PLCs; U73122 and edelfosine (also called ET-18-OCH$_3$) (*208*). Exposing cells to 40µM edelfosine, the concentration needed for full PLC inhibition (*359*), causes cell death in L6 myotubes. This has been shown in the literature before (*360–362*). However, U73122 showed no such cytotoxic side effects, and was used for future experiments.

Firstly the effect of PLC inhibition on PtdIns(4,5)P_2 levels in insulin resistant L6 myotubes was investigated. Cells were incubated with 10µM U73122 or the inactive analogue U73343, for 24 hours, in the presence of either Ang II (10nM; 24 hours) or insulin (5nM; 12 hours). 24 hour exposure to U73122 prevented the PtdIns(4,5)P_2 decrease seen in insulin resistant cells (Fig. 4.9). This restoration was not seen in insulin resistant cells incubated with U73343. Treatment with U73343 caused a decrease in PtdIns(4,5)P_2 levels in control cells (Data not shown). Due to this it was decided not to use U73343 for glucose uptake assays. Unfortunately, 24 hour exposure to U73122 induces insulin resistance by itself and insulin does not cause an increase in glucose uptake in L6 myotubes (Fig. 4.10).

4.5 Acute inhibition of PLC activity restored PtdIns(4,5)P_2 levels and partially restored insulin sensitivity

A short term exposure to U73122 was investigated, after preliminary tests shows that exposing cells to U73122 for an hour did not prevent insulin-stimulated glucose uptake (Fig. 4.11). It did however cause a decrease in basal uptake, as seen previously in adipocytes (*363*, *364*).

Incubating the insulin resistant cells with U73122 for the final hour of induction not only restores PtdIns(4,5)P_2 levels to control levels, but also partially restores insulin sensitivity in insulin- and Ang II-induced insulin resistant cells (Figs. 4.12 and 4.13 respectively). After acute U73122 treatment both insulin- and Ang II-induced insulin resistant cells show improved glucose uptake after insulin stimulation.

It should be noted that the non-insulin stimulated glucose uptake in Ang II and insulin-treated cells was significantly lower than basal uptake in untreated L6 myotubes. For insulin-induced insulin resistant L6 myotubes the basal uptake was 43.85 ± 4.7% of the untreated control. This is lower than the decreased basal glucose uptake seen in insulin-induced insulin resistant cells (52.47 ± 11.0 % of untreated control) (Fig. 3.3D), and those treated with carrier only (58.3 ± 12.4% of

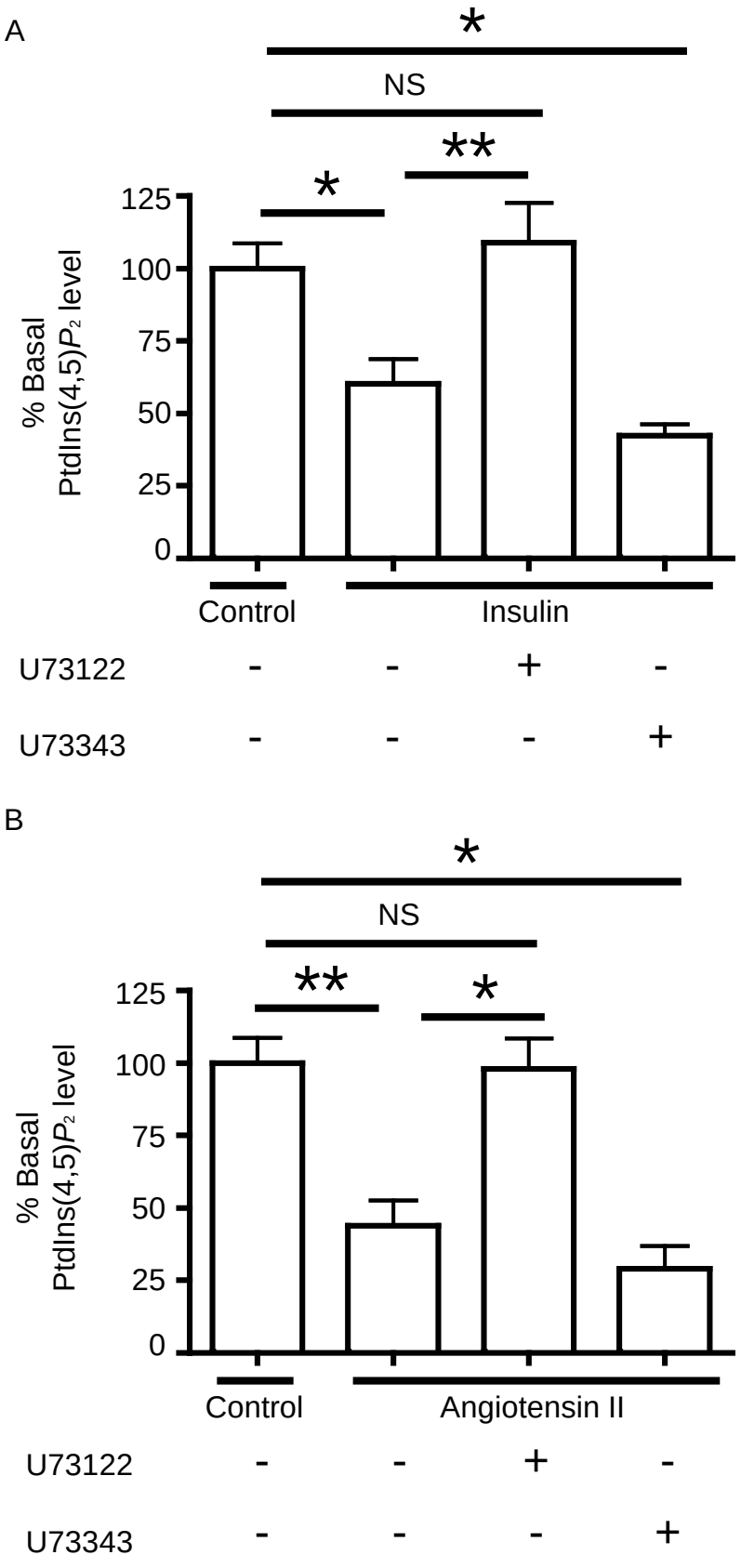

Figure 4.9 PLC inhibition restores PtdIns(4,5)P_2 levels in L6 myotubes rendered insulin resistant by exposure to insulin and Ang II. PtdIns(4,5)P_2 levels were measured in insulin sensitive (control) and insulin- (A) and Ang II- (B) induced insulin resistant L6 myotubes. U73122 or U73343 were added to the cells, during the induction of insulin resistance. Only a 24 hour exposure of U73122 restored PtdIns(4,5)P_2 levels to a similar level to control cells. (n=3-29; * P<0.05; ** P<0.01; One-way ANOVA with Bonferroni's Multi Comparison Test)

untreated control) (Fig. 3.12B). As seen in Fig. 3.12 carrier-mediated delivery of PtdIns(4,5)P_2 increases basal uptake to a similar level as untreated control, but

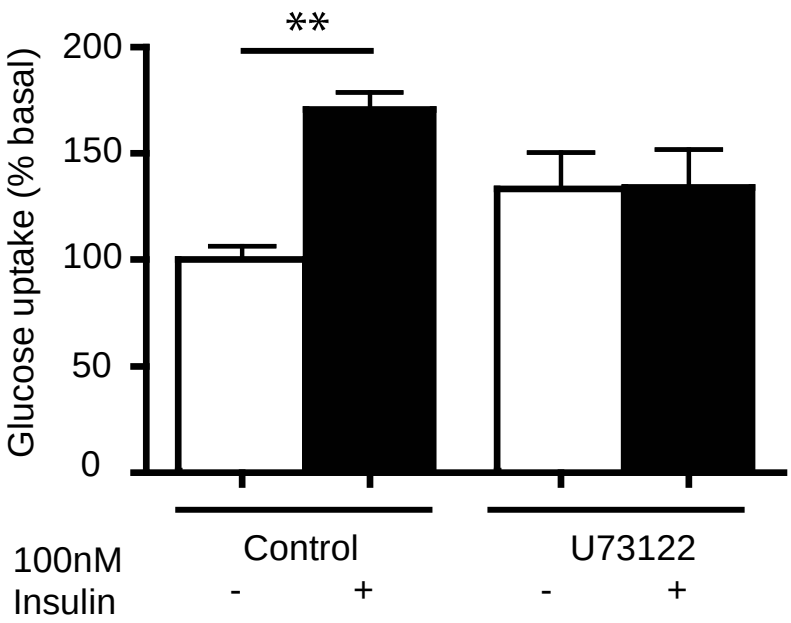

Figure 4.10 24 hour exposure to U73122 causes insulin resistance in L6 myotubes. L6 myotubes were incubated for 24 hours in the presence or absence of 10μM U73122. Glucose uptake was measured after exposure to insulin (100nM; 20 minutes). 24 hour incubation with U73122 prevented the increase in glucose uptake seen in control cells. (n=10-12; ** P<0.01; One-way ANOVA with Bonferroni's Multi Comparison Test)

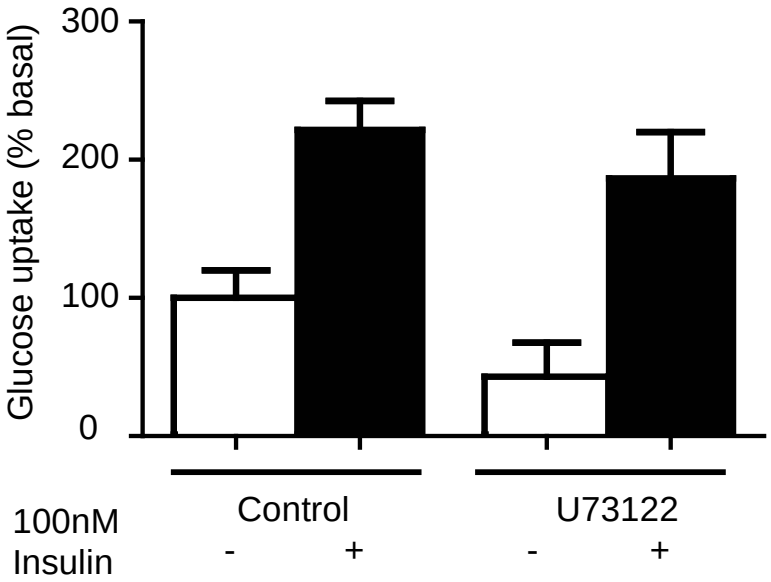

Figure 4.11 1 hour exposure to U73122 does not appear to cause insulin resistance in L6 myotubes. L6 myotubes were starved for 24 hours. For the final hour cells were incubated in the presence or absence of 10μM U73122. Glucose uptake was measured after exposure to insulin (100nM; 20 minutes). 1 hour incubation did not prevent insulin-stimulated glucose uptake. (n=2)

acute exposure to U73122 does not. This could be because U73122 may be playing a role in decreasing basal uptake similar to Fig. 4.11 and has been previously seen in the literature (*363*, *364*).

Basal glucose uptake in Ang II-treated cells exposed to U73122 was 51.65 ± 8.2% of untreated control. In the case of Ang II this mimics what was seen in the previous chapter with carrier-mediated delivery (Fig. 3.13B). Replenishing PtdIns(4,5)P_2 with carrier-mediated delivery restores insulin-stimulated glucose uptake, but basal glucose uptake was still decreased by ~40%. It is possible that Ang II is inhibiting

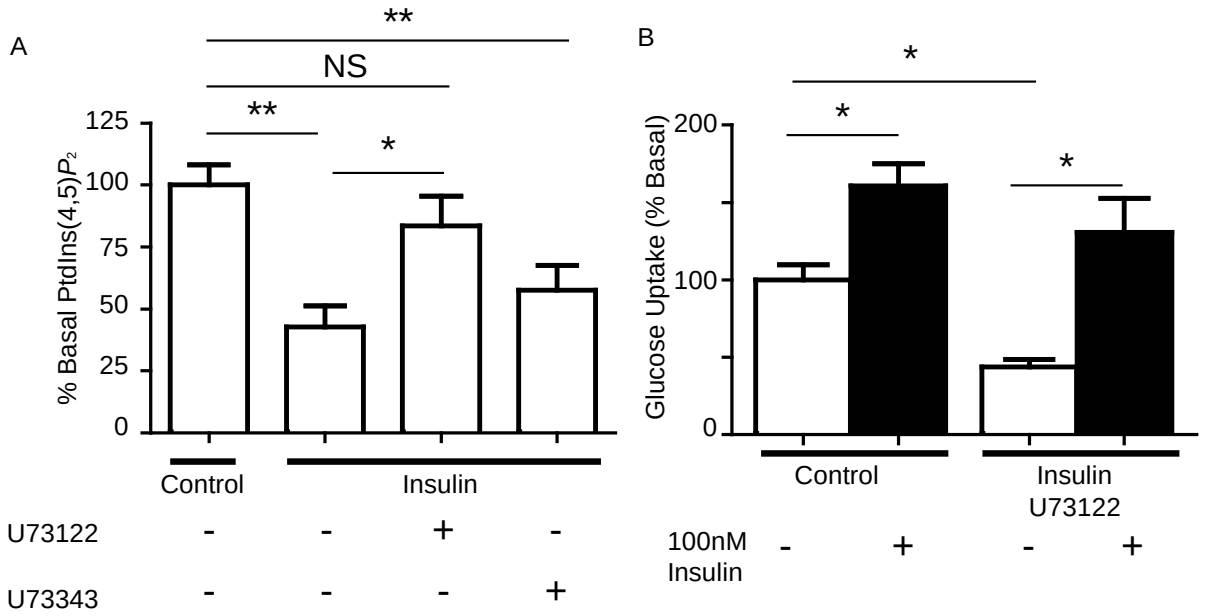

Figure 4.12 Acute inhibition of PLC restores PtdIns(4,5)P_2 levels and restores insulin sensitivity in insulin-induced insulin resistant L6 myotubes. L6 myotubes were incubated in the absence (Control) or presence of 5nM Insulin for 12 hours. Afterwards cells were incubated with either 10µM U73122 or U73343 for an hour. (A) PtdIns(4,5)P_2 levels were measured and U73122 was able to restore the PtdIns(4,5)P_2 levels to those in control cells, whereas U73343 was unable to. (n=9-11; * P<0.05) (B) Glucose uptake assays were carried out on cells in the absence or presence of insulin and U73122. The U73122 was able to partially restore the insulin-stimulated glucose uptake. (n=8-13; * P<0.05; ** P<0.005; One-way ANOVA with Bonferroni's Multi Comparison Test)

other GLUT family members (as suggested in 3.8 and Masori *et al.* (*350*)), but as this effect was seen in untreated and insulin-treated cells exposed to U73122 it is also possibly a side effect of U73122. Previous studies have shown basal glucose uptake to be decreased in 3T3-L1 adipocytes and primary rat adipocytes exposed to U73122 (*363*, *364*).

4.6 Levels of the PLCβ subfamily are increased at the plasma membrane in insulin resistant L6 myotubes

In order for PLCs to carry out their function they have to be recruited to the plasma membrane (*215*, *216*). It was decided to look at the levels of the proteins in membrane extractions from insulin sensitive and insulin resistant L6 myotubes to look for alterations in plasma membrane localisation.

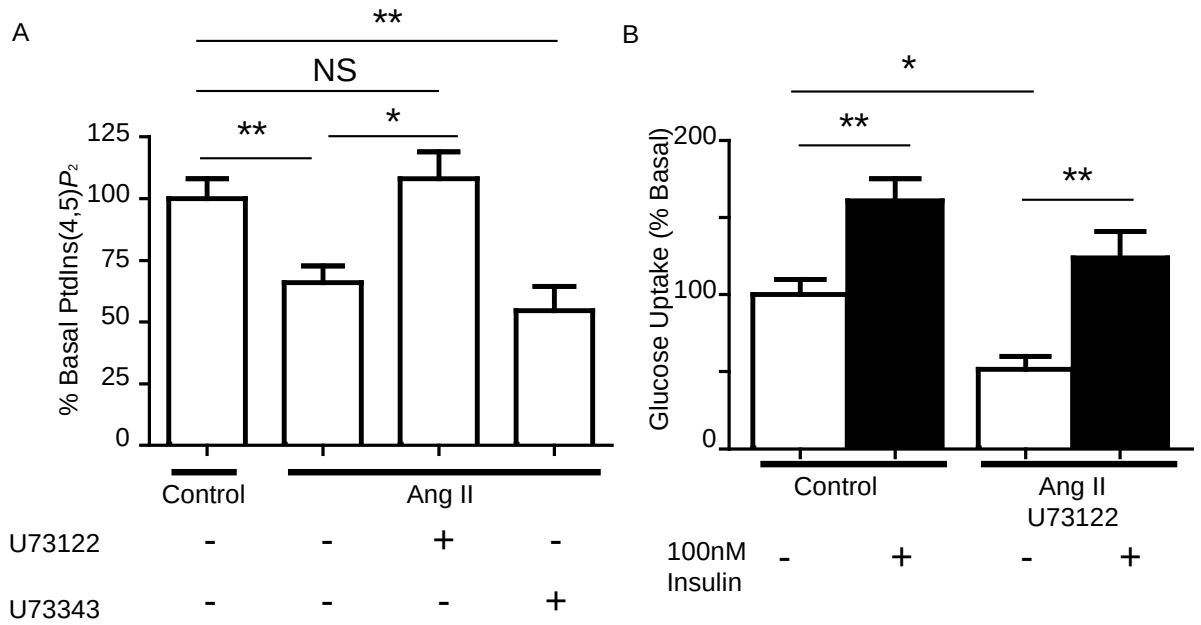

Figure 4.13 Acute inhibition of PLC restores PtdIns(4,5)P_2 levels and restores insulin sensitivity in Ang II-induced insulin resistant L6 myotubes. L6 myotubes were incubated in the absence (Control) or presence of 10nM Ang II for 24 hours. During the final hour cells were also incubated with either 10μM U73122 or U73343. (A) PtdIns(4,5)P_2 levels were measured and U73122 was able to restore the PtdIns(4,5)P_2 levels to those in control cells, whereas U73343 was unable to. (n=6-11; ** P<0.005) (B) Glucose uptake assays were carried out on cells in the absence or presence of insulin and U73122. The U73122 was able to restore insulin sensitivity, and partially restore insulin-stimulated glucose uptake (n=7-15; * P<0.05; ** P<0.005; One-way ANOVA with Bonferroni's Multi Comparison Test)

As the full mRNA and protein expression of PLCs in L6 myotubes has not been fully investigated, it was decided to use RT-PCR to determine which PLCs are expressed in these cells. RT-PCR was carried out using primers against all PLC family members, except PLCζ because this family member is only found in sperm cells (*228–230*). It was discovered that 5 PLCs are expressed in L6 myotubes at the mRNA level. These were PLCβ3, PLCβ4, PLCγ1, PLCδ1 and PLCη2 (data not shown).

Western blotting was carried out on membranes extracted from insulin sensitive and insulin resistant L6 myotubes. Unfortunately there are few commercially available antibodies against certain isoforms of PLCs. Proteintech kindly provided antibodies against PLCβ3, PLCγ1 and PLCδ1. The PLCβ4 antibody used is commercially available from Santa Cruz. There is an anti-PLCη2 antibody available from Santa

Cruz, but due to time constraints this project was not able to investigate this PLC isoform.

Unfortunately, western blotting showed that the anti-PLCγ1 antibody did not recognise any proteins of the correct molecular weight, even in whole cell extracts from L6 myotubes. However, western blotting using antibodies against PLCβ3, PLCβ4 and PLCδ1 showed some differences in the membrane localisation in membranes extracted from insulin resistant cells (Fig. 4.14). PLCβ3 is increased in membranes from Ang-II induced insulin resistant cells, but not in the membranes from insulin-induced insulin resistant cells (Fig. 4.14a). PLCβ4 levels were increased in membranes from insulin-induced insulin resistant membranes, but not in membranes from Ang II-induced insulin resistant cells (Fig. 4.14b). The PLCδ1 levels were unaffected in insulin-induced insulin resistant membranes, and levels were reduced in membranes from Ang II-induced insulin resistance (Fig. 4.14c). This suggests that different isoforms of PLCs may be responsible for the PtdIns(4,5)P_2 decrease in Ang II- and insulin-induced insulin resistance.

4.7 PtdIns(4,5)P_2 levels were not decreased in mice fed a seven week high fat diet

The final experiment was an attempt to investigate PtdIns(4,5)P_2 levels in mice fed with a high-fat diet, compared to mice fed on a control diet. Isaura Martins and Dr Catherine Lawrence kindly provided mouse tissue, and carried out the asphyxiation and saline-perfusion. Muscle was removed from the lower legs, and lipids were extracted. The phosphoinositides were purified on neomycin beads, and the level of PtdIns(4,5)P_2 investigated using a lipid-protein overlay assay (*158*).

After seven weeks of a high-fat diet, skeletal muscle PtdIns(4,5)P_2 levels were not altered from control animals (Fig. 4.15). However, as insulin resistance is a cumulative condition this could be because seven weeks of a high fat diet is not sufficient to induce severe insulin resistance, and cause the decrease in PtdIns(4,5)P_2 seen in the L6 myotubes and 3T3-L1 adipocytes, so further studies are needed.

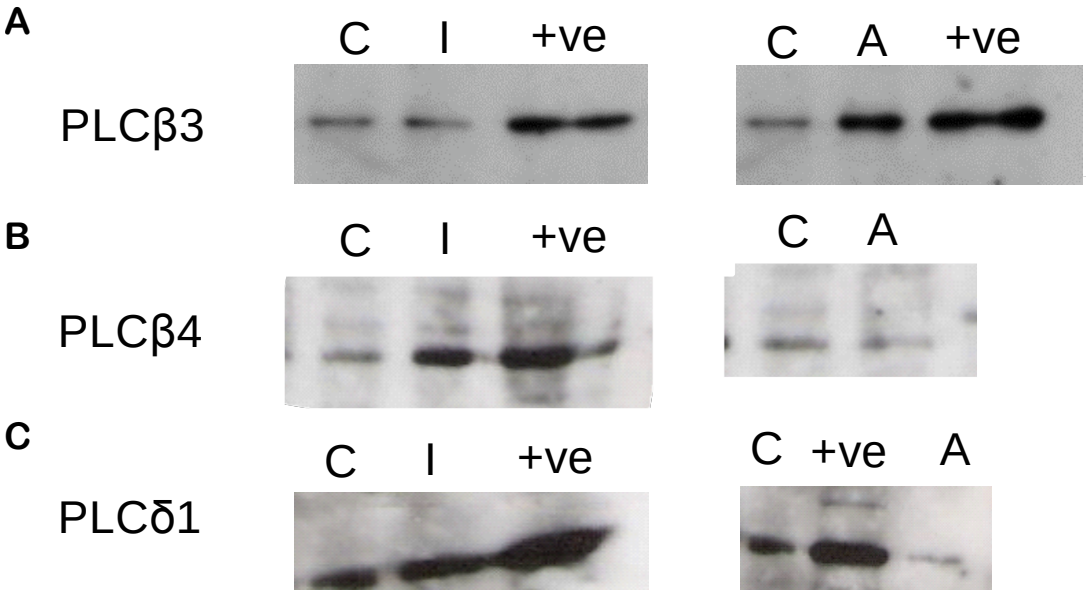

Figure 4.14 Different members of the PLCβ subfamily are increased at the plasma membrane in insulin resistance induced by insulin and Ang II. Membranes were extracted from insulin-sensitive and insulin-resistant L6 myotubes. 2.5mg protein was separated by SDS-PAGE, and the blots were probed with antibodies against PLCβ3 (A), PLCβ4 (B) or PLCδ1 (C). Levels of PLCβ3 are increased in membranes from Ang II-induced insulin resistant membranes, and levels of PLCβ4 are increased in insulin-induced insulin resistant membranes. Levels of PLCδ1 are not affected by insulin-induced insulin resistance, but appear to be lower in Ang II-induced insulin resistance. +ve; positive control.

14.8 Discussion

As demonstrated in the previous chapter levels of the lipid PtdIns(4,5)P_2 are decreased in L6 myotubes and 3T3-L1 adipocytes rendered insulin resistant by treatment with insulin (5nM; 12hours), Ang II (10nM; 24 hours), endothelin-1 (100nM; 24 hours), TNFα (40nM; 24 hours) and hydrogen peroxide (100μM; 3 hours). Importantly, restoring the levels of PtdIns(4,5)P_2 in Ang II- and insulin-induced insulin resistant L6 myotubes to those in control cells, by addition of exogenous lipid, restores the insulin sensitivity, providing evidence that PtdIns(4,5)P_2 plays a vital role in insulin-stimulated glucose uptake.

By investigating the mechanism behind the reduction in PtdIns(4,5)P_2 this chapter demonstrates that PLCs are responsible for decreasing PtdIns(4,5)P_2 in L6 myotubes exposed to Ang II and insulin. The activity of PLCs is increased, whilst the PIPkin and phosphoinositide phosphatase activity assays showed that these are unaltered in insulin resistance. By inhibiting PLCs with U73122 it is possible to

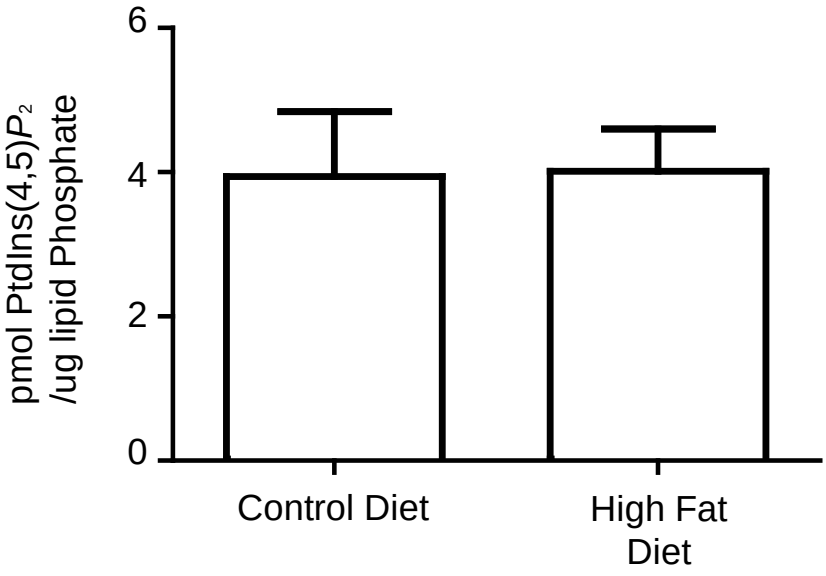

Figure 4.15 PtdIns(4,5)P_2 levels were not altered in mice fed a high fat diet for seven weeks. After eight weeks on a control diet (12% fat) male and female mice were placed on a high fat diet (60% fat). After seven weeks on the diet the animals were anesthetised under terminal anaesthesia, and muscle from the lower leg was removed, and PtdIns(4,5)P_2 levels measured. Seven weeks of a high fat diet did not affect PtdIns(4,5)P_2 levels. (n=11 (Control) and 16 (High Fat); Non-parametric Mann Whitney T-Test)

restore PtdIns(4,5)P_2 levels and also partially to restore insulin sensitivity. Western blotting suggested that the amounts of different PLC family members are increased at the plasma membrane in insulin resistance induced by different conditions. Ang II-induced insulin resistance led to an increase in PLCβ3 localisation at the membrane, whilst PLCβ4 membrane localisation is increased in insulin-induced insulin resistance.

Kinase activity assays suggest that there is no difference in the activity of PIP4kins or PIP5kins in membranes from insulin-sensitive or insulin-resistant L6 myotubes, as similar levels of PtdIns(4,5)P_2 were generated by membrane extracts from insulin-sensitive and insulin-resistant L6 myotubes. Phosphatase activity assays showed no difference in ^{32}P-PtdIns(4,5)P_2 dephosphorylation between insulin sensitive and insulin resistant membrane extracts. Originally, phosphatase activity assays showed very little basal activity, so further experiments were carried out using alamethicin. Alamethicin is a fungal peptide which forms pores in membranes. Previously, low concentrations of alamethicin have been used in PLC activity assays, where it acts a mild detergent, increasing interactions between proteins and lipids (*213*, *358*).

Finally, PLC activity assays showed that PLC activity was increased in insulin resistance. Significantly decreased levels of ^{32}P-PtdIns(4,5)P_2 were left after incubation with membrane extracts from Ang II and insulin-induced insulin resistant L6 myotubes, under conditions allowing PLC activity.

Incubating L6 myotubes with U73122 during the induction of insulin resistance (24 hours) prevented the PtdIns(4,5)P_2 decrease seen in insulin resistant cells, but this long term exposure actually caused insulin resistance. This effect of U73122 on glucose uptake has been documented before in primary rat adipocytes (*363*), where insulin-stimulated glucose uptake is completely abolished. In 3T3-L1 adipocytes U73122 treatment has also been shown to decrease insulin-stimulated glucose uptake (*363*, *364*).

Preliminary results showed that acute exposure (1 hour) to U73122 does not inhibit insulin-stimulated glucose uptake, but does decrease basal levels of glucose uptake compared to control cells. A 1 hour exposure to U73122 decreases the basal level of glucose uptake to 43.5 ± 24.4% of untreated L6 myotubes. This decreased basal glucose uptake was mirrored in insulin resistant L6 myotubes. The basal glucose uptake in Ang II-induced insulin resistant L6 myotubes was 51.7 ± 8.2% of the glucose uptake in untreated cells, and in insulin-induced insulin resistance basal glucose uptake was 43.9 ± 4.7% of the basal glucose uptake in untreated cells.

Exposing insulin-resistant cells to U73122 for one hour was enough to restore PtdIns(4,5)P_2 levels to those in control cells, and this short term exposure was also able partially to restore insulin sensitivity to insulin- and Ang II-treated cells. Insulin stimulation caused a roughly 2-fold increase in glucose uptake. This is comparable to the fold increase seen in control cells (Fig. 3.1). However, due to the decreased basal uptake the insulin-stimulated glucose uptake in U73122-treated cells was not significantly different from the basal uptake in untreated cells. This suggests that although inhibiting PLCs can improve insulin sensitivity the current inhibitors are not sufficient, due to the side effects of U73122 and edelfosine (*208*, *360–363*).

PLC isoforms can be activated by many mechanisms (see 1.4.6) and are involved in many cell signalling pathways. It is therefore likely that even a specific inhibitor would have unwanted side effects. It may be easier to target the activation of the PLCs instead, and as such it is important to understand which isoform is overactive in

insulin resistance. This study shows that PLCβ isoforms display increased plasma membrane localisation in Ang II- and insulin-induced insulin resistance. PLCβ isoforms are generally activated by GPCRs or Rho GTPases (*209*, *217*), and targeting these mechanisms may be preferable to inhibiting PLC isoforms.

It is necessary to characterise fully the expression and activity of PLC family members in L6 myotubes. RT-PCR showed that PLCβ3, PLCβ4, PLCδ1, PLCγ1 and PLCη2 are expressed at the RNA level in L6 myotubes. Western blotting has shown that levels of different PLCs are increased at the plasma membrane of insulin resistant cells. Ang II treatment leads to an increase in the recruitment of PLCβ3, whereas insulin treatment leads to the recruitment of PLCβ4. Membrane localisation of PLCδ1 does not seem to be affected in insulin-induced insulin resistance, and Ang II treatment decreases the amount of PLCδ1 membrane localisation. Unfortunately the amount of membrane PLCγ1 and PLCη2 could not be investigated due to the time constraints.

This study has begun investigating the expression of PLCs in L6 myotubes, by carrying out RT-PCR and western blotting. However, western blotting is only a preliminary step at looking at the increased PLC activity in insulin resistant cells. Specific activity assays would have to be carried out to fully elucidate which isoform, or isoforms, are to blame for the PtdIns(4,5)P_2 decrease.

As mentioned earlier, Ang II stimulation leads to the activation of PLCβ family members due to the Ang II receptor interactions with G-proteins (*365*), so it could be expected that the amount of PLCβs are increased in the plasma membrane after Ang II treatment. This project has suggested that PLCβ3 levels are increased at the plasma membrane, whereas PLCβ4 levels are not altered in membranes from Ang II-treated cells. Surprisingly, membranes from insulin-induced insulin resistant cells show an increased amount of PLCβ4. Insulin stimulation leads to the activation of PLCγ1 (*56*, *78*) but unfortunately PLCγ1 levels could not be investigated in this study. It is necessary to investigate the levels of PLCγ1 as whilst insulin stimulation activates this isoform, the effects of chronic insulin stimulation are not known.

Few studies have investigated PLC activity in insulin resistance *in vivo*. One study showed that PLC activity is increased in membranes extracted from the liver of humans suffering from type 2 diabetes (*366*), but this could not be replicated in

insulin resistant KKA(y) mice (*367*), a model for type 2 diabetes (*368*). A few studies have focussed on using knock out animals, but insulin resistance has not been investigated.

PLCβ3-deficient mice are protected from developing atherosclerosis, by increasing the susceptibility of macrophages to undergo apoptosis (*369*). Obesity and insulin resistance are risk factors for the development of atherosclerosis (*19*). It is known that Ang II and endothelin-1 play a role in the development of atherosclerosis and insulin resistance (*283–285*, *370*, *371*), and it would be interesting to investigate if the high circulating plasma levels of these peptides are activating PLCβ family members in insulin-responsive tissues, in particular skeletal muscle and adipose tissue. This could provide a link between insulin resistance and vascular diseases.

Whilst PLCβ4-deficient mice have been developed, these animals have been used for investigating PLCβ4's role in neurological signalling (*372–374*). PLCβ4 is highly expressed in the cerebellum and knockout mice exhibit locomotor ataxia (*372*, *374*). There has been no investigation into insulin resistance or hyperglycaemia in PLCβ4-deficient animals.

When fed a high fat diet, PLCδ1 knockout mice did not develop hyperglycaemia and insulin resistance (*375*). It is unknown if this protection is due directly to the role of PLCδ1 in insulin sensitivity, or because the animals were leaner and accumulated fewer lipids than control animals fed a high fat diet. Both of these factors are known to decrease the chances of inducing insulin resistance (*6*). However, in this study PLCδ1 plasma membrane levels were not altered in insulin resistant cell, and in fact in Ang II-induced membranes there are decreased levels of PLCδ1. This suggests that PLCδ1 is not playing a role in the PtdIns(4,5)P_2 decrease in Ang II- and insulin-induced insulin resistant L6 myotubes.

PLCγ1 knockout mice die during development (approximately embryonic day nine) (*376*). However, siRNA has been used to knock down the protein levels of PLCγ1 in culture models (*56*, *376*). PLCγ1-deficient fibroblasts show defects in ERK phosphorylation, but not in IRS or Akt phosphorylation (*56*). However, this is yet to be shown in cells which respond to insulin by increasing glucose uptake (i.e. skeletal muscle and adipocytes).

PLCη2 is highly expressed in the brain, and as such all work with PLCη2-deficient mice have focussed on this (*184*). PLCη2 knockout mice exhibit no noticeable phenotype (*227*) but this could be due to compensation of PLCη1 which is overexpressed in the brains of PLCη2-deficient mice (*227*, *377*). The possible role of PLCη family in insulin resistance is not known, and requires further study.

The hydrolysis of PtdIns(4,5)P_2 by PLCs leads to the production of two secondary messengers, IP_3 and DAG. DAG activates various signalling proteins including members of the PKC family (*129*, *186*, *211*), by binding to C1 domains. IP_3 releases stored Ca^{2+} into the cytoplasm (*185*). Increased levels of DAG, Ca^{2+} and activated PKCs have been suggested to play roles in insulin resistance (see 1.6.5) (*66*, *378*). However, carrier-mediated delivery of PtdIns(4,5)P_2 alone restores insulin sensitivity, suggesting that the decrease in PtdIns(4,5)P_2 is a major cause of insulin resistance.

Unfortunately, measuring PtdIns(4,5)P_2 levels in mice fed high fat diet showed no significant difference from control animals. This experiment is only preliminary, as the animals were only on a high fat diet for 7 weeks. The insulin sensitivity of these animals was not tested either, so it is not known if the animals exhibited insulin resistance. The insulin sensitivity of the animals must be ascertained. Further studies are needed on animals fed a high fat diet for a longer period of time, or on animals commonly used for studying insulin resistance and type 2 diabetes (i.e. Zucker rats).

Overall, this chapter has provided evidence that PLCs are behind the decrease in PtdIns(4,5)P_2 levels in Ang II and insulin-induced insulin resistant L6 myotubes. Using the PLC inhibitor U73122 restores PtdIns(4,5)P_2 level, and partially restores insulin resistance. Plasma membrane localisation of PLCβ3 is increased in Ang II-induced insulin resistance, and PLCβ4 plasma membrane localisation. Specific and better inhibitors are required for further studies, as U73122 affects glucose uptake, and edelfosine causes cell death.

5. General discussion

Insulin resistance can be defined as the inability of cells to respond appropriately to insulin stimulation (*3*). In skeletal muscle and adipose tissue this manifests as an inability to increase glucose uptake, as a response to insulin. Insulin resistance can be a precursor for type 2 diabetes, although it should be noted that those suffering from insulin resistance do not always develop type 2 diabetes (*5*, *7*, *10*, *11*).

In organisms, insulin resistance is induced by many independent factors (*5*, *6*). As such the actual mechanism behind insulin resistance is poorly understood, despite a huge amount of research.

5.1 Overactive PLCs decrease PtdIns(4,5)P_2 levels, potentially causing insulin resistance

This study has shown that mass levels of the phosphoinositide PtdIns(4,5)P_2 are decreased in cell models of insulin resistance, induced by exposure to Ang II, endothelin-1, hydrogen peroxide, insulin and TNFα, in both 3T3-L1 adipocytes and L6 myotubes. Previous studies have shown that insulin resistance correlated with reduced PtdIns(4,5)P_2 immunofluorescence in plasma membrane sheets from 3T3-L1 adipocytes rendered insulin resistant by exposure to insulin (*248*), endothelin-1 (*250*) and glucosamine (*249*), and insulin-induced insulin resistant L6 myotubes (*240*). This study has extended this work, confirming that treatment with Ang II, TNFα and hydrogen peroxide all produce mass PtdIns(4,5)P_2 decreases in both cell types, and that endothelin-1-treated L6 myotubes also have decreased PtdIns(4,5)P_2 levels.

Using carrier-mediated delivery of PtdIns(4,5)P_2 to restore PtdIns(4,5)P_2 levels resulted in the reversal of insulin resistance in L6 myotubes treated with Ang II and insulin. This is similar to what has been previously reported using carrier-mediated delivery of synthetic PtdIns(4,5)P_2 to insulin-induced insulin resistant cells (*240*, *248*), but this has not been shown in Ang II-induced insulin resistance before.

Experiments using membranes prepared from Ang II- and insulin-induced insulin resistant L6 myotubes have shown that the PtdIns(4,5)P_2 decrease is due to

hydrolysis by overactive PLCs. In these membranes the activity of the PIPkins and phosphoinositide phosphatases is unaffected in insulin resistance. The role of PLCs in insulin resistance has not been well investigated before, and as such the full mRNA and protein expression of PLCs has not been ascertained in L6 myotubes. This project has shown that 5 PLC isoforms are expressed at the mRNA level in L6 myotubes, and western blotting has suggested that PLCβ isoforms may be responsible for causing the PtdIns(4,5)P_2 decrease. PLCβ3 is increased at the membrane of Ang II-induced insulin resistant L6 myotubes, and that membrane PLCβ4 localisation is increased in insulin-induced insulin resistant cells. Furthermore, inhibiting PLCs by treatment with U73122 restored PtdIns(4,5)P_2 levels, and partially restores insulin sensitivity.

5.2 Further experiments

Overactive PLCs are responsible for the decreased PtdIns(4,5)P_2 in Ang II- and insulin-induced insulin resistance in L6 myotubes. This study has begun investigating PLC expression in L6 myotubes, but further work has to be carried out. RT-PCR showed expression of five PLC isoforms in L6 myotubes, but this study was only able to investigate the plasma membrane localisation of three, PLCβ3, PLCβ4 and PLCδ1. Investigating the plasma membrane localisation of PLCγ1 and PLCη2 must also be carried out. Specific activity assays could be carried out on particular isoforms to confirm that the isoform is responsible for the PtdIns(4,5)P_2 membrane decrease.

As insulin resistance is multi-factorial, but the PtdIns(4,5)P_2 decrease appears to be consistent amongst the models tested, the mechanism behind the decrease must also be investigated in other conditions known to cause insulin resistance (e.g. exposure to endothelin-1, TNFα or oxidative stress). If PLCs provide a unifying reason for the PtdIns(4,5)P_2 loss in insulin resistance, then western blotting must be carried out looking at the plasma membrane localisation of PLCs under other conditions known to induce insulin resistance. This will show if a single particular isoform or sub-family is likely to be responsible for causing the PtdIns(4,5)P_2 decrease. Evidence from this study has already suggested that PLCβ family

members are increased at the membrane in insulin resistance and may be responsible for the decrease in Ang II- and insulin-induced insulin resistance. The mechanism behind the PtdIns(4,5)P_2 decrease in 3T3-L1 adipocytes has yet to be discovered, and this must also be investigated.

As there are currently no isoform-specific PLC inhibitors available, RNA interference could be carried to out to knock down specific PLC isoforms. The effects on PtdIns(4,5)P_2 would then have to be investigated. If siRNA prevented the PtdIns(4,5)P_2 decrease in insulin resistant cells, then glucose uptake would be carried out to assess the effect this has on insulin sensitivity.

An important experiment that needs to be undertaken is whether decreasing PtdIns(4,5)P_2 by a different means can induce insulin resistance. There are potentially several methods for this, involving perturbing the mechanisms which control PtdIns(4,5)P_2 levels, namely the PIPkins, phosphoinositide phosphatase and PLCs.

Preventing the generation of PtdIns(4,5)P_2 by preventing PIPkin activity could be expected to decrease levels of this lipid. There are no good inhibitors currently available, so other methods must be employed. One method would be to use siRNA to knock down the PIPkins. However, preliminary studies have shown that siRNA against α and β type I PIPkin isoforms do not decrease protein levels in L6 myotubes and 3T3-L1 adipocytes (A Droubi and K Hinchliffe, unpublished results). Another method for decreasing PtdIns(4,5)P_2 levels would be to transfect cells with a Kinase-Dead PIPkin .

Another method of decreasing plasma membrane levels of PtdIns(4,5)P_2 would be to activate the PtdIns(4,5)P_2-specific phosphoinositide phosphatases (i.e. the 4-phosphatases I and II, the synaptojanins, OCRL1 and INPP5B). Work carried out previously has involved either overexpression (*379*) of a 5-phosphatase, or transfection with an inducible 5-phosphatase (*380*, *381*). Cells transfected with an inducible 5-phosphatase rapidly exhibited decrease PtdIns(4,5)P_2 levels upon induction, as measured by PLCδ1PH-GFP plasma membrane fluorescence (*380*, *381*).

A tetracycline-inducible PtdIns(4,5)P_2 4-phosphatase has also been generated, and has been shown to reduce global levels of PtdIns(4,5)P_2 (*197*). An increase in PtdIns5*P* levels would also be expected in cells expressing this construct, but this was not measured in this study. Increased levels of PtdIns5*P* increases glucose uptake independently of insulin (*83*, *85*), and therefore increasing levels of this lipid may produce results which are difficult to interpret. It is therefore preferable to use PtdIns(4,5)P_2 5-phosphatase, as PtdIns4*P* is not thought to play a role in glucose uptake (*83*, *84*).

PLC activation could also be investigated, and there is a pharmacological activator of PLC, *m*-3M3FBS (*208*, *382*). However, *m*-3M3FBS is known to have other side effects including PLC-independent Ca^{2+} release (*383*) and superoxide production (*384*, *385*). Both of these events occur downstream of PLC (*184*, *293*, *313*, *386*), and have been implicated in insulin resistance (*66*, *293*, *313*, *315*, *378*). This suggests that the activation of PLC using *m*-3M3FBS is unlikely to be a useful approach. Transfecting cells with a PLC isoform which constitutively targeted to the membrane may be another method of investigating overactive PLC activity.

It is also important that the findings from this study are replicated in animal models. Disappointingly, preliminary work showed that the PtdIns(4,5)P_2 decrease seen in cell culture models could not be replicated in mice fed a high fat diet for seven weeks. As shown in Figs. 3.3 and 3.4 the cell culture models express very severe insulin resistance, and pre-treatments cause a complete abolition of insulin-stimulated glucose uptake. However, *in vivo* insulin resistance is a cumulative disorder, and seven weeks may not be enough time for severe insulin resistance to be induced.

Insulin sensitivity was not investigated in these animals so it is possible that animals were not suffering from severe insulin resistance, and therefore it could be expected that the levels of PtdIns(4,5)P_2 would not be perturbed. The blood plasma levels of various factors known to be increased in insulin resistance were also not measured. If animals exhibited hyperinsulinaemia (*3*) and/or hyperglycaemia (*387*) it could be expected that the animals were insulin resistant. Similarly, increased circulating levels of Ang I) (*283*, *284*), endothelin-1 (*285*), TNFα (*286*, *287*) insulin (*19*, *287*–*289*) and FFAs (*290*), would suggest that the animals could exhibit insulin resistance.

The mice used for this study are not usually used for investigating insulin resistance, and were only fed a high fat diet. If they were to develop severe insulin resistance, it would develop insulin resistance more slowly than in other animal models usually used for investigating insulin resistance and type 2 diabetes (*311*, *388*). The majority of animal models have been specifically bred to induce obesity, hyperglycaemia and insulin resistance (*388*). It would therefore be beneficial in the future to carry out a definitive study of PtdIns(4,5)P_2 levels in animals which are known to be severely insulin resistant. If the PtdIns(4,5)P_2 levels are shown to be decreased in insulin resistant animals, then PLC activity assays would have to be carried out on skeletal muscle. This would provide evidence for a unifying mechanism behind insulin resistance.

The current inhibitors of PLC, U73122 and edelfosine, exhibit many side effects (*208*, *360–362*, *389*), and U73122 is even thought to function as a mild activator of PLC in some circumstances (*208*, *390*). In cell-free systems, using purified PLCs, U73122 stimulates the hydrolysis of PtdIns(4,5)P_2 (*390*). U73122 has been shown to activate PLCβ2, PLCβ3, and PLCγ1 as well as neither inhibiting nor activating PLCδ1 (*390*).

As PLCs are involved in many cell signalling pathways, it may be more beneficial to attempt to block the activation pathway of a specific PLC isoform. Stimulation of PLCs are also crucial for many cell signalling pathways and as such non-specific inhibition could cause many problems (*377*, *386*). PLCβ isoforms, which express increased plasma membrane localisation in Ang II- and insulin-induced insulin resistance, are activated by GPCRs or Rho GTPases (*209*, *217*). Peptides such as Ang II and endothelin-1 have high circulating plasma levels in organisms suffering from insulin resistance, obesity and type 2 diabetes (*283–285*). Ang II and endothein-1 are ligands for GPCRs, and are known to activate PLCβ family members. More research is needed into PLC signalling in insulin resistance, and if a specific isoform could be inhibited, that would be extremely beneficial to further studies.

5.3 Concluding remarks

In conclusion, this study has confirmed and extended current knowledge suggesting a causal link between decreased PtdIns(4,5)P_2 levels and insulin resistance in L6 myotubes (*240*) and 3T3-L1 adipocytes (*248–250*). After confirming that whole cell levels of PtdIns(4,5)P_2 are decreased in insulin resistance induced by several factors, the mechanism behind the decrease was then investigated in Ang II- and insulin-induced insulin resistant L6 myotubes. PLC activity was increased in plasma membranes extracts from insulin resistant cells, whereas PIPkin and PtdIns(4,5)P_2-specific phosphoinositides phosphatase activity was unaltered. Furthermore acute inhibition of PLCs, using U73122, restores PtdIns(4,5)P_2 levels and leads to the partial reversal of insulin resistance. This project has suggested that targeting PLC, or its activating mechanisms, may be a novel therapeutic target for treating insulin resistance.

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