Insulin sensitivity and nutrient utilisation in skeletal muscle

by

Yan Yan Lam

Thesis submitted in fulfilment of the requirement for
the Degree of Doctor of Philosophy

Discipline of Medicine
School of Medicine
Faculty of Health Sciences
University of Adelaide, South Australia, Australia

November 2009

Chapter 5: Effect of long-chain saturated, n-3 and n-6 polyunsaturated fatty acids on insulin-stimulated glucose uptake in skeletal muscle cells

5.1 Introduction

Epidemiological data suggest that a high-fat diet is a risk factor for type 2 diabetes (Marshall et al. 1991). In particular, saturated fat intake is associated with an impairment of insulin action and is a long-term predictor of insulin resistance and metabolic syndrome (Riccardi et al. 2004; Riserus et al. 2007). In healthy men and women aged 30 – 65 yr with BMI 22 – 32 kg/m^2, a decrease in insulin sensitivity has been observed after consuming a diet with 17% of energy from saturated fats for 90 days (Vessby et al. 2001). Elevating plasma free fatty acid levels via enteral infusion of palm oil (48% palmitic, 35% oleic and 8% linoleic acids) for 2 h resulted in a reduced insulin response to enhance glucose uptake in obese non-diabetic subjects (Clore et al. 2004). *In vitro*, incubating skeletal muscle myotubes with PA and stearic acid, the two main dietary long-chain saturated fatty acids, inhibited insulin-stimulated glucose uptake (Montell et al. 2001; Lee et al. 2006), while oleic acid and LA, the common dietary monounsaturated and polyunsaturated fatty acids respectively, were without effect. These data provide direct evidence for the role of saturated fats in mediating insulin resistance in skeletal muscle.

The inhibitory effect of saturated fats on insulin sensitivity may be mediated by the accumulation of lipid metabolites, for example ceramide and DAG in skeletal muscle. Ceramide is predominantly generated from *de novo* synthesis which involves the condensation of serine and palmitoyl-CoA, a rate-limiting step catalysed by SPT which is

highly selective for long-chain saturated fatty acyl-CoA (Hanada 2003; Straczkowski and Kowalska 2008). Obese individuals have higher intramuscular ceramide content than healthy controls and this is inversely related to insulin sensitivity (Straczkowski et al. 2007). In healthy male subjects with normal body weight and glucose tolerance, a 4 h lipid infusion (44 – 62% linoleic, 19 – 30% oleic, 7 – 14% palmitic, 4 – 11% linolenic and 1.4 – 5.5% stearic acids) was associated with insulin resistance and an increased ceramide content in skeletal muscle (Straczkowski et al. 2004). In C2C12 myotubes, PA inhibited insulin-stimulated Akt phosphorylation, an effect prevented by abolishing ceramide synthesis (Chavez et al. 2003). Conversely, incubating C2C12 myotubes with a ceramide analogue (C_2-ceramide) mimics the inhibitory effect of PA on insulin-stimulated Akt phosphorylation and glycogen synthesis (Schmitz-Peiffer et al. 1999).

DAG, another lipid metabolite, also mediates lipid-induced insulin resistance in skeletal muscle although its role is less well-defined. DAG is an intermediate in triglyceride synthesis, which is mainly derived from esterification of long-chain acyl Co-A and glycerol-3-phosphate (Timmers et al. 2008) (Section 1.3.3.2). The elevated DAG content in skeletal muscle of obese individuals is associated with decreased insulin sensitivity (Straczkowski et al. 2007). Lipid infusion (10% safflower and 10% soybean oil) induced insulin resistance in healthy subjects and the effect was associated with an elevated DAG content in skeletal muscle (Itani et al. 2002). *In vitro*, PA induced intracellular DAG accumulation and serine phosphorylation of IRS-1 in C2C12 myotubes (Coll et al. 2008). The inhibitory effect of DAG on insulin signal transduction may be mediated by the activation of DAG-sensitive nPKCs, which up-regulates the NFκB signalling pathway (Holden et al. 2008). While some nPKCs including nPKC-δ and -θ activate NFκB via the classic IKK/IκB pathway, other nPKCs are poor inducer of IKK activity and are without

effect on IκBα degradation (Catley et al. 2004). Using a DAG mimetic and a range of isoform-specific PKC inhibitors, Catley et al (2004) proposed that DAG-induced NFκB-dependent transcriptional activation may also involve the phosphorylation of cAMP-responsive element-binding protein-binding protein (CBP), a co-activator protein which facilitates transcriptional activation downstream of NFκB DNA binding. As a consequence of activating the IKK/IκB/NFκB pathway, IKK may activate mTORC1 and subsequently leads to serine phosphorylation of IRS-1 (Section 4.4). It has been proposed, however, that the effect of DAG on lipid-induced insulin resistance may be dispensable. Inhibiting *de novo* synthesis of ceramide has been consistently shown to be sufficient to restore insulin sensitivity in PA-treated skeletal muscle cells despite no change or even a further increase in DAG accumulation (Chavez et al. 2003; Powell et al. 2004; Pickersgill et al. 2007).

In contrast to the deleterious effect of saturated fats on insulin signalling, long-chain n-3 PUFAs, in particular those of marine origin including EPA and DHA, may have beneficial effects on insulin sensitivity. Epidemiological data show an inverse relationship between fish intake and the risk of developing impaired glucose intolerance and diabetes in an elderly population (Feskens et al. 1991). Supplementing an energy-restricted diet with n-3 PUFAs improved insulin response in overweight and obese young adults, an effect that was independent of weight loss and lowering of plasma triglyceride (Ramel et al. 2008). In animal models, the prevention of diet-induced insulin resistance by substitution of n-3 PUFAs for saturated or monounsaturated fat has been consistently demonstrated (Delarue et al. 2004).

The positive effect of n-3 PUFAs on insulin sensitivity may be a consequence of modifying the composition of membrane phospholipid and/or activating fatty acid oxidation. The abundance of n-3 PUFAs in membrane phospholipid, which is highly reflective of dietary fat composition, is positively associated with insulin action in skeletal muscle (Storlien et al. 1991). In rats fed with diet high in n-3 PUFAs, Liu et al (1994) showed an increase in insulin binding to its receptor in the skeletal sarcolemma which may improve insulin responsiveness. n-3 PUFAs may also improve insulin sensitivity by enhancing fatty acid oxidation. The amelioration of high-fat diet-induced insulin resistance by fish oil feeding is often associated with a decrease in hepatic lipid content (Neschen et al. 2002; Hong et al. 2003). Both *in vivo* and *in vitro* studies in liver showed that the hypolipidaemic effect of n-3 PUFAs is a result of suppressing the transcription of genes encoding for lipogenic enzymes (e.g. ACC, fatty acid synthase (FAS) and SCD) while at the same time increasing both gene expression and activities of enzymes involved in mitochondrial and peroxisomal β-oxidation (e.g. CPT-I, CPT-II and peroxisomal fatty acid oxidase) and these effects were attributed to the ability of n-3 PUFAs to function as a ligand activator of PPAR-α (Lombardo and Chicco 2006). Compared to high-fat diet-fed animals, rats fed with a 10% n-3 PUFA-enriched high-fat (28%) diet for 3 weeks had lower plasma triglyceride and this was associated with an up-regulated β-oxidation in the liver, and to a lesser extent in skeletal muscle, and an improvement in insulin sensitivity (Ukropec et al. 2003). The positive effect of n-3 PUFAs on insulin action may be attributed to the increase in fat oxidative capacity, which prevents the accumulation of lipid metabolites in peripheral tissues. This suggests that the positive effect of n-3 PUFAs on insulin sensitivity may depend on the background fatty acid profile, which is consistent with an *in vitro* study showing EPA by

itself was without effect on insulin-stimulated glucose uptake in human skeletal muscle cells (Aas et al. 2006).

n-6 PUFAs, in particular LA, contribute to the majority of PUFAs in the western diet (Schmitz and Ecker 2008). n-6 PUFAs share some common effects with n-3 fatty acids including the increase in membrane fluidity and the inhibition of lipogenic gene transcription but their effect on insulin sensitivity is unclear (Schmitz and Ecker 2008). A high-fat diet consisting primarily of LA resulted in a small and yet significant increase in insulin sensitivity in rats compared to those fed with a standard rat chow diet (Lee et al. 2006). In moderately well-controlled non-insulin-dependent diabetic patients, a 15-week intervention of substituting LA-enriched oils and margarines for saturated fat was without effect on fasting levels of blood glucose and glycosylated haemoglobin (Heine et al. 1989). LA was without effect on insulin-stimulated glucose uptake in L6 myotubes (Lee et al. 2006).

In the present study I aimed to determine:

1. *The direct effect of long-chain saturated, n-3 and n-6 polyunsaturated fatty acids on insulin sensitivity in skeletal muscle in vitro.*

   Under physiological conditions skeletal muscle is under the influence of free fatty acids as well as adipokines from adipose tissue and therefore the combined effect of fatty acids and secretory factors from either SC or IAB fat on muscle insulin action was determined. Specifically the effect of PA (16:0), LA (18:2n-6) and DHA (22:6n-3), with or without the presence of CM from SC or IAB fat, on insulin-stimulated glucose uptake in L6 myotubes was examined. I hypothesised

that PA would inhibit insulin-stimulated glucose uptake, while LA and DHA would have no effect on insulin response in L6 myotubes.

2. *The effect of the combination of PA with either DHA or LA on insulin-stimulated glucose uptake compared to that of PA alone.*

   In skeletal muscle cells, oleic acid, a monounsaturated fatty acid which does not affect insulin sensitivity, has been shown to reverse PA-induced insulin resistance when in combination with PA (Montell et al. 2001; Coll et al. 2008). Coll et al (2008) attributed the effect of oleic acid to partitioning PA towards triglyceride accumulation and mitochondrial β-oxidation and thus reducing DAG synthesis. The hypolipidaemic effect of DHA and LA has been previously demonstrated in dietary intervention studies in humans (Reaven et al. 1993; Kelley et al. 2007) but the effect of DHA and LA on PA-induced insulin resistance is unknown. I hypothesised that DHA or LA would reverse the inhibitory effect of PA on insulin-stimulated glucose uptake possibly via activating the fat oxidative pathways.

5.2 Materials and methods

5.2.1 Adipose tissue-conditioned medium (CM)

SC and IAB adipose tissue explants were obtained from two obese non-diabetic (one male and one female) and one obese type 2 diabetic (female; diet-controlled) patients undergoing gastric bypass surgery for the treatment of morbid obesity. The clinical characteristics of the tissue donors are detailed in Table 5.1. CM was generated as described in Section 2.3 and that collected after 48 h of culture was used (Section 3.3.2).

Table 5.1. Clinical characteristics of tissue donors (n = 3).

	Mean ± SD	Range
Age (yr)	62.3 ± 9.0	52 – 74
Body Mass Index (kg/m^2)	50.2 ± 3.9	47.4 – 55.8
Fasting glucose (mmol/L)	5.2 ± 0.8	4.0 – 5.8
Fasting insulin (mU/L)	5.4 ± 1.1	4.2 – 6.9
HbA1c (%)	6.1 ± 0.3	5.9 – 6.5
Fasting cholesterol (mmol/L)	4.5 ± 1.1	3.1 – 5.7
Fasting triglycerides (mmol/L)	1.6 ± 0.3	1.1 – 1.9

5.2.2 Fatty acids

Stock solutions of PA (Sigma-Aldrich #P5585), LA (Sigma-Aldrich #L1012) and DHA (Sigma-Aldrich #D2534), all at a concentration of 500 mM, were prepared in absolute ethanol and stored at -80°C until use.

Prior to each experiment, fatty acids (10 – 400μM) were conjugated with fatty acid-free BSA (Sigma-Aldrich #A8806) with a fatty acid-to-BSA molar ratio of 5:1 in α-MEM. PA was sonicated using a benchtop ultrasonic cleaner (Soniclean #250T, Thebarton, South Australia, Australia) at ~130 W for 1 min and incubated at 37°C overnight with agitation. The solution was allowed to equilibrate in an atmosphere of 5% CO_2 at 37°C for 2 h and kept at 37°C until use. DHA and LA were sonicated at ~40 W for 30 sec and incubated at 37°C for 10 min. The solutions were kept at room temperature until use. α-MEM with 0.16% ethanol (v/v) and 1% fatty acid-free BSA (w/v) was used as fatty acid-free control.

5.2.3 Myriocin

Myriocin, a fungal toxin that inhibits SPT which catalyses the rate-limiting step of ceramide *de novo* synthesis, was used to determine the role of intramuscular ceramide accumulation in skeletal muscle insulin resistance (Miyake et al. 1995). A stock solution of myriocin (Sigma-Aldrich #M1177) at a concentration of 5 mM was prepared in absolute methanol and stored at -20°C until use. The optimal concentration of myriocin to reverse PA-induced insulin resistance in L6 myotubes was determined by a dose-response trial. Myriocin at a concentration of 10 μM has previously been shown to reduce the intracellular ceramide content and negated the inhibitory effect of PA on insulin-stimulated Akt activation in skeletal muscle cells (Chavez et al. 2003; Powell et

al. 2004). In order to minimise the concentration of methanol, the vehicle for myriocin, in the cell culture, 10 µM was selected as the maximal dose of myriocin in the dose-response trial. Myriocin was added to myotube cultures 30 min prior to PA and/or CM. The concentration of methanol was kept at 0.2% (v/v) in all treatment groups.

5.2.4 Rapamycin and PDTC

Stock solutions of rapamycin (10 µM) and PDTC (0.05 M) were prepared as described in Section 4.2.3. Rapamycin and PDTC, at concentrations of 20 nM and 100 µM respectively, were added to myotube cultures 30 min prior to PA and/or CM (Section 4.3.4). The concentration of DMSO was kept at 0.2% (v/v) in all treatment groups.

5.2.5 Glucose uptake

L6 cells were cultured on 12-well plates (Section 2.2.1). Differentiated myotubes were incubated with 0 – 400 µM of PA, LA and DHA, alone and in combination, for 24 h with or without the presence of CM during the last 6 h of incubation in an atmosphere of 5% CO_2 at 37°C. The plate content was aspirated afterwards. Myotubes were then incubated with or without 100 nM insulin for 30 min, followed by the measurement of 2-deoxy-D-[^{3}H] glucose uptake as described in Section 2.7.

5.2.6 Data analysis

Data are expressed as means ± SEM. Statistical analyses were performed by one-way or two-way ANOVA with Bonferroni post hoc tests to compare treatment difference using the GraphPad Prism Program. Significance was accepted at $P < 0.05$.

5.3 Results

5.3.1 Dose-response effect of fatty acids on glucose uptake in L6 myotubes

In the controls, insulin increased glucose uptake by 52% ($P < 0.001$). Neither DHA nor LA at 10, 100, 200 and 400 μM had any effect on insulin-stimulated glucose uptake, but the insulin response was attenuated by either DHA or LA at 50 μM (Figures 5.1A and B). PA at 10 and 50 μM was without effect on insulin-stimulated glucose uptake, which was reduced with higher concentrations of PA (100 – 400 μM; Figure 5.1C). The maximal inhibitory effect of PA was observed at 400 μM, which reduced insulin-stimulated glucose uptake by 36% ($P < 0.05$) compared to the controls. Fatty acids at a concentration of 400 μM were used in subsequent studies.

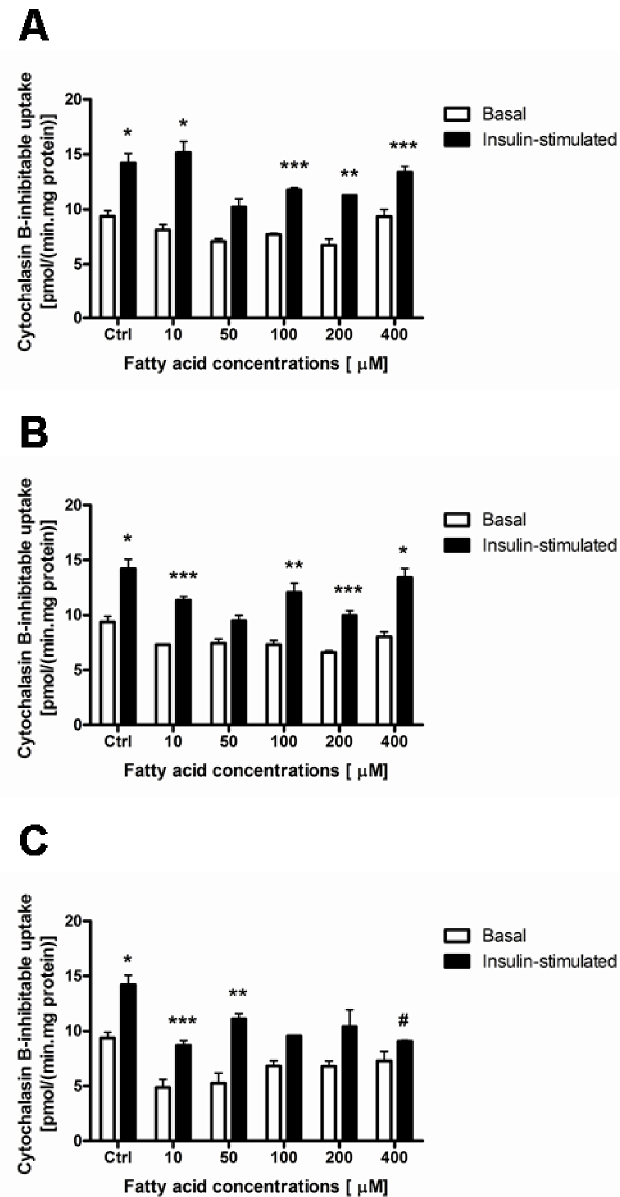

Figure 5.1. Dose-response effect of linoleic acid (A), docosahexaenoic acid (B) and palmitic acid (C) on basal and insulin-stimulated glucose uptake in L6 myotubes. L6 myotubes were cultured with fatty acids (0 – 400 μM) for 24 h, followed by a 30 min incubation at 37°C in α-MEM with or without insulin (100 nM). Myotubes were then incubated in uptake buffer containing 2-deoxy-D-[^{3}H] glucose (1 μCi/well; 10 μM) for 15 min. Experiments were completed in triplicate. Data are shown as mean ± SEM. * $P < 0.001$, ** $P < 0.01$ and *** $P < 0.05$ compared to the corresponding non-insulin-treated groups. # $P < 0.05$ compared to the corresponding control (Ctrl).

5.3.2 Effect of fatty acids on net insulin-stimulated glucose uptake in L6 myotubes

L6 myotubes were incubated with 400 μM of PA, DHA and LA, alone and in combination, for 24 h. In the controls, insulin induced a 74% ($P < 0.001$) increase in glucose uptake. PA reduced the net insulin-stimulated glucose uptake by 66% ($P < 0.001$) whereas DHA and LA were without effect. Compared to PA-treated myotubes, there was a non-significant trend for the combinations of PA with DHA and LA to increase net insulin-stimulated glucose uptake by 20% ($P = 0.45$) and 55% ($P = 0.07$) respectively (Figure 5.2).

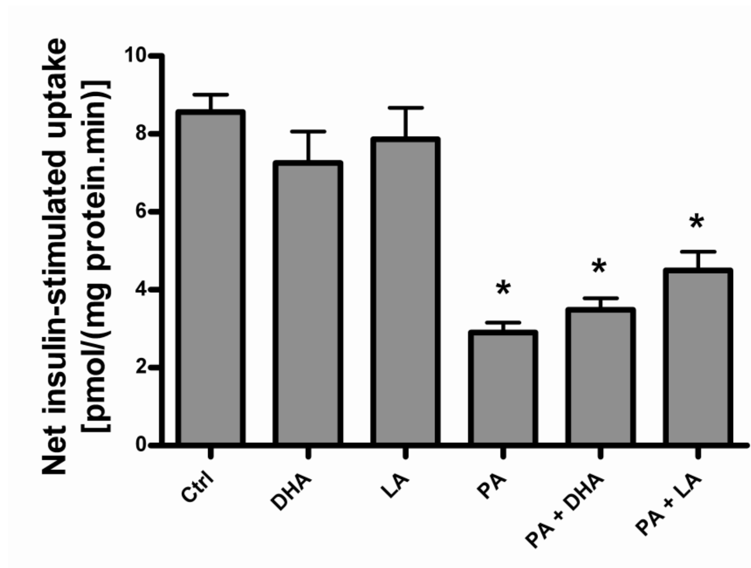

Figure 5.2. The effect of fatty acids, alone and in combination, on net insulin-stimulated glucose uptake in L6 myotubes. L6 myotubes were incubated with 400 μM of docosahexaenoic acid (DHA), linoleic acid (LA) and palmitic acid (PA), alone and in combination, for 24 h. L6 myotubes were then incubated for 30 min at 37°C in α-MEM with or without insulin (100 nM), followed by incubation in uptake buffer containing 2-deoxy-D-[^{3}H] glucose (1 μCi/well; 10 μM) for 15 min. Experiments were completed in triplicate. Data are shown as mean ± SEM. * $P < 0.001$ compared to fatty acid-free control (Ctrl).

5.3.3 Combined effect of adipose tissue-conditioned media and fatty acids on net insulin-stimulated glucose uptake in L6 myotubes

CM from IAB fat reduced net insulin-stimulated glucose uptake by 30% ($P < 0.001$) whereas CM from SC fat was without effect. Neither DHA nor LA altered the effect of CM. Compared to myotubes cultured with CM alone, the presence of PA in the culture further reduced net insulin-stimulated glucose uptake by 61 – 66% ($P < 0.001$). In cultures with either type of CM, the effect of PA was partially reversed by the combination of PA and DHA and completely reversed by the combination of PA and LA (Figures 5.3A and B). In the presence of CM from SC fat, PA and LA completely restored the insulin responsiveness to that of the controls (Figure 5.3B).

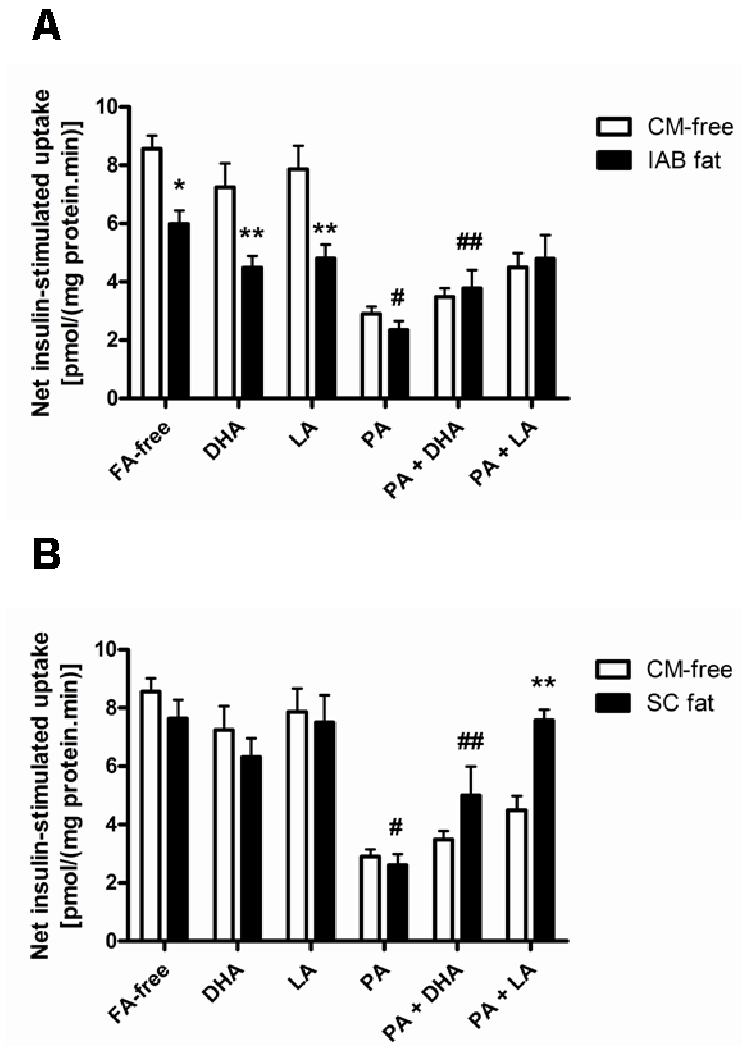

Figure 5.3. Net insulin-stimulated glucose uptake in L6 myotubes cultured with fatty acids and adipose tissue-conditioned media (CM) generated from visceral (IAB) and subcutaneous (SC) fat (n = 3/group). L6 myotubes were cultured with 400 μM of docosahexaenoic acid (DHA), linoleic acid (LA) and palmitic acid (PA), alone and in combination, for 24 h. CM from IAB (A) and SC (B) fat (concentration 1:128) were added during the last 6 h of incubation. L6 myotubes were then incubated for 30 min at 37°C in α-MEM with or without insulin (100 nM), followed by incubation in uptake buffer containing 2-deoxy-D-[^{3}H] glucose (1 μCi/well; 10 μM) for 15 min. Experiments were completed in triplicate. Data are shown as mean ± SEM. * $P < 0.001$ and ** $P < 0.01$ compared to the corresponding treatment without the presence of CM (CM-free). # $P < 0.001$ and ## $P < 0.01$ compared to the corresponding treatment without the presence of fatty acids (FA-free).

5.3.4 Role of ceramide in PA-induced insulin resistance in L6 myotubes

The optimal concentration of myriocin, a specific inhibitor of *de novo* ceramide synthesis, to reverse the inhibitory effect of PA on insulin responsiveness of L6 myotubes was determined. In PA-treated myotubes, insulin had no effect in the presence of myriocin at concentrations of 0.1 and 1 μM but increased glucose uptake by 65% ($P < 0.001$) in the presence of 10 μM myriocin (Figure 5.4A). The extent to which the inhibitory effect of PA was reversed by 10 μM myriocin, however, was not determined and therefore was validated in a subsequent study. PA reduced net insulin-stimulated glucose uptake by 76% ($P < 0.001$). The presence of 10 μM myriocin increased net insulin-stimulated glucose uptake by 215% ($P < 0.05$) and restored the insulin responsiveness similar to that in the corresponding control (Figure 5.4B). Myriocin at a concentration of 10 μM was used in the subsequent studies.

CM from IAB fat, PA and their combination reduced net insulin-stimulated glucose uptake by 26% ($P = 0.16$), 81% ($P < 0.001$) and 75% ($P < 0.001$) respectively. Myriocin did not alter the effect of CM from IAB fat. In contrast, myriocin increased net insulin-stimulated glucose uptake in myotubes cultured with PA and with a combination of PA and CM from IAB fat by 262% ($P < 0.05$) and 192% ($P < 0.05$) respectively and in both cases restored the insulin responsiveness to ~ 60% ($P < 0.05$) of the controls (Figure 5.5).

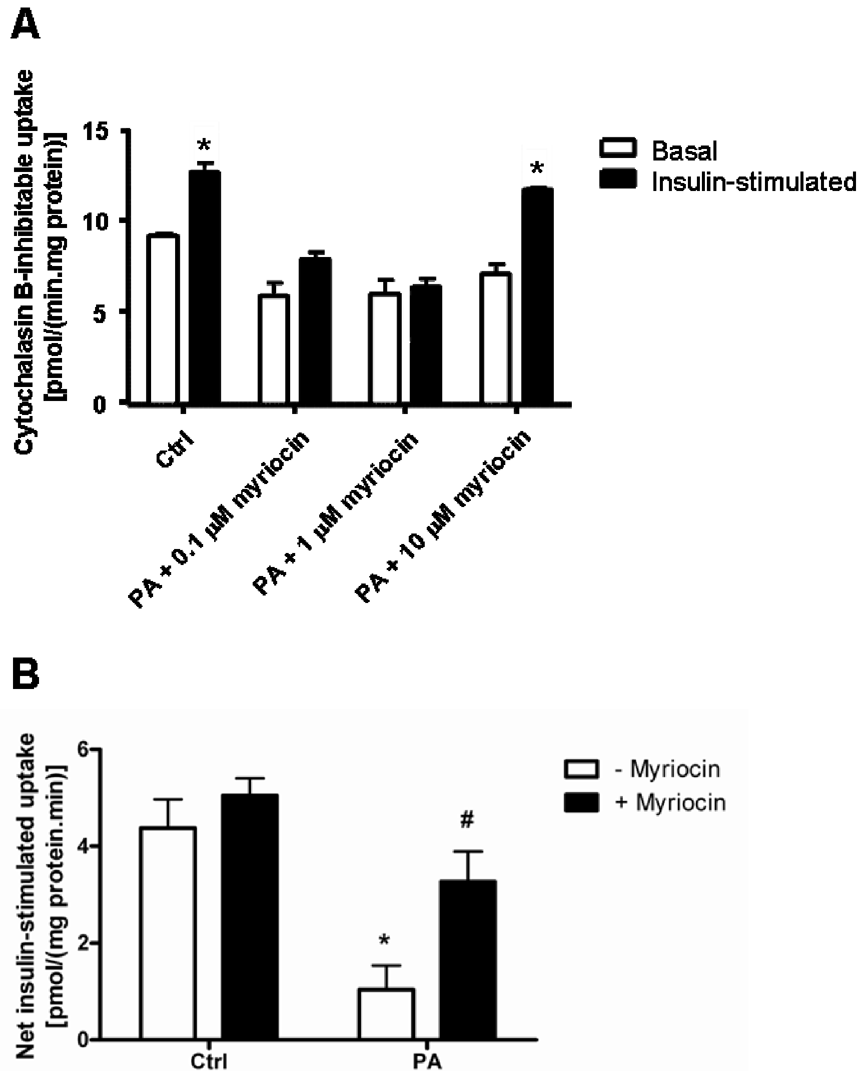

Figure 5.4. The effect of myriocin on basal and insulin-stimulated glucose uptake in palmitic acid-treated myotubes. Myriocin at concentrations of 0.1 – 10 µM (A) and 10 µM (B) was added to L6 myotubes 30 min prior to 400 µM palmitic acid (PA) and the culture was kept for 24 h. L6 myotubes were then incubated for 30 min at 37°C in α-MEM with or without insulin (100 nM), followed by incubation in uptake buffer containing 2-deoxy-D-[^{3}H] glucose (1 µCi/well; 10 µM) for 15 min. Experiments were completed in triplicate. Data are shown as mean ± SEM. A: * $P < 0.001$ compared to the corresponding basal uptake. B: * $P < 0.001$ compared to the corresponding control (Ctrl) and # $P < 0.05$ compared to the corresponding non-myriocin-treated group.

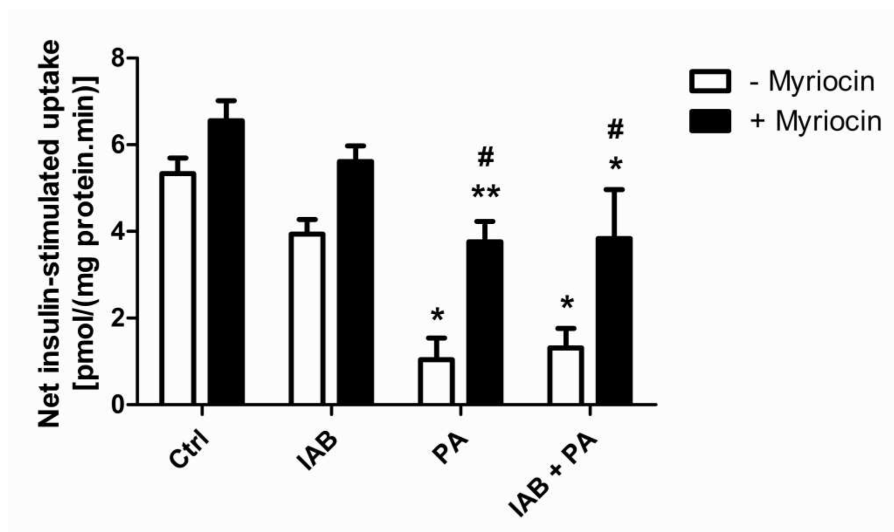

Figure 5.5. The effect of myriocin on insulin-stimulated glucose uptake in L6 myotubes cultured with visceral fat-conditioned media and palmitic acid (n = 3). L6 myotubes were incubated with 10 μM myriocin for 30 min prior to adding 400 μM palmitic acid (PA). The culture was kept for 24 h with or without visceral fat-conditioned media (IAB; concentration 1:128) during the last 6 h of incubation. L6 myotubes were then incubated for 30 min at 37°C in α-MEM with or without insulin (100 nM), followed by incubation in uptake buffer containing 2-deoxy-D-[^{3}H] glucose (1 μCi/well; 10 μM) for 15 min. Experiments were completed in triplicate. Data are shown as mean ± SEM. * $P < 0.001$ and ** $P < 0.05$ compared to the corresponding control (Ctrl). # $P < 0.05$ compared to the corresponding non-myriocin-treated group.

5.3.5 Role of mTORC1 and NFκB activation in insulin resistance in L6 myotubes

The direct involvement of the mTORC1 and NFκB signalling pathways in insulin resistance in skeletal muscle cells was determined using rapamycin and PDTC, which are specific inhibitors of the activation of mTORC1 and NFκB respectively (Section 4.3.4).

In the controls, insulin induced a 51% ($P < 0.001$) increase in glucose uptake and the effect was abolished by PA (Figure 5.6A). When mTORC1 activation was inhibited, insulin increased glucose uptake in the controls and in PA-treated myotubes by 81% ($P < 0.001$) and 57% ($P < 0.001$) respectively (Figure 5.6B). In the presence of PDTC, insulin-stimulated glucose uptake was 65% ($P < 0.001$) higher than the basal uptake in the controls and the effect of insulin was reduced to 46% ($P < 0.05$) in PA-treated myotubes (Figure 5.6C). When the activation of both mTORC1 and NFκB was inhibited, insulin increased glucose uptake in the controls and in PA-treated myotubes by 117% ($P < 0.001$) and 66% ($P < 0.001$) respectively (Figure 5.6D).

Insulin-stimulated glucose uptake was also abolished by the combined effect of PA and secretory factors from IAB fat (Figure 5.7A). In myotubes cultured with PA and CM from IAB fat, insulin increased glucose uptake by 53% ($P < 0.01$; Figure 5.7B) and 48% ($P < 0.01$; Figure 5.7C) in the presence of rapamycin and PDTC respectively. When both the mTORC1 and NFκB signalling pathways were inhibited, insulin-stimulated glucose uptake was 69% ($P < 0.001$) higher than the basal uptake (Figure 5.7D).

Taken together, rapamycin and PDTC, either alone or in combination, improved the effect of insulin on increasing glucose uptake in myotubes cultured with PA alone or in the presence of CM from IAB fat to 60 – 70% of that in the controls. These data suggest

that inhibiting the mTORC1 and NFκB signalling pathways partially and yet significantly reversed the inhibitory effect of PA and secretory factors from IAB fat on insulin responsiveness in skeletal muscle cells, although none of the inhibitors went close to restoring the insulin responsiveness to control levels.

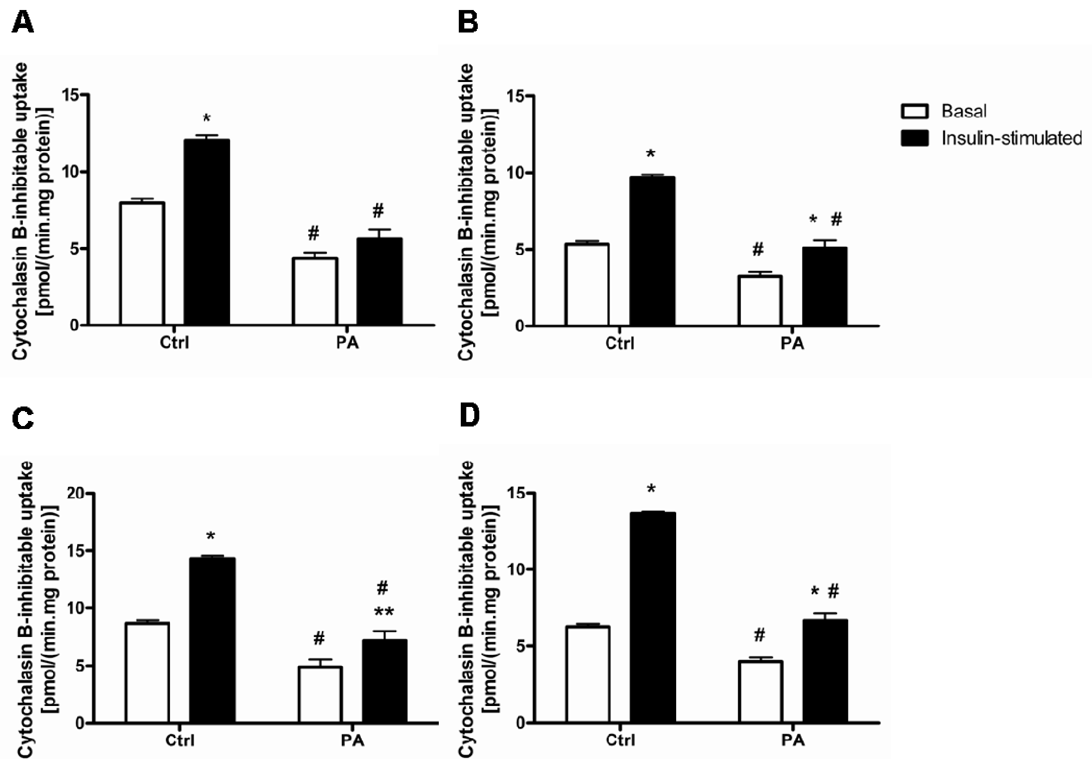

Figure 5.6. The effect of rapamycin and PDTC on basal and insulin-stimulated glucose uptake in L6 myotubes treated with palmitic acid (n = 4). L6 myotubes were pre-treated in the absence of rapamycin and PDTC (A), in the presence of 20 nM rapamycin (B), 100 µM PDTC (C) and a combination of 20 nM rapamycin and 100 µM PDTC (D) for 30 min. The inhibitors were retained when myotubes were cultured in 400 µM palmitic acid (PA) for 24 h. L6 myotubes were then incubated for 30 min at 37°C in α-MEM with or without insulin (100 nM), followed by incubation in uptake buffer containing 2-deoxy-D-[^{3}H] glucose (1 µCi/well; 10 µM) for 15 min. Experiments were completed in triplicate. Data are shown as mean ± SEM. * $P < 0.001$ and ** $P < 0.05$ compared to basal uptake of the corresponding treatment. # $P < 0.001$ compared to the corresponding control (Ctrl).

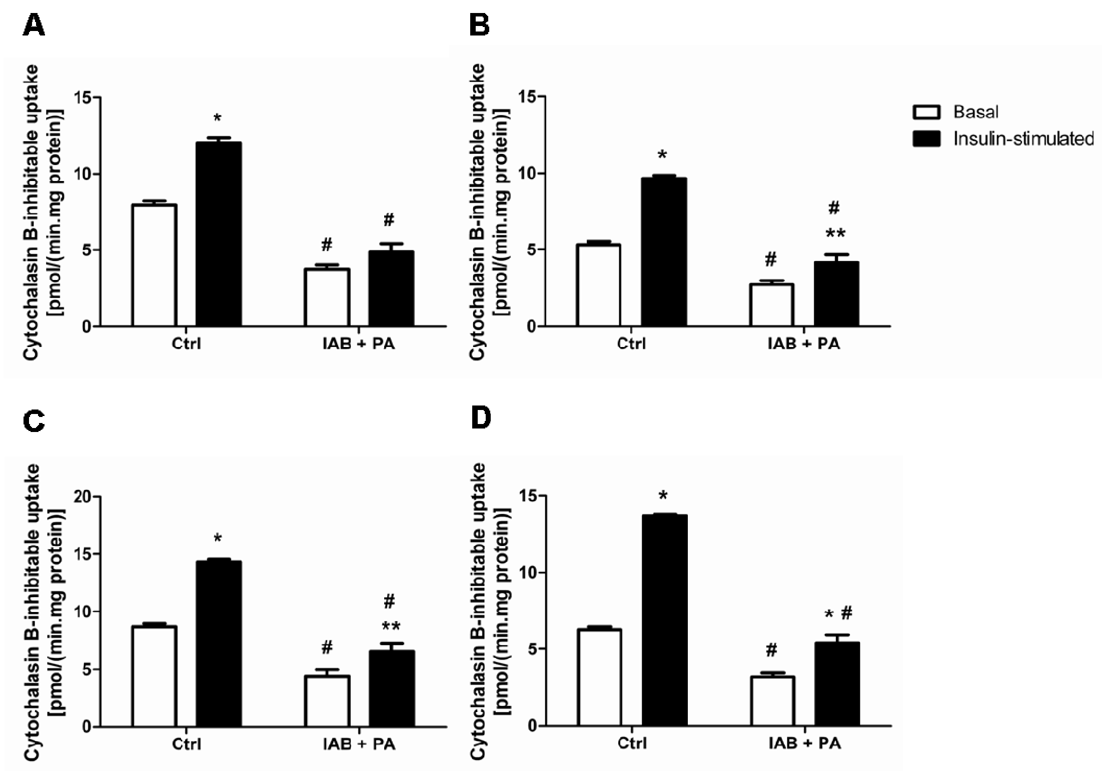

Figure 5.7. The effect of rapamycin and PDTC on basal and insulin-stimulated glucose uptake in L6 myotubes treated with visceral fat-conditioned media and palmitic acid (n = 4). L6 myotubes were pre-treated in the absence of rapamycin and PDTC (A), in the presence of 20 nM rapamycin (B), 100 µM PDTC (C) and a combination of 20 nM rapamycin and 100 µM PDTC (D) for 30 min. The inhibitors were retained when myotubes were cultured in 400 µM palmitic acid (PA) for 24 h with or without the presence of visceral fat-conditioned media (IAB; concentration 1:128) during the last 6 h of incubation. L6 myotubes were then incubated for 30 min at 37°C in α-MEM with or without insulin (100 nM), followed by incubation in uptake buffer containing 2-deoxy-D-[^{3}H] glucose (1 µCi/well, 10 µM) for 15 min. Experiments were completed in triplicate. Data are shown as mean ± SEM. * $P < 0.001$ and ** $P < 0.01$ compared to basal uptake of the corresponding treatment. # $P < 0.001$ compared to the corresponding control (Ctrl).

5.4 Discussion

The present study demonstrated the differential effect of long-chain saturated, n-3 and n-6 polyunsaturated fatty acids, and their interactions with secretory factors from IAB and SC fat, on insulin sensitivity in skeletal muscle cells *in vitro*. PA at 400 μM, a concentration comparable to that of plasma free fatty acids in humans under physiological conditions (Roden 2004), inhibited insulin-stimulated glucose uptake in L6 myotubes. The inhibitory effect of PA at similar concentrations (400 – 500 μM) on insulin sensitivity has been consistently demonstrated in skeletal muscle cells (Sinha et al. 2004; Watson et al. 2009). Ceramide is one of the lipid metabolites mediating the inhibitory effect of saturated fatty acids on insulin signal transduction by attenuating the activation of Akt, a major downstream component of the insulin signalling pathway. Possible mechanisms of ceramide-mediated insulin resistance include the activation of atypical PKC-ζ which phosphorylates and thus impairs the binding of Akt to 3-phosphoinositides (Powell et al. 2004), and the activation of PP2A which accelerates Akt dephosphorylation (Chavez et al. 2003). In myotubes cultured with 400 μM PA for 24 h, abolishing *de novo* synthesis of ceramide using a specific SPT inhibitor partially reversed (~ 40%) the effect of PA. The magnitude of this rescue effect was similar to that demonstrated in a previous study, in which the inhibitory effect of PA (750 μM; 16 h) on insulin-stimulated glucose uptake in L6 myotubes was reduced by ~ 50% (Powell et al. 2004). These data suggest that ceramide mediates part of the inhibitory effect of PA on insulin-stimulated glucose uptake. Accordingly, mechanisms other than *de novo* synthesis of ceramide must account for PA-induced insulin resistance in skeletal muscle cells.

PA has been shown to activate the IKK/IκB/NFκB pathway in skeletal muscle *in vitro* (Coll et al. 2008; Hommelberg et al. 2009). The inhibition of NFκB activation restored insulin-stimulated glucose uptake in PA-treated myotubes to 71% of the controls. Similarly, Sinha et al (2004) showed that insulin-stimulated glucose uptake in L6 myotubes cultured with 400 μM PA for 6 h was restored to ~ 80% of the controls in the presence of an IKK inhibitor. The effect of PA on inducing NFκB activity was not affected by inhibiting *de novo* synthesis of ceramide or by a ceramide analogue (Jove et al. 2005; Jove et al. 2006), suggesting that PA-induced insulin resistance is partly mediated by the NFκB signalling pathway in a ceramide-independent manner.

An alternative pathway for intramuscular metabolism of PA is the formation of DAG, which may mediate the inhibitory effect of PA on insulin sensitivity via the activation of the NFκB signalling pathway. The activation of DAG-sensitive nPKCs has been shown to increase NFκB DNA binding activity (Holden et al. 2008). Serine phosphorylation of IRS-1 by mTORC1 may be the downstream mechanism of the NFκB signalling pathway mediating the inhibitory effect of DAG on insulin signalling (Lee et al. 2008). PA inhibited insulin signal transduction in primary rat hepatocytes and the effect was associated with the activation of the mTORC1/S6K1 signalling pathway (Mordier and Iynedjian 2007). The restoration of insulin-stimulated glucose uptake in PA-treated myotubes (to ~70% of the controls) by rapamycin which inhibits mTORC1 indicates that the inhibitory effect of PA on insulin sensitivity in skeletal muscle cells is at least partly mediated by mTORC1 activation.

DAG may activate mTORC1 via the IKK/IκB/NFκB pathway similar to the effect of pro-inflammatory cytokines (Section 4.4). DAG-sensitive nPKCs may also activate the

mTORC1 signalling pathway independent of IKK by direct phosphorylation of mTORC1 via the c-Raf/MEK/ERK pathway as demonstrated in primary cultures of adult feline cardiomyocytes (Moschella et al. 2007). In the present study, inhibiting the activation of either NFκB or mTORC1 had similar effect on reversing PA-induced insulin resistance and the combined effect of the two did not further improve insulin responsiveness. These data suggest that PA may activate mTORC1 in skeletal muscle cells predominantly via the IKK/IκB/NFκB pathway. The role of the c-Raf/MEK/ERK pathway in mediating mTORC1 activation in skeletal muscle cells, if any, remains to be fully elucidated.

Taken together, the inhibitory effect of PA on insulin-stimulated glucose uptake in skeletal muscle cells may be mediated by intramuscular accumulation of both DAG and ceramide, which inhibits tyrosine phosphorylation of IRS-1 and Akt activation respectively. The possible metabolic pathways involved in PA-induced insulin resistance are summarised in Figure 5.8. The relative role of DAG and ceramide under a variety of physiological or pathological states requires further studies.

The inhibitory effect of PA is also additive to that of IAB fat on insulin sensitivity in skeletal muscle. Compared to CM from IAB fat alone, the presence of PA further reduced insulin-stimulated glucose uptake in myotubes by 39%. While the NFκB/mTORC1-dependent pathway is a common mechanism mediating both lipid- and IAB fat-induced insulin resistance in skeletal muscle cells, abolishing the activation of NFκB and mTORC1, either alone or in combination, only restored insulin-stimulated glucose uptake to ~ 70% of the controls. This is consistent with the effect of *de novo* synthesis of ceramide from PA which mediates insulin resistance independent of NFκB/mTORC1. The data suggest that insulin resistance in skeletal muscle in obesity,

which may be a consequence of an increased IAB fat and nutrient overload, is mediated by both common (NFκB/mTORC1) and distinct (ceramide) mechanisms.

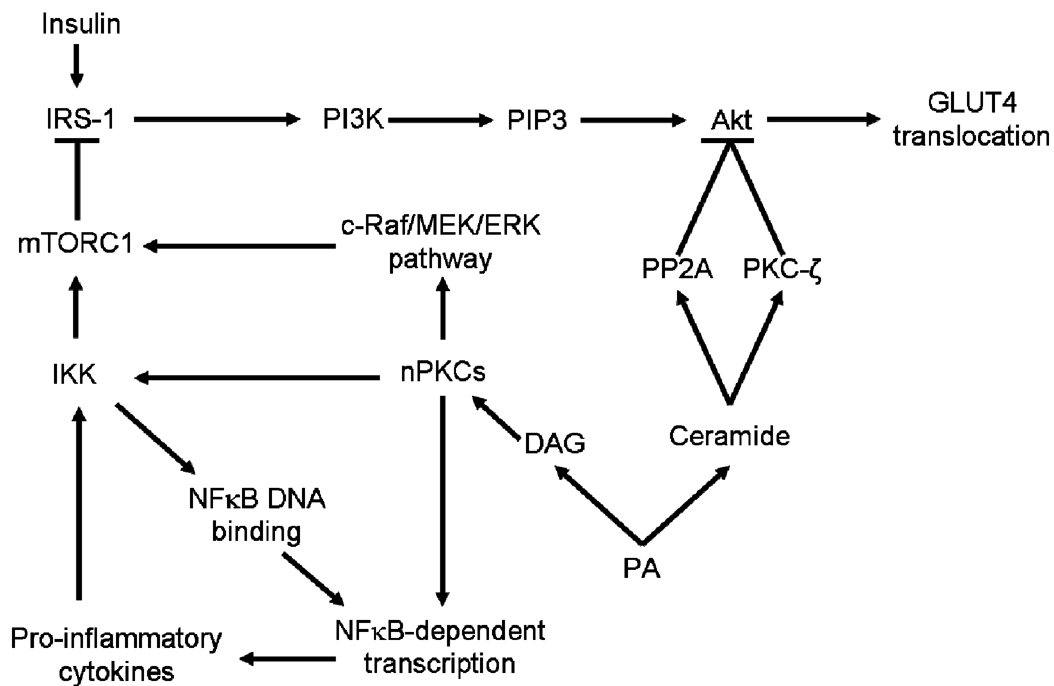

Figure 5.8. Potential mechanisms by which palmitic acid (PA) inhibits insulin-stimulated glucose uptake in skeletal muscle. PA induces insulin resistance by promoting intramuscular ceramide and diacylglycerol (DAG) accumulation. Ceramide activates protein kinase C (PKC)-ζ and protein phosphatase (PP)-2A which inhibits insulin-stimulated Akt phosphorylation. DAG activates a range of novel PKC isoforms which activates the IKK/IκB/NFκB (inhibitor kappa B kinase/inhibitor kappa B/nuclear factor kappa B) pathway. nPKCs activates mammalian target of rapamycin complex 1 (mTORC1) via either IKK or the c-Raf/MEK/ERK (c-Raf/mitogen-activated protein kinase kinase/extracellular signal-regulated kinase) pathway, which subsequently leads to the inhibitory phosphorylation of insulin receptor substrate (IRS)-1.

Consistent with the effect of n-3 (Aas et al. 2006) and n-6 (Lee et al. 2006) PUFAs documented previously, neither DHA nor LA had an effect on insulin-stimulated glucose uptake and they did not alter the effect of PA in skeletal muscle cells. In the presence of CM from either SC or IAB fat, DHA and LA reversed the inhibitory effect of PA on insulin-stimulated glucose uptake. The effect of LA in this regard was considerably greater than that of DHA. In contrast to the accumulation of lipid metabolites (DAG and ceramide) in response to PA, n-3 and n-6 PUFAs increase fatty acid oxidation in skeletal muscle (Forman et al. 1997; Ukropec et al. 2003). The effect of DHA and LA on improving or restoring insulin responsiveness in the presence of either type of CM may be a consequence of the combined effect of each fatty acid with adiponectin and/or leptin in CM together to promote fatty acid oxidation and thereby reduce substrate availability for ceramide and DAG synthesis. In rats fed with a high-fat diet, skeletal muscle-specific over-expression of CPT-I, an enzyme which up-regulates mitochondrial fatty acid uptake and thus increases fatty acid oxidation, has been shown to ameliorate lipid-induced insulin resistance and the effect is associated with a reduced membrane-to-cytosolic ratio of DAG and ceramide in skeletal muscle (Bruce et al. 2009).

The observations in this study have potential clinical implications. When added to usual diet neither EPA nor DHA supplements (4 g/day for 6 weeks) affected glycated haemoglobin, fasting insulin or insulin sensitivity in patients with type 2 diabetes (Woodman et al. 2002). Similarly, a 15-week LA-enriched (10.9% energy intake) diet had no effect on glycaemic control and carbohydrate tolerance in non-insulin-dependent diabetic patients (Heine et al. 1989). In contrast, in the context of energy restriction the inclusion of n-3 fatty acids (in the form of either fish meals 3 times per week or daily fish oil capsules) reduced fasting insulin and attenuated insulin resistance in overweight and

obese young adults (Ramel et al. 2008). The present data suggest that the rescue effect of PUFAs is minimal when PA is at a high concentration and infer that the combination of limiting saturated fat and increasing PUFAs intake may be the most effective strategies to improve insulin sensitivity, particularly in the setting of mild energy restriction or regular exercise (Hill et al. 2007).

Taken together, I showed that PA inhibited insulin-stimulated glucose uptake in skeletal muscle cells, an effect which was reversed partially by DHA and completely by LA in the presence of CM from either SC or IAB fat. The data support the role of n-3 and n-6 PUFAs on improving insulin sensitivity in skeletal muscle and highlight the potential importance of dietary fat composition in diabetes prevention and management with the implication that diets need to be very low in saturated fat and higher in PUFAs. I hypothesised that n-3 and n-6 PUFAs may improve insulin sensitivity by activating fatty acid oxidation and the possible mechanisms will be addressed in the following chapters.