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 6: Effect of long-chain saturated, n-3 and n-6 polyunsaturated fatty acids on pathways regulating energy metabolism in skeletal muscle cells

6.1 Introduction

In Chapter 5 I demonstrated the inhibitory effect of PA on insulin-stimulated glucose uptake in L6 myotubes, which may be mediated by the intramuscular accumulation of lipid metabolites including ceramide and DAG. In the presence of secretory factors from either IAB or SC fat, DHA partially and LA completely reversed the effect of PA. These data suggest the potential benefits of DHA and LA to improve insulin sensitivity in skeletal muscle. Similar positive effect of combinations of fatty acids on insulin response has previously been demonstrated. In C2C12 myotubes, PA-induced DAG accumulation and inhibition of insulin-stimulated Akt phosphorylation were reversed by co-incubation with oleic acid and the rescue effect was mediated by an increase in mitochondrial β-oxidation (Coll et al. 2008). Similarly, a combination of PA and oleic acid reduced intramuscular DAG content and restored the effect of insulin on increasing glucose uptake to ~ 70% of that in the controls in primary human myotubes (Montell et al. 2001). These data suggest that the regulation of fatty acid partitioning, in particular increasing fatty acid oxidation which reduces the availability of PA for ceramide and DAG synthesis, may be a key strategy to prevent lipid-induced insulin resistance in skeletal muscle.

The role of LA and DHA in facilitating fatty acid oxidation is supported by their hypolipidaemic effect in dietary intervention studies. An 8-week liquid diet (40% energy from fat) with 63% fatty acids as LA reduced plasma triglyceride level by 34% in healthy

hypercholesterolemic subjects (Reaven et al. 1993). Following a DHA supplementation (3 g/day) for 45 days, plasma triglyceride concentration was reduced by 24% in moderately hyperlipidaemic but otherwise healthy men (Kelley et al. 2007). In animal models, mice fed with a diet containing 12% LA (w/w) for 35 days had 41% lower plasma triglyceride than animals on a low-fat control diet and the effect was associated with an increase in hepatic activity of key enzymes of fatty acid oxidation including citrate synthase and 3-hydroxy-acyl-CoA dehydrogenase (Javadi et al. 2007). Similarly, dietary supplementation of n-3 PUFAs increased mRNA expression and activity of fatty acid oxidation enzymes (e.g. CPT and acyl-CoA oxidase) in liver and skeletal muscle, and the up-regulated β-oxidation was associated with an improvement in insulin sensitivity (Hong et al. 2003; Ukropec et al. 2003).

The hypolipidaemic effect of LA and DHA may be mediated by regulating gene transcriptions in lipid metabolism via the activation of PPAR-α, which is highly expressed in the liver and to a lesser extent in skeletal muscle (Ferre 2004). The activation of PPAR-α facilitates fatty acid oxidation by inducing the transcription of genes which encode proteins in fatty acid transport and key enzymes involved in peroxisomal and mitochondrial β-oxidation, while at the same time stimulates PDK4 gene expression which subsequently reduce PDC activity to reduce glucose oxidation (Ferre 2004). In rats fed with a high-fat diet, the administration of a PPAR-α agonist reduced triglyceride and total long-chain acyl-CoAs in liver and skeletal muscle and ameliorated whole-body and muscle insulin resistance (Ye et al. 2001). Fatty acids are natural ligand activators of PPAR-α and their ability to bind to and activate PPAR-α varies according to chain length and degree of saturation. *In vitro*, saturated fatty acids are shown to be weak activators of PPAR-α, whereas LA and DHA are among a range of

PUFAs all bind to and activate PPAR-α (Forman et al. 1997). The differential effect of fatty acids on PPAR-α activation is consistent with previous findings that unsaturated fatty acids are more readily oxidised than saturated fatty acids (DeLany et al. 2000).

Another possible mechanism for PUFAs to promote fatty acid oxidation may be the compensatory increase in lipid catabolism as a result of reduced lipogenesis. SREBPs are a family of transcription factors that activates enzymes for endogenous synthesis of fatty acids, triglycerides and cholesterol (Eberle et al. 2004). The inhibitory effect of fatty acids on SREBP-dependent gene expression increases with chain length and number of double bonds (Worgall et al. 1998). n-3 and n-6 PUFAs inhibit the gene expression and proteolytic release of nuclear SREBP-1 and therefore down-regulate the transcription of a range of lipogenic genes including FAS, ACC and SCD (Schmitz and Ecker 2008). Compared to the fat-free control group, supplementation of 10% fat (w/w) with safflower oil (65% LA) and sterol-free, menhaden fish oil (35% EPA and DHA) reduced the hepatic content of precursor and the nuclear content of mature SREBP-1 by up to 60 and 85% respectively whereas triolein (99% oleic acid) was without effect (Xu et al. 1999). *In vitro*, incubating CaCo-2 cells with LA, EPA and DHA reduced the mRNA expression of SREBP-1a and 1c, protein expression of the mature form of SREBP-1 and mRNA content of FAS and ACC whereas stearic and oleic acids were without effect (Field et al. 2002).

The inverse relationship between the expression and activity of SCD, a key enzyme regulating triglyceride synthesis by catalysing a Δ9-*cis* desaturation of saturated fatty acids, and fatty acid oxidation and insulin sensitivity has been demonstrated *in vivo*. SCD1$^{-/-}$ mice had higher expression of lipid oxidation genes and rate of β-oxidation in

the liver and showed an improved insulin sensitivity in response to high-fat feeding compared to the wild-type controls (Ntambi et al. 2002; Dobrzyn et al. 2004). In oxidative skeletal muscle, the SCD1 deficiency-induced increase in fatty acid oxidation was also associated with a decrease in intramuscular ceramide content and the mRNA expression and activity of SPT, the rate-limiting enzyme of *de novo* synthesis of ceramide (Dobrzyn et al. 2005). The relationship between SCD1 expression and fatty acid oxidation, however, is equivocal *in vitro*. A negative association has been shown between SCD1 mRNA expression and the rate of fatty acid oxidation in primary human myotubes (Hulver et al. 2005). Compared to the controls, SCD1 siRNA-treated L6 myotubes had similar rate of fatty acid oxidation but higher intracellular DAG and ceramide content in response to PA. Conversely, SCD1 over-expression resulted in a small and yet significant reduction in fatty acid oxidation and attenuated PA-induced DAG and ceramide accumulation by ~ 50% (Pinnamaneni et al. 2006).

Fatty acids of different degrees of saturation have been shown to have differential effects on SCD expression and activity and thus may in turn alter fatty acid metabolism and insulin sensitivity. Compared to the standard chow control group, rats fed with a high-fat diet consisting primarily of saturated fatty acids had higher SCD protein expression and activity in skeletal muscle whereas the opposite was observed in rats fed with a high-PUFA diet of mainly LA (Lee et al. 2006). Similarly, rats on a DHA-enriched (1.5%; w/w) low-fat diet had lower hepatic mRNA expression and activity of SCD when compared to the low-fat control group (Kramer et al. 2003). These data are consistent with the down-regulation of SCD1 mRNA expression with increasing degree of unsaturation of fatty acids demonstrated in isolated rat hepatocytes (Landschulz et al. 1994).

In the present study I aimed to determine:

1. *The direct effect of long-chain saturated, n-3 and n-6 PUFAs on the mRNA expression of key genes regulating energy metabolism in skeletal muscle in vitro.* Specifically the effect of PA, DHA and LA, with or without the presence of CM from SC or IAB fat, on mRNA expression of key genes regulating muscle energy metabolism was determined. mRNA of the genes listed below was measured. The role of these genes in energy metabolism in skeletal muscle is illustrated in Figure 6.1.

    - AMPKα1 and AMPKα2 – they encode the two catalytic subunits of AMPK which, when activated, increases mitochondrial fatty acid uptake and subsequently up-regulates β-oxidation (Thomson et al. 2007).
    - LKB1 – it encodes the upstream kinase for AMPK activation (Thomson et al. 2007).
    - PGC-1α – it induces mitochondrial biogenesis by activating mitochondrial DNA replication and transcription (Puigserver and Spiegelman 2003).
    - PDK4 – it encodes pyruvate dehydrogenase kinase which inactivates PDC and subsequently reduces glucose oxidation. It serves as an indirect indicator of fatty acid oxidation (Abbot et al. 2005).
    - SCD1 – it encodes SCD which catalyses the synthesis of monounsaturated fatty acids for tissue lipids including triglycerides (Dobrzyn and Dobrzyn 2006).
    - SPTLC1 and SPTLC2 – they encode the regulatory and catalytic subunits respectively of SPT, the rate-limiting enzyme in *de novo* synthesis of ceramide (Hanada 2003).

I hypothesised that PA would increase the mRNA content of SCD1, SPTLC2 and SPTLC2 which promote triglyceride accumulation and *de novo* synthesis of ceramide respectively, while DHA and LA would up-regulate the mRNA content of genes increasing fatty acid oxidation including AMPKα1 and α2, LKB1 and PGC-1α.

2. *The effect of combination of PA with either DHA or LA on mRNA expression of key genes regulating energy metabolism in skeletal muscle in vitro.*

   The rescue effect of DHA and LA on PA-induced insulin resistance may be mediated by activating fatty acid oxidation and subsequently reducing lipid accumulation in skeletal muscle cells. I hypothesised that the effect of DHA and LA on increasing the mRNA content of AMPKα1 and α2, LKB1 and PGC-1α would remain predominant when in the presence of PA and attenuate the effect of PA on up-regulating SCD1, SPTLC1 and SPTLC2 mRNA.

3. *The acute effect of PA, DHA and LA, alone and in combination, on protein phosphorylation of AMPKα and ACCβ in skeletal muscle in vitro.*

   Fatty acids promote fat oxidation by phosphorylating AMPKα. The activated AMPKα then phosphorylates ACCβ to inhibit the production of malonyl-CoA, which removes the inhibition on CPT-I and subsequently increases mitochondrial β-oxidation (Osler and Zierath 2008). I hypothesised that DHA and LA, alone and in the presence of PA, would increase AMPKα and ACCβ phosphorylation. In addition, the effect of fatty acids on AMPKα and ACCβ phosphorylation would correspond to their effects on mRNA content of LKB1, AMPKα1 and α2.

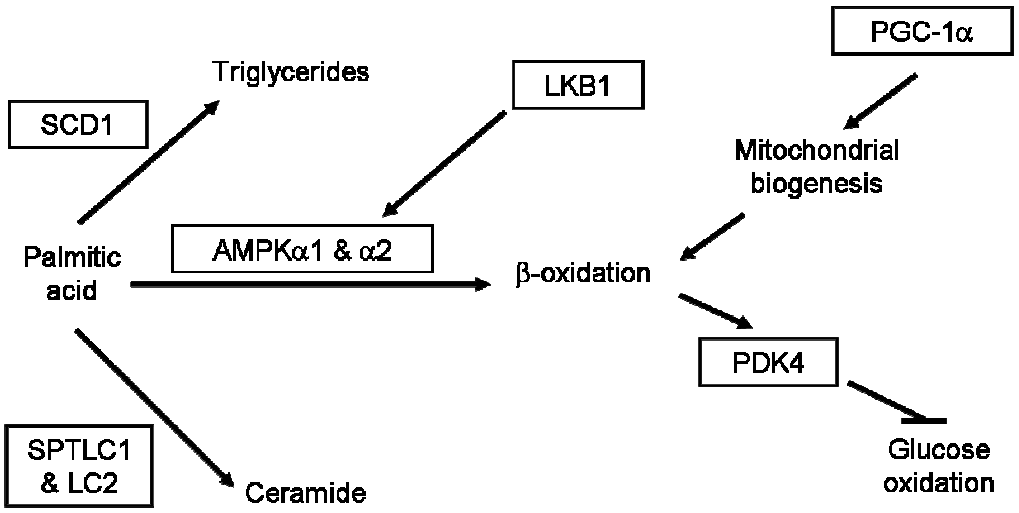

Figure 6.1. A simplified schematic model showing the key genes involved in the regulation of energy metabolism in skeletal muscle. Peroxisome proliferator-activated receptor-γ co-activator (PGC)-1α and AMP-activated protein kinase (AMPK) promote fatty acid oxidation by increasing mitochondrial biogenesis and fatty acid uptake respectively. The two catalytic subunits of AMPK are encoded by AMPKα1 and α2 and are activated mainly by LKB1. Fatty acid oxidation results in an increase in PDK4 mRNA expression which inhibits glucose oxidation. An alternative way for fatty acid metabolism is lipid accumulation. SCD1 encodes stearoy-coenzyme A desaturase which synthesises monounsaturated fatty acids for triglyceride formation. SPTLC1 and SPTLC2 encode the regulatory and catalytic subunits respectively of serine palmitoyltransferase, which catalyses the *de novo* synthesis of ceramide.

6.2 Materials and methods

6.2.1 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 (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).

6.2.2 Gene expression

L6 cells were cultured on 6-well plates (Section 2.2.1). Differentiated myotubes were cultured for 24 h with 400 μM PA, DHA and LA, alone and in combination, with or without CM from IAB and SC fat (concentration 1:128) during the last 6 h of incubation. α-MEM with 0.16% ethanol (v/v) and 1% fatty acid-free BSA (w/v) was used as control. RNA isolation and RT-PCR were performed as described in Section 2.8.1.

6.2.3 Western blotting

L6 cells were cultured on Petri dishes (Section 2.2.1) and incubated for 1 h in 400 μM PA, DHA and LA, alone and in combination, and with or without CM from IAB and SC fat (concentration 1:128). Protein extraction and immunoblots were performed as described in Section 2.9.

6.3 Results

6.3.1 Effect of fatty acids on gene expression in L6 myotubes

AMPKα2 mRNA content increased by ~ 100% in response to DHA or LA ($P < 0.05$) but decreased by 37% ($P = 0.25$) in response to PA as compared to controls. AMPKα2

mRNA content was similar to controls in response to the combination of PA and DHA and the effect of PA with LA was similar to LA alone (Figure 6.2A). While LA was without effect, there was a trend for DHA to reduce SCD1 mRNA by 33% ($P = 0.12$). PA increased SCD1 mRNA by 53% ($P = 0.13$), an effect that was abolished when combined with DHA but not LA (Figure 6.2B). DHA did not affect PDK4 mRNA content but LA increased and PA decreased PDK4 mRNA by 48% ($P = 0.06$) and 33% ($P = 0.15$) respectively. The combination of PA and DHA and that of PA and LA increased PDK4 mRNA content by 101% ($P < 0.01$) and 154% ($P < 0.001$) respectively as compared to PA alone (Figure 6.2C). DHA, LA and PA reduced PGC-1α mRNA by 26% ($P = 0.07$), 53% ($P < 0.01$) and 52% ($P < 0.01$) respectively. The combined effect of PA with either DHA or LA on PGC-1α mRNA content was not different from that of the fatty acids alone (Figure 6.2D). There were no significant changes in the mRNA content of AMPKα1, LKB1, SPTLC1 and SPTLC2 under any condition (data not shown).

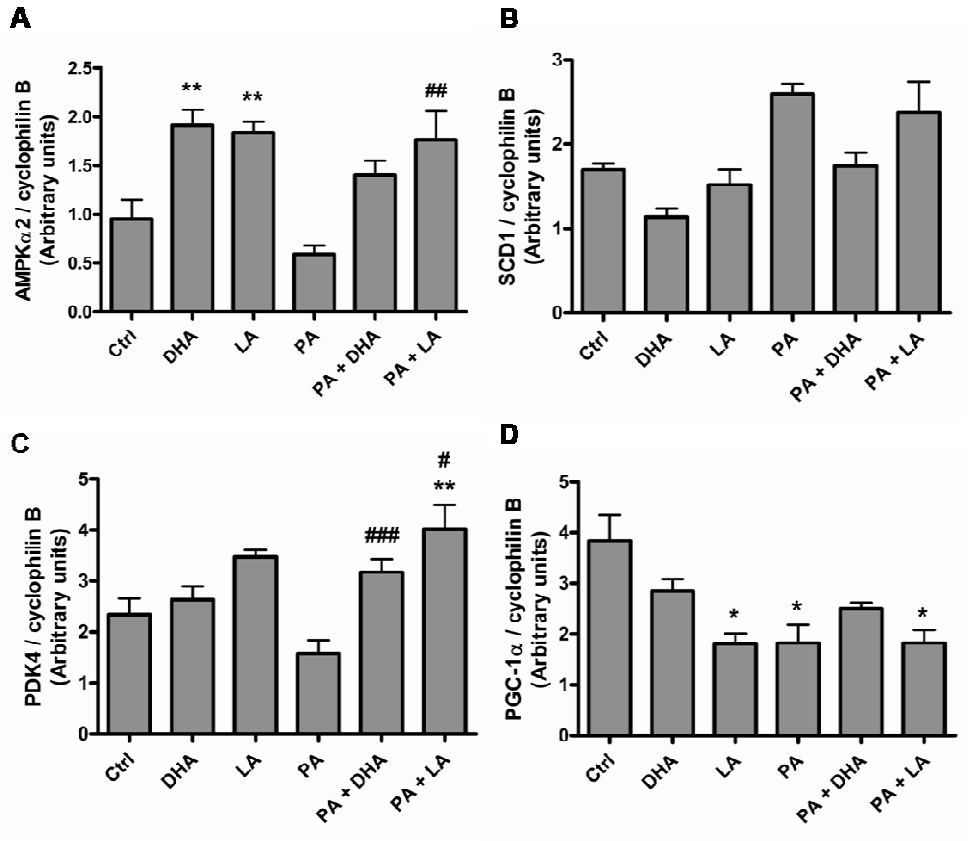

Figure 6.2. The effect of fatty acids on mRNA content of AMPKα2 (A), SCD1 (B), PDK4 (C) and PGC-1α (D) in L6 myotubes (n = 4). L6 myotubes were incubated with 400 μM of docosahexaenoic acid (DHA), linoleic acid (LA) and palmitic acid (PA), alone and in combination, for 24 h. RNA was isolated using TRIzol reagent and analysed by RT-PCR. Gene expression was normalised using cyclophilin B. Data are shown as mean ± SEM. * $P < 0.01$ and ** $P < 0.05$ compared to fatty acid-free control (Ctrl). # $P < 0.001$, ## $P < 0.01$ and ### $P < 0.05$ compared to PA.

6.3.2 Combined effect of secretory factors from adipose tissue and fatty acids on gene expression in L6 myotubes

CM from IAB and SC fat increased the mRNA content of AMPKα2 by 180% ($P < 0.05$) and 226% ($P < 0.05$) respectively (Figure 6.3A). Neither LA nor PA modified the effect of CM, whereas DHA further increased AMPKα2 mRNA by 75 – 97% ($P < 0.001$) compared to either CM alone. In the presence of CM from IAB fat, the combined effect of PA-DHA and PA-LA increased AMPKα2 mRNA content by 50 – 60% when compared to PA alone. A similar but more robust effect of PA-DHA and PA-LA on the mRNA content of AMPKα2 (78 – 117%; $P < 0.01$) was observed in cultures with CM from SC fat (Figure 6.4A).

Neither CM from IAB nor SC fat modified the mRNA content of PGC-1α (Figure 6.3B). As compared to either type of CM alone, PA reduced PGC-1α mRNA by 36 – 49% ($P < 0.01$). In cultures with CM from IAB fat, DHA and LA increased PGC-1α mRNA content by 34% ($P < 0.05$) and 44% ($P < 0.05$) respectively. There was a similar trend for DHA (25%; $P = 0.12$) and LA (29%; $P = 0.08$) to increase PGC-1α mRNA content in cultures with CM from SC fat (Figure 6.4B). In cultures with either type of CM, the combination of PA with either DHA or LA restored PGC-1α mRNA content to that in myotubes cultured with CM alone (Figure 6.4B).

CM from either IAB or SC fat was without effect on SCD1 mRNA (Figure 6.3C). In cultures with either type of CM, SCD1 mRNA was increased 85 – 104% ($P < 0.001$) by PA and decreased 37 – 41% by DHA or LA as compared to CM alone. Combinations of PA with either DHA or LA reduced the PA effect by 81 – 92% ($P < 0.01$) and 71 – 74% ($P < 0.01$) in the presence of CM from IAB and SC fat respectively (Figure 6.4C).

There was no effect of CM from either fat depots, alone or in combination with fatty acids, on mRNA content of PDK4, LKB1, AMPKα1, SPTLC1 and SPTLC2 (data not shown).

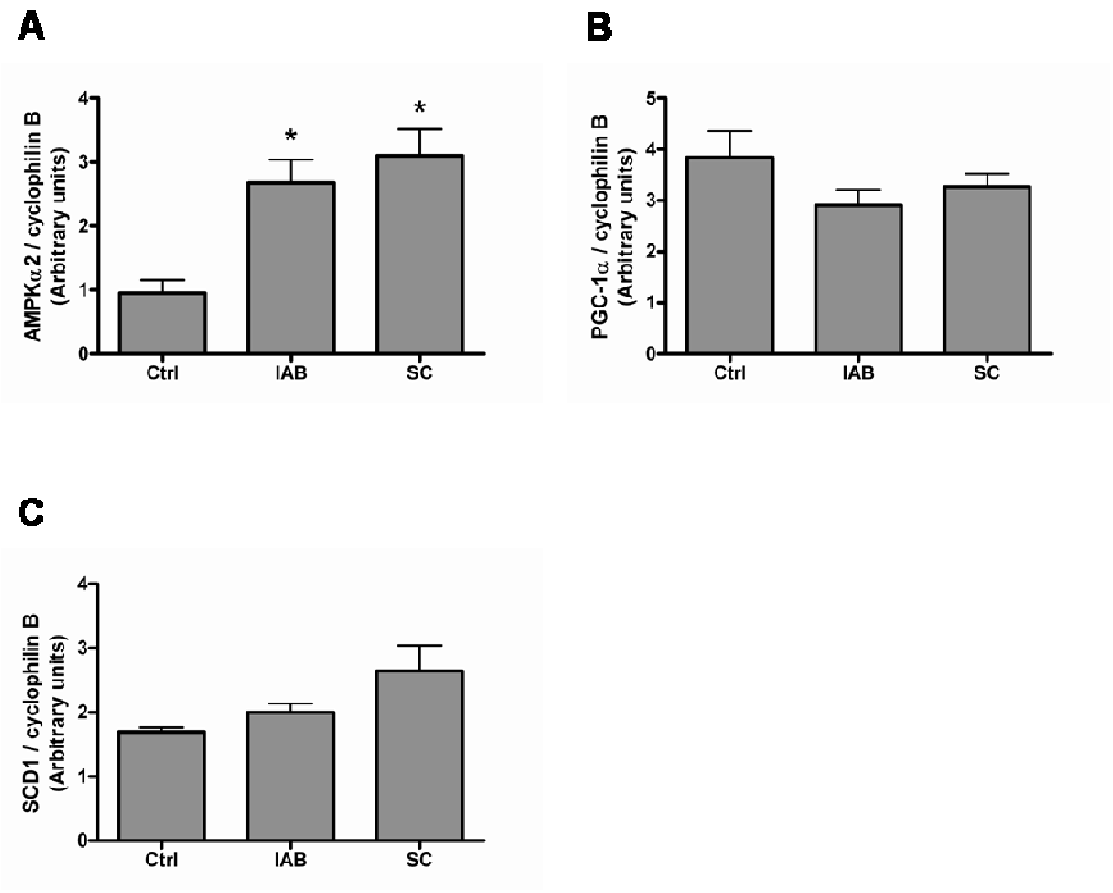

Figure 6.3. mRNA content of AMPKα2 (A), PGC-1α (B) and SCD1 (C) in L6 myotubes cultured with adipose tissue-conditioned media generated from visceral (IAB) and subcutaneous (SC) fat (n = 3). L6 myotubes were incubated with conditioned media from IAB and SC fat (concentration 1:128) for 6 h. RNA was isolated using TRIzol reagent and analysed by RT-PCR. mRNA expression was normalised using cyclophilin B. Data are shown as mean ± SEM. * $P < 0.05$ compared to control (Ctrl).

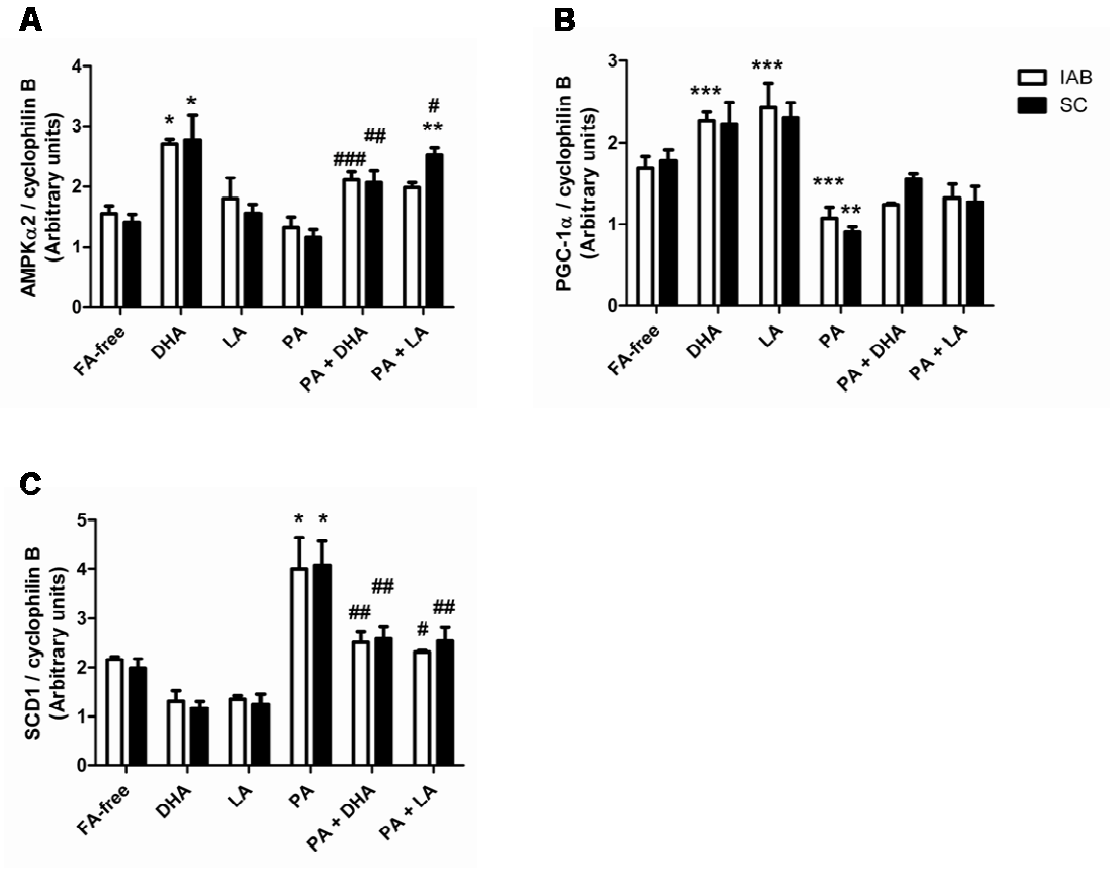

Figure 6.4. mRNA content of AMPKα2 (A), PGC-1α (B) and SCD1 (C) in L6 myotubes cultured with fatty acids and adipose tissue-conditioned media (CM) generated from visceral (IAB) and subcutaneous (SC) fat (n = 3). L6 myotubes were incubated with 400 μM of docosahexaenoic acid (DHA), linoleic acid (LA) and palmitic acid (PA), alone and in combination, with or without the presence of CM from IAB and SC fat (concentration 1:128) during the last 6 h of incubation. RNA was isolated using TRIzol reagent and analysed by RT-PCR. mRNA expression was normalised using cyclophilin B. Data are shown as mean ± SEM. * $P < 0.001$, ** $P < 0.01$ and *** $P < 0.05$ compared to the corresponding fatty acid-free co-culture (FA-free). # $P < 0.001$, ## $P < 0.01$ and ### $P < 0.05$ compared to the corresponding PA treatment.

6.3.3 Acute effect of fatty acids on protein phosphorylation in L6 myotubes

DHA, LA and PA increased the phosphorylation of AMPKα by 40% ($P = 0.17$), 92% ($P < 0.05$) and 56% ($P = 0.07$) respectively. The effect of DHA and PA appeared to be additive and the combination induced an increase in AMPKα phosphorylation of 109% ($P < 0.01$) compared to controls. The combination of LA and PA induced an 84% ($P < 0.05$) increase in the phosphorylation of AMPKα, which was not different from the effect of LA alone (Figure 6.5A).

DHA, LA and PA increased ACCβ phosphorylation by 154% ($P = 0.06$), 119% ($P = 0.08$) and 77% ($P = 0.22$) respectively. Compared to controls, the combination of DHA and PA induced a 340% ($P < 0.001$) increase in ACCβ phosphorylation whereas the combined effect of LA and PA was similar to that of LA alone (Figure 6.5B).

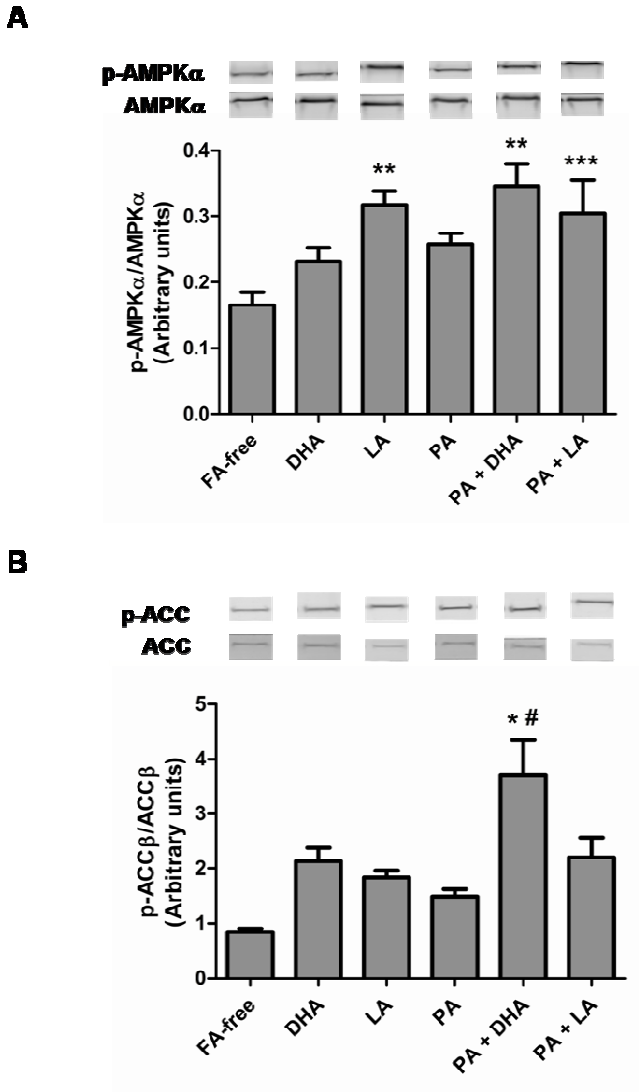

Figure 6.5. The effect of fatty acids on protein phosphorylation of AMPKα (A) and ACCβ (B) in L6 myotubes (n = 4). L6 myotubes were incubated with 400 μM of docosahexaenoic acid (DHA), linoleic acid (LA) and palmitic acid (PA), alone and in combination, for 1 h. Data are shown as mean ± SEM and are expressed as ratios of phosphorylated to total protein. * $P < 0.001$, ** $P < 0.01$ and *** $P < 0.05$ compared to fatty acid-free control (FA-free). # $P < 0.01$ compared to PA.

6.3.4 Combined effect of secretory factors from adipose tissue and fatty acids on protein phosphorylation in L6 myotubes

CM from IAB and SC fat increased the phosphorylation of AMPKα by 144% ($P < 0.01$) and 75% ($P = 0.06$) respectively (Figure 6.6A). In cultures with CM from IAB fat, DHA and LA reduced AMPKα phosphorylation by 25% ($P = 0.09$) and 43% ($P < 0.05$) respectively whereas PA was without effect. The combined effect of PA-DHA and PA-LA was similar to that of DHA and LA by themselves. In cultures with CM from SC fat, AMPKα phosphorylation was not altered by DHA but was reduced 33% ($P = 0.1$) by LA and increased 83% ($P < 0.01$) by PA. The combination of PA and DHA did not modify the effect of CM from SC fat, whereas that of PA and LA increased AMPKα phosphorylation by 22% as compared to CM alone (Figure 6.7A).

CM from IAB and SC fat increased the phosphorylation of ACCβ by 180% ($P < 0.01$) and 47% ($P = 0.31$) respectively (Figure 6.6B). None of the fatty acids, alone or in combination, modified the effect of CM from IAB fat. In cultures with CM from SC fat, LA and PA increased ACCβ phosphorylation by 200% ($P < 0.001$) and 29% ($P = 0.33$) respectively whereas DHA was without effect. Compared to that in cultures with CM from SC fat alone, the combinations of PA-DHA and PA-LA increased ACCβ phosphorylation by 114% ($P < 0.01$) and 63% ($P = 0.06$) respectively (Figure 6.7B).

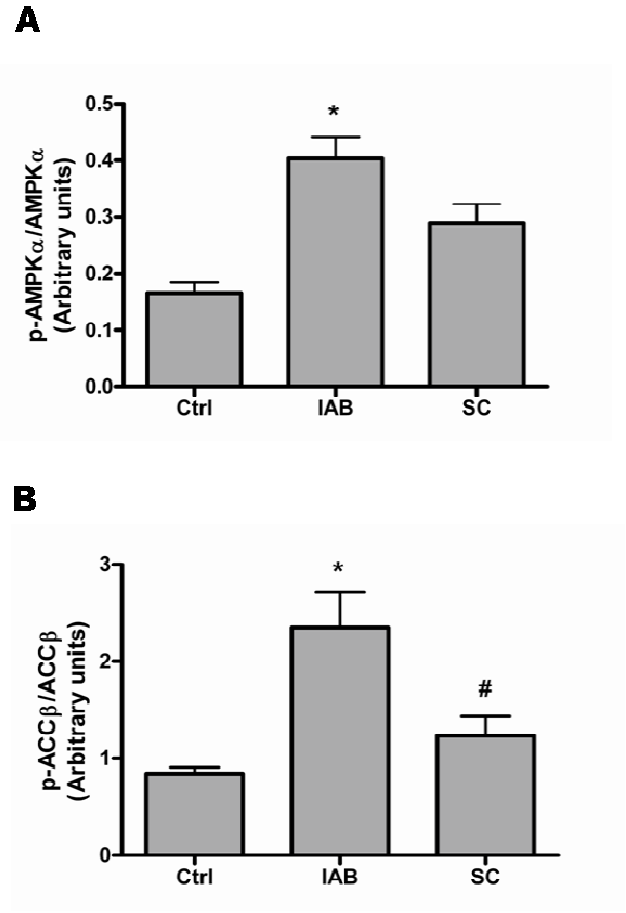

Figure 6.6. Protein phosphorylation of AMPKα (A) and ACCβ (B) in L6 myotubes cultured with adipose tissue-conditioned media from visceral (IAB) and subcutaneous (SC) fat (n = 3). L6 myotubes were incubated with conditioned media from IAB and SC fat (concentration 1:128) for 1 h. Data are shown as mean ± SEM and are expressed as ratios of phosphorylated to total protein. * $P < 0.01$ compared to control (Ctrl). # $P < 0.05$ compared to IAB fat.

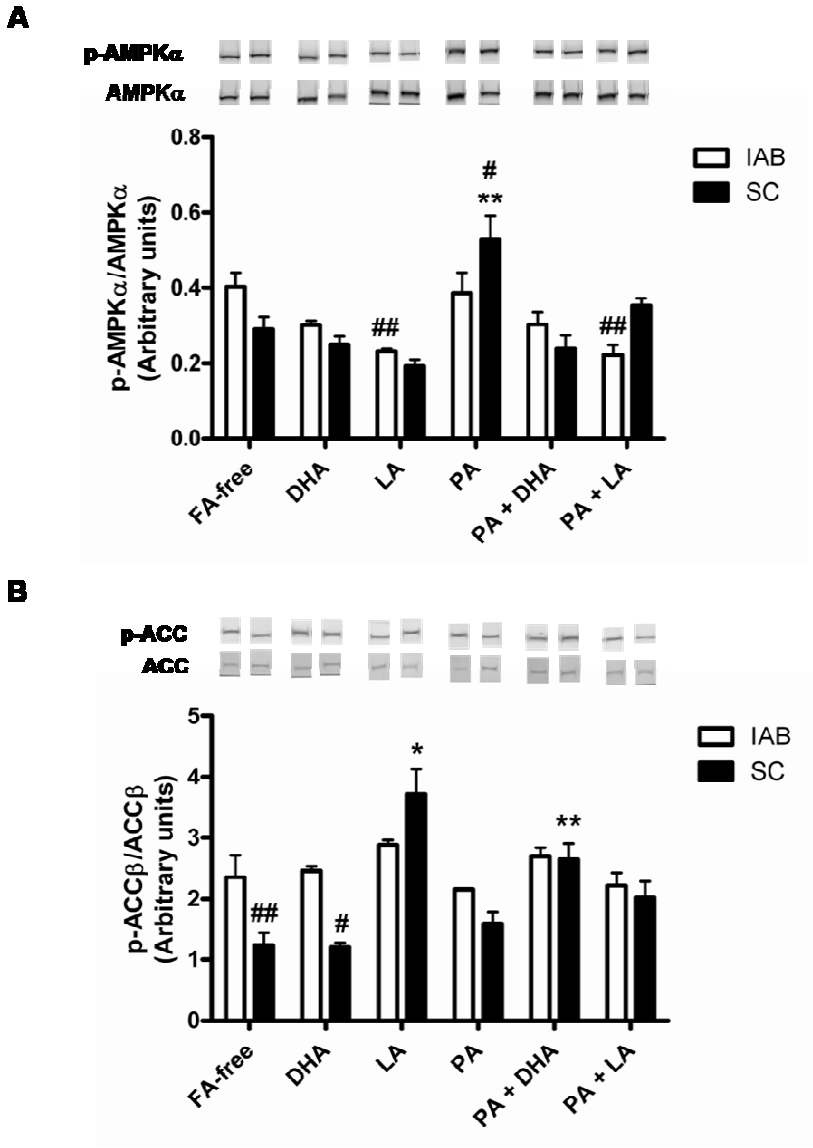

Figure 6.7. Protein phosphorylation of AMPKα (A) and ACCβ (B) in L6 myotubes cultured with fatty acids and adipose tissue-conditioned media (CM) generated from visceral (IAB) and subcutaneous (SC) fat (n = 3). L6 myotubes were incubated with conditioned media from IAB and SC fat (concentration 1:128), with or without the presence of 400 μM of docosahexaenoic acid (DHA), linoleic acid (LA) and palmitic acid (PA), alone and in combination, for 1 h. Data are shown as mean ± SEM and are expressed as ratios of phosphorylated to total protein. * $P < 0.001$ and ** $P < 0.01$ compared to the corresponding fatty acid-free co-culture (FA-free). # $P < 0.01$ and ## $P < 0.05$ compared to the corresponding treatment in CM from IAB fat.

6.4 Discussion

PA at a concentration of 400 μM induced an increase in SCD1 mRNA content in L6 myotubes. A similar effect has been shown previously to occur in L6 myotubes cultured with 200 μM PA (Voss et al. 2005) and *in vivo* in rats in response to a diet high in saturated fat (Lee et al. 2006). SCD is the rate-limiting enzyme catalysing the synthesis of monounsaturated fatty acids, mainly oleate and palmitoleate, which are the most abundant monounsaturated fatty acids in tissue lipids including phospholipids, triglycerides and cholesteryl esters (Dobrzyn and Dobrzyn 2006). Although not directly measured in this study, it has been previously shown that an increase in SCD1 mRNA content in skeletal muscle of rats fed with a high saturated fat-diet was associated with an increased intramuscular triglyceride (Lee et al. 2006). Over-expression of SCD1 in L6 myotubes results in a 40% increase in the accumulation of triglyceride (Pinnamaneni et al. 2006). In the present study, the effect of PA to increase SCD1 mRNA content suggests that PA may promote lipid accumulation in skeletal muscle.

In contrast to the effect on SCD1, PA had no effect on the mRNA content of SPTLC1 and SPTLC2, the genes encoding the regulatory and catalytic subunits of SPT which catalyses the *de novo* synthesis of ceramide. PA has been consistently shown to induce the accumulation of ceramide in skeletal muscle cells (Chavez et al. 2003; Pickersgill et al. 2007), an effect abolished by the specific SPT inhibitor myriocin in L6 myotubes without a change in the SPTLC1 protein content (Watson et al. 2009). The activity of SPT is known to be highly sensitive to substrate availability (Dobrzyn et al. 2005). Accordingly, it seems likely that PA promotes the *de novo* synthesis of ceramide by increasing SPT activity without regulating the enzyme at the transcriptional level.

There was a trend for DHA to reduce the SCD1 mRNA content in L6 myotubes. Moreover, DHA ameliorated the effect of PA on increasing SCD1 mRNA. The effect of DHA to decrease SCD1 mRNA and activity has been shown *in vivo* in the liver of rats fed with a DHA-enriched low-fat diet (0.3 g of DHA/1,000 kJ) (Kramer et al. 2003). Similarly, in spontaneously hypertensive rats a DHA-enriched diet (20 g/kg) reduced hepatic SCD activity by ~ 53% (Engler et al. 2000). The present study provides direct evidence for the effect of DHA on decreasing SCD1 mRNA content in skeletal muscle cells. In contrast to DHA, LA had no effect on SCD1 mRNA in L6 myotubes, an observation consistent with that of Lee et al (2006) who reported that the skeletal muscle of rats fed with a high-fat diet consisting primarily of LA had similar SCD1 mRNA content but lower SCD protein expression and activity compared to the controls (Lee et al. 2006). In liver, however, LA and other PUFAs including linolenic and arachidonic acids reduce SCD1 mRNA and the effect appears to be dependent on the degree of unsaturation (Ntambi 1999). These data suggest the differential effect of LA in liver as opposed to skeletal muscle.

In the mouse liver and hepatocytes, PUFAs have been shown to form a complex with a putative PUFA-binding protein, which binds to the PUFA-responsive element of the SCD genes and subsequently suppresses their transcription (Ntambi 1999). In addition, PUFAs reduce the half-life of SCD1 mRNA in 3T3-L1 adipocytes (Sessler et al. 1996). Taken together, these data highlight the tissue-specific effect of PUFAs on the regulation of SCD1 mRNA and the complexity of fatty acids as signalling molecules. Further studies are required to verify the molecular mechanisms by which fatty acids regulate SCD1 mRNA occur in skeletal muscle.

Both DHA and LA increased AMPKα2 mRNA content in L6 myotubes; PA which was without effect by itself attenuated this effect of DHA and LA. To my best knowledge, the effect of DHA and LA on AMPKα2 mRNA in skeletal muscle has not been reported previously. *In vivo* DHA has been shown to increase basal AMPK phosphorylation in skeletal muscle of *ob/ob* mice (Gonzalez-Periz et al. 2009) but in normal mice whose diet was supplemented with 5% fish oil neither the phosphorylation nor protein expression level of AMPKα in skeletal muscle changed (Dobrzyn et al. 2005). Similarly, a 4-week dietary intervention of 60% energy from safflower oil (consists primarily of LA) did not affect basal AMPK phosphorylation in rat skeletal muscle (Mullen et al. 2007).

In the present study, DHA and LA increased AMPKα and ACCβ phosphorylation in L6 myotubes after a 1 h exposure. A similar acute effect of LA at a lower concentration (250 μM) and with the same exposure duration has been documented previously (Watt et al. 2006). Literature on the acute effect of DHA on the phosphorylation of AMPKα and ACCβ on skeletal muscle cells is not available. While *in vitro* chronic studies on the effect of PUFAs on the expression of AMPK in skeletal muscle is limited, the present study provides direct evidence for the effect of DHA and LA to increase cellular AMPKα2 mRNA content and, at least acutely, to increase AMPKα and ACCβ protein phosphorylation. The acute increase in AMPKα phosphorylation has been shown to increase fatty acid oxidation in skeletal muscle *in vitro* (Fediuc et al. 2006) and *in vivo* (Bonen et al. 2007). AMPK phosphorylates and inactivates ACC, which in turn reduces malonyl-CoA synthesis and attenuates its inhibitory effect on CPT-I and subsequently promotes β-oxidation (Osler and Zierath 2008). In contrast to the acute effect of an AMPK agonist AICAR to increase AMPK activity and fatty acid oxidation (Fediuc et al. 2006; Bonen et al. 2007), chronic exposure to such an agonist does not lead to a

sustained response. For example a chronic AICAR administration (1 mg/g for 4 weeks) in rats reduced basal AMPK activity in skeletal muscle by ~ 20% and abolished the acute effect of AICAR on further increasing the AMPK activity (Winder et al. 2000). In addition, under such conditions the basal CPT-I activity was similar in AICAR-treated and control animals (Winder et al. 2000; Suwa et al. 2003). Whether the chronic effect of DHA and LA mimics the effect of AICAR on fatty acid oxidation requires further investigation.

In addition to its effect on fatty acid oxidation, AMPK also has a direct effect on increasing glucose uptake in skeletal muscle by increasing both mRNA (McGee et al. 2008) and protein (Jessen et al. 2003) content of GLUT4. However, in contrast to the acute effect of AMPKα to increase fatty acid oxidation, there is no acute effect on glucose transport (Al-Khalili et al. 2004), whereas a chronic administration of AICAR induced an increase in maximal insulin-stimulated glucose uptake in rat oxidative skeletal muscle (Jessen et al. 2003).

Given the acute effect of DHA and LA to activate pathways mediating fatty acid oxidation, it is perhaps not surprising to observe a concomitant increase in PDK4 mRNA in L6 myotubes. It has been previously shown that when fatty acid oxidation increases, PDK4 mRNA also increases in skeletal muscle (Berggren et al. 2008). PDK4 encodes PDH kinase which, by phosphorylation, inactivates PDC and down-regulates the entry of carbohydrate into the TCA cycle for oxidation and subsequently facilitates fatty acid oxidation (Sugden and Holness 2003). Taken together, these data suggest that DHA and LA increase fatty acid oxidation and glucose uptake in skeletal muscle. Accordingly, the inhibitory effect of DHA and LA on glucose oxidation promotes a shift in energy

substrate from carbohydrate to fat to prevent energy overload, which may also partition glucose towards glycogen storage in skeletal muscle. This is supported by a study from Turvey et al (2005), who reported that a 3-day intervention of a high-fat diet (75%) reduced glycogen content in skeletal muscle by ~ 20% whereas a n-3 PUFAs-enriched high-fat diet (~ 15% of the fat calories replaced by n-3 PUFAs) induced a ~ 10% increase in muscle glycogen in healthy humans.

Under physiological conditions, energy metabolism of skeletal muscle is under the influence of free fatty acids as well as adipokines. Therefore I determined the combined effect of PA, DHA, LA and secretory factors from either SC or IAB fat on the mechanisms regulating energy metabolism in skeletal muscle cells. CM from either SC or IAB fat increased the mRNA content of AMPKα2 and protein phosphorylation of AMPKα and ACCβ. The effect of CM was unlikely to be mediated by the fatty acids in the media which were present only at relatively low concentrations (Section 4.3.2). Leptin and adiponectin may be the predominant mediators of the effect of CM on AMPKα and ACCβ activation. A 2-week leptin infusion (0.5 mg/kg/day) increased AMPKα2 protein content and basal AMPK phosphorylation in rat skeletal muscle (Steinberg et al. 2003). Total AMPK protein content remained unchanged but AMPK phosphorylation was increased in skeletal muscle of rats over-expressing adiponectin (Satoh et al. 2005). *In vitro*, both leptin and adiponectin increased AMPK phosphorylation in C2C12 myotubes (Yamauchi et al. 2002; Suzuki et al. 2007). In the presence of CM from either fat depot, the combination of PA with either DHA or LA further induced the mRNA content of AMPKα2. The PA effect on inducing SCD1 mRNA was also abolished by DHA and LA. These data suggest the combination of CM, possibly mediated by leptin and adiponectin, with either DHA or LA may have an

additive effect on activating fatty acid oxidation and reducing lipid accumulation in skeletal muscle.

Taken together, the present study suggests that PA promotes lipid accumulation whereas DHA and LA attenuate lipogenesis and activate fatty acid oxidation in skeletal muscle cells. The effect of DHA and LA on improving insulin sensitivity may be a consequence of diverting fatty acids towards oxidation and subsequently reducing substrate availability for the formation of lipid metabolites including DAG and ceramide which are known to inhibit insulin signalling. Further studies to determine the effect of DHA and LA, either alone or in the presence of PA, on insulin sensitivity, rate of β-oxidation, intracellular accumulation of DAG, ceramide and triglyceride in skeletal muscle are required to elucidate the direct effect of DHA and LA on lipid metabolism and its link to the regulation of insulin sensitivity in skeletal muscle.