

Investigating the divergent regulation of skeletal muscle metabolism by
different acyl chain structures

By

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ABSTRACT

In recent decades, the prevalence of obesity and type 2 diabetes has risen dramatically. Strategies to reduce the incidence of these diseases are of great clinical relevance. The contribution of dietary fat has been central to that debate. In particular, the composition of dietary fat can influence skeletal muscle metabolism and the sensitivity to feeding and exercise adaptations. Polyunsaturated omega-3 fatty acids are linked with beneficial effects on skeletal muscle metabolism and function while saturated fatty acids have been linked with metabolic dysfunction. It is still poorly understood how differences in fatty acid structure can have contrasting effects in skeletal muscle.

It is known that omega-3 fatty acids are incorporated into skeletal muscle lipid pools, however, it is unknown what specific lipid species omega-3 fatty acids are incorporated into. In **Chapter 2** of this thesis, the effects of EPA and DHA on the lipidomic profile of skeletal muscle myotubes were explored. Although similar in structure, EPA and DHA treatment resulted in divergent lipid profiles. EPA increased the content of DPA, while DHA reduced arachidonic acid. Both omega-3 fatty acids significantly increased the saturated fatty acid content. EPA and DHA incorporated into myotubes were largely directed towards phospholipid species. The changes in lipid profiles following EPA treatment were associated with increased basal and insulin dependent glucose uptake. This increase in glucose uptake was not driven by changes in the protein abundance of glucose transporters or mitochondrial respiration. DHA did not have any impact on the metabolic measures made. These data show for the first time that EPA and DHA differentially affect glucose uptake in skeletal muscle and this effect may be associated with the differential changes in lipid profiles.

Previous studies have shown that omega-3 supplementation increase mTORC1 signalling and protein anabolism following feeding. There is evidence to suggest that EPA is the dominant n-3 fatty acid that drives muscle anabolism. These anabolic effects of omega-3 fatty acids may be driven by changes in the proteomic profile which increases sensitivity to extracellular stimuli. In **chapter 3**, the effects of EPA and DHA on protein turnover and protein expression are explored. Neither, EPA or DHA did not altered basal protein synthesis. The activation of the mTORC1 pathway in response to a combined stimulus of amino acids + serum was not altered by either EPA or DHA. EPA reduced protein breakdown and this was not related to a reduction in ubiquitinated proteins. Proteomic analysis showed that EPA and DHA differentially altered the abundance of a number of proteins. Given the significant incorporation into phospholipids, we explored how changes in membrane lipid content altered the proteins associated with membrane compartments. DHA treatment resulted in the decreased association of ribosomal proteins with the membrane while EPA induced a small increase in ribosomal proteins associated with the membrane. Gene ontology analysis showed that proteins involved in protein folding associated with cell membranes were enhanced following EPA treatment of myotubes. These results led us to hypothesis that EPA may enhance myotube protein content by altering protein fidelity

In contrast to omega-3 fatty acids, saturated fatty acids such as palmitate are linked with the dysfunction of a number of metabolic systems. A number of studies have demonstrated that palmitate causes skeletal muscle insulin resistance through the generation of lipid intermediates, such as ceramides and diacylglycerols, which inhibit insulin action. Palmitoleate, a fatty acid analogous to palimate with the addition of a

single double bond, can protect against the deleterious effect of palmitate and intrinsically improve glucose uptake. To date, no study has assessed the impact of palmitate and palmitoleate on lipid profiles. Treatment of myotubes with palmitate and palmitoleate respectively significantly increased the content of each fatty acid with myotubes, while, only modestly altering the abundance of other fatty acid species. Palmitate reduced insulin dependent glucose uptake and palmitoleate did not have any effect. PKB activation in response to insulin was unaltered by either fatty acid. Both palmitate and palmitate increased maximal mitochondrial respiration when used at a dose of 250 μ M but increasing concentration substantially reduced coupled respiration and increased proton leak. These data show that accumulation of palmitate specifically and not general lipid accumulation attenuates normal insulin action. The data also suggests that reduction in PKB activation may not be the critical mechanism for the loss of insulin stimulated glucose uptake following palmitate incorporation.

Obesity and acute increases in circulating fatty acids are linked with a reduction in the muscle protein synthetic response to insulin and amino acids. It was hypothesised that PA and PAO would have different effects on protein turnover through changes in protein synthesis and breakdown. Incorporation of palmitate into myotubes resulted in the significant decrease in the basal protein synthesis while protein breakdown was unchanged. PAO did not alter protein synthesis or breakdown. Palmitate increased phosphorylation of ribosomal protein S6, a readout of P70S6K1 activity despite reduced protein synthesis. Eif2 α phosphorylation was not altered by either fatty acid, indication no changes in endoplasmic reticulum stress. Proteomic analysis revealed that both fatty acids altered the protein abundance of a number of different proteins but no changes in proteins associated with muscle anabolism were detected. Despite

increasing anabolic signalling, increasing palmitate accumulation resulted in depressed protein synthesis independent of changes in ER stress.

Collectively, these data show that minor differences in fatty acid structure can elicit divergent metabolic activities in skeletal muscle. This may occur by altering the cell microenvironment through changes in lipid profiles, protein abundance and associated with cell membranes. The addition of a just a single double bond in the fatty acyl chain can prevent deleterious effects on glucose and protein metabolism.

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ABBREVIATIONS

4E-BP1 – Eukaryotic translation initiation factor 4E binding protein 1
AA – Arachidonic acid
ADP – Adenosine diphosphate
ALA – alpha linoleic acid
AMPK – AMP activated protein kinase
ATF6 – Activation transcription factor 6
BiP – Binding immunoglobulin protein
Bnip3 - BCL2/adenovirus E1B 19 kDa protein-interacting protein 3
BSA – Bovine serum albumin
CAMKII – Calmodulin dependent kinase 2
CD36 – Cluster of differentiation 36
COX – cyclooxygenase
CVD – Cardiovascular disease
DAG – Diacylglycerol
DHA – Docosahexaenoic acid
DHS – Donor horse serum
DM – Differentiation media
DMEM – Dulbeccos modified eagles medium
DPA – Docosapentaenoic acid
Eif2a – Eukaryotic initiation factor 2alpha
Elovl2 – Elongase 2
EPA – Eicosapentaenoic acid
ER – Endoplasmic reticulum
ESI MS/MS – Electron spray ionisation double mass spectrometry
FAME – Fatty acyl methyl esters
FATP – Fatty acid transporter proteins
FBS – Foetal bovine serum
FCCP - Carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazine
Foxo03 – Forkhead box transcription factor 3
GLUT4 – Glucose transporter 4
GLUT1 – Glucose transporter 1
GM – Growth media
GPR120 - G protein coupled receptor 120
GTP – Guanidine triphosphate
I κ B – NF-kappaB inhibitor alpha
IMCL – Intramyocellular lipid
IR – insulin resistance
IRS1 – Insulin receptor substrate 1
LA – Linoleic acid
LC/MS - Liquid chromatography mass spectrometry
LOX – lipoxygenase
LPC – Lyso-phosphatidylcholine
LPE – Lyso-phosphatidylethanolamine
MPB – Muscle protein breakdown
MPS – Muscle protein synthesis
mRNA – Messenger ribonucleic acid
mTOR – Mechanistic target of Rapamycin
mTORC1 – Mechanistic target of rapamycin complex 1

MUFA – Mono unsaturated fatty acid
MURF-1 – Muscle ring finger protein 1
NF-Kb – Nuclear factor kappa B
NRF1 – Nuclear respiratory factor 1
OCR – oxygen consumption rate
OPLS-DA – Orthogonal partial least squares – discriminant analysis
PA – Palmitate
PBS – phosphate buffer saline
PC – Phosphatidylcholine
PE – Phosphatidylethanolamine
PERK – Protein kinase R-like Er kinase
PGC-1 alpha – Peroxisome proliferator activated coactivator 1
PGF2 α – Prostaglandin 2F alpha
Pi3K – Phosphoinositide-3 kinase
PI – Phosphatidylinositol
PKB - Protein kinase B
PKC – Protein kinase C
PP2A – protein phosphatase 2A
PPAR γ – Peroxisome proliferator activated receptor gamma
P70S6K1 – Ribosomal S6 protein kinase 1
PS - Phosphatidylserine
PUFA – polyunsaturated fatty acid
SFA – Saturated fatty acid
SILAC – stable isotope labelling of amino acids in cell culture
SKM – Skeletal muscle
SPT-1 – Serine Palmitoyl transferase 1
TAG – Triacylglycerol
TCA – Trichloroacetic acid
T2D – Type 2 diabetes
TFAM – Mitochondrial transcription factor A
TNF-a – Tumour necrosis factor alpha
UPR – Unfolded protein response

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CHAPTER 1: General introduction

1.0 Introduction

In the last three decades global obesity has almost doubled to over 1.4 billion, meaning that as much as one third of westernised populations are classed as overweight/obese (World Health Organisation, 2008). Concurrent with the increase in obesity rates is an increase in obesity related disorders such as type 2 Diabetes (T2D), sarcopenic obesity and cardiovascular disease making obesity one of the major healthcare issues facing the world (World Health Organisation report, 2000). Being overweight/obese increases the risk of developing diabetes by up to 30% [1]. The cost of primary diabetes care for the UK National Health Service (NHS) is currently at £9.8 billion/annum and is expected to continue rising (NHS). Skeletal muscle is a major site of glucose disposal, accounting for approximately 30% of postprandial glucose disposal [2]. Maintaining skeletal muscle metabolic health is therefore key to maintaining glycaemic control. Strategies that improve skeletal muscle metabolic function and insulin sensitivity could therefore have a major impact on the obesity induced development of insulin resistance and diabetes and reduce health care costs and improve quality of life. Skeletal muscle mass maintenance is not only crucial to the maintenance of metabolic function, but also through the control of locomotion it is also critical to the maintenance of physical function. Skeletal muscle (depending on adiposity levels) accounts for approximately 40% of total body mass and is highly adaptable to environmental changes such as diet and physical activity levels [3], [4]. Loss of muscle mass with progressing age is an inevitable aspect of the aging process [5]. Reductions in skeletal muscle mass and metabolic function can have detrimental effects on overall health and

is a major contributing factor to the onset of disease with age [6]. Loss of muscle mass and subsequent physical function not only places the individual at greater risk of chronic disease but leads to frailty and reduced quality of life [6]. From the age of approximately 50, skeletal muscle mass declines by 0.2–0.5%/year and this loss is accelerated in a diseased state [7]. Moreover, as little as a 5% decrease in skeletal muscle mass has been associated with increased morbidity [8]. If the rate of sarcopenia (age related loss of muscle mass) can be reduced by 10% this would translate to a saving in US health care costs of \$1.1 billion per year [9]. Therefore, it is of great clinical relevance to find effective therapies for improving muscle mass and metabolic function. Recent evidence suggests that manipulating the lipid content of skeletal muscle has the capacity to alter muscle function and metabolism. For instance, enhancing the omega-3 polyunsaturated acid (PUFA) content of skeletal muscle may enhance protein anabolism and muscle strength [10], [11]. This introduction will focus on the role of multiple lipid species in skeletal muscle metabolism and function. This will be split into two sections, first the effect of omega-3 PUFAs will be reviewed and the second part will review the effects of saturated fatty acids (SFA) and monounsaturated fatty acids (MUFA).

1.1. *Dietary fatty acid sources and lipid metabolism in skeletal muscle*

As previously mentioned, skeletal muscle is highly adaptable or mal-adaptable to changes in diet composition. In particular, diets high in saturated fat have been linked with the onset of both obesity and T2D [12], [13]. However, diets high in polyunsaturated fatty acids such as the Mediterranean diet have been linked to beneficial outcomes, such as improved cardiovascular health [14], [15]. Furthermore, the traditional diet of Inuit populations which is high in omega-3 PUFAs and low in omega-6 fatty acids is associated with a lowered risk of cardiovascular disease and

improved insulin sensitivity despite being a diet very high in fat [16], [17]. Therefore, the amount and type of fat in the diet can play an important role in regulating whole body metabolic health.

Fatty acid species are classified by their varying degrees of saturation into three main classes; saturated fatty acids (SFA), monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA). SFAs are a simple carbon chain containing no double bonds, MUFAs contain one double bond and PUFAs are classified as carbon chains containing two or more double bonds. The differences in the chemical structure of these different classes can lead to different physiological effects. For example, SFA has been linked with the development of metabolic dysfunction while conversely some MUFAs and PUFAs have positive effects on metabolic function [18], [19].

At a cellular level, fatty acids are not only structurally important, as the main component of cellular membranes, but also have an important function in a number of metabolic processes such as regulating the activity of certain enzymes and by acting as signalling molecules [20]. Therefore, alterations in the composition of the muscle lipid pool may have profound effects on skeletal muscle metabolic and physical function. It is well established that skeletal muscle is sensitive to changes in dietary lipids, with a minimum 2-week alteration in dietary intake needed to significantly alter muscle lipid composition [21]–[23]. The change in cellular function brought about by changing lipid composition is possibly due to the fact that different species of fatty acids display a diverse array of structures ranging from simple saturated carbon chains to highly unsaturated carbon chains, these differences in structure are a large determinant of function. Before fatty acids can have an impact on cellular metabolism they must first be transported into the cell. However, fatty acid transport and metabolism must be tightly regulated as high intracellular levels of free fatty acids can be highly toxic due

to lipid peroxidation, theoretically, highly unsaturated fatty acids will be most readily oxidized. As a result, there are a number of transporter proteins (CD36/FATP/FABP_{pm}) and cytosolic proteins (FATP_c) that regulate uptake and subcellular localisation of fatty acids, allowing for them to be stored or metabolised effectively [24]. Fatty acid uptake is analogous to glucose uptake, in which CD36 vesicles translocate from intracellular stores to the sarcolemma and is sensitive to insulin stimulation and contraction induced activation of AMPK [24]. These transporter proteins are also known to play a role in the regulation of mitochondrial fatty acid oxidation [25]. Once transported into the cell a number of intracellular fates are possible, mainly, β -oxidation, storage in discrete lipid droplets or incorporation into cell membranes. In skeletal muscle the approximate ratio of storage to oxidation is 2:1 although this is variable depending on a number of factors such as energy expenditure and muscle fibre type [26]. Up to 90% of fatty acids transported into the cell are either stored or oxidized in soleus muscle [26]. Alternatively, fatty acids are incorporated into cellular membranes; the length of the carbon chain, number and position of double bonds of the fatty acids composing the membrane determine the physico-chemical properties such as fluidity [27]. Membranes which contain phospholipids lacking any double bonds pack tightly together reducing fluidity whereas the *cis*-kinks in the carbon chains caused by double bonds in unsaturated fatty acids pack less tightly and increase the deformability and fluidity of the membrane [28]. A change in membrane fluidity can alter the activity of membrane associated proteins, membrane receptors and vesicle budding and fusion [29], [30].

1.2. *Omega-3 Polyunsaturated fatty acids*

The omega-3 fatty acids are a group of polyunsaturated fatty acids defined by a double bond at the third carbon from the methyl end of the carbon chain. Humans do

not possess the necessary omega-3 desaturase to add a double bond at the 15th carbon of a long chain fatty acid and are, therefore, unable to endogenously synthesize alpha-linolenic acid (ALA 18:3n-3) and linoleic acid (LA 18:2n-6) making them essential fatty acids. Omega-6 PUFAs are also essential fatty acids and generally have metabolically distinct effects to omega-3 PUFAs. While the human body cannot synthesize omega-3 and omega-6 PUFAs, it does have the capacity to further metabolize these fatty acids through stages of elongation and desaturation. ALA can be metabolized to eicosapentaenoic acid (EPA 20:5n-3) and docosahexaenoic acid (DHA 22:6n-3) by $\Delta 6$ desaturase and $\Delta 5$ desaturase respectively, while LA is converted to arachidonic acid (AA 22:4n-6). However, the conversion of ALA to DHA is very inefficient with <10% conversion in females and <3% in males [31], [32]. While ALA is the preferred substrate for $\Delta 6$ desaturase, an abundance of dietary linoleic acid has been shown to suppress conversion of ALA to DHA [33], which may be a confounding factor in these studies. There is recent evidence to suggest that supplementing with stearidonic acid (18:3n-3) may improve the efficiency of conversion to DHA, indicating $\Delta 6$ desaturase as a rate limiting step [34]. There is also a degree of individual variation in the lipidome following omega-3 supplementation in humans which may be a factor in the equivocal metabolic changes measured in many human supplementation trials [35].

It is thought that hominids' diets during the Paleolithic era were high in seafood and low in seeds and vegetable oils, which led to an omega-3/omega-6 ratio of approximately 1:1 [36]. Given the likelihood that early human ancestors' diets were already high in omega-3 intake it may not have conferred any evolutionary benefit to develop the ability to synthesize omega-3 PUFAs. During the agricultural revolution, with changes to food production in the Neolithic era this $n-3/n-6$ ratio began to diverge and now in the typical western diet is thought to be as much as 20:1, with omega-3

PUFA intake predominantly from ALA [37]. Although unlikely to be a primary driver, the divergence in the $n-3/n-6$ ratio has happened concurrently with the rise in CVD and states of chronic inflammation. Briefly, omega-6 PUFAs are associated with the production of pro-inflammatory mediators while omega-3 PUFAs produce less potent inflammatory mediators and inflammatory resolving proteins and so manipulating this ratio may bring about positive health outcomes.

The potential therapeutic benefit of a diet with high omega-3 content was first observed due to the lower incidence of CVD in Greenland Inuit populations [17]. Subsequent studies observed that a period of omega-3 supplementation reduced risk factors associated with CVD, such as the lowering of plasma triacylglycerides (TAGs) and an increase in high density lipoproteins at the expense of low density lipoproteins, as well as decreasing in platelet aggregation [35], [38], [39]. Yet, when end point measures such as cardiovascular disease are taken together in a meta-analysis the results of omega-3 supplementation are equivocal [40]–[43]. We hypothesize that while a given period of omega-3 supplementation leads to a significant increase in omega-3 content of various tissues it may not be sufficient to dramatically reduce the $n-3/n-6$ ratio.

The results of studies in animals in which the $n-3/n-6$ ratio is substantially reduced have been largely positive regarding insulin sensitivity and resolving inflammation. A particularly effective model for assessing the impact of $n-3/n-6$ ratios is the fat-1 transgenic mouse model which can endogenously synthesize omega-3 PUFAs from omega-6 PUFAs [44]. The ability to convert omega-6 fatty acids to omega-3 leads to an $n-3/n-6$ ratio of approximately 1:1. This model allows for the same diet to be used in all conditions and for the comparison of two significantly different $n-3/n-6$ ratios. It is, however, difficult to discern whether any effects are due to a reduction in the overall

n-3/n-6 ratio or an increase in omega-3 PUFAs alone. Reductions in the *n-3/n-6* ratio are associated with an improvement in whole body glucose tolerance, as well as preventing the age related decline in glucose tolerance [45], [46]. Fat-1 mice were also protected from obesity related inflammatory activity and decrements in insulin sensitivity [47]. As well as improving glucose clearance, lowering the *n-6/n-3* ratio also led to an increase in insulin secretion [48]. These studies demonstrate that a balance between omega-6 and omega-3 PUFAs within the lipid pool, may have a potential role in determining the metabolic effects of omega-3 PUFAs. Studies attempting to address this ratio in humans, however, would be difficult to adequately control. A human trial investigating a Mediterranean type diet which led to a reduction in the *n-3/n-6* ratio observed that this reduction alongside other variables may confer some protection against metabolic dysfunction, providing some evidence that alterations in fatty acid content may have an effect on human health [15].

1.3. *Omega-3 PUFAs and Skeletal muscle anabolism*

There is now growing evidence that omega-3 PUFAs also have intrinsic anabolic/anti-catabolic properties in skeletal muscle. Muscle protein balance is regulated by changes in the ratio of muscle protein synthesis (MPS): muscle protein breakdown (MPB). An increase in MPS or a decrease in MPB will lead to a positive balance and ultimately hypertrophy [49]. Muscle disuse due to illness or injury is associated with severe skeletal muscle loss [50]. However, omega-3 supplementation has been shown in human, rodent and cell models to blunt the loss of skeletal muscle mass during disuse or injury [51]–[53]. It is well established that increasing amino acid availability stimulates a rise in MPS [54] and omega-3 supplementation may potentiate this response to anabolic stimuli [61] [62]. In a randomised controlled trial in healthy elderly individuals, it was observed that omega-3 PUFAs potentiated the muscle

protein synthesis (MPS) response to simulated feeding (hyperinsulemia-hyperaminoacidemia clamp) following an 8-week supplementation period (1.86 g EPA, 1.5 g DHA/day), independent of increased glucose action. Importantly, omega-3 supplementation had no effect on basal rates of MPS [56]. A follow on study also observed the same effect in the effect of omega-3 PUFA on MPS in young and middle aged healthy individuals [55]. It is proposed that the changes in these studies in MPS were independent of anti-inflammatory activity due to the healthy status of the participants. Measures of c-reactive protein, TNF α and interleukin-6 in plasma did not show any changes with fish oil supplementation. The lack of changes in plasma does not mean that changes in inflammation within the localised muscular environment did not change. The anabolic effects of omega- PUFAs is thought to be partially mediated through an increased phosphorylation of mechanistic target of rapamycin (mTOR) and downstream signalling target P70S6K1, a key regulatory pathway of protein synthesis and, by extension, muscle mass [57], [58]. Upstream of mTORC1 no changes were detected in PKB in either study, suggesting a potential direct sensitisation of mTORC1 and P70S6K1 to amino acid stimulation or alternatively an interaction of omega-3 PUFAs with other kinases involved in the activation of mTORC1. In young healthy men, 8 weeks omega-3 supplementation caused a reduction in mTOR, P70S6K1 and AMPK kinase activity following combined resistance exercise and feeding. Furthermore, omega-3 fatty acids did not enhance the MPS response to exercise and feeding, suggesting that the effects of omega-3 on MPS may be specific to feeding [59]. This study was conducted in young healthy males, where the combination of exercise and feeding may have saturated MPS precluding the ability to see any effect of fish oil supplementation. Taken together, these studies suggest that omega-3 PUFAs improve

muscle anabolism through an enhanced sensitivity to a nutritional stimulus likely independent of PKB activity.

A recent cell based study has suggested that EPA, and not DHA, is responsible for the anabolic effects of omega-3 PUFAs [60]. C₂C₁₂ myotubes treated with EPA showed elevated MPS 25% greater than the control with no effect of DHA despite P70-S6K1 phosphorylation being significantly elevated in both treatments [60]. A weakness of the study is that basal protein synthesis was not shown so the change in protein synthesis following leucine stimulation is unknown. Furthermore, leucine is a strong stimulus for mTOR signaling but does not increase protein synthesis or net protein accretion in C₂C₁₂ myotubes [61]. Meaning leucine may not be a suitable stimulus to assess changes in protein synthesis in C₂C₁₂. These data would suggest that the effect of EPA on protein synthesis is likely independent of an enhanced anabolic signal through p70S6K1. Kamolrat *et al.* (2013) [60] and others studies in cell models [62]–[64] have shown that EPA and DHA may play a role in attenuating the rate of protein degradation. The mechanism behind this effect appears to be through the inhibition of the NF-κB pathway [63]. EPA inhibits the degradation of the NF-κB complex by reducing phosphorylation of IκBα [65]. This action prevents translocation of NF-κB to the nucleus where it can induce expression of the muscle ring finger-1 gene (MURF-1) [62]. Knockdown of PPARγ significantly reduced the effect of EPA on NF-κB signalling. NF-κB is an important pathway linked with the loss of skeletal muscle mass as MURF-1 facilitates ubiquitination of muscle proteins, effectively tagging them for degradation [66], [67]. Animal models that lack MURF-1 expression are protected from atrophy [67], [68]. Further support for a role of EPA in preserving muscle mass through inhibition of the atrophy pathways, comes from earlier work in a mouse cancer cachexia model. Treatment with EPA attenuated the loss of muscle mass through the

suppression of the ubiquitin proteasome pathway [69]. Inhibition of this pathway could play an important role in the maintenance of skeletal muscle mass by offsetting periods of depressed MPS, a key contributing factor to atrophy. Contrary to other studies which observed no effect of DHA on protein breakdown, *Wang et al. (2013)* [70] observed that DHA attenuated protein degradation through the same mechanisms as EPA but with greater efficiency. These *in vitro* studies indicate a role for EPA and DHA in both protein synthesis and breakdown but few studies have made integrated measures of synthesis, breakdown and protein accretion.

There is *in vivo* evidence in humans that omega-3 PUFAs enhance the MPS response to feeding, and evidence from *in vitro* and rodent cancer models that omega-3 PUFA supplementation reduces muscle protein breakdown. Yet, it was unclear if long term omega-3 supplementation could improve muscle function. *Rodacki et al. (2012)*[11] were the first to assess if omega-3 supplementation could improve muscle function in the elderly when combined with resistance training [11]. They supplemented 45 subjects for 90 days with 2 g/day omega-3 fatty acids in combination with a 90 day progressive resistance exercise program. They found that muscle strength and neuromuscular function was significantly improved when omega-3 supplementation was combined with resistance exercise, but omega-3 alone had no effect. A potential mechanism for omega-3 PUFAs improving contractile function may be an enhanced sensitivity of the muscle to acetylcholine, a neurotransmitter that stimulates muscle contraction. It has previously been observed that fish oil supplementation led to an increase in contractility in rats [71]. Strategies to improve or maintain neuromuscular function are of great interest as decrements in motor unit number and stability may precede functional changes in muscle [72]. A study using both elderly male and female participants observed that the beneficial effects of omega-3s may be specific to females

as no effects on muscle strength were observed in older women [73]. Maintaining neuromuscular function may be an important factor in offsetting muscle loss, and there is some promising initial evidence that omega-3 PUFAs may be effective in maintaining both muscle mass and function in typically atrophic conditions. Indeed, a higher dose of omega-3 PUFAs than the dose used by *Rodacki and colleagues (2012)* [11] demonstrated that supplementing elderly individuals with 4 g of omega-3 PUFAs daily for six months preserved muscle mass and function, not due to exercise induced effects, attenuating the normal declines associated with aging [10]. The mechanisms behind this preservation in muscle mass and function may be a combination of increases in postprandial MPS and improved neuromuscular function.

Despite several studies demonstrating the beneficial effect of EPA and DHA on muscle anabolism, a study in Sprague-Dawley rats suggested that omega-3 supplementation may blunt muscle recovery following a period of atrophy inducing immobilization [74]. The results showed that while fish oil blunted the loss of muscle mass during immobilization, muscle recovery was reduced at 3 days following remobilization, however, 13 days following remobilization muscle to body weight ratio and Myosin Heavy Chain had returned to similar levels. The early phase response inhibition was attributed to a reduction in $\text{PGF2}\alpha$, a prostaglandin derived from arachidonic acid which has been shown to be important for muscle protein synthesis [75], [76]. The reduction in $\text{PGF2}\alpha$ occurred simultaneously with a reduction in P70S6K1. Thus, it may be hypothesized omega-3 fatty acids may inhibit anabolic processes by reducing $\text{PGF2}\alpha$. This may indicate that in healthy organisms without the presence of low grade inflammation that omega-3 PUFA supplementation may delay regeneration of muscle following damage.

Taken together, these studies suggest omega-3 PUFAs, particularly EPA, play a beneficial role in maintaining total protein balance (**Table 1.1**). It is thought that one of the main underlying causes of muscle wasting conditions, such as sarcopenia, is an impaired MPS response to anabolic stimuli [77]. Omega-3 PUFAs may be of potential relevance to the clinical setting as a non-pharmacological method of reducing muscle loss. Further long-term human trials are necessary to address whether omega-3 supplementation leads to muscle hypertrophy and consequent functional gains. Furthermore, the question remains whether omega-3 PUFAs not only improve the MPS response to nutrition but also increase MPS following an acute bout of resistance exercise. A recent study observed a rise in the total protein content in focal adhesion kinase, a mechanically sensitive protein, and mTOR which may indicate an enhanced capacity to respond to resistance exercise [23]. Experiments from tissue culture models indicate that EPA may be the significant contributor to changes in MPS rather than DHA, however, it has yet to be confirmed in humans if EPA underpins the changes in muscle mass and function.

Table 1. 1 Summary of studies characterising the impact of omega-3 PUFAs on skeletal muscle metabolism.

Study	Model	Protocol	Key findings
Smith et al. (2011)[55]	Human (Young)	8 weeks, 1.86 g EPA, 1.5 g DHA daily.	Augmented MPS and enhanced mTOR and p70S6K1 signalling in response to a hyperaminoacidemic-hyperinsulinemic clamp in young volunteers.
Smith et al. (2011)[78]	Human (Elderly)	8 weeks, 1.86 g EPA, 1.5 g DHA daily.	Augmented MPS and enhanced mTOR and p70S6K1 signalling in response to hyperaminoacidemia-hyperinsulinemia in elderly volunteers.
Rodacki et al. (2012)[11]	Human (Elderly)	60 days pre training and 90 days during training, 0.4 g EPA, 0.3 g DHA.	potentiated training increase in peak torque and rate of torque development (Knee extensor, flexor, plantar and dorsiflexor).
Smith et al. (2015)[10]	Human (Elderly)	6 months, 1.8 g EPA, 1.5 g DHA daily.	Ameliorated age related declines in lean muscle mass. Increased handgrip strength and 1-RM muscle strength.
You et al. (2010)[74]	Rodent	14 days enriched with 5% cod liver oil followed by 14 days immobilisation.	Reduced Myosin heavy chain loss during 14 days of hindlimb immobilisation.
Kamolrat et al. (2013)[60]	Rodent	8 weeks of chocolate derived sweets, 49.6% EPA, 50.4% DHA	Increased phosphorylation of Pi3K and p70S6K1 during aminoacidemic-insulinemic clamp.
Gingras et al. (2007)[79]	Steers	5 weeks infusion 4% menhaden oil	Enhanced insulin action alongside an increase in amino acid disposal and increased mTOR-p70S6K1 in response to hyperinsulinemic-euglycaemic-euaminoacidemic clamp.

1.4. *Omega-3 PUFAs and insulin sensitivity*

Skeletal muscle is a primary site of insulin stimulated glucose disposal [2], [80] and any dysfunction in this process can lead to the development of insulin resistance (IR), preceding T2D. A reduced sensitivity of skeletal muscle to insulin stimulation is one of the primary defects leading to the development of T2D [81]. T2D is characterized in skeletal muscle by a reduction in glucose uptake, decreased glycogen synthesis, down

regulation of fat oxidation and an expanded intramuscular (TAG) pool [82]–[84]. Lipid induced IR can be observed after only a few hours of increased plasma free fatty acids *in vitro* and in skeletal muscle within three weeks of a high fat diet in rodents [85]–[87]. In particular, an overabundance of SFA (e.g. palmitate (PA 16:0) can induce IR through the accumulation of lipid derivatives including DAGs, ceramides and long chain acyl-coAs in the cytosol, which in turn can interfere with IRS1-PKB signalling [88]–[93]. However, our understanding of the role lipid metabolites has in a clinical context are still poorly understood. Increases in these lipid derivatives, particularly ceramides, can lead to the activation of PKC isoforms which lead to the phosphorylation of serine residues on insulin receptor substrate-1(IRS) and attenuating PKB activation [94], [95]. In response to insulin, targets downstream of PKB initiate translocation of GLUT4, an insulin sensitive glucose transporter, to the membrane to facilitate transport. One of the prominent features of IR is the reduction of glucose uptake, caused by a reduction of GLUT4 at the cell surface despite no changes in total GLUT4 content [96], [97]. Compared to SFA it appears that omega-3 PUFAs appear to at least have a neutral effect on skeletal muscle insulin sensitivity with some studies also showing a beneficial effect. While meta-analyses detect little or no impact of marine derived omega-3 PUFAs on insulin sensitivity or reduction in risk of developing T2D [98]–[100] there are a number of human, animal and cell studies which support a potential role of omega-3 PUFAs in the treatment of IR/T2D.

Through alterations in muscle metabolism, and omega-3 PUFAs may protect against some of the metabolic defects induced by a high fat diet [101]. A high omega-3 index (erythrocyte concentrations of EPA and DHA) is associated with higher insulin sensitivity and lower fasting insulin levels [102]. The results of current human clinical trials however are equivocal with some showing a beneficial effect [103], [104] while

some show no effect or adverse effects [105]–[107]. Yet, a recent study has observed that addition of omega-3 PUFAs to an intravenous lipid infusion attenuated the decline in insulin stimulated glucose action in muscle as well as an increased glucose disposal compared to an omega-6 infusion, these effects were independent of changes in acyl carnitine levels; a marker of mitochondrial overload [108]. During the infusion in this study intramyocellular lipids (IMCL) significantly increased by nearly 50% in the omega-3 group suggesting that omega-3 PUFAs promote the incorporation of fatty acids in complex lipids, instead of being directed towards β -oxidation [108]. Rodents fed a 10 week high fat diet with added fish oil attenuated the decline in glucose tolerance compared to a high fat diet and this effect was accompanied by a reduction in long chain acyl-coa species as well as in some ceramide species, although the abundance of these intracellular lipids were still elevated compared to the control diet [101]. These data may be explained by the findings that pre-treatment of C₂C₁₂ cells with EPA increased the incorporation of labelled oleate into TAGs and phospholipids, with no changes in β -oxidation observed [109]. *Wensaas & colleagues* (2008) [110] also observed a marked increase in TAG synthesis following EPA treatment in isolated skeletal muscle myotubes and this effect was more pronounced in myotubes isolated from T2D individuals. EPA also reduced the amount of C:18-2 coA beyond that of control and again this effect was more pronounced in T2D myotubes. While increased levels of fatty acyl-coA in general are associated with IR and C18:2-coA accumulation is a potential indicator of IR [90], [92]. The ability of EPA to apparently increase incorporation of free fatty acids into TAGS may improve insulin sensitivity by preventing the accumulation of lipid intermediaries and the potential interference of these lipids on insulin signalling pathways. The potential for omega-3 PUFAs to increase complex lipid species in skeletal muscle is in contrast with data from other

tissues where lipid droplet formation is suppressed [111]. Increasing intracellular TAGS has previously been shown to protect against lipotoxicity in states of lipid overload [112], [113].

As well as increasing complex lipid formation, EPA may also improve both basal and insulin stimulated glucose uptake in a manner independent of PKB signaling [109], [114]. The ability to clear blood glucose is diminished in IR/T2D individuals and sustained levels of high blood glucose can lead to negative health outcomes. Aas *et al.* (2006) observed that a 24-hour treatment of skeletal muscle cells with EPA increased basal glucose uptake 2.4-fold, however, the increase with insulin stimulation was similar to that of the control. The changes in glucose uptake are likely independent of any changes in PKB signalling as Hessvik *et al.* (2010) and Aas *et al.* (2005) [109], [115] do not detect any differences in basal and insulin stimulated PKB phosphorylation. One study observed that despite phosphatidylinositol-3-kinase (PI3K) activity being down regulated PKB phosphorylation remained intact in response to insulin [116]. Potentially indicating the omega-3 PUFAs may reduce the activation threshold for signalling in response to external stimuli. DHA is also known to play a role in glucose metabolism; preventing the deleterious effects of PA on PKB phosphorylation and glucose uptake [117]. Furthermore, increased transcription of genes involved in glucose regulation may also influence the metabolic effects of EPA and DHA. Coupled with an increase in glucose uptake observed by Aas *et al.* (2006) [109] was an increase in mRNA content of GLUT1, which is involved in the regulation of basal glucose uptake [118]. Addition of fish oil to mice fed a high fat diet increased transcription of GLUT4 and IRS-1 [101]. Multiple studies have observed an increase in GLUT4 expression [114], [119], however, whether this is reflected at the protein level is still unclear with one study showing no change in GLUT4 content with a low dose of omega-3 PUFAs

[116]. These data suggest a potentially beneficial role for both EPA and DHA in glucose metabolism. Assessment of the effects of omega-3 PUFAs on cell and rodent models have returned largely positive results, however, this has yet to translate into consistent health benefits in human trials.

An alternative mechanism explaining the insulin sensitising effects of omega-3 PUFAs is through the reduction in inflammatory markers. Briefly EPA and, to a lesser extent, DHA are natural ligands for Peroxisome proliferator-activated Receptor γ (PPAR γ) and following activation of PPAR γ NF-Kb activity is suppressed reducing the release of pro-inflammatory cytokines [63]. Tumour necrosis factor- α (TNF- α) is also known to induce IR through the phosphorylation of IRS-1 on serine 307 similar to lipid intermediates [120] and EPA reduces TNF- α expression [114]. Furthermore, in macrophages and adipocytes the G-protein coupled receptor GPR120 is an omega-3 sensitive receptor that exhibits anti-inflammatory properties through the suppression of TNF-activation [121]. While this may be an important factor in clinical trials and rodent models, GPR120 expression is negligible in skeletal muscle and would therefore not explain the changes induced by omega-3 PUFAs in tissue culture models [121]. In rodents, knockout of AMPK subunit alpha 2 abrogates the improvement in hepatic insulin sensitivity by omega-3 PUFAs during a high fat diet despite studies observing little interaction between omega-3 PUFAs and AMPK [122]. It may be of interest to assess the importance of AMPK on insulin sensitizing effects of omega-3 PUFAs in skeletal muscle [123]. Skeletal muscle is highly adaptable to alterations in substrate availability and can switch between fat and glucose oxidation in response to changes in environmental conditions. Skeletal muscle mass comprises up to approximately 40% and is a significant determinant of metabolic rate. In older women, 12 weeks of omega-3 supplementation increased resting metabolic rate(14%) and energy

expenditure during exercise (10%). Moreover, fat oxidation was increased in both the resting (19%) and exercise (27%) state [124]. A follow up study in young healthy males did not find any impact of fish oil supplementation on metabolic rate or substrate metabolism

The ability to switch between different fuel sources, termed metabolic flexibility, is diminished by obesity and T2D at rest and during exercise [125], [126]. Studies have observed that the ability to oxidize fat in particular is blunted by T2D [127], [128]. In cultured muscle cells EPA and DHA treatment improved the ability to switch between fat and glucose oxidation depending on which substrate was available [115]. The studies on the effect of omega-3 PUFAs on substrate metabolism have not yielded consistent results. In healthy individuals, three weeks of omega-3 supplementation increased fat oxidation by 35% at the expense of glucose utilization in response to a bolus of glucose [129]. While a follow up study indicated a tendency towards increased fat oxidation during exercise (60% VO₂ max), however, differing modes and intensity of exercise would likely alter these results [130]. The shift towards fat utilization during exercise may be a characteristic of all PUFAs as omega-6 fatty acids suppress activation of pyruvate dehydrogenase (PDH) at the onset of exercise [131]. However, to the authors' knowledge it has yet to be determined if omega-3 also suppresses PDH activity. Another group observed time dependent changes in resting substrate selection in T2D subjects with fish oil increasing glucose utilization after one week, however, after nine weeks fat oxidation was enhanced [132]. Yet, in diabetic but non obese rats EPA alone reduced total fat oxidation by 50%, instead fatty acids were directed towards storage [114]. Furthermore, a combination of EPA and DHA in rhabdomyosarcoma cells reduced the ratio between the oxygen consumption rate: extracellular acidification rate indicating a shift towards an increase on glycolytic

reliance [119]. These data demonstrate a lack of consistency in an organism's metabolic response to omega-3 PUFA supplementation.

There has been much debate on whether mitochondrial defects are a primary factor in the development of insulin resistance (IR). Initial studies observed a reduction in mitochondrial content and size in insulin resistant and T2D individuals, accompanied by a reduction in expression of genes involved in mitochondrial biogenesis and oxidative phosphorylation [133], [134]. Yet, it has also been reported that feeding rodents a short term high fat diet increases mitochondrial content and oxidative capacity despite the development of IR [135], [136]. A number of studies have also reported normal mitochondrial function in insulin resistant populations [137], [138]. Studies which have induced severe mitochondrial dysfunction actually observe an increase in insulin sensitivity. Despite these studies indicating that mitochondrial dysfunction is not a primary driver of IR, increases in mitochondrial content and oxidative capacity may help prevent the lipid accumulation associated with the onset of IR. There is some evidence that omega-3 PUFAs may stimulate mitochondrial biogenesis through the increased mRNA expression of transcription factors such as PGC1-alpha, TFAM and NRF1 [101], [139]. This study observed an increase in mitochondrial abundance and oxidative capacity in response to a high fat diet without any added effect of fish oil [101]. Outwith the context of a high fat diet, 12 weeks of omega-3 PUFA supplementation (2 g EPA, 1 g DHA) did not alter mitochondrial abundance in humans, however, did increase ADP sensitivity [140]. It is unclear at present how the increased ADP sensitivity will alter mitochondrial function in the long term. In insulin resistant individuals omega-3 PUFAs did not alter mitochondrial abundance, maximal ADP respiration or peripheral insulin sensitivity [141]. In an aged mouse model replacing 3.4% of kcal with EPA but not DHA attenuated age related

declines in mitochondrial function through the maintenance of mitochondrial protein quality, independent from changes in mitochondrial abundance [142]. There is also evidence that both LA and ALA in an obese animal model preserve insulin signalling and oxidative capacity through differential mechanisms [140]. Interestingly, the authors observed an increase in the abundance of electron transport chain sub-units in the subsarcolemmal mitochondrial pool independent of changes in mitochondrial content with ALA [140]. This study provides some evidence that some omega-6 PUFAs have similar protective effects to omega-3 PUFAs. The currently available data suggests that any insulin sensitising effects of EPA or DHA are unlikely to occur through increases in skeletal muscle mitochondrial abundance or oxidative capacity.

Taken together, these studies suggest that both EPA and DHA may have a protective effect against fatty acid induced insulin resistance, with some potential EPA or DHA independent effects. A summary of the results from human trials on glucose homeostasis can be seen in **Table 1.2**. These effects may be mediated by anti-inflammatory actions and the increased incorporation of potentially harmful fatty acid species into complex lipids, thereby reducing the interference with signalling pathways involved in glucose metabolism. Predominantly, fatty acid accumulation induces IR partially through a reduction in PKB related signalling and the data would suggest that EPA and DHA may protect against the blunting of PKB signalling rather than intrinsically increasing PKB activity. As such, several studies have shown improvements in glucose uptake with EPA, but not DHA, independent of changes in PKB phosphorylation. As well as potential improvements in peripheral insulin sensitivity, there is some evidence that omega-3 PUFAs may improve hepatic insulin sensitivity. Changes in hepatic insulin sensitivity are of great clinical relevance as the liver is a major contributor to postprandial glucose disposal. *Lalia et al. (2015)* [141]

and colleagues observed small reduction in endogenous glucose production while subset analysis of a meta-analysis indicated an increase in hepatic insulin sensitivity [99].

Table 1. 2 Summary of studies characterising the impact of omega-3 PUFAs on glucose homeostasis.

Study	Model	Protocol	Key findings
Delarue et al. (1996)[129]	Human (healthy)	3 weeks. 1.1g EPA, 0.7g DHA daily.	Reduction in insulinemia with an increase in non-oxidative glucose metabolism. Shift towards fat oxidation following a glucose load.
Delarue et al. (2006)[103]	Human (healthy)	3 weeks. 1.1g EPA, 0.7g DHA daily.	Reduction in glucose fluxes during exercise (60% VO ₂ max). Tendency towards increase in fat oxidation during exercise.
Lalia et al. (2015)[141]	Human (insulin resistant)	6 months 3.9g EPA/DHA daily.	No change in peripheral insulin sensitivity compared to control. Small reduction in hepatic gluconeogenesis.
Fasching et al. (1991)[105]	Human (impaired glucose tolerance)	2 weeks, 3.8g EPA, 2.5g DHA daily (30 ml fish oil).	No changes in fasting plasma glucose or insulin levels. No change in glucose or insulin during hyperinsulemic clamp.
Glauber et al. (1988)[106]	Human (T2D)	4 weeks, 18g fish oil daily.	Increase in fasting plasma glucose and in response to feeding. Increased hepatic glucose production. Reduction in insulin secretion.
Popp-snijders et al. (1987)[104]	Human (T2D)	8 weeks, 3g EPA/DHA daily.	Enhanced glucose clearance during steady state infusion of glucose and insulin.

1.5. *Summary of omega-3 research*

The results from omega-3 supplementation trials have often been mixed, yielding null responses when combined in meta-analyses. We hypothesize that one of the primary variables that may limit the effect of omega-3 fatty acids is a high $n-6/n-3$ ratio. Evidently the relationship between omega-3 and omega-6 PUFAs is not simply antagonistic and different effects of fatty acids within each subclass adds to the complexity. Further studies should aim disseminate the interaction between omega-3 and omega-6 PUFAs and how this may impact metabolism. The currently available evidence suggests that omega-3 PUFAs EPA and DHA may be effective in preventing the deleterious effects of atrophic conditions or low-grade inflammation (**Figure 1.1**). The research has focused predominantly on EPA and DHA, yet it is now emerging that DPA has also been shown to have similar, and in some cases, more potent effects. There is emerging evidence that different omega-3 PUFAs has divergent metabolic functions, and further research is required to understand the different mechanisms underpinning these effects. The recent advances in the 'omic' techniques and mass spectrometry technology will allow for a comprehensive and sensitive approach to analysing the metabolic changes induced through omega-3 uptake. Currently, omega-3 PUFAs are mainly derived from marine sources. Given the increasing environmental pressures on fish populations, it brings into question the sustainability of fish as a suitable resource of omega-3. It is estimated by 2050 that the human population will reach 9.1 billion and global warming will reduce the omega-3 content in algae, reducing the total omega-3 content in fish [143]. This highlights the need to understand the mechanisms of omega-3 PUFA action which may lead to the development of an omega-3 mimetic and provide a sustainable long-term source. Further study is necessary to understand

the link between enriching omega-3 PUFAs EPA and DHA in the lipid pool with the effects on glucose and protein metabolism.

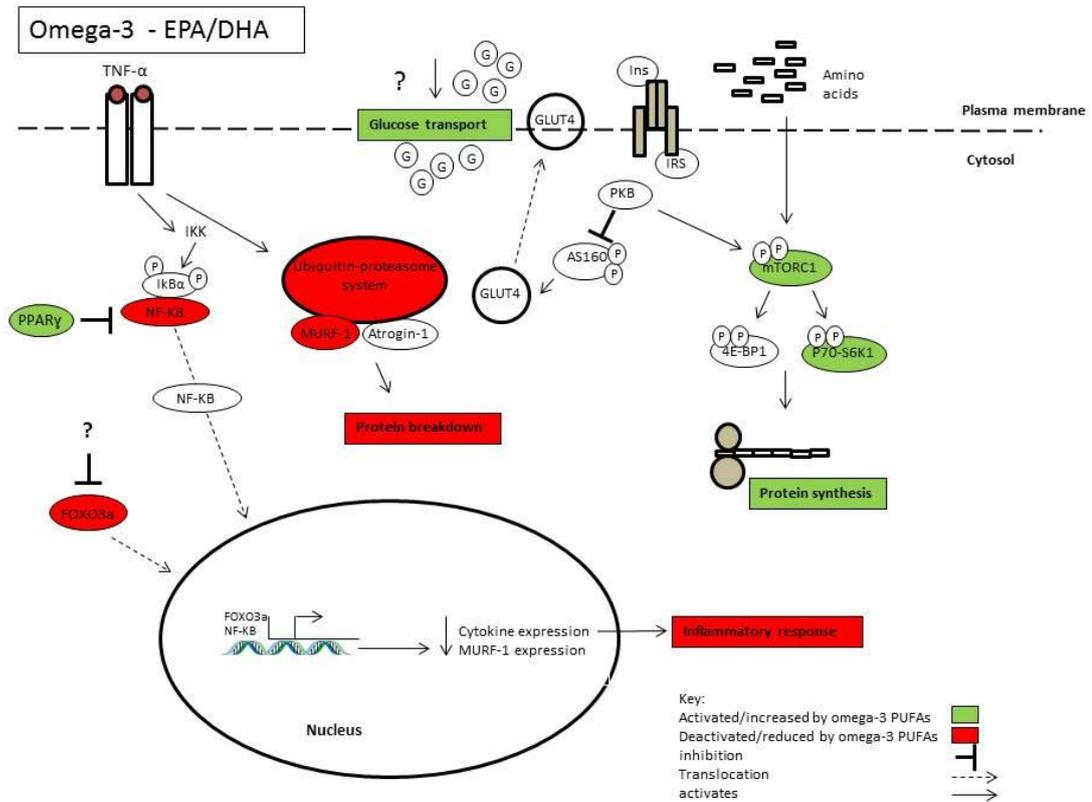


Figure 1.1. Proposed mechanisms of omega-3 PUFA action. This figure highlights the mechanisms of action by which omega-3 fatty acids may exert cellular effects. Protein synthesis in response to amino acids is enhanced through increased phosphorylation of proteins in the mTOR pathway. Omega-3 fatty acids may also reduce inflammation and protein breakdown by inhibiting the translocation of key transcription factors (NF-Kb and MURF1) to the nucleus.

1.6. Saturated fatty acids and metabolic health

Ever since *Randle & colleagues (1963)* [144] proposed that accumulation of fatty acids was linked to dysfunction in carbohydrate metabolism there has been a great deal of debate over the role of fatty acids in metabolic health. Central to that debate is the link between saturated fatty acids and a number of pathologies including cardiovascular disease and diabetes. In comparison to the PUFAs discussed in the previous sections,

saturated fatty acids are causally linked to the disruption of key anabolic processes such as protein accretion and glycogen synthesis.

It is commonly asserted that a diet high in saturated fatty acids are associated with impaired insulin sensitivity. However, the data available from human studies suggest a more ambiguous relationship. Meta-analyses indicate that replacing saturated fat with MUFA or PUFAs can have favourable effects on glucose and insulin homeostasis [145]. Yet, studies using the euglycemic-hyperinsulinemic clamp to assess insulin sensitivity have not found a significant link between SFA intake and reduced insulin sensitivity. One of the most commonly cited studies supporting the SFA hypothesis is the KANWU study [12]. This study compared diets rich in either MUFAs or SFAs for 3 months in 162 subjects (mean total fat intake was 37%). The authors found that in the SFA diet insulin sensitivity declined by 10%. Although post intervention insulin sensitivity was not significantly different from the MUFA diet group and there was no control group for comparison. As a result, the KANWU study does not provide conclusive evidence that SFA induces insulin resistance. This was a free living study where fat intake was not clamped and an interesting sub set analysis revealed that the difference in insulin sensitivity between SFA and MUFA was accentuated when fat intake was below 37%, while regardless of dietary status fat intakes of above 37% reduced insulin sensitivity. Furthermore, other studies have also observed the same results but in different populations. The LIPGENE study, reduced the dietary SFA content in 417 Europeans with metabolic syndrome and did not find any effect on the HOMA-IR index or plasma inflammatory markers [146]. Moreover, a smaller scale diet analysis study in obese individuals found no difference between diets rich in SFA or MUFA on glycemic control in spite of differential inflammatory adipose tissue gene expression [147]. Overall, the human studies do not demonstrate a clear consensus on

the effects of dietary SFA on insulin sensitivity. As discussed below, studies from tissue culture commonly demonstrate that the negative effects of PA are dampened when incubated with other fatty acids such as oleate, PAO and other unsaturated species [19], [148], [149]. In human trials the negative effects of PA may be offset by the balance of lipid species in the diet. Another confounding factor may lie in the greater susceptibility of skeletal muscle isolated from obese individuals to the deleterious effects of PA on glucose uptake compared to lean individuals [150]. Furthermore, these studies last for time periods in the range of 3 months and it is possible that only a sustained intake of SFAs may attenuate insulin sensitivity, potentially through the build-up of low grade inflammation.

1.7. *Saturated fatty acids and insulin sensitivity*

Evidence for the deleterious effects of PA on insulin sensitivity are mainly derived from animal and tissue culture studies. There is a large body of evidence demonstrating that incubation of skeletal muscle myotubes with PA(500-750 μ M) attenuates the action of insulin on glucose uptake [19], [93], [151]. The disruption of glucose homeostasis can be mitigated by co-incubation with unsaturated fatty acids species such as PAO [19]. Oversupply of PA in skeletal muscle leads to the generation of lipid intermediates such as ceramides and diacylglycerols (DAG). Palmitoyl-CoA is a substrate for Serine Palmitoyl transferase-1, which condenses palmitoyl-CoA to 3-ketohydroshingosine and is then acylated to form dihydroceramide. Ceramide is then produced through the desaturation of dihydroceramide. Ceramide accumulation results in the inhibition in a number of cellular processes such as glucose uptake and glycogen synthesis. The inhibitory action on PKB by ceramides act downstream of IRS/PI3K and occurs through alternative mechanisms in different cell types [152]. In L6 myotubes, PKB activity is

downregulated through activation of PKC isoforms while in C₂C₁₂ myotubes protein phosphatase 2A (PP2A) is activated increasing the de-phosphorylation of PKB. The mechanism of PKB inhibition by ceramide is dependent on the abundance of ceramide enriched micro domains [152]. Inhibition of SPT-1 prevents the accumulation of ceramide and is sufficient to prevent the deleterious effects of PA [153]–[155]. Ceramides are a prominent candidate for driving insulin resistance, however, not all studies report a relationship between ceramide levels and insulin sensitivity. *Skovbro and colleagues (2008)* [156] assessed muscle ceramide content across healthy, impaired glucose tolerance, T2D and endurance trained individuals and found that ceramide levels were similar across all groups, despite differing levels of insulin sensitivity. Furthermore, pharmacological/genetic inhibition of ceramide synthesis channels PA towards greater DAG synthesis resulting in impaired insulin action [157]. Alternatively, it has been observed that DAG levels, and not ceramides, are associated with acute insulin resistance following a lipid infusion in humans [88], [91]. DAGs also inhibit PKB but, unlike ceramides, act at the level of IRS/PI3K. DAGs activate PKC isoforms that increase IRS-1 serine phosphorylation which subsequently reduces IRS-1 tyrosine phosphorylation. Whether ceramide or DAG drive insulin resistance may be dependent on the lipid type. Whilst in some cell types PA induction of insulin resistance appears to be primarily ceramide driven, oversupply of linoleate (an unsaturated fatty acid) appears to induce insulin resistance through DAG mediated mechanisms [155]. It therefore appears that oversupply of a range of lipid types to a tissue with limited storage capacity can induce insulin resistance. Yet, in some contexts, unsaturated fatty acids can offset the deleterious effects of saturated fatty acids.

In a range of studies utilising a number of different cell types, unsaturated fatty acids have been shown to offset the inhibition of insulin action induced by saturated fatty

acids [19], [64], [158]. Indeed, the incubation of primary cells with a fatty acid mixture does not cause insulin resistance (as assessed by insulin induced PKB phosphorylation), even when PA is the predominant fatty acid [148]. Even minor differences in structure such as one double bond in PAO (16:1) and two extra carbons and a double bond in oleate (18:1) can have opposing effects to PA. For instance, incubation of L6 myotubes with PAO not only completely attenuated the inhibitory effects of PA on glucose uptake but also intrinsically increases glucose uptake when incubated in isolation. However, whilst basal glucose uptake was stimulated by PAO, the insulin induced response is lost [19]. Additionally, other unsaturated fatty acids such as oleate (18:1) and linoleate (structure) enhance insulin stimulated PKB and ERK1/2 activation [159]. It appears that one of the ways that unsaturated fatty acids reduce the effects of saturated fatty acids is by increasing the channelling of fatty acids into neutral lipid stores [112]. The importance of this mechanism is evident from experiments in which Triglyceride synthesis is disrupted. In the context of inhibited TAG synthesis, linoleate results in lipotoxicity. Given that the ability of skeletal muscle to store lipids is limited, channelling excess lipids into TAG would reach a saturation point where it is no longer beneficial. Yet, the channelling of SFA into neutral lipid stores may also offset some of the inflammatory effects of PA. Work from the Hundal laboratory showed that PA induced NF-Kb activation and elevated interleukin-6 mRNA levels. This effect was regulated by the extracellular regulated kinase (ERK) as inhibition ameliorated the activation of NF-Kb. Yet, the inhibitory effect of PA on glucose uptake persisted, demonstrating independent properties of PA which induce insulin resistance. Further work built on this data, showing that PAO potently repressed these inflammatory effects. This protective effect was due to increased neutral lipid storage and increased oxidative capacity. Whilst there is a limited storage

capacity for lipid in skeletal muscle, skeletal muscle does have the capacity to dramatically increase mitochondrial content. Increasing mitochondrial fatty acid oxidation may be a strategy to offset the negative effect of lipid intermediates on insulin action potentially by clearing the excess lipid through oxidation. In support of this hypothesis, studies which have induced mitochondrial uncoupling by overexpressing uncoupling protein 1 demonstrate resistance to the deleterious effects of a high fat diet [160]. There are studies which have demonstrated that obesity and type 2 diabetes lead to the reduction of mitochondrial function. Yet, high fat diets may actually lead to excessive beta-oxidation. Mice placed on a high fat diet for 5 or 20 weeks displayed increased rates of PA and Palmitoyl-CoA oxidation [136]. While in cultured myotubes, excess lipid availability resulted in increased beta-oxidation beyond the mitochondrial capacity to fully oxidise those lipids [161]. Indeed, PA exposure in culture can reduce mitochondrial function. For instance, the addition of PA (500 μ M-1mM) to myotubes increases reactive oxygen species whilst concomitantly reducing ATP levels [162]. Both PGC1 alpha expression and promoter activity were also reduced, all of the observed effects were attenuated by co incubation with oleate (500 μ M-1mM). *Nisr & colleagues (2016)* [163] also found that PA reduced oxygen consumption related to energy demand for cellular processes. Stearate (18:1) was also found to have similar properties to PA. Whilst oleate and linoleate had modest effects on ATP synthesis but did not reach statistical significance [163]. The effect of PA may depend on whether myoblasts or myotubes are used. *Patkova et al (2014)* [164] found that PA only reduced mitochondrial function in myoblasts, reducing ATP dependent oxygen consumption and maximal mitochondrial capacity. While in myotubes proton leak was increased but did not effect oxygen consumption related to ATP synthesis. It can be speculated that

these negative effects on cellular energy state could translate to impairments in anabolic processes such as muscle protein synthesis.

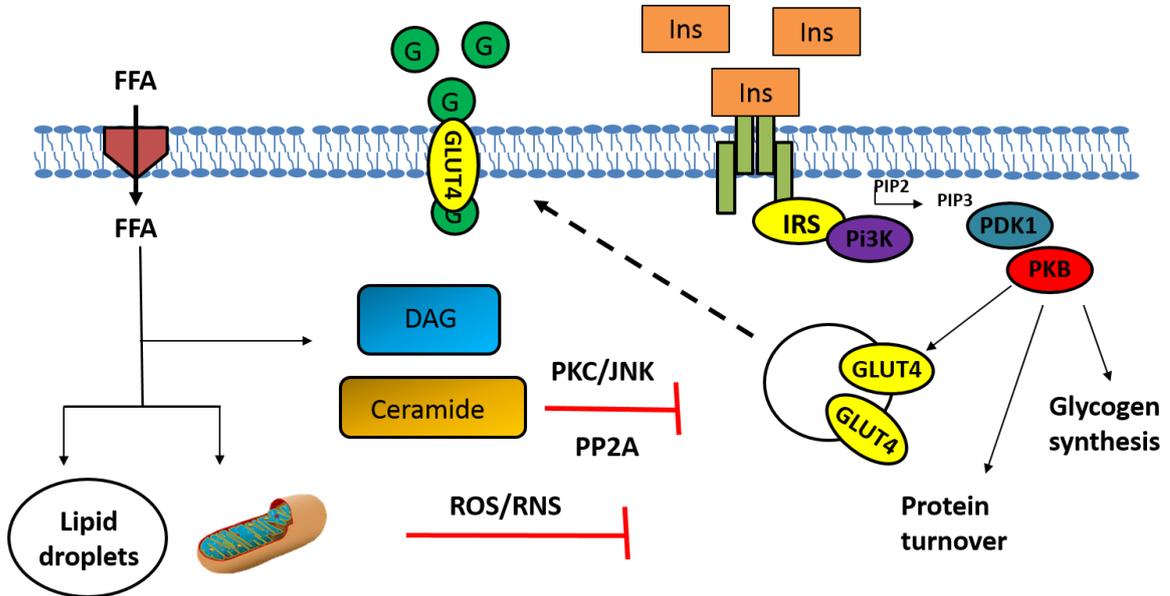


Figure 1. 2 Accumulation of FFA and increased Palmitate can disrupt insulin signalling by attenuating PKB activity. Fatty acids transported into the muscle cell are channelled towards storage in neutral lipid stores or oxidation. Accumulation of PA can provide a substrate for the production of Ceramide and DAGS which activated kinases and phosphatases that repress the action of PKB, this results in the loss of insulin stimulation glucose uptake. Mitochondrial overload by fatty acids can increase reactive oxygen species which interfere with glucose uptake

1.8. SFA and skeletal muscle anabolism

In order to maintain homeostasis, skeletal muscle is continually integrating both extracellular and intracellular signals and altering cellular function to respond to changes in the cellular environment. There is some associational data to suggest that expanded adipose mass may modulate skeletal muscle protein turnover. Obese individuals from across the age spectrum display attenuated MPS responses to elevated plasma concentrations of amino acids and insulin [165], [166]. It has been proposed that fatty acid infiltration into skeletal muscle during obesity alters signalling networks that impair the protein synthetic response to feeding in rodents [167], [168]. However, even acute exposure (6 hours) to elevated plasma lipids can lead to

impairments in muscle protein balance. Indeed, in young healthy men an acute lipid infusion (time) impairs the MPS response to infused amino acids + insulin [108]. This impaired MPS is associated with the complete attenuation of 4E-BP1 phosphorylation in response to feeding, suggesting the mechanism is via the disruption of upstream anabolic signalling. Furthermore, both acute high fat feeding (mixed) and longer term supplementation (omega-3) have been demonstrated to alter the activity of the kinases involved in muscle growth and energy sensing pathways in response to feeding and exercise [59], [169]. However, not all studies demonstrate a consensus. For instance, a similar study to *Stephens et al* observed no lipid dependent blunting of amino acid induced increases in MPS [170]. Although both Stephens and Katsanos used different doses of amino acids, one of the biggest methodological differences is the use of the hyperinsulinemic-euglycemic clamp by *Stephens et al* [108]. The mechanism of lipid action in the alteration of protein metabolic networks may therefore be regulated by insulin. Insulin additionally plays an important role in regulating skeletal muscle protein breakdown [171]. As a result, even if MPS is still sensitive to amino acids, insulin resistant muscle would fail to reduce protein breakdown in response to an anabolic stimulus. This reduction in insulin action would potentially lead to a reduction in total protein accretion, as protein breakdown would not be suppressed by feeding induced increases in insulin. These potential mechanisms are believed to partially explain the enhanced sarcopenia observed in older obese adults.

The molecular mechanisms behind the above observations are still to be fully delineated. However, we know that increasing intracellular concentrations of PA induces stress on a number of functional networks. These include alterations in lipid metabolism and storage as well as attenuation of substrate transport (Glucose, amino acids) across the plasma membrane [19], [172]. PA exposure also disrupts intracellular

signalling, in particular the PKB signalling pathway. Given that PA dysregulates PKB and downstream signalling it could be predicted that concurrent with the induction of insulin resistance that protein synthesis will also be attenuated. The currently available data on the effect of PA on muscle anabolism are limited, however, there are a number of known molecular effects of PA that are likely to interact with the regulation of protein synthesis. There is also data to suggest that fatty acids such as oleate and PAO do not have the same deleterious effects.

Upon transport into the cell, PA is converted to palmitoyl-CoA which can then be used as a precursor for ceramide production. Evidence suggests that PA is less efficiently stored in neutral lipids in muscle myotubes compared to more unsaturated lipids (oleate, linoleic, EPA, DHA) and this leads to the accumulation of lipid intermediaries such as ceramide and diacylglycerols (DAG) [112]. Previous work has shown that exogenous provision of C2 ceramide is sufficient to reduce the intracellular free amino acid pool through down-regulation of system A mediated amino acid transport, likely due to reduced membrane abundance [172]. Furthermore, downstream mTORC1 signalling and protein synthesis were attenuated as a result [172]. The authors also showed that PA derived ceramides also reduced system A transport but did not couple this to measures of MPS. In contrast, the unsaturated fatty acid PAO did not blunt amino acid uptake and is, therefore, unlikely to reduce protein synthesis. The increase of amino acid uptake in response to insulin was not dampened by increased levels of ceramide which would suggest some divergent mechanisms of PA action on insulin responsive glucose and protein metabolism. In addition to noted effects on AA transport, PA has also been noted to regulate stress pathways such as the Endoplasmic reticulum stress signalling system.

The ER is an important organelle for protein folding and modification as well as lipid synthesis and calcium homeostasis. Disruption to the normal function, termed ER stress, can lead to the aggregation of misfolded proteins and induces what is known as the 'unfolded protein response' (UPR). The UPR initiates a transcriptional programme to restore homeostasis and failure to achieve this results in apoptosis. In response to ER stress multiple proteins are activated by release from the chaperone family (BiP). Of particular interest is the protein kinase R-like ER kinase (PERK) which phosphorylates eif2 α (Ser51) which then becomes a competitive inhibitor of the guanine exchange factor eif2B reducing levels of GTP loaded eif2 and inhibiting translation initiation [173]. This conserves energy and resources to mount an ATF6 directed translational programme to enhance the levels of specific chaperone proteins required to prevent further stress induced damage. High fat feeding in animal and human models is associated with an induction of ER stress in the skeletal muscle [174]. In culture, PA is known to induce ER stress in a number of cell types, including skeletal muscle myotubes [175]. While inhibiting ceramide can prevent the development of insulin resistance, it may not be critical in SFA induced ER stress [176]. Inhibition of ceramide synthesis does not prevent the disruption of ER stress in hepatocytes [176]. Thus, PA may drive both ceramide dependant and independent reductions in protein synthesis. It could be predicted that in skeletal muscle, PA induces ER stress which leads to the phosphorylation of eif2a and subsequent inhibition of protein synthesis. Indeed, a study by *Tardif & colleagues (2014)* [177] showed a PA (375 μ M) driven and ceramide dependent reduction in muscle protein synthesis. Furthermore, PA also down-regulates MPS in L6 myotubes by a similar magnitude [163]. As we mentioned above there is also evidence that lipid overload can modify muscle protein breakdown.

The balance of protein synthesis and breakdown underpins changes in muscle protein balance. Alterations in either parameter can push the muscle into a positive protein balance leading to hypertrophy, conversely a negative protein balance will lead to atrophy. Tissue culture studies show that PA can up-regulate the activity of proteolytic systems [178]. This up-regulation of protein breakdown leads to a reduction in muscle myotube diameter after a 48 hour incubation [179]. PA is known to reduce phosphorylation of PKB and this leads to sustained nuclear retention of the Forkhead box transcription factors (FoxO3) which induces the transcription of proteins involved in degradation pathways [178]. FoxO3 transcription factors control the expression of genes involved in processes such as apoptosis, cell cycle arrest, oxidative stress resistance and muscle atrophy. In particular, expression of genes such as *Murf1*, *atrogen-1* and *Bnip3* are enhanced under atrophic conditions which lead to increased protein degradation, especially of muscle structural proteins [67], [180], [181]. The study by *Woodworth-Hobbs & colleagues (2014)* [178] showed that PA increased the mRNA abundance of *atrogen-1* and *Bnip3* when protein breakdown was also increased. This altered transcription of atrogenes is primarily driven by the attenuation of PKB phosphorylation which prevents the cytosolic sequestering of FoxO3 [178]. Another regulator of muscle atrophy is the NF- κ B pathway. NF- κ B is activated by the phosphorylation and subsequent proteasomal degradation of inhibitor of κ B kinase. Free NF- κ B then translocates to the nucleus and binds to DNA, initiating the transcription of a multitude of genes involved in cellular responses to stress. In particular, NF- κ B is known for the transcription of genes involved in inflammation. When L6 myotubes were exposed to PA NF- κ B was activated [182]. Activation of NF- κ B has been associated with muscle atrophy similar to cachexia [183].

1.9. *Summary of SFA/MUFA research*

In summary, while there is not a large body of data linking PA directly with perturbations in muscle anabolism it appears that increasing intracellular concentrations of PA can elicit an environment that leads to net protein loss and insulin resistance. PA attenuates protein synthesis while simultaneously increasing the rate of protein breakdown. There are a number of underlying mechanisms. It appears that the PKB signalling pathway is a central node in the pathology of PA driven insulin and anabolic resistance. There are multiple mechanisms which feed in to attenuate the normal function of this axis. This overview has focused on the local factors determining muscle atrophy and growth however it has not considered systemic factors such as input from the immune system which is also known to be responsive to various fatty acids. Studies also show that PA induced perturbations in anabolic signalling are prevented by unsaturated fatty acids. It therefore remains to be seen how relevant this is *in vivo* where a diet of mixed fat sources are consumed regularly leading to a mix of circulating free fatty acids and triglycerides.

1.10. *Contrasting effects of fatty acid species*

Fatty acids display a diverse range of structures which lead to a highly diverse lipid pool. As demonstrated in the previous sections, fatty acids differing in carbon chain length and unsaturation levels can have both positive and negative effects on skeletal muscle metabolism and function. For example, EPA and DHA are very similar in structure, differing by only 2 carbons and 1 double bond, but appear to have different metabolic activities in skeletal muscle [184], [185]. DPA (22:5) is an intermediate of EPA and DHA which may also have unique metabolic functions [186]. A recent study used computational modelling to demonstrate that EPA, DPA and DHA, differentially

alter membrane characteristics [187]. Similarly, PA and PAO, differ by a single double bond and this changes the way in which each fatty acid alters glucose uptake and metabolism. PA treatment of cultured myotubes attenuated insulin's effect on glucose uptake, while PAO either does not alter, or increases, glucose uptake [188]. Although the role of specific fatty acids in altering skeletal muscle metabolic processes are well characterised, their mechanisms are still poorly understood. Some of the potential ways in which fatty acids can alter skeletal muscle function will be discussed in the next section.

1.11. Remodelling the lipidome

The composition of fatty acids within skeletal muscle is determined by a number of factors. Mainly, dietary intake of fatty acids, uptake and oxidation of fatty acids in muscle and endogenous lipid production. Fatty acids can influence cell function through a number of ways. Fatty acids can alter the activity of transcription factors, such as PPAR α and SREBP-1. Specific fatty acids also serve as substrates for lipid products such as eicosanoids, prostaglandins as well as lipid intermediates such as ceramides. Furthermore, fatty acids are incorporated into phospholipids, which are the main component of membranes. Cell membranes play a critical role in maintaining a barrier between the intra and extracellular environment and regulating the entry and exit of molecules. All external signals received must be propagated through the membrane. Altering membrane fatty acid composition and physical characteristics can impact cell function

In 1972, Singer and Nicholson [189] proposed the fluid mosaic model which describes the behavior of cellular membranes. Cellular membranes are composed of an asymmetric phospholipid bilayer in which cholesterol and proteins are embedded. The membrane is not a static bilayer and membrane components can diffuse both laterally,

rotationally and flip between then inner and outer leaflets of the bilayer. These characteristics gives membranes physical properties such as fluidity and elasticity while still being structured enough to maintain the integrity of the membrane. Membrane fluidity and the movement of phospholipids is important for a number of reasons. It allows for the insertion of proteins and newly synthesized lipids into the bilayer and subsequent diffusion from the insertion point. Membrane fluidity is also important for the fusion with other membranes and mixing of the components, for example GLUT4 containing vesicles translocate to the plasma membrane in response to insulin and release GLUT4 proteins into the membrane which then diffuse laterally and open pores in the membrane. Membrane bilayers can exist in two main states. The gel phase, where the membrane is more ridged and ordered and the liquid-crystalline phase where the membrane has a looser more fluid order. One of the key determinants of membrane fluidity is the carbon length and unsaturation length of fatty acids contained within phospholipids. Different fatty acids have different melting temperatures and this alters the temperature at which a membranes shift from a gel phase to the liquid phase. Increasing carbon chain length increases the melting temperature due to the increase in surface area. Lipids, such as PA, have a higher melting temperature and membranes rich in saturated fatty acids are maintained in a more gel like rigid structure. Increasing unsaturated bonds into the carbon chain decreases the melting temperature. Unsaturated bonds introduce kinks into the structure which prevent lipids from packing closely and form less structured membranes. Monounsaturated fatty acids have a more modest effect on membrane fluidity than more highly unsaturated fatty acids. A modulator of fatty acid regulated membrane fluidity is the presence of cholesterol within membranes. The presence of

cholesterol causes tighter packing of lipids and can also segregate areas of different phases.

Omega-3 PUFAs are long chain highly unsaturated fatty acids and given these characteristics are commonly used to assess the effects of fatty acid composition on cell membrane characteristics. Omega-3 lipids are highly incorporated into all cell membranes, such as the plasma and mitochondrial membranes [190]. Alterations in the membrane composition within cells are known to alter the physico-chemical properties. An increase in unsaturation of phospholipids will increase membrane fluidity whereas greater saturation of phospholipids will induce a more rigid structure. As such, insulin resistance is associated with decreasing concentration of PUFAs in serum and muscle phospholipids [191]. Theoretically, enhanced fluidity, looser packing of phospholipids, of the membrane may facilitate increased GLUT4 vesicle fusion with the plasma membrane. Membrane composition changes may alter the micro-environment of proteins localized within the membrane causing a modification in the function of the protein [29]. There is also evidence to suggest that DHA, and to a lesser extent EPA, play a role in the formation and composition of lipid rafts [192]. Lipid rafts are insoluble fractions of the membrane composed of sphingolipids, cholesterol and proteins and are formed in order to allow the compartmentalisation of the membrane for the coordination of function by providing scaffolding to stabilise protein-protein and lipid-lipid interactions involved in signal transduction and trafficking [193]. Studies have shown in a number of cell lines that DHA and EPA are incorporated into insoluble membrane fractions and lead to the 'declustering' of lipid rafts due to the poor affinity between omega-3 PUFAs and cholesterol, leading to the expulsion of cholesterol from the lipid rafts [192], [194]. However, EPA and DHA may have divergent effects on the structure of lipid rafts with EPA incorporated into non-

raft regions and displacing cholesterol to raft regions making them more ordered, while DHA may have the opposite effect [195]. These structural changes may affect protein localisation within raft and non-raft regions, however, it is yet to be fully understood how this directly impacts on cell function. Given the important nature of signalling responses at the membrane, modification by omega-3 PUFAs could lead to a significant alteration in cellular function giving rise to changes in gene expression, cell signalling and release of bioactive metabolites. The current literature, however, focuses on immune cell lines and it remains to be studied in human skeletal muscle cells.

Given that omega-3 PUFAs interact with multiple cell signalling pathways changes in the membrane composition may be a potential convergence point for the effects of omega-3 PUFAs on metabolic outputs. The phospholipid pool also serves as the main substrate pool for production of eicosanoids. Enriching the phospholipid fraction specifically may, therefore, be necessary to elicit the beneficial effects on omega-3 PUFAs. To date, few studies have fully characterised to what extent, or even which, phospholipid fractions (i.e. phosphatidylcholines, phosphatidylserines, phosphatidylethanolamines and phosphatidylinositol) are enriched or displaced by omega-3 PUFAs in skeletal muscle and how this relates to skeletal muscle metabolism. Concurrent with the changes in membrane composition and physical characteristics, fatty acids can also serve as a substrate for lipid products and intermediates of lipid metabolism. In particular, omega-3 and omega-6 fatty acids EPA and AA are substrates for the production of prostaglandins, eicosanoids and thromboxanes. These are bioactive molecules that play a role in the modulation of inflammatory processes [196]. Eicosanoids are synthesised from 20 carbon fatty acid chains which have been released from phospholipids by phospholipase A₂ meaning both AA and EPA are

substrates for eicosanoid production. Incorporation of EPA and DHA into the substrate pool often occurs at the expense of AA reducing the potential for AA driven eicosanoid production. EPA, as a 20 carbon chain, competes with AA as a substrate for the cyclooxygenase (COX) and lipoxygenase (LOX) pathways and has also been seen to reduce the gene expression of COX-2 [197]. Dietary supplementations of omega-3 PUFAs in humans have observed decreased production of AA eicosanoids such as Prostaglandin E₂ while increasing the production of prostaglandin E₃ [198], [199]. EPA derived eicosanoids also display a reduced affinity for eicosanoid receptors rendering them up to 50-80% less biologically active than AA derived eicosanoids [200]. Both EPA and DHA also serve as substrates for resolvins, protectins and maresins which display inflammation resolving properties in both cell culture and animal models of inflammation [201], [202]. However, a recent study has called into question the production of inflammatory resolving molecules and their role in mediating inflammatory challenges in healthy humans [203]. A short term supplementation period of 17.6 g EPA and DHA/day did not result in the detection of these inflammatory resolving mediators in plasma or urine [203]. One study found that the ability of EPA to reduce Prostaglandin E₂ occurred between an intake of 1.35 and 2.75 g/day, suggesting a threshold for the anti-inflammatory effects [199]. The dose of EPA is typically lower in many studies which may be a possible factor in the equivocal changes in inflammatory markers seen in supplementation studies. Further study will be necessary to understand whether endogenous production of eicosanoids has a prominent role in the inflammatory process and if there is any potential role of eicosanoids in glucose and protein metabolism.

Fatty acids can also bind to and alter the activity of transcription factors and proteins. Multiple fatty acid species have been identified as potential ligands of the transcription

factor peroxisome proliferate activated receptor alpha (PPAR α). PPAR α is a transcription factor involved in the regulation of fatty acid oxidation and energy homeostasis [204]. Long chain omega-3 PUFAs linoleic acid (18:3) and DHA (22:6), omega-6 PUFAs arachidonic acid (20:4) and saturated species myristic (14:0) and stearic acid (18:0) have all been identified as ligands for PPAR α [205]. Prostaglandins synthesised from 20 carbon fatty acids have also been shown to be ligands for PPAR α [205]. Sterol regulatory element binding protein 1 (SREBP-1) is another transcription factor that is affected by fatty acids. SREBP-1 plays a key role in expression of genes involved in lipogenesis. Upon insulin exposure SREBP1 is cleaved from the ER and translocates to the nucleus. It has been shown that omega-3 PUFAs suppress the activation of SREBP-1 in response to insulin through the liver X receptor [206]. As well as binding to transcription factors, some fatty acids also bind to proteins. The most common fatty acid modifications are the myristoylation and palmitoylation of proteins. One of the main functions of this protein modification is the regulation of a protein's subcellular trafficking. Typically, palmitoylation of a protein targets a protein to the membrane and can specifically target a protein to lipid rafts and regulate cell signalling [207].

Collectively, this body of work demonstrates that fatty acids can alter cell function through a number of different mechanisms. Incorporation of fatty acids into complex lipid structures such as phospholipids can alter membrane characteristics and the response to extracellular stimuli. Furthermore, 20 carbon fatty acids act as a substrate for the production of prostaglandins and eicosanoids. Aside from changes in lipid structures, fatty acids can directly interact with transcription factors and induce changes in gene expression and subsequent protein abundance. Fatty acids, such as PA, can regulate protein membrane localisation by directly interacting with proteins. Thus,

fatty acids can alter the cellular environment on multiple levels. However, few studies have coupled measures of changes in lipid profiles with changes in proteomic and gene expression data.

1.11. *Mass spectrometry approaches in biology*

The generation of 'omics' datasets has been rapidly expanding in recent decades. The main concept is to better understand the underlying biology by taking a holistic view to measure the components of cells as universally as possible. This approach has been used to measure genetic sequence, mRNA, protein, lipids and metabolites. Underpinning the increase in omics studies is the advancement of available in technology. In particular, the development of mass spectrometry techniques allows the measurement of protein, lipids and metabolites with increasing resolution. The overall goal of this thesis was to further our understanding of how different fatty acyl structures alter the cellular microenvironment. To achieve this, we decided to utilise mass spec technologies to measure global changes in lipid profiles, specific changes in phospholipid composition and proteomic profiles.

Many studies analyse the changes in abundance of a selected proteins, normally associated with a specific pathway. While this provides useful information, important changes in other proteins which may be mechanistically linked are missed. Coupling isotope labelling strategies with mass spectrometry analysis allows for a more global approach to protein abundance analyses. A typical workflow for proteomics analysis requires proteins to first be extracted from the sample, proteins are then separated by molecular mass to reduce sample complexity. Proteins then undergo tryptic digestion, separated by chromatography and injected into the mass spectrometer. Multiple strategies have been developed such as isotope coded affinity tag (ICAT), isobaric tags for relative and absolute quantification (iTRAQ) and stable isotope labelling of amino

acids in cell culture (SILAC). The ICAT technique involves labelling cysteine residues with an isotopic label conjugated to a biotin molecule. This biotin molecule is then used to pull down by affinity purification of peptides containing a labelled cysteine peptide. This limits the proteomic analysis to cysteine containing peptides and reduces the sample pool for analysis. Another drawback is that each sample is labelled individually prior to pooling. This could introduce different labelling efficiencies for each sample. iTRAQ is another commonly used proteomics method. iTRAQ introduces different isobaric tag to free amine groups post protein digestion. Samples can then be multiplexed and analysed simultaneously. A major limitation of the iTRAQ method is that accurate measurement of abundance changes is limited to large changes in protein abundance [208]. The SILAC strategy overcomes these issues by introducing the isotopic label during the proliferation of cells. This method uses labelled amino acids (heavy or light labelled arginine and lysine) – need to detail specific modification, known difference in mass. Cells are grown in modified media which is free of unlabelled arginine or lysine and supplemented with modified arginine or lysine. As these are non-essential amino acid cells must use the labelled amino acid in the synthesis of new proteins. This ensures that after multiple cell population doublings, almost all proteins will contain a labelled arginine and lysine. This allows for more accurate quantification of abundance changes as multiple peptides from the same protein can be compared to ensure a similar level of change. These amino acids were also chosen as trypsin cleaves exclusively at arginine and lysine residues [209]. Thus, after tryptic digestion all peptides should contain either a labelled arginine or lysine. Prior to digestion samples are also pooled for all further steps which allows for experimental variability to be reduced. SILAC allows for the measurement of the relative change in abundance compared to both the unlabelled group and the other labelled group. The SILAC

method has a number of strengths that overcome the limitations of other methods that makes it one of the most effective ways to assess proteome changes. SILAC allows for the measurement of small changes in protein abundance as well as peptides not containing cysteine residues. Thus, SILAC proteomics provides a highly accurate method to assess relative changes in protein abundance between treatments

1.12. *Conclusion*

Overall, it appears that the metabolic effects of fatty acids are related to the presence of double bonds within the acyl chain structure. Double bonds alter the conformation of the fatty acid, altering membrane structure and lipid interaction with membrane proteins, cholesterol etc. Moreover, fatty acids are ligands for a number of enzymes/receptors. A change in the structure of the fatty acid will not only alter binding targets but also the efficiency by which those docking reactions occur [200]. Thus, different fatty acids have the capacity to substantially alter a number of cellular processes depending on the metabolic fate each fatty acid is channelled towards. Fatty acids have the capacity to alter gene expression [109], [147], [149], [210], so the metabolic activities may be reflected in the global proteomic signature of muscle cells. It is still poorly understood how fatty acids of differing chain length and unsaturation alter the myotube microenvironment by shifting lipidomic and proteomic profiles.

1.13. *Aims and objectives*

The overall aim of this thesis is to further our understanding as to how minor differences in fatty acid structure can differentially alter various aspects of skeletal muscle metabolism. Specifically, by profiling myotube metabolic phenotypes and proteomic signatures. Because our primary output was to be 3 way SILAC screens, there are 2 main branches to this thesis (**Figure 1.3**). The comparison of two n-3 PUFAs, Eicosapentaenoic acid and Docosahexaenoic acid and in another set of experiments a comparison of a saturated fat, Palmitate and an unsaturated fat Palmitoleate. The comparison of these fatty acids in two groups of experiments was necessitated by the limitation SILAC experiments being restricted to 3 way comparisons.

The main objectives are:

- 1. To characterise the incorporation of a) omega-3 fatty acids EPA and DHA and b) SFA PA and MUFA PAO into C2C12 myotube lipid pools and subsequent alterations in global lipid profiles using Gas chromatography/mass spectrometry analysis. Furthermore, analyse the storage pattern of EPA and DHA using a lipidomics approach to assess whether EPA and DHA are preferentially stored in neutral lipid stores or phospholipids.**
- 2. To determine the impact of treating c2c12 myotubes with different fatty acids (EPA,DHA,PA,PAO) on myotube insulin sensitivity. Glucose uptake will be used as a key readout of insulin sensitivity. Measures of glucose uptake will be coupled to the assessment of PKB phosphorylation and protein abundance of key glucose transporters. Secondly, assess changes in mitochondrial respiration and protein abundance of mitochondrial complex proteins.**
- 3. To measure changes in protein metabolism by assessing basal protein synthesis and breakdown in c2c12 myotubes following treatment with a) EPA or DHA and b) PA or PAO using isotopic tracers. Phosphorylation of proteins involved in the mTOR signalling pathway will also be assessed. Changes in the abundance of individual proteins will be measured using a**

SILAC proteomic screen to assess differentially regulated proteins. Where appropriate changes proteins associated with membranes will also be measured using SILAC proteomics.

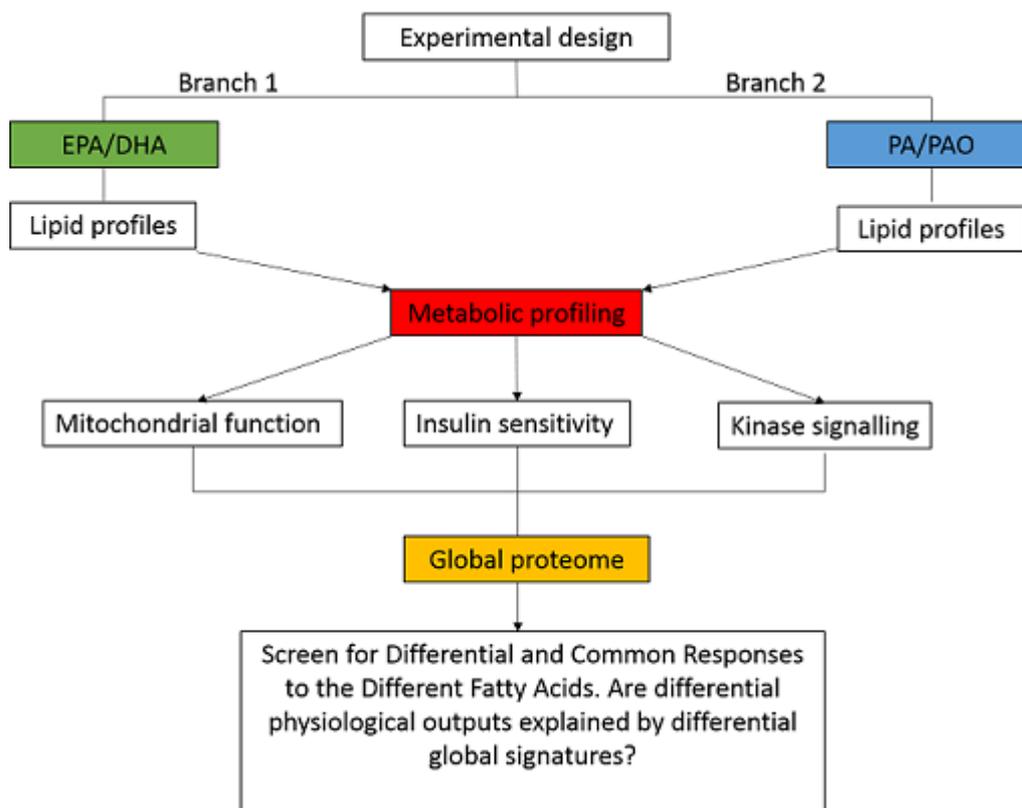


Figure 1. 3 Experimental design

CHAPTER 2: Lipid remodelling may influence the differential effects of EPA and DHA on skeletal muscle glucose uptake.

Jeromson S, Mackenzie I, Doherty MK, Whitfield PD, Dick J, Shaw A, Ashcroft S, Philp A, Galloway SD, Gallagher IJ, Hamilton DL

2.0. Abstract

There is a great deal of interest in the potential metabolic benefits of increasing omega-3 content in skeletal muscle. Studies have shown that omega-3 supplementation enhances muscle protein synthesis in response to hyperinsulinemia-hyperaminoacidemia and may improve glucose metabolism. Omega-3 fatty acids are a heterogeneous mixture of fatty acids. There is evidence that the effects of omega-3 fatty acids on skeletal muscle function and metabolism may be dependent on the specific omega-3 fatty species. EPA and DHA are the most abundant n-3 species and have differential effects on protein and glucose metabolism but the mechanisms remain unclear.

C2C12 myotubes treated with either 50 μ M EPA or DHA for 72 hours and subsequently subjected to assessment of lipid profiles, glucose uptake, mitochondria function and cell signalling. Lipid profiling of skeletal muscle myotubes revealed that EPA and DHA are substantially incorporated into the lipid pool and are directed predominantly towards phospholipid species. EPA and DHA treatment increased total omega-3 content (EPA: 951 \pm 81%, DHA: 750 \pm 5 6%) with no significant difference between groups (p=0.115). Divergent effects of EPA and DHA on individual omega-3 fatty acids were observed. EPA treatment increased EPA content (1630 \pm 23%) and DPA content (1318 \pm 199%). DHA accumulated mainly as DHA (3050 \pm 310%) with no significant changes in other omega-3 fatty acids. DHA decreased the abundance of the omega-6 species, arachidonic acid (-22 \pm 3%).

EPA increased basal glucose uptake (Con: 3.932 ± 0.206 pmol/min/mg vs EPA: 7.307 ± 0.366 pmol/min/mg) and insulin stimulated glucose uptake (Con: 7.237 ± 0.279 pmol/min/mg vs EPA: 9.996 ± 0.350 pmol/min/mg) compared to controls. DHA did not alter basal (5.041 ± 0.38 pmol/min/mg) or insulin stimulated glucose uptake (7.270 ± 0.3 pmol/min/mg). Insulin elicited a similar increase in PKB^{t308} phosphorylation following EPA or DHA treatment. This increase was not the result of increased protein abundance of GLUT1 or GLUT4 ($p > 0.05$). EPA or DHA treatment did not alter any aspect of mitochondrial respiration or abundance of respiratory complex proteins. These results show that EPA and DHA have different metabolic activities in skeletal muscle and this may be related to changes in phospholipid content.

2.1. *Introduction*

Fatty acids in skeletal muscle serve as an important substrate for energy production and play an important role in cell functions, as critical components of cell structure and regulators of signalling pathways. The lipid stores within skeletal muscle are in constant flux and are reflective of dietary intake [21]. Ectopic lipid accumulation within muscle is linked with the onset of insulin resistance [154], [211], [212]. In particular, saturated fat is causally implicated in the loss of insulin sensitivity [19], [155]. A temporal disconnect exists between the lipid accumulation in muscle and the onset of insulin resistance, suggesting there is a progressive loss of regulatory mechanisms during a high fat diet [213]. There is promising evidence to assert that the addition of polyunsaturated omega-3 lipids to the diet can protect against the decline in glucose tolerance during high fat feeding. For example, the addition of 3.4% Kcals of omega-3 lipids during a 10 week high fat diet offsets the decline in glucose control induced by high fat feeding [214]. Similar protective effects have also been observed in specific adipose tissue depots [215]. The marine derived omega-3 fatty acids eicosapentaenoic

acid (EPA 20:5) and docosahexaenoic acid (DHA 22:6) are thought to be the most bioactive omega-3 species. Supplementation of omega-3 above the recommended intake is known to enrich omega-3 content in skeletal muscle [23], [55], [59], [78].

Enrichment of EPA and DHA within skeletal muscle is known to have a number of metabolic effects. Muscle protein anabolism in response to simulated feeding is enhanced by omega-3 supplementation in both young and elderly subjects [55], [78]. Twelve weeks of omega-3 supplementation does not alter mitochondrial content but enhances ADP sensitivity [216]. While there is little consensus on the effects of omega-3 on insulin sensitivity in human models [217], there are a number of studies from rodent and tissue culture studies which support a beneficial role of omega-3 lipids in skeletal muscle glucose metabolism [83], [108], [214], [218]–[220]. A recent study in humans utilised a lipid infusion model to demonstrate that the addition of omega-3 lipids to an infusion of a lipid mixture high in omega-6 fatty acids offsets the extent of lipid induced insulin resistance [108]. This body of research highlights that the omega-3 lipid group can have both therapeutic and preventative characteristics

Despite being very similar in structure, EPA and DHA may have divergent effects on skeletal muscle metabolism. In rodents EPA, but not DHA, attenuated the decline in mitochondrial function with ageing [142]. A key study observed that in cultured myotubes EPA increased MPS in response to anabolic stimuli and reduced muscle protein breakdown (MPB) while DHA had no effect [60]. The data from the studies by Smith & colleagues (33, 34) suggest that increased mTOR-p70S6K1 signalling underpins the enhanced MPS rate in response to omega-3 supplementation, however, Kamolrat & colleagues (2013) demonstrated that EPA in cultured cells increased MPS despite P70S6k1 phosphorylation being increased by both EPA and DHA. This study

did not couple measures of protein metabolism with measures of EPA and DHA concentrations in the myotube. This precludes the ability to tell if EPA altered protein synthesis as its concentration increased to a greater extent than that of DHA. Moreover, leucine was used to stimulate protein synthesis, however, leucine may not be an appropriate stimuli to assess changes in protein synthesis [61].

Both EPA and DHA have been shown *in vitro* to down-regulate Nuclear Factor-kappa B (NF-Kb) translocation to the nucleus leading to a reduction in Muscle RING finger protein-1 (Murf-1) expression, an important mediator of muscle atrophy in cultured myotubes [62]. This effect on NF-kb is dependent on PPAR γ , as knockdown of PPAR γ abrogates the down-regulatory effect of n-3 fatty acids [63], [64]. Given that both EPA and DHA exert these metabolic effects it appears unlikely that these effects would explain the observed divergent physiological effects [64], [70].

Effects of EPA and DHA may diverge from the commonly studied mTOR pathway. Multiple laboratories have also shown that EPA increases basal glucose uptake. These studies also show that insulin stimulated glucose uptake is higher following EPA treatment but the magnitude of change is similar to control treatments. In other striated muscle models such as cardiomyocytes, EPA but not DHA increases glucose and fatty acid uptake despite similar effects on cell signalling (12). To date, whether EPA and DHA have divergent effects on glucose uptake in skeletal muscle has not been investigated. Furthermore, these studies did not assess the incorporation of EPA and DHA into the lipid pools of the muscle cell lines. Thus, it is unknown if the observed differential effects between EPA and DHA are related to different rates of accumulation within the cell. The molecular mechanisms of n-3 fatty acid action in skeletal muscle that underpin changes in glucose and protein metabolism are still poorly understood.

Given that one of the main cellular fates for fatty acids is incorporation into complex lipid species it seems logical that the differential action of EPA and DHA may be influenced by the differential effects on the cellular lipidome. To date no study has characterised the impact of EPA and DHA individually on lipidomic profiles in skeletal muscle. We therefore sought to characterize the skeletal muscle lipidome in response to EPA and DHA, assess the effects of EPA and DHA on glucose uptake and mitochondrial function. We hypothesized that EPA and DHA differentially remodel the skeletal muscle lipidome and influence downstream processes which may influence the previously observed divergent effects of EPA and DHA.

2.2. *Materials and methods*

2.2.1. *Materials*

All plastic ware for tissue culture was purchased from fisher scientific (UK). Tissue culture media and sera were purchased from Invitrogen. Fatty acids EPA and DHA (>99%, liquid form) were purchased from Sigma Aldrich (Dorset, UK). H³-DG was purchased from Hartman Analytic. All solvents were LC-MS grade (Fisher Scientific, Loughborough, UK).

2.2.2. *Cell culture*

C₂C₁₂ myoblasts were grown in Dulbecco's Modified Eagle Medium (DMEM) containing 20% foetal bovine serum and 1% penicillin/streptomycin and incubated at 37°C and 5% CO₂. Myoblasts were maintained at ~60% confluence. Differentiation was induced once confluence reached 80-90% by changing the media to differentiation media (DMEM supplemented with 2% horse serum and 1% Penicillin/Streptomycin) for 72 hours. Following 72 hours differentiation cells were treated with 50 µM EPA or 50 µM DHA conjugated to 2% fatty acid free BSA, 2% fatty acid free (FAF) BSA alone was used

a control, for 72 hours. Fatty acids were conjugated to 2% fatty acid free BSA in DM by constant agitation for 1 hour at 37°C. For lipid analysis, cell pellets were collected following 3 washes in 2% FAF BSA in PBS and centrifuged at 800 rpm for 4 minutes, excess liquid was removed and pellets were frozen in liquid nitrogen and stored at -80°C until further analysis.

2.2.3. FAME analysis

Total lipids were extracted by homogenising in 20 volumes of chloroform/methanol (2:1 v/v). Total lipids were prepared according to the method of Folch et al. [221] and non-lipid impurities were removed by washing with 0.88% (w/v) KCl. The weight of lipids was determined gravimetrically after evaporation of solvent and overnight desiccation under vacuum. Fatty acid methyl esters (FAME) were prepared by acid-catalysed transesterification of total lipids according to the method of Christie et al. Extraction and purification of FAME was performed as described by Ghioni et al. [222]. FAME were separated by gas-liquid chromatography using a ThermoFisher Trace GC 2000 (ThermoFisher, Hemel Hempstead, UK) equipped with a fused silica capillary column (ZBWax, 60m x 0.25 µm x 0.25 mm i.d.; Phenomenex, Macclesfield, UK) with hydrogen as carrier gas and using on-column injection. The temperature gradient was from 50 to 150°C at 40°C/min and then to 195°C at 1.5°C/min and finally to 220°C at 2°C/min. Individual methyl esters were identified by reference to published data (Ackman, 1980). Data were collected and processed using the Chromcard for Windows (version 2.00) computer package (Thermoquest Italia S.p.A., Milan, Italy). All experiments were carried in duplicate from 4 independent experiments. Data were represented as fold change from the respective BSA control condition, logged to log₂ and significance was determined by t-test and corrected for false discovery rate.

2.2.4. *Global lipidomic analysis of C₂C₁₂ myotubes*

Lipid extraction was performed according to the method described above. The lipids were analysed by liquid chromatography-mass spectrometry (LC-MS) using a Thermo Exactive Orbitrap mass spectrometer (Thermo Scientific, Hemel Hempstead, UK), equipped with a heated electrospray ionization probe and coupled to a Thermo Accela 1250 UHPLC system. All samples were analysed in both positive and negative ion mode over the mass to charge (m/z) range 200-2000. The samples were injected (1 µL) on to a Thermo Hypersil Gold C18 column (1.9 µm, 2.1mm x 100 mm,). Mobile phase A consisted of water containing 10 mM ammonium formate and 0.1% (v/v) formic acid. Mobile phase B consisted of 90:10 isopropanol/acetonitrile containing 10 mM ammonium formate and 0.1% (v/v) formic acid. The initial conditions for analysis were 65%A/35%B. The percentage of mobile phase B was increased to 100% over 10 min and held for 7 minutes before re-equilibration with the starting conditions for 4 minutes. The raw LC-MS data were processed with Progenesis QI v2.0 software (Non-linear Dynamics, Newcastle, UK) and searched against LIPID MAPS (www.lipidmaps.org) and the Human Metabolome Database (<http://www.hmdb.ca/>) for identification. All experiments were carried out in duplicate from three independent experiments.

2.2.5. *Phospholipid profiling of C₂C₁₂ myotubes*

In order to assess the incorporation of EPA and DHA into cellular phospholipids the lipid extracts from C₂C₁₂ myotubes were analysed by electrospray ionisation-tandem mass spectrometry (ESI-MS/MS). All analyses were performed in positive ion mode using a Thermo TSQ Quantum Ultra triple quadrupole mass spectrometer equipped with a heated electrospray ionization probe. Samples were directly infused into the ion source at a flow rate of 5 µL/min. Phosphatidylcholine and lysophosphatdilycholine

species were identified by precursor scanning for m/z 184 and phosphatidylethanolamine and phosphatidylethanolamine species were identified by neutral loss scanning for m/z 141. The data were expressed as a percentage composition of the relevant phospholipid fraction.

2.2.6. *Glucose uptake*

C₂C₁₂ myotubes were exposed to 50 μ M EPA or 50 μ M DHA for 48 h before a 2 h serum-starve. Following the 2 h serum starve cells were exposed to insulin (100 nmol/l) or vehicle control for 30 mins. Myotubes were incubated (12 min) with 10 μ mol/l 2-deoxy-D-[3 H]glucose (2DG; 24.4 kBq/ml; Hartman Analytic) at 20°C. Non-specific uptake was determined using 10 μ mol/l cytochalasin B (Sigma–Aldrich). After lysis, cell-associated radioactivity was measured (Beckman, High Wycombe, UK; LS 6000IC scintillation counter), and protein was quantified using the Bradford protein assay. Data represented are the average of six independent experiments carried out in duplicate.

2.2.7. *Cellular respiration.*

Cells were seeded in Xfe24-well cell culture microplates (Seahorse Bioscience, North Billerica, MA) at 1.0×10^4 cells/well in 100 μ l of growth medium (high glucose DMEM, 10% FBS, 1% penicillin/streptomycin) and allowed to rest at room temperature for 1 h in order to promote an even cell distribution. Cells were then incubated at 37°C and 5% CO₂ for 3 h after which 150 μ l of growth medium was added bringing the total volume to 250 μ l. Cells were then returned to the incubator for a further 48 h. Upon reaching 90% confluence cells were then differentiated for 4 days using differentiation medium (high glucose DMEM, 2% horse serum, 1% penicillin/streptomycin). Differentiation medium was replenished every other day. Following 4 days of

differentiation cells were treated for 24 h with differentiation medium containing 50 μ M of either DHA, DPA, EPA or a 2% BSA control (n=5). Prior to the assay the cells were washed once and placed in 600 μ l of Seahorse XF Base Medium (glucose 25 mM, sodium pyruvate 1 mM, glutamine 2 mM, pH 7.4) pre warmed to 37°C. The plate was then transferred to a non-CO₂ incubator for 1 hour. Following calibration oxygen consumption measurements (OCR) were performed following the sequential addition of oligomycin (1 μ M), carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (3 μ M) and antimycin A/rotenone (1 μ M). Upon completion of the assay cells were collected in lysis buffer (50 mM Tris pH 7.5; 270 mM sucrose; 1 mM EDTA; 1 mM EGTA; 1% Triton X-100; 50 mM sodium fluoride; 5 mM sodium pyrophosphate decahydrate; 25 mM beta-glycerolphosphate; 0.4% protease inhibitor cocktail; 0.4% phosphatase inhibitor cocktail; 4.8% complete™ protease inhibitor cocktail EDTA free) and centrifuged for 10 min at 8,000 *g* and the supernatant was removed for protein determination. Protein concentration was determined using the DC protein assay (Bio-Rad, Hercules, CA). Oxygen consumption rate (OCR) is reported relative to protein content (pmol/min/ μ g).

2.2.8. Cell processing

Cell lysates were collected from 6 well plates by scraping on ice in RIPA buffer [50 mmol/l Tris·HCl pH 7.5, 50 mmol/l NaF, 500 mmol/l NaCl, 1 mmol/l sodium vanadate, 1 mmol/l EDTA, 1% (vol/vol) Triton X-100, 5 mmol/l sodium pyrophosphate, 0.27 mmol/l sucrose, and 0.1% (vol/vol) 2-mercaptoethanol and Complete protease inhibitor cocktail (Roche)] followed by snap freezing on liquid nitrogen. For preparation for western blotting samples were thawed and debris was removed by centrifugation at 4°C for 15 min at 13,000 *g*. The supernatant was then removed, and

protein concentration was determined using the DC protein assay according to the manufacturer's instructions (Bio-Rad, Hercules, CA).

2.2.9. *Western blotting*

For WB, 100 µg of supernatant was made up in Lamelli sample buffer, and 15 µg of total protein was loaded per well and run at 150 V for 1 h 15 min. Proteins were then transferred onto Whatman Immobilon Nitrocellulose membranes (Fisher Scientific, Loughborough, UK) at 30 V for 2 h 15 min. Membranes were blocked in 3% BSA-Tris-buffered saline (containing vol/vol 0.1% Tween 20) for 1 h at room temperature, followed by incubation in primary antibodies [PKBthr308 (#2965), total PKB (#4691) (New England Biolabs)], Glut1, Glut4 [Santa cruz biotech], Hekokinase1, Hekokinase2 [Cell signalling] or OXPHOS mitoprofile at 4°C overnight. Membranes underwent three 5 min washes in TBST followed by incubation in the appropriate secondary antibodies [secondary horseradish peroxidase conjugated antibody was purchased from ABCAM (#6721)] for 1 h at room temperature. Membranes were again washed three times for 5 min followed by incubation in enhanced diaminobenzidine tetrahydrochloride reagent (BioRad, Herts, UK). A BioRad ChemiDoc (Herts, UK) was used to visualize and quantify protein expression. Phospho proteins were normalized to the corresponding total protein. Data are representative of 3 independent experiments carried out in duplicate.

2.2.10. *Statistics*

Statistical analyses were carried out in Graphpad Prism. Glucose uptake was analysed with a two-way ANOVA followed by Tukey's HSD test. A two-way ANOVA was used to determine the success of manipulating mitochondrial respiration. Differences in mitochondrial states between groups were assessed by a one-way ANOVA. For FAME

analyses data were assessed in R statistical packages using multiple t-tests and corrected by false discovery rate. Statistical significance was determined with a $p < 0.05$. The global lipidomic data sets were subjected to principal component analysis (PCA) and orthogonal projection latent structure-discriminant analysis (OPLS-DA) using SIMCA-P v13.0 software (Umetrics, Umea, Sweden).

2.3. Results

2.3.1. EPA and DHA substantially increase the abundance of omega-3 species but have different effects on individual omega-3 fatty acids

After exposure to 50 μ M EPA or DHA for 72 hrs C₂C₁₂ myotubes were collected for FAME analysis to determine lipid profiles. EPA and DHA significantly increased omega-3 fatty acid content from baseline values (EPA; $951 \pm 81\%$ $p=0.0014$, DHA; $750 \pm 56\%$ $p=0.0009$) with no significant difference detected between EPA and DHA treatment ($p=0.115$) (**Figure 2.1A**). The changes in omega-3 abundance are a result of differential shifts in specific omega-3 fatty acids caused by EPA or DHA treatment. The increase in omega-3 content by DHA is a result of accumulation of mainly DHA [22:6 n-3, $3050\% \pm 310\%$. While, incubation of myotubes with EPA results in the accumulation of EPA [(20:5 n-3), $1630\% \pm 23.38$] and DPA [(22:5 n-3), $1318\% \pm 199.8\%$] (**Figure 2.1B**). These data suggest that EPA is elongated to DPA whilst DHA remains largely unmodified.

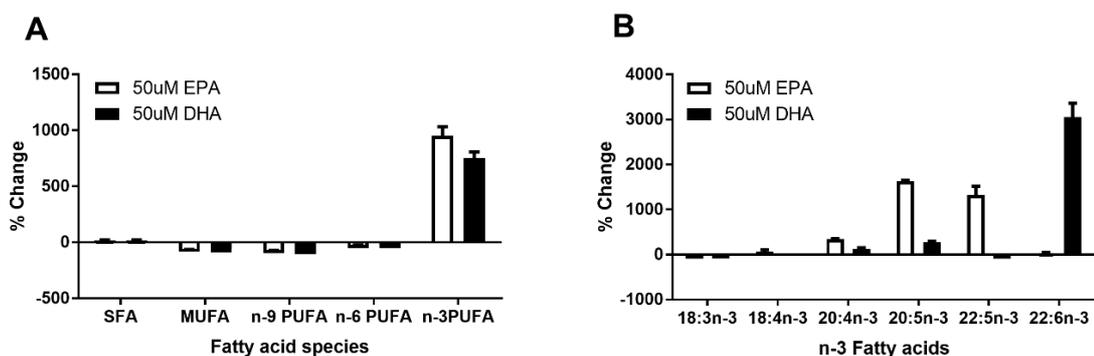


Figure 2. 1 EPA and DHA enhance n-3 abundance but differentially alter the abundance of individual omega-3 fatty acids. A) Fatty acid abundance following 72 hr treatment with either EPA or DHA determined by FAME analysis (n=4 in duplicate). B) Individual changes in omega-3 fatty acids (n=4 in duplicate). Data is plotted as mean value \pm S.E. * denotes significant difference between EPA and DHA groups ($p < 0.005$).

2.3.2. EPA and DHA divergently alter myotube lipid profiles

Upon analysis of global lipid profiles, it is evident that both EPA and DHA alter the abundance of individual fatty acids as these changes are not exclusive to n-3 fatty acids. This increase in omega-3 content was associated with significant decreases in multiple species of monounsaturated fatty acids, omega-6 and omega-9 fatty acids, with a trend for increases in saturated fatty acids (**Figures 2.1A and 2.2**). In order to clearly distinguish differential shifts in fatty acid abundance we presented the complete fatty acid profiles as fold change $[(\log_2)$ **Figure 2.2**]. The most clearly differentiated fatty acid is DPA (22:5 n-3) which demonstrates a significant $1318 \pm 200\%$ increase with EPA treatment whilst DHA treatment induces a $17.83 \pm 17.37\%$ decrease in DPA content, although this was not a statistically significant effect. Additionally there is a trend ($p=0.06$) for EPA treatment to increase the DHA content of the cells ($37.11 \pm 12.62\%$) suggesting that only a small proportion of the EPA is converted to DHA. Surprisingly arachidonic acid (20:4 n-6) was only significantly decreased by DHA treatment ($-22.35 \pm 3.174\%$) and remained unaffected by EPA treatment. Intriguingly we also observed that both EPA and DHA increased the content of the saturated fatty acid PA (**Fig 2.2**). In order to build a more complete picture of the impact of EPA and DHA on the lipidome we proceeded with a global lipidomics assessment.

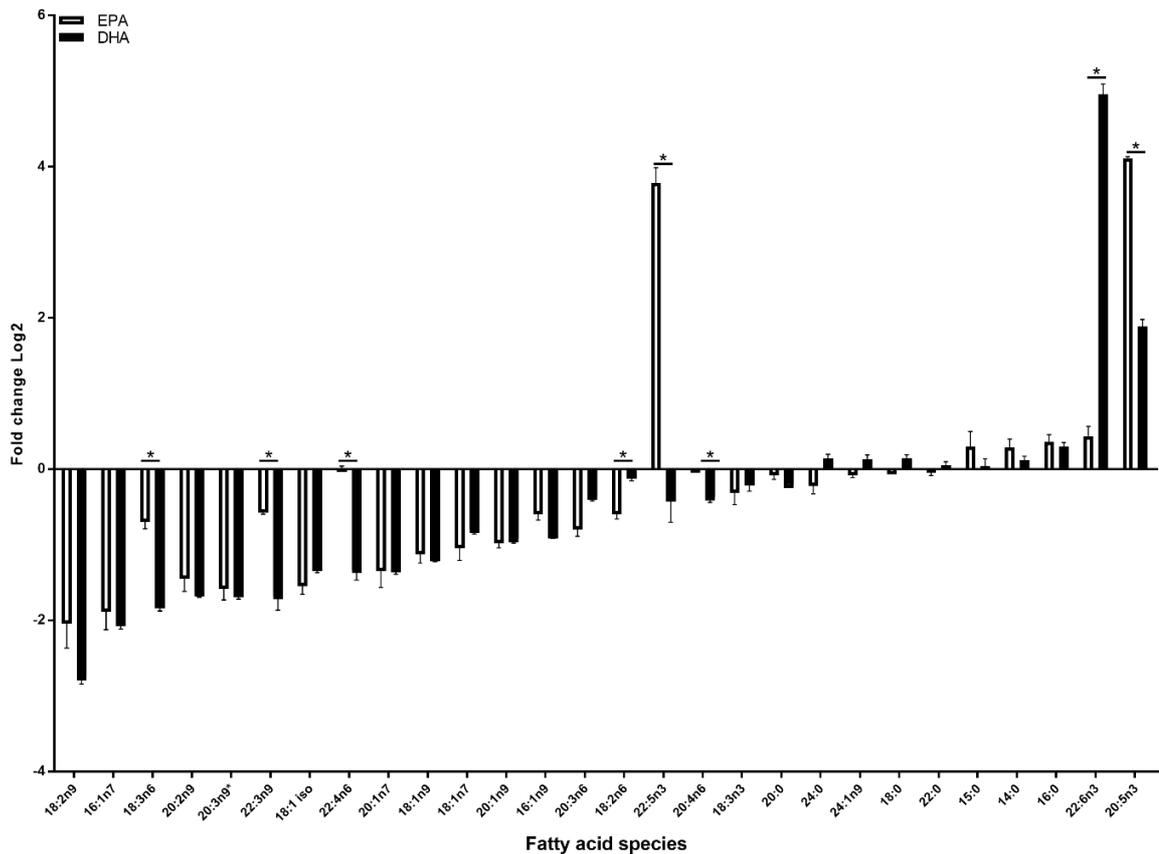


Figure 2. 2 Lipid alterations in response to EPA and DHA are not limited to omega-3 fatty acids. Fold change of individual fatty acid species (n=4 in duplicate). Data are expressed as log₂ fold change \pm S.E.

2.3.3. EPA enhances basal and insulin stimulated glucose uptake independent of increased PKB phosphorylation

Basal and insulin stimulated glucose uptake was determined by 2DG uptake. EPA incorporation significantly enhances both basal and insulin stimulated 2DG uptake indicating that EPA enhances the capacity for glucose uptake ($p < 0.05$) (**Figure 2.3A**). DHA did not have any significant effects on 2DG uptake (**Figure 2.3A**). Simultaneous treatment with both EPA and DHA displayed similar effects to EPA alone. The data suggest that there are specific characteristics of EPA that enhance the capacity glucose uptake. The observed changes in glucose uptake was not related to changes in PKB phosphorylation as basal and insulin stimulated PKB phosphorylation were similar across groups (**Figure 2.3B**). Despite insulin stimulated glucose uptake being higher following EPA treatment, the magnitude of change from basal levels was not

significantly different between groups. This suggest that the insulin sensitive signalling response leading to GLUT4 translocation to the membrane remained unchanged following fatty acid treatment.

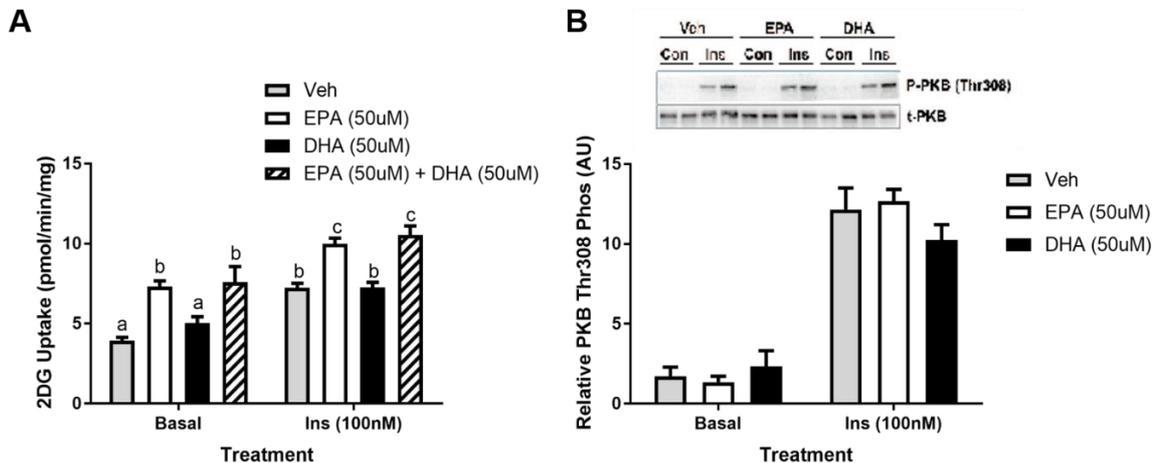


Figure 2.3 EPA and DHA have differential effects on muscle glucose uptake despite similar PKB signalling. A) Basal and insulin stimulated glucose uptake was measured by 2DG uptake. B) PKB phosphorylation was determined by western blots. Data are expressed as mean \pm S.E (n=6 in duplicate)

2.3.4. EPA and DHA do not alter the protein abundance of glucose transporters or hexokinase

To further investigate the underlying mechanism behind the observed changes in glucose uptake, we assessed if n-3 fatty acid treatment altered the abundance of proteins involved in glucose transport and metabolism. Neither fatty acid treatment altered the protein abundance of glucose transporters GLUT1 and GLUT4 ($p>0.05$) (**Figure 2.4A and B**). These measures do not, however, rule out the possibility of changes in endo/exocytic cycling of glucose transporters with the plasma membrane. Upon uptake glucose is phosphorylated to glucose-6-phosphate by hexokinase. Hexokinase is thought to be constitutively active so changes in total abundance may reflect an increased capacity to process glucose, thus maintaining low intracellular glucose levels. Treatment with EPA or DHA did not alter the abundance of Hexokinase 1 or 2 ($p>0.05$) (**Figure 2.4C and D**).

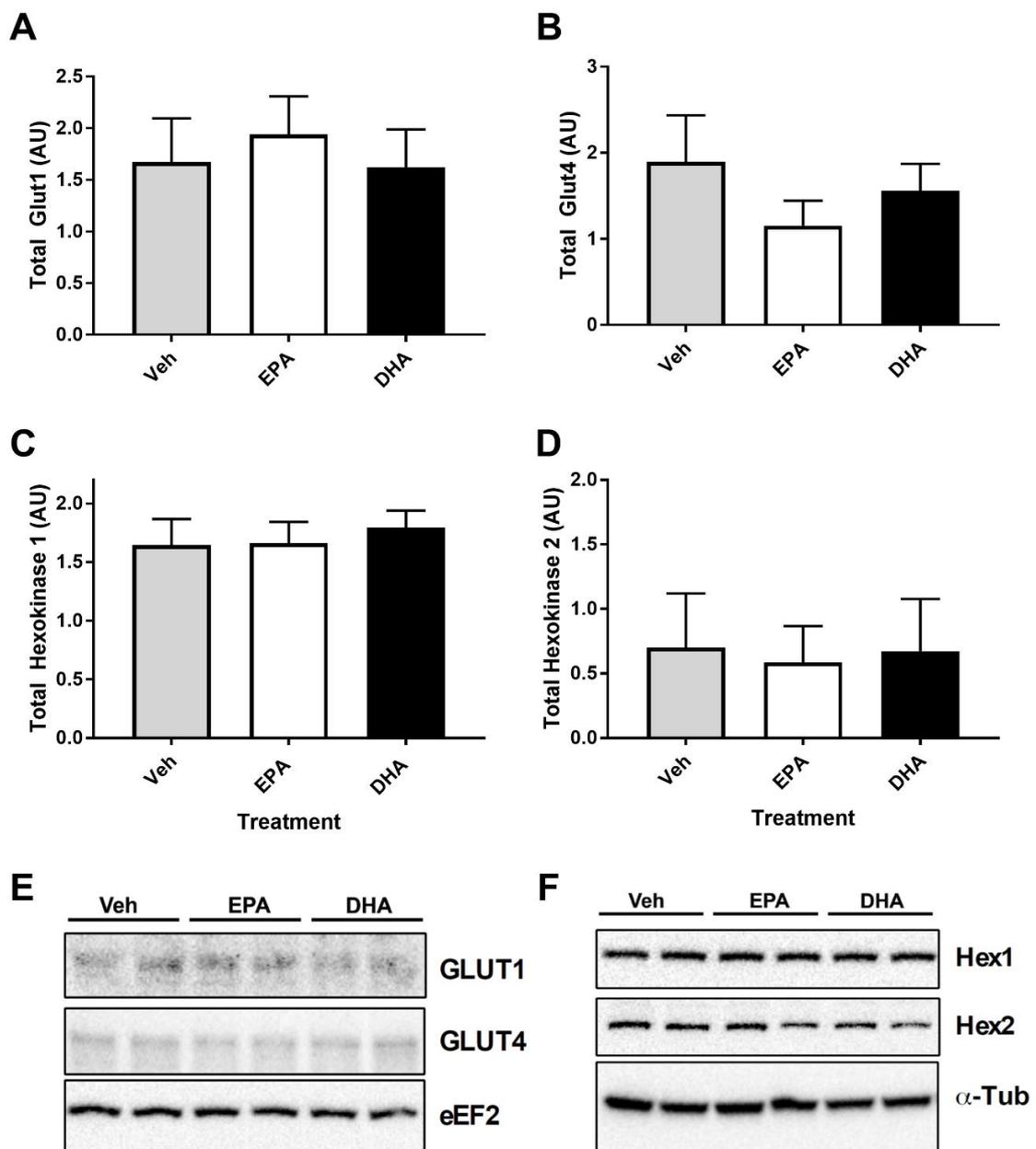


Figure 2.4 EPA and DHA do not alter total protein abundance of glucose transporters and hexokinase 1 and 2. Protein content of A) Glut1 B) Glut4 C) Hexokinase 1 and D) Hexokinase2 were measured by western blotting. Representative images are shown in E) glucose transporters and F) Hexokinases. Data are presented as mean \pm S.E (n=3 in duplicate).

2.3.5. *N-3 fatty acids do not alter mitochondrial respiration*

As no changes in glucose transporters were detected we next assessed whether changes in mitochondrial oxygen consumption may explain the increase in glucose uptake following EPA treatment. C₂C₁₂ myotubes were treated with 50 μ M EPA or DHA for 24 hours. Following treatment with multiple inhibitors/uncouplers (Oligomycin,

FCCP, rotenone/ 61 ntimycin A) were used to probe various parameters of mitochondrial function using the seahorse XF mito stress test. A two way ANOVA found a significant interaction between oxygen consumption and inhibitor compound, indicating the successful manipulation of mitochondrial function. Following fatty acid treatment no changes in cellular oxygen consumption were observed in any parameter measured (**Figure 2.5**). There is evidence to suggest that fatty acids can induce mitochondrial uncoupling, however, the current doses used in this study may not have been sufficient to observe this effect. Consistent with the lack of changes in mitochondrial function, there were no alterations in the abundance of ATP synthase and UCQR2 (Complex V and Complex III respectively) (**Figure 2.6**).

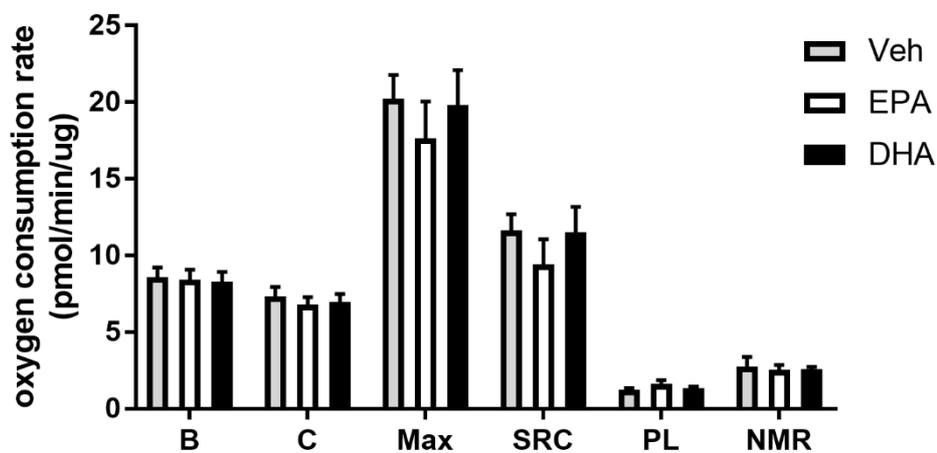


Figure 2. 5 N-3 fatty acids do not alter mitochondrial oxygen consumption. Mitochondrial function was assessed following treatment with 50 μ M EPA or DHA for 24 hours. B – baseline, C- coupled, Max – maximal respiration, proton leak, NMR – non mitochondrial respiration. Data are expressed as pmol of oxygen consumed/min/mg of protein.

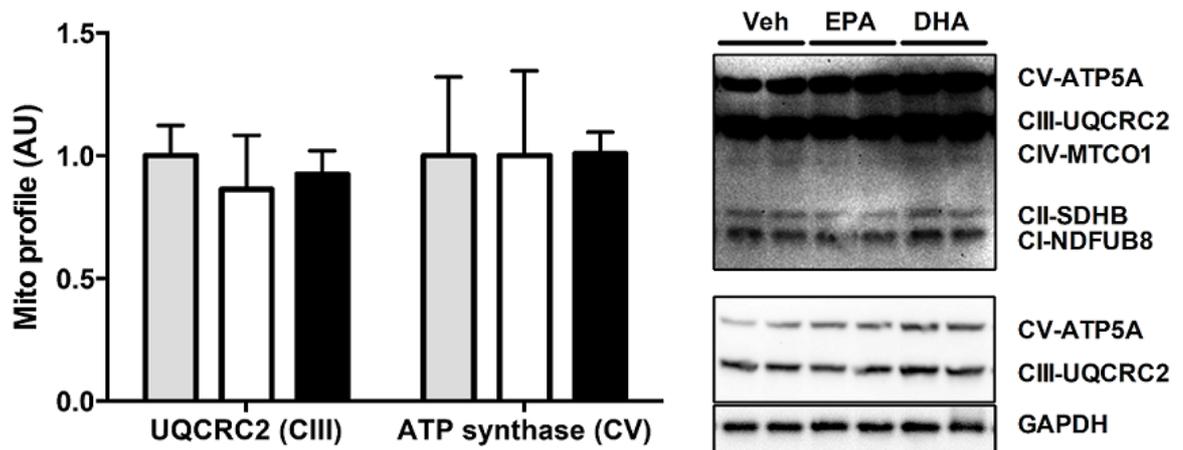


Figure 2. 6 Omega-3 treatment does not alter the abundance of proteins involved in the electron transport complex. Protein abundance of proteins in the ETC were measured by western blot. A) relative abundance of ETC proteins. B) Representative western blot images. Upper panel – long exposure. Lower panel – short exposure. Data is presented as mean values relative to a loading control (n=3 in duplicate).

2.3.6. Global lipidomic analysis reveals that EPA and DHA transition into phospholipids and divergently remodel the phospholipidome

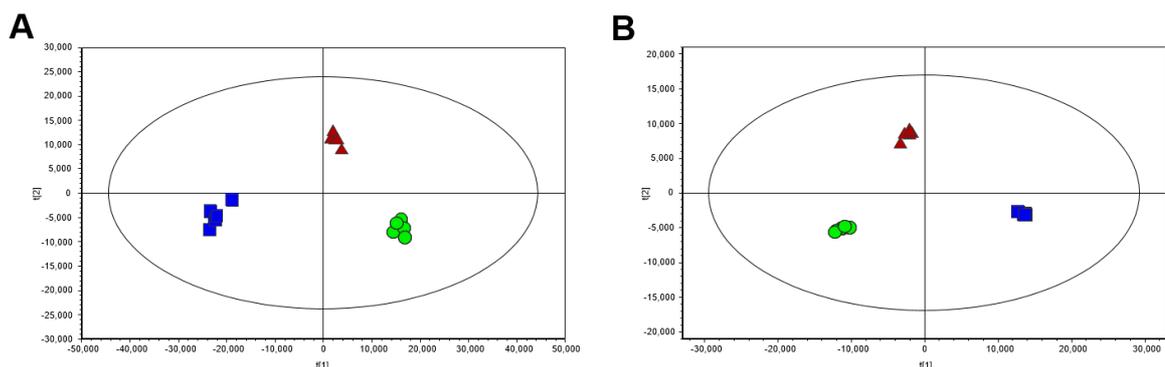


Figure 2. 7 PCA scores plots of lipid profiles generated by LC-MS in (A) positive ion and (B) negative ion modes. Cultured cells were incubated with either fatty acid free 2% BSA (green circles), fatty acid free 2% BSA pre-conjugated to 50 μ M EPA (red triangles) or 50 μ M DHA (blue squares) for 72hrs. Each point represents a single cell sample (n=6). Samples exhibiting biochemical differences caused by fatty acid treatment are represented by points in regions away from the controls.

Given that the mechanism underlying the EPA driven changes in glucose uptake was not readily apparent we assessed how the remodelling of the lipidome by fatty acid treatment may influence the observed results. Lipid extracts of myotubes treated with BSA (control), EPA or DHA were analysed by LC-MS in positive and negative ion modes, processed and subjected to multivariate data analysis. PCA of both the positive and negative ion data sets revealed that EPA and DHA supplementation caused a substantial divergence in the lipidome, effectively segregating control and treated cells

(**Fig. 7A and 7B**). Having established the existence of clustering behaviour between the sample cohorts more multivariate methods were used to characterise the specific lipid changes responsible for the observed shift in the lipidome of the EPA and DHA treated myotubes. The OPLS-DA scores and OPLS loadings 'S' curves of the positive ion data are shown in **Fig. 2.8A and 2.8B**. The results indicated that the observed variance between samples were best characterised by changes in the fatty acids contained in phospholipids, in particular PC and PE fractions. In EPA treated cells elevations in PC and PE species containing both 20:5 and 22:5 fatty acids (in agreement with FAME analysis) were observed, whereas there were relative increases in the abundance of phospholipid species with a 22:6 fatty acid in DHA treated cells. Interestingly, the analysis also revealed that both EPA and DHA treatments also resulted in an elevation of PC 32:0, a saturated species. DHA was also found to be incorporated into a number of triglyceride species. In order to fully understand the impact of EPA and DHA treatment we followed up these experiments through the targeted analysis of myotube phospholipid fractions.

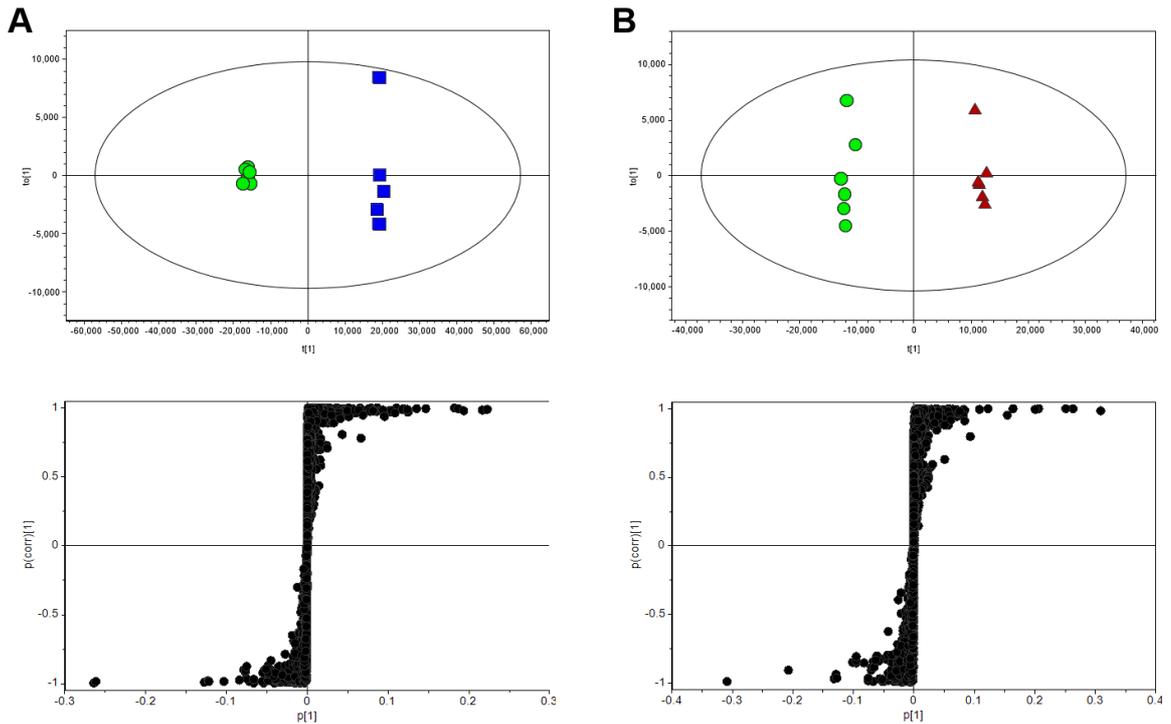


Figure 2. 8. Positive ion OPLS-DA scores plots for (A) BSA vs DHA and (B) BSA vs EPA (n=6). Green circles indicate BSA treated C_2C_{12} myotubes; blue squares indicate DHA treated C_2C_{12} myotubes and red triangles indicate EPA treated C_2C_{12} myotubes. The associated S-plots demonstrate the covariance versus the correlation in conjunction with the variable trend plots and allow easier visualization of the data. The variables that showed maximum change and, therefore strongly contributed to the class separation, are plotted at the top or bottom of the S-shaped plot. The variables that did not significantly vary are plotted in the middle. Each point represents a detected ion.

2.3.7. Targeted phospholipid analysis reveals that EPA and DHA increase the fraction of lipid species containing long chains and 5 or more double bonds at the expense of shorter chain less saturated species.

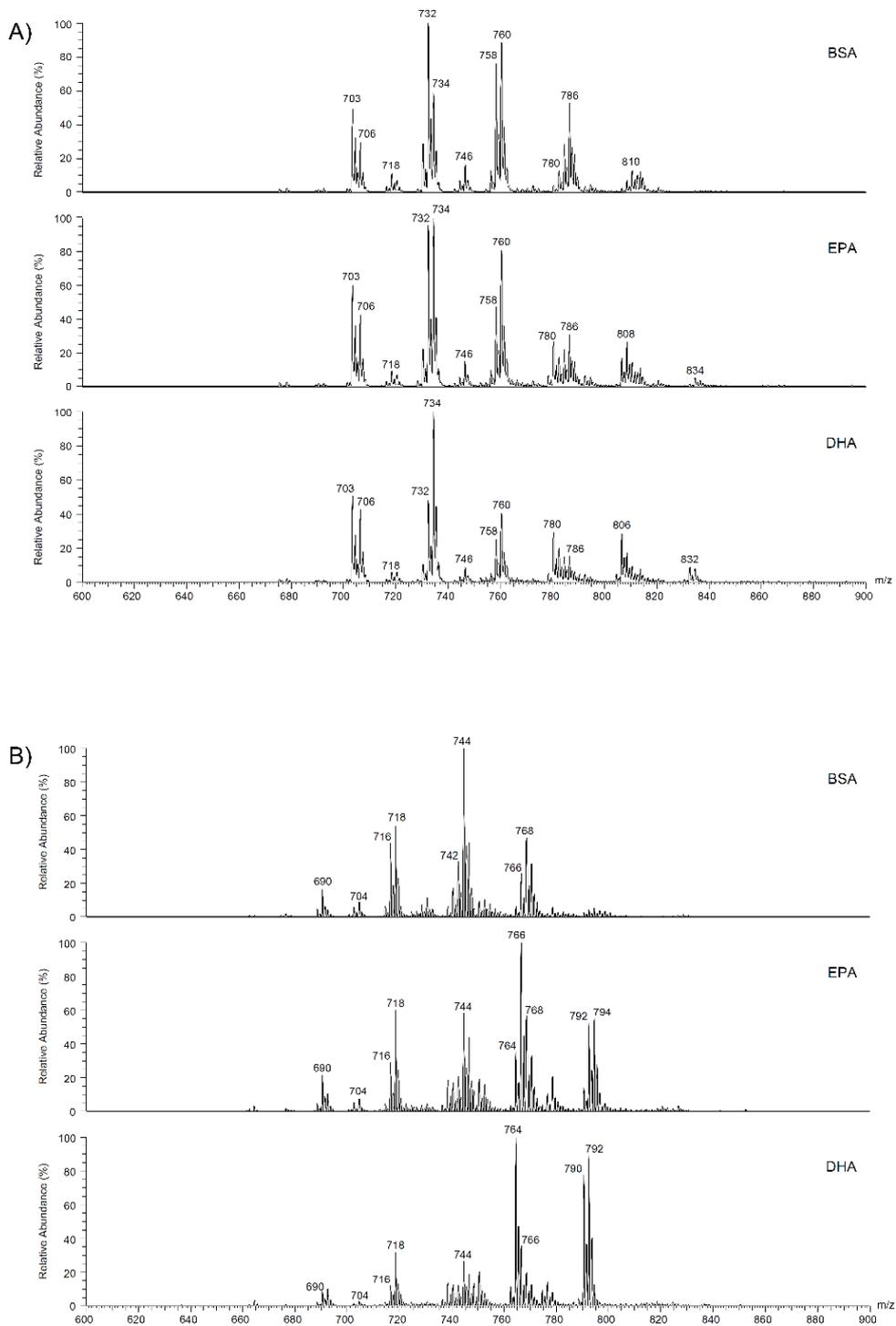


Figure 2. 9 Targeted phospholipid analysis indicates preferential incorporation of long chain polyunsaturated fatty acids into specific phospholipid classes. C₂C₁₂ myotubes were solvent extracted and molecular species of (A) phosphatidylcholine and (B) phosphatidylethanolamines were detected by ESI-MS/MS in positive-ion mode by means of a precursor ion scan for m/z 184 and a neutral loss scan of m/z 141 respectively

ESI-MS/MS was utilised to characterise the profiles of myocyte phospholipid classes. Representative mass spectra of PC and PE are shown in **Figure 2.9A and B**. The general trends (**Figure 2.10**) in both EPA and DHA were similar with the displacement of shorter chain in apparent preference for longer chain highly unsaturated fatty acids. However, the magnitude of change for these displacements was often higher with DHA. EPA, DPA and DHA were also identified as being incorporated into the PS and PI fraction in a similar fashion. Caution should be taken when interpreting the lipid remodelling in PS and PI fractions as these fractions comprise a very small proportion of phospholipids. As a result, the ion intensities detected of PS and PI species are lower and subsequent results are less robust. Interestingly, EPA and DHA induced increases in species containing saturated fatty acids. For instance DHA induced a differential increase in the lysophosphatidylethanolamine (LPE 16:0), which contains a saturated fatty acid. Whilst EPA induced an increase in LPC 16:0, 18:0 and LPE 18:0 (**For lyso-phospholid analysis, see supplementary figure S1**). It is therefore evident that in spite of significant increases in saturated fatty acids in these fractions EPA and DHA treatment still improve or maintain glucose uptake respectively. Overall, the trends directional shift towards either greater unsaturation or saturation of phospholipid classes were similar between EPA and DHA. In PC, there was an increased shift towards increased saturation of lipids. In PE species there was a decrease in saturation of phospholipids. Similarly, in PE the shift in unsaturated phospholipids was greater than the change in unsaturated PC phospholipids. Although overall trends were similar, differences emerged between EPA and DHA groups when individual phospholipids were analysed.

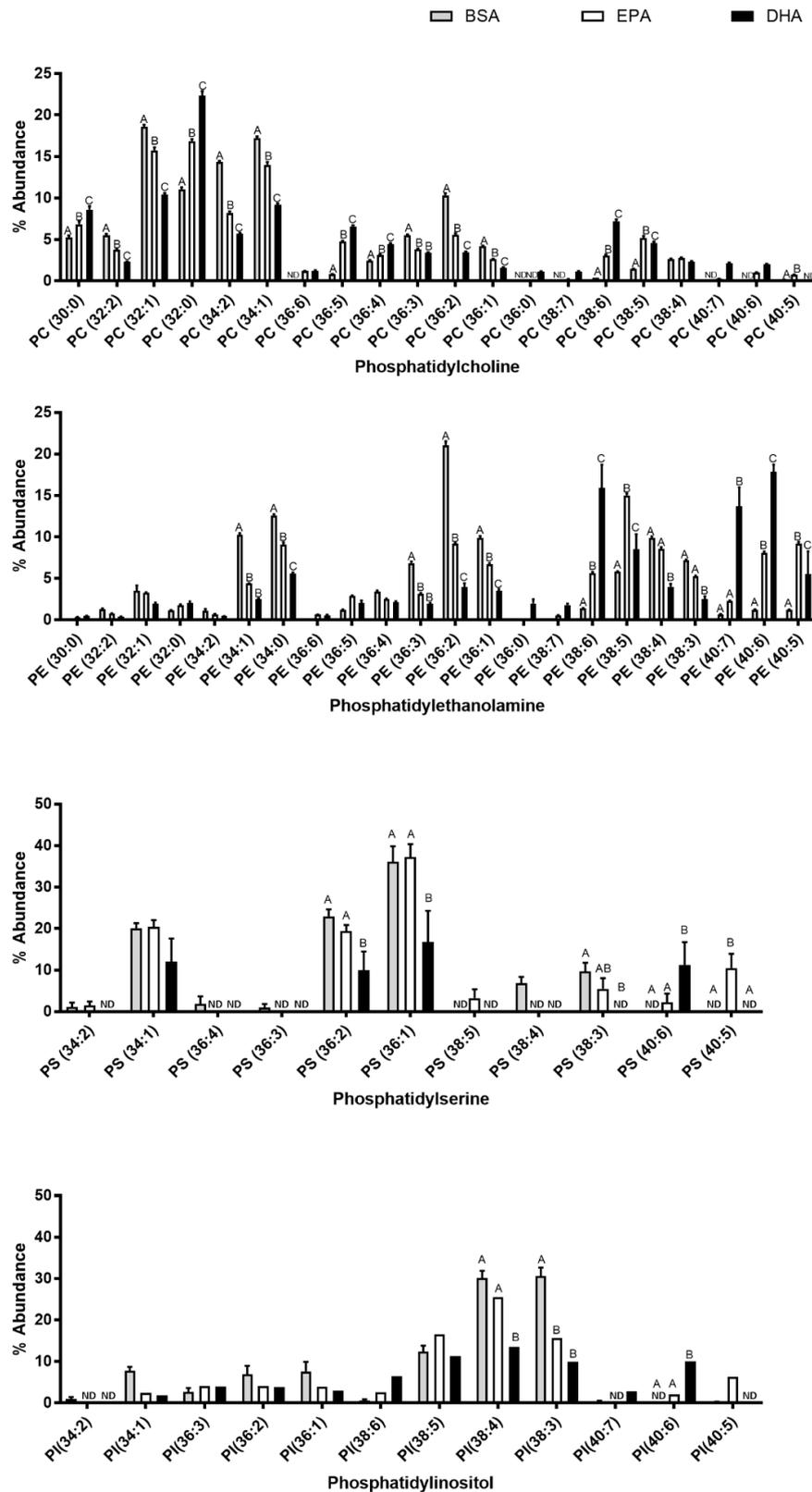


Figure 2. 10 EPA and DHA cause significant remodelling of phospholipid species. C₂C₁₂ myotubes were solvent extracted and molecular species of (A) lyso-phosphatidylcholine, (B) phosphatidylcholine, (C) lyso-phosphatidylethanolamine and (D) phosphatidylethanolamine were detected by ESI-MS/MS in positive-ion mode by means of a precursor ion scan for m/z 184 and a neutral loss scan of m/z 141 respectively. Data are presented as % abundance \pm SEM. Bars not connected by the same letter are significantly different from one another ($p < 0.05$).

2.4. Discussion

This study is the first to carry out a comprehensive analysis of the lipidomic profiles of a skeletal muscle cell line in response to two differentially bioactive n-3 fatty acids. We demonstrate the differential metabolic activities of EPA vs DHA on glucose metabolism in the C2C12 skeletal muscle cell line and provide data demonstrating the differential impact that EPA and DHA have on the skeletal muscle lipidome. Our data suggests that the bioactivity of EPA may be due to its preferential incorporation (and possibly elongation to DPA) into the phospholipid fraction where it substantially alters the long chain polyunsaturated fatty acid composition of major phospholipid classes. Increasing unsaturation of phospholipids will alter membrane organisation by maintain the membrane in a more liquid like state, increasing membrane fluidity and deformation.

EPA has a positive effect on both basal and insulin stimulated muscle glucose transport while DHA shows no significant effect. The fold change in glucose uptake from the basal to the insulin stimulated state was similar between all groups. This finding suggests that insulin response was not altered by fatty acid treatment but the mechanisms by which EPA increased glucose uptake were maintained in response to insulin. In line with this finding, PKB phosphorylation was unchanged in response to insulin by either EPA or DHA. Thus the changes in glucose uptake following EPA treatment may be independent of PKB signalling. The change in glucose uptake was also not reflected in changes in protein abundance of GLUT1, GLUT4 and hexokinase proteins involved in glucose uptake and subsequent committal to metabolic pathways. Although we did not detect any changes in total content, changes in the activity of GLUT1 and GLUT4 cannot be discounted. Moreover, we did not assess GLUT1 or GLUT4 subcellular localisation. The shift in phospholipid composition induced by EPA may have sequestered GLUT1

or GLUT4 to the membrane or altered their pore forming activity. Hexokinases are considered constitutively active so it is unlikely there were any changes in kinase activity. Furthermore, the significant remodelling of phospholipid may alter protein localisation within the cell. Alterations which bring Hexokinases within a closer vicinity to GLUT proteins may enhance the “functional coupling” of the system to sequester glucose within the cell.

We next assessed whether changes in mitochondrial function may drive increased substrate uptake. *Johnson & colleagues* [142] demonstrated that EPA but not DHA prevented the declines in mitochondrial functions in an aged rodent model. In our cultured myotube model, we did not detect any changes in mitochondrial oxygen consumption.

We hypothesised that the effects of EPA compared to DHA may be explained by differential remodelling of the lipidome. By coupling measures of lipid profiles with glucose uptake, we demonstrated that EPA increased glucose uptake and accumulated to a lesser extent than DHA. While total n-3 content was similar between treatments, EPA resulted in a larger variation in lipid species accumulating mainly as EPA and DPA and to a lesser extent DHA while DHA treatment mainly resulted in DHA accumulation with a decrease in DPA and a limited retro-conversion to EPA. The main differentially regulated fatty acids were DPA, increased in EPA and decreased in DHA, while only DHA decreased AA. It is speculated, that some of the beneficial effects of omega-3 are caused by the displacement of AA and concomitant reductions in PGF2 α production. Our data suggests that in terms of glucose uptake, changes in AA content are not required.

Interestingly DPA accumulated to a similar extent as EPA [1630% (EPA) vs 1318% (DPA)], findings consistent with previous literature in other tissues [223], [224]. Given that DPA increased to a similar extent to EPA we are unable to determine whether it is EPA or DPA that is a driver behind the metabolic effects observed. These data suggest that upon intake into the cell EPA is elongated to DPA and to a lesser extent DHA. The elongation of EPA to DPA but not DHA may be explained by the differential affinities of the desaturases and elongases involved in fatty acid metabolism. In the n-3 pathway *elovl2* catalyzes the conversion of EPA → DPA → 24:5 n-3, the precursor to DHA. However, increasing EPA concentrations is known to lower the saturation point in the conversion of DPA → 24:5 n-3 which may play a role the accumulation of DPA without being further metabolised to DHA [225]. Our data indicate that one of the primary fates of EPA and DHA was incorporation into the phospholipid fraction. In the global lipidomics screen we found EPA or DPA containing lipid species associated with the phospholipid pool whilst DHA containing species were often associated with the TAG pool, EPA containing species were rarely associated with the TAG pool. Fatty acids in the TAG pool are stored in discrete lipid droplets and therefore may be less metabolically active than the phospholipids associated with the membranes. This differential incorporation into the various lipid pools may partially explain the beneficial metabolic effects of EPA.

The potential relevance of DPA as a mediator of many of the physiological effects of n-3 supplementation is beginning to be further understood. DPA more potently inhibits platelet aggregation than EPA or DHA [226] as well as more potently stimulating endothelial cell migration than EPA or DHA [227]. In macrophages, EPA is a known inhibitor of the cyclooxygenase pathway and elongation to DPA is an important factor

in this inhibition (29). We would suggest that the elongation of EPA to DPA seen in our study may also have important physiological roles in the increase in skeletal muscle glucose transport by EPA. As with the global lipidomic analysis we observed the incorporation of long chain PUFAs into phospholipid species mainly at the expense of specific SFAs and MUFAs, however some specific SFAs were increased by both EPA and DHA. We identified multiple differentially regulated phospholipid species across PE, PC, LPE and LPC classes. In the EPA treated group a number of species were enriched by long chain PUFAs with 5 or more double bonds in addition to a number of EPA or DPA containing phospholipids in the PC, PE, PI and PS fractions. In comparison, the DHA treated group increased the long chain PUFA containing phospholipids but did not increase or increase as much as EPA the content of species containing 22:5 or 40:5. Interestingly, with DHA treatment there was a trend for an increase in the PA containing lyso-PE content above that of control and EPA. Additionally, while it appears that many phospholipids containing saturated fatty acids are displaced in favour of polyunsaturated containing phospholipids we observe a rise in PC (32:0) with both EPA and DHA, which may reflect the rise in PA observed with FAME analysis. Incorporation of PUFAs into phospholipids is known to increase membrane fluidity and we speculate that this increase may be a compensatory mechanism to maintain a base level of membrane rigidity. A minimum level of membrane rigidity is necessary To ensure a barrier is maintained around the cell and regulate the appropriate regulation of molecules crossing the membrane.

An attractive mechanism for the differential effects of EPA and DHA may lie in the reduced production of different inflammatory eicosanoids. However, only DHA reduced the total amount of AA. Additionally, the displacement of phospholipids alongside a lack of inflammatory stimulus suggests it is unlikely that this would

mediate the metabolic differences seen in this model. The G protein coupled receptor GPR120 has previously been identified as a general n-3 sensor in a number of tissues except skeletal muscle that elicits potent anti-inflammatory and consequently insulin sensitizing effects [219]. To our knowledge no such receptor exists in skeletal muscle that can discriminate between EPA and DHA deeming it unlikely that the observed differential effects are mediated by EPA or DHA through specific receptor activated signalling.

Different phospholipid species are not merely inert structural components of cellular membranes and the various roles in intracellular processes are beginning to be further understood. The significant unsaturation of phospholipids will alter the membrane structural characteristics and alter the affinities of proteins associated with the membrane. We hypothesize that the incorporation of EPA into the lipid pool and subsequent incorporation into the phospholipid fraction alters the physical characteristic of the membrane and the distribution of proteins associated with the membrane which may explain the increases in glucose uptake. PS and PE are related phospholipid species found predominantly in the inner membrane and contribute to the membrane targeting and activation and modification of protein kinases [as reviewed [228]]. It has also previously been seen that the lyso-PC, a hydrolyzed form of phospholipid, stimulated adipocyte glucose uptake and with the effect dependent upon chain length and saturation of the acyl group [229]. We observed an incorporation of long chain PUFAs (possibly EPA and DPA) into the PC, PS, PE and certain lyso-PL species and therefore cannot discount that the change in acyl chain length and unsaturation level alters the function of these PL species and leads to an increase in glucose uptake through a currently unknown mechanism.

In summary, we demonstrate that EPA and DHA display divergent metabolic activities in a skeletal muscle cell line, which may be mediated by differential remodelling the lipidome. Additionally, we hypothesize that the elongation of EPA to DPA and subsequent incorporation into the phospholipid pool is a critical event mediating the divergent physiological effects of EPA and DHA. The specific role of DPA in skeletal muscle is not yet fully understood. Given that DPA is readily retro converted to EPA and is conserved from β -oxidation to a greater degree it may be possible that DPA may serve as a more stable reservoir of EPA. The mechanism underpinning the effect of EPA on glucose uptake remains unclear, however, differential remodelling of the lipidome may influence downstream effects. Similar to the divergence in the lipidome, n-3 fatty acids may also induce differential shifts in the proteomic profile which may explain the divergent effects on metabolism.

Chapter 3: EPA and DHA alter the membrane proteome and may drive the differential effects on protein turnover.

Jeromson S, Martin CV, Rao F, Gallagher IJ, Hamilton DL

3.0. Abstract

In both young and older individuals, omega-3 supplementation improves muscle protein synthesis in response to simulated feeding. These anabolic effects may have clinical significance for the maintenance of muscle mass and strength, particularly for elderly individuals. There is evidence from *in vitro studies* that EPA and DHA have differential effects on protein anabolism in skeletal muscle cell lines. Furthermore, EPA and DHA significantly alter the lipidome and may alter the associations of proteins with cell membranes. We aimed to explore the impact of EPA and DHA on global proteomic profiles and changes in the membrane proteome. EPA treatment increased the total protein content in myotubes (Veh: 153±16.9 ug VS EPA: 237.7±44.9 ug). EPA and DHA did not alter protein synthesis (Veh:2.49±0.29 CPM/ug, EPA:2.47±0.27, DHA: 2.4±0.37) or the phosphorylation status of key proteins in the mTOR signalling pathway. EPA treatment reduced protein breakdown, while, DHA had a neutral effect. SILAC analysis of the global proteome identified divergent shifts in protein expression following EPA or DHA incorporation. Analysis of the membrane proteome revealed a reduction in ribosomal proteins following DHA treatment. EPA increased association of proteins involved in protein folding with membranous regions . We speculate that EPA may increase protein fidelity, reducing protein breakdown and increasing total protein content. These data show that EPA and DHA differentially alter the proteomic profile of skeletal muscle and this may be caused by a shift in ribosomal localisation.

3.1. Introduction

Chapter 2 of this thesis and several previous studies demonstrated that omega-3 fatty acids are readily incorporated into the skeletal muscle lipid pool. Evidence shows that manipulating skeletal muscle omega-3 content across a range of experimental models is associated with enhanced protein anabolism [55], [56], [60]. Four weeks of omega-3 supplementation is associated with increases in mTOR and focal adhesion kinase, linked with mechanotransduction (FAK) [230], [231]. Changes in the content of anabolic proteins may sensitise skeletal muscle to anabolic stimuli. Indeed, eight weeks of omega-3 supplementation in both young and elderly subjects enhanced the muscle protein synthetic response to a hyperaminoacidemic-hyperinsulinemic clamp, without altering basal protein synthesis [2],[3]. Using a similar supplementation protocol, *McGlory & colleagues* (2016)[59] demonstrated that fish oil supplementation does not enhance MPS rates when resistance exercise is performed prior to amino acid feeding in young healthy males. Despite the lack of effect on combined exercise and nutrition, increasing omega-3 intake can have functional consequences for muscle function. Increased omega-3 intake over 6 months in older individuals offset declines in strength normally associated with ageing [232]. While in elderly women, fish oil supplementation enhanced strength gains during a programme of resistance exercise training [11].

Alongside increments in MPS in response to feeding, omega-3 PUFAs have also been demonstrated to offset the deleterious effects of skeletal muscle disuse. Using a hindlimb immobilisation model, omega-3 PUFAs reduced the loss of muscle mass in rodents possibly through the preservation of the PKB-P70S6K1 anabolic signalling axis [52]. Proximal insulin signalling through the PKB-mTOR-P70S6K1 axis is also

enhanced in growing steers [79]. The fish oil supplements used in these studies are rich in EPA and DHA and despite the very similar structure, EPA and DHA may have differential effects on skeletal muscle protein metabolism. In C₂C₁₂ myotubes, EPA increased MPS after stimulation by leucine while simultaneously reducing protein breakdown [60]. Another *in vitro* study observed that both EPA and DHA reduced protein breakdown, although EPA and DHA were used at much higher concentrations than in these sets of experiments (400-600µM) [233].

There is strong evidence that omega-3 PUFAs enhance anabolic processes in skeletal muscle which may have important clinical outcomes for populations at risk of significant loss of skeletal muscle mass, such as the elderly. However, the omega-3 content in fish is declining which questions the sustainability of fish as the predominant source of omega-3 PUFAs. Current evidence suggests that EPA and DHA differentially alter protein turnover, however, it remains relatively unknown how EPA and DHA alter the proteomic signature that may provide mechanistic evidence for the observed effects of omega-3 on processes regulating protein turnover. Furthermore, the significant incorporation of EPA and DHA into phospholipids will alter the physico-chemical properties of membranes and subsequent association of integral and peripheral membrane proteins with the membrane. This suggests that alteration in membrane associated proteins may alter the response to extracellular stimuli such as increased amino acid availability. Given the differential changes in the lipid pool, the effects of EPA and DHA may be mediated by membrane critical events. Therefore, the aim of this study was to assess the effects of EPA and DHA individually on protein turnover. We also sought to characterise how altered protein turnover may be reflected by changes in the abundance of individual proteins. This information will hopefully be used as a framework to identify target pathways that could be

manipulated by small drug compounds to mimic the molecular effects of omega-3 PUFAs. A secondary aim of this study was to assess how altered membrane lipid composition influenced the proteins that are associated with the membrane.

3.2. *Materials and Methods*

3.2.1. *Materials*

All plastic ware was purchased from Fisher scientific (UK). SILAC media was purchased from Dundee cell products. Dialysed foetal bovine serum was purchased from Sigma-Aldrich (Dorset, UK) and dialysed donor horse serum was purchased from Lab tech, (Sussex, UK).

3.2.2. *Cell culture*

C₂C₁₂ myoblasts were grown in Dulbecco's Modified Eagle Medium (DMEM) containing 20% fetal bovine serum and 1% penicillin/streptomycin and incubated at 37°C and 5% CO₂. Myoblasts were maintained at ~60% confluence. Differentiation was induced once confluence reached 80-90% by changing the media to differentiation media (DMEM supplemented with 2% horse serum and 1% Penicillin/Streptomycin) for 72 hours. Following differentiation cells were treated with 50 µM EPA or 50 µM DHA conjugated to 2% fatty acid free BSA or 2% fatty acid free (FAF) BSA as a control for 72 hours for proteomics analysis. Protein synthesis and breakdown was assessed after 24 hour incubation with EPA or DHA. Fatty acids were conjugated to 2% fatty acid free BSA in DM by constant agitation for 1 hour at 37°C.

3.2.3. *Cell preparation*

Cell lysates were collected from 6 well plates by scraping on ice in RIPA buffer [50 mmol/l Tris·HCl pH 7.5, 50 mmol/l NaF, 500 mmol/l NaCl, 1 mmol/l sodium vanadate, 1 mmol/l EDTA, 1% (vol/vol) Triton X-100, 5 mmol/l sodium pyrophosphate, 0.27 mmol/l sucrose, and 0.1% (vol/vol) 2-mercaptoethanol and Complete protease

inhibitor cocktail (Roche)] followed by snap freezing on liquid nitrogen. For preparation for western blotting samples were thawed and debris was removed by centrifugation at 4°C for 15 min at 13,000 g. The supernatant was then removed, and protein concentration was determined using the DC protein assay according to the manufacturer's instructions (Bio-Rad, Hercules, CA).

3.2.4. *Protein content*

Protein content following 72 h treatment with 50µM EPA or 50µM DHA conjugated to 2% fatty acid free BSA as a carrier or 2% fatty acid free BSA alone as a control was determined by multiplying the concentration of the supernatant [as determined using the DC protein assay according to the manufacturer's instructions (Sigma Aldrich, UK)] by the total volume of supernatant collected from a 6 well plate. Data are representative of 5 independent experiments carried out in triplicate.

3.2.5. *Muscle protein synthesis and breakdown*

Protein degradation was assessed by the quantification of the released L-[2,4,³H] phenylalanine into the culture media. Following 4 days of differentiation myotubes were incubated with media containing 2.5 µCi L-[2,4,³H] phenylalanine/ml and the label was maintained for 24 hours in order to label long lived proteins. Following the labelling pulse, cells were washed 2x in PBS and incubated in cold chase media (DMEM + 2mM L-phenylalanine) for 3 hours to allow for degradation of short lived proteins. Myotubes were then treated with either 50µM EPA or DHA (DMEM, 2% horse sera, 2mM L-phenylalanine, 2% FAF-BSA) for 24 hours. Following treatment an aliquot of the media was removed and radioactivity released was assessed by scintillation counting. The remaining myotubes were then thoroughly washed with ice cold saline (0.9%) and lysed with 50mM NaOH + 1% SDS for a minimum of 30 mins at room

temperature. Residual radioactivity in cell lysates was then assessed by scintillation counting. Total radioactivity was calculated as the sum of the L-[2,4,³H] phenylalanine released into the media and the residual cell retained L-[2,4,³H] phenylalanine. Protein breakdown is presented as the fraction of the total incorporated L-[2,4,³H] phenylalanine released into the media.

Basal protein synthesis was assessed by the incorporation of L-[2,4,³H] phenylalanine into peptide chains. Following differentiation, myotubes were treated with 50µM EPA or DHA for 24 hours. At the end of the treatment period the media was removed and DMEM containing 1uCi L-[2,4,³H] phenylalanine (0.5 µCi/ml) was added for 180 mins. The reaction was stopped by 2x washes in ice cold saline (0.9%) before 3x washes with Trichloroacetic acid (TCA) (10%) to remove any unincorporated tracer. Residual TCA was then removed by rinsing cells with methanol and plates left to dry. Myotubes were then lysed in 50mM NaOH + 1% SDS for a minimum of 30 minutes. An aliquot was collected for liquid scintillation counting to assess ³H incorporation into proteins and the remaining lysate was used to determine protein content by the DC protein assay. Protein synthesis is presented as counts per minute/µg of protein. **See supplementary data for assay validation.**

3.2.6. P70S6K1 signalling in response to a serum stimulus

To assess the activation of the mTOR signalling pathway in response to a serum stimulus, the phosphorylation of P70S6K1 was measured. C₂C₁₂ myotubes were cultured as previously described. Following differentiation myotubes were treated with vehicle, EPA or DHA for 24 hours. Myotubes were then serum starved in serum free DMEM for 3 hours. Media was then changed to DMEM containing 20% foetal bovine serum for 30 or 60 minutes, cell lysates were then collected and prepared for western blotting.

3.2.7. *Western blotting*

For western blots, supernatants was made up in Lamelli sample buffer, and 10-15 µg of total protein was loaded per well and run at 150 V for 1 h 15 min. Proteins were then transferred onto Whatman Immunobilon Nitrocellulose membranes (Fisher Scientific, Loughborough, UK) at 30 V for 2 h 15 min. Membranes were blocked in 3% BSA-Tris-buffered saline (containing vol/vol 0.1% Tween 20) for 1 h at room temperature, followed by incubation in primary antibodies [pmTOR(ser2448), total mTOR, pP70S6K1(Thr389), total P70S6K1, p4E-BP1(Thr37/46), total 4E-BP1 [Cell signalling, USA]] at 4°C overnight. Membranes underwent three 5 min washes in TBST followed by incubation in the appropriate secondary antibodies [secondary horseradish peroxidase conjugated antibody was purchased from ABCAM (#6721)] for 1 h at room temperature. Membranes were again washed three times for 5 min followed by incubation in enhanced diaminobenzidine tetrahydrochloride reagent (BioRad, Herts, UK). A BioRad ChemiDoc (Herts, UK) was used to visualize and quantify protein expression. Phospho proteins were normalized to the corresponding total protein. Data are representative of 3 independent experiments carried out in duplicate.

3.2.8. *Stable isotope labelling of amino acids in cell culture*

To assess changes in protein abundance, we used the SILAC method [234]. C₂C₁₂ myoblasts were grown in Dulbeccos' modified eagles medium (DM) supplemented with 20% dialysed (10 Kda) foetal bovine serum plus labelled amino acids lysine and arginine in a humidified atmosphere of 37°C and 5% CO₂. Cells intended to act as the control group were grown in with unlabelled lysine and arginine(**light**), the EPA treatment group were grown in R6K4 media(**l-arginine-13C6 hydrochloride, l-lysine-4,4,5,5-d4 hydrochloride[medium]**) while the DHA treatment group were

grown with R10K8 containing media (**l-arginine-13C6, 15N4 hydrochloride, l-lysine-13C6,15N2 hydrochloride [Heavy]**). The use of combined labelled arginine and lysine ensures that nearly all peptides will contain a label after tryptic digestion. Cells were allowed to grow for at least 6 population doublings to ensure full incorporation of labelled amino acids. We observed that use of dialysed sera and labelled media did not affect doubling time, cell morphology or differentiation capacity. Upon reaching 90-100% confluence the media was replaced with DMEM containing 2% dialysed donor horse serum (10 Da) to induce differentiation. After 3-4 days of differentiation, myotubes were treated with either control, 50 μ M EPA or 50 μ M DHA for 72 hours (**see treatment protocol**). Changes in both the global proteome as well as proteins associated with membranes were assessed. For membrane proteome analysis, membranes were isolated using the Thermo scientific Mem per plus protein membrane extraction kit. Analysis of global and membrane proteins were then analysed by the same process.

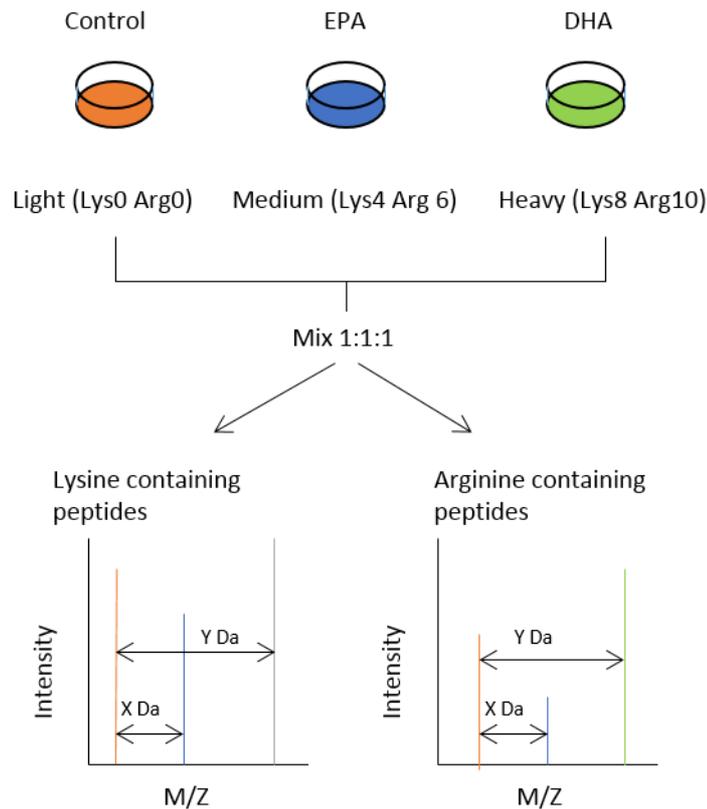


Figure 3. 1 SILAC workflow.

3.2.9. Treatment protocol

Fatty acids EPA and DHA at a concentration of 50 μ M were conjugated to 2% fatty acid free BSA by constant agitation for 1 hour at 37°C. Fatty acid treatments were made in DMEM containing the appropriate label (con-light, EPA-medium, DHA- heavy) in order to maintain the labelled proteome. Treatment was maintained for 24 or 72 hours before cell pellets were collected and frozen in liquid nitrogen before being stored at -80 until further analysis. Samples from each group were collected in triplicate and samples from each respective treatment group were pooled before processing for mass spectrometry analysis.

3.2.10. Mass spectrometry

Trypsin-digested peptides were separated using an Ultimate 3000 RSLC (Thermo Scientific) nanoflow LC system. On average 0.5 μ g of protein was loaded with a constant flow of 5 μ l/min onto an Acclaim PepMap100 nanoViper C18 trap column (100 μ m

inner-diameter, 2cm; Themro Scientific). After trap enrichment, peptides were eluted onto an Acclaim PepMap RSLC nanoViper, C18 column (75 μ m, 15 cm; ThermoScientific) with a linear gradient of 2–40% solvent B (80% acetonitrile with 0.08% formic acid) over 65 min with a constant flow of 300 nl/min. The HPLC system was coupled to a linear ion trap Orbitrap hybrid mass spectrometer (LTQ-Orbitrap Velos, Thermo Scientific) via a nanoelectrospray ion source (Thermo Scientific). The spray voltage was set to 1.2 kV, and the temperature of the heated capillary was set to 250 °C. Full-scan MS survey spectra (m/z 335–1800) in profile mode were acquired in the Orbitrap with a resolution of 60,000 after accumulation of 1,000,000 ions. The fifteen most intense peptide ions from the preview scan in the Orbitrap were fragmented by collision-induced dissociation (normalized collision energy, 35%; activation Q, 0.250; and activation time, 10 ms) in the LTQ after the accumulation of 10,000 ions. Maximal filling times were 1,000 ms for the full scans and 150 ms for the MS/MS scans. Precursor ion charge state screening was enabled, and all unassigned charge states as well as singly charged species were rejected. The lock mass option was enabled for survey scans to improve mass accuracy [209]. Data were acquired using the Xcalibur software.

3.2.11. *Quantification and bioinformatics analysis*

The raw mass spectrometric data files obtained for each experiment were collated into a single quantitated data set using MaxQuant (version 1.2.2.5) [235] and the Andromeda search engine software [236]. Enzyme specificity was set to that of trypsin, allowing for cleavage N-terminal to proline residues and between aspartic acid and proline residues. Other parameters used were: (i) variable modifications, methionine oxidation, protein N-acetylation, gln \rightarrow pyro-glu, Phospho(STY); (ii) fixed

modifications, cysteine carbamidomethylation; (iii) database: target-decoy human MaxQuant (ipi.HUMAN.v3.68); (iv) heavy labels: R6K4 and R10K8; (v) MS/MS tolerance: FTMS- 10ppm , ITMS- 0.6 Da; (vi) maximum peptide length, 6; (vii) maximum missed cleavages, 2; (viii) maximum of 84onlabel amino acids, 3; and (ix) false discovery rate, 1%. Peptide ratios were calculated for each arginine- and/or lysine-containing peptide as the peak area of 84onlabel arginine/lysine divided by the peak area of 84onlabelled arginine/lysine for each single-scan mass spectrum. Peptide ratios for all arginine- and lysine-containing peptides sequenced for each protein were averaged. Data is normalised using 1/median ratio value for each identified protein group per labelled sample.

3.2.12. *Serum dialysis*

Dialysis of FBS and DHS was carried out in CelluSep-H1 dialysis bags (Membrane filtration products, inc., Texas, USA) with molecular weight cut off points of 1 Kda, 3 Kda, 5 Kda, 10 Kda. The dialysis bags contained regenerated cellulose membranes, manufactured from natural cellulose reconstituted from cotton liners. These cellulose membranes are EDTA tested, heavy metal free, carry no fixed charge and do not adsorb most solutes. Prior to use, membranes were soaked in distilled water at room temperature for 30 minutes to remove the preservative solution. The membrane was then thoroughly rinsed with distilled water. Dialysis was carried out in independent dialysis bags with 1mL of serum per analysis. Dialysis was carried out at 4°C for 24 hours using phosphate buffered saline as a buffer in at least 200 fold excess of dialysis buffer. The buffer was changed at 3 hours, 5 hours and once prior to leaving overnight. Following dialysis serum was recovered and filtered through sterile filter units (0.1µm, Pall, NY, USA) and frozen at -20. 1mL samples were taken in duplicate from 4 independent experiments for fatty acid profiling.

3.2.13. FAME analysis

Lipid profiles of foetal bovine serum and donor horse serum which had undergone dialysis at various molecular weight cut offs (1 Kda, 3 Kda, 5 Kda, 10 Kda) were assessed by fatty acid methyl ester analysis (FAME). Total lipids were extracted by homogenising in 20 volumes of chloroform/methanol (2:1 v/v). Total lipids were prepared according to the method of Folch et al. [221] and non-lipid impurities were removed by washing with 0.88% (w/v) KCl. The weight of lipids was determined gravimetrically after evaporation of solvent and overnight desiccation under vacuum. Fatty acid methyl esters (FAME) were prepared by acid-catalysed transesterification of total lipids according to the method of Christie et al. (35). Extraction and purification of FAME was performed as described by Ghioni et al. (36). FAME were separated by gas-liquid chromatography using a ThermoFisher Trace GC 2000 (ThermoFisher, Hemel Hempstead, UK) equipped with a fused silica capillary column (ZBWax, 60m x 0.32 x 0.25 mm i.d.; Phenomenex, Macclesfield, UK) with hydrogen as carrier gas and using on-column injection. The temperature gradient was from 50 to 150°C at 40°C/min and then to 195°C at 1.5°C/min and finally to 220°C at 2°C/min. Individual methyl esters were identified by reference to published data (Ackman, 1980). Data were collected and processed using the Chromcard for Windows (version 2.00) computer package (Thermoquest Italia S.p.A., Milan, Italy). All experiments were carried out in duplicate from 4 independent experiments.

3.2.14. *Data analysis and statistics*

Proteins were considered up-regulated or down-regulated with a minimum fold change cut off of 0.2, corresponding to a 20% change in protein abundance. Ingenuity pathway analysis software was used to assess over represented pathways and pathway activation predictions. Changes in fatty acid content of FBS and DHS were

assessed by one-way ANOVA, with a tukeys post hoc test used only if significant differences were detected, using Graphpad version 7.0. P-values were adjusted for multiple comparison testing where necessary. Membrane protein abundance was considered altered if fold change if lower than 0.75 or greater than 1.25 and a significant difference between EPA and DHA ($p < 0.05$). Enrichment of biological processes was determined using gene ontology, using the whole genome of *Mus musculus* as a background reference list. Protein interactions were assessed using the STRING database (version 10).

3.3. Results

3.3.1. Serum dialysis increases the omega-3 content in donor horse serum

To prevent the dilution of the amino acid tracer pool for the SILAC protocol, myotubes must be grown in media with a modified amino acid pool which necessitates the use of dialysed FBS and DHS during cell proliferation and differentiation. Given that we aimed to assess how the modification of lipid composition affected protein expression we first sought to examine the effect of dialysis on serum lipid profiles. The lipid pool of cultured myotubes is largely dependent on the available fatty acids in the media and a significant change in the lipid profile may alter the baseline lipid profile and subsequent effects of EPA and DHA incorporation. No effect of dialysis on any lipid class in FBS was observed (**supplementary figure S1.1**). Interestingly, we found that in dialysis increased the omega-3 content in DHS (**figure S1.1B, p value < 0.05**). We used dialysed serum with a molecular weight cut off of 10 Kda (mean difference + 6.3 $\mu\text{g}/\text{mg}$ lipid) and hypothesise that a small increase would not have had a major impact on the myotubes baseline lipid profile.

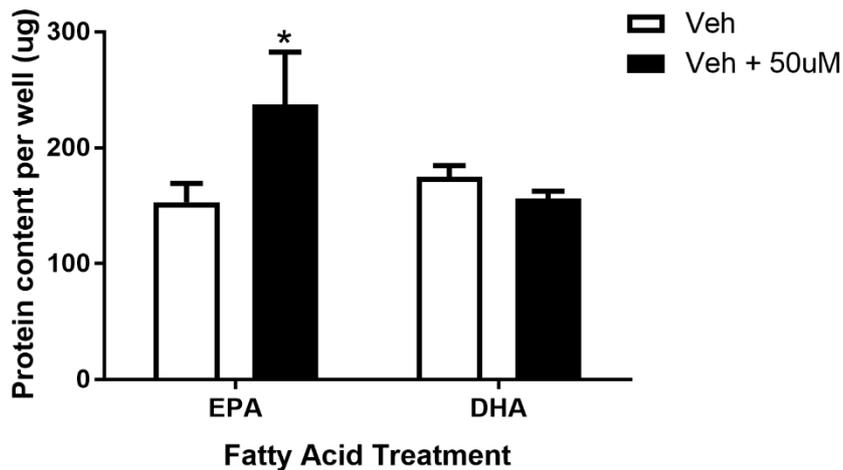


Figure 3. 2 EPA enhances protein accretion. C2C12 myotubes were treated with either EPA or DHA for 72 hours. The total amount of cellular protein was assessed by multiplying the protein concentration by the total volume of lysate. Data are presented as mean \pm S.E (n=5). * denotes significantly different from vehicle ($p<0.05$).

3.3.2. EPA enhances protein accretion through a reduction in breakdown while DHA has a neutral effect.

After a 72 hour incubation with EPA or DHA, myotubes were collected in order to determine total protein content. When myotubes were incubated with EPA total protein content was enhanced while incubation with DHA had a neutral effect (**Figure 3.2**). Protein balance is determined by the balance between synthesis and breakdown of proteins. Thus, the observed protein accretion may be reflected in changes in either protein synthesis or breakdown. In order to understand the mechanisms underlying the changes in protein content we directly assessed protein synthesis and breakdown and related signalling. Neither fatty acid had any effect on basal protein synthesis after a 24 hour incubation (**Figure 3.3A**). Anabolic signalling assessed by the phosphorylation status of mTOR, P70S6K1 and 4E-BP1 was not different between groups, corresponding with lack of changes in protein synthesis (**Figure 3.3C**). EPA reduced protein breakdown compared to both the vehicle and DHA treatments (**Figure 3.3B**). No changes were detected in the level of ubiquitin tagged proteins (**Figure 3.3D**).

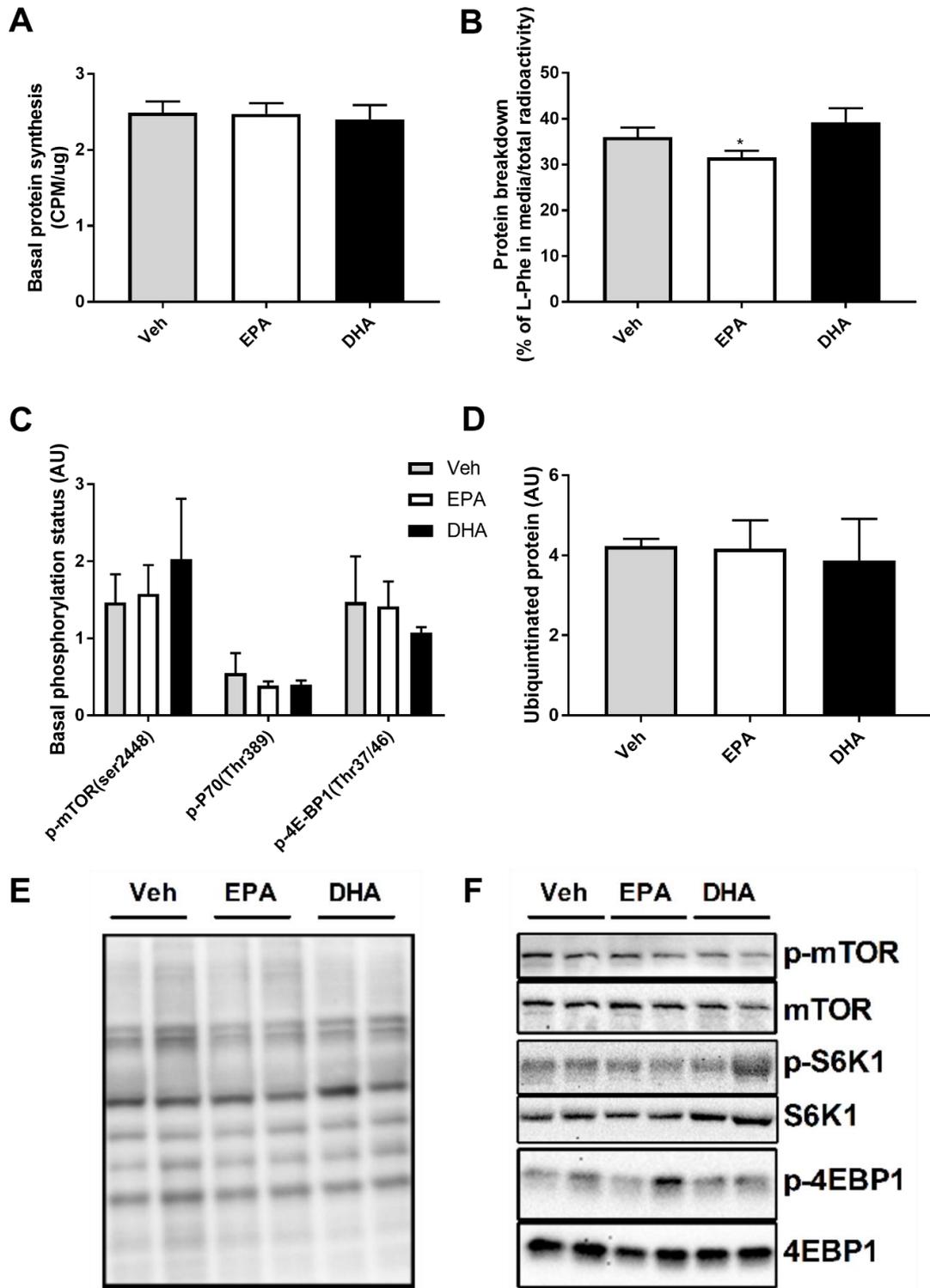


Figure 3. 3 The effect of EPA and DHA on muscle protein anabolism. A) Basal protein synthesis was measured by the incorporation of ^3H L-Phenylalanine into muscle proteins (n=5). B) Protein breakdown was assessed by conditions C) basal phosphorylation status of key proteins in the mTOR signalling pathway (n=3). D) ubiquitinated proteins tagged for degradation. E and F) Representative western blot images (n=3). P value <0.05. * denotes significant difference from vehicle and DHA.

3.3.3. *N-3 fatty acid treatment does not alter the P70S6K1 signalling response to a serum stimulus.*

In order to measure the anabolic signalling response to serum stimulation, P70S6K1 (T389) phosphorylation after a 3 hour serum starve 0, 30 and 60 minutes after addition of serum containing DMEM. Neither fatty acid treatment altered the activation or time course of P70S6K1 activation (**Figure 3.4**). These data indicate that not only has n-3 supplementation not altered basal signalling responses, but it has not altered growth factor induced P70S6K1 activation.

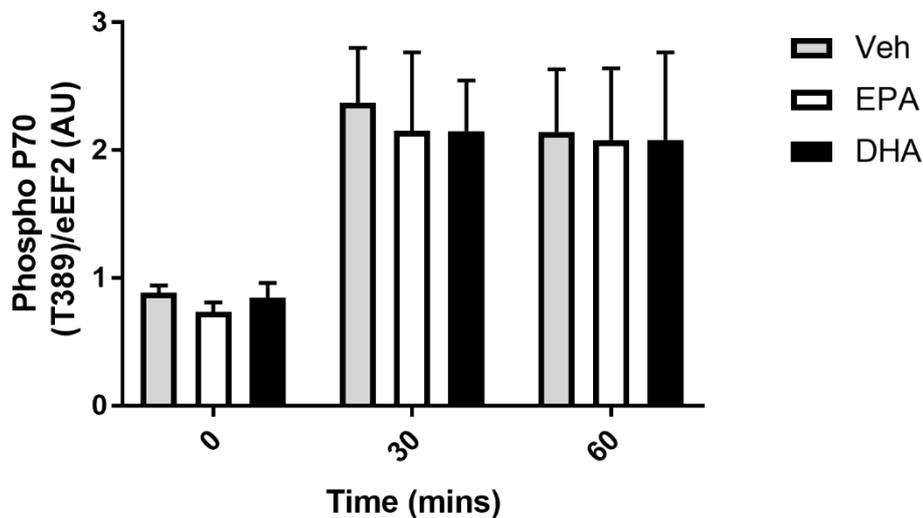


Figure 3. 4 EPA or DHA does not alter the P70S6K1 signalling response to a serum stimulus. Following treatment with EPA or DHA myotubes were stimulated with serum for 30 or 60 minutes and the anabolic signalling response was assessed by changed in P70S6K1 phosphorylation status (n=3).

3.3.4. EPA and DHA differentially alter global proteome profiles

To assess how omega-3 fatty acids altered the abundance of individual proteins we employed a SILAC model to identify changes in the global proteomic signature. The SILAC method is a semi-quantitative method that assessed relative fold changes in protein abundances from the unlabelled control group. 1563 proteins were identified and quantified in all 3 conditions (**Figure 3.5**). Proteins were considered altered if the abundance was changed by 20% (corresponding to ± 0.2 fold change). In order to discover how these alterations in protein abundance may alter cell function

overrepresentation analysis was performed. Proteins identified as altered (both up and down-regulated) were included in the same analyses. **Figure 3.6** shows that EPA and DHA alter proteins involved in different signalling pathways. EPA largely altered pathways regulating protein synthesis and breakdown, as well as the cellular effects of sildenafil and RhoA signalling (**Figure 3.6A**). While DHA altered proteins related to energy metabolism (TCA cycle), Protein ubiquitination, mitochondrial dysfunction as well as protein kinase A and integrin signalling (**Figure 3.6B**). Although this analysis does not indicate the direction of action on these pathways it does indicate the potential cellular processes affected by omega-3 incorporation.

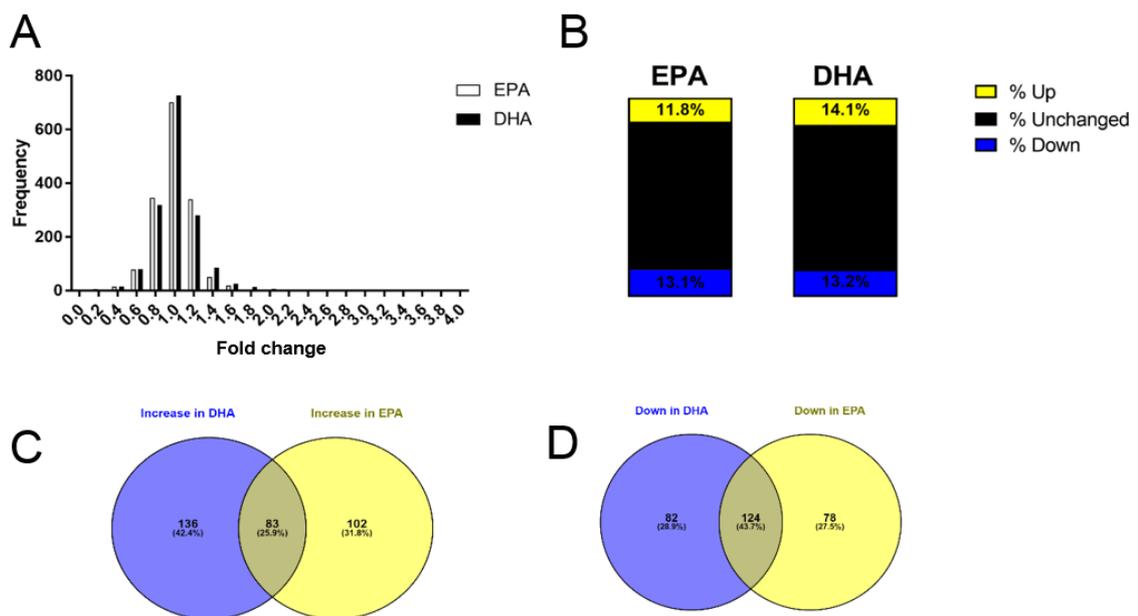


Figure 3. 5. Descriptive summary of global proteome. A) Frequency histogram of Log2 fold change B) Proportion of detected proteins altered by EPA and DHA. C) Number of proteins increased by EPA and DHA D) number of proteins decreased by EPA and DHA.

Although this proteomic analysis assessed changes in total protein content, using currently available knowledge of positive and negative regulators, the activation or inhibition of some pathways can be predicted. Investigation of potentially activated pathways revealed a common influence of omega-3 fatty acids on actin cytoskeleton

signalling and the RhoGDI pathway. DHA incorporation is also predicted to inhibit a number of growth factor related pathways (**Table 3.1.**). The prediction of changes in the RhoGDI and actin cytoskeleton pathways may indicate a novel role of omega-3 fatty acids in GTP regulated processes in skeletal muscle.

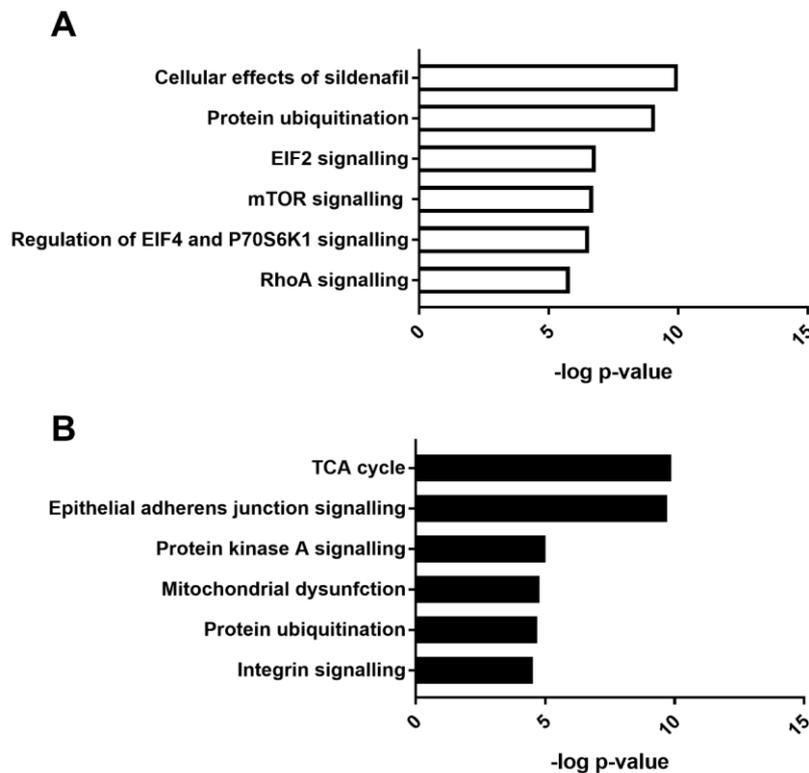


Figure 3. 6 Over representation analysis. A) Cellular processes overrepresented in EPA treatment group. B) cellular processes overrepresented in DHA treatment group.

Table 3. 1 Pathway activation prediction analysis.

EPA (z-score)		DHA (z-score)	
Activated pathways	Inhibited pathways	Activated pathways	Inhibited pathways
RhoGDI signalling (2.333)	Regulation of actin based motility (-2.121)	RhoGDI signalling (2.646)	Actin cytoskeleton signalling (-3.71)
	Actin cytoskeleton signalling (-2.041)	Intrinsic prothrombin activation (-2)	ILK signalling (-2.837)
			CXCR4 signalling (-2.646)
			PAK signalling(-2.499)
			PDGF signalling (2.499)
			IL-8 signalling (-2.236)
			EGF signalling (-2)
			HGF signalling (-2)
			VEGF signalling (-2)

3.3.5. Altered composition of phospholipids changes the membrane-associated proteome.

Table 3.2 Overrepresented biological processes in EPA membrane proteome.

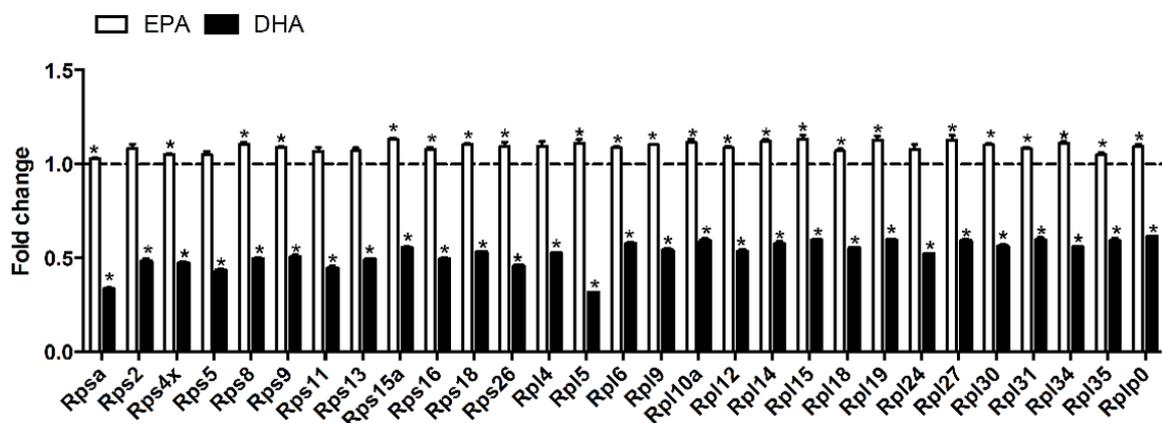
Go accession	Biological process	P-value	Genes (mean fold change)
GO:0006457	Protein folding	4.76E-03	Fkbp10 (1.49), Hspa4L (0.65), P3h1 (1.29), Txndc5 (1.30), Prdx4 (1.30), Ppib (1.34), Ppic (1.45)

Over 3000 proteins associated with the membrane compartment were identified in the SILAC screen. These results were filtered down to 625 proteins with a coefficient of variance $\leq 5\%$ to describe consistent changes in membrane abundance. Proteins were considered enriched or reduced in the membrane with a fold change cut off of 0.25. Proteins similarly affected by EPA and DHA were removed from analysis. Proteins with altered abundance were then subject to gene ontology analysis for biological processes. Although a number of individual proteins were altered by EPA, there was not a major association of these proteins with cell processes.

Membrane proteins altered by EPA were associated with protein folding ($p = 4.76E-03$) (**Table 3.2**). EPA also increased Calumenin in membranes which also plays a role in calcium sensitive protein folding. DHA altered proteins associated with a number of processes, primarily related to oxidative metabolism and ribosomal formation (**Table 3.3**). Further examination of ribosomal proteins revealed that DHA induced the significant reduction in ribosomal proteins associated with both small and large subunits at the membrane. Conversely, EPA induced a small rise in ribosomal proteins (**Figure3. 7**).

Table 3. 3 Overrepresented biological processes in DHA membrane proteome

Go accession	Biological process	P-value	Genes (log ₂ mean fold change)
GO:0015986	ATP synthesis coupled transport	8.68E-04	<i>Stoml2</i> (1.32), <i>Tcirg1</i> (1.26), <i>Atp5a1</i> (0.62), <i>Atp5i</i> (0.61), <i>Atp5f1</i> (0.58), <i>Atp5o</i> (0.46)
GO:0006084	Acetyl-CoA metabolic process	3.65E-02	<i>Pdha1</i> (1.37), <i>Acaa2</i> (0.67), <i>Dld</i> (0.65), <i>Pdhx</i> (0.60), <i>Acat1</i> (0.64)
GO:0042775	Mitochondrial ATP synthesis coupled electron transport	1.55E-03	<i>Uqcrc1</i> (1.54), <i>Uqcrb</i> (1.43), <i>Cox4i1</i> (1.38), <i>Coq9</i> (1.27), <i>Dld</i> (0.65), <i>Ndufa10</i> (1.49), <i>Mtch2</i> (1.36)
GO:0042255	Ribosome assembly	6.05E-04	<i>Rpsa</i> (0.33), <i>Rpl6</i> (0.58), <i>Ddx3x</i> (0.68), <i>Rpl12</i> (0.54), <i>Rps2</i> (0.48), <i>Rpl5</i> (0.31), <i>Rpl24</i> (0.52), <i>Rps5</i> (0.44)
GO:0006412	Translation	7.24E-05	<i>Rpl35</i> (0.59), <i>Aars2</i> (0.58), <i>Rps8</i> (0.49), <i>Rpl10a</i> (0.58), <i>Rpl34</i> (0.56), <i>Rpsa</i> (0.33), <i>Rps16</i> (0.50), <i>Mrpl47</i> (0.59), <i>Mrpl13</i> (0.55), <i>Mrpl24</i> (0.54), <i>Rpl18</i> (0.55), <i>Rpl6</i> (0.58), <i>Rplp0</i> (0.61), <i>Ddx3x</i> (0.68), <i>Tufm</i> (0.5), <i>Rps4x</i> (0.47), <i>Rpl4</i> (0.53), <i>Rpl12</i> (0.54), <i>Rpl14</i> (0.57), <i>Rpl15</i> (0.59), <i>Rps11</i> (0.45), <i>Rps9</i> (0.49), <i>Rps2</i> (0.48), <i>Mrpl15</i> (0.52), <i>Rpl5</i> (0.31), <i>Rpl19</i> (0.60), <i>Rpl24</i> (0.52), <i>Rps5</i> (0.44), <i>Mrpl21</i> (0.53), <i>Rps13</i> (0.49)
GO:0006417	Regulation of translation	2.33E-05	<i>Hnrnpa2b1</i> (0.68), <i>Ddx39b</i> (0.67), <i>Ddx3x</i> (0.68), <i>Srrt</i> (0.33), <i>Fxr1</i> (0.57), <i>Rps9</i> (0.49), <i>Ppp1ca</i> (0.68), <i>Elavl1</i> (1.35), <i>GAPDH</i> (1.87), <i>Rps5</i> (0.44), <i>Trap1</i> (0.67), <i>Eif5</i> (0.75), <i>Syncrip</i> (0.63)
GO:0046907	Intracellular transport	8.12E-03	<i>Sar1a</i> (1.63), <i>Hnrnpa2b1</i> (0.68), <i>Stoml2</i> (1.32), <i>Dctn2</i> (0.72), <i>Rpsa</i> (0.33), <i>Ddx39b</i> (0.67), <i>Sptbn1</i> (0.62), <i>Hspa4</i> (0.54), <i>Nup62</i> (0.54), <i>Bicd2</i> (0.64), <i>Timm44</i> (1.38), <i>Anxa2</i> (0.73), <i>Ywhaq</i> (0.73), <i>Rab6a</i> (1.32), <i>Vcp</i> (0.52), <i>Trap1</i> (0.67), <i>Uso1</i> (0.64), <i>Actr2</i> (0.62), <i>Atp5o</i> (0.46), <i>Nup155</i> (0.42), <i>Hsp90aa1</i> (0.64), <i>Os9</i> (1.26), <i>Copb2</i> (2.19), <i>Anp32b</i> (0.53)

**Figure 3. 7. Ribosomal protein association with membranes.** Ribosomal proteins detected in the membrane proteome screen. Data is presented as fold change. Data is representative of n=3.

3.4. Discussion

The aim of this study was to assess the differential effects of EPA and DHA on protein metabolism. If changes in protein metabolism were reflected by changes in the proteomic profile of myotubes. We characterised the shift in global proteomic signatures following EPA or DHA incorporation and subsequently how omega-3 driven lipid remodelling altered the membrane associated proteome. We show that EPA and DHA also differentially alter the abundance of individual proteins and divergently changed proteins associate with different cell processes. Furthermore, we characterise the shift in proteins associated with the membrane in response to EPA or DHA.

Our results show that a 72 hour incubation of C₂C₁₂ myotubes with EPA results in an increased accretion of protein. These changes were due to a reduction in protein breakdown and an unchanged rate of protein synthesis. Previous studies have shown that both EPA and DHA exert a protective effect when myotubes are in the presence of deleterious stimuli such as PA and TNF- α [64], [179]. The present study demonstrates that EPA has effects on basal protein breakdown in the absence of a catabolic stimulus. These findings are in agreement with data from *Kamolrat & colleagues (2013)*[60]. While the magnitude of change in protein breakdown was moderate (approximately 5%) this measure was made after 24 hours for technical reasons. We speculate that a sustained decrease in protein breakdown for 72 hours while synthesis is maintained would confer an increased total protein content. One of the main pathways for protein breakdown is the ubiquitin/proteasome pathways. Ubiquitin molecules are added to proteins which tag them for degradation. We were unable to detect any changes in the amount of proteins tagged with ubiquitin. However, increased levels of P62 (gene – SQSTM1) were detected in the proteome screen and

differentially altered by EPA and DHA. P62 functions as an adaptor protein that links ubiquitin tagged proteins with the autophagy machinery [237]. Subsequently, P62 is degraded in the lysosome and may be used as an indicator of autophagic flux [238]. However, EPA decreases protein levels of P62 while DHA increases P62 (**Figure 3.8**). This would be indicative of an increase and decrease respectively of autophagic flux, in contrast with observed changes in protein breakdown. Levels of P62 may also be affected by changes in gene expression and other cellular perturbations. We were unable to validate the changes in P62 by western blotting. More work is necessary to understand the mechanisms of the EPA induced depression of protein breakdown. One possible mechanism by which EPA may work is the maintenance of protein quality, reducing the targeted turnover of misfolded or damaged protein aggregates [107]. Atrophy is thought to be primarily driven by depression in protein synthesis so it remains to be seen how relevant small depressions in muscle protein breakdown would be in human models.

We also assessed how EPA and DHA altered the individual abundance of proteins. Given, that DHA altered the abundance of a number of proteins without altering protein synthesis or breakdown, the results are not necessarily causally related. The abundance of proteins can be altered by both changes in the rate of turnover and gene expression. Both EPA and DHA are ligands for transcription factors such as PPAR α and can inhibit the activation of SREBP-1 [205], [206]. The observed changes in the proteome may be due to alterations in the activity of transcription factors. Coupling our proteomic screen with micro array analysis would allow for the determination of whether changes in the proteomic profile are due to gene expression changes or turnover rates of proteins.

The proteins altered by EPA which associated with a number of pathways involved in protein metabolism. DHA associated with pathways involved in energy metabolism, integrin signalling and protein ubiquitination. Analysis of the proteomics revealed that proteins altered by EPA were associated with the processes regulating cell growth and protein turnover such as the mTOR and ubiquitin pathways. However, analysis of basal mTOR pathway activation by western blot did not reveal changes in the phosphorylation of mTOR or P70S6K. To test if these changes may only be observed in response to a stimulus, we performed a time course of P70S6K phosphorylation in response to a serum stimulus. This experiment did not show any changes in P70S6K1 activation following EPA or DHA treatment. Kamolrat et al (2013)[184] observed that EPA and DHA enhanced P70S6K1 phosphorylation in response to leucine. In response to a serum stimulus consisting of amino acids and growth factors we did not observe any differences between groups on the phosphorylation of P70S6K1. In C₂C₁₂ myotubes leucine stimulates anabolic signalling but not protein synthesis [61]. It is possible that the combination of amino acids and growth factors saturated the P70S6K1 signalling response in our study and prevented any differences from being observed.

Human and trials have observed augmented signalling in response to amino acids and insulin in response to omega-3 fatty acids. It is possible that the effects are specific to these stimuli. Given that these stimuli converge on common pathways it is not known how this regulation would occur upstream of the mTOR pathway. Although we only assessed the abundance of proteins the IPA software can predict the activation or inhibition of pathways. Based on the protein expression profile it is predicted that EPA and DHA both inhibit actin cytoskeleton signalling. Omega-3 supplementation has been shown to alter the expression of cytoskeleton related genes in whole blood [239].

Furthermore, actin remodelling is critical for insulin sensitive glucose uptake [240]. Given that no decline the insulin driven glucose uptake was observed, inhibition of this pathway did not appear to have any effect (See chapter 2). Multiple growth factor receptor mediated signalling was predicted to be downregulated by DHA. Previous studies support a role for omega-3 fatty acids in reducing epidermal growth factor signalling and it appears this might extend to similar growth factor activated pathways in skeletal muscle [241]. This is thought to be one of the mechanisms by which omega-3 fatty acids act to inhibit proliferation of cancer cells. Additionally, we observed a differential effect of EPA and DHA on the abundance of collagen proteins. EPA reduced collagen proteins while they were increased by DHA. The accumulation of collagen within the extracellular matrix is associated with insulin resistance [242] (**Figure 3.8A**). However, DHA increased collagen protein abundance without altering insulin sensitivity, so changes in collagen abundance do not appear to have affected the phenotype observed in our work. One limitation of this proteomic approach is that due to running samples on gels prior to mass spectrometry analysis the data is biased towards the detection of cytosolic proteins. Coupling changes in protein abundance with changes in post translational modifications and gene expression would give a more holistic view as to how individual fatty acids alter myotubes phenotypes

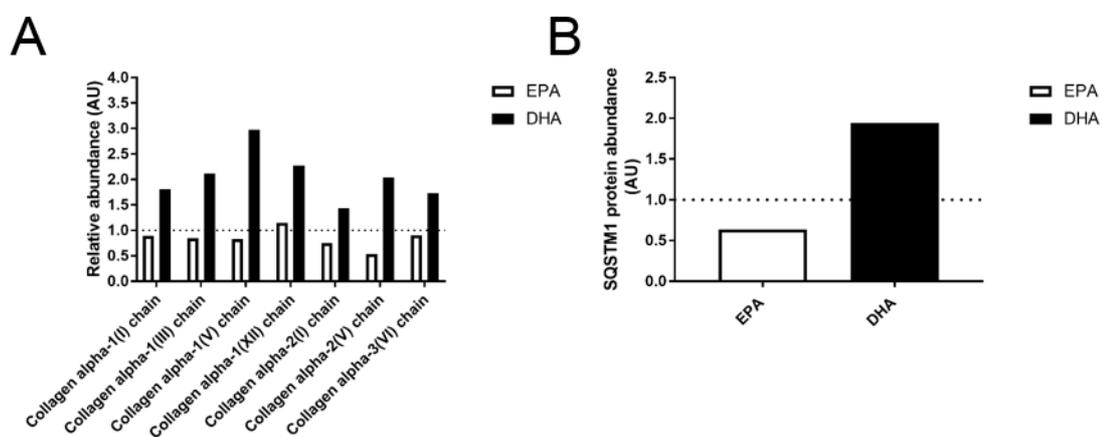


Figure 3. 8 Abundance of a) collagen proteins and b) P62, detected in the global proteomic screen.

In the previous chapter, EPA and DHA were shown to be incorporated into phospholipid species. Phospholipids are found primarily in the membrane, these data indicate that EPA and DHA have the potential to alter membrane properties and potentially the membrane associated proteome. We show that EPA and DHA divergently alter the membrane associated proteome. This is likely due to the changes in phospholipid composition following EPA or DHA treatment. There are a number of mechanisms by which proteins can bind to the membrane which are influenced by the fatty acid composition of the lipid bilayers. Either by altering interactions between fatty acyl chains and proteins or the structure of lipid rafts. We hypothesised that the alteration of protein-membrane interactions may link lipid remodelling to downstream metabolic effects. Gene ontology analysis indicated that proteins associated with protein folding at the membrane were overrepresented following EPA incorporation (**Table 3.1**). There is experimental evidence that a number of the proteins identified with this process interact, as probed by the STRING database (**Figure 3.9**). Furthermore, analysis of ribosomal proteins indicated a small shift of ribosomal proteins at the membrane. We propose a mechanism in which protein folding is enhanced, reducing damaged or misfolded proteins and reducing protein breakdown which results in increased protein accretion. Further experimental work is needed to assess this hypothesis. Indeed, previous work has shown that EPA, but not DHA, maintained protein quality in aging mice [107]. In comparison, DHA significantly reduced the abundance of a number of ribosomal proteins with the membrane (**Figure 3.7**). If total ribosomal content is unchanged then this would indicate an increase in cytosolic ribosomes. Proteins that enter the secretory pathways or integral membrane proteins are synthesised in the ER while other proteins are translated in the cytosolic

ribosome pool [243]. This differential shift in ribosomal compartmentalisation may partially explain the divergent changes in protein expression profiles.

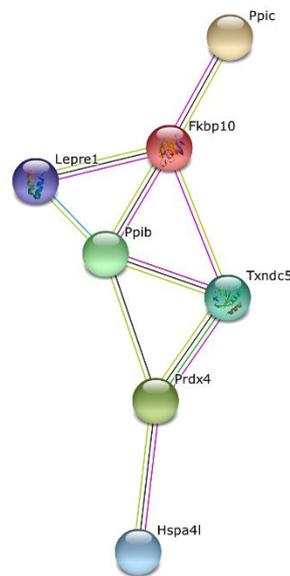


Figure 3. 9 STRING diagram representing protein-protein interactions. Levels of confidence key – curated database (Blue), experimental evidence (Pink), text mining (Yellow), Co-expression (Black)

DHA also altered the abundance of proteins involved in ATP coupled proton transport and acetyl-CoA metabolism, in agreement with the global proteome analysis. The proteins identified with ATP synthesis coupled transport were mainly downregulated proteins in the ATP synthase complex. This would be expected to manifest as a reduced ability to generate ATP yet no changes were observed in ATP synthase dependent oxygen consumption, as measured in the previous chapter. The effects of DHA may not have been severe enough to observe at basal levels. Although maximal respiration is measured during the mito stress test, it is induced by uncoupling so would not detect any changes in ATP synthesis. Previous studies have observed that omega-3 fatty acids can alter mitochondrial function, altering ADP kinetics without altering maximal respiration [190]. It remains to be seen if this reduction in ATP synthase proteins would alter the mitochondrial response to cellular stress or changing substrate availability.

In summary, we demonstrate that protein turnover is differentially altered by EPA and DHA. EPA reduces protein breakdown which may be due to an increase in protein folding at the endoplasmic reticulum. No changes in protein synthesis or activation of the mTOR signalling pathway were observed with either EPA or DHA. Furthermore, no changes in the phosphorylation status of P70S6K1 following serum stimulation were detected. The changes in phospholipid composition described in the previous chapter induces a shift in proteins associated with cellular membranes. The changes in global or membrane specific proteome did not always reconcile with the metabolic measures such as mTOR activation with EPA or mitochondrial function with DHA. It may be that changes in these processes are only observable under specific stimuli that have not been explored in this work. Building on this work, future experiments should investigate the mRNA transcripts associated with different ribosomal pools and the regulation of ribosomal protein localisation. The metabolic effects were not readily explained by changes in the global proteome but the membrane associated proteome may link lipid remodelling following omega-3 supplementation to the observed metabolic effects in human models.

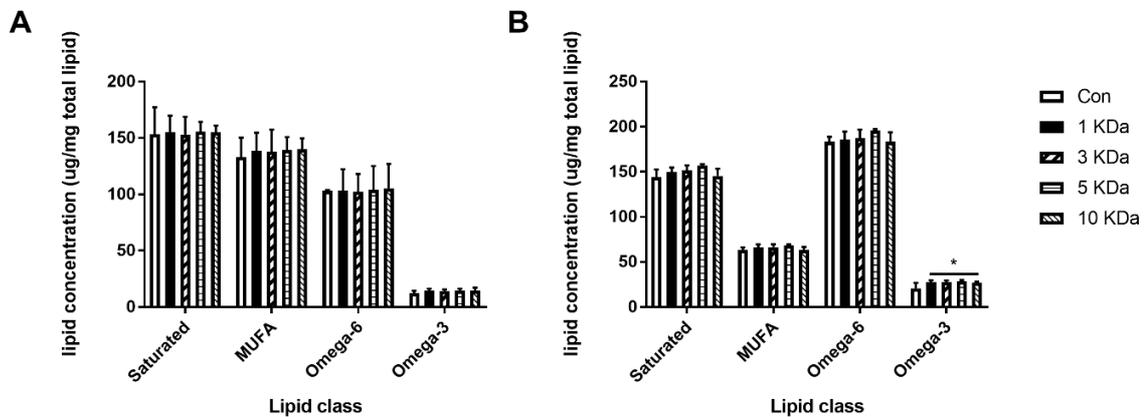
3.5. *Supplementary figures*

Figure S3 1. The effect of serum dialysis on the lipid composition of a) fetal bovine serum and b) donor horse serum. Graph represents N=4. Data expressed as mean \pm SD. * indicates significant difference from control of that lipid class.

Muscle protein synthesis/breakdown assay development:

Linearity of L-[2,4,³H] phenylalanine incorporation into muscle proteins was determined by assessing the time course of labelled L-[2,4,³H] phenylalanine associated with proteins. Myotubes were serum starved for 2 hours in serum free DMEM. Media was then substituted for DMEM or Growth media + 10% FBS with labelled L-[2,4,³H] phenylalanine (0.5 μ Ci/ml) and measures of protein synthesis were taken at 60,120,180 and 240 minutes. Incorporation of L-[2,4,³H] phenylalanine was linear in both conditions over 240 minutes (R^2 – DMEM (0.98), GM (0.97), **Figure S1.**). Although a divergence over time can be observed between both conditions, FBS contains an unknown amount of unlabelled L-Phenylalanine. This means the tracer pool is diluted, underestimating the true rate of incorporation over time.

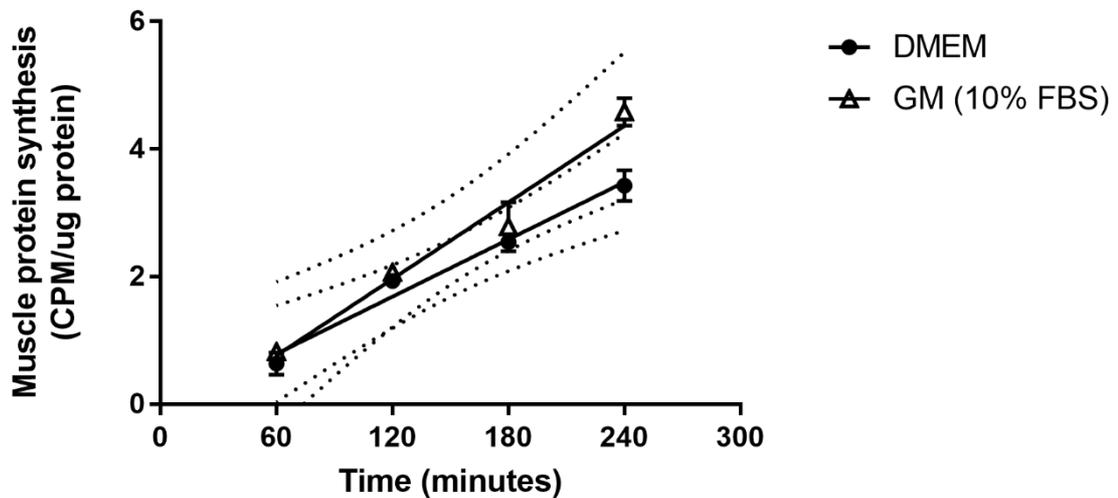


Figure S3.2 Validation of muscle protein synthesis assay. Incorporation of L-[2,4,³H] phenylalanine in the basal state or in the presence of 10% growth serum.

Protein breakdown:

Protein breakdown was assessed in the presence of 3 different media compositions in order to ensure measures of protein breakdown were sensitive to changes in media composition. Protein breakdown was measured after incubation of myotubes with differentiation media (2% DHS), serum free DMEM or growth media (10% FBS + 100nM insulin). DM was used as a baseline, DMEM alone increased protein breakdown while incubation with GM + insulin suppressed protein breakdown. These results demonstrate that the protein breakdown assay was able to detect expected changes in protein breakdown.

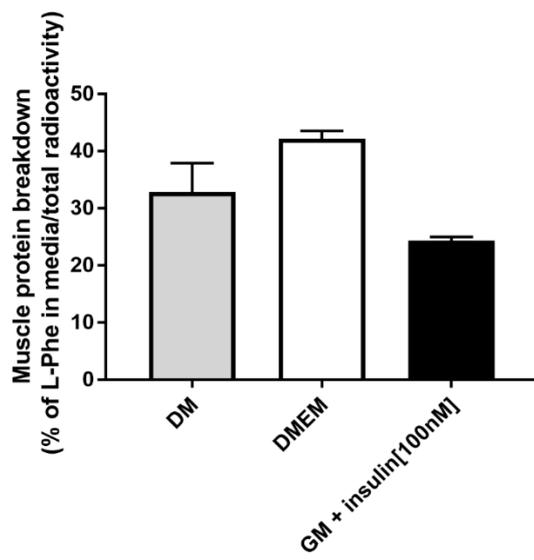


Figure S3. 3 Protein breakdown is sensitive to culture media.

Chapter 4: Palmitate induces insulin resistance despite conserved insulin driven PKB phosphorylation.

Jeromson S, Shaw A, Cruz AM, Beall C, Gallagher IJ, Hamilton DL

4.0. *Abstract*

Saturated fatty acids have been linked with the development of metabolic disease and insulin resistance. The saturated fatty acid PA is a precursor of ceramides, which attenuate insulin action through a reduction in PKB activity. Studies have shown that PAO, a monounsaturated fatty acid that differs by a single double bond, can prevent the deleterious effects of PA. Few studies have assessed the impact of palmitate or PAO on the lipid composition of skeletal muscle myotubes. The aims of this study was to assess the effects of PA and PAO on myotube lipid profiles, glucose uptake and mitochondrial respiration. C₂C₁₂ myotubes were treated with PA or PAO (750 μ M) for 16 hours. PA and PAO abundance increased significantly (181 \pm 10% and 470 \pm 41% respectively) after a 16 hour treatment with only modest effects on other fatty acid species. PA attenuated insulin dependent glucose uptake (Veh: 4.38 \pm 0.58 pmol/min/mg PA: 2.88 \pm 0.57 pmol/min/mg) but did not decrease insulin stimulated PKB phosphorylation. A dose response study (250,500 and 750 μ M) of the effects of PA and PAO on mitochondrial respirations revealed that at 250 μ M palmitate (Control: 135% of baseline, PA: 165.3%) and PAO (181.1% of baseline) increased maximal mitochondrial respiration. Fatty acid concentrations of 500 and 750 μ M decreased ATP synthesis coupled respiration and increased proton leak. These data demonstrate that at lower concentrations PA and PAO can improve mitochondrial function. Furthermore, attenuation of PKB activation may not be critical for the deleterious effects of PA.

4.1. Introduction

High fat diets are implicated in the onset of obesity and insulin resistance. In contrast to highly unsaturated long chain fatty acids, saturated fatty acids are associated with deleterious effect on a number of aspects of metabolism in skeletal muscle. In particular, PA (16:0) and PA derivatives are linked with the deterioration of insulin sensitivity. There is a large body of evidence from animal and tissue culture studies that implicate a causal role for saturated fat in the inhibition of aspects of skeletal muscle metabolic regulation [93], [244]. *In vivo* human studies, however, present a more ambiguous relationship between saturated fat intake and insulin sensitivity [145].

One of the central mechanisms linking PA to the attenuation of metabolic function lies in the conversion of PA to lipid intermediates such as ceramides and diacylglycerols (DAG) [157], [245], [246]. Both ceramides and DAGs act via different mechanisms but result in the inability of insulin to activate PKB, resulting in attenuated glucose uptake/glycogen synthesis [188]. DAGs act at the level of IRS/Pi3k, activating PKC isoforms which increase IRS serine phosphorylation, reducing IRS activation of PKB. While, ceramides inhibit PKB activation also by activating PKC isoforms and protein phosphatases (PP2A) which reduce phosphorylation of PKB [152], [159], [247]. Additionally, PA disrupts signalling cascades involved in inflammation and reactive oxygen species [149], [248]. Co-treatment of cells with both PA and PAO is sufficient to prevent PA driven metabolic dysfunction [188]. Moreover, PAO alone has been shown to improve basal glucose uptake. Thus, PAO may have intrinsic benefits other than counteracting the effects of PA. The effects of PA and PAO on insulin sensitivity has been investigated by multiple laboratories. However, few studies have measured the

accumulation of PA and PAO within myotubes following treatment and changes in the abundance of other fatty acid species.

Another factor that may impact on metabolic function and substrate utilisation, contributing to the accumulation of lipid intermediaries, is mitochondrial function. Prior studies have observed that muscle from obese individuals are more susceptible to the deleterious effects of PA than lean individuals [244]. This may be influenced by impaired mitochondrial function, reducing the ability to oxidise fat. Yet, insulin resistance may be characterised by excessive beta-oxidation [161]. However, high fat diets have been shown to increase mitochondrial biogenesis while concurrently inducing insulin resistance [136], [249]. This is thought to be due to increased CaMKII phosphorylation driven by an increase in reactive oxygen species [250]. Despite increases in mitochondrial content, incubation of muscle *in vitro* with PA at higher concentration (500-750 μ M) causes a decline in mitochondrial function [163], [164]. Another study observed that the effects of PA were dependent on whether mitochondrial function was assessed in myoblasts or myotubes [164]. Furthermore, insulin improves mitochondrial function by reducing proton leak and this effect is prevented by PA [163]. These studies all used concentrations of PA ranging between 500-750 μ M. *Woodsworth-hobbs & colleagues* [178] observed that PKB inhibition increases with increasing PA. It remains to be seen whether a similar effect on mitochondrial dysfunction occurs with lower doses of PA.

The aims of this study were to establish how PA and PAO, differing in structure by one double bond, differentially altered lipid profiles and how PA or PAO altered insulin stimulated glucose uptake and PKB phosphorylation. In addition, we aimed to assess a

dose response effect of fatty acids on multiple parameters of mitochondrial function at three different doses (250 μ M, 500 μ M, 750 μ M). We hypothesise that the incorporation of PA into myotubes will compromise insulin sensitive glucose and PKB phosphorylation in addition to impairing mitochondrial function. We hypothesised that the addition of PAO would not however have the same deleterious effect on glucose uptake.

4.2. *Materials and methods*

4.2.1. *Cell culture*

C₂C₁₂ myoblasts were grown in Dulbecco's Modified Eagle Medium (DMEM) containing 20% foetal bovine serum and 1% penicillin/streptomycin and incubated at 37°C and 5% CO₂. Myoblasts were maintained at ~60% confluence. Differentiation was induced once confluence reached 80-90% by changing the media to differentiation media (DMEM supplemented with 2% horse serum and 1% Penicillin/Streptomycin) for 5-6 days. Following differentiation, myotubes were treated with 750 μ M PA or PAO for 16 hours. For fatty acid preparation, Palmitate (PA) or Palmitoleate (PAO) were dissolved in 100% ethanol at a stock solution of 100mM. Fatty acids were then added to the appropriate treatment media + 2% BSA to a working solution. Fatty acid-BSA complexes were prepared by constant vigorous shaking at 37°C for 1 hour prior to use in experiments.

4.2.2. *FAME analysis*

Total lipids were extracted by homogenising in 20 volumes of chloroform/methanol (2:1 v/v). Total lipids were prepared according to the method of Folch et al. [221] and non-lipid impurities were removed by washing with 0.88% (w/v) KCl. The weight of lipids was determined gravimetrically after evaporation of solvent and overnight desiccation under vacuum. Fatty acid methyl esters (FAME) were prepared by acid-

catalysed transesterification of total lipids according to the method of Christie et al. Extraction and purification of FAME was performed as described by Ghioni et al. (36). FAME were separated by gas-liquid chromatography using a ThermoFisher Trace GC 2000 (ThermoFisher, Hemel Hempstead, UK) equipped with a fused silica capillary column (ZBWax, 60m x 0.32 x 0.25 mm i.d.; Phenomenex, Macclesfield, UK) with hydrogen as carrier gas and using on-column injection. The temperature gradient was from 50 to 150°C at 40°C/min and then to 195°C at 1.5°C/min and finally to 220°C at 2°C/min. Individual methyl esters were identified by reference to published data (Ackman, 1980). Data were collected and processed using the Chromcard for Windows (version 2.00) computer package (Thermoquest Italia S.p.A., Milan, Italy). All experiments were carried out in duplicate from 4 independent experiments.

4.2.3. *Glucose uptake*

C₂C₁₂ myotubes, were exposed to 750µM PA or PAO complexed with 2% fatty acid free BSA or 2% fatty acid free BSA as a control for 48 h before a 2 hour serum-starve. Following the 2 hour serum starve cells were exposed to insulin (100 nmol/l) or vehicle control for 30 mins. Myotubes were incubated (12 min) with 10 µmol/l 2-deoxy-D-[3 H]glucose (2DG; 24.4 kBq/ml; Hartman Analytic) at 20°C. Non-specific uptake was determined using 10 µmol/l cytochalasin B (Sigma–Aldrich). After lysis, cell-associated radioactivity was measured (Beckman, High Wycombe, UK; LS 6000IC scintillation counter), and protein was quantified using the Bradford protein assay. Data represented are the average of 4 independent experiments carried out in duplicate.

4.2.4. *Cell respiration*

C₂C₁₂ myoblasts were cultured in XFe96 microplates at a density of 1600 cells/well. Differentiation was induced once cells had reached 80-90% confluence and media was

changed daily for 5 days, with myotubes being used for experiments on day 6. Cells were treated with 250 μ M, 500 μ M or 750 μ M PA or PAO in 1x Earle's buffered saline solution (5.5 mM glucose) for 16 hours. Myotubes were placed in base XF medium (5mM glucose, 2mM L-glutamine, 2.5mM sodium pyruvate and the pH adjusted (with sodium hydroxide) to 7.4 at 37°C) and supplemented with PA or PAO. The plate was then transferred to a non-CO₂ incubator for 1 hour. Following basal measures of oxygen consumption Oligomycin (1 μ M), FCCP (2 μ M) and antimycin A/rotenone (1 μ M) were added sequentially. The data presented is the average of 4 cycles after each mitochondrial manipulation. Acute changes in oxygen consumption were assessed following the addition of either PA or PAO at the indicated concentrations. Cells were then lysed with 50mM NaOH and protein content determined by the Bradford method. Oxygen consumption rates are presented as pmol/min/mg.

4.2.5. *Cell processing*

Cell lysates were collected from 6 well plates by scraping on ice in RIPA buffer [50 mmol/l Tris·HCl pH 7.5, 50 mmol/l NaF, 500 mmol/l NaCl, 1 mmol/l sodium vanadate, 1 mmol/l EDTA, 1% (vol/vol) Triton X-100, 5 mmol/l sodium pyrophosphate, 0.27 mmol/l sucrose, and 0.1% (vol/vol) 2-mercaptoethanol and Complete protease inhibitor cocktail (Roche)] followed by snap freezing on liquid nitrogen. For preparation for western blotting samples were thawed and debris was removed by centrifugation at 4°C for 15 min at 13,000 g. The supernatant was then removed, and protein concentration was determined using the DC protein assay according to the manufacturer's instructions (Bio-Rad, Hercules,CA).

4.2.6. *Western blotting*

For WB, 100µg of supernatant was made up in Lamelli sample buffer, and 15 µg of total protein was loaded per well and run at 150 V for 1 h 15 min. Proteins were then transferred onto Whatman Immunobilon Nitrocellulose membranes (Fisher Scientific, Loughborough, UK) at 30 V for 2 h 15 min. Membranes were blocked in 3% BSA-Tris-buffered saline (containing vol/vol 0.1% Tween 20) for 1 h at room temperature, followed by incubation in primary antibodies [PKBthr308 (#2965), total PKB (#4691) (New England Biolabs) or OXPHOS mitoprofile at 4°C overnight. Membranes underwent three 5 min washes in TBST followed by incubation in the appropriate secondary antibodies [secondary horseradish peroxidase conjugated antibody was purchased from ABCAM (#6721)] for 1 h at room temperature. Membranes were again washed three times for 5 min followed by incubation in enhanced chemiluminescence reagent (BioRad, Herts, UK). A BioRad ChemiDoc (Herts, UK) was used to visualize and quantify protein expression. Phospho proteins were normalized to the corresponding total protein. Data are representative of 3 independent experiments carried out in duplicate.

4.2.7. *Data analysis and statistics*

Statistical analyses were carried out in GraphpadPrism v 7.0. Changes in lipid species were determined by two-way ANOVA followed by a bonferonni multiple comparisons test. Differentially changed lipid species were assessed by multiple t-tests corrected for false discovery rate (5%). A two-way ANOVA was used to determine the success of manipulating mitochondrial respiration. Changes in oxygen consumption rate in each mitochondrial state between groups at each concentration used were determined by a one-way ANOVA followed by a bonferonni multiple comparisons test where appropriate.

4.3. *Results*

4.3.1. Palmitate and Palmitoleate exert similar effects on lipid profiles

To assess incorporation of PA and PAO into muscle lipids, myotubes were collected following a 16 hour incubation and lipid profiles analysed by FAME analysis. Total saturated fat content was significantly increased following PA treatment ($p < 0.05$) while also decreasing monounsaturated species ($p < 0.05$) (**Figure 4.1A**). The increase in total saturated lipid content was driven primarily by an increase in PAO alone (% change: $181\% \pm 10\%$, $P < 0.0001$) (**Figure 4.1B**). Although total MUFA species were decreased, PA actually increased the content of PAO, albeit modestly. Treatment of myotubes with PAO significantly increased total MUFA content and reduced saturated fatty acid content (**Figure 4.1A**). Again, the increase in MUFA was due to a substantial increase in PAO content (% change: $470\% \pm 41$, $P < 0.0001$) (**Figure 4.1B**). Despite the larger relative change in PAO content, PA composed a larger proportion of total fatty acids in the lipid pool. This is largely determined by the greater PA content prior to incubation with PA or PAO. Given that changes in Saturated or MUFA content were primarily driven by changes only in the treatment fatty acids suggests that PA or PAO are not readily converted to other fatty acid species. The different relative changes in relative changes of PA and PAO content treatment groups may be explained by different rates of uptake or oxidation.

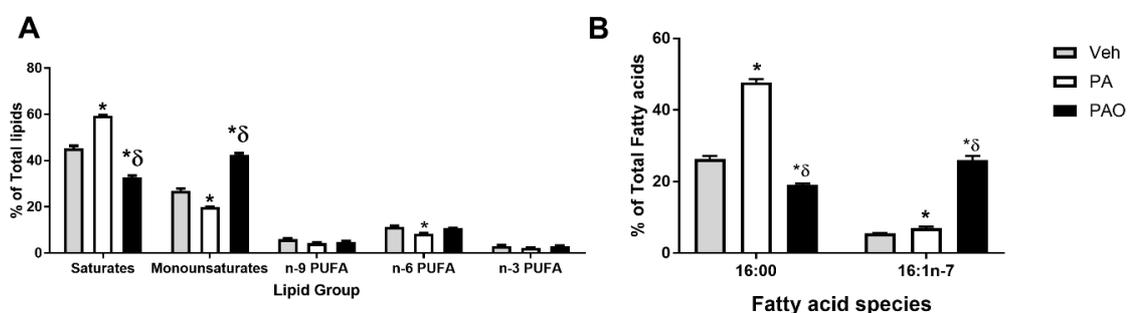


Figure 4. 1 Palmitate and Palmitoleate induced lipid profiles in C₂C₁₂ myotubes. **A.** Incorporation of PA and PAO were assessed by FAME analysis. Data is expressed as % of lipid pool and representative of $n=3$ in duplicate. **B** Relative change in PA(16:00) and PA (16.1n-7) abundance. * denotes significantly different from control and treatment group. δ indicates significant difference between PA and PAO.

To determine differential changes in individual fatty acid species following PA or PAO treatments, log₂ fold changes were plotted and divergent responses assessed by multiple t-tests (**Figure 4.2**). The only differential response between the two fatty acids were the expected increases in PA and PAO in each respective treatment group. Both PA and PAO decreased a number of saturated and monounsaturated fatty acids and polyunsaturated fatty acids. Given the high concentrations of PA and PAO used [750µM] it is likely that the decreases in a number of fatty acid species are due to reduced uptake of these fatty acids from the cell media.

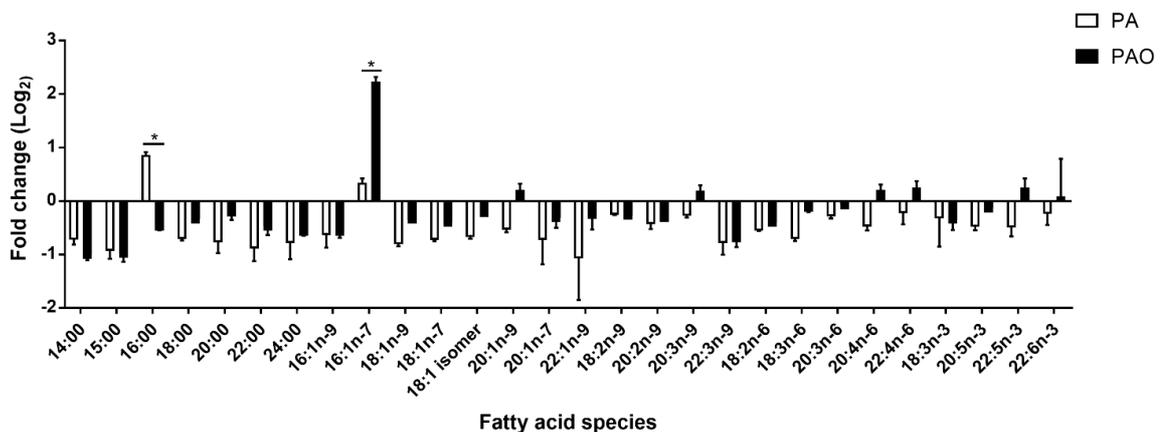


Figure 4. 2. Differential changes in fatty acids species following PA or PAO incorporation. Changes in fatty abundance were plotted as log₂ fold to assess divergent changes in fatty acid species. * denotes significant difference between groups. Data are representative of n=3 (in duplicate).

4.3.2. Palmitate causes insulin resistance despite normal PKB phosphorylation

Insulin sensitivity was assessed by measuring glucose uptake and PKB phosphorylation following treatment with either PA or PAO. PA or PAO did not have any effect on basal glucose uptake. PA attenuated the increase in glucose uptake driven by insulin while myotubes treated with PAO were unaffected ($p < 0.05$) (**Figure 4.3A**). The attenuation of PKB phosphorylation in response to insulin is often associated with a reduction in glucose uptake. In this study PKB^(thr308) phosphorylation (basal and

insulin stimulated) was unchanged by PA or PAO despite the loss of insulin sensitive glucose uptake in the PA condition (**Figure 4.3B**).

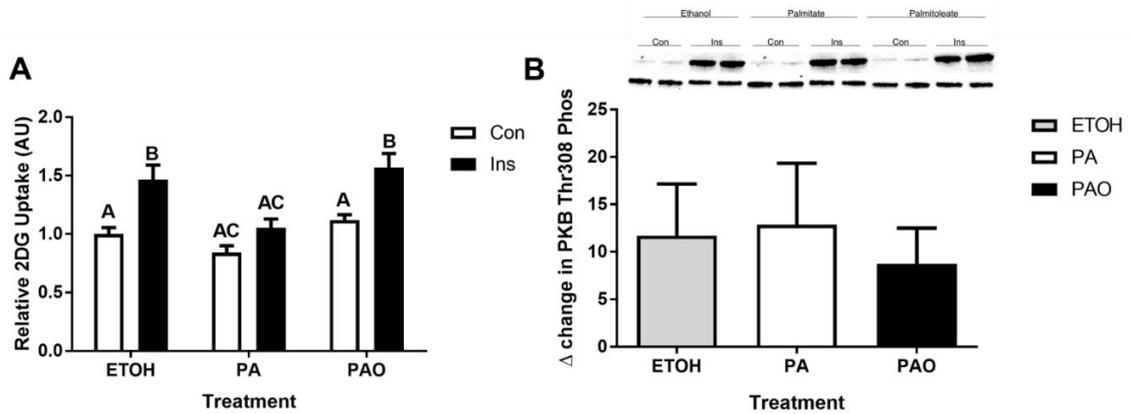


Figure 4. 3 Palmitate inhibits insulin sensitive glucose uptake with no change in PKB phosphorylation. A) glucose uptake was measured by uptake of ^3H -Deoxyglucose. Data is representative of n=6 (in duplicate). Bars that do not share a letter are considered significantly different. B) PKB(thr308) phosphorylation was assessed by western blotting. Data are representative of n=3 (in duplicate).

4.3.3. Palmitate disrupts mitochondrial function

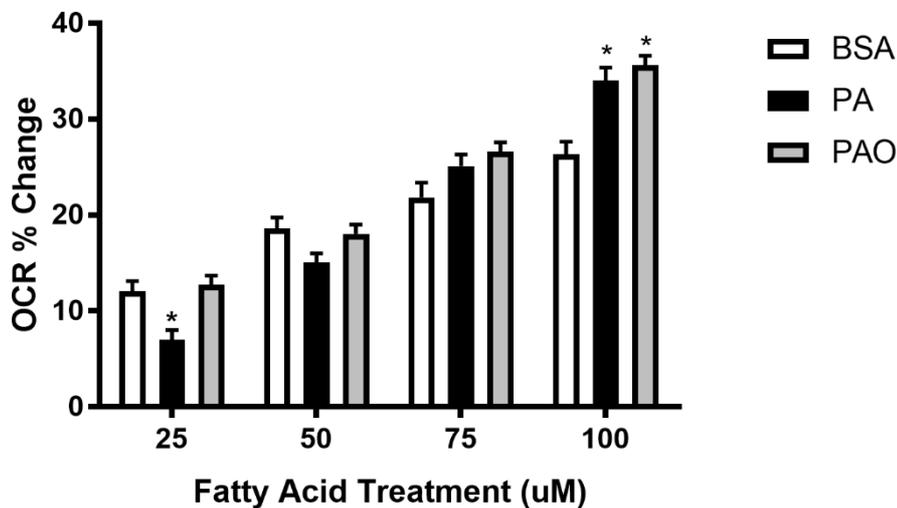


Figure 4. 4. Acute changes in oxygen consumption. Changes in the rate of oxygen consumption were measured immediately after exposure to varying doses of PA and PAO. Data are representative of n=20. * denotes significant difference from control (p-values < 0.05).

Both PA and PAO were administered at the same concentration yet the capacity to exert effects on cell metabolism may be related to how readily oxidised each fatty acid is. The intracellular concentration of fatty acids will reflect the balance between uptake and

oxidation. Measurement of oxygen consumption after the acute exposure reflects the oxidation of exogenous free fatty acids/uncoupling of respiration. A dose response relationship was assessed in OCR changes following the additions of PA or PAO up to a concentration of 100 μ M (**Figure 4.4**). A significant % change in OCR in BSA and PAO groups was observed compared to PA ($p < 0.05$, 5.7 mean % change between PA: PAO) was observed at 25 μ M. At 100 μ M, oxygen consumption was increased by both PA and PAO above that of the BSA control suggesting an increased oxidation of exogenous fatty acids. However, no differences between PA and PAO were observed.

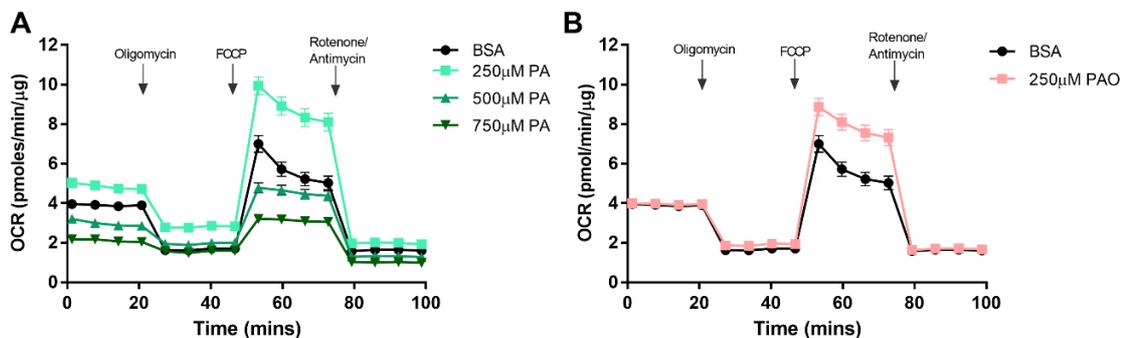


Figure 4.5. Mitochondrial oxygen consumption traces. C₂C₁₂ myotube oxidative capacity was assessed using a seahorse bioanalyser and mitochondrial stress test. A) Changes in mitochondrial respiration following PA treatment. B) Mitochondrial respiration following PAO treatment.

We next assessed how a 16 hour incubation with PA and PAO altered parameters of mitochondrial function. Following treatment of C₂C₁₂ myotubes with 250 μ M, 500 μ M and 750 μ M PA or PAO for 16 hours mitochondrial function was assessed using the XF Seahorse mito stress test. Due to technical difficulties we were unable to obtain readings for mitochondrial function at doses of 500 and 750 μ M PAO. PA caused the progressive decline in mitochondrial function with increasing concentration (**Figure 4.5A**). Oxygen consumption related to ATP synthesis was reduced by all doses with concomitant increases in proton leak (**Figure 4.6A, E**). The most deleterious effects were observed at doses of 500 and 750 μ M PA. Maximal respiration was reduced, as a

result, mitochondria had very little spare respiratory capacity. Interestingly, 250 μ M PA actually increased maximal respiration and spare respiratory capacity. Similarly, 250 μ M PAO also enhanced maximal respiration (**Figure 4.5B**). Unlike PA, PAO did not alter ATP synthase dependent OCR indicating that PAO was not negatively affecting mitochondrial function. There was a significant increase in proton leak in the PAO group, however, this was a small increase and is unlikely to have major effects on mitochondrial function.

Abundances of proteins in the electron transport chain were assessed by western blot to assess if the reduction in ATP synthase activity was due to a decreased abundance of critical proteins (**Figure 4.6**). The protein abundances of ATP synthase (subunit alpha) and a complex 3 protein UQCRC2 were unaltered by either fatty acid treatment. This suggests either an intrinsic defect with the energy producing machinery or a reduced attenuation of energy consuming processes reducing energy demand for metabolic processes.

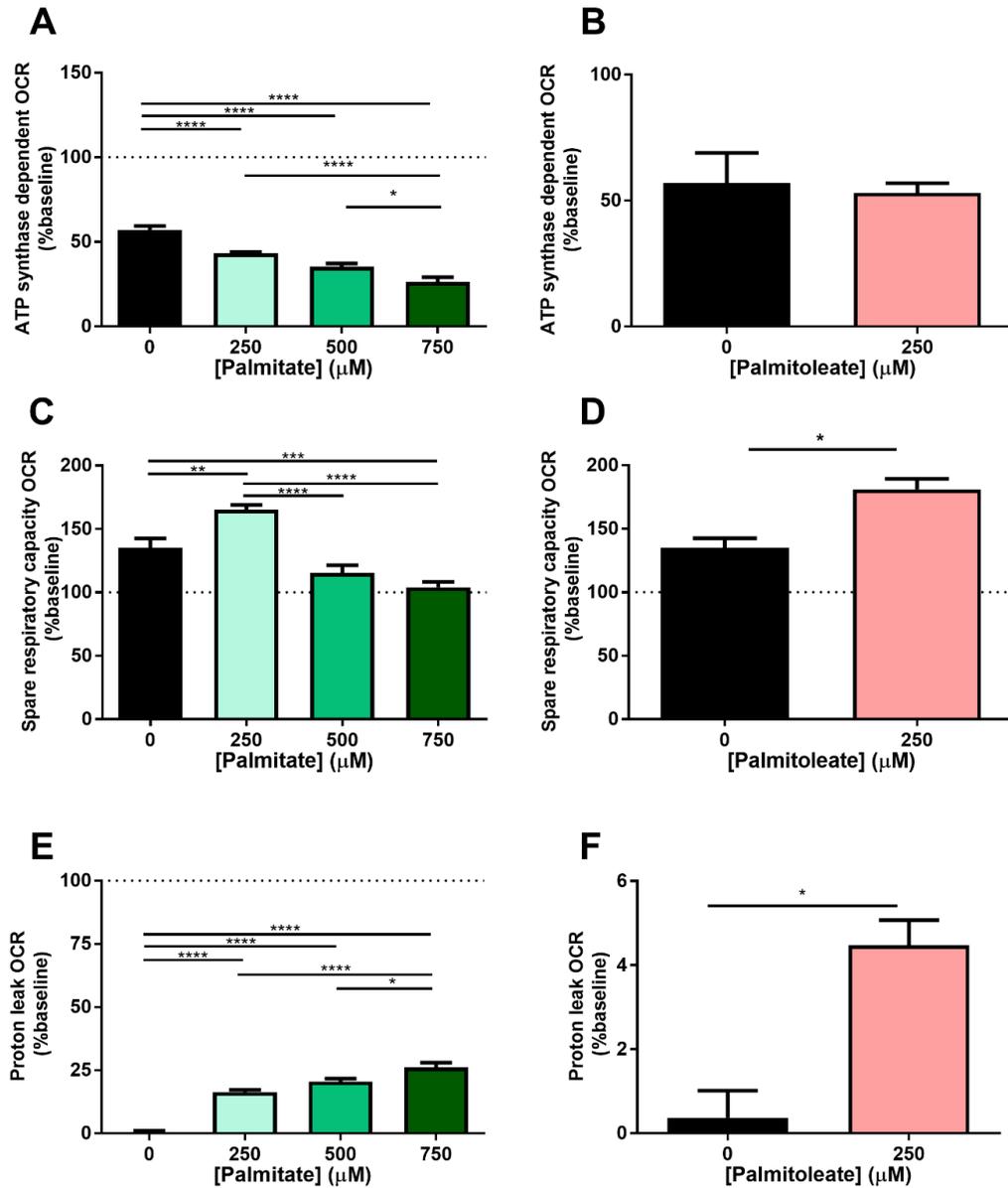


Figure 4.6. Effects of Palmitate and Palmitoleate on parameters of mitochondrial function. (A,C,E) effect of Palmitate on ATP synthesis, spare respiratory capacity and proton leak, respectively. (B,D,F) Effect of Palmitoleate on ATP synthase, spare respiratory capacity and proton leak, respectively. Data are representative of n=26-28. Data is presented as % of baseline OCR. Number of * denotes smaller p value.

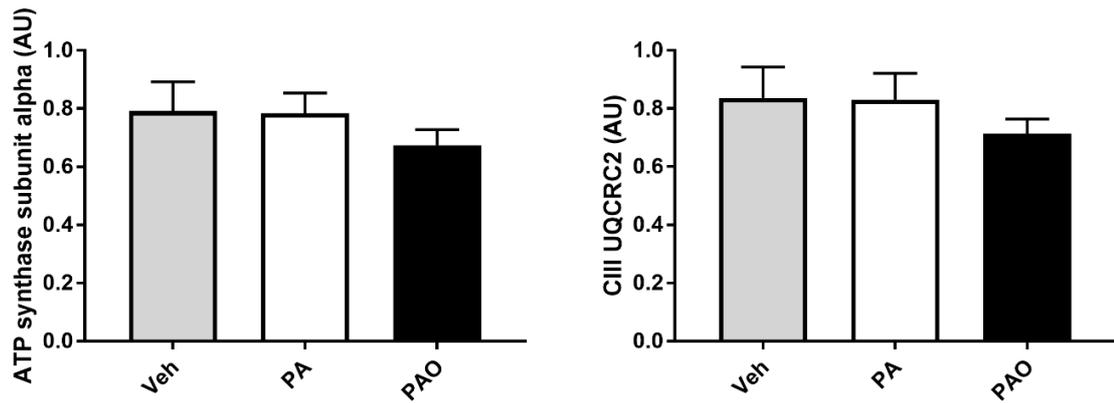


Figure 4. 7 . Protein abundances of electron transport chain complexes. A – ATP synthase alpha (Complex V). B – UQCRC2 (Complex III). Data are representative of n=3 in duplicate. Data are presented as mean \pm S.E.M

4.4. Discussion

This study demonstrates that incubation of C₂C₁₂ myotubes with 750 μ M PA or PAO leads to a significant incorporation of these fatty acids into the lipid pool and results in divergent metabolic responses. One of the key findings of this study demonstrated that PA can suppress insulin stimulated glucose uptake without attenuating PKB phosphorylation. Basal glucose uptake was not altered by PA but insulin failed to stimulate an increase in glucose uptake. PAO did not alter glucose uptake or PKB phosphorylation. Interestingly, assessment of mitochondrial function following PA or PAO treatment revealed that at 250 μ M, maximal and spare respiratory capacity were both increased. At higher doses (500-750 μ M) PA increased proton leak significantly. This disruption in mitochondrial function by PA may play a role in reducing energy availability and reducing the myotubes capacity to modify glucose uptake appropriately in response to insulin.

We observed that both PA and PAO were significantly incorporated into the myotube lipid pool following a 16 hour incubation with a 750 μ M bolus of either PA or PAO.

Compared to previous chapters, neither fatty acid induced substantial changes in the abundance of other saturated and unsaturated fatty acid species. The only differentially altered fatty acids were the expected changes in PA and PAO. Myotubes were treated with PA and PAO in PBS + 5mM glucose, meaning no other fatty acids were present in the media. Given the treatment media and lack of substantial changes in lipid profiles, the data suggests that neither PA or PA is readily converted to other fatty acids through elongation and unsaturation reactions. The change in abundance likely reflects the balance between uptake and oxidation. PA is the most abundant fatty acids in C2C12 myotubes and PA abundance doubled following treatment. PAO had a lower concentration in the vehicle group but increased to a greater relative extent following PAO treatment than PA. The different changes in abundance following treatment using the same fatty acid concentrations may be explained by differential changes in oxidation of PA and PAO. To assess potential differences in oxidation we measured acute changes in oxygen consumption following the addition of increasing doses of either PA or PAO. The only difference in OCR between PA and PAO was observed at 25 μ M. At doses of 100 μ M both PA and PAO increased OCR above control but no difference was detected between PA and PAO. These data suggest that oxidation of exogenous PA and PAO are similar in C₂C₁₂ myotubes. Fatty acid uptake is largely mediated by transporter proteins, given the media used, the uptake of PA or PAO would not be limited by competitive uptake [251]. A recent study has demonstrated that addition of the unsaturated fatty acid ALA to the diet alters the abundance of fatty acid transporters at the membrane in mouse skeletal muscle [252]. It has yet to be investigated if MUFAs such as PAO exert similar effects on fatty acid transporter membrane localisation.

Following the addition of PA we observed that insulin stimulated glucose uptake was attenuated with no effect of PA on basal glucose uptake. This finding is in agreement with a number of previous studies which observed a deleterious effect of 750 μ M PA on insulin stimulated glucose uptake [151], [188]. In contrast to these studies, however, we did not observe any suppression in insulin stimulated PKB phosphorylation by PA. This is a curious finding as PKB activation by insulin and downstream signalling is key for initiating the translocation of GLUT4 containing vesicles to the membrane. The lack of suppression of PKB phosphorylation would also suggest that in our model PA did not activate PKC isoforms or PP2A which inhibit PKB activation either directly or by inhibiting upstream signalling. This led us to speculate that the deleterious effects of PA may lie downstream of PKB. PKB phosphorylates AS160 and inhibits its GTPase activity resulting in the movement of GLUT4 vesicles to the myotube membrane. Under the experimental conditions used, PA may act directly on AS160. Alternatively, the inhibition of glucose uptake in response to insulin may be mediated by an increased saturation of phospholipids. Although we did not directly measure PA content in phospholipids, the large rise in PA content would likely mean an increased incorporation in phospholipids. Increasing phospholipid saturation would cause the denser packing of phospholipids and a more gel-like membrane structure. The denser packing would decrease membrane deformability and interfere with the fusion of GLUT4 vesicles with the membrane [253]. More rigid packing could also interfere with the lateral diffusion of GLUT4 molecules along the membrane and their pore forming activity. If downstream PKB signalling is intact, then this may explain the loss of insulin stimulated glucose uptake. More comprehensive profiling of PKB pathway activation will be necessary to corroborate this hypothesis.

While many previous studies observe attenuated PKB phosphorylation, the PKB response to insulin is not completely ablated. To the author's knowledge, only Woodworth-hobbs et al (2014) demonstrate complete suppression of PKB phosphorylation at 750 μ M PA [178]. For instance, Dimopolous & colleagues (2006) observe an increase in PKB phosphorylation after insulin stimulation, albeit reduced compared to the control group [188]. Given that GLUT4 abundance at the membrane saturates at 5% of maximal insulin stimulated PKB phosphorylation, the suppression of PKB activation by PA may not be the driver of decreased glucose uptake [254]. Furthermore, PA does not induce insulin resistance when co-incubated with a lipid mixture ([148]). These data question whether a reduction in PKB activation is critical to the disruption of GLUT4 translocation to the membrane induced by PA. Since we did not observe an interaction between reduced PKB phosphorylation and glucose uptake, the mechanism by which glucose uptake is suppressed may lie downstream of PKB or intrinsic defects in glucose transport.

PA is known to induce reactive oxygen species production, of which mitochondria are a major source of production [255]. There is now available data that demonstrate the effect of individual fatty acids on multiple parameters of mitochondrial function [163], [164]. We utilised the Seahorse analyser to perform a mitochondrial stress test following pre-treatment with 3 different doses of PA and PAO (250 μ M, 500 μ M, 750 μ M). Technical issues prevented the assessment of PAO at doses above 250 μ M. We observed the progressive decline in mitochondrial function with increasing doses of PA. In particular, PA caused a large increase in mitochondrial proton leak. Increased proton leak can lead to the increase in reactive oxygen species. Reactive oxygen species have been linked with a reduction in glucose uptake[256]. Our results are in agreement with previous findings in cultured skeletal muscle myotubes that PA

reduced ATP synthesis related oxygen consumption, with a concomitant increase in proton leak [163]. Although not directly measured, we predict the ATP availability would be reduced following PA treatment. In contrast, other studies have observed that PA only altered mitochondrial respiration when C₂C₁₂ cells were in the myoblast stage [164]. Reconciling experimental differences is difficult but the contrasting results may be due to differences in mitochondrial capability and basal energy demand. Unlike PA, PAO (250 μ M) induced a significant but small rise in proton leak that did not compromise ATP linked oxygen consumption. We speculate, however, that at higher doses a decline in mitochondrial oxygen consumption would occur. Interestingly, at the lowest dose used (250 μ M) both PA and PAO increased maximal respiration and therefore spare respiratory capacity. The increase in maximal oxygen consumption could not be fully explained by increasing proton leak. Myotubes would treated with these relatively lower doses of fatty acids would therefore likely have an increased capacity to alter metabolic rate in response to cellular stress. While the mechanism underlying the increase in maximal respiratory capacity is poorly understood it may not be related to changes in mitochondrial abundance. The protein content of proteins involved in oxidative phosphorylation were unchanged by PA or PAO treatment. The increase in maximal capacity may be related to changes in mitochondrial fission/fusion.

Insulin stimulated glucose uptake requires the translocation of GLUT4 containing vesicles and fusion of those vesicles with the membrane [257]. Membrane fusion is a multistep process involving the movement, docking and fusion with the membrane, key to this process are SNARE proteins [258], [259]. Multiple SNARE complexes are present in each vesicle fusion event and requires approximately 10 ATP molecules per SNARE complex. Extrapolating to the large number of vesicles containing GLUT4, this

would be an energetically costly process [260], [261]. With mitochondrial function being severely compromised at 750 μ M PA, vesicle movement and fusion may have been compromised. The signal for vesicle translocation lies downstream of PKB so would potentially explain how glucose uptake is attenuated despite PKB phosphorylation being unaffected.

In summary, we show that both PA and PAO are significantly incorporated into the lipid pool but only PA is associated with the reduction in insulin stimulated glucose uptake. Both basal and insulin stimulated PKB phosphorylation were unaffected by fatty acid treatment. Instead, we speculate that compromised mitochondrial function or altered phospholipid compositions may disrupt insulin stimulated glucose uptake. Our data is in contrast to a number of previous studies. The major difference between this study and others is the treatment media used. Prior data from our lab showed that PA driven insulin resistance was only detectable when PBS+ 5mM glucose was used as a treatment media. Other substrates in the media may influence the effect of fatty acids on myotube metabolism. Given the significant disruption of metabolic processes by PA but not PAO, we next sought to examine the effect of these fatty acids on protein metabolism and proteomic profile to further understand the metabolic changes.

Chapter 5: Palmitate attenuates protein synthesis despite an increase in anabolic signalling.

Jeromson S, Shaw A, Martin CV, Gallagher IJ, Hamilton DL

5.0. *Abstract*

Maintenance of skeletal muscle mass is critical for metabolic health and activity. Obesity and ectopic accumulation of lipids within skeletal muscle is associated with a reduction in the MPS response to insulin and amino acids. There is still little understanding in how individual fatty acids can alter muscle anabolism. PA and PAO differ in structure by a single double bond but there is evidence to suggest that these fatty acids may have different effects on protein turnover. The primary aim of this study was to study the effect of incorporation of PA or PAO on protein synthesis, breakdown, anabolic signalling and shifts in protein expression in C₂C₁₂ myotubes. Treatment of C₂C₁₂ myotubes with PA resulted in attenuated basal protein synthesis ($62.9 \pm 12.6\%$ of control) while PAO did not have a significant effect ($79.2 \pm 15\%$ compared to vehicle). Rates of protein breakdown were unaltered by PA or PAO treatment. Assessment of anabolic signalling revealed that PA increased phosphorylation of ribosomal protein S6. Neither PA nor PAO affected measures of endoplasmic reticulum stress. Proteomic analysis also revealed that PA and PAO differentially alter the proteomic profile of skeletal muscle. PA and PAO altered the expression of proteins involved in purine nucleotide synthesis and cell-cell adhesion. These results show that PA attenuated protein synthesis despite an increase in anabolic signalling and no change in endoplasmic reticulum stress. Despite being very similar in structure PA and PAO have differential effects on protein turnover and protein expression.

5.1. Introduction

Skeletal muscle mass maintenance is determined by the balance between protein synthesis and breakdown. Maintaining muscle mass is crucial for locomotion, metabolic health and longevity [262]. Conditions such as obesity and type 2 diabetes have been associated with the accelerated loss of muscle mass [263], [264]. Dietary fat has been implicated as playing a role in attenuated protein anabolism. Ectopic lipid accumulation within skeletal muscle is thought to contribute to the attenuation of muscle anabolism. In rodents, fatty acid accumulation impairs activation of signalling pathways and subsequently attenuates protein synthesis [202],[203]. Moreover, obese individuals display an attenuated MPS response to elevations in plasma insulin and amino acids [165], [166]. Activity of protein kinases within skeletal muscle is sensitive to regulation by different fatty acids [59], [169]. In young healthy men, an acute lipid infusion impaired the MPS response to insulin and amino acids [265]. This suppression of MPS was associated with the downregulation of 4E-BP1 phosphorylation with increasing amino acid concentration. However, not all studies observe an effect of fat on protein synthesis. *Katsanos & colleagues (2011)* [166] did not observe any effect of a high fat infusion on rates of MPS. Thus, there is still not a strong consensus on how fat or individual fatty acids alter protein synthesis in humans. Given, that saturated fat is linked with a negative effect on insulin action, it is logical to hypothesise that saturated fat could also causally disrupt in protein turnover.

There is evidence from *in vitro* models that PA can alter multiple aspects of cellular function that would serve to suppress protein synthesis. PA is known to inhibit glucose uptake and this effect extends to other substrates, such as amino acids [172]). System A transport facilitates the sodium dependant uptake of neutral amino acids [266].

Exogenous provision of C2 ceramide reduces system A mediated amino acid transport, an effect mimicked by PA [172]. This may lead to a reduction in amino acid availability for incorporation into peptide chains. Furthermore, PA can induce ER stress which leads to the phosphorylation of Eif2 α (Ser51) inhibiting translation initiation [173], [175]. Similar to insulin sensitivity, unsaturated fatty acids do not have the same effect. It has yet to be investigated if the structurally similar fatty acid PAO has the same effect on protein metabolism as PA. As well as altering the synthesis of proteins, fatty acids can also affect the rate at which proteins are broken down. PA inhibition prevents the sequestering of FoxO proteins within the cytoplasm by PKB. As a result, FoxO proteins translocate to the nucleus and initiate expression of atrogenes; Murf1, atrogen-1 and Bnip3 [67], [178]. Furthermore, a 48 hour incubation with PA resulted in decreased muscle myotube diameter, likely linked to the increased protein degradation of muscle structural proteins [179]. This shift in protein metabolism following PA treatment has been shown to alter the expression of a number of proteins involved in cellular processes such as lipid metabolism, protein synthesis and ER stress [267]. The co-incubation of PA with oleate can counteract the changes in protein expression caused by PA. Given that saturated fatty acids and polyunsaturated fatty acids can differentially alter protein expression it is likely that monounsaturated fats will have independent effects on the abundance of proteins. The alteration in the proteome profile may contribute to the understanding of how different fatty acids differentially alter skeletal muscle cellular function.

PA has the potential to influence multiple aspects of protein turnover. Thus, PA may induce a shift in the proteomic signature of individual protein abundance. This shift in protein abundance could alter the metabolic phenotype of skeletal muscle which would serve to alter changes in metabolism in response to intra/extracellular stimuli.

Previous studies have only assessed the proteome following PA alone and co-incubation with oleate treatments [267]. We therefore aimed to investigate the proteomic profile in response to PA and PAO using SILAC labelling. Furthermore, we investigated the effects of different fatty acid species on measures of protein synthesis and breakdown and associated signalling. We hypothesised that PA would induce a negative protein balance by altering protein synthesis and breakdown, while PAO would display a neutral effect.

5.2. *Materials and methods*

5.2.1. *Cell culture*

C₂C₁₂ myoblasts were grown in Dulbecco's Modified Eagle Medium (DMEM) containing 20% foetal bovine serum and 1% penicillin/streptomycin and incubated at 37°C and 5% CO₂. Myoblasts were maintained at ~60% confluence. Differentiation was induced once confluence reached 80-90% by changing the media to differentiation media (DMEM supplemented with 2% horse serum and 1% Penicillin/Streptomycin) for 5-6 days. Following differentiation, myotubes were treated with 750µM PA or Palmitoleate for 16 hours. For fatty acid preparation, Palmitate (PA) or Palmitoleate (PAO) were dissolved in 100% ethanol at a stock solution of 100mM. Fatty acids were then added to the appropriate treatment media + 2% BSA to a working solution. Fatty acid-BSA complexes were prepared by constant vigorous shaking at 37°C for 1 hour prior to use in experiments.

5.2.2. *Cell processing*

Cell lysates were collected from 6 well plates by scraping on ice in RIPA buffer [50 mmol/l Tris·HCl pH 7.5, 50 mmol/l NaF, 500 mmol/l NaCl, 1 mmol/l sodium vanadate, 1 mmol/l EDTA, 1% (vol/vol) Triton X-100, 5 mmol/l sodium pyrophosphate, 0.27

mmol/l sucrose, and 0.1% (vol/vol) 2-mercaptoethanol and Complete protease inhibitor cocktail (Roche)] followed by snap freezing on liquid nitrogen. For preparation for western blotting samples were thawed and debris was removed by centrifugation at 4°C for 15 min at 13,000 g. The supernatant was then removed, and protein concentration was determined using the DC protein assay according to the manufacturer's instructions (Bio-Rad, Hercules, CA).

5.2.3. *Western blotting*

For western blotting, 100µg of supernatant was made up in Lamelli sample buffer, and 15 µg of total protein was loaded per well and run at 150 V for 1 h 15 min. Proteins were then transferred onto Whatman Immobilon Nitrocellulose membranes (Fisher Scientific, Loughborough, UK) at 30 V for 2 h 15 min. Membranes were blocked in 3% BSA-Tris-buffered saline (containing vol/vol 0.1% Tween 20) for 1 h at room temperature, followed by incubation in primary antibodies [PKBthr308 (#2965), total PKB (#4691) (New England Biolabs) or OXPHOS mitoprofile at 4°C overnight. Membranes underwent three 5 min washes in TBST followed by incubation in the appropriate secondary antibodies [secondary horseradish peroxidase conjugated antibody was purchased from ABCAM (#6721)] for 1 h at room temperature. Membranes were again washed three times for 5 min followed by incubation in enhanced chemiluminescence reagent (BioRad, Herts, UK). A BioRad ChemiDoc (Herts, UK) was used to visualize and quantify protein expression. Phospho proteins were normalized to the corresponding total protein. Data are representative of 3 independent experiments carried out in duplicate.

5.2.4. *Muscle protein synthesis and breakdown*

Protein degradation was assessed by the quantification of the released L-[2,4,³H] phenylalanine into the culture media. Following 4 days of differentiation myotubes were incubated with medium containing 2.5 μ Ci L-[2,4,³H] phenylalanine/ml and the label was maintained for 24 hours in order to label long lived proteins. Following the pulse the cells were washed 2x in PBS and incubated in cold chase media (DMEM + 2mM L-phenylalanine) for 3 hours to allow for degradation of short lived proteins. Myotubes were then treated with either 750 μ M PA or PAO (PBS+ 5mM glucose, 2mM L-phenylalanine, 2% FAF-BSA) for 24 hours. Following treatment an aliquot of the media was removed and radioactivity released was assessed by scintillation counting. The remaining myotubes were then thoroughly washed with ice cold saline (0.9%) and lysed with 50mM NaOH + 1% SDS for a minimum of 30 mins at room temperature. Residual radioactivity in cell lysates was then assessed by scintillation counting. Total radioactivity was calculated as the sum of the L-[2,4,³H] phenylalanine released into the media and the residual cell retained L-[2,4,³H] phenylalanine. Protein breakdown is presented as the fraction of the total incorporated L-[2,4,³H] phenylalanine released into the media.

Basal protein synthesis was assessed by the incorporation of L-[2,4,³H] phenylalanine into peptide chains. Following differentiation, myotubes were treated with 750 μ M PA or PAO for 16 hours (PBS + 5mM glucose). DMEM + fatty acids were used for the last 2 hours of the fatty acid treatment in order to prevent the stimulation of protein synthesis by the addition of amino acid containing DMEM. At the end of the treatment period the media was removed and DMEM containing 1 μ Ci L-[2,4,³H] phenylalanine (0.5 μ Ci/ml) was added for 180 mins. The reaction was stopped by 2x washes in ice cold saline (0.9%) before 3x washes with trichloroacetic acid (TCA) (10%) to remove any unincorporated tracer. Residual TCA was then removed by rinsing cells with

methanol and plates left to dry. Myotubes were then lysed in 50mM NaOH + 1% SDS for a minimum of 30 minutes. An aliquot was collected for liquid scintillation counting to assess ^3H incorporation into proteins and the remaining lysate was used to determine protein content by the DC protein assay. Protein synthesis is presented as counts per minute/ μg of protein.

5.2.5. *Stable isotope labelling of amino acids in cell culture*

Proteomic profiles were profiled using the SILAC labelling method [234]. C₂C₁₂ myoblasts were grown in differentiation media (DM) supplemented with 20% dialysed (10 KDa) foetal bovine serum plus labelled amino acids lysine and arginine in a humidified atmosphere of 37°C and 5% CO₂. Cells intended to act as the control group were grown in with unlabelled lysine and arginine(**light**), the PA treatment group were grown in R6K4 media(**l-arginine-13C6 hydrochloride, l-lysine-4,4,5,5-d4 hydrochloride[medium]**) while the PAO treatment group were grown with R10K8 containing media (**l-arginine-13C6, 15N4 hydrochloride, l-lysine-13C6,15N2 hydrochloride [Heavy]**). The use of combined labelled arginine and lysine ensures that nearly all peptides will contain a label after tryptic digestion. Cells were allowed to grow for at least 6 population doublings to ensure full incorporation of labelled amino acids. We observed that use of dialysed sera and labelled media did not affect doubling time, cell morphology or differentiation capacity. Upon reaching 90-100% confluence the media was replaced with DMEM containing 2% dialysed donor horse serum (10 Da) to induce differentiation. Following differentiation, myotubes were treated with either PA or PAO for 16 hours and collected for proteome analysis.

5.2.6. *Mass spectrometry*

Trypsin-digested peptides were separated using an Ultimate 3000 RSLC (Thermo Scientific) nanoflow LC system. On average 0.5 μg of protein was loaded with a constant

flow of 5 $\mu\text{l}/\text{min}$ onto an Acclaim PepMap100 nanoViper C18 trap column (100 μm inner-diameter, 2cm; Themro Scientific). After trap enrichment, peptides were eluted onto an Acclaim PepMap RSLC nanoViper, C18 column (75 μm , 15 cm; ThermoScientific) with a linear gradient of 2–40% solvent B (80% acetonitrile with 0.08% formic acid) over 65 min with a constant flow of 300 nl/min . The HPLC system was coupled to a linear ion trap Orbitrap hybrid mass spectrometer (LTQ-Orbitrap Velos, Thermo Scientific) via a nanoelectrospray ion source (Thermo Scientific). The spray voltage was set to 1.2 kV, and the temperature of the heated capillary was set to 250 $^{\circ}\text{C}$. Full-scan MS survey spectra (m/z 335–1800) in profile mode were acquired in the Orbitrap with a resolution of 60,000 after accumulation of 1,000,000 ions. The fifteen most intense peptide ions from the preview scan in the Orbitrap were fragmented by collision-induced dissociation (normalized collision energy, 35%; activation Q, 0.250; and activation time, 10 ms) in the LTQ after the accumulation of 10,000 ions. Maximal filling times were 1,000 ms for the full scans and 150 ms for the MS/MS scans. Precursor ion charge state screening was enabled, and all unassigned charge states as well as singly charged species were rejected. The lock mass option was enabled for survey scans to improve mass accuracy [209]. Data were acquired using the Xcalibur software.

5.2.7. *Quantification and bioinformatics analysis*

The raw mass spectrometric data files obtained for each experiment were collated into a single quantitated data set using MaxQuant (version 1.2.2.5) [235] and the Andromeda search engine software [236]. Enzyme specificity was set to that of trypsin, allowing for cleavage N-terminal to proline residues and between aspartic acid and proline residues. Other parameters used were: (i) variable modifications, methionine oxidation, protein N-acetylation, $\text{gln} \rightarrow \text{pyro-glu}$, Phospho(STY); (ii) fixed

modifications, cysteine carbamidomethylation; (iii) database: target-decoy human MaxQuant (ipi.HUMAN.v3.68); (iv) heavy labels: R6K4 and R10K8; (v) MS/MS tolerance: FTMS- 10ppm , ITMS- 0.6 Da; (vi) maximum peptide length, 6; (vii) maximum missed cleavages, 2; (viii) maximum of labeled amino acids, 3; and (ix) false discovery rate, 1%. Peptide ratios were calculated for each arginine- and/or lysine-containing peptide as the peak area of labeled arginine/lysine divided by the peak area of nonlabeled arginine/lysine for each single-scan mass spectrum. Peptide ratios for all arginine- and lysine-containing peptides sequenced for each protein were averaged. Data were normalised using 1/median ratio value for each identified protein group per labelled sample.

5.2.8. *Data analysis and statistics*

Statistical analyses were carried out using Graphpad Prism (V7). Changes in protein synthesis, breakdown and signalling between groups were assessed using a One-way ANOVA followed by multiple comparisons tests. SILAC proteomics results were initially filtered for experimental replicates within 1 standard deviation. Protein abundance was considered altered with a fold change ≥ 1.2 or ≤ 0.8 . Overrepresentation analysis was assessed using gene ontology. The whole mus musculus genome was used as a background list.

5.3. *Results*

5.3.1. *Palmitate attenuated protein synthesis but not protein breakdown*

To assess protein metabolism we utilised tritiated L-phenylalanine as a tracer to measure both synthesis and breakdown of skeletal muscle proteins. Protein synthesis is assessed by the incorporation of labelled phenylalanine into amino acid chains. In order to accurately assess protein synthesis, the fatty acid treatment protocol had to be slightly modified in order to prevent the amino acid stimulation of MPS when the

tracer media was used. Only the first 14 hours of the PA/PAO treatment were completed in PBS + 5mM glucose with the last 2 hours being completed using DMEM + 750 μ M PA/PAO. This was to allow protein synthesis to return to basal levels following the introduction of amino acids. Moreover, L-phenylalanine is not an essential amino acid, therefore any changes in protein synthesis are unlikely to be driven by the addition of the small amounts of this tracer. Following fatty acid treatment, PA significantly reduced basal protein synthesis by approximately 40% (**Fig 5.1**). PAO also induced a small decline in MPS but was not significantly different from control. Surprisingly, despite MPS being attenuated RpS6 phosphorylation (a readout of P70S6K1 activity) was increased by PA. Eif2 α (Ser51) phosphorylation, a marker of ER stress, was unchanged by any fatty acid treatment.

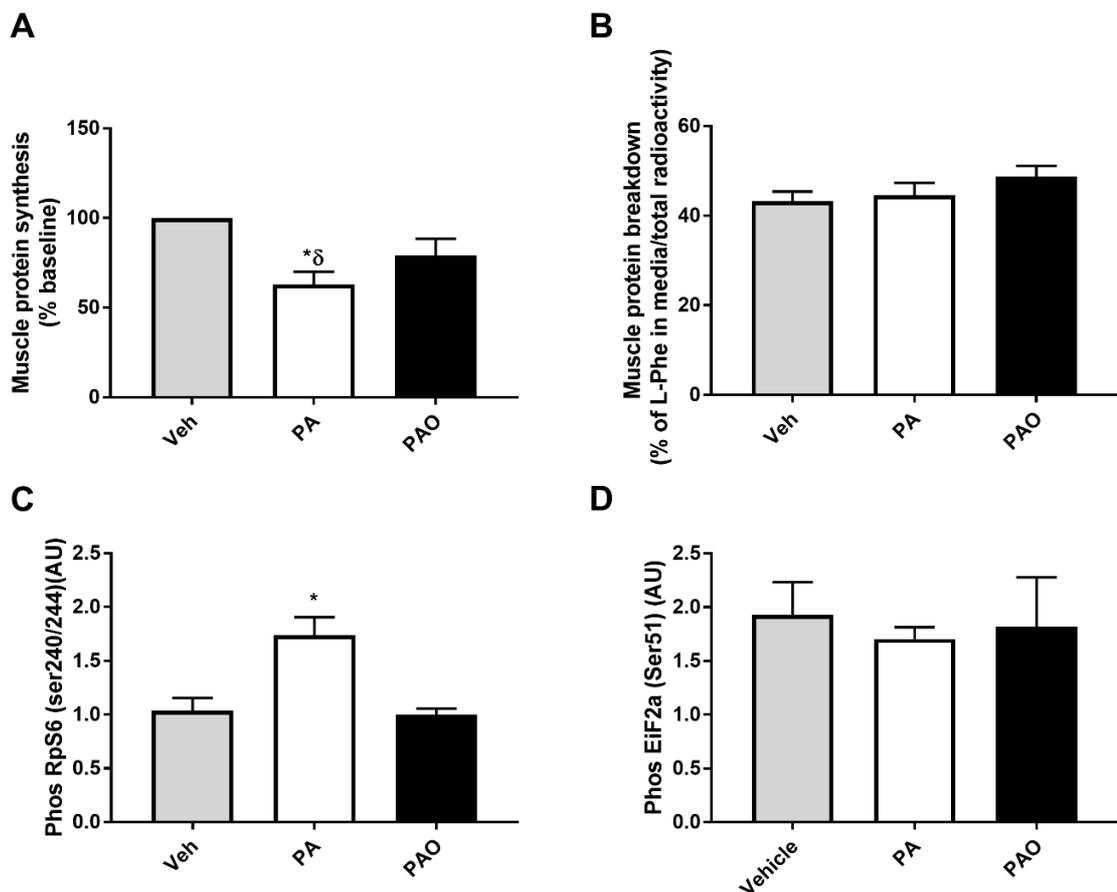


Figure 5. 1 Effect of PA and PAO on protein metabolism. A) muscle protein synthesis was assessed by L-[2,4,³H] Phenylalanine into protein chains. Data is representative of n=5 and presented as % of baseline \pm S.E.M. B) protein breakdown was assessed by measuring release of L-[2,4,³H] Phenylalanine into media. Data is representative of n=8 and presented as % of total radioactivity \pm S.E.M. C)

Phosphorylation of RpS6 (Ser240/244) as assessed by western blotting. Data is representative of n=3 and presented as mean fold change \pm S.E.M. D) phosphorylation of Eif2 α (Ser51) was assessed by western blotting. Data is representative of n=3 and presented as mean fold change \pm S.E.M.

5.3.2. *Altered rates of protein turnover results in divergent proteomic profiles*

Due to the finding that PA downregulated protein synthesis and PAO had no effect, we hypothesised that this change in protein turnover would result in divergent proteomic profiles. We therefore examined if changes in protein turnover altered the abundance of individual proteins and subsequently what cell processes these altered proteins were associated with. We identified 2079 proteins in the SILAC screen. Due to the high variability between replicates, only proteins with replicates within one standard deviation were used for further analysis, leaving 532 proteins. Proteins altered in abundance by \pm 20% were considered to be changed by fatty acid treatment. We observed that only a small number of proteins were altered in abundance by either fatty acid treatment (**Figure 5.2**). In the PA group ATR serine/threonine kinase displayed the biggest relative change with a 7-fold reduction in abundance. In the PAO treatment group, Cytochrome P450, subfamily F had the largest magnitude of all proteins with a log₂ fold change of 1.59. A full list of genes altered by both fatty acid treatments can be seen in **Table 1 and 2**. Gene ontology analysis on proteins altered revealed that purine nucleotide synthesis was overrepresented (P = 3.07E-02). Proteins altered after PAO treatment associated with cell-cell adhesion (p = 2.16E-02) and cell organisation (p = 1.09E-02).

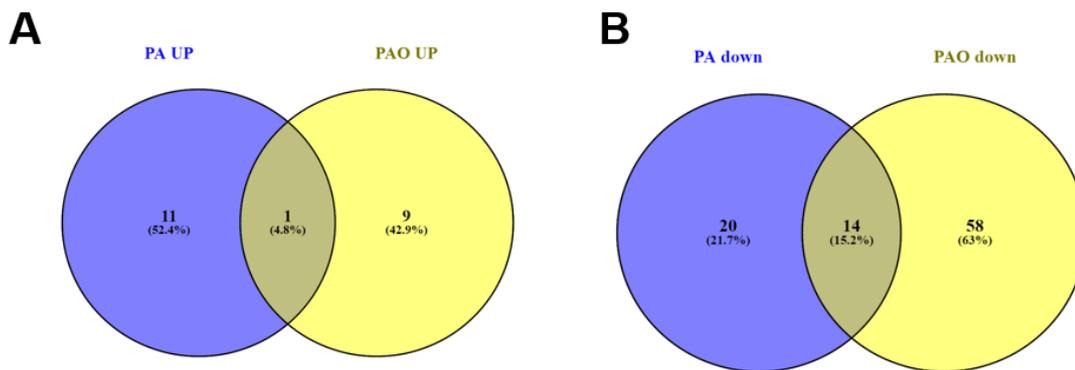


Figure 5. 2 Venn diagram of common and divergent changes in protein abundance following PA/PAO treatment. A) comparison of proteins upregulated in each fatty acid treatment B) comparison of proteins downregulated by each fatty acid treatment.

Table 5. 1 List of proteins altered by PA

PA	
Upregulated	RPL19, VCAN, ADSL, CYP2F1, EPB41L1, PSME2, RPL18, BPNT1, NDRG1, CNOT1, LRBA, SORCS2
Downregulated	CYC1, CTSA, CANX, ATP1B3, SEC63, SUCLG1, SYPL1, NCSTN, BAG3, PSMD3, KIAA1033, SAE1, NME1, DNAJA1, TRAP1, SCCPDH, Crip2, HSD11B1, Pdlim3, MT-ND5, ATP5J2, ATP2A1, CTSD, TIMM50, PBXIP1, NAP1L1, GCDH, POR, PRKAR1A, ATP5L, ZMPSTE24, XRN2, ATR

Table 5. 2 List of proteins altered by PAO

PAO	
Upregulated	CYP2F1, BZW1, EIF4G, RENBP, DLG1, Tpm4, STIP1, HGS, PTGFRN, HSD11B1
Downregulated	HDLBP, PRKACB, ENO3, MAT2A, CTSD, HDGF, SCCPDH, UPF1, STRAP, GBA, Slc25a1, MT-ND5, RSU1, PPA1, CKM, CKAP5, PEBP1, PGLS, ITGB1, TM9SF3, MAP1B, UNC45B, TIMM50, AMCAR, C12orf10, Cyb5r3, PPP1CA, EIF3I, PURB, LAP3, AIMP2, KIAA1033, MAP7D1, ATP5J2, PSME4, CSNK2A1, PARVB, ATP2A1, DYSF, DRG2, POR, CDH13, SUGT1, ADPRH, CRYAB, SYPL1, FLOT1, ITGA7, APOBEC2, BASP1, ARL6IP5, TRIM72, ANKDR2, TRIP12, GPC1, ZMPSTE24, AP2M1, CACNG1, SERBP1, ACSL4, YBX3, CDH2, ABCC1, NID1, NAP1L1, SORBS2, TRAP1, DIAPH1, Pdlim3, ATP2A3, KLH41

5.4. Discussion

Previous studies have shown that PA can have deleterious effects on myotube diameter and amino acid transport, which indicate a disruption in protein anabolism [172], [179]. In this study we provide some mechanistic insight into how PA can inhibit aspects of protein turnover. We show that following a 16 hour treatment PA reduces rates of basal protein synthesis. PAO treated myotubes displayed a small decline in

protein synthesis but was not significantly different from control. These of different effects of PA and PAO on protein metabolism mirror the differential effects on insulin sensitivity. The attenuation in protein synthesis by PA was unlikely to be due to changes in ER stress. Surprisingly, PA enhanced phosphorylation of ribosomal protein S6, a downstream marker of P70S6K1 activation. Furthermore, we show that PA and PAO alter the proteomic profile of C₂C₁₂ myotubes.

In muscle tissue, protein is in a constant state of turnover. Protein expression is regulated by a number of different variables but ultimately acts to coordinate changes in protein abundance in order to appropriately respond to both internal and external changes in environment. The loss of protein content can result in the development of metabolic dysfunction. In skeletal muscle, factors that result in a negative protein balance leads to atrophy. Fatty acid infiltration into muscle can have a negative impact on glucose metabolism. We show here that PA attenuates protein synthesis but does not impact protein breakdown, while PAO has a no effect. A previous study observed that PA treatment induced a progressive loss of myotube diameter in C₂C₁₂ cells [178]. Our results indicate that the loss of myotube diameter may be due to a reduction in protein synthesis rather than elevated protein breakdown. *Hyde & Colleagues (2005)*[172] observed that PA derived ceramides reduced system A amino acid transport so it is logical that PA incorporation results in the attenuation of protein synthesis. To understand the mechanism underlying the reduced protein synthesis we assessed signalling markers of anabolic signalling and ER stress. In response to ER stress the unfolded protein response is initiated which halts initiation of protein synthesis to restore cell homeostasis. In particular, the kinase PERK is activated which phosphorylates Eif2 α (Ser51) which subsequently inhibits protein translation. We did not observe any changes in Eif2 α (Ser51) phosphorylation following treatment with

either PA or PAO. Our data contrasts with that of previous reports which observed that PA treatment induced ER stress in skeletal muscle myotubes [175]. Our study used PBS + 5mM glucose while [175] used typical differentiation media (2% Serum in DMEM). The differences in the available exogenous substrate pools and substrate utilisation will alter the effects of PA on protein and glucose metabolism. ER stress can be induced by the aggregation of misfolded proteins. The reduction in the rate of newly synthesised proteins caused by PA would reduce the load on the protein folding machinery and may offset a reduction in protein folding capacity, thus preventing ER stress. Measures of mTORC1 pathway activity were assessed by measuring phosphorylation of downstream targets. We hypothesised that since protein synthesis was reduced that mTORC1 related signalling would be similarly reduced. Surprisingly, phosphorylation of RpS6, a downstream target of P70S6K1, was enhanced following PA activation. The uncoupling of protein synthesis to anabolic signalling is not without precedent. Termed the “muscle full effect”, anabolic signals remain elevated despite changes in MPS being transient [268]. This work shows that protein synthesis does not always follow the same pattern as signalling pathway activation. In Beta-cells acute PA treatment increased mTORC1 signalling and mRNA translation. However, a 72 hour PA treatment resulted in ER stress and the initiation of the unfolded protein response [269]. We assessed MPS and signalling after 16 hours and it is possible that we missed an acute rise in MPS prior to MPS declining below control. Given that PA can reduce amino acid transport and basal protein synthesis is reduced. We also hypothesise that the increase in MPS in response to anabolic stimuli would also be attenuated compared to control and PAO treated myotubes. Future experiments should aim to assess the temporal nature of the effect of PA on muscle anabolism. In a skeletal muscle model, PA was observed to only phosphorylate P70S6K1 without having any effect on 4E-BP1.

We did not assess the phosphorylation status of 4E-BP1 but our findings would likely be in agreement given that RpS6 is a downstream target of P70S6K1. A recent study found that PA attenuated MPS by a similar magnitude, using a different methodological approach [163]. This study did not couple changes in protein synthesis to anabolic signalling measures. Similar to insulin stimulated glucose uptake, protein synthesis is also an energetically costly process. In the previous chapter we showed that at the dose of PA used for protein anabolism measurements mitochondrial function is severely compromised, in particular ATP synthase dependent OCR. As a result of the reduced capacity to meet energy demands, protein synthesis may have been reduced in accordance with energy availability, despite the activation of anabolic signalling pathways. Due to the significant uncoupling of mitochondrial respiration to ATP synthesis it is unlikely that the changes in mitochondrial function are due to a reduced energy demand for protein synthesis. Treatment media lacking amino acids would have led to myotubes relying on intracellular recycling of amino acids to generate new proteins. If PA disrupted autophagy then this would result in a reduced rate of protein synthesis. In order to further understand the phenotype of PA and PAO treated myotubes we followed up these experiments with a SILAC global proteome analysis.

Overall, the effects of PA and PAO on protein abundance identified in the SILAC screen revealed changes in a small number of proteins. PAO altered the most proteins, mainly through a reduction in protein abundance. Gene ontology analysis did not identify many cell processes that would be affected by changes in the abundance of these proteins. Analysis of proteins altered by PA revealed enriched processes for purine nucleotide biosynthesis. While PAO proteins associated with cell-cell adhesion and organelle organisation. Yet analysis of these singular proteins may indicate critical

changes in some cellular processes. Indeed, the largest protein downregulated by PA was the serine/threonine kinase ATR. This kinase plays a critical role in the DNA damage response and activates signalling cascades to repair DNA [270]. The 7-fold decrease in this kinase may prevent the appropriate response to DNA damage and increase apoptosis. PA is known to induce mitochondrial DNA damage due to an increase in ROS production and if ATR also functions on mitochondrial DNA then this may prevent mtDNA repair [164]. Moreover, Protein kinase A was reduced by 30%. It is unknown if this loss of PKA would have an observable effect on metabolism. A recent study by *Chen & colleagues (2016)* [267] used a similar SILAC design. This work assessed the proteome following incubation with PA and a co-incubation of PA and Oleic acid. This study identified Cox-2, a prostaglandin synthase, as a potential effector of PA effects. The increase in Cox-2 was reversed by co-incubation with OA. Thus, the protective effects of unsaturated fatty acids may be due to the reversal of changes in protein abundance induced by PA. Furthermore, given that PA can alter RpS6 phosphorylation in the absence of any stimulus we speculate that the effects may also be driven by changes in post translational modifications (PTM) and repression of changes in PTM, as is the case with PKB. The different experimental methods used by this study and Chen et al was the different vehicle media used. This key difference prevents a full comparison of the effects of PA on global protein expression. One limitation to our model is that the vehicle media did not contain amino acids. This means that the cells would have to rely on internal protein recycling as a source of amino acids for synthesis of new proteins. The design of the MPS assay means that would not have detected any detrimental effect of reduced amino acid availability on rates of protein turnover. Therefore, we may have missed changes in gene expression

induced by PA and PAO that were not seen at the protein level due to limited substrate availability for new proteins.

In summary, PA incorporation into C₂C₁₂ myotubes alters protein metabolism by attenuating muscle protein synthesis. In contrast, PAO does not significantly alter any aspect of protein synthesis or breakdown. These findings contrast with some of the previous literature which observe that PA can increase protein breakdown. Surprisingly, the attenuation in protein synthesis was not reflected by decreased anabolic signals but RpS6 phosphorylation was actually enhanced. We also did not detect any changes in the phosphorylation of ER stress marker Eif2a which inhibits protein synthesis. PAO did not alter any measured aspect of anabolic or ER stress signalling. It appears that the addition of a single bond into the fatty acid chain is sufficient to attenuate the deleterious effect of PA on skeletal muscle protein anabolism.

Chapter 6: General discussion

6.0. *General discussion*

There is a great deal of interest in the how the composition of dietary fat can elicit alterations in glucose and protein metabolism. In the past few decades, the proportion of individuals diagnosed with conditions such as type-2 diabetes has risen dramatically. Up to 10% of the UK population are estimated to be living with diabetes (Diabetes UK).. The consumption of a diet high in fat has been associated with the accumulation of lipids in peripheral tissues which subsequently disrupts normal metabolic function. Furthermore, animal and cell studies have linked a high fat intake with insulin resistance, altered protein turnover and defects in lipid metabolism [165], [265]. The composition of the fat in the diet can also modify the impact of fat on metabolism. SFA are implicated as having deleterious effects on insulin sensitivity and protein metabolism. While, mono and polyunsaturated fatty acids can offset the negative effects of SFA and intrinsically improve aspects of glucose and protein metabolism. This supposition is strongly supported by molecular data from cell culture and rodent models. However, human studies have provided a more ambiguous relationship between dietary fatty acid composition and alterations in metabolic function. In particular, there is a great deal of interest in understanding the beneficial effects of omega-3 incorporation into skeletal muscle. There have also been multiple human trials which have attempted to tease out the relationship between SFA and insulin resistance.

It is still not fully understood how the fatty acid composition of the diet interacts with other environmental variables to impact on metabolic function. The discussion of this

work will be split into 2 main parts; **part A** will discuss the effects of n-3 fatty acids EPA and DHA on glucose and protein metabolism, while **part B** will discuss the effects of PA and PAO on glucose and protein metabolism. The common and differential effects of EPA,DHA,PA and PAO will be examined in **part C**

The body of experimental work presented in this thesis was undertaken in order to add to the understanding of how subtle differences in fatty acid structure and unsaturation can elicit divergent effects on insulin sensitivity and protein metabolism. In order to achieve those aims we designed experiments to meet the following objectives:

- 1. To characterise the incorporation of a) omega-3 fatty acids EPA and DHA and b) SFA PA and MUFA PAO into C2C12 myotube lipid pools and subsequent alterations in global lipid profiles using Gas chromatography/mass spectrometry analysis. Furthermore, analyse the storage pattern of EPA and DHA using a shotgun lipidomics approach to assess whether EPA and DHA are preferentially stored in neutral lipid stores or phospholipids.**
- 2. To determine the impact of treating c2c12 myotubes with different fatty acids (EPA,DHA,PA,PAO) on myotube insulin sensitivity. Glucose uptake will be used as a key readout of insulin sensitivity. Measures of glucose uptake will be coupled to the assessment of PKB phosphorylation and protein abundance of key glucose transporters. Secondly, assess changes in mitochondrial respiration and protein abundance of mitochondrial complex proteins.**
- 3. To measure changes in protein metabolism by assessing basal protein synthesis and breakdown in c2c12 myotubes following treatment with a) EPA or DHA and b) PA or PAO using isotopic tracers. Phosphorylation of proteins involved in the mTOR signalling pathway will also be assessed. Changes in the abundance of individual proteins will be measured using a SILAC proteomic screen to assess differentially regulated proteins. Where appropriate changes proteins associated with membranes will also be measured using SILAC proteomics.**

These three aims were addressed through two sets of parallel experiments. In the first set of experiments we examined the effects of two n-3 PUFAs EPA and DHA on lipid

profiles and metabolic measures such as glucose uptake, protein anabolism and mitochondrial function in C₂C₁₂ myotubes. To explore the mechanism underlying the observed changes, we coupled these experiments to global and membrane specific proteomic assessments. In the second branch of experiments, we examined the effects of the saturated fatty acid PA and mono unsaturated fatty acid PAO on glucose uptake, protein metabolism and mitochondrial function. We also examined the proteomic profile following PA or PAO treatment of C₂C₁₂ myotubes.

Part A

It is now well characterised that omega-3 consumption results in the incorporation of n-3 fatty acids into the skeletal muscle pool [23], [55], [56], [59]. These studies collectively demonstrate that 4-8 weeks of supplementation (5g n-3, 3g EPA, 2g DHA) result in the significant incorporation into the muscle lipid pool and the phospholipid specific pool. It appears that omega-3 content saturates after an approximate two-fold increase in both total and phospholipid pool. This incorporation of n-3 PUFAs into skeletal muscle is thought to bring about positive health outcomes such as increased strength, and increased anabolic sensitivity to aminoacidemia and hyperinsulinemia [11], [55], [56], [232]. These health benefits are thought to be the result of potentiated activation of the mTOR-P70S6K1 signalling axis in skeletal muscle. In young trained individuals omega-3 supplementation did not augment the MPS in response to combined feeding and resistance exercise [59]. A surprising finding of this study was that the activities of PKB, P70S6K1 and AMPK α 2 were suppressed by fish oil supplementation [59]. In an animal model of hindlimb suspension omega-3 fatty acids ameliorated the loss of muscle mass, however recovery after reloading was delayed [52], [74]. These studies collectively demonstrate that omega-3 fatty acids can have a significant physiological impact on skeletal muscle protein metabolism. Omega-3 fatty

acids comprise a heterogeneous mixture of fatty acids of which EPA and DHA are the prominent fatty acids. Commonly, supplementation studies use a combination of EPA and DHA to demonstrate the effects of n-3 PUFAs on physiology. This prevents the ability to decipher whether EPA or DHA are, individually or in combination, driving the observed metabolic effects. An interesting study using C₂C₁₂ myotubes compared the anabolic effects of EPA and DHA individually [184]. The results of this study suggest that in skeletal muscle EPA increases protein synthesis and reduces protein breakdown, DHA had no effect. In **Chapters 2 and 3** of this thesis the potential mechanisms underlying the differential effects of EPA and DHA were explored.

Work using C₂C₁₂ myotubes and cardiomyocytes demonstrate that EPA and DHA differentially alter both protein and glucose metabolism [184], [271]. In cardiomyocytes EPA, but not DHA, increased glucose uptake [271]. Two independent labs have observed that in skeletal muscle EPA increases basal and insulin stimulated glucose uptake but it was not previously known if DHA had a similar effect [109], [272]. It is demonstrated in **Chapter 2** that in line with previous findings EPA enhances glucose uptake, in contrast, DHA does not alter basal or insulin stimulated glucose uptake. We show that EPA elicits an increase in glucose uptake at even lower concentrations than used by *Aas & colleagues* and *Figueras et al (2011)* [109], [272]. The divergent effect on glucose uptake are curious findings given the similarities in the structure of EPA and DHA. Changes in the mRNA abundance of glucose transporters GLUT1 and GLUT4 have been previously observed following EPA treatment of cultured myotubes [109], [272]. However, in the experiments in **Chapter 2**, any increase in glucose transporter expression was not reflected at the protein level. Assessing the localisation of GLUT1 and GLUT4 after treatment with EPA or DHA would have provided stronger evidence that alterations in glucose transporters did not play a role

Changes in glucose uptake were unlikely to be explained by increased conversion of glucose to glucose-6-phosphate as hexokinase 1 and 2 abundance were not altered. Hexokinases are considered constitutively active, so a change in HK content would indicate changes in kinase activity. Thus, protein abundance can be used as a readout of abundance and activity [273]. A recent study in older women observed a significant relationship between circulating levels of DHA and insulin dependent glucose uptake in skeletal muscle. Our data and that of others would suggest that DHA does not play a causal role in this association in muscle and cardiac tissue [274]. Although DHA did not alter glucose uptake or PKB phosphorylation, it cannot be discounted that DHA may alter glucose utilisation in human models. No changes in mitochondrial respiration were observed in **Chapter 2** but substrate utilisation was not assessed. The mechanism explaining how EPA increased glucose uptake was not readily apparent from measures of glucose transporter abundance, PKB signalling or mitochondrial function.

Given that fatty acids are either broken down for energy or stored in complex lipid species as an energy reserve and as membrane components it was hypothesised that the metabolic effects of n-3 fatty acids are dependent on the incorporation into complex lipid species. Therefore, the divergent physiological effects of n-3 PUFAs may be explained by differential remodelling of the lipidome. Following extensive measures of global lipid profiles, our data are in line with this hypothesis. A major difficulty in lipid remodelling experiments is directly linking changes in multiple lipid species in various lipid compartments with physiological effects.

Following EPA and DHA treatment of myotubes, total omega-3 content increased up to 10-fold. This is significantly higher enrichment than has typically been observed *in vivo*

in human skeletal muscle. Both EPA and DHA resulted in the significant increase in total n-3 content, with up to a 10-fold increase each. There are multiple plausible reasons as to the ability of myotubes to incorporate n-3 PUFAs to a greater degree than in vivo skeletal muscle. N-3 PUFAs are known to be incorporated into a number of tissues such as red blood cells, adipose tissue and skeletal muscle. Therefore, there is “competition” between tissues for the uptake of available n-3 fatty acids. Moreover, differences in lipid turnover rates and uptake capacity can alter the accumulation of n-3 PUFAs within tissue. Rates of fatty acid oxidation are known to differ depending on fibre type composition [26]. Studies looking at the impact of n-3 PUFAs in human muscle are largely conducted in the *vastus lateralis*, a mixed fibre type muscle. It is possible that n-3 content and metabolic effects may act in a fibre type dependent manner. Furthermore, we used EPA and DHA at a concentration of 50µM. This concentration is within the physiological range of omega-3 concentrations in individuals who consume regular oily fish [275]. Although 50µM is not a supra-physiological concentration the available n-3 PUFA pool for uptake into skeletal muscle is unlikely be as high as 50µM. Speculatively, It is possible that in order to have an impact on glucose uptake and insulin sensitivity, the intracellular omega-3 concentration may need to be higher than the concentration needed to elicit changes in protein anabolism . This could contribute to the explanation as to why human studies show a beneficial effect on protein anabolism in the fed state but not on insulin sensitivity. We used EPA and DHA in the free fatty acid form bound to albumin. There is evidence that the method of delivery (i.e in TAGs or phospholipids) can affect the bioavailability and physiological effects [276]. In this study EPA and DHA concentrations in plasma rose to a greater extent when omega-3 PUFAs were provided as phospholipids rather than TAGs. This greater rise in plasma concentration was

correlated with greater reductions of steatosis and improvements in glucose metabolism.

Although both EPA and DHA induced significant increases in n-3 content, the composition of individual n-3 fatty acids differed between both treatment groups. We observed the expected changes in EPA and DHA with each respective treatment but EPA induced the increase in DPA (22:5), an intermediate fatty acid between EPA and DHA. There is interesting mechanistic data to explain why EPA can be elongated to DPA without being further converted to DHA [225]. The elongases' affinity for DPA saturates with increasing accumulation of DPA. In DHA treated myotubes retro conversion to EPA was observed without accumulating as DPA. The remaining question, is whether DPA can play a role in the metabolic effects of n-3 PUFAs in skeletal muscle. We speculate that the conversion of EPA to DPA may be a critical factor in the beneficial effects of EPA. Corroborating this hypothesis, *Norris & colleagues (2012)*[277] observed the blunting of EPAs effect in macrophages when the conversion to DPA was inhibited. Inhibiting this conversion in our model is difficult due to the lack of specificity of elongases and desaturases. While certain enzymes have a higher affinity towards different fatty acids, they are not exclusive. Thus, we could not be confident that any effects seen by blocking the conversion of EPA to DPA were not due to off target effects. Furthermore, other experimental work from our lab showed that treatment of C₂C₁₂ myotubes with DPA resulted in the similar accumulation of both DPA and EPA, with limited conversion to DHA (Data not shown). Indeed, *in vivo* work in rats demonstrated that DPA supplementation results in the accumulation of DPA and EPA in a number of tissues [278]. A parallel study from the same group then demonstrated using radioactively labelled fatty acids that DPA accumulates to a

greater extent than EPA in skeletal muscle [279]. Likely due to the reduced oxidation of DPA compared to EPA. These studies suggest different incorporation patterns hint at potential differential metabolic effects. N-3 PUFAs are known substrates for conversion to bioactive lipid intermediates such as prostaglandins, maresins and resolvins. A short supplementation study with EPA, DPA and DHA caused a differential shift in plasma levels of resolvins and maresins with little overlap between groups [280]. This may partially explain a mechanism for the differential effects of n-3 PUFAs. There is, however, little current understanding of the role lipid mediators could play in enhancing glucose metabolism. However, it should be noted that a group did not observe any changes in n-3 derived lipid mediators after a short term supplementation with a very high dose of n-3 PUFAS [203]. Given that EPA and DPA are interconvertible, it is difficult to tease out divergent physiological effects. One study collected a time course of EPA and DHA levels in hepatic tissue and observed that after 8 hours of incubation DPA has accumulated significantly without interconversion to EPA. Using this time point the authors demonstrated that DPA down-regulated expression of lipogenic genes such as Sterol regulatory element binding protein (SREBP-1), fatty acid synthase (FASn) and acetyl-CoA carboxylase 1 (ACC1) [281]. These effects are similar to previous reports that EPA and DHA both downregulate lipogenic gene expression [282], [283]. Surprisingly in our model, lipid profiling revealed that both EPA and DHA treated myotubes had elevated levels of saturated fatty acids.

The observation that both EPA and DHA treatment resulted in an increase in saturated fatty acids was unexpected. Most predominant, was an approximate 20% shift in the saturated fatty acid PA. Although we cannot confirm the underlying cause of this shift we speculate that it may be a compensatory mechanism due to the significant increase in myotube unsaturation levels. PA is often linked with a reduction in insulin

stimulated glucose uptake. Yet, an increase in PA did not appear to have any negative effects on glucose uptake or protein turnover in the context of incubation in EPA/DHA. As mentioned previously, n-3 PUFAs downregulate lipogenic genes in hepatic tissue [282], [283]. It is unlikely that EPA and DHA promote lipogenesis in skeletal muscle. The membrane proteome screen conducted in **Chapter 3** detected a significant decrease in SREBP-1 association with the membrane following DHA treatment. The cleavage of SREBP-1 from the membrane is a significant step in producing the mature form of the protein [284]. If expression is unchanged then DHA may activate SREBP-1 and subsequent lipogenic gene transcription. Another plausible scenario for the accumulation of saturated fats, is the reduction in saturated fat oxidation due to the large increase in the presence of n-3 fatty acids. Due to the increased number of double bonds, n-3 fatty acid species are more susceptible to oxidation [285]. We did not observe any changes in mitochondrial oxygen consumption but that does not discount that there were any shifts in substrate utilisation. Increased n-3 availability may have shifted fat utilisation to preferentially oxidise n-3 fatty acids. However, both EPA and DHA increased substantially (~10-fold increase) so it is unlikely that both fatty acids were metabolised for energy production to a great extent. Another potential reason that PA levels increased globally and at the phospholipid level is the compensatory response to the increased unsaturation levels. The regulatory mechanisms that control phospholipid fatty acid composition remain to be fully defined.

Incorporation of n-3 PUFAs into phospholipid species is known to induce a shift in physico-chemical properties such as membrane fluidity and melting temperature. Furthermore, both EPA and DHA are both incorporated into lipid raft like domains [195]. Lipid raft domains are highly ordered structures high in sphingomyelin and cholesterol that are thought to act as scaffolding domains for protein signalling [286].

Therefore, modulation of lipid raft composition has the capacity to alter downstream signalling. Indeed, in colonocytes, DHA suppressed downstream endothelial growth factor signalling to ERK1/2, STAT3 and mTOR signalling pathways [287]. It has been previously shown that PUFAs are incorporated into lipid rafts and modify the structure of these microdomains [288]. PUFA rich lipid rafts have a poor affinity with cholesterol and depending on the specific PUFA species, membranes can have both PUFA rich/sterol poor and sterol rich/PUFA poor micro domains [288]. Follow up work observed that EPA and DHA differentially integrate into lipid rafts, with DHA demonstrating a higher incorporation capacity [195]. The phospholipid species that n-3 PUFAs are incorporated in can also determine spatio-location within the membrane and segregation in raft regions [289]. DHA incorporated into PC segregate into raft regions, while incorporation into PE increases incorporation into non raft regions [195]. Not only do n-3 PUFA alter the fatty acid composition but also the function of phospholipid species. In **Chapter 2** we observed the significant incorporation of EPA and DHA into all lipid fractions. However, we observed differences in the relative abundance of n-3 containing phospholipids between species. Of the two most abundant phospholipid groups, PE and PC, we observed that PE had the highest abundance of unsaturated species and displayed the greatest relative increase in species containing unsaturated fatty acids. In contrast, PC species had the highest saturated content and largest decrease in saturated content, with the exception of PC 30:00 and PC 32:00. In light of these data and the observed phospholipid remodelling, we hypothesised that the incorporation of EPA and DHA into phospholipids would alter the membrane associated proteome. Differential shifts in proteins associated with the membrane may explain the contrasting effects of EPA and DHA.

To understand how changes in membrane phospholipid altered the membrane associated proteome we utilised a SILAC labelling model followed by the isolation of membranous regions. Despite the similar patterns in phospholipid composition the membrane association of a number of proteins were differentially altered. The most striking results from this screen was the reduction in membrane associated ribosomal proteins following DHA treatment. There was a small increase in membrane ribosomal proteins following EPA treatment. Changes in membrane association likely reflect shifts in the ER and to a lesser extent mitochondrial ribosomal pool. *Siekavitz & Palade (1956)* [290] demonstrated that mRNA transcripts associated with ER bound ribosome largely code for secreted and integral membrane proteins while cytosolic ribosomes translate cytosolic/nuclear proteins [291], [292]. If total ribosome abundance remained stable, then this may represent a divergent shift in the type of proteins being translated following EPA or DHA incorporation. Indeed, in **Chapter 2**, a global SILAC protein screen identified a number of differentially expressed proteins in EPA or DHA treated myotubes. Complementing these data with microarray analysis would help to identify whether changes in protein abundance are due to increased transcription or changes in the turnover of these proteins.

As well as changes in gene expression, the rate at which individual proteins are recycled influence the total abundance of any given protein within the cell. Using a global SILAC screen we observed a number of differentially expressed proteins following either EPA or DHA treatment. Further analysis of these data revealed that differentially altered proteins were associated with different cellular processes. Thus, differential shifts in the proteome in response to EPA and DHA incorporation may explain the observed divergent effects on metabolism.

Overall, our data on protein turnover agree with previous literature [293]. In response to an increased availability of leucine, EPA but not DHA, increases the protein synthesis response in C2C12 myotubes. Here we show that in the basal state, neither EPA nor DHA alters protein synthesis. Following serum starvation, myotubes were stimulated with 20% FBS growth media and P70S6K1 phosphorylation was assessed as a marker of the anabolic response. In contrast to previous reports, neither fatty acid altered P70S6K1 phosphorylation. This suggests the effect of omega-3 on muscle anabolism is determined by the nature of the anabolic stimulus. We also observed a reduction in protein breakdown, again in agreement with previous reports. This change in protein turnover resulted in a significant increase in protein content after 72 hours of treatment. In an animal model, omega-3 fatty acids offset the loss of muscle mass following hind limb immobilisation [52]. Our work and the work of others in tissue culture models suggest that this protective effect may be driven mainly by EPA. Following resuspension in the same cohort, the fish oil group displayed a delayed response in muscle growth. This delayed recovery was associated with a reduction in prostaglandin F2 α compared to the control group [74]. This may be the result of phospholipid remodelling reducing the precursor pool for production of this prostaglandin species. It should be noted, that by the end of the experimental period, mice in the omega-3 supplementation group had recovered to a similar extent than control mice. Thus omega-3 PUFAs may only be detrimental in the early phases of recovery when acute inflammation is a necessary stimulus for recovery. Under atrophic conditions, the attenuating effect of EPA on protein breakdown may lead to the accumulation of damaged proteins. A reduction in protein breakdown may not be advantageous for effective recovery.

In summary, we provide evidence to suggest that EPA is the main driver of the beneficial effects of n-3 fatty acids on protein accumulation and glucose uptake in skeletal muscle. The elongation of EPA to n-3 DPA may be a critical step in eliciting effects on cell metabolism. In contrast, DHA does not mediate any observable differences from the vehicle treatment and does not appear to have any deleterious effect in respect to the metabolic parameters measured. These effects may be influenced by the EPA specific alteration of fatty acids species contained in phospholipids and the changes in proteins associated with phospholipids.

Part B

In the second experimental arm of this thesis the effects of saturated fatty acid PA and mono unsaturated fatty acid PAO on glucose and protein metabolism were assessed. This comparison allows for the assessment of the importance of double bonds within a fatty acid carbon chain. PA is often associated with deleterious effects on muscle glucose metabolism. It is often thought that PA treatment disrupts normal metabolic function through the accumulation of lipids. Yet PAO, when used at the same concentration does not induce the same defects [19]. Moreover, when PA and PAO are co-incubated the deleterious effects of PA are attenuated. These antagonistic effects make it difficult to assess the effect of PA or PAO on insulin sensitivity *in vivo*. Doubling the saturated fat content of the diet can alter gene transcription [147] but does not alter insulin sensitivity. *Ex vivo* assessment of muscle glucose uptake showed that PA reduced insulin stimulated glucose uptake and obesity increased sensitivity to this effect [244]. To date, the understanding of PA effect in muscle has relied on information from tissue culture models.

Very little information is available regarding the effects of PA and PAO on the overall lipid profiles in skeletal muscle. Multiple studies present data regarding the metabolic

effects of PA but it is not known how well it is incorporated into the lipid pool. We first assessed the remodelling of the lipid pool following treatment with 750 μ M PA or PAO for 16 hours. Unlike n-3 fatty acids neither PA nor PAO had a major impact on the abundance of other fatty acids. Generally, MUFA species are displaced in favour of accumulation of PA or PAO. Although, the magnitude of these changes were smaller than the changes observed in the omega-3 lipid profiling experiments. This is surprising given that PA and PAO were used at a significantly higher concentration. Although differences in the relative fold change were observed between PA and PAO, the absolute incorporation was significantly greater in the PA group. This would not suggest that fatty acid transporters have different affinities for fatty acid species. Although we did not make a direct measure, it is likely that the two-fold increase in total lipid content would induce an increase in saturated fatty acid containing phospholipid species. Increasing the saturated fatty acid of membrane phospholipids would theoretically increase the rigidity of phospholipids due to the increase in melting temperature of the membrane. This could reduce the fusion of proteins translocating to the membrane. This is one of the potential mechanisms by which PA could downregulate glucose uptake in response to insulin.

Following these measurements, we made assessments of glucose uptake in the basal state and insulin stimulated state, alongside PKB phosphorylation. In agreement with previous reports, PA attenuated insulin stimulated glucose uptake but did not affect basal uptake. However, unlike these studies we did not observe any changes in PKB phosphorylation in response to insulin. This demonstrates that there are multiple mechanisms by which PA attenuates insulin driven glucose uptake. As discussed in **Chapter 4** PKB activation may not be the most critical factor in PA's reduction in glucose uptake. Multiple studies show that the capacity to phosphorylate PKB is still

maintained following PA treatment, albeit to a lesser extent ([19], [247], [294]). PKB may only require a small increase in phosphorylation to saturate GLUT4 translocation to the membrane [254]. Common in phosphorylation amplification cascades, a small increase in the activity of an upstream regulator can still have significant downstream effects on downstream signalling. Thus, the critical deleterious effect of PA may lie downstream of PKB. Current thinking suggests that the conversion of PA to ceramides activate PKC isoforms and protein phosphatases which disrupt PKB activation. We did not assess ceramide levels but given that there were no changes in PKB phosphorylation it could be speculated that ceramides did not impact PKB phosphorylation or did not increase enough to interfere with PKB phosphorylation. These results also highlight that PA may act downstream of PKB or through PKB independent mechanisms to interfere with insulin stimulated glucose uptake. One such possibility may be the down regulation of Sortilin, which regulates GLUT4 cycling in C₂C₁₂ myotubes [295]. Following these experiments, we assessed the effect of PA and PAO on mitochondrial oxidative capacity. At 750 μ M PA significantly attenuated oxidative capacity, with increasing proton leak and compromised ATP synthesis. It could be hypothesised that the reduction in ATP production would activate energy stress pathways, such as AMPK activation, which increase glucose uptake and shift energy production to rely on glycolysis. Due to the acidic nature of fatty acids it is difficult to measure the rate of extracellular acidification to assess changes in glycolysis. We did not assess any changes in glucose transporter capacity or localisation following PA or PAO treatment which may play a role. It remains to be seen whether defects in oxidative phosphorylation precede decrements in protein synthesis.

The decrements in insulin sensitivity caused by PA may only be present in the context of energy overload within skeletal muscle [296]. For example, providing 2DG, a non-metabolizable form of glucose, instead of glucose in culture media abrogates the deleterious effect of PA on insulin sensitivity. In our experimental model, PA and PAO treatment were given in media lacking serum, amino acids and low in glucose (5mM). Given these conditions, we are able to show that in C₂C₁₂ myotubes PA induces insulin resistance in conditions of reduced energy availability. Thus, the insulin resistance induced in our model is due to specific properties of PA and not energy overload of the myotubes.

While there has been a significant amount of investigation into the effects of PA and PAO on glucose metabolism, there is little information regarding the effects of these fatty acids on muscle protein anabolism in human models. PA is known to induce ER stress in myotubes and this subsequently induces a signal which feedbacks to inhibit protein synthesis [175]. Thus, it was hypothesised that PA would inhibit protein synthesis. Indeed, we and other groups have recently shown that in skeletal muscle myotubes PA attenuates basal protein synthesis [163]. Our data suggest that the reduced protein synthesis was not due to ER stress as EIF2 α (ser51) phosphorylation was unchanged following PA treatment. Interestingly, the attenuation in synthesis of new proteins was also not due to reduced P70S6K1 signalling as RpS6 phosphorylation was increased. There are data to suggest that a negative feedback loop following P70S6K1 activation which inactivates the upstream regulators which can negatively affect glucose uptake [297]. However, if this was the case then it would be expected to manifest as a reduction in PKB phosphorylation. Since we did not see any changes in PKB phosphorylation it does not appear that this feedback loop is having any effect. From our data it is not readily apparent the disconnect between anabolic signalling and

protein synthesis. We did not detect any changes in protein breakdown in either fatty acid treatment group, It may be that PA enhances the protein breakdown response only in the presence of catabolic stimuli such as TNF α .

Similar to **Chapter 3**, we performed a global SILAC analysis to build a more complete picture of the protein expression changes. Gene ontology analysis did not reveal any enrichment for major anabolic or glucose metabolic pathways. It could be the case that proteins identified in the screen may have novel functions in the regulation of glucose uptake. *Chen & colleagues (2016)* using a similar experimental design, showed that PA upregulated Cox-2 and this effect was reversed by co-incubation with oleic acid. It remains to be seen if changes in prostaglandin synthesis plays a causal role in the metabolic effects of PA. We were unable to detect Cox-2 or perilipin proteins in our screen so it is difficult to compare results. This is likely due to the different treatment medias used to deliver PA and PAO in myotubes. The effects of PA and PAO are likely to be different when in the presence of lipids, growth factors and amino acids. However, this experiment was designed to understand the changes in glucose and MPS observed using PBS + 5mM glucose as a vehicle media. We propose that the effects of PA are largely driven by changes in post translational modifications (PTM). EPA and DHA have been shown in vivo to have differential effects on PTMs and it is likely that comparing unsaturated fatty acids with saturated fatty acids would demonstrate even larger differences [107]. Further work should assess the phospho-proteome to assess changes in kinase signalling following insulin treatment.

Part C

Although direct experimental comparisons are difficult using these fatty acids we can compare the overall effects. From the data gathered it suggests that EPA and PA have unique effects on skeletal muscle myotube function. There is now a large amount of

evidence that longer chain, highly unsaturated fatty acids have beneficial and protective effects against shorter chain saturated fatty acids. In particular, the n-3 PUFA EPA both increases glucose uptake and reduces protein breakdown while DHA which differs by 2 carbons and 1 double bond does not display any of these effects (in our hands). The MUFA PAO, similar to DHA, did not have any effect on glucose uptake or protein metabolism. It appears that the previously observed protective effects may only be evident in the presence of an inflammatory or catabolic stimulus. Interestingly, both PA and PAO increased maximal mitochondrial capacity when used at concentrations of 250 μ M. The effects of EPA and DHA on mitochondrial function were only assessed at 50 μ M. It would be interesting to assess if EPA and DHA had similar effects on maximal mitochondrial respiration when used at higher concentrations.

Our main hypothesis was that the divergent effects of different fatty acid species were driven in part by the fatty acid specific shift in the myotube lipid pool. Both PA and PAO treatment results in increases in those fatty acid species, however, very few other fatty acids were displaced or increased as a result. In contrast, EPA and DHA had a significant impact on the abundance on MUFAs (decrease) and SFA (increase in some fatty acids). Moreover, there were divergent effects between EPA and DHA in important species such as arachidonic acid (AA) and DPA. The differences in lipid remodelling between experimental branches may have been related to the length of fatty acid treatment (72 vs 16 hours). However, prior studies have observed changes in lipid content after a short incubation with fatty acids [298] and we do not predict that extended incubation with PA or PAO would alter the observed effects. Assessing the time course of each individual fatty acid incorporation would assist in answering this question.

A shift in the lipidomic profile influences the proteins associated with the membrane which has the potential to alter the cellular response to extracellular stimuli such as

insulin and amino acids. The fatty acid composition of skeletal muscle can also determine the rate of protein turnover. EPA increases protein accumulation over 72 hours, largely due to a reduction in protein breakdown. The data from this thesis suggest that shorter chain SFA do not alter protein breakdown but attenuate basal rates of protein synthesis. In light of the body of data, increasing the unsaturated fatty acid content, in particular with EPA and DPA, can have protective and beneficial effects on skeletal muscle metabolism. While saturation of the lipid pool can have deleterious effects on muscle metabolism.

We also profiled the proteome of myotubes following treatment with EPA, DHA, PA or PAO. We then assessed the association of altered proteins with biological processes. Treating myotubes with omega-3 PUFAs EPA and DHA elicited alterations in a large number of proteins which associated with the mTOR pathway, EIF2 signalling, protein ubiquitination and actin cytoskeleton dynamics. Namely, processes all involved in cell growth and protein turnover. In contrast, proteomic profiling of PA and PAO treated myotubes revealed changes in the abundance of only a few proteins. Furthermore, gene ontology analysis of these proteins revealed that these proteins did not associated with a substantial number of cell processes. Only purine nucleotide biosynthesis was enriched by PA and cell-cell adhesion by PAO. These proteomic profiles suggest that highly unsaturated fatty acids EPA and DHA have a greater impact on protein expression and turnover. The different treatment medias used in each experimental arm preclude definitive evidence of this.

Limitations and future approaches

This thesis has added novel information about the effects of fatty acids of differing chain length and saturation. It is however, important to acknowledge that there are limiting factors to every experimental model. Two of the main physiological outputs

we assessed in this thesis were glucose uptake and muscle anabolism. In order to further explore the mechanisms underlying the observed changes we utilised a global SILAC labelling proteomics approach with the hypothesis that alterations in protein abundance may sensitise or desensitise muscle to extracellular glucose or protein. This approach is a highly sensitive way to assess changes in the abundance of proteins [234]. Global proteomics are often labelled as an unbiased approach compared to western blotting for individual protein abundance, however, there are certain caveats to this point. Proteomic screens are unlikely to capture the entire proteome, thus this approach only reduces bias. Labelled lysine and arginine amino acids are added to the cell media during proliferation and differentiation and are subsequently incorporated into proteins with high efficiency. Light, medium and heavy labels are used which allows up to 3 groups to be compared at one time. Prior to infusion into the mass spectrometer proteins undergo tryptic digestion. Trypsin cleaves at lysine and arginine residues which results in every peptide containing a labelled lysine or arginine. While there are a number of strengths of using this method there are some drawbacks. Prior to tryptic digestion the samples are separated on a 2D gel. Due to many membrane proteins containing hydrophobic domains, it can lead to the formation of protein aggregates and subsequent mobility issues when ran on a gel. Furthermore, proteins with isoelectric points and molecular weight outwith the median of the proteome can be difficult to quantify. This biases protein detection to that of cytosolic proteins and may impact or mask the detection of potential changes in protein abundance that may explain the phenotype of cells following fatty acid treatment. Given this bias, following our finding that omega-3 incorporation resulted in significant phospholipid remodelling we conducted a membrane proteomic screen. By isolating the membrane fraction prior to separation, membrane proteins are enriched thus allowing for easier

identification. This screen revealed a number of differentially altered proteins that were not detected in the global proteomic screen (**Chapter 2**).

Skeletal muscle has a number of mechanisms that couple extracellular stimuli with the appropriate signalling and physiological response. Responses to extracellular stimuli such as insulin and amino acid availability are governed by post translational modifications[250], [268], [299]. We chose to initially assess global changes in protein abundance to identify gene expression networks sensitive to fatty acids with subtle differences in structure. Moreover, we observed that EPA and PA altered aspects of basal glucose and protein metabolism. We also observed that in response to insulin EPA enhanced glucose uptake compared to DHA while PA inhibited glucose uptake. Future studies should couple the observed changes in protein abundance with phospho-proteomics in the basal and insulin stimulated state. Furthermore, in **Chapter 2** we observed that protein breakdown is altered by EPA.. Unlike previous reports, we did not observe any potentiating effect of EPA or DHA on P70S6K1 phosphorylation in response to a serum stimulus. It is possible that this effect may be dependent on the presence of insulin [170], [265]. These experiments were completed in a C₂C₁₂ cell line which were insulin sensitive in terms of glucose uptake but protein synthesis was not responsive to increased insulin availability. This replicates the observation in human skeletal muscle that MPS is not sensitive to increasing concentrations of insulin [171]. Previous studies in C₂C₁₂ myotubes have shown an effect of insulin on MPS and this highlights that even immortalised cell lines are not always homogeneous. Furthermore, studies in humans have shown that omega-3 fatty acids can induce modest changes in gene expression but it remains to be seen if gene expression is specific to either EPA or DHA [210].

One of the most significant limitations of this thesis is the inability to directly compare the effects of all four fatty acids. The experimental design necessitated that the project be completed in two independent arms. This also meant that different concentrations and vehicle media were used in each experimental arm. Using the same fatty acid concentrations and media conditions across all experiments would mean that some of the observed metabolic changes would not have been observed. Furthermore, at a concentration of 750 μ M, EPA and DHA would be toxic to the cells while at 50 μ M PA and PAO would be unlikely to have any significant effects. Moreover, previous media testing from our lab was only able to detect a PA induced reduction in glucose uptake when PBS+5mM glucose was used as the vehicle media. Each set of experiments required different media conditions which further precludes running a 4-way comparison in the same set of experiments. Furthermore, the maximum number of groups that can be compared in one SILAC experiment is three. This limits the conclusions of these studies about the function of different fatty acid structures, it is difficult to design a direct metabolic comparison without confounding variables such as lipotoxicity induced cell death. One strength of these experiments is that DHA and PAO had neutral effects, acting as a secondary fatty acid control group. This allowed us to delineate that the observed effects were due to differences in fatty acid structure and not simply fatty acid overload.

To address these limitations, future experiments should aim to validate the main results of the mass spectrometry experiments. The SILAC proteomic screen identified a number of differentially altered proteins. Western blotting for some of the main targets identified in this screen would validate these results. Additionally, one of the main trends that emerged from the membrane proteomic data suggested a shift in ribosomal protein abundance. Subcellular fractionation of the cytosolic and membrane

compartment followed by western blotting for ribosomal proteins would add further insight into these results. If this shift in ribosomal content was confirmed then ribosomal profiling would allow for the identification of mRNA transcripts associated with specific ribosomal pools. Due to the lack of changes in the lipidome following PA or PAO treatment, we did not follow this work up with the assessment of phospholipid analysis. Coupling the metabolic changes in PA/PAO treated cells with extensive phospholipid analysis may allow for a better comparison with EPA/DHA treated cells.

Future philosophy

The body of experimental work presented in this thesis demonstrates that different carbon chain lengths and number of double bonds of fatty acid species can have a significant impact on skeletal muscle myotube metabolic function. We provide further evidence that EPA is more metabolically active in skeletal muscle than DHA, when assessing glucose and protein metabolism. We provide novel data that suggest these differential effects are driven by lipid specific remodelling of the lipidome and changes in phospholipid structure and associated proteome. Moreover, by comparing PA and PAO we show that a single double bond can influence the metabolic effects of fatty acids in skeletal muscle. These results, whilst interesting, raise a number of questions that will hopefully be resolved in the future.

1. How are specific fatty acid species channelled towards different metabolic fates?
2. How does EPA increase glucose uptake independently of changes in PKB phosphorylation and glucose transporter abundance?
3. Do n-3 fatty acids modify the actions of insulin on signalling pathway activation and gene expression?
4. Do EPA and DHA differentially alter protein turnover in human models under conditions of suboptimal anabolic stimuli?

5. Further define how PA supresses protein synthesis despite increases in P70 related signalling

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