The role of the perilipin proteins in regulating intramuscular lipid metabolism: effect of exercise training

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Abstract

Physical inactivity is associated with dysregulation of intramuscular triglyceride (IMTG) metabolism leading to the accumulation of fatty acid metabolites and insulin resistance. This thesis aims to provide new information concerning the role of a subset of proteins associated with the metabolic regulation of IMTG-containing lipid droplets (LDs), known as the perilipin (PLIN) proteins, predominantly using immunofluorescence microscopy. Chapter 2 demonstrates that PLIN2-containing LDs are preferentially targeted for breakdown during moderate-intensity exercise in lean, sedentary males. Chapter 3 reveals that 6 months of endurance training (ET) in obese type 2 diabetes patients enhances PLIN2 content in type I fibres. Chapter 4 demonstrates that improvements in IMTG metabolism, including increased expression of PLIN2 and PLIN5, occur in response to both ET and sprint interval training (SIT) in lean, sedentary males. In Chapter 5 data is provided to show that RT also improves IMTG metabolism and insulin sensitivity in lean, sedentary males. Chapter 6 documents the effectiveness of a new constant-workload SIT protocol with regards to improving insulin sensitivity in obese individuals. In conclusion, this thesis generates novel data that contributes to our understanding of the underlying mechanisms that govern the relationship between IMTG metabolism and insulin sensitivity.

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Abstracts, Conference Communications and Publications

During the period of postgraduate study at the University of Birmingham, data from the current thesis resulted in the following abstracts:

Wagenmakers AJM, **Shepherd SO**, van Loon LJC, Shaw CS (2012). Exercise effects on fat metabolism and insulin sensitivity in human muscle. *Endocrine Abstracts* 28, S5.2.

Shepherd SO, Cocks M, Tipton KD, Ranasinghe AM, Barker TA, Burniston JG, Wagenmakers AJM, Shaw CS (2011). High intensity interval and traditional endurance training are equally effective in enhancing intramuscular triglyceride metabolism and improving insulin sensitivity in sedentary males. *16th Proceedings of the European College of Sport Science*.

Cocks M, **Shepherd SO**, Shaw CS, Fisher J, Ranasinghe AM, Barker TA, Tipton KD, Wagenmakers AJM. High intensity interval and traditional endurance training are equally effective in improving muscle microvascular function in sedentary males. *16th Proceedings of the European College of Sport Science*.

Shepherd SO, Cocks M, Tipton KD, Wagenmakers AJM, Shaw CS (2010). Improvements in insulin sensitivity and whole-body fat oxidation after a period of high-intensity interval training. *Br J Sports Med* 44, 14 i11 doi:10.1136/bjsm.2010.078972.32.

Cocks M, **Shepherd SO**, Tipton KD, Wagenmakers AJM, Shaw CS (2010). High-intensity interval training improves microvascular and macrovascular function and insulin sensitivity. *Br J Sports Med* 44, 14 i9 doi:10.1136/bjsm.2010.078972.27.

During the period of postgraduate study, the data in the thesis also resulted in the following conference communications:

Physiological Society Main Meeting, Oxford, UK, July 2011 – Poster communication, 1st prize - Preferential utilisation of perilipin 2 associated intramuscular triglycerides during one hour of moderate intensity endurance-type exercise.

European College of Sport Science (ECSS) Annual Conference, Liverpool, UK, July 2011 – Oral communication - High intensity interval and traditional endurance training are equally effective in enhancing intramuscular triglyceride metabolism and improving insulin sensitivity in sedentary males.

International Sports Science & Sports Medicine Conference (ISSSM), Newcastle, UK, August 2010 – Poster communication - Improvements in insulin sensitivity and whole-body fat oxidation after a period of high-intensity interval training.

The following papers were also published during the period of postgraduate study:

Shepherd SO, Cocks M, Tipton KD, Ranasinghe AM, Barker TA, Burniston JG, Wagenmakers AJM, Shaw CS (2012). Sprint interval and traditional endurance training increase net intramuscular triglyceride breakdown and expression of perilipin 2 and 5. *J Physiol*. (under review).

Cocks M, **Shepherd SO**, Shaw CS, Fisher J, Ranasinghe AM, Barker TA, Tipton KD, Wagenmakers AJM (2012). High intensity interval and endurance training are equally effective in increasing muscle microvascular density and eNOS content in sedentary males. *J Physiol*. DOI:10.1113/jphysiol.2012.239566.

Shaw CS, **Shepherd SO**, Wagenmakers AJM, Hansen D, Dendale P, van Loon LJC (2012). Prolonged exercise training increases type I muscle fibre type specific intramuscular lipid content and perilipin 2 expression in type 2 diabetes. *Am J Physiol Endocrinol Metab*. DOI:10.1152/ajpendo.00272.2012.

Cocks M, **Shepherd SO**, Shaw CS, Achten J, Costa ML, Wagenmakers AJM (2012). Immunofluorescence microscopy to assess enzymes controlling NO availability and microvascular blood flow in skeletal muscle. *Microcirculation*. DOI: 10.1111/j.1549-8719.2012.00199.

Shepherd SO, Cocks M, Tipton KD, Ranasinghe AM, Barker TA, Burniston JG, Wagenmakers AJM, Shaw CS (2012). Preferential utilisation of perilipin 2 associated intramuscular triglycerides during one hour of moderate intensity endurance-type exercise. *Experimental Physiology* 97(8), 970-980.

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Chapter 1

General Introduction

1.1 Defining the 'normal' human phenotype

At the most simplistic level, the survival of homo sapiens is dependent on the ability to reproduce, feed, and perform physical activity. Indeed, the procurement of food for our hunter-gatherer ancestors of the Late-Paleolithic era (50,000 – 10,000 BC) was highly dependent on the capacity to perform physical activity (Bramble and Lieberman, 2004). Therefore, an inability to perform high levels of physical activity resulted in insufficient nutrition and ultimately mortality, and it is likely that the gene pool of these individuals would be extinguished before the reproductive age was reached. In addition, food procurement was not always guaranteed (Diamond, 2003), indicating that our hunter-gatherer ancestors will have undergone periods of food abundance interspersed with periods of famine. Accordingly, a cyclic relationship between food abundance and famine would have existed, in addition to a cycle of physical activity and rest. Neel (Neel, 1962) proposed that these environmental pressures would have shaped the human genome over thousands of years, such that certain 'thrifty' genes were incorporated into the human genome as they inferred a survival advantage over less 'thrifty' ones. A 'thrifty' genotype was characterised by an exceptional efficiency for food intake, storage and utilisation. Thus, individuals possessing a 'thrifty' genotype would be best equipped to adapt to the oscillating periods of food abundance and famine, and physical activity and rest (Booth et al., 2002; Neel, 1999). Interestingly, the present-day human genome appears to have remained unchanged from this period (Cordain et al., 1998), and therefore the 'physically active genotype' should be considered 'normal' (Booth and Lees, 2006).

The hypothesis that our genotype was selected to support obligatory physical activity is in line with the proposal that 'thrifty' genes were selected to promote greater efficiency of food utilisation (Neel, 1962). In accordance, 'thrifty' genes particularly target the regulation of the two major sources of fuel storage in humans: carbohydrate and fat (or glycogen and triglyceride) (Chakravarthy and Booth, 2004). A cycle of glycogen depletion and repletion is consistent with environmental oscillations between food abundance and famine. However, the need to preserve muscle glycogen for the purpose of providing rapid energy for immediate tasks to enable survival was also of high priority (Chakravarthy and Booth, 2004), highlighting the importance of triglyceride as a substrate source. Therefore, the capacity to

cycle between glycogen and triglyceride as a substrate source supports the 'physically active genotype' that is fundamental for the existence of our species.

It is becoming increasingly apparent that the 'physically active genotype' selected over 10,000 years ago is not compatible with the relatively sedentary nature of our current lifestyle. In fact, it is now almost a decade ago that the U.S. Centers for Disease Control (CDC) first specified physical inactivity as a *cause* of metabolic disease (Mokdad et al., 2004), which include obesity, insulin resistance and type 2 diabetes, and ultimately cardiovascular disease. Notably, exercise capacity is the strongest predictor of mortality risk compared to more clinical variables or established risk factors, such as hypertension, smoking or diabetes (Myers et al., 2002). In addition, low muscular fitness or strength is associated with greater risk of mortality and increased incidence of metabolic syndrome (Jurca et al., 2005). Increasing the level of physical activity can reduce the risk of mortality by an equivalent (Paffenbarger et al., 1993) or greater magnitude (Blair et al., 1995) compared to the modification of other risk factors, such as giving up smoking. Given that 'thrifty' genes were selected to promote efficient food storage and utilisation making physical activity possible, it is perhaps not surprising that common metabolic disorders are linked to the dysregulation of substrate metabolism due to physical inactivity. This PhD thesis will predominantly focus on one substrate source, skeletal muscle triglyceride, and the regulation of its metabolism. In addition, the therapeutic effect of exercise training in its various forms will be considered with regards to skeletal muscle triglyceride metabolism, and its association with insulin sensitivity.

1.2 Insulin-mediated glucose homeostasis

Carbohydrates ingested during a meal are broken down to their most basic constituents during the digestion process, and are absorbed through the gut and into the circulation as glucose. In response to a postprandial rise in plasma glucose concentrations, insulin is secreted from the pancreas into the circulation. Insulin binds to its receptors on the plasma membrane of skeletal muscle, liver and adipose tissue, and promotes glucose removal from the circulation into these tissues. Postprandial glucose uptake into these tissues maintains plasma glucose levels at approximately 5 $mmol.l^{-1}$, and circumvents a situation in which chronic hyperglycaemia may occur. On entering skeletal muscle, liver

or adipose tissue, glucose can be used to provide energy through glycolytic processes, or be shunted towards storage as glycogen (or in adipose tissue converted to palmitate through de novo lipogenesis or used to provide the glycerol backbone of triacylglycerol (TAG)).

Skeletal muscle is the major site of glucose uptake, with skeletal muscle being responsible for ~80% of glucose removal from the circulation in healthy lean insulin sensitive individuals during a hyperinsulinaemic euglycemic clamp (DeFronzo et al., 1981; Thiebaud et al., 1982). When studied under more physiological conditions, such as following a mixed meal after a ~15 h overnight fast, skeletal muscle glucose uptake remains high, accounting for ~50% of glucose disposal (splanchnic glucose disposal accounts for the other 50%) (Capaldo et al., 1999). Therefore, it is logical that insulin will stimulate skeletal muscle glucose uptake through the direct action of insulin on the plasma membrane of skeletal muscle fibres, and significantly contribute to the maintenance of whole body glucose homeostasis. In addition, insulin is also able to stimulate the recruitment of the microvascular system, thereby promoting greater delivery of insulin and other substrates (i.e. glucose) to the muscle fibre (for recent reviews see (Barrett et al., 2009; Barrett et al., 2011; Clark, 2008; Muniyappa et al., 2007). Whilst acknowledging the potential importance of insulin action on the microvascular system in mediating insulin-mediated glucose disposal, this thesis will predominantly focus on intramuscular metabolic factors determining insulin action on skeletal muscle fibres and glycemic control.

1.2.1 Insulin-mediated glucose uptake into skeletal muscle

Glucose uptake into skeletal muscle occurs primarily through facilitated diffusion, a process in which the glucose transporter proteins play a fundamental role. Together, 14 glucose transporter isoforms have been identified (Uldry and Thorens, 2004), although glucose transporter 4 (GLUT4) is the predominant insulin responsive isoform required for glucose uptake into skeletal muscle (Watson and Pessin, 2001). Under fasted conditions GLUT4 resides in intracellular cytosolic microvesicles, but in the fed state (i.e. under conditions leading to elevated plasma insulin concentrations) GLUT4 microvesicles translocate to the plasma membrane where the GLUT4 protein is deposited and ultimately facilitates glucose uptake into the muscle fibre. The mechanism by which insulin promotes

GLUT4 redistribution to the plasma membrane has been well studied, and it now appears that that the process of GLUT4 translocation is dependent on multiple intracellular signalling events.

The cascade of signalling events which results in GLUT4 translocation to the plasma membrane is initiated by insulin binding to the extracellular α-subunit of the insulin receptor (IR), thus stimulating tyrosine autophosphorylation of the transmembrane IR β-subunit and activation of the intrinsic tyrosine kinase (Fig. 1.1). In turn, a number of substrate proteins are phosphorylated and recruited to the IR, including the insulin receptor substrate (IRS) proteins. Specifically, phosphorylation of Tyr^{960} on the IR β-subunit recruits and binds the phosphotyrosine binding (PTB) domain of IRS-1. Furthermore, IR tyrosine kinase activity phosphorylates IRS-1 on a number of tyrosine residues which subsequently bind with high affinity to *src*-homology (SH) 2 (SH2) domains of signal transducing proteins. Phosphorylation of IRS-1 on Tyr^{608} and Tyr^{628} residues generates the major docking site for the signal transducing protein phosphoinositide (PI) 3-kinase (PI3-K) (Esposito et al., 2001). PI3-K is composed of a p85 regulatory subunit, which contains two SH2 domains that bind to phosphopeptides of IRS-1, and a p110 catalytic subunit. PI3-K subsequently catalyzes the phosphorylation of PI at the D-3 position of the inositol ring to produce phosphatidylinositol-3-phosphates, and in particular phosphatidylinositol-3,4,5-triphosphate (PI-(3,4,5)-P_3 or PIP_3). PIP_3 directly interacts with the pleckstrin homology (PH) domain of phosphoinositide-dependent kinase 1 (PDK1), protein kinase B (Akt/PKB), and atypical protein kinase C isoforms (aPKC), which recruits these proteins to the plasma membrane. Upon recruitment, PDK1 promotes phosphorylation of both Akt/PKB and aPKC isoforms on threonine residues located in their catalytic domain. In particular, phosphorylation of Akt/PKB on Thr^{308} and Ser^{473} residues results in its activation, and activated Akt/PKB downstream targets include glycogen synthase kinase-3 (GSK-3) and TBC 1 domain family, member 4 (TBC1D4, also known as AS160). Akt/PKB inactivates GSK-3 through phosphorylation of the Ser^{9} residue in parallel to protein phosphatase-1 activation, which subsequently relieves inhibition of the enzyme glycogen synthase leading to its activation and enhanced glycogen synthesis. Under basal conditions, the GTPase-activating protein domain of TBC1D4 retains Rab proteins in an inactive GDP-bound state that prevents GLUT4 translocation to the plasma membrane. Upon insulin stimulation, activated Akt/PKB phosphorylates TBC1D4, suppressing the GTPase activity, potentially through the binding of 14-3-3

protein (Geraghty et al., 2007). As a consequence, Rab proteins become GTP-loaded leading to reorganisation of the cytoskeleton that is required for GLUT4 mobilisation and glucose uptake (Vind et al., 2011).

Insulin-mediated GLUT4 translocation is also possible through an IRS-PI3-K-independent pathway (Fig. 1.1), as pharmacological inhibition of PI3-K does not completely block insulin-mediated glucose uptake. Similarly, pharmacological activation of intermediates of the IRS-PI3-K pathway does not necessarily promote glucose uptake (reviewed in (Kanzaki, 2006). Consequently it is apparent that complete activation of insulin-mediated GLUT4 translocation leading to glucose uptake requires an integrated response from the PI3-K-dependent and PI3-K-independent pathways. More specifically, tyrosine activation of IR stimulates the recruitment of c-CBl to the IR by two adaptor proteins, APS and CAP, in order to form an APS-CBl-CAP complex. This activation of the CBl protein subsequently promotes activation of TC10, which is required for GLUT4 translocation (Bogan, 2012).

Following translocation to specific fusion sites, GLUT4 vesicles are required to undergo a process of facilitated fusion with the plasma membrane in order to allow glucose uptake. In this process, a number of SNAP receptors (SNAREs) on the target membrane (t- or Q-SNAREs) interact with SNARE proteins on the transport vesicle membrane (v- or R-SNAREs). Briefly, the v-SNARE vesicle-associated membrane protein-2 (VAMP2) attaches to the t-SNAREs, SNAP23 and syntaxin 4, leading to the formation of a SNARE complex. Consequently, the plasma membrane and GLUT4 vesicle membrane are brought into close proximity, promoting the fusion of the lipid bilayers and incorporation of GLUT4 within the plasma membrane. Insulin regulates this fusion process through a number of interacting proteins that either facilitate or promote the assembly of the SNARE complex. For example, insulin-stimulated activation of aPKC promotes phosphorylation of Munc18c leading to its dissociation from syntaxin 4 (Jewell et al., 2011). In addition, activated aPKC phosphorylates VAMP2 (Farese and Sajan, 2010), permitting its association with syntaxin 4 and enabling the SNARE complex to form and facilitate GLUT4 fusion. The reader is directed to two excellent reviews (Bryant and Gould, 2011; Leney and Tavare, 2009) for a further, detailed overview of the role of SNARE proteins in insulin-stimulated glucose uptake.

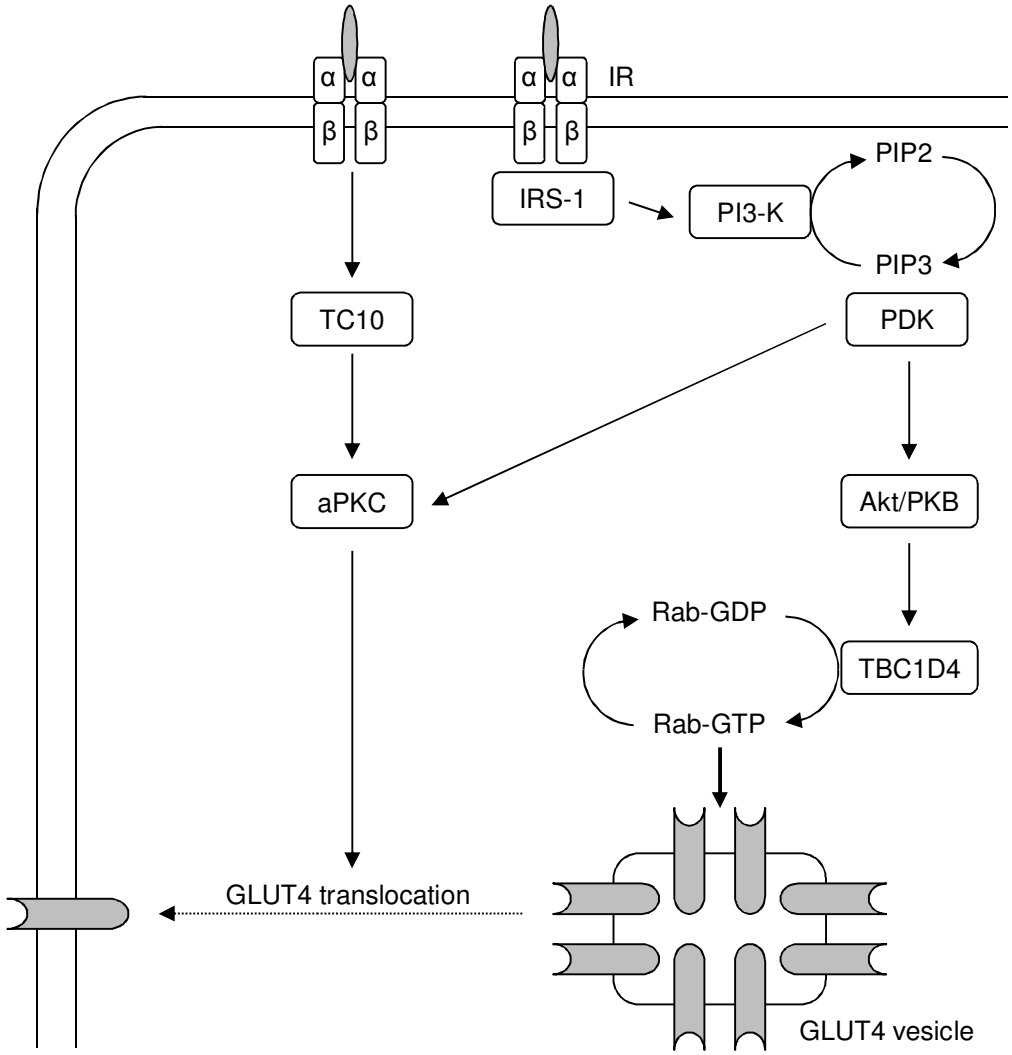


Figure 1.1 Insulin-mediated glucose uptake into skeletal muscle

aPKC atypical protein kinase C, *Akt/PKB* protein kinase B, *GLUT4* glucose transporter 4, *IR* insulin receptor, *IRS-1* insulin receptor substrate-1, *PDK* phosphoinositide-dependent kinase 1, *PI3-K* phosphoinositide 3-kinase, *PIP2* phosphatidylinositol 4,5-bisphosphate, *PIP3* phosphatidylinositol-3,4,5-triphosphate, *Rab-GDP* Rab guanosine disphosphate, *Rab-GDP* Rab guanosine triphosphate, *TBC1D4* TBC 1 domain family member 4.

1.2.2 Contraction-mediated glucose uptake into skeletal muscle

Skeletal muscle glucose uptake is also increased during exercise via a mechanism that is insulin independent. Like physiological increases in insulin, muscle contraction stimulates the translocation of GLUT4 from the intracellular vesicles to the plasma membrane and t-tubules, thereby permitting glucose uptake into the muscle fibre. In contrast to insulin, muscle contraction does not activate components of the proximal insulin signalling cascade, as concluded from the observation that incubation of rat soleus muscle with wortmannin, a PI3-K inhibitor, has no impact on contraction-mediated GLUT4 translocation (Lund et al., 1995). Furthermore, electrically stimulated contraction of isolated skeletal muscle has no effect on the activity of IRS-1, PI3-K or Akt/PKB (Goodyear et al., 1995; Wojtaszewski et al., 1996).

During muscle contraction an increase in the AMP to ATP ratio leads to activation of AMP-activated protein kinase (AMPK) (Wojtaszewski et al., 2000b). Importantly, AMPK activation by 5-aminoimidazole-4-carboxamide-1-β-D-ribonucleoside (AICAR; an AMP analogue) also enhances GLUT4 translocation to the plasma membrane and glucose uptake (Kurth-Kraczek et al., 1999; Merrill et al., 1997), whereas AICAR-stimulated glucose uptake is not effected by incubation with wortmannin (Hayashi et al., 1998), indicating that AMPK activation, like muscle contractions, leads to GLUT4 translocation independent of an effect on the proximal insulin signalling components. However, it is important to acknowledge that 60% of contraction-mediated glucose uptake is maintained when AMPK is knocked out (Mu et al., 2001), suggesting that other intracellular factors, independent of AMPK, may also stimulate glucose uptake. To this end, calcium (Ca^{2+}) released from the sarcoplasmic reticulum during muscle contraction forms a complex with calmodulin (CaM) that subsequently binds to and activates Ca^{2+}/CaM-activated kinase (CAMK). CAMKII, which is the predominant isoform present in skeletal muscle, may provide a link between contraction-mediated Ca^{2+} release and GLUT4 translocation, since blocking CAMK activation reduces glucose uptake (Wright et al., 2005; Wright et al., 2004). In addition, increases in Ca^{2+} concentration in response to muscle contraction lead to activation of PKC which may play an important role in exercise-induced glucose uptake, since pharmacological inhibition of PKC (using calphostin C) reduces contraction-mediated glucose uptake (Ihlemann et al., 1999). However, the lack of isoform specificity of calphostin C precludes

identification of the responsible PKC isoforms. Subsequent studies demonstrated that PKC activity was increased in rat skeletal muscle in response to electrical stimulation, and this coincided with activation of conventional and novel PKC isoforms (Richter et al., 2003). However, neither the conventional or novel PKC isoforms are activated during exercise in human skeletal muscle (Rose et al., 2004). Consequently, it has been proposed that the increase in PKC activity in response to exercise is attributed to activation of atypical PKC (Perrini et al., 2004; Rose et al., 2004), although this may not be crucial for contraction-mediated glucose uptake (Sajan et al., 2010). Rather, it is suggested that increased atypical PKC activation in response to exercise may potentially enhance the action of insulin (Maarbjerg et al., 2011). It is also worth noting that several mitogen-activated protein kinase (MAPK) isoforms (extracellular related kinase 1/2 (ERK1/2), p38, and c-Jun N-terminal kinase (JNK)) are activated in response to muscle contraction (Sakamoto and Goodyear, 2002). More specifically, the increase in atypical PKC activation in response to exercise is paralleled by increased ERK activity (Nielsen et al., 2003), although blocking ERK activation does not completely inhibit contraction-mediated glucose uptake (Wojtaszewski et al., 1999). Phosphorylation of p38 MAPK is also increased concomitant with enhanced contraction-induced glucose uptake, and inhibition of p38 MAPK blocks this increase (Chambers et al., 2009). However, the p38 inhibitor used in this study has previously been reported to directly interact with and inhibit GLUT4 (Jensen and Richter, 2012), and therefore may not conclusively identify a role for p38 MAPK in contraction-mediated glucose uptake. Clearly, we do not have a complete understanding of how these intracellular signals 'blend' to provide a specific increase in the level of glucose transport in response to muscle contraction.

One downstream target of AMPK and CAMKII is AS160/TBC1D4, providing a link by which these intracellular kinases may promote GLUT4 translocation independent of insulin action. Importantly, AMPK has been shown to phosphorylate TBC1D4 (Kramer et al., 2006a; Treebak et al., 2006; Treebak et al., 2010), whereas expression of a mutant form of TBC1D4 reduces contraction-mediated glucose uptake (Kramer et al., 2006b). In addition, it has been reported that TBC1D4 contains a calmodulin binding domain, and mutation of this domain reduces contraction-mediated glucose uptake (Kramer et al., 2007). Furthermore, a single bout of resistance exercise enhances TBC1D4 phosphorylation in the post exercise period (Dreyer et al., 2008), and TBC1D4 phosphorylation was

increased following 60 and 90 min of moderate-intensity endurance exercise (Treebak et al., 2007). TBC1D1 shares 50% of the identity of TBC1D4, but importantly, the GAP domains are 79% identical, suggesting that TBC1D1 also plays a role in regulating GLUT4 translocation (Sakamoto and Holman, 2008). Like TBC1D4, AMPK also phosphorylates TBC1D1 (Taylor et al., 2008), and TBC1D1 phosphorylation is increased in response to exercise (Frosig et al., 2010; Jessen et al., 2011). Importantly, contraction-induced glucose uptake is impaired in skeletal muscle of TBC1D1-deficient mice (Szekeres et al., 2012), providing evidence that TBC1D1 plays a significant role in contraction-mediated increases in glucose uptake. It is clear that further work is needed in this area to fully understand the mechanism by which muscle contraction stimulates GLUT4 translocation, but it appears that TBC1D4 and TBC1D1 play a key role in this process.

1.3 Lipid metabolism in skeletal muscle

1.3.1 Lipid storage in the human body

Subcutaneous and deep visceral adipose tissue depots are the predominant sites for the storage of fat as TAG in the human body. More specifically, the average non-obese male (~70 kg) stores between 9 and 15 kg (equating to an energy store of 80,000 to 140,000 kcal) of TAG in adipose tissue (van Loon, 2004). Adipose tissue TAG concentration is increased during periods of caloric excess resulting in expansion of adipose tissue mass. In contrast, periods of starvation or famine lead to a reduction in adipose tissue TAG concentration and adipose tissue mass, due to the hydrolysis of TAG leading to fatty acid (FA) mobilisation, in order to meet the body's energy requirements. Consequently, it is appreciated that TAG stored within adipose tissue represents a dynamic pool of FAs.

Although adipose tissue is the predominant site for TAG storage, nearly all cell types possess the capacity to store FAs as TAG in intracellular lipid droplets (LDs). LDs are encased by a phospholipid monolayer, which also contains cholesterol and a large number of proteins, and in addition to TAG, LDs have the capacity to store diacylglycerol (DAG), retinyl esters, cholesterol esters, and free cholesterol (Meex et al., 2009). Although their structure infers a prominent role in TAG storage, LDs also have a function in vesicle trafficking and cell signalling capabilities. Furthermore, the physiological role of individual LDs is determined by a number of factors including morphology,

subcellular localisation, and the specific proteins associated with the LD. For example, both cardiac and skeletal muscle tissue are oxidative tissues and contain a large number of small, discrete LDs (ranging from 100 nm up to 1 μm in size), whereas the cytoplasm of an individual adipocyte is occupied entirely by the LD (up to 100 μm). Importantly, the balance between TAG synthesis and TAG breakdown determines the LD size and number, and therefore their intracellular concentration, which in turn is reflective of both nutritional and training status.

The concentration of intramuscular triglyceride (IMTG) is generally in the range between 2 and 10 $mmol.kg^{-1}$ wet weight, equating to a mixed muscle TAG content of approximately 0.2 kg (an energy store of 1,850 kcal) (van Loon, 2004; Watt et al., 2002b; Wendling et al., 1996), and is predominantly influenced by energy intake, dietary composition and training status. Indeed, the consumption of a high-fat diet, either short-term (<67 h) (Bachmann et al., 2001; Johnson et al., 2006; Zderic et al., 2004) or for a more prolonged period (>7 days) (Schrauwen-Hinderling et al., 2005; Van Proeyen et al., 2011a; Van Proeyen et al., 2010), increases the concentration of IMTG. In addition, directly increasing plasma free fatty acid (FFA) concentrations through a lipid and heparin infusion enhances IMTG storage (Boden et al., 2001). A number of cross-sectional reports also demonstrate that one of the metabolic adaptations to a period of endurance training is greater IMTG storage (reviewed in (Shaw et al., 2010), which appears to parallel greater glycogen storage in the endurance-trained state. Furthermore, morphological evidence reveals that IMTG are predominantly located in close proximity to the mitochondria (Hoppeler, 1999; Shaw et al., 2008). Given that FFAs liberated from IMTG via hydrolysis are metabolised in the mitochondria, this apparent morphology provides evidence that IMTG is a readily available pool of FA which can be used as an oxidative substrate source during exercise in active individuals.

1.3.2 Intramuscular triglyceride as a substrate source during exercise

Total fat oxidation rates are increased approximately 10-fold compared to resting values during moderate-intensity exercise in trained individuals (Romijn et al., 1993; van Loon et al., 2001), and it is therefore plausible that the IMTG pool provides a readily available source of FAs for mitochondrial β-oxidation, especially considering the physical IMTG-mitochondria interactions mentioned previously

(Hoppeler, 1999; Shaw et al., 2008). In order to estimate the contribution of the IMTG pool to total fat oxidation during an acute exercise bout a number of early studies employed indirect calorimetry measures in combination with the infusion of a ^{13}C-labelled FA tracer (Romijn et al., 1993; van Loon et al., 2001; van Loon et al., 2003; van Loon et al., 2005b; Watt et al., 2002a). Using this technique it is possible to measure total fat and plasma FFA oxidation and calculate the contribution of other fat sources (i.e. the sum of IMTG and lipoprotein-derived TAG) to total fat oxidation from the difference between the two. However, this method makes the assumption that the contribution of lipoprotein-derived TAG oxidation is relatively small (~5-10%) following an overnight fast under normal dietary conditions (van Loon, 2004; Watt et al., 2002b). Stable isotope methodology has been employed to demonstrate that the rate of non-plasma FA oxidation (i.e. IMTG and lipoprotein-derived TG) is greatest during exercise between 40 and 65% $VO_{2\,max}$, and accounts for ~30-50% of total fat oxidation (Romijn et al., 1993; van Loon et al., 2001).

Although the use of stable isotopes provides an indirect estimate of the contribution of the IMTG pool to total fat oxidation during exercise, it is essential to directly quantify net changes in IMTG concentration following an acute exercise bout. This can be achieved non-invasively through the use of ^{1}H magnetic resonance spectroscopy (^{1}H-MRS), which permits the quantification of both IMTG and extramyocellular lipid (EMCL) concentrations. More precisely, this technique is based on the observation that the resonance signal from methylene and methyl protons of TAG appear as multiple, but separate, peaks on the proton spectrum of skeletal muscle and are representative of either IMTG or EMCL (see van Loon, 2004). Using this technique, it is consistently reported that IMTG concentration is significantly reduced (~20-40%) in response to moderate-intensity exercise (reviewed in van Loon, 2004), and therefore provides convincing evidence that the IMTG pool contributes to total fat oxidation during exercise in active individuals. Using ^{1}H-MRS it has also been established that a short-term (48 h) high-fat diet enhances IMTG utilisation during exercise, which was attributed to a greater pre-exercise IMTG concentration (Johnson et al., 2003; Zderic et al., 2004). However, using this technique to quantify exercise-induced changes in IMTG content in sedentary, obese individuals and type 2 diabetes patients is difficult, due to the presence of a high EMCL signal which interferes with the quantification of the IMTG signal.

While both stable isotope and ^{1}H-MRS methodology provide some evidence that the IMTG pool contributes to total fat oxidation during moderate-intensity exercise in active individuals, direct evidence obtained through the measurement of net changes in IMTG concentration using the biochemical extraction technique in skeletal muscle tissue are less clear. In accordance, a significant net reduction in IMTG concentration following exercise is reported in a number of studies (Essen-Gustavsson and Tesch, 1990; Phillips et al., 1996c; Sacchetti et al., 2002; Watt et al., 2002a), while others do not observe a significant decrease (Bergman et al., 1999; Kiens et al., 1993; Kiens and Richter, 1998; Wendling et al., 1996). These inconsistent observations may be attributed to methodological issues, particularly a large between-biopsy variability (20-26%) (van Loon, 2004; Watt et al., 2002b), indicating that exercise-induced changes in IMTG concentration of less than ~25% do not always reach statistical significance. It is postulated that the source of this variability is the presence of a variable amount of EMCL deposits in freeze-dried muscle samples, although whether significant pools of EMCL exist remains to be established (Guo, 2001; van Loon, 2004). Nevertheless, failure to eliminate EMCL prior to performing biochemical IMTG extraction leading to contamination of the muscle sample will result in the overestimation of IMTG concentration. Notably, the majority of reports that demonstrate a significant net decrease in IMTG content following exercise are performed in trained individuals who exhibit low EMCL concentrations (Szczepaniak et al., 1999). Thus, data from studies employing the biochemical TAG extraction technique suggest that IMTG oxidation contributes significantly to total fat oxidation during exercise only in trained individuals.

Using either ^{1}H-MRS or biochemical TAG extraction only permits the quantification of mixed muscle TAG concentration and therefore does not allow an appreciation of fibre-specific differences and subcellular distribution of intramuscular lipid deposits. Therefore, immunofluorescence microscopy methods have been developed which aim to discriminate between IMTG content in type I and type II fibres. By combining an antibody stain for slow myosin heavy chain type I with a lipid stain, such as oil red O, this method has been used to demonstrate that IMTG content is approximately 3-fold greater in type I compared to type II fibres (Koopman et al., 2001; van Loon et al., 2003). Furthermore, use of immunofluorescence microscopy reveals that IMTG is deposited in greater abundance in subsarcolemmal regions of the individual muscle fibres (Shaw et al., 2008; Stellingwerff et al., 2007;

van Loon et al., 2004). However, it is also worth noting that immunofluorescence microscopy only provides a semi-quantitative analysis of IMTG concentration, as the efficacy of the staining procedure and image-acquisition settings strongly determines the area fraction stained (i.e. the commonly reported unit of IMTG concentration). Thus, analytical controls should be implemented to minimise variability in IMTG concentration which may confound 'true' intervention and/or population differences. When accurately controlled, this technique has been successfully employed to show 2 h of moderate-intensity cycling induces a >60% net decline of the intramuscular lipid pool specific to type I fibres (van Loon et al., 2003). Further studies have since demonstrated that IMTG concentration is significantly depleted in type I fibres following both endurance-type exercise (De Bock et al., 2008; De Bock et al., 2007; De Bock et al., 2005; van Loon et al., 2005b; Van Proeyen et al., 2011a; Van Proeyen et al., 2011b) and resistance exercise (Koopman et al., 2006). IMTG utilisation during exercise is strongly determined by resting IMTG concentration (Stellingwerff et al., 2007), and type I fibres exhibit a greater oxidative capacity, due to a larger mitochondrial density. Thus it is logical that utilisation of the intramuscular lipid pool during exercise is greatest in type I fibres. Interestingly, mixed-muscle IMTG concentration determined through immunofluorescence microscopy correlates well with values obtained using biochemical TAG extraction (Stellingwerff et al., 2007). However, this relationship disappears when used to quantify IMTG utilisation during exercise (Stellingwerff et al., 2007), as the rate of IMTG utilisation is higher in type I than in type II fibres. In summary, the use of immunofluorescence microscopy demonstrates the importance of considering fibre-specific responses in order to accurately determine the contribution of IMTG oxidation during exercise. Importantly, when the data from studies employing different techniques to study IMTG concentration are pooled, it is consistently demonstrated that IMTG provides a readily available fuel source for trained athletes undertaking moderate-intensity exercise.

1.3.3 Healthy individuals exhibit a high rate of IMTG turnover

As well as demonstrating an ability to use IMTG as a fuel during moderate-intensity exercise, active individuals demonstrate high IMTG fractional synthetic rates compared to sedentary individuals (Bergman et al., 2010), Thus, regular depletion of IMTG stores during exercise and subsequent replenishment during post-exercise recovery illustrates that the intramuscular lipid pool exhibits a high

rate of turnover in trained athletes. Notably, in healthy active individuals complete turnover of the IMTG pool under resting conditions is estimated to take approximately 29 h (Sacchetti et al., 2004). Fundamentally, the turnover of IMTG is a constantly-occurring process, the rate of which will subsequently determine the IMTG concentration at any given time. More specifically, IMTG turnover rate is a function of the relationship between FA uptake, esterification, hydrolysis and oxidation, and therefore an appreciation of the mechanisms that regulate the storage and oxidation of IMTG is important.

(i) IMTG hydrolysis

The hydrolysis of IMTG occurs in three consecutive steps and is dependent on the interaction of at least three different lipases in order to sequentially cleave a FA from the glycerol backbone (Fig. 1.2). Lipolysis is initially catalyzed by adipose triglyceride lipase (ATGL) which converts TAG to diacylglycerol (DAG). Hormone-sensitive lipase (HSL) is the predominant lipase responsible for hydrolyzing DAG to monoacylglycerol (MAG), after which MAG lipase liberates the final FA. HSL is a cytosolic protein, which is most abundantly expressed in adipose tissue, and to a lesser extent in skeletal muscle and cardiac tissue (Kraemer et al., 1993; Langfort et al., 1999). Notably, HSL expression in skeletal muscle is greater in type I fibres compared to type II fibres (Langfort et al., 1999; Peters et al., 1998) and therefore correlates with the fibre-specific capacity for IMTG oxidation. In addition, skeletal muscle HSL activity is increased in response to exercise at varying intensities (Watt et al., 2003a; Watt et al., 2003b), although this does not necessarily result in significant IMTG hydrolysis (Watt et al., 2004). Nevertheless, HSL appears to be required for complete TAG hydrolysis since HSL-null mice demonstrate DAG accumulation (Haemmerle et al., 2002). Furthermore, HSL demonstrates the greatest affinity for DAG, although hydrolase activity of HSL is not limited to DAG (Taschler et al., 2011; Zimmermann et al., 2004). In skeletal muscle, the hydrolase activity of HSL accounts for ~60% of total neutral hydrolase activity at rest, whereas during exercise increased HSL activity has been reported to account for almost all of the total lipase activity (Watt et al., 2004), indicating the importance of HSL activation in the lipolytic process. In general, HSL activation is a hormonal- and contraction-mediated process. Firstly, adrenaline concentrations are increased during exercise which activates β-adrenergic receptors leading to protein kinase A (PKA) stimulation and

serine phosphorylation (S^{563} and S^{660}) of HSL (Watt et al., 2006). Upon phosphorylation, cytosolic HSL is redistributed to LDs, a process which appears to be crucial for complete hydrolase activity of HSL (Prats et al., 2006; Wang et al., 2009), and has been shown to occur in isolated rat soleus muscle following adrenaline incubation or electrically-stimulated muscle contraction (Prats et al., 2006). However, it is not known whether HSL translocation to LDs occurs in response to dynamic exercise in either rodent or human skeletal muscle. Notably, muscle contraction also enhances AMPK activity leading to ser^{565} phosphorylation of HSL, which acts to reduce HSL activity. In addition, the observation that fatty acyl-CoA accumulation occurs at high exercise intensities (>90% $VO_{2\ max}$) may also allosterically inhibit HSL activity (Kiens et al., 1999). These opposing pathways appear to indicate the presence of a mechanism by which HSL activity can be adjusted relative to exercise intensity and duration (Meex et al., 2009; Watt et al., 2006).

HSL knockout mice demonstrate unaltered basal TAG-hydrolase activity (Haemmerle et al., 2002), although ~40% of basal total neutral lipid hydrolase activity is not accounted for by HSL (Watt et al., 2004). In addition, given the high affinity of HSL for DAG hydrolysis, and that HSL knockout mice accumulate DAG in their muscles (Haemmerle et al., 2002), it is apparent that another lipase is responsible for the hydrolysis of TAG. ATGL, first detected in adipose tissue, demonstrates a relatively high affinity for TAG hydrolysis (Zimmermann et al., 2004), and therefore appears the primary candidate for this role. Although most abundantly expressed in adipose tissue, ATGL is also expressed in skeletal muscle and cardiac tissue (Zimmermann et al., 2004), and, like HSL, appears to be most abundant in type I compared to type II muscle fibres (Jocken et al., 2008). Importantly, overexpression of ATGL in non-adipocyte cells enhances the degradation of TAG (Smirnova et al., 2006), whereas ATGL knockout mice demonstrate reduced TAG lipolysis leading to IMTG accumulation (Haemmerle et al., 2006), underlining the importance of ATGL to the liberation of FA stored in intracellular LDs. The regulation of ATGL activity is poorly understood in skeletal muscle. In adipose tissue the activity of ATGL is enhanced upon binding with its co-activator, comparative gene-identification 58 (CGI-58) (Lass et al., 2006), and reduced by G(0)/G(1) switch gene 2 (G0S2) (Yang et al., 2010). Notably, CGI-58 and G0S2 are both expressed in skeletal muscle (Lass et al., 2006; Yang et al., 2010), although their role *in vivo* is yet to be established. However, the importance of

CGI-58 to ATGL activation in skeletal muscle is demonstrated by the fact that individuals who have a mutation of CGI-58 have an increased IMTG content in their muscle (Lass et al., 2006).

Although the role and regulation of TAG and DAG hydrolases is relatively well-understood, at least in adipose tissue, little information is available regarding the lipase activity against MAG. MAG hydrolases act to cleave the remaining FA from the glycerol backbone (Fig. 1.2), and would therefore presumably be important for complete TAG hydrolysis in skeletal muscle, as has been demonstrated in adipose tissue (Taschler et al., 2011). Despite this gap in our knowledge, it is clear that efficient regulation of TAG hydrolysis is fundamental to increase lipolysis during exercise and preserve high rates of TAG turnover in trained individuals.

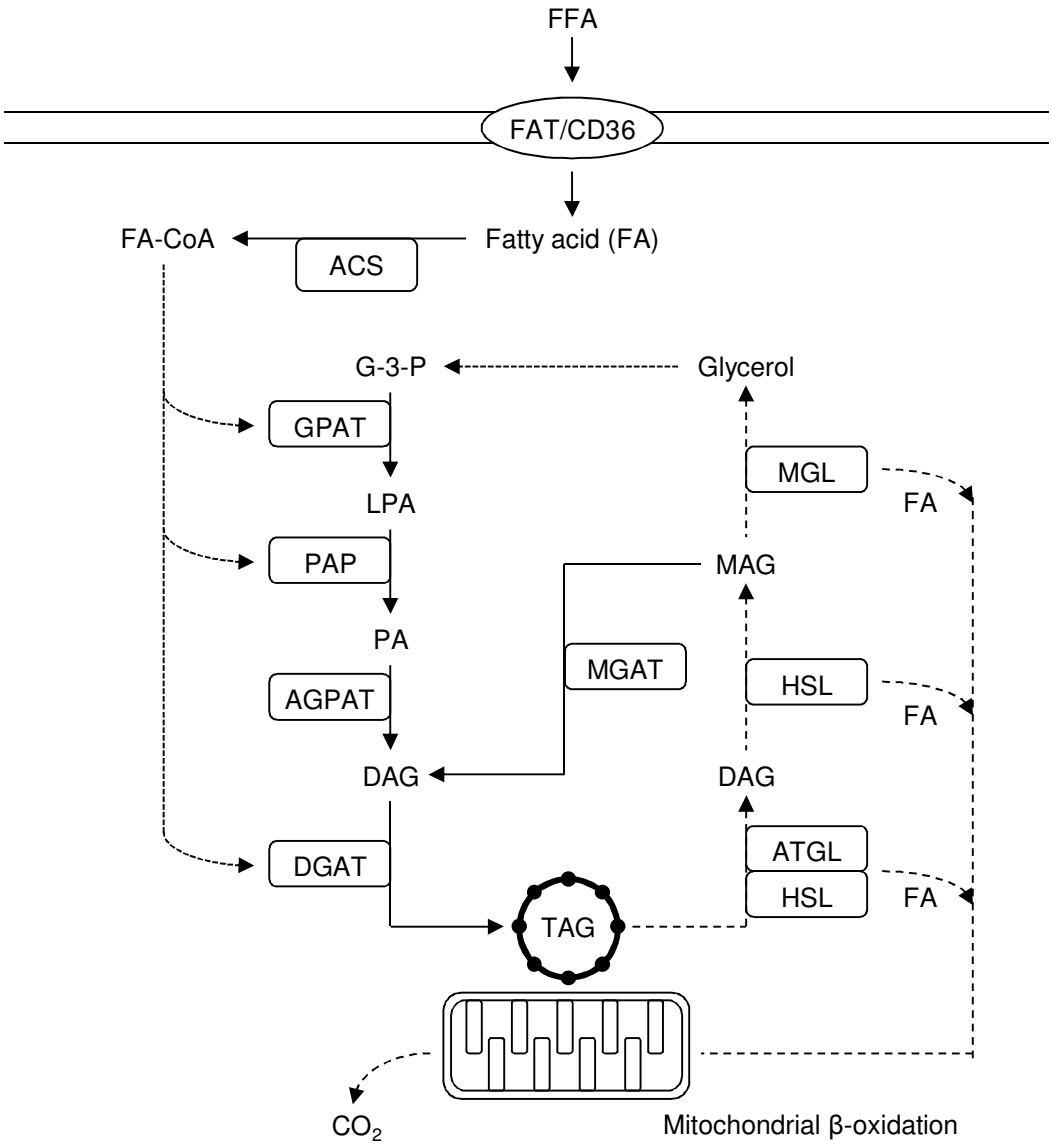


Figure 1.2 TAG synthesis and hydrolysis in skeletal muscle

AGPAT 1-acylglycerol-3-phosphate acyltransferase, *ATGL* adipose triglyceride lipase, *DAG* diacylglycerol, *DGAT* diacylglycerol transferase, *FA*-CoA fatty acyl-CoA, *FAS* fatty acyl-CoA synthetase, FAT*/CD36* fatty acid transporter, *FFA* free fatty acid, *GPAT* glycerol-3-phosphate acyltransferase, *HSL* hormone sensitive lipase, *LPA* lysophosphatidic acid, *MAG* monoacylglycerol, *MGAT* monoacylglycerol transferase, *PAP* phosphatidic acid phosphatase, *TAG* triacylglycerol.

(ii) Proteins associated with IMTG

Intracellular LDs display a large number of proteins associated with their phospholipid monolayer (Zhang et al., 2011) surrounding the LD like a coat (Shaw et al., 2009). The most well-characterised family of these proteins are known as the PAT/perilipin proteins. The nomenclature originates from the founding members perilipin, ADRP (adipose differentiation related protein/adipophilin) and TIP-47 (tail interacting protein of 47kDa), and all present a common NH_2-terminal motif. Two other proteins, S3-12 and OXPAT, are also included in the PAT/perilipin family, as they demonstrate similar sequence homology and/or function to the original PAT/perilipin proteins. In this thesis the PAT proteins will be referred to as perilipin (PLIN) 1 to 5 corresponding to perilipin, ADRP/adipophilin, TIP-47, S3-12 and OXPAT, respectively, in line with a recent call for a unifying nomenclature (Kimmel et al., 2010). Importantly, each PLIN protein possesses a unique tissue distribution and subcellular compartmentalization, indicating that they each play distinct roles in the regulation of LD metabolism.

PLIN1 is the most well-characterised PLIN protein and is exclusively expressed in adipose tissue and steroidogenic cells (Londos et al., 1995), although PLIN1 mRNA have been detected at low levels in skeletal muscle (Gjelstad et al., 2012). In adipocytes and steroidogenic cells PLIN1 appears to regulate the storage and hydrolysis of lipid stored in LDs. Notably, PLIN1 knockout mice demonstrate enhanced basal TAG hydrolysis in isolated adipocytes, which appears to protect against diet-induced obesity (Tansey et al., 2001). In addition, HSL-stimulated lipolysis in isolated adipocytes was reduced (Tansey et al., 2001), indicating that PLIN1 not only functions to reduce lipase activity under basal conditions, but also coordinates recruitment and/or activation of lipases under lipolytic conditions. In support, PKA phosphorylates PLIN1 on serine residues leading to the translocation of HSL to PLIN1-coated LDs in 3T3-L1 adipocytes (Granneman et al., 2007). Therefore, PLIN1 phosphorylation appears to permit HSL access to the stored TAG. In addition, it has been established using COS-7 cells that PLIN1 located at the LD binds and sequesters CGI-58 under basal conditions preventing ATGL activation; however, PKA phosphorylation of PLIN1 releases CGI-58 enabling its association with, and subsequent activation of ATGL (Granneman et al., 2009). Thus, it appears that PLIN1 plays a fundamental role in regulating LD metabolism in adipocytes.

As PLIN1 protein expression does not extend to human skeletal muscle (Alsted et al., 2009; Phillips et al., 2005), this suggests that other perilipin proteins may be required to regulate TAG storage and hydrolysis in this tissue. Two potential candidates are PLIN2 and PLIN3, since HSL redistribution to LDs coated with these proteins occurs in isolated rat soleus muscle following adrenaline incubation or electrically-stimulated muscle contraction (Prats et al., 2006). However, the precise functional role of PLIN2 in skeletal muscle is yet to be established. Interestingly, protein expression of PLIN2 closely correlates with IMTG concentration in Zucker diabetic fatty rats (Minnaard et al., 2009) and endurance trained athletes (Minnaard et al., 2009; Shaw et al., 2009), indicating that the relative expression of PLIN2 may determine the capacity for intracellular TAG storage. In this respect, increasing the expression of PLIN2 enhances the TAG content of fibroblasts (Imamura et al., 2002), HEK 293 cells (Listenberger et al., 2007), hepatic stellate cells (Fukushima et al., 2005), and COS-7 cells (Gao and Serrero, 1999), whereas PLIN2-null mice demonstrate reduced hepatic TAG concentrations compared to their wild-type littermates (Chang et al., 2006). These observations suggest that PLIN2 may either promote TAG synthesis or reduce TAG lipolysis as both will lead to increases in TAG content. Indeed, it has been reported that PLIN2 may limit the access of lipases, such as ATGL, to the stored TAG, thereby reducing the rate of TAG turnover (Bell et al., 2008; Listenberger et al., 2007). Notably, only ~two-thirds of LDs in skeletal muscle contain PLIN2 (Shaw et al., 2009), suggesting that other proteins may be associated with the remaining LD pool. Two potential proteins, PLIN3 and PLIN4, are expressed in skeletal muscle, although to a much lesser degree than they are in adipose tissue (Wolins et al., 2001; Wolins et al., 2003). In addition, they appear to translocate from a pre-existing cytosolic pool to nascent LDs, which form when FA availability is enhanced in adipocytes (Bulankina et al., 2009; Wolins et al., 2005). Importantly, PLIN3 and PLIN4 are both stable in the cytosol, unlike PLIN2 which is degraded when TAG concentration is reduced in Chinese hamster ovary cells (Xu et al., 2005). Furthermore, knock-down of PLIN3 in PLIN2-null fibroblasts reduces the number of LDs (Sztalryd et al., 2006), and it is therefore suggested that PLIN3 may also protect against lipolysis and promote TAG storage. However, the precise role of PLIN3 and PLIN4 in skeletal muscle remains to be established.

PLIN5 is highly expressed in oxidative tissues, including skeletal muscle, cardiac tissue, brown adipose tissue and liver (Wolins et al., 2006b), and it appears from cell culture studies that PLIN5 may be important for the accumulation of TAG, since expression of PLIN5 in Chinese hamster ovary cells (Dalen et al., 2007) or COS-7 cells (Wolins et al., 2006b) increases TAG concentration. In contrast, PLIN5-null mice demonstrate reduced TAG concentrations in the heart compared to wild-type controls (Kuramoto et al., 2012). Similarly to PLIN2, these observations suggest that PLIN5 may either promote TAG synthesis or decrease TAG lipolysis, thereby leading to an increase in TAG concentration. However, it is interesting to note that PKA-stimulation of alpha mouse liver 12 cells overexpressing PLIN5 promotes TAG hydrolysis, increasing the availability of FAs for β-oxidation (Wang et al., 2011b) Taken together, these findings suggest that PLIN5 may be a major regulator of intracellular TAG turnover. More specifically, PLIN5 overexpression in COS-7 cells and Chinese hamster ovary (CHO) cells reveals that this protein may bind either CGI-58 or ATGL (but not both simultaneously) under basal conditions, reducing the TAG hydrolase activity of ATGL, and therefore limiting lipolysis (Granneman et al., 2011; Wang et al., 2011a). However, phosphorylation of PLIN5 under PKA-stimulating conditions causes the release of ATGL and CGI-58 and is proposed to enhance TAG hydrolysis (Granneman et al., 2011; Wang et al., 2011a). Notably, PLIN5 also mediates the physical interaction of LDs with the mitochondria (Bosma et al., 2011; Wang et al., 2011b), and therefore given the role of PLIN5 in controlling TAG hydrolysis, it is suggested that this PLIN protein channels FA released by lipolysis into the mitochondria for β-oxidation. However, at the start of this project no information was available as to the exact role of PLIN5 in skeletal muscle.

(iii) IMTG synthesis

The exact process through which intracellular LDs are formed remains to be fully established, especially in skeletal muscle, although the prevailing model hypothesises that neutral lipid deposited between the leaflets of the endoplasmic reticulum (ER) forms an expanding lens of neutral lipid which is eventually budded into the cytoplasm as a nascent LD (reviewed by (Beller et al., 2010a; Brasaemle and Wolins, 2012; Ducharme and Bickel, 2008). This model is derived from morphological evidence demonstrating that LDs are surrounded by the ER (Tauchi-Sato et al., 2002; Wolins et al., 2005), and that a number of lipid synthesising enzymes are associated with the ER fraction (see (Brasaemle and

Wolins, 2012; Takeuchi and Reue, 2009). In addition, several enzymes involved in the initial esterification of FA moieties to TAG are associated with the mitochondrial fraction (Brasaemle and Wolins, 2012; Takeuchi and Reue, 2009). Fatty acyl-CoA synthetase (FAS) resides in both the mitochondria and ER, and activates FAs for further metabolism through either β-oxidative pathways or TAG synthesis (Fig. 1.2) (Coleman et al., 2000). Fatty acyl-CoA's used for TAG synthesis are first attached to a glycerol-3-phosphate backbone to generate lysophosphatidic acid (LPA), a process which is catalyzed by a key lipogenic enzyme, glycerol-3-phosphate acyltransferase (GPAT). Four GPAT isoforms exist, but differ by their subcellular distribution, such that GPAT1 and GPAT2 localise to the mitochondria, whereas GPAT3 and GPAT4 are observed at the ER (Gimeno and Cao, 2008). Notably ~90% of total GPAT activity in skeletal muscle is attributed to GPAT1 (Park et al., 2002). A second fatty acyl-CoA is subsequently transferred to LPA by a 1-acylglycerol-3-phosphate acyltransferase (AGPAT) to generate phosphatidic acid (PA). Next, the lipin family of enzymes, which confer phosphatase activity, convert PA to DAG. This reaction is reversible through the action of a DAG kinase, which is important as PA is a precursor for phospholipid biosynthesis. Thus, the production of DAG serves as a key point for determining the use of FAs for either TAG storage, growth (phospholipid biosynthesis) or energy production (β-oxidation) (Brasaemle and Wolins, 2012). In the fed state, TAG synthesis proceeds through the action of diacylglycerol transferase (DGAT) catalyzing the addition of the final fatty acyl-CoA to DAG. Two DGAT isoforms exist and both are expressed in skeletal muscle although they demonstrate different subcellular distributions, such that DGAT1 is localised to the ER, whereas DGAT2 is observed at the ER, mitochondria and LD (Bosma et al., 2012b). Importantly, DGAT1 is the predominant isoform expressed in skeletal muscle (Cases et al., 1998; Cases et al., 2001), suggesting that TAG synthesis in this tissue occurs primarily within the ER. To summarise, this brief overview demonstrates that LD formation and storage of TAG is highly dependent on a number of lipogenic enzymes, whose coordinated interaction is a key determinant of the rate of IMTG turnover (Fig. 1.2).

1.3.4 Cellular energetics as a regulator of IMTG use during exercise

As described in the previous section, IMTG utilisation (and whole body fat oxidation) is greatest during an acute bout of moderate-intensity exercise. However, it is important to address the question of how

the proportion that IMTG contribute to total fat oxidation is regulated during moderate-intensity exercise. A number of lines of evidence point at plasma FFA availability as being an important determinant. Indeed, Watt *et al.* (2002) reported that IMTG utilisation was greatest during the first 2 h of a 4 h exercise bout performed at 57% VO_{2max}, whereas the contribution of plasma FFA to total fat oxidation increased during the second half of the exercise bout. Notably, the decrease in IMTG utilisation after 2 h of exercise coincided with an increase in plasma FFA concentration (Watt et al., 2002a). This observation has since been replicated during 2 h of exercise at 50% of maximum aerobic workload (W_{max}) (van Loon et al., 2003). In another study performed by van Loon *et al.* (van Loon et al., 2005b), reducing plasma FFA availability through administration of a nicotinic acid analog (acipimox; inhibitor of adipose tissue lipolysis) enhanced the utilisation of IMTG during 2 h of exercise at 50% of maximum aerobic workload. Unfortunately, van Loon *et al.* (2005) failed to observe any changes in AMPK activation, acetyl-CoA carboxylase (ACC) phosphorylation, or HSL serine phosphorylation between normal and low plasma FFA availability trials, which may otherwise have explained the increase in IMTG utilisation. Nevertheless, these studies do provide evidence to suggest that plasma FFA availability is a key factor determining the contribution of IMTG utilisation to total fat oxidation during low to moderate intensity exercise.

An important observation from the study of van Loon *et al.* (2005) was that although acipimox administration increased the relative contribution of IMTG utilisation to total fat oxidation during exercise, absolute rates of fat oxidation were actually reduced (the energy deficit being accounted for by an increase in glycogen use). Therefore, it appears that plasma FFA availability may regulate the relative contribution of carbohydrate and fat oxidation to total energy expenditure during exercise. The 'glucose-FFA cycle', first proposed by Randle et al (Randle et al., 1963), aimed to explain the reduction in the rate of muscle carbohydrate oxidation when plasma FFA availability was high. Specifically, this theory proposed that increased plasma FFA availability could enhance the rate of fat oxidation and reduce the rate of carbohydrate oxidation by suppressing pyruvate dehydrogenase complex (PDC) activity, via an increase in the acetyl-CoA/free CoA (CoASH) ratio and NADH/NAD^+ ratio. Furthermore, an increase in the cytosolic citrate concentration was proposed to suppress the activity of phosphofructokinase (PFK), resulting in an increase in glucose-6-phosphate, which would

subsequently suppress the activity of hexokinase and glycogen phosphorylase. Therefore, this theory proposed that plasma FFA availability was the key factor determining the relative utilisation of carbohydrate and fat during exercise.

In support of the 'glucose-FFA cycle' (Randle et al., 1963) it has been shown that increasing plasma FFA availability through an intralipid-heparin infusion increases the rate of fat oxidation during exercise (Hawley et al., 2000; Horowitz et al., 1997). However, no significant reductions in plasma FFA concentrations are observed when moving from moderate to high intensity exercise (Romijn et al., 1993; van Loon et al., 2001), as would be expected if plasma FFA availability was the determining factor regulating an increase in carbohydrate oxidation during high intensity exercise. Indeed, it is likely that blood flow is actually greatest during high intensity exercise, suggesting that FFA supply to skeletal muscle would also be greatest during high intensity exercise. It is important to note that Romijn *et al.* (Romijn et al., 1995) did observe a decrease in palmitate rate of appearance (a measure of plasma FFA availability) during exercise performed at ~85% VO_2 max; however, an intralipid infusion to increase plasma FFA availability only partially rescued the rate of fat oxidation during exercise. Taken together, these data suggest that plasma FFA availability does not regulate the relative contribution of carbohydrate and fat oxidation to total energy expenditure during high intensity exercise.

Although plasma FFA availability is not significantly altered when moving from moderate to high intensity exercise, and FFA delivery to skeletal muscle may actually be greater due to an increase in blood flow, this may not necessarily lead to an increase in FA uptake. Indeed, it is logical that a reduced FA uptake would limit the availability of FA for oxidation in the mitochondria. FA uptake into skeletal muscle requires transporter proteins (i.e. FAT/CD36, FABP) that reside at endosomal membranes at rest, and translocate to the plasma membrane upon muscle contraction (Bonen et al., 2000; Bradley et al., 2012). Recently, it has been reported that FA uptake in FAT/CD36 knockout mice is reduced at rest and during exercise compared to their wild-type counterparts, and that this occurs concomitant with a decrease in fat oxidation (McFarlan et al., 2012), suggesting that fat oxidation rates are highly dependent on FA uptake into skeletal muscle. However, plasma membrane

FAT/CD36 concentration was increased concomitant with enhanced FA uptake in giant vesicles prepared from contracting muscle in an intensity-dependent manner (Bonen et al., 2000), suggesting that FA entry into skeletal muscle is not limited as exercise intensity increases. In support, Kiens *et al.* (Kiens et al., 1999) reported that skeletal muscle long chain fatty acid (LCFA) concentrations were increased in response to exercise performed at 90% VO_2 max compared to 65% VO_2 max. Taken together, these data demonstrate that the decrease in fat oxidation observed during high intensity, submaximal exercise is not due to a reduction in the intramyocellular FA availability.

Given that the oxidation of FA's (through β-oxidation) occurs within the mitochondria, it is possible that limiting the entry of FA into the mitochondria may explain how fat oxidation is regulated. Importantly, medium chain fatty acids (MCFA) can enter the mitochondria directly, whereas LCFA require a transport mechanism in order to cross through the outer and inner mitochondrial membranes. Based on this characteristic, two studies have investigated the oxidation of MCFA and LCFA under conditions of high glycolytic flux, either by pre-exercise glucose feeding (Coyle et al., 1997) or by moving from a low (40% VO_2 max) to a high (80% VO_2 max) exercise intensity (Sidossis et al., 1997). Infusion of a ^{14}C-labeled MCFA (octanoate) and a ^{13}C-labeled LCFA (oleate) allowed quantification of the proportion of MCFA and LCFA taken up and oxidised during exercise. During exercise at 80% VO_2 max LCFA oxidation was reduced, whereas MCFA oxidation, which does not rely on a mitochondrial transport mechanism, was unchanged compared to exercise at 40% VO_2 max (Sidossis et al., 1997). The authors concluded that LCFA oxidation was limited by the mitochondrial transport mechanism for LCFA, which involves the enzyme carnitine palmitoyl transferase-1 (CPT-1). Therefore, increasing concentrations of malonyl-CoA during high intensity exercise would inhibit CPT-1 activity, thereby reducing LCFA transport into the mitochondria. However, no evidence is available to support the notion that malonyl-CoA concentrations are increased in human skeletal muscle as exercise intensity increases (Odland et al., 1998); rather, malonyl-CoA concentrations may actually decrease during exercise in an intensity-dependent manner (Dean et al., 2000). Thus, while it appears that LCFA transport into the mitochondria may limit fat oxidation during high intensity exercise this cannot be attributed to the suppression of CPT-1 activity by an increase in malonyl-CoA concentrations. In addition, H^+ accumulation as a result of high glycolytic flux during high intensity exercise may

contribute to the reduction in fat oxidation through a pH-mediated downregulation in CPT-1 activity (Starritt et al., 2000).

It is important to consider that performing high intensity, submaximal exercise is associated with an increase in the ratios of AMP/ATP and ADP/ATP, and that these metabolic disturbances stimulate glycolytic flux. Specifically, an increase in AMP and ADP will lead to allosteric activation of the key glycolytic enzymes glycogen phosphorylase, PFK and PDHa. Thus, during high intensity exercise, the flux through PDC will be in excess of the flux through the tri-carboxylic acid (TCA) cycle leading to an accumulation of acetyl-CoA (van Loon et al., 2001). As acetyl-CoA is a precursor for malonyl-CoA, an increase in acetyl-CoA concentration could theoretically lead to greater malonyl-CoA production; however, it has already been concluded that malonyl-CoA concentrations are not increased during high intensity exercise. An alternative fate for the excess acetyl-CoA is the formation of acetylcarnitine, a reaction catalysed by the enzyme carnitine acetyltransferase (CAT). In this way, free carnitine provides a buffer for the excess acetyl-CoA, and ensures that a viable pool of CoASH is maintained which is important for the continuation of PDC and TCA flux. However, as a consequence of this buffering mechanism for acetyl-CoA, the free carnitine pool is significantly reduced. Therefore, it is proposed that a reduction in the availability of carnitine through the process of acetyl group buffering limits the capacity for carnitine to participate in the transport of LCFA into the mitochondria through CPT-1. In support, it has been shown that acetylcarnitine concentrations are increased during high intensity, submaximal exercise concomitant with a reduction in free carnitine and a decrease in fat oxidation (van Loon et al., 2001). In contrast, acetylcarnitine and free carnitine concentrations are significantly lower and higher, respectively, during moderate-intensity exercise, suggesting that the flux through PDC is similar or only marginally greater than the flux through the TCA cycle (van Loon et al., 2001). Accordingly, total fat oxidation (and the contribution of IMTG utilisation) is greatest during moderate-intensity exercise when there is sufficient carnitine available to participate in LCFA transport into the mitochondria. Overall, current evidence suggests that the contribution of fat oxidation (and IMTG utilisation) to total energy expenditure during exercise is regulated primarily by the glycolytic and PDC flux.

1.4 Physical inactivity – a major contributor to chronic disease

The present-day lifestyle is far removed from that in which our "physically active genotype" originated. In accordance, recent advances in technology have increased the use of motorised transport, and an occupational shift from labour-intensive work (e.g. farming, construction, and mining) to sedentary office-based jobs has occurred. Further, pressure in the workplace has increased, such that less time is prioritised for physical activity concomitant with greater consumption of energy-dense foods with poor nutritional value. In fact, in 2008, only 39% of men and 29% of women met the current physical activity guidelines which suggest that individuals should perform at least 150 min of exercise per week (Health Survey for England, (2010). Therefore, at least ~65% of the UK population do not achieve the levels of physical activity that are required to remain free of chronic disease. Interestingly, a recent report by the World Health Organisation (WHO) stated that physical inactivity was a leading cause of mortality worldwide (WHO, 2010).

Leading a physically inactive lifestyle is associated with the development of a number of metabolic disorders including obesity, insulin resistance, metabolic syndrome, type 2 diabetes and cardiovascular disease. Obesity is the result of an imbalance between energy intake and expenditure, and is therefore considered a consequence of physical inactivity coupled with the consumption of an energy dense diet. Physical inactivity is also associated with insulin resistance, which is characterised by an inability of tissues to respond to a physiological increase in plasma insulin concentration. In healthy individuals, insulin is secreted from the pancreas in response to carbohydrate ingestion, and is responsible for the clearance of glucose from the plasma into peripheral tissues, such as skeletal muscle. However, in the insulin resistant state physiological insulin secretion becomes insufficient to stimulate glucose uptake into skeletal muscle, resulting in greater insulin secretion in order to return to a state of euglycaemia. Further progression of insulin resistance to type 2 diabetes is characterised by prolonged periods of postprandial hyperglycaemia and hyperinsulinaemia, where high levels of insulin are inadequate to reduce plasma glucose concentrations following a meal. Notably, the progression to type 2 diabetes correlates strongly with BMI (Edelstein et al., 1997), indicating that obesity (and physical inactivity) is a primary risk factor for the development of type 2 diabetes.

1.5 Lipid-induced insulin resistance of skeletal muscle

Experimentally, a shift from a state of high physical activity to a sedentary condition decreases insulin-stimulated glucose disposal into skeletal muscle and promotes greater abdominal fat storage, as evidenced from studies in rodents (Kump and Booth, 2005) and humans (see (Booth et al., 2008). As skeletal muscle accounts for ~50% of glucose disposal following ingestion of a mixed meal (Capaldo et al., 1999), skeletal muscle glucose uptake is a key determinant of whole-body insulin sensitivity. Since physical inactivity is also associated with increased adiposity, it is likely that greater lipid storage may also contribute to reduced insulin sensitivity.

1.5.1 Lipid 'overflow' hypothesis

Adipose tissue is the primary organ controlling the plasma concentrations of FFA and TAG in the fasted and postprandial states, and plasma FFA concentrations are greatest following an overnight fast (typically 0.3 to 0.6 $mmol.l^{-1}$). Ingestion of a mixed meal increases the plasma glucose concentration resulting in insulin secretion from pancreatic β-cells. The subsequent increase in plasma insulin concentrations has three predominant effects: 1) suppression of adipose tissue hormone sensitive lipase (HSL) activity reducing FFA release into the circulation, 2) activation of lipoprotein lipase (LPL) in the capillaries of adipose tissue, thereby stimulating hydrolysis of chylomicron-TAG, releasing FA that are subsequently taken up by adipocytes for storage as TAG, and 3) suppression of LPL activity in the capillary bed of skeletal muscle. It is important to note that a proportion of FA liberated from chylomicron-TAG always escape esterification into adipose tissue and contribute to the plasma FFA pool, which is termed 'spillover'. Fundamentally, through its ability to buffer the lipid flux adipose tissue is suggested to be important in protecting non-adipose tissues, such as the liver, skeletal muscle, heart and pancreatic β-cells, from the potentially damaging effects of elevated plasma concentrations of FFA and TAG (Frayn, 2002).

In obese subjects displaying large visceral adipose tissue stores, there is considerable evidence to suggest that the ability of adipose tissue to buffer the lipid flux is compromised, an observation that is commonly associated with insulin resistance (Bays et al., 2004; Frayn, 2002; Lewis et al., 2002). In particular, the obese phenotype is reported to exhibit impaired insulin-mediated suppression of

adipose tissue lipolysis, resulting in greater appearance of FFA liberated from adipocytes in the circulation (Coppack et al., 1992; Hickner et al., 1999). This increase in FFA appearance in the plasma has been linked to insulin resistance, since high-fat feeding for 56-67 h enhances plasma FFA concentrations concomitant with reduced insulin sensitivity (Johnson et al., 2006; Pehleman et al., 2005). Furthermore, elevating plasma FFA concentrations through infusion of an intralipid/heparin emulsion in humans decreases insulin-stimulated glucose uptake in skeletal muscle (Bachmann et al., 2001; Boden and Chen, 1995; Itani et al., 2002; Kelley et al., 1993; Roden et al., 1999). However, rates of insulin-stimulated glucose disposal were only reduced after 2 hours of lipid infusion, suggesting that plasma FFA per se do not directly interfere with insulin action. This is consistent with the observation that rates of adipose tissue FFA release in obese individuals are normal when adjusted relative to total fat mass (Bickerton et al., 2008; Campbell et al., 1994; Fabbrini et al., 2009; McQuaid et al., 2011; Robinson et al., 1998). Consequently, plasma FFA concentrations may not differ between lean and obese individuals (Bickerton et al., 2008; McQuaid et al., 2011; Reeds et al., 2006), indicating that the elevated plasma insulin concentrations observed in insulin-resistant individuals may actually be sufficient to suppress FFA release from adipose tissue. Rather, it appears that the capacity of adipose tissue to store plasma-derived TAG is impaired in obese individuals (McQuaid et al., 2011), which may be due to a reduced LPL rate of action (per unit of fat tissue) in the capillary bed of adipose tissue (Frayn, 2002; Karpe et al., 2011). As a result, there is an increase in TAG which has not undergone lipolysis in the plasma, and it is suggested that the elevated plasma TAG concentrations are more closely related to insulin resistance than increased FFA concentrations (Bickerton et al., 2008; Karpe et al., 2011). In this respect, TAG remnants of chylomicron-TAG are deposited in the liver and provide substrate for the production of very low-density lipoproteins containing TAG (VLDL-TAG), and increased assembly and secretion of VLDL-TAG is a common feature of obesity and insulin resistance (Karpe et al., 2011; Reilly and Rader, 2003). Accordingly, increased plasma TAG and FFA concentrations during an intralipid/heparin infusion are associated with insulin resistance (Bachmann et al., 2001), and it is postulated that FFAs 'spillover' into non-adipose tissues in order to maintain plasma lipid homeostasis. In particular, an increase in intracellular TAG concentration is observed in the liver and skeletal muscle. Consequently, the obese insulin resistant state is characterised by reduced insulin-stimulated suppression of hepatic glucose

output and glucose uptake into skeletal muscle. Accordingly, a link between lipid accumulation in non-adipose tissues in obesity and hyperglycaemia in insulin resistance has been identified.

Several lines of evidence are available to support the notion that lipid accumulation in non-adipose tissues is an important event in the development of insulin resistance. Firstly, lipodystrophy is a condition that is characterized by partial or complete loss of adipose tissue. As a result, excess calorie ingestion in lipodystrophic mice or humans promotes lipid storage in non-adipose tissues, such as skeletal muscle and liver, concomitant with reduced insulin sensitivity (Gavrilova et al., 2000; Kim et al., 2000a). A-ZIP/F-1 mice exhibit a severe form of lipoatrophic diabetes, but transplantation of adipose tissue from wild-type mice into these mice reverses hyperglycaemia, reduces insulin concentrations and improves skeletal muscle insulin sensitivity (Gavrilova et al., 2000; Kim et al., 2000a). Significantly, these adaptations occurred concomitant with a decrease in lipid storage in skeletal muscle and liver tissue (Kim et al., 2000a), underlining the importance of adipose tissue to maintain skeletal muscle insulin sensitivity. Secondly, thiazolidinedione (TZD) treatment of obese Zucker rats activates peroxisome proliferator γ (PPARγ) receptors in adipocytes, leading to adipocyte differentiation. As a consequence, lipid stored in both skeletal muscle and liver is redistributed to adipocytes, concomitant with improved insulin sensitivity (Hallakou et al., 1997). Further evidence is available from human studies in both healthy individuals and type 2 diabetic patients, whereby supplementation of the TZD, Pioglitazone, improves insulin sensitivity in line with a reduction in muscle lipid concentration (Rasouli et al., 2005; Teranishi et al., 2007). Together, these reports demonstrate that the accumulation of lipid in non-adipose tissues, such as liver and skeletal muscle, contributes to the development of insulin resistance.

1.5.2 IMTG accumulation, per se, is not responsible for insulin resistance

IMTG concentration is negatively associated with insulin-stimulated glucose uptake in rodents (Storlien et al., 1991) and humans (Pan et al., 1997), in as much as elevated IMTG content provides a risk factor for the development of insulin resistance and/or type 2 diabetes. Accordingly, greater IMTG concentrations are observed in insulin-resistant obese individuals and obese type 2 diabetes patients when compared to lean, insulin-sensitive controls (Goodpaster et al., 2001; van Loon et al., 2004).

However, this relationship becomes distorted when endurance-trained athletes are included in the comparison as they also demonstrate elevated IMTG concentrations, yet remain exquisitely insulin sensitive, in a phenomenon known as the "athlete's paradox" (Goodpaster et al., 2001; van Loon et al., 2004). Therefore, only when comparing individuals who are sedentary does IMTG content negatively correlate with insulin sensitivity. In fact, elevated IMTG content in endurance-trained athletes may actually predict greater insulin sensitivity (Thamer et al., 2003). Thus it appears that the quantity of FA stored within intramuscular LDs, per se, does not directly impair insulin sensitivity.

The relationship between IMTG concentration and insulin sensitivity may be partly explained by the fibre type composition of skeletal muscle. Type I muscle fibres are reported to exhibit a three to four-fold greater IMTG content than type II fibres (He et al., 2001; van Loon et al., 2003; van Loon et al., 2004). This may be an important observation given that skeletal muscle of obese individuals and/or type 2 diabetes patients demonstrates a reduced proportion of type I fibres when compared to lean individuals or BMI-matched controls (Nyholm et al., 1997; Tanner et al., 2002), although this is not a universal finding (van Loon et al., 2004). Nevertheless, it is well-documented that endurance-trained athletes exhibit a large proportion of type I fibres (Andersson et al., 2000). Therefore, fibre type composition combined with fibre-specific lipid content may partly explain the paradoxical relationship between IMTG concentration and insulin sensitivity in trained athletes, obese individuals and type 2 diabetes patients. In this respect, it was reported that mixed muscle IMTG content was ~78% greater in trained athletes compared to type 2 diabetes patients (van Loon et al., 2004). However, when corrected for fibre type composition, ~43% of the difference in IMTG content between groups was explained by the greater proportion of type I fibres observed in trained athletes, with the remainder attributed to greater IMTG content in type I fibres in athletes. Therefore, muscle fibre composition only partly explains the differences between groups in the relationship between IMTG content and insulin sensitivity. It is also worth considering that IMTG concentration is a function of both the number and volume of TAG-containing LDs. In this respect, it appears that the elevated IMTG concentration in trained athletes is attributable to a greater number of LDs, whereas no difference in LD volume was observed when compared to obese individuals or type 2 diabetes patients (van Loon et al., 2004).

1.5.3 IMTG metabolism as a determinant of insulin sensitivity

(i) An imbalance in IMTG turnover leads to IMTG accumulation

Elevated IMTG storage in endurance-trained individuals appears to represent one of a number of metabolic adaptations to endurance training (Amati et al., 2011; Goodpaster et al., 2001; van Loon et al., 2004), increasing the capacity to use IMTG as a substrate source during exercise. Accordingly, longitudinal training studies (De Bock et al., 2008; Phillips et al., 1996b; Schrauwen et al., 2002; Van Proeyen et al., 2011a; Van Proeyen et al., 2011b) and cross-sectional comparisons between trained and untrained individuals (Coggan et al., 2000; Klein et al., 1994) demonstrate that the IMTG pool makes a significant contribution to total substrate oxidation during exercise following endurance training. On the other hand, it appears that IMTG does not provide a readily available fuel source during exercise in sedentary, obese and/or type 2 diabetes patients (Blaak et al., 2000; Schrauwen et al., 2002). This finding appears logical given that elevated plasma FFA concentrations, as observed in obese individuals and/or type 2 diabetes patients, may inhibit IMTG utilisation during exercise (van Loon et al., 2005b). Notably, administration of acipimox to inhibit adipose tissue lipolysis thereby reducing plasma FFA concentrations enhanced the utilisation of IMTG during exercise (van Loon et al., 2005b). Therefore, in obese individuals and/or type 2 diabetes patients the elevated plasma FFA availability and FA uptake inhibit the capacity for IMTG utilisation, and as a result IMTG accumulation occurs. In trained individuals a harmonious relationship between FA uptake, esterification, hydrolysis and oxidation is apparent resulting in high rates of IMTG turnover. However, in obese individuals and/or type 2 diabetes patients FA uptake is greater than the rate of FA oxidation, implying that the rate of IMTG turnover is low as well.

Not only is a low rate of IMTG turnover associated with an increase in IMTG content, but the functional imbalance between IMTG storage, lipolysis and FA oxidation may also result in the accumulation of FA metabolites. These metabolites possess signalling properties that appear to downregulate the insulin signalling cascade. The most well-studied FA metabolites thought to possess the capacity to negatively impact insulin signalling include DAGs, long chain fatty acyl-CoAs (LCFA-CoA) and ceramides. DAG is an intermediate in both IMTG synthesis and hydrolysis, and is increased in obesity-induced insulin resistance in animals (Turinsky et al., 1990a) and lipid-induced insulin

resistance in humans (Itani et al., 2002). However, cross-sectional studies comparing lean and obese individuals (Jocken et al., 2010; Thrush et al., 2009) or normal glucose tolerant and obese insulin resistant individuals (Coen et al., 2010; Perreault et al., 2010) do not report significant differences in skeletal muscle DAG concentration. Nevertheless, DAG concentration may be an important predictor of insulin resistance (Bergman et al., 2012; Moro et al., 2009; Straczkowski et al., 2007). More precisely, DAG saturation is greater in sedentary lean and obese individuals or those with insulin resistance compared to athletes or normal glucose tolerant controls (Bergman et al., 2012; Bergman et al., 2010; van Hees et al., 2011), although observations to the contrary are also reported (Amati et al., 2011). Most recently it has been reported that the degree of FA saturation in the DAG fraction extracted from the muscle plasma membrane fraction of individuals with obesity and type 2 diabetes was greater than of trained athletes, and that it showed a negative association with insulin sensitivity (Bergman et al., 2012). Thus, while total DAG is not consistently increased in the insulin resistant state, insulin resistance induced by FA metabolites may be dependent on the accumulation of certain DAG species at specific intracellular locations, in this case the compartment in which the proximal insulin signalling intermediates reside.

Ceramides provide structural support for biological membranes, and require long chain saturated FA for their biosynthesis. Furthermore, ceramide accumulation is reported in obese individuals and type 2 diabetes patients compared with lean controls (Adams et al., 2004; Coen et al., 2010; Moro et al., 2009; Straczkowski et al., 2007; Thrush et al., 2009), although reports to the contrary also exist (Skovbro et al., 2008). Interestingly, ceramide content is reported to be greater in type I compared to type II fibres in healthy lean individuals (Nordby et al., 2010) and obese subjects with or without type 2 diabetes (Kristensen et al., 2012). However, as with DAG, ceramide FA saturation may be more important than total ceramide content, as this is increased in obese compared to lean individuals (Adams et al., 2004; Moro et al., 2009; Thrush et al., 2009). Furthermore, 4-6 h intralipid infusion, which mainly consists of unsaturated lipid, induces insulin resistance without significant elevations in ceramide content (Hoeg et al., 2011; Itani et al., 2002; Vistisen et al., 2008). Thus, a high degree of FA saturation appears to be an important factor in the mechanism by which ceramide accumulation leads to skeletal muscle insulin resistance. Much less is known about the effects of long-chain fatty

acyl-CoAs. If the rate of IMTG synthesis or FA β-oxidation is not matched to FA uptake then fatty acyl-CoA accumulation occurs in either the cytosolic or membrane fraction. Notably, rats fed a high-fat compared to a standard chow diet (Ellis et al., 2000), or obese compared to lean humans (Hulver et al., 2003) demonstrate long chain fatty acyl-CoA accumulation. In addition, insulin-sensitising interventions reduced intramuscular long chain fatty acyl-CoA concentrations (Bajaj et al., 2010; Bajaj et al., 2005; Houmard et al., 2002). However, contrary to these reports, no differences in long chain fatty acyl-CoA concentrations were observed between obese individuals, type 2 diabetes patients or trained athletes (Bruce et al., 2003). Taken together, further studies are required to elucidate the exact role that lipid metabolite species play in the development of skeletal muscle insulin resistance.

It is clear that further investigations are required in order to determine the species, saturation status and subcellular localisation of the exact lipid species responsible for inducing obesity-related insulin resistance in skeletal muscle. Nevertheless, it is logical that their accumulation may be a function of a mismatch between FFA uptake and IMTG synthesis, or an increased rate of IMTG hydrolysis which is not matched by an increased FA oxidation rate. Therefore, further understanding of the mechanistic processes underlying LD synthesis, hydrolysis and oxidation, including the role of LD-associated proteins will help provide an explanation for the underlying cause of lipid-induced insulin resistance.

(ii) Control of FA uptake, IMTG synthesis and hydrolysis, and FA oxidation in insulin resistance

The expression of the lipid transporter, fatty acid translocase (FAT/CD36), appears to be similar in obese individuals and lean controls, at least when assessed using whole muscle homogenates (Aguer et al., 2010; Bonen et al., 2004; Holloway et al., 2007). Like the glucose transporter, GLUT4, FAT/CD36 is able to cycle between intracellular depots and the plasma membrane in response to muscle contraction and insulin (Bonen et al., 2000; Luiken et al., 2002). However, under conditions of chronically elevated plasma FFA concentrations, as in obesity, it has been shown that permanent relocation of FAT/CD36 to the plasma membrane fraction occurs (Aguer et al., 2010; Bonen et al., 2004). As a result, the capacity for plasma-derived FA uptake may be enhanced in the insulin resistant state.

Despite plasma FFA availability and FA uptake being elevated in obese individuals and/or type 2 diabetes patients, an enhanced capacity for IMTG synthesis would direct FA towards storage, reducing their potential to negatively impact insulin signalling. Notably, rodent studies report improved insulin sensitivity when DGAT1, a key rate-limiting enzyme in IMTG synthesis, is overexpressed in skeletal muscle (Liu et al., 2009; Liu et al., 2007; Timmers et al., 2011). However, reports comparing lean and obese individuals do not demonstrate significant differences in the expression of GPAT1 or DGAT1 (Li et al., 2011; Thrush et al., 2009). Furthermore, DGAT1 mRNA or protein expression does not differ between trained athletes and sedentary controls (Amati et al., 2011; Bergman et al., 2010; Schmitt et al., 2003), and subsequently it has been reported that 8-16 weeks of endurance training does not significantly alter DGAT1 protein expression in lean (Alsted et al., 2009) or obese individuals (Dubé et al., 2011). In contrast, acute exercise increases protein expression of both GPAT1 and DGAT1 while preventing insulin resistance induced by an overnight lipid-heparin infusion (Schenk and Horowitz, 2007). Thus, GPAT1 and DGAT1 protein expression appears to respond to changes in plasma FFA availability when preceded by exercise-induced increases in IMTG lipolysis leading to transient decreases in IMTG content. However, obese individuals and/or type 2 diabetes patients exhibit chronically elevated plasma FFA concentrations, yet GPAT1 and DGAT1 protein expression is not significantly different when compared to lean, healthy individuals. Accordingly, it is speculated that a failure to upregulate IMTG synthesis when plasma FFA availability and uptake is increased may negatively impact insulin sensitivity. Indeed, DGAT1 knockout mice demonstrate reduced insulin sensitivity concomitant with a greater concentration of lipid metabolites (Liu et al., 2007). However, whether an impaired capacity for IMTG synthesis (relative to FA availability) plays a causative role in the development of lipid-induced insulin resistance requires further investigation.

The rate of IMTG turnover is not only determined by the capacity for IMTG synthesis, but also IMTG lipolysis. Direct evidence obtained *in vivo* using microdialysis suggests that skeletal muscle lipolysis is impaired in obese individuals (Blaak et al., 2004). However, IMTG lipolysis is largely under the control of two lipases, ATGL and HSL, which differ in their affinity for TAG and DAG, respectively (Zechner et al., 2012). As such, a mismatch in the activity of HSL relative to ATGL will result in the accumulation of FA metabolites. In accordance, ATGL protein expression is increased in insulin resistant obese

individuals relative to lean controls, whereas a proportionate increase in HSL protein expression is not observed (Badin et al., 2011; Jocken et al., 2010). As a result, DAG hydrolase activity is reduced relative to TAG hydrolase activity (Jocken et al., 2010; Moro et al., 2009), a factor which is estimated to explain 54% and 38% of the increase in DAG and ceramide accumulation in obesity, respectively (Moro et al., 2009). However, DAG accumulation is not always associated with a reduced DAG to TAG hydrolase ratio (Jocken et al., 2010), and skeletal muscle ATGL and HSL protein expression is not consistently different between insulin resistant and insulin sensitive individuals (Coen et al., 2010; Li et al., 2011). Thus, whether impairments in IMTG lipolysis promote the accumulation of lipid metabolites that contribute to lipid-induced insulin resistance remains to be determined.

The activation of ATGL and HSL requires a number of other proteins, at least in cell culture studies, such as CGI-58, G0S2, and the perilipin proteins (Granneman et al., 2009; Granneman et al., 2011; Wang et al., 2011a; Wang et al., 2011b). As such, a reduced DAG to TAG hydrolase ratio may be attributable to a disproportionate activation of ATGL or HSL. However, CGI-58 protein expression does not differ in skeletal muscle of lean and obese individuals (Jocken et al., 2010), and skeletal muscle expression of G0S2 is yet to be investigated. The predominant role of the PLIN proteins appears to be as a protective coat for stored TAG against the action of intracellular lipases, such as ATGL or HSL. Specifically, expression of PLIN2 in human embryonic kidney cells or AML12 cells limits the interaction of ATGL with TAG-containing LDs, thereby reducing the rate of TAG hydrolysis (Bell et al., 2008; Listenberger et al., 2007). In addition, using COS-7 cells it was observed that PLIN5 binds either CGI-58 or ATGL (but not both simultaneously) under basal conditions, which may reduce the activation of ATGL by CGI-58 (and therefore the TAG hydrolase capacity of ATGL), thus limiting the rate of lipolysis (Granneman et al., 2011; Wang et al., 2011a). Taken together these observations suggest that the PLIN proteins appear to be important in protecting stored TAG from unregulated lipolysis, which may generate insulin resistance-inducing lipid metabolites. In line with this proposal, downregulation of PLIN2 in AML12 liver cells decreases insulin sensitivity, as evidenced by reduced insulin stimulated Akt phosphorylation (Bell et al., 2008). In humans, limited evidence is available to discern a relationship between PLIN protein expression and insulin sensitivity. Indeed, PLIN2 protein expression has been reported to be similar between type 2 diabetes patients and BMI-matched

controls, although pharmacological treatment with insulin-sensitizing drugs may increase (Phillips et al., 2005) or decrease perilipin 2 expression (Minnaard et al., 2009). Interestingly, PLIN3 expression is reported to be greater in obese individuals compared to lean controls, when corrected for IMTG concentration (Li et al., 2011). Furthermore, PLIN5 protein expression was enhanced in trained athletes compared to sedentary lean and obese controls, and PLIN5 expression correlated with IMTG concentration (Amati et al., 2011). Thus, the expression of PLIN proteins might represent an adaptive response to altered intramuscular lipid content. However, given that PLIN5 is present on the LD and mitochondria (Bosma et al., 2011; Wang et al., 2011b), this suggests that PLIN5 may be a key regulator of IMTG-derived FA delivery to the mitochondria for oxidation.

A reduced capacity to oxidise FA liberated from IMTG-containing LDs may also contribute to lipid metabolite accumulation and impaired insulin signalling. Importantly, a reduced skeletal muscle oxidative capacity, as assessed through measurements of the common mitochondrial markers citrate synthase (CS), β-hydroxyacyl-CoA dehydrogenase (β-HAD) and cytochrome *c* oxidase (COX), correlates with a decrease in the capacity for FA oxidation (Simoneau et al., 1999). In accordance, cross-sectional studies report that obese individuals and/or type 2 diabetes patients exhibit a reduced oxidative capacity (Amati et al., 2011; Bruce et al., 2003; Goodpaster et al., 2001). However, whether the reduced oxidative capacity is due to intrinsic defect in the mitochondrial oxidative machinery or simply the result of a decrease in mitochondrial density requires further investigation. Indeed, a number of reports implicate the latter as the underlying cause of a reduced capacity for FA oxidation (He et al., 2001; Holloway et al., 2007; Kelley et al., 2002; Kim et al., 2000b; Ritov et al., 2005), while other reports suggest that there is an intrinsic defect in the mitochondrial oxidative machinery (Liang et al., 1997; Petersen et al., 2004). In addition, it is suggested that mitochondrial density within the subsarcolemmal region of the muscle fibre is where the greatest reduction is observed, compared to lean controls (Ritov et al., 2005), although other studies attribute the reduction in mitochondrial content to the intermyofibrillar region of the muscle fibre (Chomentowski et al., 2011; Holloway et al., 2007). Nevertheless, substantial evidence exists to indicate that obese individuals have a reduced oxidative capacity, presumably due to a lack of regular physical activity, and this may be a more important determinant of insulin sensitivity than an increased IMTG concentration (Bruce et al., 2003).

In summary, an imbalance between the processes liberating FA (IMTG lipolysis and FA uptake) and those processes that consume FA (IMTG synthesis and mitochondrial β-oxidation) may lead to an increase in the concentration of FA metabolite species, thereby contributing to the development of skeletal muscle insulin resistance. It appears that in trained athletes, who exhibit regular depletion and resynthesis of the IMTG pool, this balance is maintained to a greater extent than in sedentary obese individuals and type 2 diabetes patients and therefore trained athletes remain highly insulin sensitive. However, the mechanisms regulating the efficient balance between FA liberation and consumption in trained individuals are not well understood.

1.5.4 Lipid-induced impairments of insulin signalling in skeletal muscle

As already described, it is generally accepted that lipid-induced insulin resistance is associated with the accumulation of FA metabolites, which include long chain fatty-acyl CoAs, DAG, and ceramides (Samuel et al., 2010). Although controversy still exists as to whether absolute intracellular DAG concentration, saturation, or subcellular localisation (or a combination of all three) is the key factor in determining insulin sensitivity in skeletal muscle, DAG is implicated in the development of insulin resistance due to its ability to activate novel protein kinase C (PKC) isoforms (δ, ε, ζ, θ, and μ). Accordingly, high fat feeding in rodents (Schmitz-Peiffer et al., 1997) and lipid infusion studies conducted in humans (Itani et al., 2002) suggest that PKC activation, as measured through PKC translocation to the plasma membrane, is increased concomitant with elevated DAG concentrations and a reduced rate of glucose disposal. Notably, PKC-θ knockout mice are protected against the detrimental effects of a lipid infusion, indicating the importance of PKC activation in the development of lipid-induced insulin resistance (Kim et al., 2004). Furthermore, PKC-θ is increased in the muscle plasma membrane fraction of obese individuals (Itani et al., 2000) and type 2 diabetes patients (Itani et al., 2001). Moreover, a recent study has reported that PKC-ε activation is positively associated with the plasma membrane concentration of DAG, which itself is negatively associated with insulin sensitivity (Bergman et al., 2012). Taken together, these reports suggest that DAG-induced translocation of PKC to the plasma membrane is an important feature of insulin resistance, presumably by increasing the proximity of PKC to components of the insulin signalling cascade. In accordance, it appears that DAG-activated novel PKCs target the IRS proteins (Fig. 1.3), either

directly or through upstream activation of the stress kinases IκBα kinaseβ (IKKβ) and c-Jun NH2-terminal kinase (JNK). Importantly, enhanced PKC-θ activation is associated with reduced tyrosine phosphorylation and increased serine phosphorylation of IRS-1 in response to a lipid infusion in humans (Yu et al., 2002). A plethora of studies have now established the predominant serine phosphorylation sites which are targeted by novel PKCs and stress kinases which include ser^{307}, $ser^{636/639}$, and ser^{1101} (reviewed in (Boura-Halfon and Zick, 2009; Gual et al., 2005). Given that serine phosphorylation of IRS-1 generally inhibits IRS1 tyrosine phosphorylation and subsequent downstream activation of PI3-K and Akt/PKB, it is postulated that insulin-stimulated GLUT4 translocation is consequently reduced (Fig. 1.3).

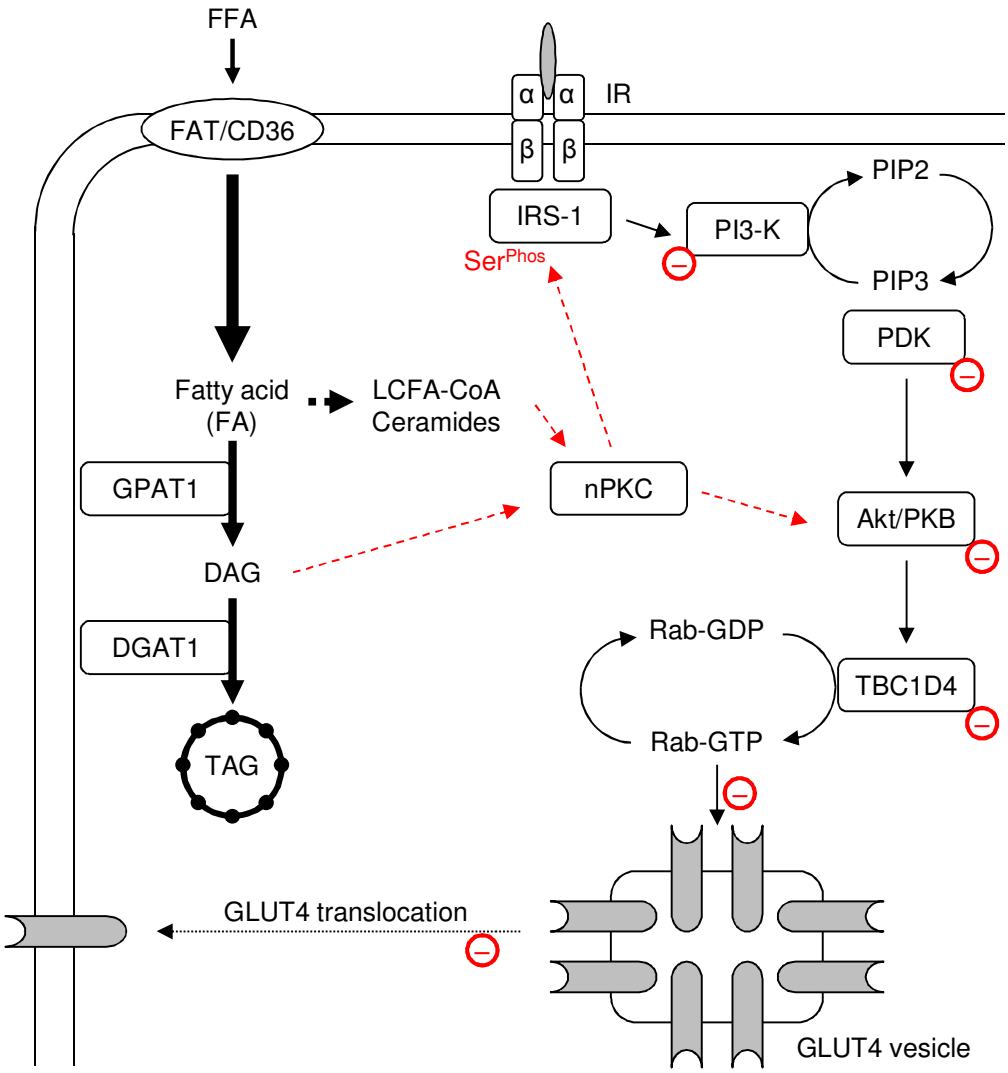


Figure 1.3 Lipid-induced insulin resistance in skeletal muscle

Akt/PKB protein kinase B, *DAG* diacylglycerol, *DGAT1* diacylglycerol acyltransferase, *FAT/CD36* fatty acid transporter, *FFA* free fatty acid, *GLUT4* glucose transporter 4, *GPAT1* glycerol-3-phosphate acyltransferase, *IR* insulin receptor, *IRS-1* insulin receptor substrate-1, *LCFA-CoA* long chain fatty acyl CoA, *nPKC* novel protein kinases C, *PDK* phosphoinositide-dependent kinase 1, *PI3-K* phosphoinositide 3-kinase, *PIP2* phosphatidylinositol 4,5-bisphosphate, *PIP3* phosphatidylinositol-3,4,5-triphosphate, *Rab-GDP* Rab guanosine disphosphate, *Rab-GDP* Rab guanosine triphosphate, *TAG* triacylglycerol, *TBC1D4* TBC 1 domain family member 4.

Ceramide accumulation is also reported to inhibit insulin signalling as it initiates a signal that leads to dephosphorylation and inactivation of Akt/PKB. Indeed, incubation of C2C12 skeletal muscle cells or L6 myotubes with the saturated FA palmitate leads to elevated ceramide concentrations concomitant with an impaired capacity for Akt/PKB activation (Pickersgill et al., 2007; Schmitz-Peiffer et al., 1999; Watson et al., 2009). It is now established that ceramide directly activates protein phosphatase 2A (PP2A) leading to dephosphorylation of Akt/PKB already phosphorylated at thr^{308} and ser^{473} residues, resulting in inactivation of Akt/PKB (reviewed in (Lipina and Hundal, 2011; Summers, 2006). Furthermore, ceramide can activate the aPKC isoforms ζ and λ, leading to serine phosphorylation of the Akt/PKB pleckstrin homology, thus reducing its affinity towards PIP_3 and ultimately preventing Akt/PKB translocation to the plasma membrane (reviewed in (Lipina and Hundal, 2011; Summers, 2006). As a result of these defects in insulin signalling at IRS-1 and Akt/PKB, GLUT4 translocation from its intracellular vesicles is reduced, and insulin-stimulated glucose uptake is subsequently decreased. In accordance, GLUT4 translocation to the plasma membrane fraction was not increased following insulin stimulation in skeletal muscle obtained from type 2 diabetic patients (Zierath et al., 1996).

1.6 Exercise training

1.6.1 Exercise as a therapeutic agent to combat insulin resistance

Booth and colleagues have proposed that the current human genotype originates from a physically active lifestyle (Booth et al., 2002; Booth et al., 2008; Booth and Lees, 2006; Chakravarthy and Booth, 2004), although the environmental pressures which formed our current genotype are absent from present day living, and as such, our lifestyle is relatively sedentary by comparison. Most notably, a sedentary, 'physically inactive' lifestyle is estimated to cause 13% of the burden of disease from type 2 diabetes and 11% from coronary heart disease (Lee et al., 2012). Therefore, increasing the quantity of physical activity that we perform will significantly improve metabolic health.

Large-scale lifestyle intervention studies provide some insight as to the impact of physical activity for reducing the risk of developing type 2 diabetes. Lifestyle intervention studies generally invite individuals to incorporate at least 30 minutes of exercise into their day, decrease body mass by

around 7%, and reduce total and saturated dietary fat intake. A review of the literature by Sigal and colleagues (2004) reveals that lifestyle interventions reduce the risk of developing type 2 diabetes by ~50%. However, it must be acknowledged that lifestyle intervention studies do not allow the relative importance of either physical activity or diet to be determined. Accordingly, large-scale physical activity intervention studies provide some indication of the effect of incorporating regular physical activity into daily living on the risk of developing metabolic disease (reviewed in (Bassuk and Manson, 2005). For example, walking briskly for 30 minutes per day, 5 days per week, reduced the risk of developing type 2 diabetes by 25% over 8 years of follow up in a group of more than 70,000 initially healthy US women (Hu et al., 1999), or by 34% over 7 years of follow up in a group of 38,000 US female health professionals (Weinstein et al., 2004). Similarly, of 897 Finnish men, those who performed at least 40 minutes of moderate-to-vigorous intensity activity per week were 56% less likely to develop type 2 diabetes over 4 years of follow up (Lynch et al., 1996). Importantly, regular physical activity may also be beneficial for individuals who already demonstrate symptoms of metabolic disease, such as type 2 diabetes patients (reviewed in (Gordon et al., 2009; Sigal et al., 2004). Taken together, these observations provide strong support for undertaking regular physical activity, preferably performed as part of a structured exercise training programme, as a viable therapeutic intervention to enhance metabolic health and reduce the risk of developing metabolic and cardiovascular disease.

It is important to acknowledge that the rate of insulin-stimulated glucose clearance, a measure of insulin sensitivity, is improved following a single bout of endurance exercise (Brestoff et al., 2009; Wojtaszewski et al., 2000a; Wojtaszewski et al., 1997). Similarly, an acute bout of resistance exercise has been reported to improve glucose tolerance and insulin sensitivity in both insulin-sensitive and insulin-resistant individuals (Fenicchia et al., 2004; Koopman et al., 2005; Venables et al., 2007). Notably, improvements in insulin sensitivity following an acute bout of exercise appear to persist for up to 48 h (Mikines et al., 1988; Perseghin et al., 1996). However, following a period of chronic exercise training, improvements in insulin sensitivity are observed beyond those detected following a single bout of exercise (Perseghin et al., 1996), thereby supporting epidemiological reports of the beneficial health effects of performing regular exercise.

1.6.2 Exercise training-induced improvements in insulin signalling

Regular exercise training in the form of prolonged bouts of aerobic exercise or resistance-type exercise have both been shown to directly improve insulin signalling capacity. Importantly, both endurance and resistance training is associated with an enhanced capacity for skeletal muscle glucose uptake (Frosig et al., 2007; Holten et al., 2004), and it appears that this adaptation is highly dependent on the capacity for GLUT4 translocation to the plasma membrane (Frosig and Richter, 2009). In this regard both endurance and resistance training increase the protein expression of GLUT4 (Frosig et al., 2007; Holten et al., 2004; Ivy, 2004; Stuart et al., 2010). However, insulin-stimulated GLUT4 translocation, and therefore glucose uptake, is also dependent on a number of insulin signalling intermediates. Interestingly, IRS-1 protein expression does not seem to be enhanced with exercise training in humans (Frosig et al., 2007; Holten et al., 2004; Yu et al., 2001). Rather, it appears that serine phosphorylation of IRS-1 is reduced in response to regular exercise training (Glynn et al., 2008; Langlais et al., 2011), therefore increasing the capacity for insulin-stimulated IRS-1 tyrosine phosphorylation. In addition, PI3K protein expression is unchanged following exercise training (Frosig et al., 2007; Holten et al., 2004), although PI3K activity was increased in response to 7 days of endurance training in sedentary males (Houmard et al., 1999), and greater in trained compared to untrained males (Kirwan et al., 2000). In contrast, insulin-stimulated IRS-1 associated PI3K activity was reduced following 3 weeks of knee extensor exercise training (Frosig et al., 2007). Taken together, these results demonstrate that improved proximal insulin signalling is predominantly attributed to enhanced activity and/or phosphorylation of IRS-1 and PI3K, rather than changes in protein expression.

As previously stated, Akt/PKB is a critical node in insulin signalling as it can affect many cellular processes, including protein synthesis, glucose uptake and glycogen storage. Both endurance and resistance training stimulate increased protein expression and insulin-stimulated activity of Akt/PKB in parallel to enhanced glucose uptake (Frosig et al., 2007; Holten et al., 2004; Vind et al., 2011). In addition, TBC1D4 protein expression and phosphorylation is increased in response to endurance training (Frosig et al., 2007; Vind et al., 2011). Thus, exercise training appears to enhance both the expression and activation of the distal insulin signalling intermediates, leading to an increase in

GLUT4 translocation and a greater capacity for glucose uptake. The capacity for glycogen synthesis is also enhanced following both endurance and resistance training, as glycogen synthase protein expression and activity are elevated (Holten et al., 2004; Vind et al., 2011). Increased intramuscular glycogen storage is a key adaptation to exercise training, as incorporating glucose taken up from the circulation into glycogen stores maintains the cellular gradient for subsequent glucose disposal, and is therefore associated with insulin sensitivity.

1.6.3 Exercise training-induced changes in lipid metabolism

(i) Changes to IMTG utilisation during exercise following exercise training

One of the most common adaptations to endurance exercise training is an increased capacity to oxidise fat as a substrate source during exercise, thereby reducing the reliance on carbohydrate oxidation to meet the energy demands (see (van Loon, 2004). Furthermore, it is well established that IMTG provides a readily available substrate source during moderate-intensity exercise in trained individuals (see (van Loon, 2004). Studies using FA tracer methodology have reported that 12 weeks of endurance training in lean, sedentary individuals increases the use of TAG (sum of muscle plus lipoprotein-derived TAG) during 1 h of moderate-intensity exercise (Schrauwen et al., 2002). Of additional importance was the observation that the increase in total fat oxidation following endurance training was predominantly attributed to the enhanced oxidation of TAG (sum of muscle plus-lipoprotein-derived TAG), while plasma FA oxidation rates did not change (Schrauwen et al., 2002). In addition, several other studies employing FA tracer methodology are available to substantiate the observation that IMTG use during exercise is enhanced following exercise training (Martin et al., 1993; Phillips et al., 1996b). Studies that directly measure IMTG content in muscle biopsies obtained before and after exercise also demonstrate that IMTG use during moderate intensity exercise is greater following 6 to 12 weeks endurance training (De Bock et al., 2008; Hurley et al., 1986; Van Proeyen et al., 2011a; Van Proeyen et al., 2011b), although this is not a universal finding (Bergman et al., 1999; Kiens et al., 1993). In addition, when assessed in a fibre type specific manner, the increase in IMTG use appears to be specific to type I fibres, especially when training in the fasted state (De Bock et al., 2008; Van Proeyen et al., 2011a; Van Proeyen et al., 2011b). However, the consumption of a high-fat diet during training may also increase IMTG use in type IIa fibres (Van Proeyen et al., 2011a). Thus,

longitudinal training studies are consistent with acute reports from trained individuals suggesting that IMTG use during exercise is enhanced following exercise training.

Enhanced IMTG utilisation during exercise following exercise training may be explained by greater expression of enzymes responsible for IMTG hydrolysis and/or an increased capacity for and/or efficiency for oxidation of FA liberated from IMTG stores. In accordance, endurance exercise training is associated with an increased expression of ATGL in human skeletal muscle (Alsted et al., 2009; Yao-Borengasser et al., 2010), although HSL expression does not appear to be enhanced following exercise training in either rats (Enevoldsen et al., 2001) or humans (Alsted et al., 2009). Probably the most well-established adaptation to exercise training is an enhanced skeletal muscle oxidative capacity. This is a logical adaptation to exercise training, given that skeletal muscle oxidative capacity correlates with the ability to oxidise fat (Simoneau et al., 1999) and exercise training increases fat oxidation (see van Loon, 2004). Notably, the training-induced increase in oxidative capacity has been determined using a number of markers, including the activity of mitochondrial enzymes such as citrate synthase and 3-hydroxyacyl-CoA dehydrogenase, protein expression of mitochondrial proteins such as cytochrome *c* oxidase, *ex vivo* respiration capacity of isolated mitochondria, or point-counting of mitochondria from electron microscopy images. In accordance, protein expression or activity assays of mitochondrial enzymes provide a relatively robust measure of oxidative capacity, although they do not provide data on the morphology or cellular distribution of mitochondria. Using electron microscopy it is reported that following 10 weeks of endurance training in type 2 diabetes patients and BMI-matched controls the increase in mitochondrial density occurred predominantly in the subsarcolemmal region of the muscle fibre (Nielsen et al., 2010). Furthermore, endurance training may increase the volume of individual mitochondria, and also their proximity to LDs (Tarnopolsky et al., 2007). This adaptation would presumably provide an efficient system in which FA liberated from intracellular LDs are efficiently shuttled to the mitochondria to undergo β-oxidation. Indeed, evidence is available to suggest that in trained individuals the mitochondria becomes a 'reticulum', such that a number of mitochondria form a functional network (Hood, 2001), enabling efficient generation of ATP at all cellular locations requiring energy. Through this mechanism, an increase in mitochondrial density and formation of a mitochondrial reticulum reduces the 'energy stress' (AMP and ADP production) that is

generated at the same exercise intensity, reducing the inhibition placed on the capacity to oxidise fat by the glycolytic flux (through the mechanisms discussed in 1.3.3).

Although endurance training consistently enhances skeletal muscle oxidative capacity, mitochondrial adaptations to resistance training are less clear. In accordance, some studies report an increase in oxidative capacity following a period of resistance training (Balakrishnan et al., 2010; Pesta et al., 2011; Tang et al., 2006), whereas others observe no change (Bell et al., 2000; Green et al., 1999), or even a decrease (Chilibeck et al., 1999; MacDougall et al., 1979). The variable findings may be attributed to the population studied, training protocol employed, frequency and duration of training, or the degree of hypertrophy observed, with less hypertrophy occurring in short interventions and interventions with a lighter workload.

(ii) Changes in IMTG concentration following exercise training

Cross-sectional studies consistently report that endurance trained individuals demonstrate significantly higher IMTG concentrations than untrained controls (Amati et al., 2011; Bergman et al., 2010; Goodpaster et al., 2001; van Loon et al., 2004). Furthermore, IMTG use during exercise strongly correlates with resting IMTG concentration (Stellingwerff et al., 2007; van Loon et al., 2003). Therefore, as endurance training enhances the rate of IMTG utilisation during exercise, the elevated IMTG concentration observed in endurance trained athletes should be regarded as a key metabolic adaptation to endurance training. Although the data obtained from cross-sectional studies are clear, longitudinal studies employing endurance training interventions are less consistent. Interestingly, some studies report significant increases in IMTG content (Dubé et al., 2008; Dubé et al., 2011; Kiens et al., 1993; Phillips et al., 1996b; Pruchnic et al., 2004; Schrauwen-Hinderling et al., 2003; Tarnopolsky et al., 2007; Van Proeyen et al., 2011a; Van Proeyen et al., 2010), whereas others report either a decrease (Alsted et al., 2009; Bergman et al., 1999; Bruce et al., 2004; Bruce et al., 2006; Solomon et al., 2008) or no change (De Bock et al., 2008; Hurley et al., 1986; Meex et al., 2010; Van Proeyen et al., 2011b) in IMTG concentration, in both lean and obese insulin-resistant individuals. The discrepant findings may be attributed to the population studied, training protocol, diet, and the method used for IMTG analysis.

Despite the inconsistent findings obtained from longitudinal studies, it is clear that endurance trained athletes exhibit large IMTG stores. However, the expression of IMTG synthesising enzymes, such as DGAT1 or GPAT1, does not seem to be upregulated when comparing trained and untrained individuals (Amati et al., 2011; Bergman et al., 2010) or following a period of endurance training (Alsted et al., 2009). Interestingly, DGAT1 and GPAT1 protein expression is increased in the period following exercise (Schenk and Horowitz, 2007), suggesting that acute upregulation of the IMTG synthesising enzymes could at least enhance the maximal capacity for IMTG synthesis in the post-exercise period, and therefore partly explain the enhanced IMTG concentration associated with regular exercise training. It is also important to acknowledge that an increase in the protein content of FAT/CD36 following exercise training (Perry et al., 2008; Talanian et al., 2010) could enhance the maximal capacity for uptake of FA into skeletal muscle, thereby delivering FA for an increase in IMTG synthesis. However, as FAT/CD36 mediates passive diffusion of FA into skeletal muscle, the rate of FA uptake will also depend on the FA concentration gradient between arterial blood and skeletal muscle. Taken together, these observations demonstrate that an increase in IMTG concentration will only occur when substrate concentration and enzyme activation is optimal.

It is also worth noting that ET enhances the expression of the rate-limiting enzyme responsible for the conversion of saturated FAs into monounsaturated FAs, known as steroyl-CoA desaturase-1 (SCD-1). Indeed, 6 weeks of ET increased both the mRNA and protein expression of SCD-1 in soleus muscle of rodents (Dobrzyn et al., 2010). In human skeletal muscle, SCD-1 protein expression is upregulated following an acute bout of exercise (Schenk and Horowitz, 2007), suggesting that desaturation of incoming FAs is a key process during post-exercise IMTG resynthesis. Furthermore, endurance trained athletes demonstrate elevated SCD-1 mRNA expression compared to sedentary controls (Bergman, 2010). Given that saturated FA metabolites have recently been proposed to be important in the development of insulin resistance (Bergman et al., 2012; Bergman et al., 2010; van Hees et al., 2011), enhancing SCD-1 expression may play a key role in maintaining lower concentrations of saturated FA metabolites.

(iii) Improved insulin sensitivity following exercise training is associated with an enhanced rate of IMTG turnover

An increased rate of IMTG turnover is proposed to be associated with improved insulin sensitivity following exercise training. Notably, ET increases the protein expression and/or activity of the lipolytic enzymes (ATGL and HSL), thereby facilitating greater rates of IMTG lipolysis (Alsted et al., 2009; Yao-Borengasser et al., 2010). Furthermore, ET enhances skeletal muscle mitochondrial density, providing a greater capacity to oxidise FAs liberated from IMTG as a substrate source during moderate-intensity exercise (see van Loon, 2004). In addition, ET increases the capacity for FA uptake by augmenting FAT/CD36 protein expression (Perry et al., 2008; Talanian et al., 2010), and in the period following a bout of exercise the protein expression and/or activity of the lipogenic enzymes (DGAT1 and GPAT1) is increased (Schenk and Horowitz, 2007), leading to replenishment of the IMTG stores. Therefore, during a period of prolonged ET involving repeated bouts of endurance-type exercise, it is likely that the activity of the processes liberating FA (IMTG lipolysis and FA uptake) and the activity of those processes that consume FA (IMTG synthesis and mitochondrial β-oxidation) remain continuously elevated. Consequently, the concentration of FA metabolites, such as DAGs and ceramides, remains low and therefore it is postulated that a high IMTG turnover rate contributes to the ET-induced improvement in insulin sensitivity (Moro et al., 2008; Shaw et al., 2010; van Loon and Goodpaster, 2006). This hypothesis is supported by the observation that 16 weeks ET in overweight non-diabetic individuals improves insulin sensitivity in line with a reduction in the concentration of DAG and ceramide (Dubé et al., 2008; Dubé et al., 2011). In lean sedentary individuals, a common adaptation to short term ET (< 12 weeks) is an increase in IMTG content concomitant with improved insulin sensitivity (Phillips et al., 1996b; Van Proeyen et al., 2010). It should be noted, however, that improved insulin sensitivity following exercise training is not always accompanied by significant IMTG accretion. In this regard, 12 weeks of exercise training in type 2 diabetes patients did not increase IMTG concentration, although insulin sensitivity was enhanced (Meex et al., 2010). Thus, it is postulated that in sedentary populations who exhibit large pre-training IMTG concentrations, exercise training resets the balance between IMTG synthesis and IMTG hydrolysis, leading to a decrease in FA metabolites at a higher range of IMTG concentrations (Shaw et al., 2010; van Loon and Goodpaster, 2006).

1.6.4 Use of high intensity interval training to improve metabolic health

High intensity interval training (HIT) is defined as brief, intermittent bursts of vigorous exercise, interspersed by periods of rest or active recovery (low intensity exercise). Interestingly, when work- or energy expenditure-matched to traditional endurance-type exercise it has been reported that HIT can induce similar, if not superior, improvements in performance and metabolic health parameters in both healthy and diseased populations (Tjonna et al., 2009; Wisloff et al., 2007). Furthermore, HIT is perceived to be more enjoyable than traditional endurance training at least in young active males (Bartlett et al., 2011), and therefore taken together it appears that HIT provides a viable alternative to more traditional forms of exercise training.

The physiological and metabolic benefits of HIT have been reported in studies employing a number of different forms of HIT. In this respect, high intensity aerobic interval training, which requires subjects to perform 10 x 4 min exercise at 90% $VO_{2\ max}$ interspersed with 2 min rest, is reported to improve oxidative capacity, protein expression of the fat and glucose transporter proteins, and rates of fat oxidation during steady state exercise in untrained males and females (Perry et al., 2008; Talanian et al., 2007). A different method of high intensity aerobic interval training was pioneered by Wisløff and colleagues, in which subjects performed ~4 x 4 min exercise at 90-95% HR_{max} interspersed with 3 min active recovery, and has been extensively applied to a variety of patient populations (Moholdt et al., 2009; Rognmo et al., 2004; Schjerve et al., 2008; Tjonna et al., 2008; Tjonna et al., 2009; Wisloff et al., 2007). This approach practiced for 4 to 12 weeks led to improvements in aerobic capacity, endothelial function, body composition, blood pressure, and insulin sensitivity, and reversed left ventricular remodelling in overweight and obese individuals, as well as those recovering from heart failure or coronary artery bypass (Moholdt et al., 2009; Rognmo et al., 2004; Schjerve et al., 2008; Tjonna et al., 2008; Tjonna et al., 2009; Wisloff et al., 2007). Furthermore, the beneficial adaptations to high intensity aerobic interval training were often more pronounced and longer-lasting than those observed using more traditional exercise approaches (Moholdt et al., 2009; Tjonna et al., 2009).

Gibala and colleagues established an alternative approach to HIT (known as sprint interval training, *SIT*) which was based on a Wingate test, and requires subjects to perform repeated 30 s 'all out' effort

cycling exercise against a supra-maximal resistance. During one session subjects perform 4-6 Wingate tests, separated by ~4 min rest, which equates to 2-3 min exercise over a 20-30 min period. This research group initially demonstrated that just 6 sessions of SIT over a 2 week period improved skeletal muscle oxidative capacity, as measured by enhanced activity and/or protein expression of mitochondrial enzymes (Burgomaster et al., 2005; Gibala et al., 2006), a finding that has since been replicated over 6 weeks of HIT (Burgomaster et al., 2007; Burgomaster et al., 2006; Burgomaster et al., 2008). Most notably, when compared to traditional endurance training consisting of moderate-intensity cycling for 40-60 min, 5 times per week for 6 weeks, it appears that the magnitude of improvement in skeletal muscle oxidative capacity is similar to that observed following 6 weeks of SIT (Burgomaster et al., 2008). In addition, similar increases in resting glycogen content, whole-body fat oxidation during steady state exercise, and time trial performance were also observed (Burgomaster et al., 2008). Furthermore, several studies have reported improvements in insulin sensitivity following 2 weeks of SIT in sedentary lean or overweight individuals (Babraj et al., 2009; Richards et al., 2010; Whyte et al., 2010). Importantly, this form of HIT requires one fifth of the time commitment, and a ~90% lower weekly training volume, compared to traditional endurance training. Given that the most commonly cited barrier to participating in regular exercise is a "lack of time" (Stutts, 2002), the observation that SIT and ET induce similar physiological and metabolic adaptations has clear implications for exercise prescription.

Despite the potency of Wingate-based SIT to stimulate beneficial metabolic adaptations, it does require specialized equipment, high levels of motivation and may induce nausea during the initial sessions of the training programme, suggesting that it may not be tolerable or appealing for all people. Consequently, Gibala and colleagues established a new 'constant-load' HIT protocol, which requires subjects to perform 8-12 60s bouts of cycling at a constant workload which equates to ~90% HR_{max}, interspersed with 60s rest (Little et al., 2010). Notably, 2 weeks of 'constant-load' HIT induced significant improvements in skeletal muscle oxidative capacity (Little et al., 2010). This adaptation may be, in part, due to greater mRNA and protein expression of peroxisome-proliferator activated receptor γ coactivator (PGC)-1α, which is considered the 'master regulator' of mitochondrial biogenesis, following HIT (Little et al., 2011a; Little et al., 2011b; Little et al., 2010). Improvements in

insulin sensitivity have also been reported following 2 weeks of 'constant-load' HIT in overweight individuals (Hood et al., 2011). In addition, a recent study has reported that 2 weeks of 'constant-load' HIT in type 2 diabetes patients significantly reduced postprandial glucose excursions and 24 h average blood glucose concentrations, as assessed using continuous glucose monitoring (Little et al., 2011a). Furthermore, HIT improved skeletal muscle oxidative capacity and increased GLUT4 protein expression (Little et al., 2011a). Taken together, it appears that HIT provides a practical alternative to traditional ET to improve physiological and metabolic health in both a healthy and a diseased population.

Although it is becoming clear that HIT enhances insulin sensitivity in a number of populations, how this compares to ET remains to be examined. Furthermore, HIT studies have so far focused on glucose metabolism to explain how this training mode enhances insulin sensitivity, although it is clear that lipid metabolism is also a key determinant of insulin action in skeletal muscle. Therefore, further research is required to elucidate the impact of HIT on the relationship between skeletal muscle lipid metabolism and insulin sensitivity.

1.7 Thesis overview

Chapter 1 provides an overview of how a sedentary lifestyle leads to the development of insulin resistance and other metabolic disorders, and outlines the effectiveness of regular exercise training as a therapeutic tool to improve skeletal muscle insulin sensitivity and reduce the risk of developing type 2 diabetes. In addition, the introductory chapter identifies a number of gaps in our knowledge which require further investigation and have been chosen as the topics of the experimental chapters. To this end, the studies in this thesis were designed to generate new knowledge on the role of PLIN2 and PLIN5 in skeletal muscle, and also to investigate the effect of acute exercise or various exercise training modes on IMTG metabolism and insulin sensitivity.

Section 1.3.3 of the introduction concluded that PLIN2 is reported to play a regulatory role in TAG lipolysis in cultured cells, although the precise role of PLIN2 in skeletal muscle remains to be elucidated. **Chapter 2** therefore aimed to generate novel data to support the theory that PLIN2 also

plays a regulatory role in IMTG lipolysis. To this end, Chapter 2 investigated the hypothesis that PLIN2-containing LDs are preferentially targeted for breakdown during exercise. To test this hypothesis, the effect of a single bout of moderate-intensity cycling exercise on the colocalisation between PLIN2 and IMTG using immunofluorescence microscopy methods was investigated.

In section 1.6.3 it was explained that ET alters the balance between IMTG synthesis and hydrolysis such that a net increase in IMTG concentration occurs concomitant with improved insulin sensitivity. However, at this time no studies had examined the impact of prolonged ET on PLIN2 protein expression in skeletal muscle. **Chapter 3** therefore investigated the hypothesis that ET in type 2 diabetes patients would preferentially augment PLIN2 protein expression in type I muscle fibres. Given that the results of Chapter 2 suggest the PLIN2 association with IMTG plays a role in the utilisation of IMTG during endurance-type exercise, it was expected that PLIN2 expression would be enhanced following ET.

Like PLIN2, PLIN5 has also been implicated in the regulation of TAG hydrolysis in cultured cells *in vitro* (Wang et al., 2011a; Wang et al., 2011b), although whether a similar function exists in skeletal muscle remains to be elucidated. Furthermore, the use of sprint interval training (SIT) as a time-efficient training strategy to induce positive metabolic adaptations has recently received much interest, although whether changes in IMTG metabolism occur following SIT remains unknown. Therefore, **Chapter 4** first investigated the hypothesis that both PLIN2 and PLIN5 play a role in the well-established increase in IMTG breakdown during exercise following ET in sedentary young men. **Chapter 4** also aimed to establish whether SIT also enhances IMTG breakdown during moderate-intensity exercise and increases the protein expression of PLIN2 and PLIN5.

Chronic resistance training induced qualitative changes in skeletal muscle that are responsible for the observed improvements in insulin sensitivity. However, whether the improvements in IMTG metabolism that contribute to ET-induced increases in insulin sensitivity also occur following RT has not yet been investigated. **Chapter 5** therefore investigated whether RT augments IMTG content and mitochondrial density, and enhances IMTG breakdown during endurance-type exercise concomitant with improvements in insulin sensitivity. It was hypothesised that repeated bouts of resistance

exercise undertaken during a RT programme would result in a net increase in IMTG content and enhance the capacity for IMTG breakdown during exercise.

In Chapter 4 it was shown that SIT was an effective training mode to enhance IMTG content and IMTG breakdown during exercise concomitant with improved insulin sensitivity. In **Chapter 6**, a pilot study was conducted to investigate the effects of a new, more practical and time-efficient constant-workload SIT strategy. The effect of this protocol was investigated with regards to IMTG content, PLIN2 and PLIN5 protein expression, and oxidative capacity, and whether changes in these variables translated in an improved aerobic capacity, insulin sensitivity, and cardiovascular risk profile, in a group of young, previously sedentary obese males. It was hypothesised that 4 weeks constant-load SIT would elicit an enhanced IMTG content, PLIN2 and PLIN5 protein expression, and oxidative capacity, and that these changes would mirror improvements in insulin sensitivity and cardiovascular risk profile.

Chapter 7 (general discussion) summarises the main findings and conclusions generated by the experimental chapters. Based on these findings, and together with data reported in the most recent literature, a number of future experiments aiming to generate new information on the mechanisms underlying the improvements metabolic health following exercise training are suggested.

Preferential utilisation of perilipin 2 associated intramuscular triglycerides during one hour of moderate intensity endurance-type exercise

2.1 Abstract

The lipid droplet (LD)-associated protein perilipin 2 (PLIN2) appears to colocalise with LDs in human skeletal muscle fibres, although the function of PLIN2 in the regulation of intramuscular triglyceride (IMTG) metabolism is currently unknown. Here we investigated the hypothesis that the presence of PLIN2 in skeletal muscle LDs helps to increase lipolysis during exercise. We, therefore, measured exercise-induced changes in IMTG and PLIN2 distribution and changes in their colocalisation. Muscle biopsies from the *vastus lateralis* were obtained from 7 lean, untrained males (22±2 years, BMI 24.2±0.9 $kg.m^{-2}$, VO_{2peak} 3.35±0.13 $L.min^{-1}$) before and after 1 h of moderate intensity cycling at ~65% VO_{2peak}. Cryosections were stained for perilipin 2, IMTG, and myosin heavy chain type I and viewed using widefield and confocal fluorescence microscopy. Exercise induced a 50±7% decrease in IMTG content in type I fibres only (P<0.05) but no change in PLIN2 content. Colocalisation analysis showed that the fraction of PLIN2 associated with IMTG was 0.67±0.03 before exercise, which was reduced to 0.51±0.01 post-exercise (P<0.05). Further analysis revealed that the number of PLIN2-associated LDs was reduced by 31±10% after exercise (P<0.05), whereas the number of PLIN2-null LDs was unchanged. No such changes were seen in type II fibres. In conclusion, this study shows that PLIN2 content in skeletal muscle is unchanged in response to a single bout of endurance exercise. Furthermore, the PLIN2 and IMTG association is reduced post-exercise, apparently due to preferential utilisation of PLIN2-associated LDs. These results confirm the hypothesis that the PLIN2 association with IMTG is related to the utilisation of IMTG as a fuel during exercise.

2.2 Introduction

Lipid droplets (LDs) are storage depots for sequestered fatty acids and are important in maintaining energy homeostasis within cells, especially when energy provisions are low or an increase in energy consumption occurs. The current view suggests that LDs are a dynamic cellular organelle with a variety of functions (for recent reviews see (Beller et al., 2010b; Ducharme and Bickel, 2008; Fujimoto et al., 2008; Guo et al., 2009), due to the distinct array of proteins that have been found to be associated with the phospholipid monolayer of the LD. The most well recognised are the PAT proteins (Perilipin, ADRP/adipophilin, TIP-47, S3-12 and OXPAT, referred to in this paper as perilipin 1, 2, 3, 4, and 5, (Kimmel et al., 2010). Perilipin 1 is the most well-characterized protein and is reported to be exclusively expressed in adipocytes and steroidogenic cells (Londos et al., 1995). Its expression is linked to triacylglycerol (TAG) accumulation, with suggestions made that it regulates the activity of lipolytic enzymes, such as hormone-sensitive lipase (HSL) and adipose triglyceride lipase (ATGL) (Granneman et al., 2007; Wang et al., 2009). Perilipin 2 (PLIN2) was originally discovered as an early marker of adipocyte differentiation, although it is now known to be expressed in other tissues, including skeletal muscle (Brasaemle et al., 1997). It is suggested that PLIN2, like perilipin 1, serves to increase the storage of TAG, decreasing TAG turnover through a reduction of lipolytic rates, which is supported by evidence from PLIN2 overexpression in murine fibroblasts and PLIN2 loss of function studies in AML12 cells (Bell et al., 2008; Imamura et al., 2002). In particular, PLIN2 expression has been shown to decrease the association of ATGL with LDs in non-adipose cell lines leading to reduced rates of lipolysis under basal conditions (Listenberger et al., 2007; Wang et al., 2011a). In skeletal muscle, little is known about the function of PLIN2 with regards to the regulation of IMTG metabolism, both at rest and during exercise.

LDs in skeletal muscle provide a readily available store of energy during exercise in endurance-trained athletes, a view that is supported by studies measuring IMTG turnover using metabolic tracers (Sacchetti et al., 2002; van Loon et al., 2003). In addition, reductions in IMTG content during prolonged (~2 h duration) endurance exercise have consistently been observed using magnetic resonance spectroscopy (~30% decrease) (Brechtel et al., 2001; Decombaz et al., 2000; Decombaz et al., 2001) and fluorescence microscopy techniques (~60%) (De Bock et al., 2005; van Loon et al.,

2003). Fluorescence microscopy permits fibre type-specific observations to be made, and studies employing this technique have demonstrated that the reduction in IMTG after an acute exercise bout occurs exclusively in type I muscle fibres (De Bock et al., 2005; van Loon et al., 2003).

A recent study from our laboratory has described a fibre type-specific distribution of PLIN2, where there is a two-fold greater concentration of PLIN2 in type I muscle fibres of trained individuals (Shaw et al., 2009). This observation underlines the importance of acknowledging fibre type when investigating IMTG metabolism (van Loon et al., 2003; Watt et al., 2002b). In addition, approximately 60% of IMTG are found to colocalise with PLIN2 (Shaw et al., 2009), suggesting that the IMTG pool is heterogenous with regard to their protein composition. These observations offer a unique opportunity to investigate the role of PLIN2 content and LD association in the regulation of IMTG metabolism in skeletal muscle during exercise. In this study we therefore aim to investigate the hypothesis that the PLIN2 association with IMTG is related to IMTG utilisation during an acute bout of endurance exercise.

2.3 Methods

Subjects and pre-experimental testing

Seven lean, untrained males (22 ± 2 years, BMI 24.2 ± 0.9 $kg.m^{-2}$) (see Table 2.1 for characteristics) volunteered to take part in the study, which was approved by the Black Country NHS Research Ethics Committee (West Midlands, UK) and conformed with the *Declaration of Helsinki*. Written, informed consent was obtained from volunteers following a verbal and written explanation of the nature and risks involved in the experimental procedure. A general health questionnaire assessed all participants as healthy but all engaged in less than two 30 min sessions of physical activity per week in the preceding year. At least one week prior to the experimental trial all participants performed a progressive exercise test to exhaustion on an electronically braked cycle ergometer in order to determine peak oxygen uptake ($VO_{2\ peak}$) using an on-line gas collection system (Oxycon Pro, Jaeger, Germany). Briefly, subjects initially cycled at 95 W for 3 minutes after which the workload was increased by 35 W every 3 minutes until subjects could not maintain a cadence of > 50 rpm, at which point the test was terminated. VO_{2peak} was taken as the highest value obtained in the last 30-seconds of the test. A workload equivalent to 65% VO_{2peak} was calculated and used during the experimental trial. Body composition was analysed using Dual X-ray Absorptiometry (DXA). QDR software (Hologic Inc., Beford, MA) was used to analyse fat mass and fat-free mass. Relative fat mass was calculated using absolute fat mass as a proportion of total mass.

Study protocol

On the day of the experimental trial, subjects arrived at the laboratory after an overnight fast (> 10 h) to complete a 60 min cycle ride at ~65% VO_{2peak}. Prior to and immediately following the exercise bout a muscle biopsy was taken from the *vastus lateralis*. Briefly, 1% lidocaine (~5 ml) was applied to the skin and fascia of the muscle, after which two small incisions were made approximately 2 cm apart. A muscle biopsy (~100 mg) was taken from the distal incision using the Bergström needle technique. The leg from which the biopsy was taken was randomised in order to counter any bias of the dominant limb. A portion of the muscle sample was immediately dissected free of any fat and connective tissue, embedded in Tissue-Tek OCT Compound (Sakura Finetek Europe, The Netherlands) and frozen in

liquid nitrogen-cooled isopentane. The remaining muscle tissue (~40mg) was immediately snap-frozen in liquid nitrogen. Subjects cycled on an electronically braked cycle ergometer for 60 min (at ~65% VO_{2peak}) maintaining a constant cadence throughout. Heart rate was recorded every 5 minutes, and gas collection (5 min collection period) was performed at 15 minute intervals (t = 15, 30, 45, 60 min) using an on-line gas collection system (Oxycon Pro, Jaeger, Germany), in order to determine rates of carbohydrate and fat oxidation. A second muscle biopsy was taken immediately post-exercise from the proximal incision. Whole body rates of carbohydrate and fat oxidation were estimated from VO_2 and VCO_2 values, using the equations used by Jeukendrup and Wallis (Jeukendrup and Wallis, 2005).

Three days prior to the experimental trial, all subjects were requested to refrain from vigorous physical activity, as well as avoid excessive alcohol or caffeine intake. In addition, subjects were provided with all food and drinks to be consumed in the 24 h preceding the experimental trial. The diet was of a standard macronutrient composition (50% CHO, 35% fat, 15% protein), but adjusted for each individuals habitual caloric intake, assessed through the completion of a 3 day weighed-intake diet diary.

Muscle samples analysis

Immunofluorescence staining: Serial 5 µm sections were cut at -30°C on to plain glass slides and left for a maximum of 1 h to air dry prior to treatment. Sections of 5 µm thickness were chosen in order to reduce the background signal from out of focus light which improves the quality of image analysis. Paired samples (pre and post-exercise) were mounted on to the same slide to reduce the variation in staining intensity between sections. Slides were fixed in 3.7% formaldehyde for 1 h, rinsed 3x30 s in deionised water, followed by permeabilisation in 0.5% Triton-X 100 for 5 min. After washing slides in PBS for 3x5 min, sections were incubated with appropriate primary antibodies for 45 min. Following a further 3x5 min PBS wash, sections were incubated with appropriately targeted secondary fluorescent conjugated antibodies for 30 min. To visualise IMTG, the well-established neutral lipid dye oil red O staining protocol in combination with immunofluorescence was used (Koopman et al., 2001). In this respect, oil red O solution was applied to washed (3x5 min PBS) sections for 30 min following secondary antibody incubation. Slides were finally rinsed 3x30 s in deionised water followed by a 10

min rinse with slow running tap water, after which coverslips were mounted with a glycerol and mowiol 4-88 solution in 0.2 M Tris buffer (pH 8.5) (including 0.1% DABCO anti-fade medium).

Fibre type determination was undertaken through incubation of muscle sections with mouse anti-myosin for slow twitch fibres (A4.840-c, DSHB, University of Iowa, US, developed by Dr. Blau) followed by the application of Alexa Fluor goat-anti mouse IgM 488. Positively stained fibres were classified as type I fibres, and those with no staining were classified as type II fibres. As a marker of the cell border, the basal lamina was visualised by first incubating sections with a mouse anti-collagen type IV antibody (M3F7, DSHB, University of Iowa, US, developed by Dr. Furthmayr), followed by treatment with Alexa Fluor goat anti-mouse IgG_1 350. A working solution of oil red O was freshly produced for each staining procedure and consisted of 100 mg oil red O in 20 ml 60% triethylphosphate (Sigma-Aldrich, UK). Twelve ml of working solution was added to 8 ml deionised water and filtered twice to remove any oil red O crystals. PLIN2 was visualised using a monoclonal mouse anti-ADRP antibody (American Research Products, USA), followed by treatment with either Alexa Fluor GAMIgG_1 488 (for co-staining with IMTG) or Alexa Fluor GAMIgG_1 594 (for co-staining with MHC I). Control slides were included where the appropriate secondary antibody for PLIN2 (Alexa Fluor GAMIgG_1 488) or oil red O was omitted in order to confirm the absence of non-specific staining and/or bleed through into the opposing channel (Scriven et al., 2008). The specificity of the PLIN2 antibody was confirmed in a competition experiment where a PLIN2 recombinant peptide (Genway Biotech, Inc, CA, USA) was incubated with the primary antibody, and subsequently the positive fluorescent signal for PLIN2 was removed (Fig. 2G and H). In addition, immunoblot analysis of human skeletal muscle using the PLIN2 antibody revealed a band at ~50 kDa (Fig. 2I), in accordance with the previously identified molecular weight (Brasaemle et al., 1997).

Image capture, processing and data analysis: Images of cross-sectional orientated fibres for the fibre type-specific determination of IMTG and PLIN2 content were captured using a widefield Nikon E600 microscope with a 40x 0.75 NA objective, coupled to a SPOT RT KE colour 3 shot CCD camera (Diagnostic Instruments Inc., MI, USA). Both DAPI UV (340-380 nm) and FITC (465-495 nm) excitation filters were used to view the Alexa Fluor 350 and 488 fluorophores, respectively. In addition,

a Texas red (540-580 nm) excitation filter was used to view sections stained with oil red O or the Alexa Fluor 594 fluorophore. The use of 3 excitation filters and I dichroic and 1 emission filter ("Pinkel" Triple Set, Semrock, Kettering, UK) allowed for semi-automated sequential image capture. Digital images to specifically assess the size of IMTG and PLIN2 containing droplets, as well as the colocalisation of IMTG and PLIN2, were obtained using an inverted confocal microscope (Leica DMIRE2, Leica Microsystems) using a 63x 1.4 NA oil immersion objective. An argon laser was used to excite the Alexa Fluor 488 fluorophore, while both the Alexa Fluor 594 fluorophore and oil red O was excited using a Helium-Neon laser.

Image processing was undertaken using Image-Pro Plus 5.1 software (Media Cybernetics, MD, USA). An intensity threshold was uniformly selected to represent positive signal for IMTG and PLIN2 identification. Widefield images were used to assess fibre-type specific content of both IMTG and PLIN2. Accordingly, between 6 and 10 widefield images were used per muscle section, resulting in a total of 98 ± 8 fibres analysed per muscle cross-section (39 ± 6 type I fibres, 59 ± 9 type II fibres). IMTG and PLIN2 content was expressed as the percentage of the total fibre area positively stained. Images captured using confocal microscopy were used to identify changes in mean LD size and density. Mean LD size was calculated by dividing the total number of objects by the total area stained. As there were no detectable changes in the relative lipid area stained in type II fibres, only type I fibres were used when assessing changes in LD area. Images of PLIN2 were also captured in the texas red channel (when using the Alexa Fluor GAMIgG_1 488 secondary antibody) in order to confirm there was no bleed through of the positive signal for PLIN2.

For colocalisation analysis of PLIN2 and IMTG, sequential images captured using confocal microscopy at 8x digital magnification were used. Positive signal for IMTG and PLIN2 was identified through the selection of a uniform intensity threshold. Based on these thresholds, binary images were generated for both IMTG and PLIN2, which were subsequently used for colocalisation analysis using Image-Pro Plus 5.1 software. A binary image was created to define the overlapping area between the IMTG and PLIN2 images. The total number of overlapping objects was expressed relative to the total number of PLIN2 objects, as a measure of colocalisation (Fig. 3). No signal above the threshold was

detected in the opposing channel for each negative control. In order to determine whether the degree of colocalisation observed was statistically different from that expected for a random distribution, we generated a series of randomized images from non-corresponding images of IMTG and PLIN2. In this respect, each PLIN2 image was randomly assigned to an IMTG image under the same experimental conditions, thus preserving the spatial and intensity distribution of the objects, but removing the correlation between the green and red images (Fig. 3). Consequently it was found that the degree of colocalisation occurring by chance (random distribution) was far less than that observed when using actual images (Fig. 4).

Western blot analysis: Snap frozen muscle tissue was powdered in liquid nitrogen and mixed on ice for 2 hours in eppendorf tubes containing lysis buffer (1x RIPA buffer, Cell Signalling, including 1 complete mini protease inhibitor tablet, Roche Diagnostics, Germany) followed by homogenization for ~30 s at slow speed (Polytron) and centrifugation for 20 min at 4°C and 10,000 G. The supernatant was removed and a small portion used to determine protein concentration (Pierce BCA assay kit). Samples were subsequently diluted to a final concentration of 3 $\mu g.\mu l^{-1}$, and equal quantities of protein (45 µg) were separated by electrophoresis on 12% SDS-polyacrylamide gels and transferred to nitrocellulose membranes at 25 V for 1.5 hours. Ponceau S staining was performed to assess transfer efficacy and subsequently imaged using a ChemiDoc XRS+ system (Bio-Rad, UK). This image was used to quantify the total protein stain of each lane, and this was used as a measure of equal loading when the inter-lane variation was less than 10%. Membranes were subsequently blocked for 1 h in 5% non-fat dried milk (NFDM) in phosphate-buffered saline with 0.1% Tween (PBST) followed by incubation overnight at room temperature with an anti-adipophilin (American Research Products, MA, USA) antibody diluted in 3% NFDM in PBST. The membranes were then washed (3 x 5 min PBST), blocked in 5% NFDM for 1 h, followed by incubation for 1 h at room temperature with appropriate horseradish peroxidase-conjugated IgG secondary antibodies diluted in 3% NFDM in PBST. Antibody binding was detected using enhanced chemiluminescence HRP detection reagent (GE Healthcare, UK) and imaging and band quantification was performed using a ChemiDoc XRS+ system (Bio-Rad, UK).

Statistics

All data are expressed as means ± S.E. To identify significant fibre type and time differences for IMTG and PLIN2, we applied a repeated measures analysis of variance (ANOVA), with the within subject factors as fibre type and time. This analysis was also applied to measure significant changes in IMTG and PLIN2 colocalisation, but also included the between subject factor as image type (i.e. actual or randomised). A paired t-test was applied to measure significant differences between IMTG and PLIN2 object density and size, before and after exercise. In order to investigate specific relationships between variables, a simple linear regression analysis was used. Significance was set at the 0.05 level of confidence.

Table 2.1. *Subject Characteristics*

Age (yrs)	22 ± 2
Height (m)	1.78 ± 0.01
Body mass (kg)	76.7 ± 3.1
BMI ($kg.m^{-2}$)	24.2 ± 0.9
$VO_{2\ peak}$ ($l.min^{-1}$)	3.35 ± 0.13
W_{max} (W)	235 ± 13
Basal plasma glucose ($mmol.1^{-1}$)	5.4 ± 0.2
2h OGTT glucose ($mmol.l^{-1}$)	6.4 ± 0.6
FFM (kg)	54.3 ± 1.8
FM (kg)	13.3 ± 1.4

Data provided are means ± S.E. (n = 7). *BMI* body mass index, W_{max} maximum workload, *OGTT* oral glucose tolerance test, *FFM* free fat mass, *FM* fat mass.

2.4 Results

Substrate Oxidation

Carbohydrate and fat oxidation rates during the 60 min constant load cycle at 66 ± 2% $VO_{2\ peak}$ (53 ± 3% W_{max}) are shown in Table 2.2. Fat oxidation increased over the cycling bout, such that absolute fat oxidation was significantly increased at 30, 45 ($P < 0.05$), and 60 min ($P < 0.01$) compared to the value obtained at 15 min, while no changes in absolute carbohydrate oxidation were observed. In addition, RER was significantly reduced at 45 ($P < 0.05$) and 60 min ($P < 0.01$) compared to RER at 15 min.

IMTG and PLIN2 content

Pre-exercise, IMTG content (expressed as the % of the total fibre area positively stained) was greater in type I fibres compared to type II fibres (type I 3.80 ± 0.41, type II 0.74 ± 0.10, $P < 0.001$, Fig. 2.1G). Similarly, an approximately two-fold greater concentration of PLIN2 was found in type I fibres relative to type II fibres (type I 1.92 ± 0.20, type II 0.87 ± 0.02, $P = 0.005$, Fig. 2E).

Cycling at ~65% VO_{2peak} for 60 min induced a 50 ± 7% decrease in IMTG content in type I fibres only (pre 3.80 ± 0.41%, post 1.90 ± 0.39%, $P < 0.001$, Fig. 2.1G). Using confocal images, it was determined that the reduction was accounted for by both a 50 ± 9% decrease in LD density (pre 0.043 ± 0.010, post 0.022 ± 0.006 LDs.μm^{-2}, $P = 0.042$) and an 11 ± 4% decrease in LD size (pre 0.58 ± 0.02, post 0.52 ± 0.03 μm^2, $P = 0.012$). No significant change in IMTG content was observed in type II fibres post-exercise ($P = 0.331$). PLIN2 content was unchanged after 60 min steady state cycling in both type I fibres (pre 1.92 ± 0.20%, post 1.78 ± 0.18%, $P = 0.192$, Fig. 2.2E) and type II fibres (pre 0.87 ± 0.02%, post 0.74 ± 0.07%, $P = 0.228$). Accordingly, no significant changes in PLIN2 density (pre 0.032 ± 0.003, post 0.033 ± 0.006 PLIN2.μm^{-2}, $P = 0.736$) were observed. PLIN2 content, quantified through immunoblotting of whole muscle homogenates, also remained unchanged following exercise, and showed a strong positive correlation with mixed muscle PLIN2 content (when type I and type II fibres were pooled) determined from immunofluorescence images ($R = 0.61$, $P = 0.018$, Fig. 2.2F). The IMTG content at rest positively correlated with IMTG breakdown during exercise ($R = 0.86$,

$P < 0.001$). In addition, a positive correlation was observed between resting IMTG content and PLIN2 content (R = 0.69, $P = 0.01$). As a result, PLIN2 content was positively associated with IMTG breakdown during exercise (R = 0.55, $P = 0.044$) when type I and type II fibres were pooled.

IMTG and PLIN2 association

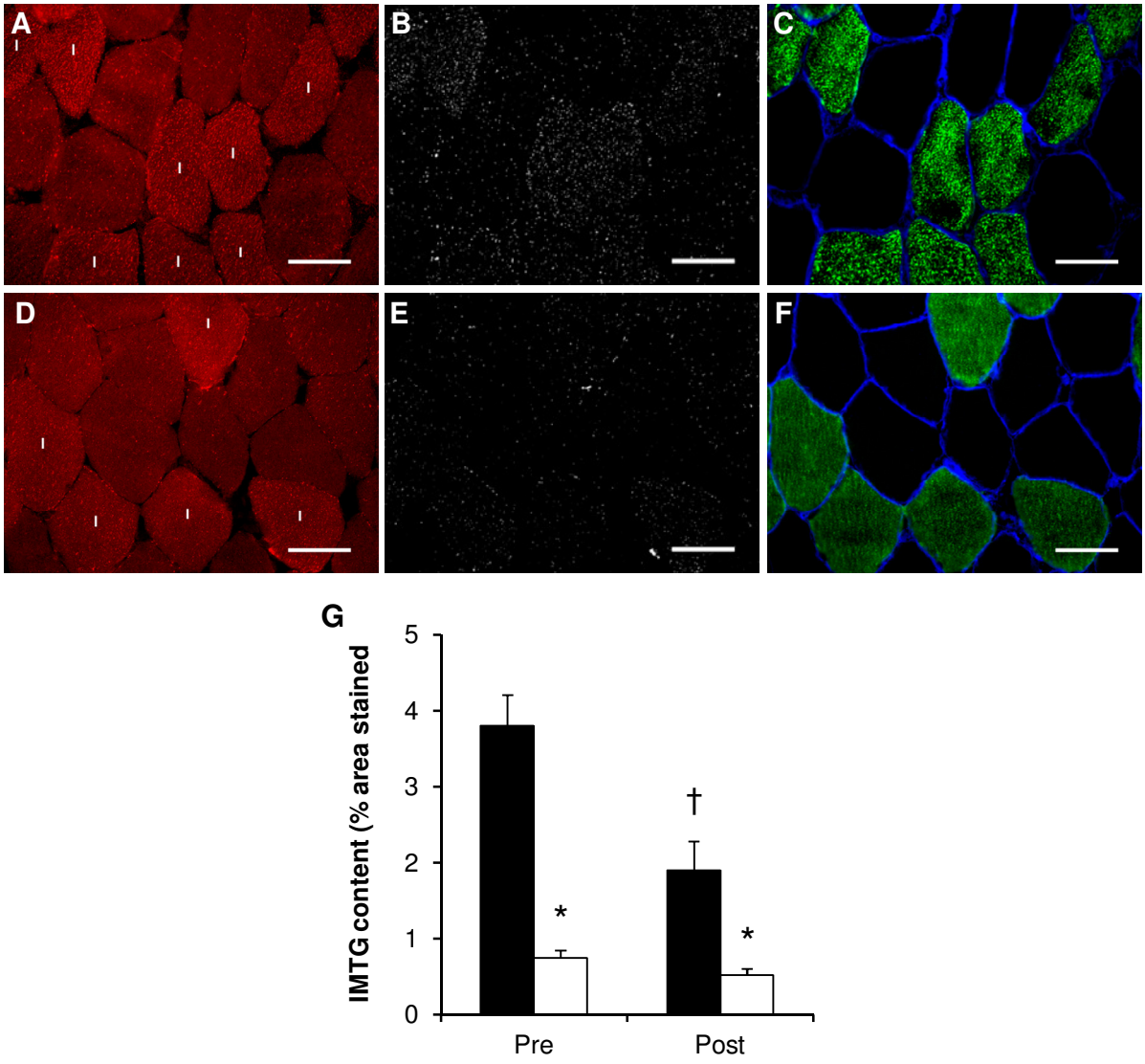
In the first instance, colocalisation was measured by expressing the number of overlapping objects relative to the total number of PLIN2 objects. Pre-exercise, the fraction of PLIN2 associated with IMTG was similar in type I and type II fibres (0.67 ± 0.03, 0.59 ± 0.04, respectively; $P = 0.906$), and this was significantly different from the fraction obtained between randomised images (0.10 ± 0.02, $P < 0.001$). To this end, the fraction of IMTG associated with PLIN2 prior to exercise was 0.71 ± 0.02 and 0.67 ± 0.05 in type I and type II fibres respectively ($P = 0.673$, Figs. 2.3A-D and 2.4). In response to exercise, the association of PLIN2 and IMTG in type I fibres was reduced by 24 ± 4% ($P = 0.001$, Fig. 2.3E-H and 2.4), whereas no significant changes were observed in type II fibres. Thus, post-exercise the fraction of PLIN2 associated with IMTG was 0.51 ± 0.01. Furthermore, a significant difference between actual and randomised images was obtained ($P < 0.001$, Fig. 2.4A), indicating that any changes occurring in response to the exercise were 'true' changes, and did not occur by chance. As a change in PLIN2 association with IMTG was observed in response to exercise, we next investigated the number of LDs either associated (PLIN2-LD) or not associated (PLIN2-null-LD) with PLIN2, both before and after exercise. Pre-exercise, there was a significant difference between the number of PLIN2-LD (0.165 ± 0.012 LD.μm^{-2}) compared to the number of PLIN2-null-LD in type I fibres (0.110 ± 0.012 LD.μm^{-2}, $P = 0.001$, Fig. 2.5A). Post-exercise, the number of PLIN2-LD was reduced by 31 ± 10% ($P = 0.02$), while the number of PLIN2-null-LD was unchanged in type I fibres ($P = 0.621$, Fig. 2.5A). In accordance, the quantity of PLIN2 not associated with IMTG was increased post-exercise in type I fibres ($P = 0.023$, Fig. 2.5B). The number of PLIN2-LD, PLIN2-null-LD and the quantity of PLIN2 not associated with IMTG was significantly different between type I and type II fibres ($P < 0.001$), but no change was observed in response to exercise for any of these variables in type II fibres (Fig. 2.5C and D). As before, there was a significant difference between the values obtained for PLIN2-LD and PLIN2-null-LD across actual and randomized images ($P < 0.001$).

Table 2.2. *Substrate utilization during 60 min of moderate-intensity cycling*

	Time (min)				
	15	30	45	60	Mean
VO_2 ($l.min^{-1}$)	2.03 ± 0.17	2.29 ± 0.09	2.32 ± 0.09	2.40 ± 0.08	2.26 ± 0.09
VCO_2 ($l.min^{-1}$)	1.85 ± 0.14	2.06 ± 0.08	2.06 ± 0.08	2.08 ± 0.07	2.01 ± 0.06
RER	0.92 ± 0.01	0.89 ± 0.02	0.88 ± 0.01*	0.87 ± 0.02†	0.89 ± 0.01
CHO oxidation ($g.min^{-1}$)	1.78 ± 0.13	1.81 ± 0.16	1.71 ± 0.08	1.65 ± 0.16	1.75 ± 0.09
Fat oxidation ($g.min^{-1}$)	0.29 ± 0.07	0.42 ± 0.07*	0.44 ± 0.06*	0.53 ± 0.07†	0.41 ± 0.06

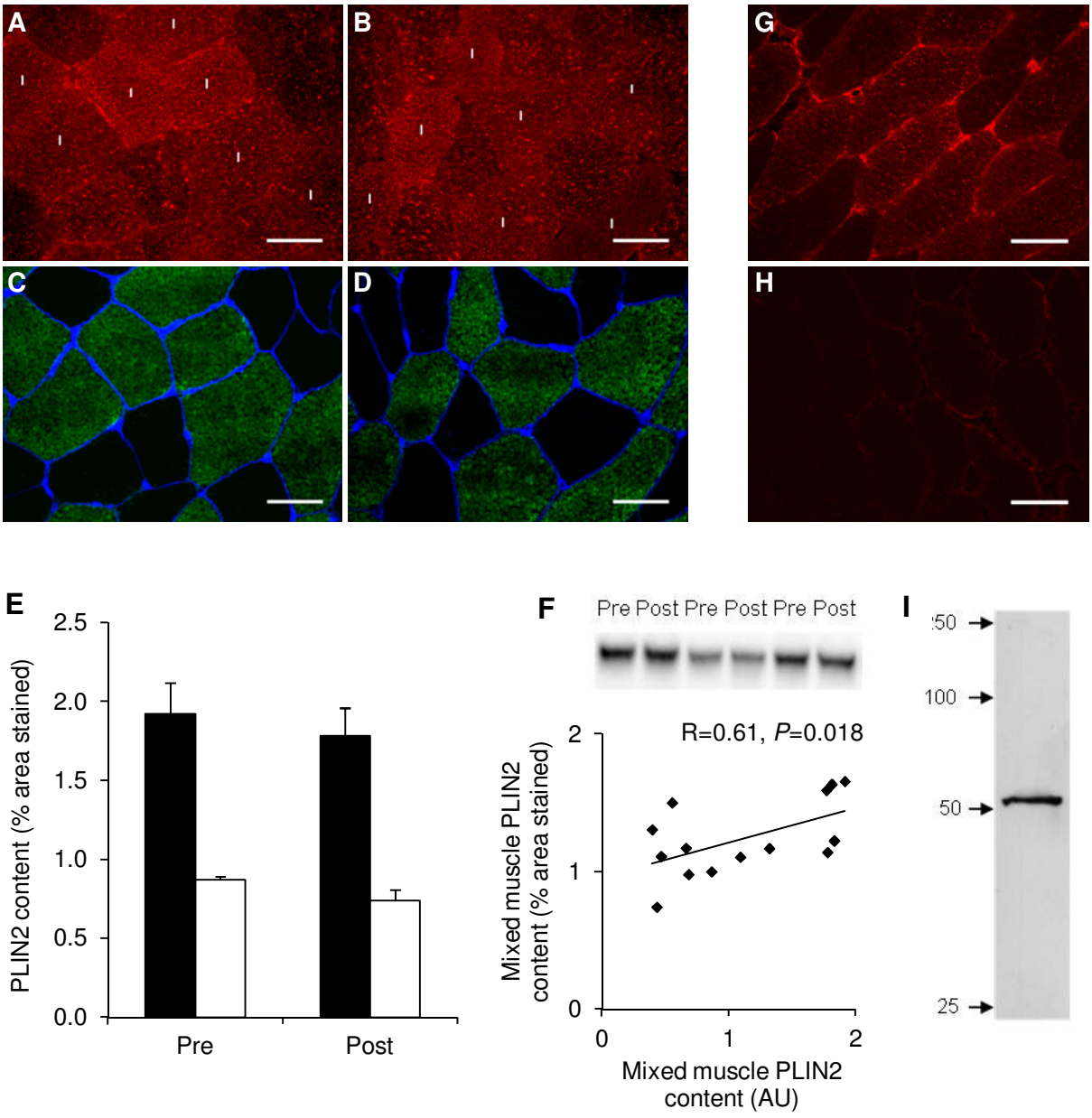
Values obtained during 60 min of moderate-intensity cycling at ~65% $VO_{2\ peak}$. Data provided are means ± S.E. (n = 7). *RER* respiratory exchange ratio, *CHO* carbohydrate. * $P < 0.05$, † $P < 0.01$ versus value at 15 min.

Fig. 2.1. Fibre specific IMTG content visualized through immunofluorescence microscopy and quantified before and immediately after exercise.



Representative immunofluorescence images of IMTG *(pre A, post D)*, the ORO signal obtained after the intensity threshold was applied during quantitation (showing the LDs in white) *(pre B, post E)* and myosin heavy chain type I combined with collagen IV *(pre C, post F)* from one participant pre- and post-exercise. Type I muscle fibres are indicated with "I" in images *A* and *D*, all other fibres are assumed type II fibres. White bar = 50µm. Fibre type specific IMTG *(G)* content quantified from immunofluorescence images before and after 1 hr constant load cycling at ~65% $VO_{2\ peak}$. Closed bars represent type I fibres, open bars represent type II fibres. Values are presented as means ± S.E (*n*=7). * $P < 0.01$ versus type I fibres, † $P < 0.01$ versus pre-exercise.

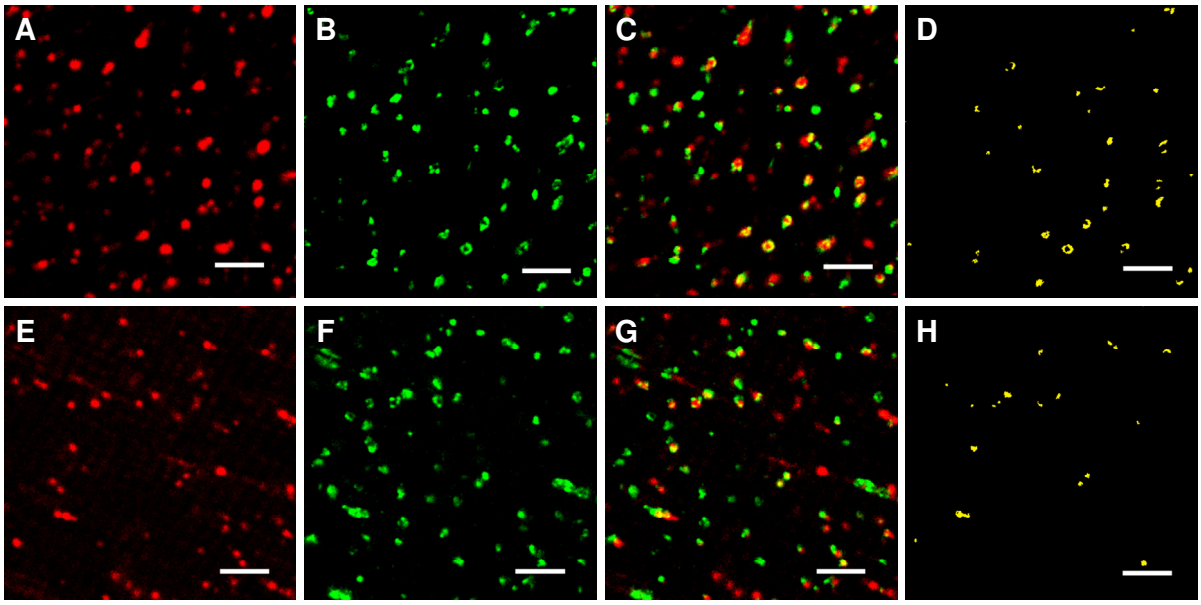
Fig. 2.2 Fibre specific PLIN2 content visualized through immunofluorescence microscopy and quantified before and immediately after exercise.



Representative immunofluorescence images of PLIN2 *(pre A, post B)* and myosin heavy chain type I combined with collagen IV *(pre C, post D)* from one participant pre- and post-exercise. Type I muscle fibres are indicated with "I" in images *A* and *B*, all other fibres are assumed type II fibres. White bar = 50µm. Fibre type specific PLIN2 *(E)* content quantified from immunofluorescence images before and after 1 hr constant load cycling at ~65% $VO_{2\ peak}$. Closed bars represent type I fibres, open bars represent type II fibres. Values are presented as means ± S.E (*n*=7). * $P < 0.01$ versus type I fibres.

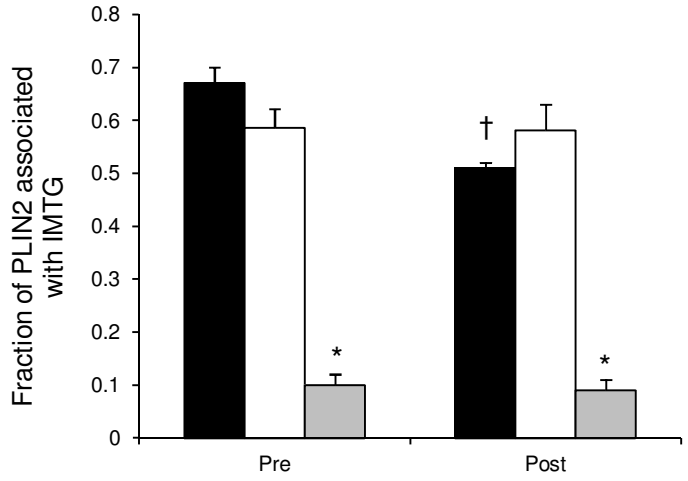
(F) PLIN2 content in pre and post exercise samples was also determined using immunoblotting, and correlated well with PLIN2 content (when type I and type II fibres were pooled) determined using fluorescence microscopy. *(G)* The specificity of the PLIN2 antibody was confirmed in a competition experiment where a PLIN2 recombinant peptide (Genway Biotech, Inc, CA, USA) was incubated with the antibody, and subsequently the punctate pattern typical of PLIN2 positive staining was removed *HI)*. Immunoblot analysis of human skeletal muscle using the PLIN2 antibody revealed a band at ~50 kDa *(I)*.

Fig. 2.3. IMTG and PLIN2 colocalisation images visualized using immunofluorescence microscopy before and immediately after exercise.



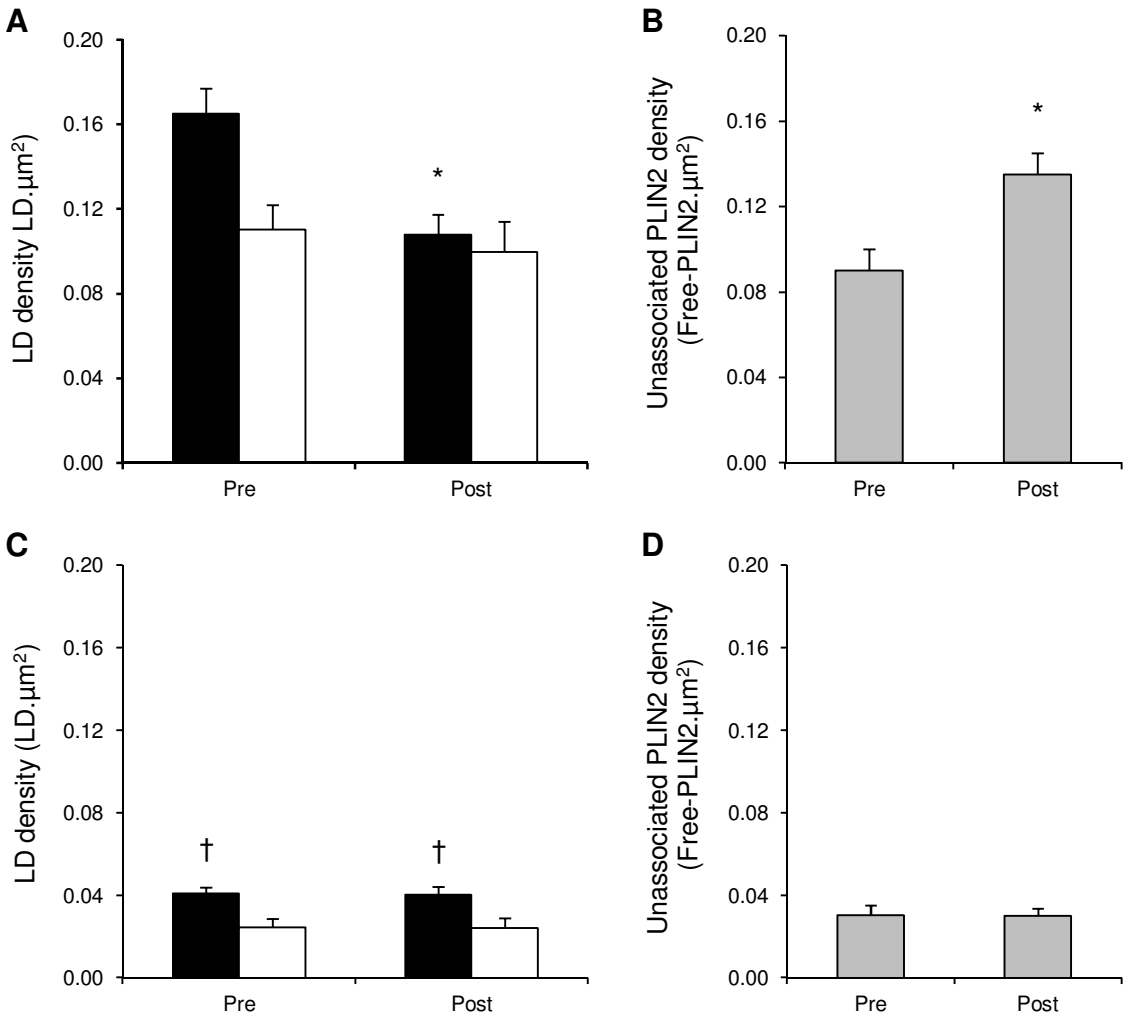
Representative confocal microscopy images of IMTG *(A pre, E post)*, PLIN2 *(B pre, F post)*, and the subsequent merged images *(C pre, G post)* used to calculate colocalisation, obtained from one participant. Images *D* and *H* (pre and post exercise, respectively) show the overlaying areas from both IMTG and PLIN2 images, used to identify the relative association of PLIN2 with IMTG. White bar = 5µm.

Fig. 2.4. Fraction of PLIN2 associated with IMTG before and after exercise.



Relative association of PLIN2 with IMTG pre- and post-exercise. Colocalisation analysis was performed in actual images in type I fibres (closed bars) and type II fibres (open bars), and statistically compared to randomized images (grey bars) where colocalisation would occur by chance. Values are presented as means ± S.E (*n*=7). * $P < 0.001$ versus actual images, † $P < 0.05$ versus pre-exercise.

Fig. 2.5. PLIN2 and IMTG association before and after exercise.



The number of LDs associated (PLIN2-LD) (closed bars) or not associated (PLIN2-null-LD) (open bars) with PLIN2 before and after exercise in type I fibres *(A)* and type II fibres *(C)*. The quantity of PLIN2 that is not associated with LDs (grey bars) in type I *(B)* and type II fibres *(D)* before and after exercise. Values are presented as means ± S.E (n=7). * $P < 0.05$ versus pre-exercise, † $P < 0.05$ versus PLIN2-null-LD.

2.5 Discussion

In this study we investigated the hypothesis that the PLIN2 association with IMTG is related to IMTG utilisation during an acute bout of endurance exercise. Accordingly, we demonstrate for the first time that although PLIN2 content is unchanged immediately post-exercise, the reduction in IMTG results in a decrease in the PLIN2 association with IMTG in type I muscle fibres. In addition, the data suggest that PLIN2-associated LDs are preferentially depleted over those LDs not associated with PLIN2.

PLIN2 presents a fibre type-specific distribution in skeletal muscle obtained from humans in the rested, fasted state, with a two-fold greater concentration observed in type I fibres (Shaw et al., 2009), a finding confirmed in the current study. The observation of IMTG colocalisation with PLIN2 demonstrates that PLIN2 is contained on the LD surface as reported previously in adipocytes (Brasaemle et al., 1997) and skeletal muscle (Prats et al., 2006; Shaw et al., 2009). The fraction of IMTG associated with PLIN2 (~0.67 in type I fibres and ~0.58 in type II fibres) indicates that the majority, but not all, IMTG contains PLIN2. Therefore, it appears that different subpopulations of LDs with a particular perilipin protein composition exist in muscle. This is in agreement with several *in* vitro observations that suggest that the protein composition of the LD could influence its function (Ducharme & Bickel, 2008; Fujimoto *et al.*, 2008; Wolins *et al.*, 2006). Using the techniques described in the present study it is not possible to discount the possibility that small, undetectable levels of PLIN2 are present on all LDs. Nevertheless, in our model two pools of IMTG can be described: 1) PLIN2-associated LDs (PLIN2-LD), and 2) LDs where no PLIN2 was detected (PLIN2-null-LD). This apparent heterogeneity of LD protein composition in skeletal muscle corroborates a previous report from our lab (Shaw et al., 2009).

Endurance exercise in trained individuals stimulates a reduction in IMTG content, which is specific to type I muscle fibres (De Bock et al., 2005; Stellingwerff et al., 2007; van Loon et al., 2003). We report that 60 min steady state cycling induced a significant (~50%) fibre-specific decrease in IMTG content, which is consistent with previous reports using fluorescence microscopy demonstrating an approximate 60% reduction following 2-3 h of endurance exercise (De Bock et al., 2005; Stellingwerff

et al., 2007; van Loon et al., 2003). In addition, our observation confirms that IMTG also contributes to total fat oxidation in untrained individuals (Coggan et al., 2000). As there was no change in PLIN2 content in response to exercise, the fraction of PLIN2 associated with IMTG was significantly reduced ~24% following exercise in type I fibres. Conversely, no change in either IMTG or PLIN2 content was observed in type II fibres in response to exercise, and therefore the fraction of PLIN2 associated with IMTG remained unchanged. Thus, from our data, we can suggest that the change in PLIN2 association with IMTG in type I fibres is due to the decrease in PLIN2-associated IMTG containing LDs observed following exercise. In order to validate our finding, we compared the fraction of PLIN2 associated with IMTG in 'actual' images to the fraction observed in a subset of randomized images, as previously described (Lachmanovich et al., 2003). The degree of colocalisation between the randomized images was only a fraction of that observed in the 'actual' images and provides evidence that the observed colocalisation in the basal and post exercise biopsies was not due to chance colocalisation.

The degree of IMTG breakdown during exercise was related to basal IMTG concentrations, in agreement with previous studies (Steffensen et al., 2002; Stellingwerff et al., 2007; van Loon et al., 2003), and also basal PLIN2 content. This suggests that the IMTG and PLIN2 content are related and that greater PLIN2 expression is related to enhanced IMTG utilisation during exercise. To further investigate this relationship, we investigated the number of PLIN2-LD and PLIN2-null-LDs before and after exercise. Interestingly, in type I fibres 60 min steady state exercise induced a significant reduction in the number of PLIN2-LD, with no apparent change in the number of PLIN2-null-LD. Therefore, it appears that PLIN2-LD are preferentially utilised, compared to the PLIN2-null-LD, in response to moderate-intensity exercise in type I fibres. These data confirm the hypothesis that in skeletal muscle the PLIN2 association with IMTG may be a factor mediating IMTG utilisation during an acute bout of endurance exercise. The precise mechanisms regulating exercise-induced lipolysis in skeletal muscle are poorly understood, particularly in comparison to adipocytes (reviewed in (Lass et al., 2011). In isolated rat skeletal muscle, the colocalisation of HSL, a key lipolytic enzyme, with PLIN2 was enhanced in response to muscle contraction and epinephrine (Prats et al., 2006), though this does not provide substantial evidence that PLIN2 is able to activate HSL. Nevertheless, in

combination with the results of the present study, it does suggest that PLIN2-associated LDs are targeted to undergo lipolysis and subsequent oxidation during exercise compared to the PLIN2-null-LDs. In adipocytes or CHO cells expressing perilipin 1, it has been demonstrated that a key step in achieving maximal lipolysis is phosphorylation of perilipin 1, which recruits HSL to the LD and also releases CGI-58, the co-activator of ATGL (Sztalryd et al., 2003; Wang et al., 2009). However, perilipin 1 is absent from skeletal muscle (Londos et al., 1999), and so far, PLIN2 phosphorylation has only been reported in HeLa cells (Bartz et al., 2007). Studies in CHO cells transfected with PLIN2 demonstrate that HSL binds to PLIN2 in the basal state probably via its PAT-1 domain, the sequence that is common to most perilipins (Londos et al., 1995; Wang et al., 2009). There are also suggestions that PLIN2 can bind CGI-58 (Wang et al., 2011a) . These results suggest that the PLIN2-LD may be at an advantage, compared to the PLIN2-null-LD, when activation of lipolysis is required given the likelihood of HSL and CGI-58 localisation to those LDs. Thus, our results are compatible with a mechanism in which PLIN2 provides a structural function for HSL activation.

As we have only considered PLIN2, we cannot discount the probability that other perilipin proteins which are expressed in skeletal muscle may be associated with either the PLIN2-LD or the PLIN2-null-LD pools, as has been previously shown in rodent skeletal muscle (Ducharme and Bickel, 2008; Thiele and Spandl, 2008). Clearly the exact role of PLIN2 and the other perilipins in the regulation of skeletal muscle LD metabolism remains to be determined and warrants further investigation.

In the pre exercise state the fraction of PLIN2 associated with IMTG was ~0.67 and ~0.58 in type I and type II fibres, respectively, indicating that a proportion of PLIN2 is not bound to IMTG, which appears contrary to the general notion that PLIN2 is found predominantly at the LD surface (Bell et al., 2008; Prats et al., 2006; Wolins et al., 2006b). Nevertheless, a recent study has proposed that PLIN2 may cycle between the cytosolic and LD fraction (Wang et al., 2009), and PLIN2 has also been observed at the endoplasmic reticulum membrane (Robenek et al., 2006). Furthermore, as exercise induced a reduction in the number of LD only (in type I fibres) and no change in PLIN2, it is worth noting that as a result a larger proportion of PLIN2 is not associated with the LD after exercise. As our data only concerns PLIN2 content immediately following exercise, we cannot establish how the exercise-

induced IMTG decrease influences PLIN2 content in the period following exercise. *In vitro* data from CHO cells suggests that a decrease in TAG concentration promotes PLIN2 ubiquitination and degradation through proteolysis (Xu et al., 2005). Our data suggest that this process was not detectable during the first hour of exercise using immunofluorescence microscopy.

In conclusion, the reduction in IMTG during moderate intensity exercise in type I muscle fibres is due to the preferential utilisation of LDs associated with PLIN2. This adds to the evidence that perilipin proteins regulate IMTG metabolism in skeletal muscle, and suggests that the perilipin composition of the LDs in skeletal muscle is important in determining the utilisation of IMTG during exercise.

Prolonged exercise training increases intramuscular lipid content and perilipin 2 expression in type I muscle fibres of patients with type 2 diabetes

3.1 Abstract

The aim of the present study was to investigate changes in intramuscular triglyceride (IMTG) content and perilipin 2 (PLIN2) expression in skeletal muscle tissue following 6 months of endurance type exercise training in type 2 diabetes patients. 10 obese, male type 2 diabetes patients (age 62±1 y, BMI 31±1 kg/m^2) completed 3 exercise sessions per week, consisting of 40 min of continuous endurance type exercise at 75% $VO_{2\ peak}$, for a period of 6 months. Muscle biopsies collected at baseline and after 2 and 6 months of intervention were analysed for IMTG content and PLIN2 expression using fibre type specific immunofluorescence microscopy. Endurance type exercise training reduced trunk body fat by 6±2% and increased whole-body oxygen uptake capacity by 13±7% (P<0.05). IMTG content increased two-fold in response to the 6 months exercise training in both type I and type II muscle fibres (P<0.05). A three-fold increase in PLIN2 expression was observed from baseline to 2 and 6 months of intervention in the type I muscle fibres only (1.1±0.3, 3.4±0.6, and 3.6±0.6% fibre stained, respectively; P<0.05). Exercise training induced a 1.6-fold increase in mitochondrial content after 6 months of training in both type I and type II muscle fibres (P<0.05). In conclusion, this is the first study to report that prolonged endurance type exercise training increases the expression of PLIN2 alongside increases in IMTG content in a type I muscle fiber type specific manner in type 2 diabetes patients.

3.2 Introduction

Skeletal muscle insulin resistance is a defining characteristic of type 2 diabetes and is associated with intramuscular triglyceride (IMTG) accumulation. The concept of lipid-induced insulin resistance was initially derived from cross-sectional studies which demonstrated a correlation between high IMTG concentrations and insulin resistance (Pan et al., 1997). However, trained endurance athletes are generally highly insulin sensitive despite having substantially elevated IMTG levels (Goodpaster et al., 2001; van Loon et al., 2004). Consequently, IMTG accumulation appears to relate to insulin resistance when accompanied by a sedentary lifestyle and low oxidative capacity (Bruce et al., 2003). It is postulated that a low turnover of the intramuscular lipid pool and a resultant elevation in the concentration of lipid metabolites, such as diacylglycerol and ceramides, mediates impairments in the insulin signaling pathway which are responsible for reduced insulin sensitivity (Moro et al., 2008).

The benefits of prolonged endurance type exercise training on cardiovascular and metabolic health have been well established (Hansen et al., 2010), and provide a basis for prescribing exercise in the prevention and treatment of type 2 diabetes (Praet and van Loon, 2007). Exercise training interventions that enhance oxidative capacity and improve the storage and packaging of IMTG are likely to facilitate the improvement in skeletal muscle insulin sensitivity. In accordance, recent studies demonstrate an increase in mitochondrial density and intrinsic mitochondrial function in response to prolonged endurance type exercise training in type 2 diabetes patients (Hey-Mogensen et al., 2010; Phielix et al., 2010). The impact of endurance type exercise training on IMTG content is less clear, with studies showing an increase (Dubé et al., 2008; Pruchnic et al., 2004), no change (Bruce et al., 2006), or a decrease (Bruce et al., 2004; Solomon et al., 2008) in muscle lipid storage in older obese individuals and obese type 2 diabetes patients. Changes in IMTG deposition can be assessed by the use of biochemical TG extraction of muscle tissue as well as immunohistochemical analyses of oil red O-stained muscle cross sections (Stellingwerff et al., 2007). The latter approach has shown a 3-4 fold greater lipid content in type I versus type II muscle fibres (van Loon, 2004). This method has been applied frequently to evaluate fibre type specific differences in IMTG content across different populations and in response to exercise (van Loon et al., 2003; van Loon et al., 2004). Therefore it is

important to utilize techniques that allow IMTG content and associated proteins to be analysed in a muscle fibre type specific manner.

Lipid droplets (LDs) containing IMTG are viewed as a dynamic organelle which play a role in a variety of cellular functions including lipid homeostasis and cell signaling (for recent reviews see (Farese and Walther, 2009; Goodman, 2008). This notion is supported by the discovery of a family of proteins associated with the phospholipid monolayer of LDs, referred to as the perilipin proteins (numbered 1 to 5; (Kimmel et al., 2010). Perilipin 1 is relatively well-characterized and appears to regulate lipolysis through its interaction with lipases and co-activators at the surface of the LD (Granneman et al., 2009; Wang et al., 2009), however its expression is reported to be limited to adipocytes and steroidogenic cells (Londos et al., 1999). Perilipin 2 (formerly known as adipocyte differentiation-related protein; ADRP or adipophilin) (PLIN2) on the other hand is ubiquitously expressed and present in skeletal muscle tissue. PLIN2 content is closely related to IMTG concentrations and is more abundantly expressed in the type I muscle fibres (Brasaemle et al., 1997; Minnaard et al., 2009; Shaw et al., 2009). Although the exact function of PLIN2 remains to be established, *in vitro* data suggest that its presence on the lipid droplet surface can limit the LD-association with adipose triglyceride lipase (ATGL) (Bell et al., 2008; Listenberger et al., 2007). Therefore, TG accumulation in cells expressing PLIN2 has been attributed to the subsequent lowering of basal lipolytic rates which also promotes tissue insulin sensitivity (Bell et al., 2008). In agreement, human studies demonstrate that PLIN2 gene expression is higher in insulin sensitive versus insulin resistant individuals (Coen et al., 2010) and improvements in insulin mediated glucose disposal in response to weight loss and the pharmacological treatment of type 2 diabetes alters the expression of PLIN2 in skeletal muscle (Minnaard et al., 2009; Phillips et al., 2005). However, the impact of prolonged endurance type exercise training on fibre type specific PLIN2 protein expression remains to be assessed.

We hypothesized that prolonged endurance type exercise training increases muscle lipid storage and upregulates the expression of PLIN2. Given the importance of considering muscle fibre type when investigating IMTG and related proteins, we applied immunofluorescence microscopy techniques to

investigate muscle fibre type specific changes in IMTG, PLIN2 and cytochrome *c* oxidase (COX) content following 2 and 6 months of endurance type exercise training in type 2 diabetes patients.

3.3 Methods

Participants

Ten type 2 diabetes patients participated in the current study (62 ± 1 y, BMI 31.2 ± 0.9 $kg.m^{-2}$). Participants had been diagnosed for at least 12 months, were all being treated with oral blood-glucose-lowering medication and were sedentary. The study was approved by the medical ethics committee of the Virga Jesse Hospital, Belgium and written informed consent was obtained from all participants. The patients in the current study were part of a larger project (clinical trial registration: ISRCTN32206301) investigating the impact of prolonged endurance type exercise training in a cohort of fifty type 2 diabetes patients, described in detail elsewhere (Hansen et al., 2009).

Study design

Participants completed a 6 month endurance type exercise training program. Prior to commencement of the study, and after 2 and 6 months of the intervention, oxidative capacity, body composition and oral glucose tolerance were assessed as described previously (Hansen et al., 2009). Muscle biopsies were taken from the *vastus lateralis* in the morning and following an overnight fast and were analysed for mitochondrial content, IMTG, and PLIN2 expression. The measurements at 2 and 6 months were performed at least 4 d after the last exercise session. Oral blood glucose and/or lipid-lowering medication were stopped 3 d prior to these measurements.

Training intervention

Participants undertook 3 supervised training sessions per week in the rehabilitation centre of the hospital. Each exercise session consisted of walking, cycling, and cross-country ski-type exercise and was performed for 40 min at a heart rate corresponding to exercise performed at 75% of $VO_{2\ peak}$. The relationship between $VO_{2\ peak}$ and heart rate was reassessed after 2 months, and training intensity was adjusted accordingly.

Immunohistochemistry

Muscle samples were dissected free of fat and connective tissue, before being embedded in Tissue-Tek OCT Compound (Sakura Finetek Europe, The Netherlands) and frozen in liquid nitrogen-cooled isopentane. Cryosections of 5 µm thickness were fixed in 3.7% formaldehyde and permeabilised for 5 min in 0.5% Triton-X 100. Sections were then incubated for 1 h with a mouse monoclonal anti-ADRP/perilipin2 antibody (Progen, Germany) as described previously (Shaw et al., 2008; Shaw et al., 2009). As a key protein in the electron transport chain, identification of cytochrome C oxidase using a mouse monoclonal anti-OxPhos Complex IV (COX) antibody (Invitrogen, UK) was also used as a marker of the mitochondrial network of skeletal muscle. Fibre type determination was achieved through incubation of muscle sections with mouse anti-myosin heavy chain type I (A4.840-c, DSHB, developed by Dr. Blau). Sections were then incubated with either an Alexa Fluor goat anti-mouse IgG_{2a} 594 (for OxPhos Complex IV) or an Alex Fluor goat anti-mouse IgG_1 594 (for PLIN2) in combination with an Alexa Fluor goat anti-mouse IgM 488 (for MHC I) (Invitrogen, UK) for 30 min. Coverslips were mounted with a glycerol and mowiol 4-88 solution in 0.2 M Tris buffer (pH 8.5) (including 0.1% DABCO anti-fade medium). When IMTG visualisation was undertaken, the neutral lipid dye oil red O staining protocol in combination with immunofluorescence was used (van Loon et al., 2003). In this respect, oil red O was applied to sections for 30 min following incubation with antibodies for fibre type determination.

Image capture, processing and data analysis

Image capture was performed in a blinded fashion on a widefield Nikon E600 microscope with a 40x 0.75 NA objective, coupled to a SPOT RT KE colour 3 shot CCD camera (Diagnostic Instruments Inc., USA) for the fibre type-specific determination of IMTG and PLIN2. FITC (465-495 nm) and Texas Red (540-580 nm) excitation filters were used to view the Alexa Fluor 488 and 594 fluorophores, respectively. The Texas Red excitation filter was also used to view sections stained with oil red O. An inverted confocal laser scanning microscope (Leica DMIRE2, Leica Microsystems) with a 63x 1.4 NA oil immersion objective was used to obtain digital images of mitochondria, IMTG and PLIN2. A Helium-Neon laser was used to excite the Alexa Fluor 594 fluorophore and oil red O, and an argon laser was used to excite the Alexa Fluor 488 fluorophore.

Image processing was undertaken using Image-Pro Plus 5.1 software (Media Cybernetics, MD, USA). Widefield images were used to assess fibre-type specific content of IMTG and PLIN2. Confocal images were used to assess COX content, LD size and to visualize the subcellular distribution of PLIN2. Fibres positively stained for MHC I were considered type I muscle fibres and non-stained fibres were considered type II muscle fibres. Identification of COX, IMTG and PLIN2 was achieved through the selection of an intensity threshold that was used uniformly for all images in that series. COX, IMTG and PLIN2 content was expressed as the percentage fibre area positively stained. IMTG and PLIN2 density were expressed as number of positively stained 'spots' corrected for fibre area (μm^2). Mean LD size was calculated by dividing the total number of objects by the total area stained. A total of 100 ± 12, 72 ± 6 and 86 ± 7 fibres were analysed per muscle cross-section for COX, IMTG and PLIN2 analysis respectively.

Statistics

All data are expressed as means±SEM. Significance was set at the 0.05 level of confidence. Changes in whole-body characteristics, exercise capacity, body composition and insulin sensitivity were analysed were using a one-way repeated measures ANOVA, with the within-subject factor as '*time*' (0 vs 2 vs 6 months). Changes in COX, IMTG and PLIN2 were assessed using a two-way repeated measures ANOVA, with two within-subject factors '*fibre*' (type I vs type II fibres) and '*time*' (0 vs 2 vs 6 months). Significant main effects or interactions were assessed using Bonferroni adjustment post hoc analysis.

3.4 Results

Participants

Participant characteristics are displayed in Table 3.1. Significant reductions in body mass and BMI were observed with training ($P < 0.05$; Table 3.1) which were accompanied by a reduction in relative trunk fat and leg fat percentage of 6±2% and 5±2% post-training, respectively ($P < 0.05$). Maximal oxygen uptake demonstrated a significant increase over time ($P < 0.05$) and was 16 ± 3% higher after 2 months of training (from 23.4 ± 1.5 to 27.1 ± 1.7 $mL \cdot kg^{-1} \cdot min^{-1}$, $P < 0.01$), and remained 13 ± 7% higher compared to baseline values after 6 months of intervention (26.5 ± 1.9 $mL.kg^{-1}.min^{-1}$, $P = 0.08$). Although the total cohort showed significant improvements in glycemic control, as shown by reduced levels of HbA_{1c} (Hansen et al., 2009), in this small subcohort of 10 subjects the decrease in HbA_{1c} following training failed to reach significance (7.0 ± 0.4, 6.7 ± 0.3 and 6.5 ± 0.2%, for 0, 2 and 6 months respectively; $P = 0.20$). No significant changes in fasting plasma glucose and insulin concentrations, 2 h post-OGTT glucose concentrations or HOMA index of insulin sensitivity were observed in response to training ($P > 0.05$).

Table 3.1 *Subject characteristics*

Variable	Time			Effect of time (*P* value)
	Baseline	2 months	6 months	
Age (years)	62 ± 1	-	-	
Height (m)	1.72 ± 0.02	-	-	
Body weight (kg)	92.9 ± 3.4	91.7 ± 3.4	91.3 ± 3.4	<0.05
Body mass index ($kg.m^{-2}$)	31.2 ± 0.9	30.8 ± 0.9	30.7 ± 0.9	<0.05
Insulin sensitivity				
Fasting glucose ($mmol.L^{-1}$)	9.2 ± 0.7	9.0 ± 0.7	8.7 ± 0.7	0.632
2 h glucose ($mmol.L^{-1}$)	17.9 ± 1.6	16.5 ± 1.6	15.5 ± 1.7	0.367
Fasting insulin ($\mu IU.mL^{-1}$)	17.8 ± 2.3	16.8 ± 2.3	17.7 ± 2.1	0.855
HOMA index	7.5 ± 1.4	7.0 ± 1.5	7.1 ± 1.2	0.865
HbA_{1c} (%)	7.0 ± 0.4	6.7 ± 0.3	6.5 ± 0.2	0.200
Exercise capacity				
$VO_{2\ peak}$ ($L.min^{-1}$)	2.15 ± 0.14	2.46 ± 0.15*	2.41 ± 0.19	<0.05
$VO_{2\ peak}$ ($mL.kg^{-1}.min^{-1}$)	23.4 ± 1.5	27.1 ± 1.7*	26.5 ± 1.9	<0.05
W_{max} (W)	180 ± 12	189 ± 11	189 ± 11	0.134
Body composition				
% Trunk fat	38.4 ± 1.4	37.3 ± 1.7	35.8 ± 1.3*	<0.01
% Leg fat	23.2 ± 1.6	22.4 ± 1.5	21.8 ± 1.3	<0.05

Data provided represent means ± S.E. (n=10). * P<0.05 vs. baseline.

Immunohistochemical analysis

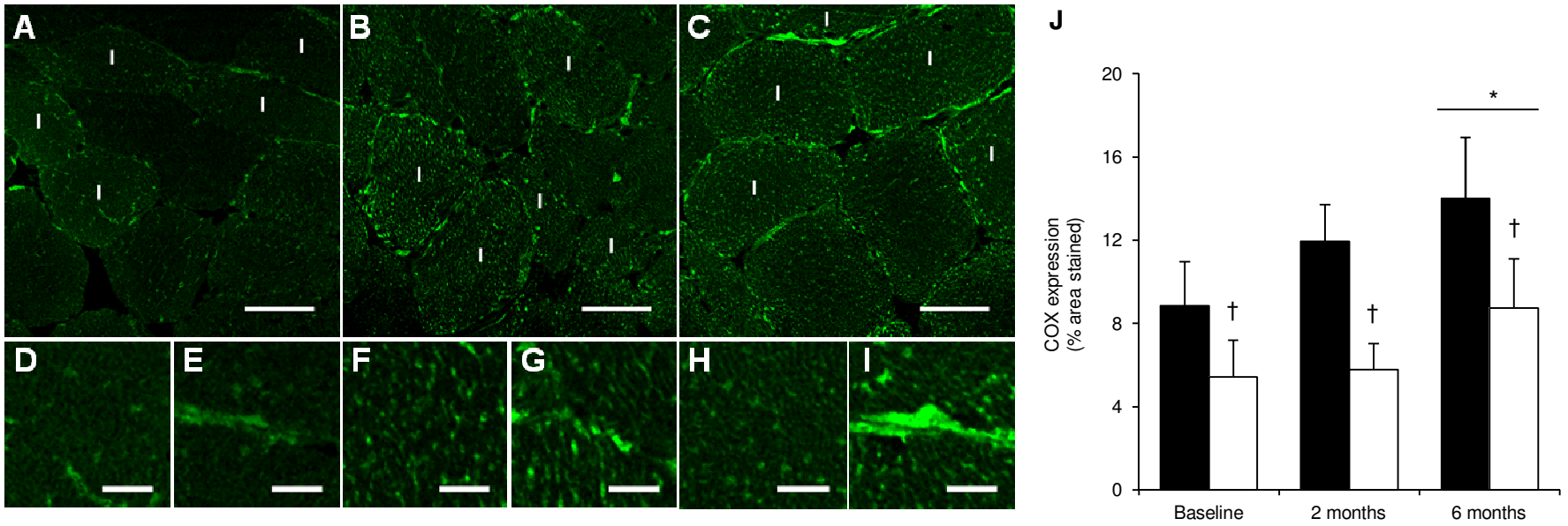
Cytochrome c oxidase: Muscle fibre type specific COX expression was significantly greater in type I compared with type II muscle fibres at all time points ($P < 0.01$; Fig. 3.1). Six months of endurance type exercise training induced a time-dependent increase in COX expression in both type I and type II fibres ($P < 0.01$). Following 6 months of exercise training, COX expression was higher than baseline in both type I (8.9 ± 2.1 vs. 14.0 ± 2.9% fibre stained) and type II muscle fibres (5.4 ± 1.8 vs. 8.8 ± 2.4% fibre stained). The confocal micrographs of COX stained muscle fibres at baseline, 2 months and 6 months are presented in Fig. 3.1D-I. These images demonstrate greater COX density in both intermyofibrillar and subsarcolemmal regions of the muscle fibres after 6 months of training, and are most prominent in the subsarcolemmal regions.

Intramuscular triglyceride: IMTG content, expressed as the area fraction stained, differed between type I and type II muscle fibres at each time point (Fig. 3.2C; $P < 0.01$). Six months of endurance type exercise training induced a time dependent increase in IMTG content in both type I and II muscle fibres. IMTG content had increased ~1.9-fold in type I fibres (2.3 ± 0.4 vs 4.3 ± 0.5%; $P < 0.01$) and type II fibres (0.9 ± 0.1 vs 1.7 ± 0.3%; $P<0.01$) following 6 months of training. The increase in IMTG content was mirrored by significant increases in IMTG density after 6 months of training in type I fibres (0.051 ± 0.007 and 0.079 ± 0.008 LDs/μm^2 for 0 and 6 months, respectively; $P < 0.01$) but not in type II muscle fibres (0.022 ± 0.002 and 0.031 ± 0.003 LDs/μm^2 for 0 and 6 months, respectively; $P = 0.056$). No significant changes in IMTG content or density were apparent after 2 months of training in either fibre type ($P > 0.05$). There were no differences in lipid droplet size as determined by confocal microscopy between fibre types or in response to endurance type exercise training ($P > 0.05$).

PLIN2 expression: At baseline, PLIN2 expression calculated as the percentage area stained did not differ between type I and type II muscle fibres (Fig. 3.4; $P > 0.05$). Six months of exercise training induced a time dependent increase in PLIN2 expression in type I muscle fibres only ($P < 0.05$). In comparison to baseline, PLIN2 expression in type I muscle fibres had increased ~3-fold after 2 months of training (1.1 ± 0.3 vs 3.6 ± 0.6%, respectively; $P < 0.05$) with no further increase observed after 6 months of training. This fibre type specific training response resulted in greater PLIN2 expression in

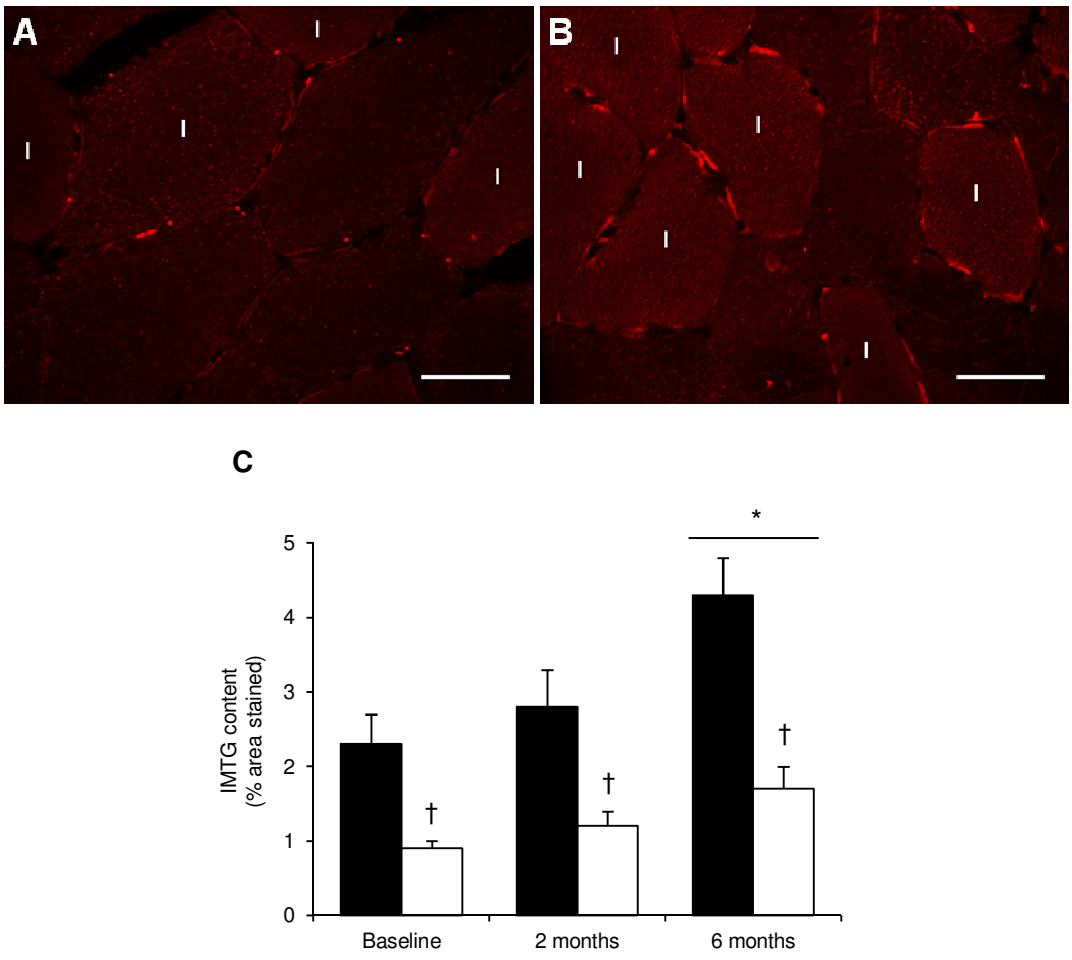
type I compared with type II muscle fibres after 2 and 6 months of training ($P<0.05$). Representative immunofluorescence images of PLIN2 expression in type I and type II muscle fibres at baseline and after 2 and 6 months of training are shown in figure 3. In comparison to baseline, PLIN2 density in type I fibres also increased ~2-fold after 2 and 6 months (0.018 ± 0.003, 0.033 ± 0.004 and 0.035 ± 0.005 PLIN2 objects/μm^2 for 0, 2 and 6 months respectively; $P < 0.05$) whereas PLIN2 density in type II muscle fibres did not change ($P > 0.05$). Therefore, PLIN2 density was higher in type I than type II muscle fibres after 2 and 6 months of training only. Higher magnification images of PLIN2 were obtained using confocal laser scanning microscopy and are shown in Fig. 3.3J-L. These images show a clear increase in PLIN2 expression after 6 months of training. These images also demonstrate that distinct rings of PLIN2 can frequently be observed and are more abundant after prolonged exercise training.

Fig. 3.1. Effect of 6 months exercise training on fibre type-specific COX expression



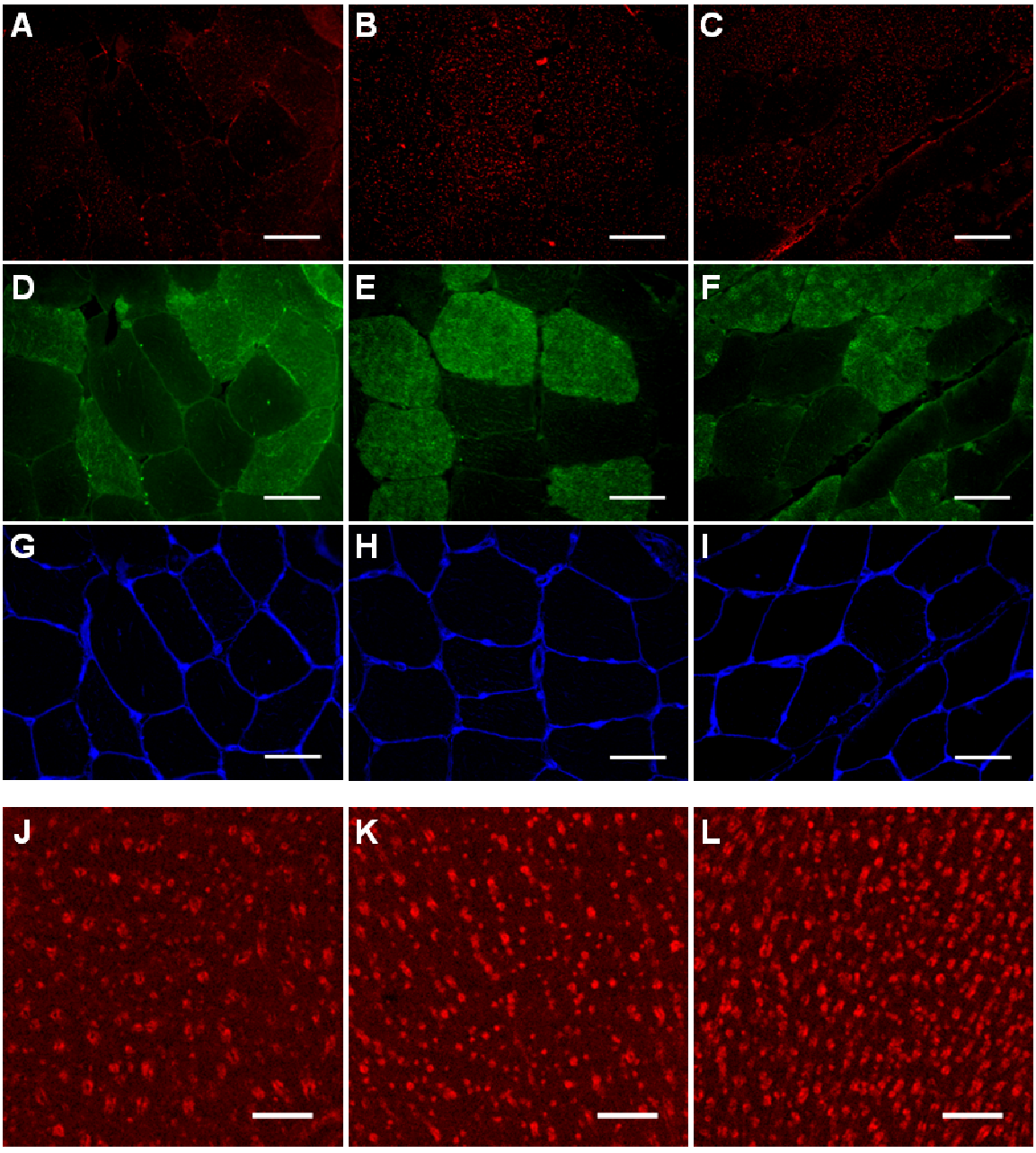
Representative confocal images of COX expression in skeletal muscle at baseline *(A)*, and after 2 *(B)* and 6 months *(C)* of exercise training. White bar = 50 µm. Representative images of the intermyofibrillar *(D, F, H)* and subsarcolemmal *(E, G, I)* mitochondrial regions at baseline, and after 2 and 6 months of exercise training, respectively. White bar = 1 µm. Fibre type-specific COX expression (expressed as % of fibre stained) at baseline, and after 2 and 6 months of exercise training, in type I (closed bars) and type II fibres (open bars). Values are presented as means ± S.E. * $P < 0.05$ vs. Baseline. † $P < 0.001$ vs. type I fibres.

Fig. 3.2. Effect of 6 months exercise training on fibre type-specific IMTG content



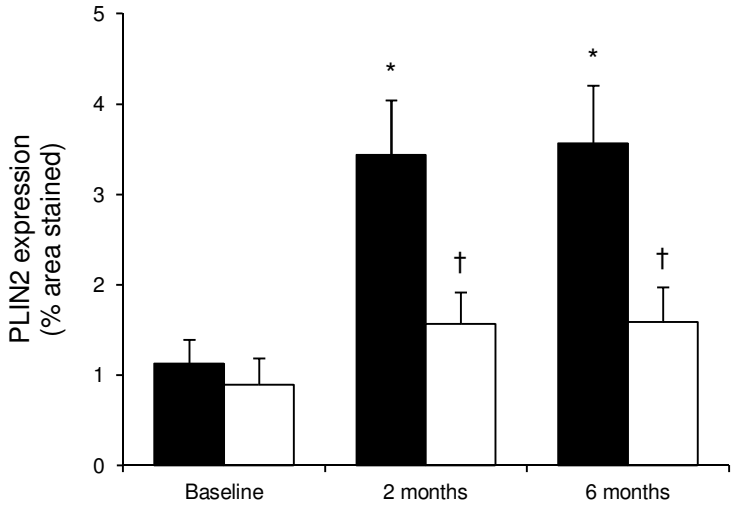
Representative immunofluorescence images of IMTG at baseline *(A)* and after 6 *(B)* of exercise training. Type I fibres are indicated with "I", all other fibres are assumed type II fibres. White bar = 50 µm. *(C)* IMTG content was measured at baseline, and after 2 and 6 months of exercise training in type I (closed bars) and type II fibres (open bars). Values are presented as means ± S.E. * $P < 0.05$ vs. Baseline. † $P < 0.001$ vs. type I fibres.

Fig. 3.3. Immunofluorescence images of fibre type-specific PLIN2 protein expression.



Representative images of PLIN2 at baseline *(A)*, and after 2 *(B)* and 6 *(C)* months of exercise training, stained using anti-PLIN2 in combination with anti-myosin heavy chain type I *(D, E, F, respectively)* and wheat germ agglutinin 350 (WGA350) *(G, H, I, respectively)* and viewed and quantified with widefield immunofluorescence microscopy. White bar = 50 µm. Representative confocal images of PLIN2 at baseline *(A)*, and after 2 *(B)* and 6 *(C)* months of exercise training; rings of perilipin staining are clearly visible in all images. White bar = 10 µm.

Fig. 3.4. Effect of 6 months exercise training on PLIN2 protein expression



Fibre type-specific COX expression (expressed as % of fibre stained) at baseline, and after 2 and 6 months of exercise training, in type I (closed bars) and type II fibres (open bars). Values are presented as means ± S.E. * $P < 0.05$ vs. Baseline. † $P < 0.001$ vs. type I fibres.

3.5 Discussion

Prolonged endurance type exercise training is known to improve insulin-stimulated glucose uptake and glycaemic control in type 2 diabetes patients (Hansen et al., 2010). In this study we demonstrate that endurance type exercise training also increases both IMTG deposition and COX expression, which are higher in type I muscle fibres. In accordance, we show for the first time that training induces a greater expression of PLIN2 in type I muscle fibres.

Insulin sensitivity is enhanced by regular physical activity which explains why significant improvements in glycaemic control were observed in the previous study after 6 months of endurance type exercise training in a large cohort of type 2 diabetes patients (Hansen et al., 2009). In the subset of participants used in this study there was no significant change in glycaemic control as evident from the HbA_{1c} levels after 6 months of training (Table 3.1). Nevertheless a decline from 7.0 ± 0.4% down to 6.5 ± 0.2% in HbA_{1c} is of great clinical significance, as it would translate into a >10% reduction in the risk of premature death, a 5-10% reduction in the risk of myocardial infarction and a ~20% reduction in the risk of microvascular disease (Manley, 2003).

Skeletal muscle oxidative capacity and whole body fatty acid oxidation are good predictors of muscle insulin sensitivity (Bruce et al., 2003; Goodpaster et al., 2001; Goodpaster et al., 2003; Kelley et al., 1999). Obese individuals with insulin resistance and type 2 diabetes commonly display a reduced capacity for oxidative metabolism (Boushel et al., 2007; Kelley et al., 2002; Ritov et al., 2005). Thus, it is likely that increased oxidative capacity following exercise interventions are mechanistically linked to improvements in metabolic health in this population. Accordingly, we observed a ~1.6-fold increase in COX expression in skeletal muscle following 6 months of endurance type exercise training (Fig. 3.1J). The increase in COX expression in this subset of patients is in agreement with the 50% increase in COX and citrate synthase activities observed in the full cohort of patients reported previously (Hansen et al., 2009). We extend on these previous data by the application of immunofluorescence microscopy, allowing us to assess oxidative capacity in a muscle fiber type specific manner. Furthermore, we also assessed subcellular localisation of the observed increases in oxidative capacity (Fig. 3.1D-I). The present work shows that increases in the content of the mitochondrial enzyme COX can be observed

in both the subsarcolemmal and intermyofibrillar region of the type I muscle fibres. In agreement with previous data investigating mitochondrial content following a 10 week training intervention in type 2 diabetes patients using transmission electron microscopy (Nielsen et al., 2010), we show that increased COX expression is prominent in subsarcolemmal regions of type I fibres after prolonged endurance type exercise training.

The exercise training-induced increase in skeletal muscle oxidative capacity was accompanied by a ~2-fold elevation in skeletal muscle lipid deposition in both type I and type II muscle fibres (Fig. 3.2C). This is the first study to show a type I muscle fibre specific increase in IMTG content following prolonged exercise training in type 2 diabetes patients. These findings tend to be in line with several recent studies demonstrating IMTG accretion coupled to increased oxidative capacity in older, obese insulin resistant individuals following 12-16 weeks of exercise training (Dubé et al., 2008; Pruchnic et al., 2004). Although IMTG content is already elevated in obese type 2 diabetes patients, these levels still remain below those observed in endurance-trained athletes who are highly insulin sensitive (Goodpaster et al., 2001; van Loon et al., 2004). The high IMTG content in combination with a reduced oxidative capacity in type 2 diabetes patients likely mediates the reduction in muscle insulin sensitivity rather than merely elevated IMTG stores. Accordingly, exercise training-induced increases in mitochondrial content, coupled to IMTG accretion appear to enhance insulin sensitivity. For example, a recent study has demonstrated that training-induced increases in IMTG concentrations and improvements in insulin sensitivity are coupled to a reduction in the concentration of diacylglycerol and ceramide (Dubé et al., 2011). Therefore it has been hypothesized that the process of IMTG synthesis consumes the lipid metabolites that are precursors to IMTG and impair skeletal muscle insulin signaling. In further support, the high IMTG synthesis rates observed in the period after endurance type exercise protects against the development of insulin resistance during (intra)lipid infusion (Schenk and Horowitz, 2007). The present study adds to this growing body of evidence by demonstrating greater IMTG storage and improved glycaemic control in response to 6 months training in type 2 diabetes patients. Some studies employing a shorter training duration have failed to observe a significant increase in type 1 muscle fibre IMTG content following training in type 2 diabetic patients (Meex et al., 2010). Therefore, it is possible that a more prolonged intervention, such as the 6 month

endurance training programme applied in the current study, is required before increases in IMTG deposition are observed in type 2 diabetes patients. The duration of the training intervention, in addition to the method of IMTG analysis, may also explain the discrepancy across the many studies investigating changes in IMTG content.

The increase in total IMTG content following training in the present study was accompanied by an increase in the number of LDs in type I fibres, whereas there was no change detected in LD size. This is in agreement with a previous electron microscopy study in young males and females, where the increase in total IMTG content with training was due to an increase in LD density while LD size remained unchanged (Tarnopolsky et al., 2007). IMTG expansion through an increase in the number of smaller LDs would befit a metabolic advantage as the surface area available for the interaction of lipolytic enzymes with the regulatory proteins contained on the LD surface would be enhanced. This would maximize the capacity for rapid LD turnover, allowing more efficient lipid mobilization and therefore oxidation during exercise.

One of the regulatory proteins that reside on the surface of the LD monolayer is PLIN2. In the current study, despite observing a ~2-fold higher IMTG concentration in the type I muscle fibres, there was no difference in PLIN2 expression between type I and type II muscle fibres prior to endurance type exercise training (Fig. 3.4). However, training induced a significant increase in PLIN2 expression in type I muscle fibres. The perilipins are important in the packaging of lipid droplets and *in vitro* studies demonstrate that PLIN2 expression increases cellular TG and improves insulin sensitivity. The presence of PLIN2 at the LD surface appears to limit the association of ATGL with the LD surface, reduce basal lipolytic rates and therefore promote TG storage (Bell et al., 2008; Listenberger et al., 2007).

We show that when type 2 diabetes patients are physically active, type I muscle fibres exhibit a greater expression of PLIN2 than type II muscle fibres. This is in agreement with our previous observations of a greater PLIN2 expression in the type I muscle fibres of sedentary individuals and trained cyclists (Shaw et al., 2009; Shepherd et al., 2012). The increase in PLIN2 expression in type I

muscle fibres is likely to result in enhanced coverage of the LD surface with PLIN2. This adaptation would limit rates of basal lipolysis and promote IMTG storage in the basal state. Furthermore, hormone sensitive lipase translocates to PLIN2-coated LDs during muscle contraction and adrenaline stimulation (Prats et al., 2006) and PLIN2-associated LDs are depleted during exercise (Shepherd et al., 2012). We hypothesize that an increase in PLIN2 surface coverage of the LD, along with the greater total LD surface area available and the enhanced mitochondrial density, would aid the mobilization and oxidation of the IMTG pool during exercise. This proposed improvement in the regulation of IMTG turnover both at rest and during exercise may go some way to explaining why insulin sensitivity can be enhanced despite further accumulation of IMTG with training. However, it should be noted that neither intramuscular lipolysis nor lipid oxidation rates were assessed in the present study, therefore further research is required to fully explore the relationship between changes in PLIN2 expression and intramuscular lipid oxidation.

A non-exercise control group was not included in the present study, however reductions in fat mass, and improvements in VO_{2max} and muscle oxidative capacity are not seen in a similar time frame in non-exercising controls (Church et al., 2010; Short et al., 2003). Therefore we can be confident that the related changes in PLIN2 expression and IMTG storage in the present study are specific adaptations to the exercise intervention. As PLIN2 is one of four perilipin proteins present in skeletal muscle, additional investigations examining other perilipins are required to fully understand the role of IMTG metabolism in the development insulin resistance and the insulin sensitizing effect of endurance type training.

In conclusion, prolonged endurance type exercise training increases intramuscular lipid storage in a muscle fibre type dependent manner in type 2 diabetes patients. Importantly, the increase in IMTG content is accompanied by a type I muscle fiber specific increase in PLIN2 expression. The greater PLIN2 expression following prolonged endurance type exercise training in combination with increased oxidative capacity may explain the improved turnover of the skeletal muscle lipid pool with regular physical activity, and likely contributes to the improvements in skeletal muscle insulin action and subsequent glycaemic control.

Sprint interval and traditional endurance training increase net intramuscular triglyceride breakdown and expression of perilipin 2 and 5

4.1 Abstract

Intramuscular triglyceride (IMTG) utilization is enhanced by endurance training (ET) and is linked to improved insulin sensitivity. This study first investigated the hypothesis that ET-induced increases in net IMTG breakdown and insulin sensitivity are related to increased expression of perilipin 2 (PLIN2) and perilipin 5 (PLIN5). Secondly, we hypothesized that sprint interval training (SIT) also promotes increases in IMTG utilization and insulin sensitivity. Sixteen sedentary males performed 6 weeks of either SIT (4-6, 30s Wingate tests per session, 3d.week^{-1}) or ET (40-60 min moderate-intensity cycling, 5d.week^{-1}). Training increased resting IMTG content (SIT 1.7-fold, ET 2.4-fold; $P<0.05$) concomitant with parallel increases in PLIN2 (SIT 2.3-fold, ET 2.8-fold; $P<0.01$) and PLIN5 expression (SIT 2.2-fold, ET 3.1-fold; $P<0.01$). Pre-training, 60 min cycling at ~65% pre-training VO_{2peak} decreased IMTG content in type I fibres (SIT 17±10%, ET 15±12%; $P<0.05$). Following training, a significantly greater breakdown of IMTG in type I fibres occurred during exercise (SIT 27±13%, ET 43±6%; $P<0.05$), with preferential breakdown of PLIN2- and particularly PLIN5-associated lipid droplets. Training increased the Matsuda insulin sensitivity index (SIT 56±15%, ET 29±12%, main effect $P<0.05$). No training x group interactions were observed for any variables. In conclusion, SIT and ET both increase net IMTG breakdown during exercise and similar increases in PLIN2 and PLIN5 protein expression. The data are consistent with the hypothesis that increases in PLIN2 and PLIN5 are related to the mechanisms that promote increased IMTG utilization during exercise and improvements in insulin sensitivity following 6 weeks SIT and ET.

4.2 Introduction

High intramuscular triglyceride (IMTG) concentrations are associated with insulin resistance in sedentary obese individuals and type 2 diabetes patients (Goodpaster et al., 2001; Pan et al., 1997; Phillips et al., 1996a). However, the athletes' paradox describes a state of elevated IMTG storage alongside high levels of insulin sensitivity in endurance trained athletes (Goodpaster et al., 2001; van Loon et al., 2004). Endurance training also increases oxidative capacity and promotes a shift towards greater IMTG utilization during exercise (Schrauwen et al., 2002). Therefore, the capacity to oxidise IMTG as a fuel source is believed to be mechanistically important for the preservation of high insulin sensitivity in the face of elevated intramuscular lipid storage (Bruce et al., 2003; Goodpaster et al., 2001; van Loon and Goodpaster, 2006). The overriding current hypothesis is that high rates of IMTG oxidation during exercise allows the regular turnover of the intramuscular lipid pool and prevents the accumulation of fatty acid metabolites, such as long-chain acyl-CoA, diacylglycerol and ceramides, which are believed to blunt insulin sensitivity (Moro et al., 2008; Shaw et al., 2010; van Loon and Goodpaster, 2006). Therefore, understanding the mechanisms regulating IMTG lipolysis and IMTG-derived FA oxidation during exercise remain important.

Recent attention has focused on the role of the perilipins (PLINs), a family of lipid droplet (LD) proteins, of which perilipin 2 (PLIN2) and 5 (PLIN5) are expressed in skeletal muscle, but who's precise role is not fully understood. PLIN2 is ubiquitously expressed in the human body (Brasaemle et al., 1997) and its content is ~2-fold higher in type I muscle fibres compared to type II fibres, which mirrors the fibre type distribution of IMTG (Shaw et al., 2009). In non-muscle cells, PLIN2 expression regulates basal lipolytic rates *in vitro* by limiting the interaction of adipose triglyceride lipase (ATGL) with the LD (Bell et al., 2008; Listenberger et al., 2007). However, in intact rat skeletal muscle in response to lipolytic stimuli (electrically induced contractions and adrenaline) there is an increase in colocalisation of hormone sensitive lipase (HSL) with PLIN2 and of HSL with LDs (Prats et al., 2006). Furthermore, we recently reported that PLIN2-containing LDs are depleted in human skeletal muscle during moderate intensity exercise (Chapter 2). PLIN5, on the other hand, is highly expressed in oxidative tissues (Dalen et al., 2007; Wolins et al., 2006a; Yamaguchi et al., 2006). Cell culture studies demonstrate that PLIN5 expression limits basal lipolysis more effectively than PLIN2 (Wang et al.,

2011a). Interestingly, these studies also show that PLIN5 expression (but not PLIN2) recruits ATGL and its coactivator, CGI-58, to the LD surface under basal conditions, and it is believed that PLIN5 phosphorylation releases CGI-58 to bind ATGL to stimulate lipolysis in response to PKA-activation (Wang et al., 2011a). Skeletal muscle PLIN5 expression is higher in athletes (Amati et al., 2011) and increases following training in lean and obese individuals (Peters et al., 2012). Collectively, the results from the limited number of studies so far suggest that both PLIN2 and PLIN5 are important in regulating IMTG lipolytic rates, but that PLIN5 may be the key PLIN regulating IMTG lipolysis and net IMTG breakdown during exercise.

Sprint interval training (SIT) promotes skeletal muscle adaptations and improvements in insulin sensitivity comparable to those induced by ET (Burgomaster et al., 2008; Gibala et al., 2012). In particular, skeletal muscle oxidative capacity is enhanced following SIT (Burgomaster et al., 2007; Burgomaster et al., 2008; Burgomaster et al., 2005; Hood et al., 2011; Little et al., 2011a; Little et al., 2010), and may partly explain the SIT-induced improvements in whole-body insulin sensitivity (Babraj et al., 2009; Hood et al., 2011; Little et al., 2011a; Richards et al., 2010). However, it has yet to be investigated whether such improvements in insulin sensitivity occur alongside increases in net IMTG breakdown during exercise and enhanced skeletal muscle expression of PLIN2 and PLIN5.

Given the importance of studying IMTG metabolism in a fibre type specific manner (van Loon, 2004), in the current study we investigated IMTG metabolism and the content and localization of PLIN2 and PLIN5 following different training interventions using immunofluorescence microscopy. We hypothesized that increased expression of PLIN2 and PLIN5 plays a role in the well-established increase in net IMTG breakdown during exercise following ET. In addition, we investigated the hypothesis that SIT also increases the capacity to breakdown IMTG during moderate intensity exercise, the protein expression of PLIN2 and PLIN5 and insulin sensitivity.

4.3 Methods

Ethical approval

Sixteen healthy, sedentary males volunteered to take part in the study (for characteristics see Table 1), which was approved by the Black Country NHS Research Ethics Committee (West Midlands, UK) and conformed with the Declaration of Helsinki. Written, informed consent was obtained from volunteers following a verbal and written explanation of the nature and risks involved in the experimental procedure. All subjects were healthy (free of any known metabolic or cardiovascular disease) and engaged in less than two 30 min sessions of physical activity per week in the preceding year. Percutaneous muscle biopsies taken in this study before and after each training mode, under basal conditions and after 60 min of moderate-intensity exercise have been used both for the measurements described in this manuscript and for measurements of endothelial eNOS content, eNOS serine^{1177} phosphorylation, NOX2 content and capillarization. The latter are reported in a parallel manuscript (M. Cocks *et al.*, under review). Measures such as insulin sensitivity and $VO_{2\ peak}$ also made in the volunteers are relevant for the interpretation of both studies and are presented in both manuscripts.

Pre-experimental procedures

At least 5 days prior to entering the study subjects performed a progressive exercise test to exhaustion on an electronically braked cycle ergometer (Lode BV, Groningen, The Netherlands) in order to determine peak oxygen uptake ($VO_{2\ peak}$) using an on-line gas collection system (Oxycon Pro, Jaeger, Germany). The test consisted of initially cycling at 95 W, followed by sequential increments of 35 W every 3 minutes until cadence was reduced to < 50rpm, at which point the test was terminated. $VO_{2\ peak}$ was taken as the highest value obtained in the last 30-seconds of the test. During this visit subjects were also allocated to their training intervention. The 16 subjects were divided into pairs, with the best possible match for age, BMI and $VO_{2\ peak}$, with one member from each pair randomly assigned to SIT and the other to the ET group. As none of the volunteers were familiar with SIT exercise, the subjects in the SIT group were familiarised to this exercise mode through the

performance of one 30 s all out effort (Wingate test, (Bar-Or, 1987)) on a cycle ergometer against a load equivalent to 0.075 kg per kg body mass.

Experimental procedures

Experimental procedures performed before (pre) and after (post) training were identical, and were undertaken across two consecutive days. Post-training experimental procedures were performed > 48h after the final exercise training session in order to minimize any acute effects of the last exercise training bout.

Day 1: All subjects reported to the laboratory after an overnight fast (> 10 h), having been instructed to refrain from performing vigorous exercise in the preceding 48 h period. On arrival, a 20G cannula was inserted into the antecubital vein of one arm and a 3-way stop cock attached to allow for multiple blood sampling and flushing of the cannula. After a 20 mL baseline sample was obtained, subjects consumed a beverage containing 75 g glucose (using 82.5g dextrose monohydrate obtained from Roquette, UK) dissolved in water made up to 300 ml. Further 5 mL blood samples were obtained after 30, 60, 90 and 120 minutes, and collected into EDTA-containing vacutainers. Isotonic saline (3 mL) (B Braun, UK) was used to keep the cannula patent every 15 minutes during the 2 h test. Plasma samples for each time point were obtained through centrifugation (10-minutes at 3500 rpm at 4°C) and stored at -80°C for subsequent analysis. Immediately following the oral glucose tolerance test (OGTT), body composition analysis was performed using Dual energy X-ray Absorptiometry (DXA). QDR software (Hologic Inc., MA, US) was used to analyse fat mass and fat-free mass in individual limbs and the trunk region of each subject. Relative fat mass was calculated using absolute fat mass as a proportion of total region mass.

Day 2: On arrival at the laboratory after an overnight fast (> 10 h), the thigh of one leg was prepared for muscle biopsy collection. Subjects were randomised as to which leg they received the biopsy from pre-training; the contralateral leg was sampled post-training. Briefly, local anaesthetic (1% lidocaine, B Braun, UK) was administered under the skin and over the fascia of the vastus lateralis before two incisions were made approximately 2 cm apart. A muscle biopsy (~100mg) was taken using the

Bergström technique (Bergström, 1975) from the distal incision prior to exercise. Skeletal muscle samples were first blotted to remove excess blood, and visible fat and collagen were removed through dissection. A portion of the muscle tissue was then prepared for immunohistochemical analysis by embedding in Tissue-Tek OCT Compound (Sakura Finetek Europe, The Netherlands) and frozen in liquid nitrogen-cooled isopentane. The remaining muscle tissue (~40mg) was snap frozen in liquid nitrogen. Subsequently, subjects underwent 60 min of steady state exercise on an electronically braked cycle ergometer set at a workload equivalent to 65% pre-training $VO_{2\ peak}$. Heart rate and rate of perceived exertion (RPE) were recorded every 5 minutes, while gas collection (5 min collection period) was performed at 15 minute intervals (t = 15, 30, 45, 60 min) using an on-line gas collection system (Oxycon Pro, Jaeger, Germany), in order to determine rates of carbohydrate and fat oxidation. A second muscle biopsy was taken immediately post-exercise from the proximal incision.

For all experimental trials, subjects were provided with all food and drinks to be consumed in the preceding 24 h period. The diet was of a standard macronutrient composition (50% carbohydrate, 35% fat, 15% protein), but adjusted for each individuals habitual energy intake, assessed through the completion of 3 day diet diary. When completing diet diaries, subjects were provided with electronic scales in order to increase the accuracy of portion size determination. All subjects were asked to continue their habitual dietary and physical activity patterns during the training period, again completing a 3 day diet diary post-training in order to assess any differences in energy or macronutrient intake over the training period.

Training interventions

Sprint Interval Training (SIT): The SIT training protocol was similar to the protocol used by Burgomaster et al (Burgomaster et al., 2008). Subjects performed 30 s 'all out' sprints (Wingate test) on a cycle ergometer against a load equivalent to 0.075 kg per kg body mass and with 4.5 minutes recovery between each test. During the recovery period subjects cycled against a small load (30 W) maintaining a cadence of below 50 rpm. All participants trained 3 times a week for 6 weeks, and were excluded from the study if they were absent from more than 2 sessions. Initially participants performed

4 Wingate tests per training session, increasing to 5 tests in weeks 3 and 4, and finishing with 6 tests per session in weeks 5 and 6.

Endurance Training (ET): Subjects in the ET group trained 5 times a week over the 6 week training period, and were excluded from the study if they were absent from more than 3 training sessions. All subjects cycled at a workload equivalent to ~65% $VO_{2\ peak}$ for 40 minutes in the first 2 weeks, increasing to 50 minutes in the following 2 weeks, and 60 minutes in the final 2 weeks. $VO_{2\ peak}$ was reassessed after 3 weeks of training and workload adjusted accordingly.

Muscle sample analysis

Immunofluorescence staining: Serial 5 µm sections were cut at -30°C on to ethanol-cleaned glass slides. Sections were fixed in 3.7% formaldehyde for 1 h, rinsed briefly in deionised water, and permeabilised in 0.5% Triton-X 100 for 5 min, followed by 3 x 5 min washes in PBS. Subsequently, slides underwent incubation with appropriate antibodies for 45 min, after which, a further 3 x 5 min PBS wash preceded incubation of sections with appropriately targeted secondary fluorescent conjugated antibodies for 30 min. IMTG (lipid droplets) were visualised using neutral lipid dye oil red O staining (Koopman et al., 2001). As such, oil red O solution was applied to washed (3 x 5min PBS) sections for 30 min following secondary antibody incubation. Slides were then rinsed briefly in deionised water, followed by a 10 min rinse with slow running tap water, after which coverslips were mounted with a glycerol and mowiol 4-88 solution in 0.2 M Tris buffer (pH 8.5) (including 0.1% DABCO anti-fade medium).

Cell border visualisation was achieved through incubation of sections with a wheat germ agglutinin Alexa Fluor 350 conjugate (Cat. #W11263, Invitrogen, Paisley, UK). Muscle fibre type was determined using a mouse anti-myosin antibody for slow twitch fibres (A4.840-c, DSHB, developed by Dr. Blau) followed by the application of Alexa Fluor goat anti-mouse IgM 488 or 350. Fibres containing positive staining were classified as type I fibres, while those with no staining were classified as type II fibres. The oil red O working solution was freshly produced for each staining procedure and consisted of 100 mg oil red O in 20 ml 60% triethylphosphate (Sigma-Aldrich, UK). Twelve ml of working solution was

added to 8 ml deionised water and filtered twice to remove any oil red O crystals. Mitochondria were visualised using a mouse monoclonal anti-OxPhos Complex IV primary antibody (Cat. #459600, Invitrogen, Paisley, UK), followed by an Alexa Fluor goat anti-mouse IgG_{2a} 594 secondary antibody. PLIN2 visualisation was achieved using a mouse monoclonal anti-adipophilin primary antibody (Cat. #03-610102, American Research Products, MA, USA), followed by an Alexa Fluor goat anti-mouse IgG_1 594 secondary antibody. On occasions when IMTG staining with oil red O was undertaken in parallel with PLIN2, an Alexa Fluor goat-anti mouse IgG_1 488 secondary antibody was used. PLIN5 was visualised using a guinea pig polyclonal anti-OXPAT primary antibody (Cat. #GP31, Progen Biotechnik, Germany) followed by application of an Alexa Fluor goat-anti guinea pig IgG 488 secondary antibody. All Alexa Fluor secondary antibodies were obtained from Invitrogen (Paisley, UK). The specificity of the PLIN5 antibody was confirmed in a competition experiment in which a PLIN5 recombinant peptide (Cat. #NB110-60511PEP, Novus Biologicals, Cambridge, UK) was incubated with the PLIN5 primary antibody, subsequently resulting in removal of the positive fluorescent signal for PLIN5 (Supplementary fig. 1).

Image capture, processing and data analysis: Images of cross-sectional orientated sections were used for the determination of fibre-type differences in the content of IMTG, mitochondria, and PLIN2 and PLIN5. Images were captured using a Nikon E600 microscope with a 40x 0.75 NA objective, coupled to a SPOT RT KE colour 3 shot CCD camera (Diagnostic Instruments Inc., MI, USA). Both DAPI UV (340-380 nm) and FITC (465-495 nm) excitation filters were used to view the Alexa Fluor 350 and 488 fluorophores, respectively. In addition, a Texas red (540-580 nm) excitation filter was used to view sections stained with oil red O or the Alexa Fluor 594 fluorophore. The use of 3 excitation filters and 1 dichroic and 1 emission filter ("Pinkel" Triple Set, Semrock, Kettering, UK) allowed for semi-automated sequential image capture. Digital images to specifically assess the size and number of IMTG and PLIN2 and PLIN5 objects were obtained using an inverted confocal microscope (Leica DMIRE2, Leica Microsystems) using a 63x 1.4 NA oil immersion objective. An argon laser was used to excite the Alexa Fluor 488 fluorophore, while both the Alexa Fluor 594 fluorophore and oil red O was excited using a Helium-Neon laser. The same system was used to capture digital images and to assess IMTG and PLIN2 or PLIN5 colocalisation.

Image processing was undertaken using Image-Pro Plus 5.1 software (Media Cybernetics, MD, USA). Fibre-type distribution of IMTG, PLIN2 and PLIN5 and mitochondria was assessed using widefield images. To this end, between 6 and 10 widefield images were used per muscle section, resulting in 104 ± 5 fibres analysed per muscle cross-section (42 ± 3 type I fibres, 62 ± 3 type II fibres). Mitochondrial fluorescence staining intensity was used to indicate changes in mitochondrial content after training. An intensity threshold was uniformly selected to represent a positive signal for IMTG, and PLIN2 and PLIN5. The content of IMTG and PLIN2 and PLIN5 were expressed as the positively stained area fraction relative to the total area of each muscle fibre. Images captured using confocal microscopy were used to identify changes in mean IMTG, PLIN2 and PLIN5 size and density. Density was calculated as the number of IMTG objects relative to area. The area of individual IMTG (lipid droplets), PLIN2 or PLIN5 objects was used as a measure of size.

Colocalisation analysis was performed separately for PLIN2 or PLIN5 with IMTG. This analysis was restricted to type I fibres as a previous publication from our laboratory has shown that changes in colocalisation with IMTG do not occur in type II fibres in response to the exercise bout used in the present study (Shepherd et al., 2012). In this respect, sequential images of either PLIN2 or PLIN5 and IMTG were captured using confocal microscopy (as described) with 8x digital zoom. Positive signal for either PLIN protein and IMTG was obtained through the selection of a uniform intensity threshold. Binary images were created based on the selected threshold and subsequently used for colocalisation analysis. A colocalisation map was generated, displaying the merged images, with the overlapping area subsequently extracted to create a separate binary image. The total number of objects in this image was expressed relative to the total number of PLIN2 (or PLIN5) as a measure of colocalisation (Fig. 4). The extracted objects were also used as a measure of the number of PLIN associated-LDs (PLIN-LDs), and the number of extracted objects were subsequently subtracted from the total number of LDs to quantify the number of LDs not associated with PLIN (PLIN-null-LDs). Several controls were performed to check for bleed through, non specific secondary antibody binding and autofluorescence before colocalisation analysis was performed. No positive staining was observed in the opposite channel when single staining (IMTG, PLIN2 and PLIN5) was performed and omission of the primary or

secondary antibody, and oil red O abolished all positive signal in the relevant channel discounting the influence of tissue autofluorescence or non-specific secondary antibody binding. In addition, we performed the same colocalisation analysis on a series of non-corresponding PLIN2 (or PLIN5) and IMTG images, in order to determine whether the level of random colocalisation was statistically different from that obtained for the true corresponding images, as previously described (Lachmanovich et al., 2003).

Western blot analysis: Snap frozen muscle tissue was powdered in liquid nitrogen and transferred to eppendorf tubes containing lysis buffer (1x RIPA buffer, Cell Signalling, including 1 complete mini protease inhibitor tablet, Roche Diagnostics, Germany). Samples were mixed on ice for 2 hours and then homogenized on ice for ~30 s at slow speed (Polytron), followed by centrifugation for 20 min at 4°C and 10,000 G. The supernatant was removed and a small portion used to determine protein concentration (Pierce BCA assay kit). Samples were subsequently diluted to a final concentration of 3 $\mu g.\mu l^{-1}$, using homogenization buffer and Laemmli sample buffer (Sigma) and boiled for 5 min. Equal quantities of protein (45 µg) were separated by electrophoresis on 12% SDS-polyacrylamide gels and transferred to nitrocellulose membranes at 25 V for 1.5 hours. Ponceau S staining was performed to assess transfer efficacy and subsequently imaged using a ChemiDoc XRS+ system (Bio-Rad, UK). This image was used to quantify the total protein stain of each lane, and this was used as a measure of equal loading when the inter-lane variation was less than 10%. Membranes were subsequently destained (0.1M NaOH) and blocked for 1 h in 5% non-fat dried milk (NFDM) in phosphate-buffered saline with 0.1% Tween (PBST) followed by incubation overnight at room temperature with either the anti-adipophilin (Cat. #03-610102, American Research Products, MA, USA) or anti-OXPAT (Cat. #GP31, Progen Biotechnik, Germany) primary antibody diluted in 3% NFDM in PBST. The membranes were then washed 3 times in PBST, blocked in 5% NFDM in PBST for 1 h, followed by incubation for 1 h at room temperature with appropriate horseradish peroxidase-conjugated IgG secondary antibodies diluted in 3% NFDM in PBST. Antibody binding was detected using enhanced chemiluminescence HRP detection reagent (GE Healthcare, UK) and imaging and band quantification was performed using a ChemiDoc XRS+ system (Bio-Rad, UK).

Blood Sample Analysis

Plasma glucose concentrations were determined spectrophotometrically using an ILab-600 semi-automatic analyzer in combination with the glucose oxidase kit (Instrumentation Laboratory Ltd UK, Warrington, UK). Insulin concentrations during the OGTT were determined using a commercially available direct insulin ELISA kit (Invitrogen, Paisley, UK). Insulin sensitivity index (Matsuda and DeFronzo, 1999) was calculated based on plasma glucose and insulin values.

Calculations and statistical analysis

Rates of whole body fat and carbohydrate oxidation ($g.min^{-1}$) were calculated from the VO_2 and VCO_2 values collected at 15, 30, 45 and 60 minutes during the steady state cycling exercise. The calculations were made assuming protein oxidation was negligible, and according to previously published equations (Jeukendrup and Wallis, 2005):

Carbohydrate oxidation ($g.min^{-1}$) = $4.210 \cdot VCO_2 - 2.962 \cdot VO_2$

Fat Oxidation = $1.695 \cdot VO_2 - 1.701 \cdot VCO_2$

Insulin sensitivity was calculated using the Matsuda index (Matsuda and DeFronzo, 1999), using the following equation:

$$\text{Matsuda (ISI)} = \frac{10{,}000}{\sqrt{(FPG \times FPI) \times (P\bar{G} \times P\bar{I})}}$$

where FPG is fasting plasma glucose concentration, FPI is fasting plasma insulin concentration, $P\bar{G}$ is mean plasma glucose concentration (over 2 h OGTT), and $P\bar{I}$ is mean plasma glucose concentration.

All data are expressed as means ± S.E.M. Significance was set at the 0.05 level of confidence. Changes in body composition, exercise capacity, insulin sensitivity and substrate utilization were assessed using a 2-factor repeated measures ANOVA, with the between-subject factor '*group*' (SIT vs. ET) and the within-subject factor '*training*' (pre- vs. post-training). Changes in mitochondrial density and PLIN2 and 5 content were assessed using a mixed 3-factor repeated measures ANOVA using the within-subject factors '*fibre*' (type I versus type II fibres) and '*training*' and between-subject (*group*)

factor used in the 2-factor analysis. To inspect differences in IMTG, we hypothesised *a priori* that IMTG content would be significantly different between type I and type II fibres. Thus, we used a 3-factor repeated measures ANOVA with the between-subject factor as '*group*', and the within-subject factor's '*training*' and '*time*' (pre- versus post-exercise). The same three-factor repeated measures ANOVA and between-subject and within-subject factors were used when assessing differences in colocalisation, following an *a priori* hypothesis that colocalisation would be significantly different between actual and randomised images, based on a previous study (Lachmanovich et al., 2003). Significant main effects or interactions were assessed using Bonferroni adjustment post hoc analysis. Improvements in training performance (SIT group; e.g. mean power) were examined using a paired t test analysis. A Pearson's bivariate correlation analysis was used to investigate specific relationships between variables.

4.4 Results

Exercise capacity, body composition, substrate utilization and insulin sensitivity

At baseline, no differences between groups were observed for any of the variables mentioned in Table 4.1. In response to training VO_{2peak} was significantly improved irrespective of training type (SIT 7 ± 2%, ET 15 ± 3%, main effect for condition, $P < 0.001$, Table 4.1). The difference between the modes of exercise did not reach statistical significance ($P = 0.064$). W_{max} was also increased after training, with no difference between groups (main effect for condition, $P < 0.001$). Fat free mass increased significantly post-training, with no difference between groups (main training effect, $P = 0.039$). The observed decrease in relative fat mass (Table 4.1) did not reach statistical significance, with no difference between groups (main training effect, $P = 0.075$). Glucose area under the curve (AUC) was reduced post-training (SIT 17 ± 2%, ET 12 ± 2%; main training effect, $P < 0.001$, Table 4.1), with no difference between groups. Similarly, training reduced insulin AUC (SIT 33 ± 3%, ET 18 ± 5%; main training effect, $P < 0.001$, Table 4.1), with no difference between groups. In accordance, training improved insulin sensitivity, calculated using the Matsuda index (Matsuda and DeFronzo, 1999) (SIT 56 ± 15%, ET 29 ± 12%; main training effect, $P < 0.01$, Table 4.1), with no difference between groups. Carbohydrate (absolute and relative) and fat (relative only) oxidation, as well as RER, were all significantly altered compared to pre-training following ET only ($P < 0.05$, Table 4.2), with no effect apparent following SIT. RER tended to be lower in the SIT group (P = 0.055) before training. When considering the effectiveness of SIT, the first and final training sessions were examined. Specifically, mean power output increased during training by 14 ± 3% ($P = 0.001$) and peak power output increased by 21 ± 6% ($P = 0.01$).

Table 4.1. Subject characteristics

	SIT		ET	
	Pre	*Post*	*Pre*	*Post*
Age (yrs)	22 ± 1		21 ± 1	
Height (m)	1.74 ± 0.02		1.77 ± 0.03	
Body mass (kg)	75.1 ± 3.0	75.2 ± 3.1	70.8 ± 4.4	70.8 ± 4.5
BMI ($kg.m^{-2}$)	24.8 ± 0.8	24.8 ± 0.9	22.6 ± 1.6	22.6 ± 1.2
$VO_{2\ peak}$ ($l.min^{-1}$) *	3.13 ± 0.15	3.37 ± 0.18	2.93 ± 0.30	3.40 ± 0.38
$VO_{2\ peak}$ ($l.min^{-1}.kg^{-1}$) *	41.9 ± 1.8	45.1 ± 2.3	41.7 ± 4.1	48.2 ± 5.0
W_{max} (W) *	221 ± 11	241 ± 14	218 ± 11	253 ± 16
FFM (kg) *	52.5 ± 1.9	53.6 ± 1.8	50.7 ± 1.5	51.1 ± 2.7
FM (kg)	14.2 ± 1.6	13.6 ± 1.5	12.3 ± 1.5	12.2 ± 1.9
Insulin AUC ($\mu IU.ml^{-1}.min^{-1}$) *	8847 ± 1140	5792 ± 688	8434 ± 880	6813 ± 711
Glucose AUC ($mg.dl^{-1}.min^{-1}$) *	17634 ± 710	14551 ± 398	16835 ± 992	14783 ± 950
ISI-Matsuda *	3.9 ± 0.3	5.8 ± 0.4	3.7 ± 0.5	4.7 ± 0.7

Data provided are means ± S.E. (n = 8 per group). *BMI* body mass index, W_{max} maximum workload, *OGTT* oral glucose tolerance test, *FFM* fat free mass, *FM* fat mass, *AUC* area under the curve, *ISI* insulin sensitivity index. * Indicates main effect for training ($P < 0.05$), such that Pre ≠ Post. No interaction effects (training x group) were observed for any of the variables.

Table 4.2. Substrate utilization during 60 min cycling at ~65% pre-training $VO_{2\ peak}$

	SIT		ET	
	Pre	*Post*	*Pre*	*Post*
Heart rate ($b.min^{-1}$) *	164 ± 3	147 ± 3	165 ± 3	138 ± 4
VO_2 ($l.min^{-1}$) *	2.22 ± 0.11	2.12 ± 0.12	2.19 ± 0.14	2.10 ± 0.13
VCO_2 ($l.min^{-1}$) *	1.94 ± 0.09	2.01 ± 0.13	1.97 ± 0.11	1.81 ± 0.10
CHO oxidation				
($g.min^{-1}$)	1.61 ± 0.07	1.56 ± 0.13	1.80 ± 0.12	1.37 ± 0.07 †
(% of total oxidation)	62.3 ± 2.0	63.2 ± 4.4	70.9 ± 4.3	56.6 ± 3.1 †
Fat oxidation				
($g.min^{-1}$)	0.46 ± 0.04	0.42 ± 0.06	0.36 ± 0.07	0.49 ± 0.05
(% of total oxidation)	39.5 ± 1.9	38.6 ± 4.3	31.0 ± 4.3	45.1 ± 3.1 †
RER	0.88 ± 0.01	0.88 ± 0.01	0.90 ± 0.01	0.86 ± 0.01 †

Values obtained during 60 min of moderate-intensity cycling at ~65% pre-training $VO_{2\ peak}$. Data provided are means ± S.E. (n = 8 per group). *RER* respiratory exchange ratio, *CHO* carbohydrate. * Main effect for training ($P < 0.05$) such that Pre ≠ Post (no significant differences between training interventions). † Interaction effect (training x group; $P < 0.05$), such that the effect of ET was significantly different from the effect of SIT.

Analysis of mitochondrial content

Immunofluorescence images demonstrated that COX protein expression (fluorescence intensity), representing mitochondrial density, was greater in type I fibres than type II fibres pre and post-training (main effect of fibre; $P < 0.001$, Table 4.3). No group differences were apparent at baseline. A main effect of training was observed, with increases in mitochondrial density in both type I fibres (SIT 39 ± 4%, ET 46 ± 16%) and type II fibres (SIT 39 ± 3%, ET 50 ± 14%; $P < 0.05$, Table 4.3). No difference between groups was apparent following training.

Table 4.3. Effect of 6 weeks of SIT or ET on fibre type specific mitochondrial content

COX expression	SIT		ET	
(fluorescence intensity)	*Pre*	*Post*	*Pre*	*Post*
Type I fibres	54.1 ± 4.6	74.4 ± 4.0†	60.1 ± 7.7	81.5 ± 5.9†
Type II fibres*	41.1 ± 3.0	56.8 ± 3.5†	42.4 ± 4.8	60.4 ± 5.1†

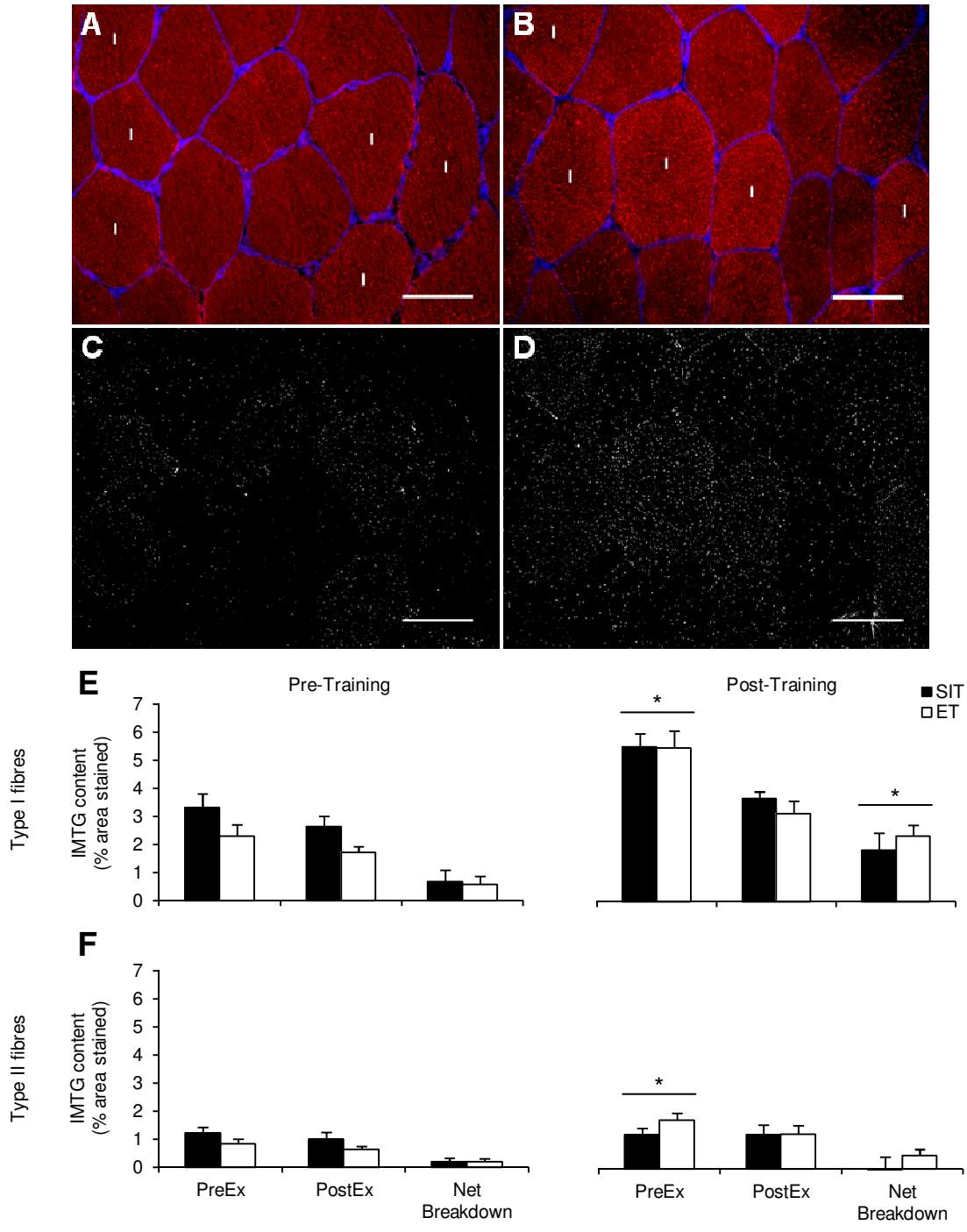
Mitochondrial density, quantified from immunofluorescence images of COX in type I and type II fibres obtained before and after 6 weeks of SIT or ET. Values are presented as means ± S.E. (n = 8 per group). * Main fibre effect ($P < 0.05$ vs. type I fibres). † Main training effect ($P < 0.05$ vs. pre-training).

Intramuscular triglyceride analysis

Our *a priori* hypothesis that IMTG content is greater in type I fibres compared to type II fibres meant that we examined IMTG fibre-type specific training effects separately. There were no differences in resting IMTG content between groups at baseline. A significant main effect of training was observed with increased resting IMTG content in type I fibres after both interventions (expressed as % fibre stained) (SIT 1.9-fold, ET 2.9-fold, main training effect ; $P < 0.05$, Fig. 4.1E). This finding was attributed to a significant rise in IMTG density, expressed as the number of LDs per area, after SIT (pre-training, 0.065 ± 0.009; post-training, 0.087 ± 0.010 μm^2) and ET (pre-training, 0.064 ± 0.009; post-training, 0.109 ± 0.012 μm^2). A main effect of training was observed for IMTG content in type II fibres after both interventions (SIT 1.4-fold, ET 2.5-fold, main training effect, $P < 0.05$, Fig. 4.1F). This increase in IMTG content was again attributable to an increase in IMTG density. No changes in IMTG size were observed in response to training. There were no interaction effects between training and group in any of the measures of resting IMTG content.

Before training, absolute IMTG content in type I fibres decreased significantly in response to 60 min steady state cycling, with no difference between groups (SIT 17 ± 9%, ET 15 ± 12%, main effect of time; $P < 0.05$, Fig. 4.1E). Following training, net IMTG breakdown during exercise in type I fibres was also significantly increased (SIT 27 ± 13%, ET 43 ± 5%; main effect of time; $P < 0.05$, Fig. 4.1E). In comparison to pre-training, net IMTG breakdown in type I fibres was significantly greater following training (training x time interaction; $P < 0.05$) with no difference in net IMTG breakdown between groups. Both pre- and post-training, the reduction in IMTG content in type I fibres was attributed to decreases in IMTG density after SIT (pre-training, 21 ± 13%; post-training, 38 ± 7%) and ET (pre-training, 20 ± 17%; post-training, 32 ± 8%). No significant breakdown of IMTG occurred during the exercise bout in type II fibres either before or after training (Fig. 4.1F).

Fig. 4.1. Effect of 6 weeks of SIT and ET on fibre type specific IMTG content and net IMTG breakdown during exercise.



Representative immunofluorescence images of IMTG (stained red) in combination with WGA to identify the cell border (stained blue) in skeletal muscle, pre *(A)* and post *(B)* 6 weeks of SIT. Type I fibres are indicated with a "I", all other fibres are assumed type II fibres. White bar = 50µm. Images

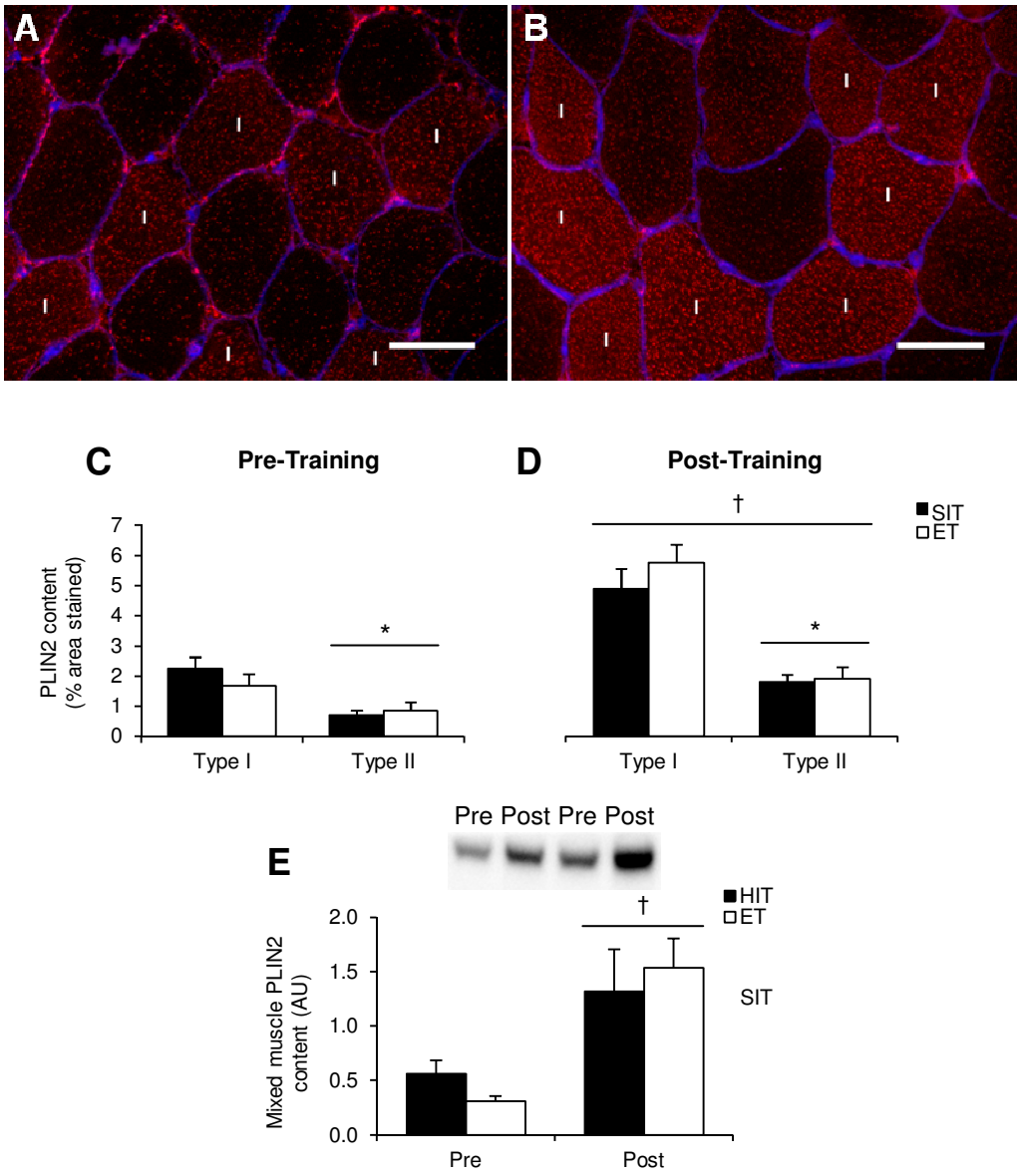
(C) and *(D)* display the ORO signal obtained after the intensity threshold was applied during quantitation (showing the LDs in white), before and after SIT, respectively. IMTG content, quantified from immunofluorescence images of oil red O-stained muscle sections, before *(Pre-Training)* and after *(Post-Training)* 6 weeks of SIT (closed bars) or ET (open bars). IMTG content was measured before *(PreEx)* and after *(PostEx)* 60 min steady state cycling, and net IMTG breakdown calculated *(Breakdown)*, in type I *(E)* and type II fibres *(F)*. Values are presented as means ± S.E. (n = 8 per group). * Main effect of training intervention ($P < 0.05$ vs. pre-training).

PLIN2 and PLIN5 analysis

Immunofluorescence images show that PLIN2 content (expressed as % fibre stained) in type I fibres was significantly greater than in type II fibres both before and following training, irrespective of intervention ($P < 0.001$, Fig. 4.2C & D). A main effect of training was observed, with an increase in PLIN2 content in both type I (SIT 2.1-fold, ET 3.4-fold, Fig. 4.2C & D) and type II fibres (SIT 2.5-fold, ET 2.2-fold, main training effect, $P < 0.001$). This finding was attributed to an increase in PLIN2 density in both type I (SIT: pre 0.057 ± 0.008, post 0.092 ± 0.010 μm^2, ET: pre 0.051 ± 0.012, post 0.100 ± 0.009 μm^2) and type II fibres (SIT: pre 0.020 ± 0.004, post 0.044 ± 0.009 μm^2, ET: pre 0.012 ± 0.002, post 0.031 ± 0.006 μm^2) (main training effect, $P < 0.001$). No significant condition and group interactions were observed for either PLIN2 content or density. PLIN5 content (expressed as % of fibre stained) was significantly greater in type I fibres than type II fibres at all time points, irrespective of intervention ($P < 0.001$, Fig. 4.3C & D). A main effect of training was observed for PLIN5 content, with significant increases occurring in both fibre types after training (type I fibres: SIT 2.2-fold, ET 2.8-fold, type II fibres: SIT 2.2-fold, ET 3.4-fold, main training effect; $P < 0.001$, Fig. 4.3C & D). This finding was attributed to an increase in PLIN5 density in both type I (SIT: pre 0.054 ± 0.005, post 0.079 ± 0.007 μm^2, ET: pre 0.039 ± 0.003, post 0.107 ± 0.011 μm^2) and type II fibres (SIT: pre 0.009 ± 0.001, post 0.018 ± 0.004 μm^2, ET: pre 0.005 ± 0.001, post 0.025 ± 0.005 μm^2) (main training effect, $P < 0.001$). Again, no significant condition and group interaction effects were observed.

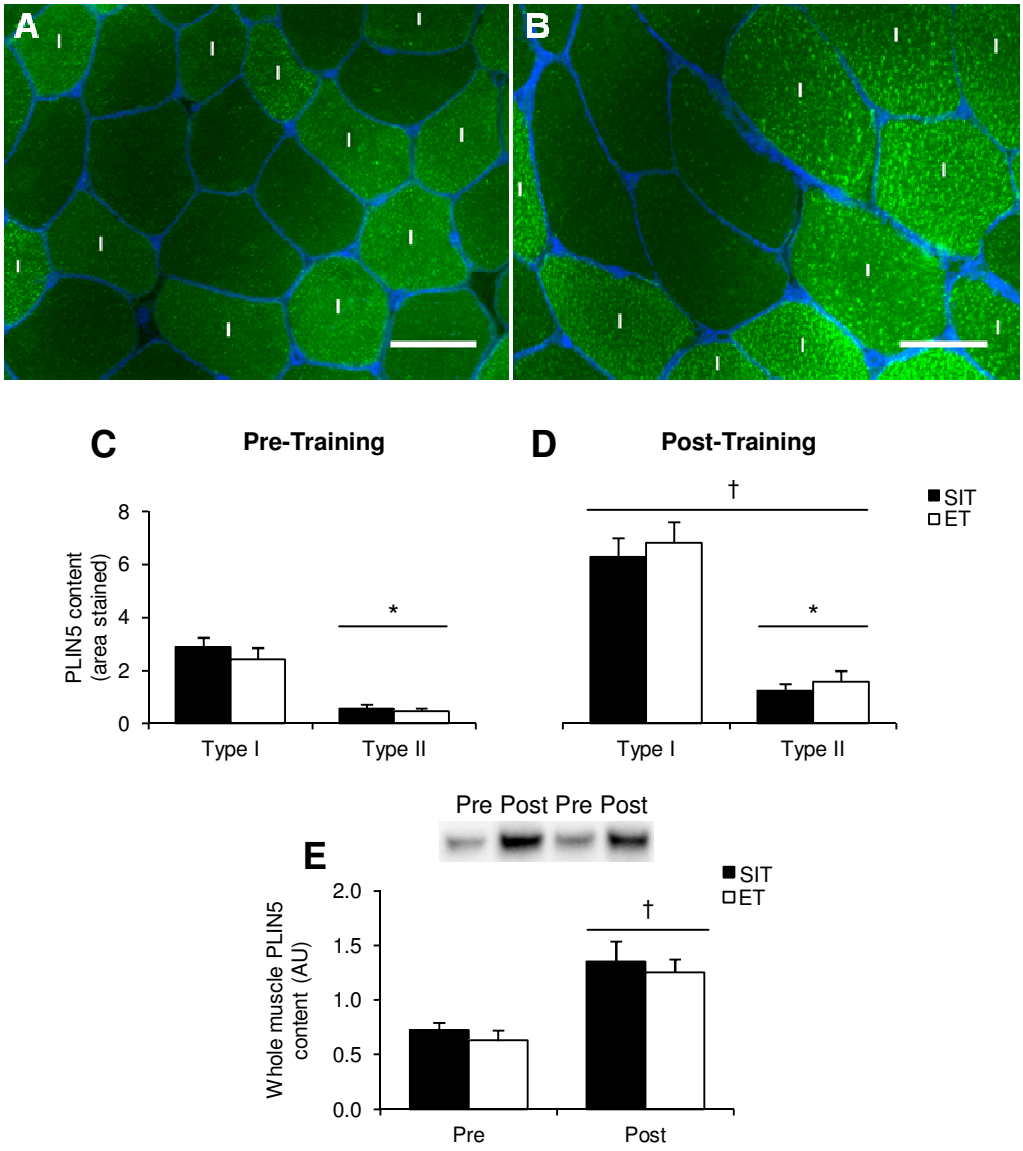
We performed immunoblotting of whole muscle homogenates in order to confirm the finding of an increase in both PLIN2 and PLIN5 content in response to training using immunohistochemical methodology. Immunoblot analysis revealed a main effect of training for PLIN2 content (SIT 3.9-fold, ET 5.8-fold; $P = 0.02$, Fig 4.2E), with no difference between groups. PLIN5 protein expression was also increased following training (SIT 2.0-fold, ET 2.4-fold, main training effect; $P = 0.01$, Fig 4.3E), again with no difference between groups.

Fig. 4.2. Effect of 6 weeks of SIT and ET on fibre type specific PLIN2 expression.



Representative immunofluorescence images of PLIN2 (stained red) in combination with WGA to identify the cell border (stained blue) in skeletal muscle, pre *(A)* and post *(B)* 6 weeks of SIT. Type I fibres are indicated with a "I", all other fibres are assumed type II fibres. White bar = 50µm. PLIN2 expression, quantified from immunofluorescence images of PLIN2, in type I and type II fibres obtained before *(C)* and after *(D)* 6 weeks of SIT (closed bars) or ET (open bars). PLIN2 expression quantified from immunofluorescence images correlated with PLIN2 expression determined following immunoblotting of whole muscle homogenates *(E)*. Values are presented as means ± S.E. (n = 8 per group). * Main effect of fibre type ($P < 0.05$ vs. type I fibres). † Main effect of training intervention ($P < 0.05$ vs. pre-training).

Fig. 4.3. Effect of 6 weeks of SIT and ET on fibre type specific PLIN5 expression.



Representative immunofluorescence images of PLIN5 (stained green) in combination with WGA to identify the cell border (stained blue) in skeletal muscle, pre *(A)* and post *(B)* 6 weeks of SIT. Type I fibres are indicated with a "I", all other fibres are assumed type II fibres. White bar = 50µm. PLIN5 expression, quantified from immunofluorescence images of PLIN5, in type I and type II fibres obtained before *(C)* and after *(D)* 6 weeks of SIT (closed bars) or ET (open bars). PLIN5 expression quantified from immunofluorescence images correlated with PLIN5 expression determined following immunoblotting of whole muscle homogenates *(E)*. Values are presented as means ± S.E. (n = 8 per group). * Main effect of fibre type ($P < 0.05$ vs. type I fibres). † Main effect of training intervention ($P < 0.05$ vs. pre-training). ‡ Significant correlation ($P < 0.05$).

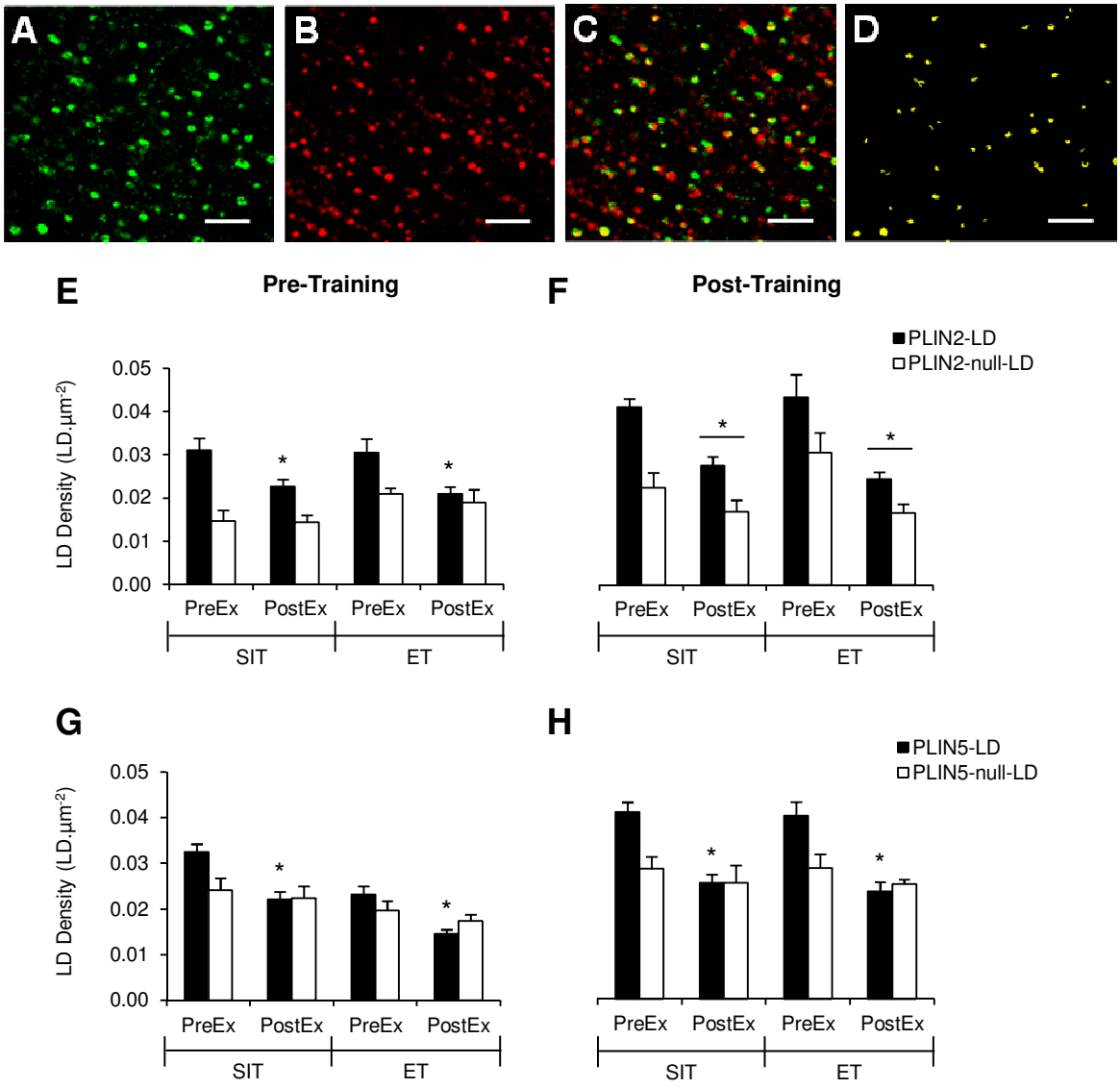
Colocalisation of PLIN2 and PLIN 5 with IMTG

Before training, the fraction of PLIN2 colocalised with IMTG in resting muscle was similar between groups (SIT 0.58 ± 0.04, ET 0.61 ± 0.04; $P = 0.96$). This relationship was unchanged in response to both training interventions. Pre-training, 60 min steady state exercise induced a significant reduction in the fraction of PLIN2 colocalised with PLIN2 (SIT 30 ± 6%, ET 23 ± 4%; main training effect, $P < 0.01$). This reduction was the result of a decrease in IMTG content and an unchanged PLIN2 content. Post-training, a significantly larger reduction in the fraction of PLIN2 associated with IMTG was observed in response to the exercise bout (SIT 37 ± 7%, ET 40 ± 4%; $P = 0.01$) with no difference between groups (data not shown). As a change in PLIN2 association with IMTG was observed following steady state exercise both before and after training, we investigated the number of LDs either associated (PLIN2-LD) or not associated (PLIN2-null-LD) with PLIN2 at each time point. Both before and after training, the number of PLIN2-LD was greater than the number of PLIN2-null-LD before exercise. Pre-training, 60 min steady state exercise induced a reduction in the number of PLIN2-LD (SIT 22 ± 10%, ET 28 ± 6%; $P = 0.01$, Fig. 4.4E & F), whereas the number of PLIN2-null-LD was unchanged. In contrast, following training, exercise induced a significant reduction in both the PLIN2-LD (SIT 32 ± 6%, ET 39 ± 6%; $P < 0.001$) and the PLIN2-null-LD (SIT 24 ± 12%, ET 35 ± 12%; $P = 0.01$, Fig. 4.4E & F). No group interactions were detected with either condition or time for variables of PLIN2 and IMTG association, indicating that observations were statistically similar between groups.

Prior to training, the fraction of PLIN5 colocalised with IMTG was similar between groups (SIT 0.58 ± 0.04, ET 0.50 ± 0.04; $P = 0.20$), and following both training interventions this relationship was unchanged. Pre-training, 60 min steady state exercise significantly reduced the fraction of PLIN5 associated with IMTG (SIT 36 ± 4%, ET 33 ± 2%; main effect of time, $P < 0.01$), which was the result of a reduction in IMTG with no change in PLIN5 content. Following training, the exercise-induced reduction in the fraction of PLIN5 colocalised with IMTG tended to be larger compared to pre-training, which bordered on statistical significance (SIT 40 ± 3%, ET 41 ± 3%; $P = 0.06$), and with no difference between groups (data not shown). As above, we subsequently investigated the absolute number of LDs either associated (PLIN5-LD) or not associated (PLIN5-null-LD) with PLIN5 at each time point. Pre-training, the number of PLIN5-LD was larger in the SIT than the ET group ($P < 0.05$), but no

difference in the number of PLIN5-null-LD was apparent. Despite this discrepancy, a similar reduction in PLIN5-LD was observed in response to exercise (SIT 32 ± 4%, ET 35 ± 4%; $P < 0.01$, Fig. 4.4G & H), with no change in the number of PLIN5-null-LD, and no difference between groups for either variable. Following training, exercise induced a reduction in the number of PLIN5-LD only (SIT 38 ± 3%, ET 41 ± 5%; $P < 0.01$, Fig. 4.4G & H), with no difference between groups.

Fig. 4.4. Analysis of the colocalisation of PLIN2 and PLIN5 with LDs pre and post 60 min steady state cycling, before and after 6 weeks of SIT or ET.



Representative immunofluorescence images of PLIN5 *(A)*, IMTG *(B)*, and the merged images *(C)* with the yellow areas describing regions of overlap between PLIN5 and IMTG images. The yellow objects were then extracted *(D)* and the number expressed relative to the total number of PLIN5 objects as a measure of colocalisation. The same procedure was used to obtain values of colocalisation between PLIN2 and IMTG. WSITe bar = 5µm. The number of PLIN2-LD (closed bars) and PLIN2-null-LD (open bars) were quantified before (PreEx) and after exercise (PostEx), prior to *(E)* and following *(F)* training in both the SIT and ET groups. The same analysis was repeated for PLIN5 for pre *(G)* and post training *(H)*. * Main effect of exercise bout ($P < 0.05$ vs. pre-exercise).

Correlation analyses

Due to the lack of group x training interactions in all variables, significant training effects were detected independent of exercise modality. Therefore, the data from the SIT and ET interventions (type I and type II fibres, pre and post training) were pooled for correlation analysis (Table 4.4). Both PLIN2 (R = 0.68; $P < 0.001$) and PLIN5 content (R = 0.72; $P < 0.001$) showed strong positive correlations with IMTG content. A very strong positive correlation was observed between pre-exercise IMTG concentrations and the decrease in IMTG content in response to 60 min steady state exercise (R = 0.81, $P < 0.001$). In addition, PLIN2 (R = 0.57, $P = 0.001$) and PLIN5 content (R = 0.63, $P < 0.001$) were strongly associated with net IMTG breakdown. Insulin sensitivity were modestly associated with PLIN2 (R = 0.40; $P = 0.02$) and PLIN5 content (R = 0.42; $P = 0.02$), as well as IMTG content (R = 0.53; $P = 0.01$).

Table 4.4. Bivariate correlation analysis

	IMTG	Net IMTG breakdown	Insulin sensitivity
PLIN2	0.68‡	0.57‡	0.40*
PLIN5	0.72‡	0.63‡	0.42*
IMTG		0.81‡	0.53†

IMTG, PLIN2 and PLIN5 represent resting content. Net IMTG breakdown refers to absolute change in IMTG concentration from pre to post-exercise. Values represent Pearson's squared correlation coefficients (R values) for relationships between variables. Due to the lack of group x training interactions in all variables, it was assumed that the exercise training was driving the changes independent of exercise modality. Therefore, the data from both intervention groups was pooled for correlation analysis. Significant correlations: $^{*}P < 0.05$, $^{†}P < 0.01$, $^{‡}P < 0.001$.

4.5 Discussion

This is the first study to report that greater protein expression of PLIN2 and PLIN5 is associated with larger IMTG stores and enhanced net IMTG breakdown during exercise in type I muscle fibres following a period of traditional ET. This observation is consistent with our first hypothesis. We also provide novel evidence that PLIN5-LDs are utilized during an acute bout of exercise irrespective of training status, whereas after a period of training both PLIN2-LDs and PLIN2-null-LDs are utilized during an acute bout of exercise. In support of our second hypothesis, we demonstrate for the first time that like traditional ET, SIT also enhances IMTG content and net breakdown during 60 min of moderate intensity exercise and leads to increases in the protein expression of PLIN2 and PLIN5. This common metabolic response following both SIT and ET is also evident in the utilization of PLIN5-LDs, PLIN2-LDs and PLIN2-null-LDs. Taken together, these adaptations may contribute to the mechanisms underlying the observed improvements in insulin sensitivity following SIT and ET.

We report that ET leads to increases in IMTG content in both type I and type II fibres, and is accompanied by increased mitochondrial density, corroborating the findings of previous studies (Dubé et al., 2011; Pruchnic et al., 2004; Schrauwen-Hinderling et al., 2003; Tarnopolsky et al., 2007). In addition before training, 60 min moderate-intensity cycling induced a small but significant decrease in IMTG in type I fibres. Importantly, we report that ET leads to increased rates of whole body fat oxidation during exercise at 65% pre-training VO_{2peak} and that this is accompanied by large increases in net IMTG breakdown primarily in type I fibres. Previous studies combining IMTG content and A-V balance measurements using [U-^{13}C]-palmitate demonstrate that oxidation of plasma fatty acids does not completely account for fat oxidation during exercise, indicating that other fat sources contribute to total fat oxidation (Sacchetti et al., 2002; van Hall et al., 2002; van Loon et al., 2003). Therefore, it is reasonable to assume that fatty acids liberated through the net reduction in IMTG content contribute to total fat oxidation during exercise in lean but sedentary individuals and the IMTG contribution to total fat oxidation is increased following ET. The latter observation is consistent with a number of recent training studies also examining net IMTG breakdown in a fibre type specific manner (Van Proeyen et al., 2011a; Van Proeyen et al., 2011b).

Our data demonstrate that net IMTG breakdown during exercise is positively correlated with resting IMTG concentration. This corroborates the findings of several other reports in trained individuals (Coggan et al., 2000; Stellingwerff et al., 2007; van Loon et al., 2003) and indicates that the ET-induced increase in IMTG content contributes to the increase in net IMTG breakdown during endurance type exercise. However, it is worth noting that this is only one of a number of adaptations which likely explain the increase in IMTG breakdown during exercise following ET (Shaw et al., 2010; van Loon, 2004). The increase in IMTG content was due to a greater number of LDs rather than an increased volume of pre-existing LDs. This conclusion is in agreement with a previous report using transmission electron microscopy (Tarnopolsky et al., 2007). As smaller LDs maintain a higher surface area to volume ratio, they also provide a greater surface area for the interaction of lipolytic enzymes with the IMTG substrate and regulatory proteins on the LD surface. As a result, the mobilization of FA for oxidation during exercise would be enhanced.

Using immunofluorescence microscopy we previously reported that trained athletes display a ~two-fold greater PLIN2 concentration in type I compared to type II muscle fibres (Shaw et al., 2009). The present data extends this finding to sedentary individuals, and further reveals a ~four-fold difference in PLIN5 protein expression when comparing type I and type II fibres. In addition, we report that ET enhances the intramuscular content of both PLIN2 and PLIN5, assessed through immunoblotting of whole muscle homogenates. The findings are partly in agreement with a recent study showing increased PLIN5, but not PLIN2, protein abundance in the muscle of lean and obese males following 12 weeks of endurance training (Peters et al., 2012) and with a cross-sectional study that reported a ~seven-fold higher PLIN5 content in older athletes compared to age-matched sedentary controls (Amati et al., 2011). However, our study is the first to examine changes in the intramuscular expression of the PLIN proteins in response to training interventions in a fibre type specific manner using immunofluorescence microscopy. Accordingly, we demonstrate that, like the changes in IMTG, the increase in PLIN2 and PLIN5 protein expression occurs in both type I and type II muscle fibres.

In agreement with data obtained in rodent (Minnaard et al., 2009) and human skeletal muscle (Amati et al., 2011; Peters et al., 2012), both PLIN2 and PLIN5 expression positively correlated with IMTG

content suggesting that the expression of these PLIN proteins is proportional to IMTG content in lean, healthy individuals. Future research will be required to clarify if the increase in PLIN expression is the primary event driving the increase in IMTG content (which is the mechanism that we propose) or the consequence of the increase in IMTG content. Furthermore, the fraction of IMTG associated with either PLIN2 or PLIN5 at rest was similar pre and post training in type I fibres (~0.64 and ~0.59, respectively). These values are consistent with previous reports in rat (Macpherson et al., 2012; Prats et al., 2006) and human skeletal muscle (Shaw et al., 2009; Shepherd et al., 2012)(Chapter 2). Although the physiological significance of this partial colocalisation is currently unknown, this finding is in agreement with a number of observations demonstrating that the protein composition of LDs may vary (Ducharme and Bickel, 2008; Fujimoto et al., 2008; Wolins et al., 2005; Wolins et al., 2006b). To further investigate this relationship we assessed the colocalisation of these PLINs with LDs before and after 60 min moderate intensity endurance exercise. For each PLIN we describe two pools of LDs; 1) PLIN2-associated LDs (PLIN2-LD) and LDs that do not contain PLIN2 (PLIN2-null-LD) and 2) PLIN5-associated LDs (PLIN5-LD) and LDs that do not contain PLIN5 (PLIN5-null-LD). In the untrained state, 60 min moderate intensity endurance exercise induced a significant decrease in the number of PLIN2-LD and PLIN5-LD, with no change in the number of PLIN2-null-LD and PLIN5-null-LD. These observations confirm a recent report from our laboratory showing a preferential utilization of PLIN2-LD, and not PLIN2-null-LD, in response to moderate intensity endurance exercise in sedentary individuals (Chapter 2), and provides novel evidence to suggest that a similar relationship exists for PLIN5. After 6 weeks ET, a preferential decrease in the number of PLIN2-LD and PLIN5-LD also occurred in response to 60 min moderate intensity endurance exercise. Interestingly, in the trained state exercise also induced a decrease in the content of PLIN2-null-LD after 60 min moderate intensity endurance exercise, whereas the content of PLIN5-null-LD did not change. This is the first observation to suggest that PLIN5 is more important than PLIN2 in mediating the hydrolysis of intramuscular lipid stores during exercise and is in line with emerging evidence concerning the precise role of PLIN5 in lipolysis in cultured cells. Indeed, PLIN5 is the only PLIN thus far that has been shown to bind ATGL, and its coactivator, CGI-58. In this respect, overexpression of PLIN5 in cultured cells recruits ATGL and CGI-58 under basal conditions, while expression of PLIN2 does not have this effect (Wang et al., 2011a). Furthermore, PLIN5 is phosphorylated in response to protein kinase A (PKA)-activation, and the

expression and PKA-induced activation of PLIN5 stimulates triacylglycerol hydrolysis in Chinese hamster ovary cells, whereas rates of lipolysis are unaffected by PKA activation in cells overexpressing PLIN2 (Wang et al., 2011a; Wang et al., 2011b). It is also important to note that electrical stimulation and adrenaline stimulation of rat skeletal muscle (Prats et al., 2006) has been reported to increase the colocalisation of HSL with PLIN2. However, it is not known whether this association occurs in man *in vivo* and whether it increases rates of lipolysis. It is also worth acknowledging that we only assessed colocalisation of either PLIN2 or PLIN5 with the LD's, and therefore cannot discriminate LDs containing both PLIN2 and PLIN5 from those LDs containing only PLIN2 or PLIN5. Nevertheless, the observation that the number of PLIN5-LD is reduced following exercise, while no reduction occurs in the number of PLIN5-null-LD appears to underpin the important role of PLIN5 in LD lipolysis and subsequent oxidation in skeletal muscle. In line with this conclusion, observations from various cell culture models and rat skeletal muscle preparations suggest that PLIN5 also recruits mitochondria to the surface of LD's, thus stimulating the formation of functional complexes between mitochondria and LDs (Bosma et al., 2011; Wang et al., 2011b). Accordingly, PKA activation in alpha mouse liver 12 (AML12) cells expressing PLIN5 has recently been shown to promote triacylglycerol hydrolysis and efficient channeling of liberated fatty acids to the mitochondria for β-oxidation (Wang et al., 2011b). IMTG and mitochondria in skeletal muscle of endurance athletes are observed in close proximity (Shaw et al., 2008), and ET enhances the spatial interaction between IMTG and mitochondria (Tarnopolsky et al., 2007). Collectively these observations suggest that increased expression and PKA activation of PLIN5 may also contribute to an increased net IMTG breakdown during moderate intensity endurance exercise by mediating stronger interactions between IMTG and mitochondria.

It is worth noting that there was no net IMTG breakdown in type II fibres during 60 min moderate intensity exercise pre or post 6 weeks of training. This observation is in line with previous studies in well-trained athletes performing 2-3 h moderate-intensity exercise (De Bock et al., 2005; Stellingwerff et al., 2007; van Loon et al., 2003). However, PLIN2 and PLIN5 protein expression was significantly increased in type II fibres, raising the question as to why IMTG breakdown was not enhanced in parallel as seen in the type I fibres. However, as the exercise was performed at a moderate intensity,

it is likely that only a small proportion of the type II fibres were recruited, and this therefore may explain why no net IMTG breakdown was observed in type II fibres. In addition the higher PLIN2 and PLIN5 content after training in both fibre types may also serve other metabolic roles such as to mediate IMTG storage under non-exercise conditions.

Both PLIN2 and PLIN5 also appear to play an important role in limiting rates of lipolysis in the basal state, since expression of either PLIN2 or PLIN5 in cultured cells reduces basal lipolytic rates compared to when PLIN2 is knocked down or PLIN5 is not expressed (Bell et al., 2008; Listenberger et al., 2007; Wang et al., 2011a). Specifically, PLIN2 expression reduces the association of ATGL with the LD (Bell et al., 2008; Listenberger et al., 2007), and PLIN5 binds ATGL under basal conditions thereby reducing its activity (Wang et al., 2011a). Furthermore, it has recently been shown that PLIN5 expression in AML12 cells reduces basal triacylglycerol hydrolysis and promotes palmitate incorporation into LD triacylglycerol (Wang et al., 2011b). If such mechanisms exist *in vivo*, these observations suggest that PLIN5 and/or PLIN2 may play a key role in the coupling of TAG hydrolysis to the metabolic demand for fatty acids. As mentioned, ET increases the net breakdown of IMTG during exercise, therefore during an ET intervention IMTG concentrations are reduced on a regular basis. In addition, IMTG synthesis is upregulated in the period following IMTG-depleting exercise (Schenk and Horowitz, 2007). Therefore, undertaking repeated bouts of exercise during an exercise training programme likely results in high rates of IMTG synthesis (Bergman et al., 2010). As a result of this combination of training adaptations in the enzymes controlling IMTG metabolism, insulin resistance-inducing-FA metabolites have been suggested to be maintained at a lower concentration in skeletal muscle, thereby preserving higher insulin sensitivity in trained individuals. (Dubé et al., 2008; Dubé et al., 2011).

The most well-documented adaptations to SIT are an improved VO_{2peak} and an enhanced skeletal muscle oxidative capacity (reviewed (Gibala et al., 2012), both of which are increased by a similar magnitude as ET (Burgomaster et al., 2008). We also demonstrate that SIT and ET improve VO_{2peak} in sedentary individuals as previously described (Burgomaster et al., 2008), although in the present study the improvement in VO_{2peak} tended to be greater in the ET group ($P = 0.064$). Like ET, SIT also

increased mitochondrial density, as previously reported (Burgomaster et al., 2008), and we provide novel data showing that SIT leads to higher IMTG content in type I and type II muscle fibres. In contrast to ET, no changes in whole body fat oxidation rates were observed following SIT. This finding is in contrast to the findings of Burgomaster *et al.* (2008), who reported an increase in fat oxidation during exercise following SIT. The SIT group tended to exhibit a lower RER than the ET group pre-training, suggesting that rates of fat oxidation were higher in the SIT group before training, despite the groups being matched for physical activity level, aerobic capacity and fat mass, and diet was controlled in the 48 h prior to the exercise bout. Nevertheless, we observe clear increases in IMTG breakdown in type I fibres during moderate-intensity exercise in response to SIT, suggesting that the source of oxidised fat is shifted from plasma fatty acids towards IMTG. Importantly, the expression of both PLIN2 and PLIN5 correlated with IMTG content, net IMTG breakdown in type I fibres during exercise, and insulin sensitivity. Therefore, the data suggests that the increase in IMTG content and net breakdown during exercise that results from the increased expression of PLIN2 and PLIN5 likely maintains low intramuscular fatty acid metabolite concentrations leading to improvements in insulin sensitivity following both ET and SIT. Accordingly, the present study adds to the growing body of evidence that SIT is a time-efficient exercise intervention to improve insulin sensitivity in healthy individuals and patient populations (Babraj et al., 2009; Hood et al., 2011; Little et al., 2011a; Richards et al., 2010).

It is important to note that the primary aim of the study was to investigate exercise training-induced increases in net IMTG breakdown during 60 min moderate-intensity exercise and PLIN2 and PLIN5 protein expression. However, it is likely that the number of subjects in the present study is not sufficiently high to detect between groups differences for some of the measured variables. Therefore, although many of the training effects are of a similar magnitude, larger-scale studies are required to determine which exercise mode is superior in increasing the main metabolic outcome measures (IMTG breakdown during exercise and PLIN2 and PLIN5 expression) and/or in increasing whole body insulin sensitivity and aerobic exercise capacity. Studies involving a larger number of participants should be completed before advice can be given about which exercise training mode is the best for the general population and for specific patient populations.

In conclusion, PLIN2 and PLIN5 expression are upregulated in response to ET, and the upregulation of these proteins appears to be important to facilitate the enhanced rates of net IMTG breakdown during exercise. In addition, PLIN2 and PLIN5 expression may contribute to an increase in IMTG content, which likely results in lower fatty acid metabolite concentrations, and in turn may drive the ET-induced improvements in insulin sensitivity. Despite the large differences in duration and energy expenditure between SIT and ET, we provide novel evidence indicating that SIT induces similar improvements in net IMTG breakdown, PLIN2 and PLIN5 protein expression, aerobic exercise capacity and insulin sensitivity as traditional ET. This study, therefore, generates novel information on the mechanism by which net IMTG breakdown during endurance type exercise increases following both modes of exercise training, and provides evidence that SIT provides a time-efficient exercise alternative to achieve improvements in aerobic fitness and insulin sensitivity.

Chapter 5

Resistance training enhances skeletal muscle oxidative capacity and increases intramuscular triglyceride breakdown during exercise in type I and type II fibres of sedentary males

5.1 Abstract

Increases in oxidative capacity and intramuscular triglyceride (IMTG) mobility are fundamental adaptations to endurance training and are mechanistically linked to enhanced skeletal muscle insulin sensitivity. Currently, it is not known whether resistance training (RT) also increases mitochondrial density and IMTG utilisation during endurance-type exercise. Thirteen sedentary males (20±1 yrs, 24.8±0.8 $kg.m^{-2}$) performed 6 weeks of whole-body RT (3 times per week), after which changes in VO_{2peak} and insulin sensitivity (Matsuda index) were assessed. Muscle biopsies from the *m. vastus lateralis* were obtained from 8 individuals before and after 60 min steady state cycling at ~65% pre-training VO_{2peak}. Immunofluorescence microscopy was used to assess muscle fibre type-specific changes in mitochondrial density, IMTG content and expression of PLIN2 and PLIN5. RT increased VO_{2peak} (8±3%), mitochondrial density (46±13% and 61±13% in type I and II fibres respectively; $P<0.05$) and the Matsuda insulin sensitivity index (47±6%; $P<0.05$). In type I fibres IMTG (52±11%; $P<0.05$) and PLIN2 content was increased (107±19%; $P<0.05$) and PLIN5 content tended to increase (54±22%; $P=0.054$) post-training. In type II fibres PLIN2 content increased (57 ± 20%; $P<0.05$), and IMTG (46 ± 17%; $P=0.1$) and PLIN5 (44 ± 24%; $P=0.054$) content tended to increase post-training. Pre-training, net IMTG breakdown occurred during endurance-type exercise in type I fibres only (30±8%; $P<0.05$). Post-training, IMTG breakdown occurred in both type I and II fibres (43±5%, 37±5%, respectively; $P<0.05$). The results demonstrate for the first time that RT enhances mitochondrial density and increases IMTG utilisation in both type I and type II fibres during endurance-type exercise in previously sedentary males.

5.2 Introduction

Elevated intramuscular triglyceride (IMTG) concentrations have been linked to a reduced skeletal muscle insulin sensitivity in sedentary and obese individuals and type 2 diabetes patients (Pan et al., 1997; Phillips et al., 1996a; van Loon et al., 2004). Endurance training (ET) is also associated with elevated IMTG concentrations, although muscle insulin sensitivity remains high in a phenomenon termed the 'athlete's paradox' (Goodpaster et al., 2001; van Loon et al., 2004). As ET increases the breakdown of IMTG during exercise (Schrauwen et al., 2002; Van Proeyen et al., 2011a; Van Proeyen et al., 2011b)(Chapter 4), the capacity to oxidise IMTG is suggested to be mechanistically linked to the preservation of muscle insulin sensitivity alongside elevated IMTG concentrations (Bruce et al., 2003; Goodpaster et al., 2001; van Loon and Goodpaster, 2006). The prevailing hypothesis is that regular breakdown and resynthesis of the IMTG pool during exercise training leads to a metabolic state in which a high IMTG content is combined with low concentrations of fatty acid metabolites, such as long chain acyl-CoA, diacylglycerol (DAG) and ceramides, which are thought to otherwise reduce insulin sensitivity (Moro et al., 2008; Shaw et al., 2010; van Loon and Goodpaster, 2006).

Resistance training (RT) is also associated with improvements in insulin sensitivity in healthy individuals (Miller et al., 1994), those with impaired glucose tolerance (Eriksson et al., 1998), and overweight and obese individuals with type 2 diabetes (Hills et al., 2010; Holten et al., 2004). Accordingly it is recommended that RT is included in exercise programmes which aim to reduce the risk of developing metabolic disease (Gordon et al., 2009; Ratamess et al., 2009). As skeletal muscle is the predominant site for insulin-stimulated glucose uptake, improvements in insulin sensitivity following RT are often attributed to skeletal muscle hypertrophy (Eriksson et al., 1997; Poehlman et al., 2000; Takala et al., 1999) and/or a greater abundance of insulin signalling intermediates and GLUT4 (Holten et al., 2004; Iglay et al., 2007; Krisan et al., 2004). Such adaptations would increase the total capacity for glucose uptake and glycogen synthesis in skeletal muscle and improve insulin-mediated glucose clearance from the blood. However, whether the described improvements in IMTG metabolism that contribute to ET-induced improvements in insulin sensitivity also occur following RT has not yet been investigated.

An increased capacity for IMTG breakdown is linked to a greater skeletal muscle oxidative capacity and mitochondrial density following ET (Moro et al., 2008; van Loon, 2004). However, whether RT enhances skeletal muscle oxidative capacity is equivocal, as some (Balakrishnan et al., 2010; Pesta et al., 2011; Tang et al., 2006), but not all studies (Bell et al., 2000; Chilibeck et al., 1999; Green et al., 1999; MacDougall et al., 1979) report an increased mitochondrial density following RT. In contrast, a number of studies have convincingly shown that net IMTG breakdown occurs during a single session of resistance exercise (Creer et al., 2005; Essen-Gustavsson and Tesch, 1990; Koopman et al., 2006). Consequently, the possibility that RT will have a similar effect as ET in terms of being able to increase IMTG content and the ability to breakdown IMTG during subsequent endurance-type exercise cannot be excluded. Therefore, we first hypothesize that RT will increase IMTG content and mitochondrial density, and enhance IMTG breakdown during endurance-type exercise. We (Chapter 4), and others (Amati et al., 2011; Peters et al., 2012), have reported that ET augments the expression of two lipid droplet (LD)-associated proteins, perilipin 2 (PLIN2) and perilipin 5 (PLIN5), in skeletal muscle, and that the increase in these proteins occurs in parallel to the increase in IMTG content (Chapter 4). Although the precise role of these proteins in skeletal muscle is not known, data obtained *in vitro* suggests that PLIN2 and/or PLIN5 compete with lipases at the LD surface to downregulate basal lipolysis and upregulate exercise or PKA stimulated lipolysis (Bell et al., 2008; Imamura et al., 2002; Listenberger et al., 2007; Wang et al., 2011a; Wang et al., 2011b; Wolins et al., 2006b). Accordingly, we recently reported that both PLIN2 and PLIN5-containing LDs are preferentially utilised during moderate intensity exercise in sedentary individuals (Chapter 2 & 4). Therefore, we further hypothesize that increased IMTG content and breakdown during exercise following RT would occur alongside increases in PLIN2 and PLIN5 protein expression.

To investigate these hypotheses we employed immunohistochemical methodology described in Chapter's 2, 3 and 4 to obtain muscle fibre type-specific information on muscle mitochondrial density, IMTG content and the protein expression of PLIN2 and PLIN5 in percutaneous muscle biopsies of the *m. vastus lateralis* obtained from sedentary males before and after 6 weeks of RT.

5.3 Methods

Subject Characteristics

Thirteen healthy, lean sedentary males (see Table 5.1 for characteristics) provided written, informed consent following a verbal and written explanation of the nature and risks involved in the study, which was approved by the Black Country NHS Research Ethics Committee (West Midlands, UK). All subjects were free of known metabolic or cardiovascular disorders assessed using a general health questionnaire. In addition, subjects had performed less than two 30 min sessions of physical activity per week in the preceding year, and were not involved in a structured exercise programme.

Pre-experimental procedures

All subjects initially underwent a progressive exercise test to exhaustion on an electronically braked cycle ergometer (Lode BV, Groningen, The Netherlands) in order to determine peak oxygen uptake ($VO_{2\ peak}$), as previously described (Chapter 2 & 4). After sufficient recovery, individual one repetition maximum's (1RM) were determined for all exercises to be performed during the training programme, which included chest press, leg press, latissimus pull down, leg extension, leg curl, shoulder press, bicep curls, and tricep extensions. For each exercise a short warm up was performed, which included 6 repetitions at an estimate of 50% 1RM, 4 repetitions at ~70% 1RM, and 2 repetitions at ~80% 1RM, with a 2 min recovery period between each set. An estimate of 1RM was made based on the maximum number of repetitions performed for a specific load, using the following equation (Bryzicki, 1998):

$$1RM = \frac{Load}{(1.0278 - [0.0278 \times repetitions])}$$

Subsequently, each exercise was performed at the estimated 1RM until only 1 repetition was possible, at which point the load was determined to be 1RM. Sufficient rest was provided between each 1RM effort. Post-training 1RM was determined for leg press, chest press, leg extension and shoulder press, as a measure of programme efficacy. All exercises were performed on commercially available exercise machines (Cybex International Inc., MA, USA).

Experimental procedures

Pre and post-training testing procedures were identical in all respects, and were undertaken across two consecutive days. On both occasions, subjects were instructed to refrain from performing vigorous exercise in the preceding 48 h period. In addition, subjects were provided with all food and drinks to be consumed in the preceding 24 h. The diet was of a standard macronutrient composition (50% carbohydrate, 35% fat, 15% protein), but adjusted for each individual's habitual caloric intake, assessed through the completion of a 3 day weighed-intake diet diary. During the exercise training period subjects were asked to maintain their habitual physical activity and dietary patterns, with the latter being assessed through completion of a 3 day diet diary post-training.

Day 1: Subject's reported to the laboratory after an overnight fast (>10 h) to undergo a 2 h oral glucose tolerance test (OGTT) as previously described (Chapter 4). Plasma glucose and insulin concentrations were determined and subsequently used to calculate an index of insulin sensitivity (Matsuda and DeFronzo, 1999), as previously reported (Chapter 4). Body composition analysis was performed immediately following the OGTT using Dual energy X-ray Absorptiometry (DXA). Regional fat mass and fat-free mass of each subject was determined using specialist software (QDR software, Hologic Inc., MA, USA). Relative fat mass was calculated for each region using absolute fat mass as a proportion of total mass.

Day 2: 8 out of the 13 subject's taking part in the study agreed to provide muscle samples. These subject's again reported to the laboratory after an overnight fast (>10 h) at ~7am, and the thigh of one leg was prepared for muscle biopsy collection. Two incisions were made approximately 2 cm apart. The leg that was to undergo biopsy was randomised in the pre-training condition; the contralateral leg was sampled post-training. A resting muscle biopsy was obtained from the distal incision of the vastus lateralis using the Bergström technique and prepared for immunohistochemical analysis, as described previously (Chapter 2 & 4). Subsequently, subjects underwent 1 h of steady state cycling at a workload equivalent to 65% pre-training $VO_{2\ peak}$ on an electronically braked cycle ergometer (Lode BV, Groningen, The Netherlands). A second muscle biopsy was taken immediately post-exercise from the proximal incision.

Training procedures

The training programme was designed to increase strength and induce hypertrophy, reflecting the most recent ACSM position stand for strength training in healthy adults (Ratamess et al., 2009). Specifically, subjects undertook three training sessions per week for six weeks in the resistance training laboratory. During each session a total of 9 exercises were performed: chest press, leg press, lat pull down, leg curl, leg extension, shoulder press, bicep curl, tricep extension, abdominal crunches. For each exercise, subjects performed 3 sets consisting of 12 repetitions per set. In session 1, the workload for each exercise was equal to 50% of each individuals 1RM. In sessions 2 and 3 this was increased to 60 and 70% of 1RM respectively. The workload was further increased to 80% 1RM in session 4, at which point the workload was fixed. In order to progress, subjects had to achieve 3 complete sets of 12 repetitions, after which the workload was increased 5 lb. Importantly, subjects were encouraged to work to fatigue on the final set of each exercise, irrespective of the number of repetitions achieved. All training sessions were supervised to ensure programme adherence and form quality was maintained.

Muscle analysis

Serial 5 µm cryosections from pre and post training muscle biopsies were cut at -30°C and transferred on to ethanol-cleaned glass slides and stained for fibre type determination of IMTG (oil red O), cytochrome *c* oxidase (COX), PLIN2, and PLIN5 content using immunofluorescence, as described previously (Chapter 2, 3, & 4). Oxidative capacity was also estimated by determining maximal succinate dehydrogenase (SDH) activity. Accordingly, cryosections were incubated in 0.2M phosphate buffer (13 ml 0.2 M NaH_2PO_4 + 87 ml 0.2 M Na_2HPO_4, pH 7.6) containing 0.1 M Na succinate and 1.2 mM nitro-blue tetrazolium (all chemicals obtained from Sigma, UK) for 60 min at 37ºC. Slides were rinsed (3 x 30s deionised water), after which the unbound nitro-blue tetrazolium was removed with three consecutive washes in increasing solutions of acetone (30%, 60%, and 90%). Slides were air dried for 15 min followed by incubation with appropriate primary and secondary antibodies for determination of fibre type (as described in Chapter 2, 3 & 4). Capture of immunofluorescence-stained cross-sectional orientated images was performed as previously reported (Chapter 2, 3 & 4). Brightfield images of cross-sectional orientated sections displaying fibre type-

specific distributions of SDH activity were captured using an Olympus BX61 microscope with a 20x 0.50 NA objective coupled to a SPOT RT3 colour camera (Diagnostic Instruments Inc., MI, USA). All image processing was performed using Image-Pro Plus 5.1 software (Media Cybernetics, MD, USA). Both widefield and brightfield images were used to assess the fibre specific distribution of IMTG, PLIN2, PLIN5 and mitochondria. Analysis was performed for 97 ± 9 fibres per muscle cross-section (38 ± 4 type I fibres, 59 ± 6 type II fibres). COX and SDH staining intensity was used to indicate changes in mitochondrial content after training. An intensity threshold was uniformly selected to represent positive signal for IMTG, PLIN2 and PLIN5. The content of IMTG, PLIN2 and PLIN5 was expressed as the positively stained area fraction relative to the area of each muscle fibre. Confocal microscope images of IMTG, PLIN2 and PLIN5 were used to identify changes in size and density (calculated as the number of objects relative to area).

Statistics

All data are expressed as means ± S.E. Significance was set at the 0.05 confidence level. Changes in parameters of exercise capacity, body composition, and insulin sensitivity were assessed using a paired t-test analysis. A two-factor repeated measures ANOVA was used to assess mitochondria, and PLIN2 and 5 content, using the two within subject factors as *'training'* (pre- versus post-training) and *'fibre'* (type I versus type to II fibres). To inspect differences in IMTG, we hypothesised *a priori* that IMTG content would be significantly different between type I and type II fibres. Thus, we employed a two-factor repeated measures ANOVA, using the within subject factors *'training'* (pre- versus post-training) and *'time'* (pre- versus post-exercise). Significant main effects or interactions were assessed using Bonferroni adjustment post hoc analysis. In order to investigate specific relationships between variables, a simple linear regression analysis was used.

5.4 Results

Whole-body measures (n=13)

Exercise capacity, body composition and insulin sensitivity (Table 5.1): The effectiveness of the RT programme employed in the current study was underlined through the observed improvements in 1RM for leg press, chest press, leg extension and shoulder press (43 ± 7%, 34 ± 4%, 35 ± 5% and 34 ± 4%, respectively; $P < 0.001$). Training also induced an 8 ± 3% ($P = 0.015$) improvement in $VO_{2\ peak}$ and a 7 ± 2% increase in W_{max} ($P = 0.001$). No changes in BMI or body mass were observed following training. Both an increase in free-fat mass (FFM) (3 ± 1%; $P = 0.010$) and a decrease in fat mass (FM) (8 ± 3%; $P = 0.036$) occurred in response to training, which corresponded to an 8 ± 2% decrease in relative fat mass. Training induced a 12 ± 3% ($P = 0.023$) and 24 ± 6% ($P < 0.001$) decrease in glucose and insulin area under the curve (AUC), respectively. Insulin sensitivity, calculated using the Matsuda index, was improved 47 ± 6% ($P < 0.001$) following training.

Table 5.1. *Subject characteristics*

	Pre-Training	Post-Training
Age (yrs)	20 ± 1	
Height (m)	1.76 ± 0.03	
Weight (m)	76.7 ± 3.3	76.1 ± 3.1
BMI ($kg.m^{-2}$)	24.8 ± 0.8	24.6 ± 0.7
Exercise capacity		
Leg press 1RM (kg) *	194 ± 10	278 ± 15
Chest press 1RM (kg) *	54 ± 3	71 ± 3
Leg extension 1RM (kg) *	119 ± 5	158 ± 5
Shoulder press 1RM (kg) *	47 ± 2	64 ± 2
$VO_{2\,peak}$ ($l.min^{-1}$) *	3.24 ± 0.16	3.46 ± 0.21
$VO_{2\,peak}$ ($l.min^{-1}.kg^{-1}$) *	42.3 ± 1.4	45.5 ± 1.7
W_{max} (W) *	216 ± 11	231 ± 12
Body composition		
FM (kg) *	16.0 ± 1.4	14.8 ± 1.4
FFM (kg) *	51.1 ± 2.0	52.8 ± 2.1
% FM *	22.9 ± 1.7	21.0 ± 1.6
Whole-body insulin sensitivity		
Glucose AUC ($mg.dl^{-1}.120\ min^{-1}$) *	16329 ± 724	14567 ± 826
Insulin AUC ($\mu U.ml^{-1}.120\ min^{-1}$) *	9548 ± 1006	7484 ± 863
ISI-Matsuda *	2.9 ± 0.3	3.8 ± 0.3

Data provided are means ± S.E. (n=13). *BMI* body mass index, W_{max} maximum workload, *FFM* fat free mass, *FM* fat mass. Body composition data was obtained using a DXA scan. * indicates main training effect ($P < 0.05$), such that Pre ≠ Post.

Skeletal muscle sample analysis (n=8)

Intramuscular triglyceride analysis (Figure 5.1): To investigate the *a priori* hypothesis that IMTG content is greater in type I fibres versus type II fibres, we examined training effects separately for the 2 fibre types. Training increased IMTG content in type I fibres (52 ± 11%; P = 0.01) and tended to increase IMTG content in type II fibres (46 ± 17%; P = 0.1). The increase in IMTG content was attributed to greater IMTG density in both type I (pre 0.049 ± 0.008 LDs.μm^{-2}, post 0.074 ± 0.008 LDs.μm^{-2}; P = 0.047) and type II fibres (pre 0.013 ± 0.004 LDs.μm^{-2}, post 0.027 ± 0.005 LDs.μm^{-2}; P = 0.07). No significant changes in lipid droplet size were observed.

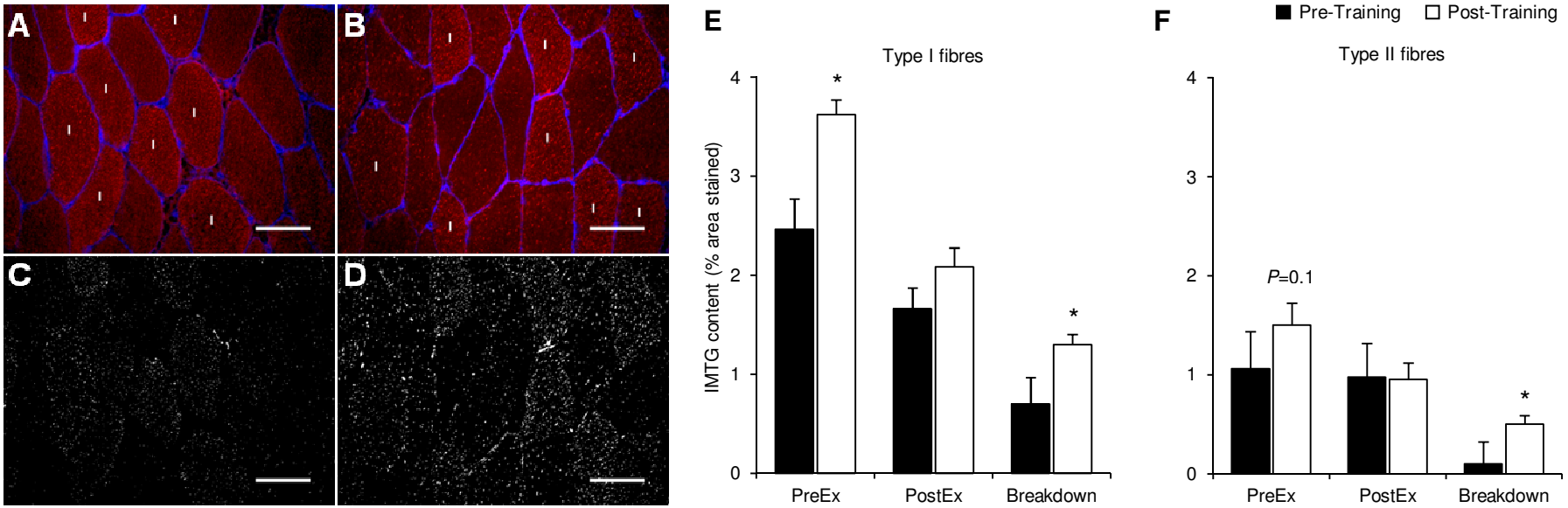
Pre-training, 60 min steady state cycling induced a 30 ± 8% decrease in IMTG content in type I fibres (main time effect; P = 0.04), with no changes apparent in type II fibres. Post-training, significant IMTG breakdown during steady state exercise occurred in both type I (43 ± 5%, main time effect; P < 0.01) and type II fibres (37 ± 5%,main time effect; P = 0.01). In comparison to pre-training, net IMTG utilisation in type I and type II fibres was significantly greater following training (training x time interaction; P < 0.05). All reported changes in IMTG content in response to exercise were attributed to significant reductions in IMTG density, with no changes in IMTG size observed.

Mitochondrial analysis (Figure 5.2): Immunofluorescence images of the mitochondrial marker COX revealed a greater mitochondrial density (expressed as fluorescence intensity) in type I fibres compared to type II fibres both pre and post-training (P < 0.001). Mitochondrial density was significantly increased in response to training in both type I and type II fibres (46 ± 13% and 61 ± 13%, respectively; P < 0.001). The magnitude of change in mitochondrial density was significantly greater in type II fibres compared to type I fibres (P = 0.02). In addition, brightfield images demonstrated that SDH activity (expressed as optical density) was significantly greater in type I fibres compared to type II fibres both pre and post-training (P < 0.01). Furthermore, SDH activity in both type I and type II fibres was significantly increased following training (17 ± 5%, 23 ± 5%, respectively; P < 0.001).

PLIN2 and PLIN5 analysis (Figure 5.3): Both PLIN2 and PLIN5 content (expressed as a % of fibre stained) was significantly greater in type I fibres compared to type II fibres, irrespective of training state

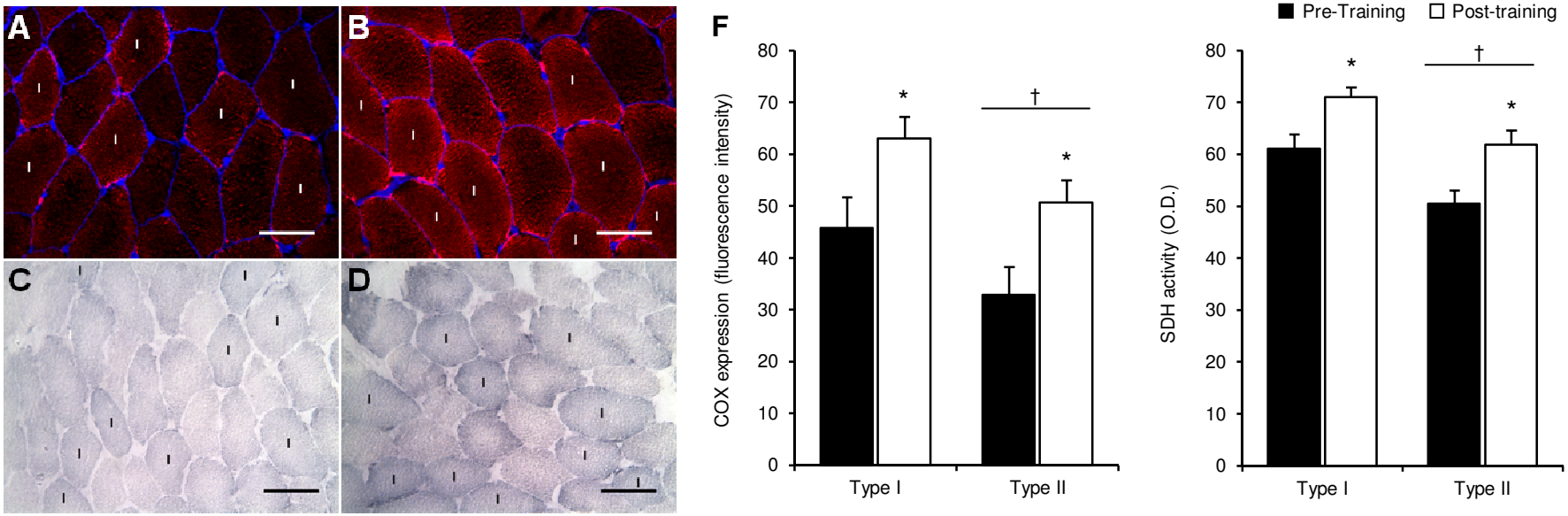
(main fibre effect; $P < 0.001$). Training induced a significant increase in PLIN2 content in both type I and type II fibres (107 ± 19% and 57 ± 20%, respectively, main training effect; $P < 0.01$). This was attributed to a greater PLIN2 density in both fibre types (type I fibres: pre-training 0.046 ± 0.005 PLIN2.μm^{-2}, post-training 0.085 ± 0.009 PLIN2.μm^{-2}, type II fibres: pre-training 0.011 ± 0.003 PLIN2.μm^{-2}, post-training 0.015 ± 0.004 PLIN2.μm^{-2}; main training effect; $P < 0.001$). PLIN5 content tended to increase following training in both type I and type II fibres (58 ± 22% and 44 ± 24%, respectively, main training effect; $P = 0.054$). This was again attributable to a greater PLIN5 density in both fibre types (type I fibres: pre-training 0.060 ± 0.007 PLIN5.μm^{-2}, post-training 0.093 ± 0.008 PLIN5.μm^{-2}; type II fibres: pre-training 0.007 ± 0.003 PLIN5.μm^{-2}, post-training 0.019 ± 0.004 PLIN5.μm^{-2}, main training effect; $P = 0.026$).

Fig. 5.1. Effect of 6 weeks RT on fibre type specific IMTG content and utilisation during exercise



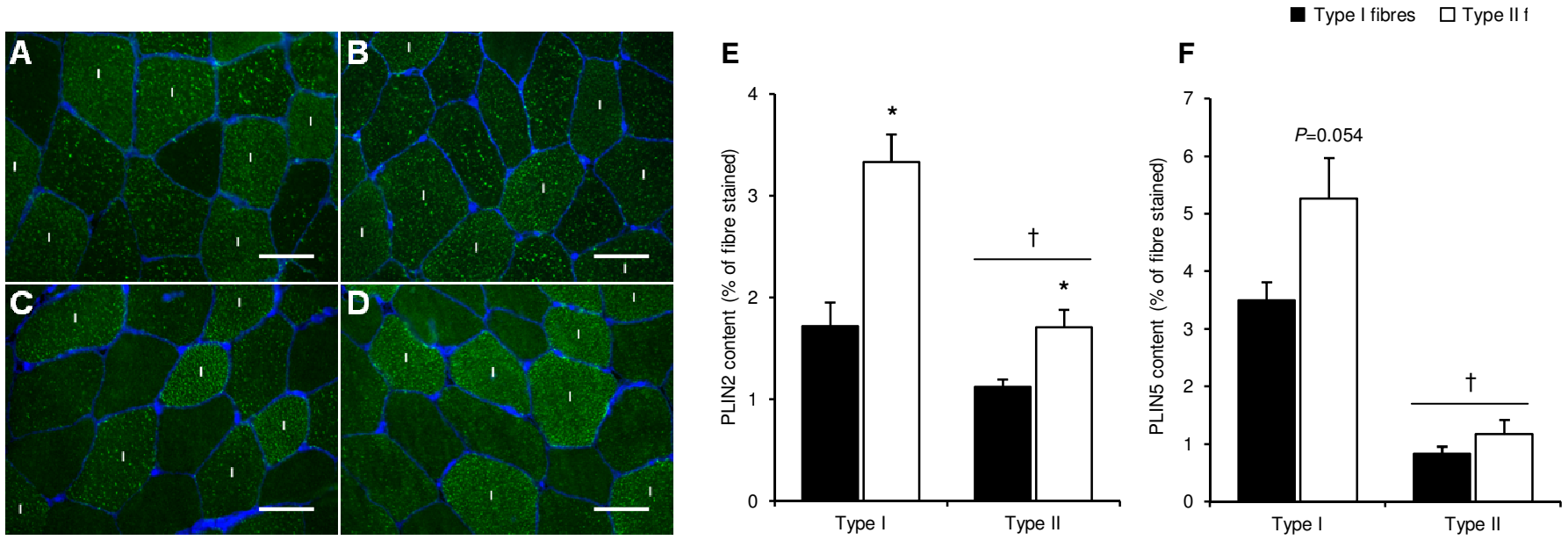
Representative immunofluorescence images of IMTG (stained red) in combination with WGA to identify the cell border (stained blue) in skeletal muscle, pre *(A)* and post *(B)* 6 weeks of RT. Type I fibres are indicated with "I", all other fibres are assumed type II fibres. White bar = 50µm. Images *(C)* and *(D)* display the ORO signal obtained after the intensity threshold was applied during quantitation (showing the LDs in white), before and after RT, respectively. IMTG content quantified from immunofluorescence images of oil red O-stained muscle sections, before (closed bars) and after (open bars) 6 weeks of RT, in type I *(E)* and type II *(F)* fibres. IMTG content was measured before *(PreEx)* and after *(PostEx)* 60 min steady state cycling at 65% pre-training $VO_{2\ peak}$, and net IMTG breakdown calculated *(Breakdown)*. Values are presented as means ± S.E. (n = 8). Type I fibres: Training effect; P = 0.024, time effect; P < 0.01, training x time interaction; P = 0.04. Type II fibres: training effect; P = 0.098, time effect; P < 0.041, training x time interaction; P = 0.033. * Significant training effect (P < 0.05).

Fig. 5.2 Effect of 6 weeks of RT on fibre type-specific mitochondrial density



Fibre type specific immunofluorescence visualisation of COX (stained red) in combination with WGA to identify the cell border (stained blue) in skeletal muscle, *(A)* pre and *(B)* post 6 weeks of RT. Type I fibres are indicated with a "I", all other fibres are assumed type II fibres. White bar = 50µm. *(C)* Mitochondrial content, quantified from immunofluorescence images of COX, obtained before and after 6 weeks of RT in type I fibres (closed bars) and type II fibres (open bars). Training effect; $P < 0.001$, fibre effect; $P < 0.001$. Fibre specific visualisation of SDH activity in skeletal muscle, *(C)* pre and *(D)* post 6 weeks of RT. Black bar in panel C and D = 100µm. *(F)* SDH activity in type I fibres (closed bars) and type II fibres (open bars). COX protein expression and SDH activity was significantly different between type I and type II fibres at all time-points ($P < 0.05$). Values are presented as means ± S.E ($n = 8$). Training effect, $P = 0.001$; fibre effect, $P < 0.001$. * Significant training effect, † significant fibre effect ($P < 0.05$).

Fig. 5.3 Effect of 6 weeks of RT on fibre type specific PLIN2 and PLIN5 content



Fibre type specific visualisation of PLIN2 (stained green) *(A: Pre, B: Post)* and PLIN5 *(C: Pre, D: Post)* in combination with WGA to identify the cell border (stained blue) in skeletal muscle, following 6 weeks of RT. Type I fibres are indicated with a "I", all other fibres are assumed type II fibres. White bar = 50µm. Fluorescence images were used to quantify PLIN2 *(E)* and PLIN5 *(F)* content in type I (closed bars) and type II (open bars) fibres. Values are presented as means ± S.E (n = 8). *(E)* Training effect, $P < 0.001$; fibre effect, $P = 0.002$; training x fibre interaction, $P = 0.017$. *(F)* training effect; $P = 0.054$, fibre effect, $P < 0.001$; training x fibre interaction; $P = 0.06$. * Significant training effect, † significant fibre effect ($P < 0.05$).

Correlation analyses

For correlation analyses, data obtained for type I and type II fibres, pre and post-training were pooled (Table 5.2). Pre-exercise IMTG content showed a strong positive correlation with PLIN2 content (R = 0.55; $P < 0.001$), PLIN5 content (R = 0.61; $P < 0.001$) and COX intensity (R = 0.58, $P < 0.001$). Pre-exercise IMTG content (R = 0.66; $P < 0.001$), PLIN5 content (R = 0.50; $P = 0.003$), COX intensity (R = 0.60; $P < 0.001$) and SDH intensity (R = 0.43; $P = 0.02$) were all strongly associated with IMTG utilisation, whereas PLIN2 content (R = 0.36; $P = 0.044$) was moderately associated with IMTG utilisation. Insulin sensitivity was moderately associated with IMTG content (R = 0.39; $P = 0.037$) and strongly associated with COX intensity (R = 0.71; $P < 0.001$), but did not correlate with PLIN2 or PLIN5 expression.

Table 5.2. *Bivariate correlation analysis*

	IMTG	Net IMTG breakdown	Insulin sensitivity
IMTG		0.66‡	0.39*
PLIN2	0.55‡	0.36*	0.14
PLIN5	0.61‡	0.51†	0.24
COX	0.58‡	0.60‡	0.71‡

IMTG, PLIN2 and PLIN5 content and COX fluorescence intensity represent pre-exercise values. IMTG utilisation refers to the absolute change in IMTG concentration from pre to post-exercise. Values represent Pearson's squared correlation coefficients (R values) for relationships between variables. All data obtained for type I and type II fibres, pre and post-training were pooled for correlation analysis. Significant correlations: *$P < 0.05$, †$P < 0.01$, ‡$P < 0.001$.

5.5 Discussion

This is the first study to show that 6 weeks of RT increases IMTG content, muscle mitochondrial oxidative capacity, and net IMTG breakdown during endurance-type exercise, in line with our first hypothesis. The increase in IMTG content and breakdown following RT was also accompanied by greater expression of PLIN2 and PLIN5, consistent with our second hypothesis. Both PLIN2 and PLIN5 are present in the protein coat surrounding LDs (Brasaemle et al., 1997; Wolins et al., 2006b) and have recently been shown to play a role in the training-induced increases in IMTG breakdown during endurance-type exercise (Chapter 4). Together, these observations confirm the underlying hypothesis of the study that improvements in insulin sensitivity following 6 weeks RT are, at least in part, attributable to increased mobility of the IMTG pool and an enhanced skeletal muscle oxidative capacity. It should be noted, however, that the increase in mitochondrial density and IMTG utilisation during endurance-type exercise is significant after 6 weeks of RT in both type I and type II fibres. In contrast, increases in IMTG breakdown are restricted to type I fibres following 6 weeks of ET and sprint interval training (SIT) (Chapter 4). This observation is likely to be important as it implies that a combination of ET and RT is required to iincrease IMTG breakdown, oxidative capacity and insulin sensitivity of type II fibres.

Regular breakdown and resynthesis of IMTG during exercise training is postulated to increase IMTG content whilst maintaining low concentrations of fatty acid metabolites, such as long chain acyl-CoA, DAG and ceramides (Moro et al., 2008; Shaw et al., 2010; van Loon and Goodpaster, 2006), the accumulation of which leads to increased IRS-1 serine phosphorylation and Akt/PKB dephosphorylation (Gual et al., 2005; Summers, 2006), thereby reducing insulin-mediated glucose uptake. An increased rate of IMTG turnover is proposed to contribute to the improvement in insulin sensitivity observed following both ET and HIT in sedentary individuals (Chapter 4), and we hypothesized that a similar mechanism contributes to the enhanced insulin sensitivity following RT in the present study. In support, we report for the first time that 6 weeks of whole-body RT in sedentary individuals enhances IMTG content in type I fibres, with a strong trend for an increase in type II fibres. A number of studies have previously shown that IMTG are reduced following an acute bout of resistance exercise (Creer et al., 2005; Essen-Gustavsson and Tesch, 1990; Koopman et al., 2006),

and that rates of IMTG synthesis increase in the period following endurance-type exercise-induced IMTG depletion (Schenk and Horowitz, 2007). Therefore, our data are compatible with the hypothesis that repeated depletion and subsequent resynthesis of IMTG stores with each bout of resistance exercise leads to a higher set-point for IMTG content after 6 weeks of RT.

To investigate the impact of RT on IMTG breakdown, we examined IMTG breakdown in type I and type II fibres during the acute bout of endurance-type exercise both before and after RT. Before training, 60 min steady state cycling induced a ~30% decrease in IMTG content in type I fibres only, confirming that IMTG contribute to energy expenditure during exercise in young, sedentary individuals (Coggan et al., 2000; Shepherd et al., 2012). Following RT, endurance-type exercise at the same absolute workload induced a significantly greater decline in IMTG content than that observed at baseline, with a ~43% and ~37% decrease occurring in type I and type II fibres, respectively. Thus, we provide clear evidence that, in a similar manner to the well described effects of ET and HIT (Hurley et al., 1986; Schrauwen et al., 2002; Van Proeyen et al., 2011b)(Chapter 4), 6 weeks of RT also improves IMTG utilisation during endurance-type exercise. In lean, physically active individuals, IMTG utilisation during exercise is strongly associated with pre-exercise IMTG content (Stellingwerff et al., 2007; van Loon et al., 2003), a finding that is replicated in the present study. Therefore, the RT-induced elevation in IMTG concentration at least partly explains the enhanced IMTG utilisation during endurance-type exercise following RT. However, this relationship is not observed in obese type 2 diabetes patients who display large concentrations of IMTG but low rates of IMTG utilisation during exercise (Blaak et al., 2000; Schrauwen et al., 2002; van Loon et al., 2003), indicating that a high IMTG content alone does not determine the degree of IMTG breakdown during exercise.

Importantly, 6 weeks of RT also enhanced skeletal muscle oxidative capacity, which occurred concomitant with a significant improvement in maximal aerobic exercise capacity. As skeletal muscle oxidative capacity is strongly determined by fibre type composition, we used both immunofluorescence and brightfield microscopy to measure fibre-type specific changes in oxidative capacity. Accordingly, we demonstrate that both COX protein content and SDH activity are significantly increased following RT, and these increases occur in both type I and type II muscle fibres. Interestingly, the magnitude of

the increase in COX expression is greater in type II fibres compared to type I fibres, which is likely explained by the primary recruitment of type II fibres during resistance exercise. The finding of an increase in oxidative capacity following RT is in agreement with a number of previous studies (Balakrishnan et al., 2010; Pesta et al., 2011; Tang et al., 2006), although it should be noted that some studies show no change (Bell et al., 2000; Green et al., 1999) or even a decrease in mitochondrial content following prolonged RT (Chilibeck et al., 1999; MacDougall et al., 1979). Nevertheless, in line with the present study, an acute bout of resistance exercise is known to enhance rates of mitochondrial protein synthesis and the expression of PGC1α (Wilkinson *et al.*, 2008; Burd *et al.*, 2011). However, the stimulation of mitochondrial protein synthesis following an acute bout of resistance exercise is lost following 10 weeks of RT (Wilkinson *et al.*, 2008). Thus, it could be speculated that an increase in oxidative capacity is an early (<6 weeks) adaptation to RT in sedentary individuals. Given that fat oxidation rates are dependent on the capacity for β-oxidation and therefore mitochondrial density (Simoneau *et al.*, 1999), the elevations in oxidative capacity observed in the present study likely provide an enhanced capacity for fatty acid oxidation, thus enabling an increased contribution of the expanded IMTG pool.

The oxidation rate of IMTG-derived fatty acids is also dependent on rates of IMTG lipolysis, and the regulation of basal and stimulated lipolysis is determined by the activation and/or localisation of the lipolytic enzymes. Expression of either PLIN2 or PLIN5, two LD-associated proteins, in cultured cells reduces the rate of basal TAG lipolysis (Listenberger *et al.*, 2007; Bell *et al.*, 2008; Wang *et al.*, 2011a), and increases palmitate incorporation into LD TAG (Wang *et al.*, 2011b; Bosma *et al.*, 2012a). In contrast, protein kinase A (PKA)-activation in PLIN5-expressing alpha mouse liver 12 (AML12) cells increases TAG hydrolysis, releasing fatty acids for β-oxidation (Wang *et al.*, 2011b). Moreover, adrenaline stimulation of rat skeletal muscle increases the colocalisation of HSL with PLIN2 (Prats *et al.*, 2006), and PLIN5 is able to bind ATGL, and its co-activator, CGI-58 (Granneman *et al.*, 2011; Wang *et al.*, 2011a). Taken together, these data suggest that PLIN2 and PLIN5 may mediate the interaction of lipolytic enzymes with the LD, and therefore contribute to the regulation of the rate of lipolysis. Our observations (Chapter 2 & 4) that PLIN2 and PLIN5-containing-LDs are preferentially broken down during endurance-type exercise support this role, and the protein expression of both

PLIN2 and PLIN5 is enhanced following both ET and HIT, in line with the observed increase in IMTG breakdwon during exercise (Chapter & 4). In the present study we found that 6 weeks RT in sedentary individuals increases the protein expression of PLIN2 and PLIN5 in both type I and type II fibres. This is the first study to report an effect of RT on PLIN expression at the protein level, although it was recently reported that 11 weeks of RT had no effect on the mRNA expression of any of the PLINs in skeletal muscle (Gjelstad *et al.*, 2012). Nevertheless, it is notable that in the present study when all pre-exercise data was pooled, IMTG content positively correlated with the expression of PLIN2 and PLIN5, in agreement with previous observations in rodent (Minnaard *et al.*, 2009) and human skeletal muscle (Amati *et al.*, 2011)(Chapter 4). Furthermore, both PLIN2 and PLIN5 expression showed a positive correlation with IMTG breakdown during exercise. Although the precise significance of this relationship is unknown, it is likely that greater PLIN2 and PLIN5 expression preserves the PLIN coverage of the expanded LD surface area, thereby maintaining adequate lipolytic control over the LD pool at rest and during exercise. In addition, it is worth acknowledging that recent data obtained in rat cardiomyocytes and human and rat skeletal muscle demonstrate that PLIN5 also mediates the spatial interaction of the LD with the mitochondrial network (Bosma *et al.*, 2011; Wang *et al.*, 2011b), thereby increasing the efficiency by which FA released by LD lipolysis are channelled into the mitochondria for β-oxidation (Wang *et al.*, 2011b). Previously it has also been observed that the physical distance between LD and mitochondria is smaller in skeletal muscle of ET-individuals (Tarnopolsky *et al.*, 2007). Therefore, as RT induced an increase in mitochondrial density, the number of LDs and the expression of PLIN5, it is likely that RT also may improve the physical interaction between LD and mitochondria that results in a more efficient channelling of FA released by lipolyis into the muscle for β-oxidation in the current study.

The 6 week whole-body RT programme employed in the present study induced a small but significant elevation in total fat free mass. As such, it should be noted that the increase in skeletal muscle mass will increase the total capacity for glucose disposal, and therefore contributes to the improvement in insulin sensitivity. RT has also been shown to enhance the total number of GLUT4 transporters and insulin signalling intermediates per muscle mass unit, independent of an increase in muscle mass (Holten *et al.*, 2004; Krisan *et al.*, 2004; Iglay *et al.*, 2007). Consequently, we do not claim that an

increase in IMTG mobility is the only mechanism by which RT improves insulin sensitivity. Rather, this study highlights the underappreciated contribution of IMTG metabolism to the well described improvements in insulin sensitivity with RT. Furthermore, additional muscular adaptations such as increased mitochondrial density and PLIN protein expression are likely to be important to support such improvements in IMTG metabolism and insulin sensitivity.

In conclusion, this study confirms the effectiveness of a RT intervention in sedentary males for improving insulin sensitivity and supports the current recommendations that RT should be included as one of the components in mixed exercise programmes which aim to reduce the risk of developing sarcopenia and metabolic disease, or programmes that aim to maintain muscle mass and strength in frail individuals.

Constant-workload sprint interval and traditional endurance training both increase insulin sensitivity and expression of perilipin 2 and 5 in obese males

6.1 Abstract

In Chapter 4 it was demonstrated that in previously sedentary young males both Wingate-based sprint interval training (SIT) and traditional endurance-based training (ET) enhance skeletal muscle oxidative capacity, intramuscular triglyceride (IMTG) concentration and whole-body insulin sensitivity. Evidence was also presented demonstrating that increases in perilipin 2 (PLIN2) and perilipin 5 (PLIN5) protein expression were instrumental to increases in net IMTG breakdown observed after both training modes. The current study first investigated the hypothesis that constant workload SIT (repeated 30s sprints at 200% W_{max}) also improves insulin sensitivity and VO_{2peak} in sedentary obese males. Secondly, this study investigated the hypothesis that constant workload SIT and traditional ET enhance IMTG concentration and PLIN2 and PLIN5 expression. Sixteen sedentary obese males performed 4 weeks of either SIT (4-7x 30s sprints at 200% W_{max}, 3d.wk^{-1}) or ET (40-60 min cycling at ~65% $VO_{2\ peak}$, 5d.week^{-1}). Training increased skeletal muscle oxidative capacity (SIT 47±6%, ET 38±75; *P*<0.05) and the Matsuda insulin sensitivity index (SIT 13±5%, ET 23±4%; *P* < 0.05). Training also increased PLIN2 (SIT 92±19%, ET 82±23%; *P*<0.05) and PLIN5 protein expression (SIT 54±15%, ET 36±10%; *P*<0.05) in type I fibres, whereas IMTG concentration only showed a trend to increase (SIT 51±21%, ET 31±25%; *P*=0.086). Fasting plasma cholesterol concentration was reduced following training (SIT 13±6%, ET 10±7%; *P*<0.05), and fasting plasma triglyceride concentration tended to decrease (SIT 16±8%, ET 10±7%; *P*=0.06). In conclusion, the data in this study show that 4 weeks constant workload SIT or ET both lead to increases in insulin sensitivity in sedentary obese males. The data are also consistent with the hypothesis that increased expression of PLIN2 and PLIN5, leading to improved regulation of IMTG, is instrumental in the underlying mechanism.

6.2 Introduction

Improvements in insulin sensitivity typically observed in response to endurance training (ET) are associated with an increased skeletal muscle oxidative capacity, enhanced intramuscular triglyceride (IMTG) concentration, and a greater capacity to oxidise IMTG during exercise (reviewed in van Loon & Goodpaster, 2006; Moro *et al.*, 2008; Shaw *et al.*, 2010). In accordance, overweight and obese individuals undertaking ET for 16 weeks or more demonstrate increased IMTG storage in parallel to improved insulin sensitivity and/or glycaemic control (Dubé *et al.*, 2008; Dubé *et al.*, 2011)(Chapter 3), a relationship which may be mechanistically linked to reduced intramuscular concentrations of specific diacylglycerol (DAG) and ceramide species (Bruce *et al.*, 2006; Dubé *et al.*, 2008; Amati *et al.*, 2011; Dubé *et al.*, 2011). Improvements in insulin sensitivity have also been observed in response to sprint interval training (SIT) in sedentary lean individuals (Babraj *et al.*, 2009; Richards *et al.*, 2010), and following constant-load high intensity interval training in lean sedentary individuals (Hood *et al.*, 2011) and obese type 2 diabetes patients (Little *et al.*, 2011a). However, in these studies no attempt has been made to investigate whether changes in IMTG metabolism are potentially instrumental in the underlying mechanism. In Chapter 4 of this thesis it was demonstrated for the first time that 6 weeks of SIT in lean, sedentary, insulin sensitive individuals enhances the content and breakdown of IMTG during moderate-intensity exercise in parallel with increased protein expression of the lipid droplet (LD)-associated proteins, perilipin 2 (PLIN2) and perilipin 5 (PLIN5). No studies have yet investigated the potentially beneficial effects of SIT and ET on IMTG content, perilipin protein expression and insulin sensitivity in obese, sedentary individuals who likely demonstrate pre-existing elevated IMTG stores (van Loon *et al.*, 2004; Amati *et al.*, 2011) and are at an increased risk of developing insulin resistance and type 2 diabetes.

The SIT protocol traditionally consists of 4-6 30 s Wingate cycling tests interspersed with 4.5 minutes rest, performed on three separate occasions per week (reviewed in Gibala *et al.*, 2012). This equates to 2-3 min exercise per session, and a total weekly exercise commitment of 6-9 min (Table 6.1). Notably, the resistance during a Wingate test is determined by a combination of the subject's body weight (equivalent to 7.5% of body mass) and the number of revolutions achieved per minute (cadence). Therefore, Wingate-based SIT requires an 'all-out' effort against a very high resistance on

a specialized cycle ergometer, necessitating high levels of internal and external motivation and expensive equipment. As a result, the practicality of Wingate-based SIT for the general population is questionable, especially in overweight and obese individuals who may benefit most from the effects of SIT. In line with this notion, a number of previous studies have employed a constant load high intensity interval training protocol to induce positive metabolic adaptations (Talanian *et al.*, 2007; Perry *et al.*, 2008; Little *et al.*, 2010). Specifically, these studies have employed intervals ≥1 min in duration performed at an intensity of 90-100% peak power output, and therefore energy production will be predominantly met by aerobic metabolism (Medbo & Tabata, 1993). In contrast, anaerobic energy production predominates during shorter (≤ 30s) high intensity exercise (Medbo & Tabata, 1993), although the contribution from oxidative phosphorylation increases during consecutive bouts of high intensity exercise (Parolin *et al.*, 1999). The present study aimed to employ a practical high intensity interval training protocol that retained both the metabolic stimulus and time saving benefits associated with Wingate-based SIT. To this end, a protocol employing repeated bouts of high intensity interval training performed at a constant workload equivalent to 200% peak power output was chosen for investigation in the present study.

Obese individuals exhibit increased plasma concentrations of triglyceride and total cholesterol (Pi-Sunyer, 2002; Mokdad *et al.*, 2003), and these factors are associated with a greater risk of developing cardiovascular disease (Hokanson & Austin, 1996). ET has been shown to reduce the plasma concentrations of triglyceride and cholesterol (Durstine & Haskell, 1994), although whether high intensity interval training has a similar effect is yet to be investigated. Notably, in Chapter 4 it was reported that like moderate-intensity ET, 6 weeks of Wingate-based SIT in sedentary lean males improved insulin sensitivity. Therefore, to test the effectiveness of the constant-workload SIT protocol developed in the present study, the hypothesis that 4 weeks of constant-workload SIT would improve insulin sensitivity, VO_{2peak}, and reduce cardiovascular disease riskin sedentary obese males was first investigated. It was also reported in Chapter 4 that improvements in IMTG metabolism following both SIT and ET likely contribute to the underlying mechanism of an increase in insulin sensitivity. The second hypothesis therefore investigated whether constant workload SIT would enhance IMTG concentration and the protein expression of PLIN2 and PLIN5.

6.3 Methods

Subjects and ethical approval

Sixteen healthy, sedentary obese (BMI >30 $kg.m^{-2}$) males volunteered to take part in the study (see table 2 for characteristics), which was approved by the Black Country NHS Research Ethics Committee (West Midlands, UK). Following a verbal and written explanation of the nature and risks involved in the experimental procedure, written, informed consent was obtained from all volunteers. Prior to entering the study, all subjects underwent a medical screening procedure to check that there were no underlying cardiovascular abnormalities and they were free of any known metabolic disease. Specifically, all subjects completed a general health questionnaire, after which a resting 12 lead ECG was performed by a medical professional, who also obtained a brief medical history from each subject. Following this examination, volunteers were deemed eligible if they had also engaged in less than two 30 min sessions of physical activity per week in the preceding year.

Experimental procedures

Subject's initially performed a progressive exercise test to volitional exhaustion on an electronically braked cycle ergometer (Lode BV, Groningen, The Netherlands) in order to determine peak oxygen uptake (VO_{2peak}) and maximum workload (W_{max}), as previously described (Chapters 2, 4, 5). During this visit subjects were allocated to their training intervention. Accordingly the 16 subjects were divided into pairs with the best possible match for age, BMI and VO_{2peak}, with one member from each pair randomly assigned to SIT and the other to the ET group. Following sufficient rest, those subjects assigned to the SIT group were familiarised to the SIT protocol through the performance of one 30 s constant-workload sprint on a cycle ergometer. The load that the subject's pedalled against during each sprint was equivalent to 200% W_{max} achieved during the progressive exercise test.

At least 3 days following the progressive exercise test subject's reported to the laboratory after an overnight fast (> 10 h) and the thigh of one leg was prepared for muscle biopsy collection. The leg undergoing muscle biopsy before training was randomised such that the contralateral leg was sampled post-training. A resting muscle biopsy was obtained and prepared for immunohistochemical

analysis as previously described (Chapters 2, 4, 5). Following the muscle biopsy, a 2 h oral glucose tolerance test was performed and plasma glucose and insulin concentrations determined as previously reported (Chapters 4 & 5). Plasma glucose and insulin concentrations were subsequently used to calculate an index of insulin sensitivity (Matsuda & DeFronzo, 1999). An ILab-600 semi-automatic spectrophotometric analyzer was used to determine non-esterified fatty acid (NEFA), triglyceride and total cholesterol concentrations using compatible commercially available kits (NEFA: Randox Laboratories Ltd, Co. Antrim, UK, triglyceride and cholesterol: Instrumentation Laboratory Ltd UK, Warrington, UK). Following the OGTT, body composition analysis was performed using Dual energy X-ray Absorptiometry (DXA), and analyzed for fat mass and fat-free mass. All experimental procedures were repeated at least 48 h following completion of the training period in order to negate any acute effects of the last exercise training bout, and were identical in all respects to those performed before training.

Subjects were provided with all food and drink to be consumed in the 24 h period preceding the experimental procedures. The diet was of a standard macronutrient composition (50% CHO, 30% fat, 20% protein), but adjusted for each individuals habitual energy intake, assessed through the completion of 3 day diet diary. When completing diet diaries, subjects were provided with electronic scales in order to increase the accuracy of portion size determination. All subjects were asked to continue their habitual dietary and physical activity patterns during the training period.

Training interventions

High intensity interval training (SIT): The SIT protocol in the present study was adapted from the protocol previously used by Burgomaster *et al*. (2008) and ourselves (Chapter 4). From our previous study (Chapter 4), we recognised that Wingate-based SIT elicited a mean power output that was equivalent to ~200% maximal workload (W_{max}) achieved during a progressive exercise test to determine VO_{2peak}. Thus, to closely match the work performed during a Wingate test, we developed a SIT protocol that maintains both the metabolic stimulus and the time saving benefits of Wingate-based SIT (Table 6.1). Specifically, subjects in the present study performed repeated 30s sprints on a cycle ergometer against a constant load equivalent to 200% of their W_{max}, as determined during the

progressive exercise test. Each 30s sprint was separated by 2 minutes recovery, during which subjects cycled against a small workload (30 W) maintaining a cadence of < 50 rpm. All participants trained three times a week for four weeks, and were excluded from the study if they were absent from more than two sessions. Participants initially performed four 30 s sprints per training session during week one, after which an additional sprint was included on each consecutive week, such that seven 30 s sprints were performed in the final week of training. Heart rate was recorded throughout each session.

Table 6.1. *Comparison of SIT protocol*

Variable	Wingate SIT (Burgomaster *et al*., 2005)	60 s constant-load SIT (Little *et al*., 2010)	30 s constant-workload SIT (present study)
Protocol	30 s 'all out' effort	60 s constant-load	30 s constant-workload
Training Intensity	Resistance equivalent to 7.5% body mass	100% W_{max}	200% W_{max}
Rest	4.5 min	75 s	2 min
Repetitions	4-7	8-12	4-7
Time commitment:			
Intervals per session	2-3.5 min	8-12 min	2-3.5 min
Total time per session	20-35 min	18-26 min	10-18 min
Total time per week	60-105 min	60-90 min	30-60 min
Energy expenditure	~225 kJ	~210 kJ	~235 kJ

W_{max} maximum workload, *kJ* kilojoules

Endurance training (ET): Subjects in the ET group trained 5 times per week over the 4 week training period, and were excluded from the study if they were absent from more than 2 training sessions. All subjects cycled at a workload equivalent to ~65% $VO_{2\ peak}$ for 40 minutes during the first 7 sessions, increasing to 50 minutes during sessions 8 to 14, and 60 minutes during sessions 15 to 20. $VO_{2\ peak}$ was reassessed after 2 weeks of training and workload adjusted accordingly.

Muscle analysis

Pre- and post-training muscle biopsies were cryosectioned (5 µm) at -30°C on to ethanol-cleaned glass slides and stained for fibre type determination of IMTG (oil red O), cytochrome *c* oxidase (COX), PLIN2, and PLIN5 content using immunofluorescence, as previously described (Chapter 5). Capture of cross-sectional orientated images was identical in all respects to that described previously (Chapter 5). All image processing was undertaken using Image-Pro Plus 5.1 software (Media Cybernetics, MD, USA). Specifically, widefield images were used to assess the fibre specific distribution of IMTG, PLIN2, PLIN5 and mitochondria. In this respect 8 ± 2 images were used per muscle section, resulting in 98 ± 8 fibres analysed per time-point for each subject. The COX staining intensity was used to indicate changes in mitochondrial content after training. An intensity threshold was uniformly selected to represent positive signal for IMTG, PLIN2 and PLIN5. The positively stained area fraction was expressed as a percentage of the total area of each muscle fibre, and was used as a measure of IMTG, PLIN2 and PLIN5 content. Images captured using confocal microscopy were used to assess changes in mean IMTG, PLIN2 and PLIN5 size and density. Density was calculated as the number of IMTG, PLIN2 or PLIN5 objects relative to area. The mean area of individual IMTG (LDs), PLIN2 or PLIN5 objects was used as a measure of size.

Statistics

All data are expressed as means ± S.E.M. Significance was set at the 0.05 level of confidence. A two-factor repeated measures ANOVA was used to assess training-induced changes in parameters of exercise capacity, body composition, and insulin sensitivity, using the between-subject factor '*group*' (SIT vs. ET) and the within-subject factor '*training status*' (pre- vs. post-training). A three-factor repeated measures ANOVA was used to assess changes in COX, IMTG, PLIN2 and PLIN5 content using the within-subject factors '*fibre type*' (type I versus type II fibres) and '*training status*' and between-subject (*group*) factor used in the 2-factor analysis. Bonferroni adjustment post hoc analysis was employed to assess significant main effects or interactions.

6.4 Results

Exercise capacity, body composition, and insulin sensitivity (Table 6.2)

At baseline, no differences between groups were observed for any of the variables mentioned in Table 6.2. The ET group lost 2.2±0.5 kg in response to training (2±1%, group x training interaction; $P < 0.01$), which corresponded to a significant decrease in BMI (2±1%, group x training interaction; $P = 0.01$). No significant changes in weight or BMI occurred in the SIT group. Fasting plasma total triglyceride concentration showed a trend to decrease in response to training (SIT 16±8, ET 10±7%, $P = 0.058$). Both fasting cholesterol concentration (SIT 13±6%, ET 7±5%, main training effect; $P = 0.017$) and fasting NEFA concentration (SIT 21±11%, ET 9±7%, main training effect; $P = 0.023$) was significantly reduced following training. Training induced a significant increase in absolute VO_{2peak} (SIT 7±3%, ET 11±3%, main training effect; $P = 0.01$), and this increase remained when VO_{2peak} was expressed relative to body weight (SIT 7±3%, ET 12±4%, main training effect; $P = 0.01$), with no difference between groups. W_{max} was also increased after training, with no difference between groups (SIT 13±3%, ET 11±4%, main training effect; $P < 0.001$). Absolute fat mass decreased in response to ET only (5±1%; group x training interaction; $P < 0.01$), as did relative fat mass (4±1%; group x training interaction; $P < 0.01$), but no significant changes occurred following SIT. Glucose area under the curve (AUC) was reduced post-training (SIT 7±2%, ET 11±5%, main training effect; $P = 0.003$) with no difference between groups. Similarly, training reduced insulin AUC (HIT 14±3%, ET 17±9%, main training effect; $P = 0.006$). Accordingly, training improved insulin sensitivity, calculated using the Matsuda index (Matsuda & DeFronzo, 1999) (HITSIT 13±5%, ET 23±4%; main training effect, $P = 0.001$, Table 2), with no difference between groups.

Table 6.2. *Subject characteristics*

	Variable	SIT		ET		Training effect	Training x group interaction
		Pre	*Post*	*Pre*	*Post*	(*P* value)	(*P* value)
	Age (yrs)	24 ± 2		26 ± 2			
	Height (m)	1.75 ± 0.03		1.84 ± 0.03			
	Weight (kg)	109.7 ± 4.5	109.4 ± 4.5	113.3 ± 5.5	111.1 ± 5.6*	0.003	0.016
	BMI ($kg.m^{-2}$)	35.8 ± 0.8	35.7 ± 0.8	33.7 ± 1.5	33.1 ± 4.4*	0.004	0.019
Body composition	FM (kg)	32.0 ± 3.0	31.6 ± 3.1	32.5 ± 2.7	30.9 ± 2.7*	<0.001	0.016
	FFM (kg)	66.2 ± 1.9	66.3 ± 1.9	69.8 ± 3.3	70.1 ± 3.2		
	% FM	31.4 ± 2.0	30.9 ± 2.2	30.9 ± 1.8	29.6 ± 1.7*	0.002	0.049
Exercise capacity	$VO_{2\ peak}$ ($L.min^{-1}$)	3.69 ± 0.15	3.97 ± 0.24	3.94 ± 0.14	4.36 ± 0.22	0.002	
	$VO_{2\ peak}$ ($L.min^{-1}.kg^{-1}$)	33.9 ± 1.2	36.3 ± 1.6	35.1 ± 1.5	39.8 ± 2.7	0.002	
	W_{max} (W)	214 ± 14	245 ± 15	249 ± 16	276 ± 16	<0.001	
	200% W_{max} (W)	429 ± 28	491 ± 31	499 ± 32	552 ± 33	<0.001	
Blood lipid profile	Triglycerides ($mmol.L^{-1}$)	1.69 ± 0.17	1.37 ± 0.16	1.63 ± 0.22	1.41 ± 0.21	0.058	
	Total cholesterol ($mmol.L^{-1}$)	6.0 ± 0.5	5.1 ± 0.3	5.4 ± 0.4	4.9 ± 0.3	0.017	
	NEFA ($mmol.L^{-1}$)	0.49 ± 0.04	0.35 ± 0.03	0.49 ± 0.04	0.44 ± 0.04	0.023	
Whole-body insulin sensitivity	Fasting glucose ($mmol.L^{-1}$)	5.5 ± 0.3	5.6 ± 0.2	5.9 ± 0.4	5.6 ± 0.2		
	Fasting insulin ($\mu IU.ml^{-1}$)	23.5 ± 1.7	23.6 ± 2.2	19.6 ± 2.6	20.4 ± 2.5		
	Glucose AUC ($mmol.L^{-1}.min^{-1}$)	996 ± 50	915 ± 46	998 ± 70	880 ± 63	0.003	
	Insulin AUC ($\mu IU.ml^{-1}.min^{-1}$)	11492 ± 1140	12607 ± 1264	16517 ± 828	13597 ± 1339	0.006	
	ISI-Matsuda	1.8 ± 0.1	2.0 ± 0..2	1.8 ± 0.1	2.1 ± 0.2	0.001	

Data provided are means ± S.E.M. (n=8 per group). *BMI* body mass index, W_{max} maximum workload, *FFM* fat free mass, *FM* fat mass, *NEFA* non-esterified fatty acids, *AUC* area under the curve, *ISI* insulin sensitivity index. Body composition data was obtained using a DEXA scan. * Significant training x group interaction, ET vs. SIT ($P < 0.05$).

Muscle analysis

Mitochondrial density (Table 6.3): Immunofluorescence images of the mitochondrial marker COX revealed a greater mitochondrial density (expressed as fluorescence intensity) in type I fibres compared to type II fibres both pre and post-training (main effect of fibre; $P < 0.001$). In response to training, mitochondrial density was significantly increased in both type I and type II fibres (32 ± 4%, 47 ± 8%, respectively, main training effect; $P = 0.01$).

Table 6.3. Effect of 4 weeks of SIT or ET on fibre type specific mitochondrial content

COX expression	SIT		ET	
(fluorescence intensity)	*Pre*	*Post*	*Pre*	*Post*
Type I fibres	37.3 ± 4.3	48.0 ± 6.1†	34.3 ± 1.4	46.5 ± 2.8†
Type II fibres*	22.6 ± 3.0	33.6 ± 4.2†	20.6 ± 1.7	28.0 ± 2.4†

Mitochondrial density (expressed as fluorescence intensity), quantified from immunofluorescence images of COX in type I and type II fibres obtained before and after 6 weeks of SIT or ET. Values are presented as means ± S.E. ($n = 8$ per group). * Main fibre effect ($P < 0.05$ vs. type I fibres). † Main training effect ($P < 0.05$ vs. pre-training).

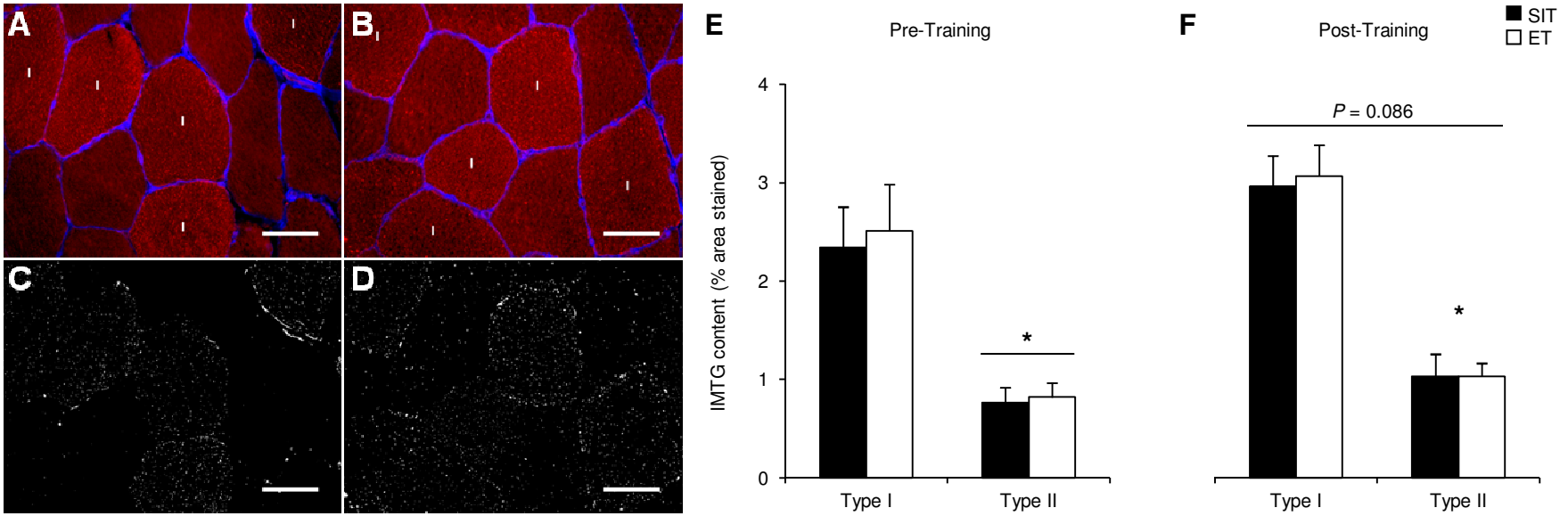
IMTG analysis (Fig. 6.1): IMTG content in type I fibres (expressed as % area stained) was significantly greater than that observed in type II fibres both pre and post-training (main fibre effect; $P < 0.001$). IMTG content showed a trend to increase in response to training in both type I (SIT 48±21%, ET 49±28%) and type II fibres (SIT 55±38%, ET 62±49%) (main training effect; $P = 0.086$), with no difference between groups. LD density (number of LD per area) significantly increased in response to training in both type I (SIT pre 0.079±0.018 LD.μm^{-2} vs. SIT post 0.085±0.011 LD.μm^{-2}, ET pre 0.047±0.007 LD.μm^{-2} vs. ET post 0.068±0.010 LD.μm^{-2}) and type II fibres (SIT pre 0.022±0.005 LD.μm^{-2} vs. SIT post 0.043±0.009 LD.μm^{-2}, ET pre 0.017±0.008 LD.μm^{-2} vs. ET post 0.020±0.004 LD.μm^{-2}, main training effect; $P = 0.033$), with no difference between groups. No significant changes in LD size were observed in response to training in type I fibres (SIT pre 0.48±0.06 μm^2 vs. SIT post

0.51±0.06 μm^2, ET pre 0.37±0.03 μm^2 vs. ET post 0.36±0.02 μm^2) or type II fibres (SIT pre 0.50±0.04 μm^2 vs. SIT post 0.41±0.05 μm^2, ET pre 0.33±0.03 μm^2 vs. ET post 0.32±0.03 μm^2) (main training effect; $P = 0.3$), with no difference between groups.

PLIN analysis: PLIN2 content in type I fibres (expressed as % area stained) was significantly greater than that observed in type II fibres both pre and post-training, irrespective of training type (main fibre effect; $P < 0.001$, Fig. 6.2). A training x fibre interaction was observed in response to training, such that PLIN2 content significantly increased in type I fibres (SIT 92±19%, ET 87±23%; $P < 0.001$), but the change did not reach statistical significance in type II fibres (SIT 17±11%, ET 40±10%; $P = 0.186$, Fig. 6.2). No group interactions with fibre type or training mode were observed for PLIN2 content. The increase in PLIN2 content in type I fibres was attributed to a greater PLIN2 density (SIT pre 0.054±0.005 PLIN2.μm^{-2} vs. SIT post 0.075±0.007 PLIN2.μm^{-2}, ET pre 0.069±0.023 PLIN2.μm^{-2} vs. ET post 0.105±0.028 PLIN2.μm^{-2}, training x fibre; $P = 0.01$).

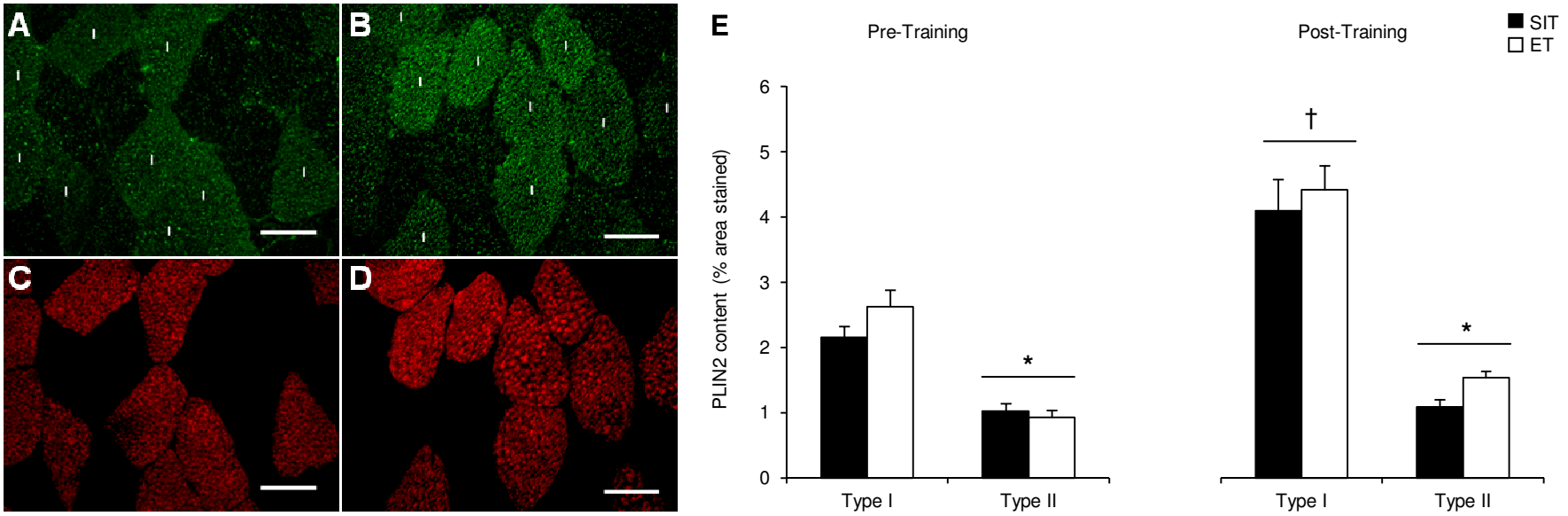
PLIN5 content in type I fibres was significantly greater than observed in type II fibres both pre- and post-training, with no difference between groups (main fibre effect; $P < 0.001$, Fig. 6.3). A training x fibre interaction was observed in response to training, such that PLIN5 content increased in type I fibres (SIT 54±15%, ET 36±10%; $P = 0.003$), but not in type II fibres ($P = 0.287$, Fig. 6.3). No group interactions with fibre type or training mode were observed for PLIN5 content. The increase in PLIN5 content in type I fibres was attributed to a greater PLIN5 density (SIT pre 0.057±0.008 PLIN5.μm^{-2} vs. SIT post 0.076±0.010 PLIN5.μm^{-2}, ET pre 0.050±0.009 PLIN5.μm^{-2} vs. ET post 0.078±0.014 PLIN5.μm^{-2}, training x fibre; $P = 0.006$).

Fig. 6.1 Effect of 4 weeks constant-workload SIT and ET on fibre-specific IMTG content



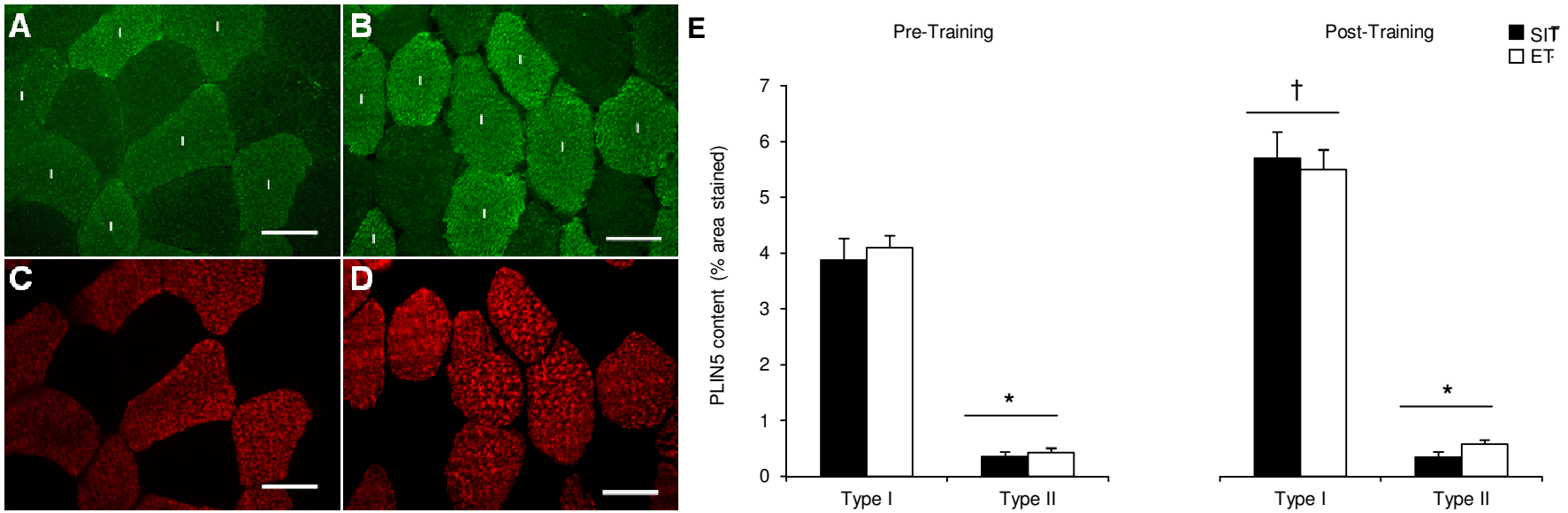
Representative immunofluorescence images of IMTG (stained red) in combination with WGA to identify the cell border (stained blue) in skeletal muscle, pre *(A)* and post *(B)* 4 weeks of SIT. Type I fibres are indicated with "I", all other fibres are assumed to be type II fibres. White bar = 50µm. Images *(C)* and *(D)* display the ORO signal obtained after the intensity threshold was applied during quantitation (showing the LDs in white), before and after HIT, respectively. *(E)* IMTG content, quantified from immunofluorescence images of oil red O-stained muscle sections, in type I and type II fibres before and after 4 weeks of HIT (closed bars) and ET (open bars). Values are presented as means ± S.E.M. (n=8 per group). * Main effect of fibre ($P < 0.05$ vs. type I fibres).

Fig. 6.2 Effect of 4 weeks constant-workload SIT and ET on fibre-specific PLIN2 expression



Representative immunofluorescence images of PLIN2 (stained green) in skeletal muscle, pre *(A)* and post *(B)* 4 weeks of SIT, and corresponding images *(C, D)* of MHC I (stained red), used for fibre type determination. Type I fibres are indicated with a "I", all other fibres are assumed type II fibres. White bar = 50µm. *(E)* PLIN2 content, quantified from immunofluorescence images of PLIN2-stained muscle sections, in type I and type II fibres before and after 4 weeks of SIT (closed bars) and ET (open bars). Values are presented as means ± S.E (n=8 per group). * Main effect of fibre ($P < 0.05$ vs. type I fibres). † Training x fibre interaction ($P < 0.05$ vs. type I fibres pre-training).

Fig. 6.3 Effect of 4 weeks constant-workload SIT and ET on fibre-specific PLIN5 expression



Representative immunofluorescence images of PLIN5 (stained green) in skeletal muscle, pre *(A)* and post *(B)* 4 weeks of SIT, and corresponding images *(C, D)* of MHC I (stained red), used for fibre type determination. Type I fibres are indicated with a "I", all other fibres are assumed type II fibres. White bar = 50µm. *(E)* PLIN5 content, quantified from immunofluorescence images of PLIN5-stained muscle sections, in type I and type II fibres before and after 4 weeks of SIT (closed bars) and ET (open bars). Values are presented as means ± S.E (n=8 per group). * Main effect of fibre ($P < 0.05$ vs. type I fibres). † Training x fibre interaction ($P < 0.05$ vs. type I fibres pre-training).

Correlation analyses

For correlation analyses, data obtained for type I and type II fibres, pre and post-training were pooled (Table 6.4). IMTG content showed a positive correlation with PLIN2 content (R = 0.56; $P < 0.01$), PLIN5 content (R = 0.48; $P < 0.05$) and COX intensity (R = 0.51, $P < 0.01$). Insulin sensitivity was strongly associated with COX intensity (R = 0.65; $P < 0.001$), but did not correlate with IMTG content PLIN2 or PLIN5 expression.

Table 6.4. *Bivariate correlation analysis*

	IMTG	Insulin sensitivity
IMTG		0.23
PLIN2	0.56†	0.14
PLIN5	0.48*	0.17
COX	0.51†	0.65‡

IMTG, PLIN2 and PLIN5 content and COX fluorescence intensity represent pre-exercise values. IMTG utilisation refers to the absolute change in IMTG concentration from pre to post-exercise. Values represent Pearson's squared correlation coefficients (R values) for relationships between variables. All data obtained for type I and type II fibres, pre and post-training were pooled for correlation analysis. Significant correlations: *$P < 0.05$, $^{\dagger}P < 0.01$, $^{\ddagger}P < 0.001$.

6.5 Discussion

This study is the first to report that 4 weeks of constant-workload SIT improves insulin sensitivity in sedentary, obese males, in line with the first hypothesis. Further, this improvement was accompanied by both an increased aerobic exercise capacity (VO_{2peak}) and greater mitochondrial density, as well as a reduction in fasting plasma free fatty acids and total cholesterol concentration. In support of the second hypothesis, expression of both PLIN2 and PLIN5 was enhanced in type I muscle fibres following both constant-workload SIT and ET, although this occurred without a significant increase in IMTG content. Together, these results suggest that greater expression of PLIN2 and PLIN5 contribute to the mechanism that leads to improvements in insulin sensitivity following 4 weeks of constant-workload SIT and ET.

The 4 week constant-workload SIT protocol, using 30s exercise bouts at 200% W_{max}, employed in the present study elicited similar heart rate responses (~95% HR_{max}) to those observed using Wingate-based SIT in Chapter 4 (data not shown). The new protocol of constant-workload SIT successfully increased skeletal muscle mitochondrial density concomitant with an improved aerobic exercise capacity, which is consistent with a number of previous short term training studies in lean and obese individuals using both Wingate-based SIT (Burgomaster *et al.*, 2005; Gibala *et al.*, 2006; Burgomaster *et al.*, 2008; Whyte *et al.*, 2010) and 60 s constant-load HIT protocols (Little *et al.*, 2010; Hood *et al.*, 2011; Little *et al.*, 2011a). Notably, mitochondrial density increased similarly in type I and type II fibres, in line with a previous study performed in lean sedentary individuals (Chapter 4), demonstrating that the 30s constant-workload SIT protocol employed in the present study is also an effective intervention to enhance skeletal muscle oxidative capacity in obese individuals. This is an important finding, given that obese individuals and/or obese type 2 diabetes patients commonly display a reduced muscle oxidative capacity (Kelley *et al.*, 2002; Ritov *et al.*, 2005; Toledo *et al.*, 2006), which appears to be due to a reduced mitochondrial density rather than diminished function (Boushel *et al.*, 2007; Holloway *et al.*, 2007), and is strongly associated with the development of insulin resistance (Bruce *et al.*, 2003). In this respect, improvements in oxidative capacity in response to 4 weeks constant-workload SIT in the present study were paralleled by enhanced insulin sensitivity. This finding is consistent with previous reports describing improved insulin sensitivity in lean, healthy

individuals undertaking Wingate-based SIT (Babraj *et al.*, 2009; Richards *et al.*, 2010)(Chapter 4) and overweight individuals and obese type 2 diabetes patients undertaking 60 s constant-load SIT (Hood *et al.*, 2011; Little *et al.*, 2011a). Thus, the 30s constant-workload SIT protocol described in the present study provides an effective stimulus to induce improvements in insulin sensitivity in young, obese individuals.

A high flux of fatty acids being delivered to and transported into skeletal muscle leads to reductions in insulin sensitivity (Bachmann *et al.*, 2001). In addition, high fasting plasma triglyceride concentrations are associated with insulin resistance and cardiovascular disease, as a result of greater lipid delivery and uptake into skeletal muscle. Notably, there was a significant reduction in fasting plasma fatty acid and total cholesterol concentration, in addition to a trend for a reduction in the fasting plasma total triglyceride concentration ($P = 0.058$) in response to training. These observations are consistent with previous reports in sedentary lean and obese individuals undertaking 12 weeks of ET (Schrauwen *et al.*, 2002; Solomon *et al.*, 2009), and for the first time illustrate the beneficial impact of constant-workload SIT on risk factors of cardiovascular disease.

Contrary to our hypothesis, IMTG concentration was not significantly increased following either constant-workload HIT or traditional moderate-intensity ET, although there was a trend for a main effect of training to enhance IMTG concentration ($P = 0.086$). This finding is in contrast to Chapter 4 where 6 weeks of Wingate-based SIT and ET resulted in similar increases in IMTG content in lean, sedentary individuals (Chapter 4). Notably, obese individuals and/or type 2 diabetes patients demonstrate increased IMTG stores in response to long term ET (>16 weeks) (Dubé *et al.*, 2008; Dubé *et al.*, 2011)(Chapter 3), indicating that further increases in IMTG concentration with training occur in those individuals who may already exhibit elevated IMTG stores (van Loon *et al.*, 2004; Amati *et al.*, 2011). This observation suggests that the duration of the training period may have been insufficient to induce significant increases in IMTG content, despite observing similar improvements in fibre-specific mitochondrial density using constant-workload SIT and ET compared to those previously reported for Wingate-based SIT and ET using the same number of subjects (Chapter 4). Further inspection of the data in the present study revealed that two individuals from each group failed to

increase their IMTG content in type I fibres (Fig. 2), suggesting that a lack of statistical power may also contribute to the current finding. Indeed, calculating the power required to obtain a significant increase in IMTG concentration using the observed variation in the current data set revealed that 12 participants per group would have been needed to observe a main effect of training. Despite the lack of an increase in IMTG concentration following training, a significant increase in IMTG density occurred, which is in line with previous reports following both SIT and ET interventions (Tarnopolsky *et al.*, 2007)(Chapter 4). Since a greater number of IMTG-containing LDs would present an enhanced surface area available for the interaction with lipolytic enzymes with the regulatory proteins contained on the LD surface, this finding indicates a shift to a more metabolically flexible intracellular lipid store.

Two of the regulatory proteins contained within the phospholipid monolayer of IMTG-containing LDs are PLIN2 and PLIN5. In the present study it was found that young, insulin-sensitive obese individuals demonstrate ~two-fold greater PLIN2 protein expression in type I compared to type II fibres, a finding that is consistent with previous observations in lean sedentary (Chapter 2, 4, 5) and trained (Shaw *et al.*, 2009) individuals. In addition, a large (~10-fold) difference in PLIN5 expression between type I and type II fibres was observed before training. Importantly, the expression of PLIN2 and PLIN5 in type I fibres is significantly enhanced in response to constant-workload SIT in the present study. Furthermore, the increase in PLIN2 and PLIN5 expression following constant-workload HIT is similar to that induced by traditional moderate-intensity ET. This finding is consistent with a previous report demonstrating similar increases in both PLIN2 and PLIN5 expression in response to Wingate-based HIT and traditional ET in lean sedentary individuals (Chapter 4). In contrast, a recent study reported that only PLIN5 was enhanced in response to 12 weeks ET in obese males, whereas no change in PLIN2 expression was observed (Peters *et al.*, 2012). Although the observation regarding PLIN2 expression contradicts the finding of the present study, this may be reconciled by differences in the methodology employed (immunofluorescence microscopy vs. immunoblotting in Peters *et al.*, 2012). Indeed, given that training-induced increases in the expression of PLIN2 and PLIN5 was specific to type I fibres in the present study, this highlights the need to investigate proteins associated with IMTG metabolism in a fibre specific manner.

The data of the present study suggest that exercise training-induced increases in the expression of PLIN2 and PLIN5 appear to precede significant increases in IMTG concentration. This observation is in line with the observation in Chapter 3 of this thesis, demonstrating that PLIN2 expression is enhanced following 2 months of ET in type 2 diabetes patients, whereas a significant increase in IMTG content was only detected following 6 months of ET. Collectively these data suggest that the enhanced expression of the PLIN proteins is an early training adaptation that is necessary for increased IMTG content to occur in response to training interventions. In support of this hypothesis, cultured cells overexpressing PLIN2 or PLIN5 exhibit lower rates of basal lipolysis compared to when PLIN2 or PLIN5 is not expressed or knocked-down, and as a result cellular TAG concentration is increased (Imamura *et al.*, 2002; Wolins *et al.*, 2006b; Listenberger *et al.*, 2007; Bell *et al.*, 2008; Wang *et al.*, 2011a; Wang *et al.*, 2011b). Indeed, it appears that upregulating PLIN2 and/or PLIN5 expression is a prerequisite for greater TAG storage, since PLIN2 knockdown in myotubes prevents TAG accumulation and LD formation upon oleate or palmitate incubation (Bosma *et al.*, 2012a). Furthermore, PLIN5 overexpression in alpha mouse liver 12 cells enhances palmitate incorporation into TAG leading to an increase in cellular TAG concentration (Wolins *et al.*, 2006b; Wang *et al.*, 2011b). Therefore, it is likely that augmenting PLIN2 and PLIN5 expression is first required in order to facilitate an increase in IMTG content in response to both constant-workload SIT and ET. Notably, overexpression of PLIN2 in vivo promotes intracellular lipid storage as TAG rather than DAG, thereby maintaining insulin sensitivity (Bosma *et al.*, 2012a). Therefore, greater PLIN2 and/or PLIN5 expression following exercise training would presumably help maintain low concentrations of specific DAG and ceramide species leading to improved insulin sensitivity, as observed following ET (Dubé *et al.*, 2008; Dubé *et al.*, 2011). In accordance, we propose that enhanced expression of PLIN2 and PLIN5 contributes to the improvements in insulin sensitivity observed in the current study in obese individuals following constant-workload SIT and ET.

As PLIN2 and PLIN5 content is increased in the present study, while IMTG content is not significantly enhanced, this suggests that a greater PLIN2:IMTG and PLIN5:IMTG ratio exists following training. Presumably this implies that the LD surface coverage by PLIN2 and PLIN5 is enhanced following training, indicating that the capacity for lipolytic enzymes to target the LD and breakdown IMTG during

a bout of moderate-intensity exercise is increased. Future research should investigate whether IMTG breakdown is already increased after 4 weeks of SIT and ET in obese individuals and whether further increases in IMTG content and the PLIN:IMTG ratio occurs after more prolonged training interventions also leading to higher rates of IMTG breakdown during exercise. In addition, it will be of interest to determine whether SIT or ET is superior with regards to improving the primary outcome measure of the present study, insulin sensitivity. Indeed, although many of the training effects in the present study were of a similar magnitude between the two groups (including insulin sensitivity), it is clear that larger-scale studies are required in order to determine which training mode is superior for inducing positive metabolic adaptations.

In conclusion, this study demonstrates that a new protocol of constant-workload SIT, using 30 s exercise bouts at 200% W_{max} and thus preserving the time-efficient nature of the Wingate-based HIT protocol, provides an effective stimulus to induce improvements in whole body insulin sensitivity in sedentary obese individuals. Furthermore, in accordance with previous studies the type I fibre-specific increase in PLIN2 and PLIN5 expression may be instrumental to the improvement in insulin sensitivity. Finally, this study is the first to show that a SIT protocol achieves measurable and similar reductions in the fasting plasma fatty acid, TG and cholesterol concentration as ET and leads to a reduction in these risk factors for cardiovascular disease after only 4 weeks. Therefore, this study adds to the growing evidence that SIT may provide an effective tool to improve metabolic health in sedentary obese individuals.

Chapter 7

General Discussion

7.1 Overview

Significant changes in lifestyle have occurred over the last 40 years, such that performing regular physical activity is no longer a priority, while the consumption of energy-dense food with poor nutritional value has increased. As a result, the prevalence of many major non-communicable diseases has increased, including obesity, insulin resistance, metabolic syndrome, type 2 diabetes and cardiovascular disease. The challenge to researchers is therefore to develop therapeutic interventions based on a thorough understanding of the underlying mechanisms. Skeletal muscle is the predominant site of glucose disposal following the ingestion of a mixed meal (Ferrannini *et al.*, 1985; Capaldo *et al.*, 1999), and therefore plays a key role in maintaining glucose homeostasis. However, dysregulation of lipid metabolism in skeletal muscle is associated with reductions in glucose tolerance and insulin sensitivity. In contrast, acute and chronic exercise training is associated with improvements in glucose tolerance and insulin sensitivity, due in part to an increased capacity for IMTG breakdown and enhanced regulation of the turnover of the intramuscular lipid pool. However, the underlying mechanisms regulating the turnover of the IMTG pool were only partially understood at the start of the PhD project. In particular, very little information was available concerning the potential importance of the PLIN proteins in regulating both basal and stimulated lipolysis of intracellular lipid stores. In addition, whether more time-efficient modes of exercise training, such as sprint interval training (SIT), elicit similar improvements in lipid metabolism and insulin sensitivity as traditional moderate-intensity endurance training (ET) had not been studied. Consequently the aims of this thesis were 1) to investigate the effect of a single bout of moderate-intensity cycling exercise on the association between perilipin 2 (PLIN2) and intramuscular triglyceride (IMTG), 2) to assess the effect of ET on the expression of PLIN2 in type 2 diabetes patients, 3) to investigate whether PLIN2 and PLIN5 play a role in the ET-induced increase in IMTG utilisation during exercise in previously sedentary individuals, and if similar adaptations are observed following SIT, 4) to establish whether RT also enhances content and breakdown via a similar mechanism as ET and SIT, and 5) to investigate whether ET and constant-workload SIT-induced improvements in insulin sensitivity in obese individuals are associated with similar adaptations in IMTG metabolism as seen in previously sedentary individuals.

7.2 The role of PLIN2 and PLIN5 in TAG hydrolysis

Studies performed under *in vitro* conditions in cultured cells have established that the perilipin (PLIN) proteins play a regulatory role in the hydrolysis of triacylglycerol (TAG) (outlined in Chapter 1.3.3ii). However, whether the PLIN proteins perform a similar function in skeletal muscle *in vivo* is currently not known. Based on the evidence that IMTG in type I fibres provides a substrate source during exercise (Chapter 1.3.2), Chapter 2 demonstrates that LDs containing PLIN2 within the phospholipid monolayer are preferentially broken down during an acute bout of moderate-intensity endurance exercise in 7 sedentary males, compared to those LDs which do not contain PLIN2. Therefore, the presence of PLIN2 in the protein coat of LDs appears to be one factor mediating IMTG breakdown during exercise. Chapter 4 subsequently confirms this finding, and also establishes that a similar relationship exists for PLIN5, such that LDs containing PLIN5 are preferentially broken down during an acute bout of exercise, compared to those LDs not containing PLIN5. As discussed in both Chapter 2 and 4, the assays used in these studies do not permit identification of LDs that contain both PLIN2 and PLIN5, compared to those LDs that only contain PLIN2 or PLIN5. Furthermore, whether other PLIN proteins, such as PLIN3 and PLIN4, are also found in the protein coat of LDs in skeletal muscle was not investigated. Nevertheless, the findings of Chapters 2 and 4 do suggest that LDs containing either PLIN2 or PLIN5 may confer an advantage over those LDs not containing PLIN2 or PLIN5 when activation of lipolysis is required. Therefore, the results of Chapter's 2 and 4 are consistent with the mechanisms proposed by *in vitro* work in which PLIN2 and PLIN5 mediate the interaction of the lipolytic enzymes with the LD, thereby regulating the rate of IMTG hydrolysis. It has recently been reported that PLIN5 also recruits mitochondria to the surface of LDs (Bosma *et al.*, 2011; Wang *et al.*, 2011b). Thus it appears that PLIN5 regulates the rate of TAG hydrolysis, and ensures that fatty acids liberated from intracellular TAG stores are efficiently channelled to mitochondria to undergo β-oxidation.

7.3 The effect of exercise training on skeletal muscle PLIN2 and PLIN5 expression and insulin sensitivity

7.3.1 Endurance training

A number of cross-sectional and longitudinal studies report that prolonged traditional moderate-intensity ET leads to an increase in IMTG concentration and concomitantly enhances the capacity to break down IMTG during exercise (Chapter 1.6.3). Chapter 4 confirms that 6 weeks ET induces these adaptations in lean, sedentary individuals. In addition to augmenting IMTG concentration, 6 weeks ET also leads to an increase in the protein expression of PLIN2 and PLIN5 in skeletal muscle in both type I and type II fibres. A recent study also demonstrates, using immunoblotting methodology, that PLIN5 is increased in lean individuals following 12 weeks of ET (Peters *et al.*, 2012). However, the same study also reported a decrease in PLIN2 protein expression following ET (Peters *et al.*, 2012), which is in contrast to the increase in PLIN2 protein content described in Chapter 4 and observed using both immunoblotting and immunofluorescence methodology.

It is perhaps more relevant to understand the relationship between IMTG concentration and the expression of the PLIN proteins. Indeed, it has been previously reported that the expression of PLIN2 and PLIN5 correlates with IMTG concentration (Minnaard *et al.*, 2009; Amati *et al.*, 2011; Peters *et al.*, 2012), and more evidence was obtained to support this observation in Chapters 4, 5 and 6 of this thesis. In addition, PLIN2 and PLIN5 expression is greater in type I compared to type II fibres in both lean and obese sedentary individuals (Chapters 2, 4, 5 & 6) (Shaw *et al.*, 2009), and is consistent with the fibre type specific distribution of IMTG (Koopman *et al.*, 2001; Malenfant *et al.*, 2001). Notably, the fraction of IMTG associated with either PLIN2 or PLIN5 in type I fibres was similar before and after 6 weeks ET (Chapter 4), and significant breakdown of PLIN2-containing LDs and PLIN5-containing LDs during exercise was observed both before and following ET in type I fibres. Given that the association of PLIN2 and/or PLIN5 with IMTG appears to be one factor mediating IMTG breakdown (Chapters 2 & 4), an increase in the expression of PLIN2 and PLIN5 is therefore presumably required to enable greater use of IMTG as a substrate source during exercise following ET. A number of studies performed in cultured cells *in vitro* also indicate that expression of PLIN2 and/or PLIN5 is

required to maintain low rates of basal lipolysis (Dalen *et al.*, 2007; Listenberger *et al.*, 2007; Bell *et al.*, 2008; Wang *et al.*, 2011a; Wang *et al.*, 2011b). Given that ET enhances the LD surface area available for the interaction of lipolytic enzymes with the stored TAG, presumably an increase in PLIN2 and PLIN5 expression is required to preserve the coverage of the LD surface area, thereby maintaining low basal lipolytic rates. Taken together these observations suggest that PLIN2 and/or PLIN5 may play a key role in efficiently coupling the rate of TAG hydrolysis to the metabolic demand for fatty acids.

Chapter 3 demonstrates that obese type 2 diabetes patients do not exhibit a fibre type difference in PLIN2 expression, as observed in lean individuals (Shaw *et al.*, 2009)(Chapters 2 and 4), whereas IMTG concentration is greater in type I compared to type II fibres, as previously reported (Koopman *et al.*, 2001; Malenfant *et al.*, 2001). Thus a disparity between PLIN2 expression and IMTG concentration in type I fibres appears to exist in type 2 diabetes patients. Given the potential role of PLIN2 in IMTG hydrolysis, this observation may explain, at least partly, why type 2 diabetes patients are unable to break IMTG down as a substrate source during exercise (van Loon *et al.*, 2005a). Similarly, Chapter 6 demonstrates that the protein expression of both PLIN2 and PLIN5 is increased following ET in obese individuals, whereas IMTG concentration is not significantly enhanced. This provides further evidence to suggest that in sedentary obese individuals the expression of PLIN2 and PLIN5 is not well aligned to the concentration of IMTG and therefore potentially limits IMTG breakdown before training.

The athlete's paradox describes a state in which endurance trained athletes combine elevated IMTG concentrations with high levels of insulin sensitivity (Goodpaster *et al.*, 2001; van Loon *et al.*, 2004; Amati *et al.*, 2011). In contrast, elevated IMTG concentrations in sedentary obese individuals and type 2 diabetes patients are associated with insulin resistance (Phillips *et al.*, 1996a; Pan *et al.*, 1997; Goodpaster *et al.*, 2001). Consequently, it is believed that the flexibility of the IMTG pool to respond to the metabolic demands placed on skeletal muscle, rather than the absolute concentration of IMTG per se, more accurately determines insulin sensitivity (van Loon & Goodpaster, 2006; Moro *et al.*, 2008). To this end, an imbalance between free fatty acid uptake, IMTG synthesis, IMTG lipolysis and fatty

acid oxidation is associated with the accumulation of fatty acid metabolites, such as long chain acyl-CoA, DAG and ceramide, which subsequently contribute to the development of insulin resistance (van Loon & Goodpaster, 2006; Moro *et al.*, 2008; Shaw *et al.*, 2010). Thus, tight regulation of IMTG hydrolysis and IMTG synthesis in response to changes in intramuscular fatty acid concentration and the demand for fatty acids for energy production may be important in order to maintain high levels of insulin sensitivity. In obese individuals and type 2 diabetes patients, where the expression of PLIN2 and PLIN5 is low for the high IMTG content, it is plausible that intracellular lipases may have unregulated access to the stored TAG, leading to a mismatch between TAG hydrolysis and the oxidation of liberated fatty acids. As a result, insulin resistance-inducing fatty acids metabolites will accumulate and contribute to the development of insulin resistance. Importantly, in both obese individuals (Chapter 6) and type 2 diabetes patients (Chapter 3), prolonged ET was able to increase the expression of both PLIN2 and PLIN5. Presumably, this adaptation would have corrected the shortfall in PLIN2 and/or PLIN5 expression in relation to IMTG content, thereby enhancing the regulation of IMTG hydrolysis at rest and during exercise, which in turn may drive the ET-induced improvements in insulin sensitivity.

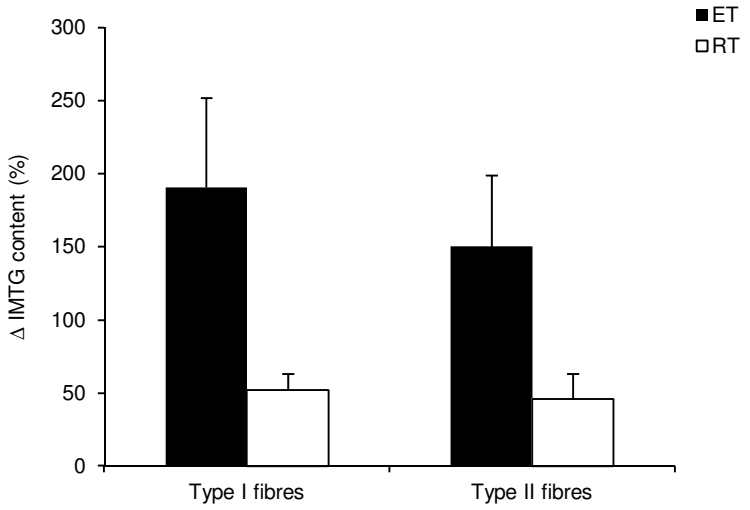
7.3.2 Resistance training

Chapter 5 aimed to investigate whether improvements in IMTG metabolism contribute to the well-documented increase in insulin sensitivity that occurs in response to chronic RT (Miller *et al.*, 1994; Eriksson *et al.*, 1997; Holten *et al.*, 2004; Gordon *et al.*, 2009; Hills *et al.*, 2010). 6 weeks of whole-body RT in lean, sedentary males enhanced insulin sensitivity concomitant with an increase in resting IMTG concentration and breakdown during an acute bout of moderate-intensity endurance-type exercise. In addition, the protein expression of both PLIN2 and PLIN5 was enhanced, and the oxidative capacity of the muscle was also increased. Taken together, these findings suggest that RT also leads to tighter regulation of IMTG hydrolysis at rest and during exercise, and that this adaptation, similar to ET, contributes to the improvements in insulin sensitivity.

The finding of an increase in IMTG concentration following RT is in contrast to a recent study that reported no effect of 11 weeks RT on IMTG content (Ngo *et al.*, 2012). However, this study combined

lower-body ET with upper-body RT and sampled the *vastus lateralis* and *deltoid* muscles, respectively, in order to directly compare the effect of ET and RT in one individual. Thus, it is not reasonable to directly compare the results from this study to those obtained in Chapter 5. Furthermore, it is difficult to draw definitive conclusions from this study since large differences exist between upper and lower body muscles with regards to metabolism and fibre type differences (Mygind, 1995; Van Hall *et al.*, 2003). However, the study of Ngo et al. (2012) does highlight an interesting comparison. Consequently, the results of Chapters 4 and 5 (ET and RT, respectively) are compared in Fig. 7.1, as the results of these studies were obtained using the same experimental conditions.

Fig. 7.1 Comparison of ET and RT induced changes in IMTG content



Relative change (%) in IMTG content in type I and type II fibres in response to 6 weeks of ET (closed bars) or RT (open bars) in lean sedentary males.

Figure 7.1 demonstrates that when the two forms of exercise training are compared, ET promotes the greatest increase in IMTG content. Nevertheless, the magnitude of the increase in the Matsuda index of insulin sensitivity is similar between the groups (ET 29 ± 12%, RT 37 ± 12%). Therefore, whilst both forms of exercise training are similarly effective in enhancing insulin sensitivity, improvements in IMTG metabolism may explain a greater proportion of the increase in insulin sensitivity observed following ET. However, as discussed in Chapter 5, improvements in IMTG metabolism should also be considered a contributing factor to the increase in insulin sensitivity following RT.

7.3.3 High intensity interval training

Improvements in insulin sensitivity have been observed in response to HIT in lean sedentary (Babraj *et al.*, 2009; Richards *et al.*, 2010) and overweight individuals (Hood *et al.*, 2011), and in type 2 diabetes patients (Little *et al.*, 2011a). However, the mechanisms underlying the improvements in insulin sensitivity have not been well-studied. Chapter 4 aimed to investigate whether improvements in IMTG metabolism may contribute to the observed increases in insulin sensitivity following SIT. In response to 6 weeks of Wingate-based SIT in sedentary, lean males, insulin sensitivity was improved concomitant with an increase in IMTG concentration and an enhanced capacity for IMTG breakdown during moderate-intensity exercise was observed. Furthermore, SIT increased skeletal muscle oxidative capacity, as previously reported (Burgomaster *et al.*, 2005; Gibala *et al.*, 2006; Burgomaster *et al.*, 2008), and also augmented the protein expression of both PLIN2 and PLIN5, and together these adaptations appear to be mechanistically linked to the observed improvement in IMTG breakdown during exercise. Therefore, like ET, the improvements in insulin sensitivity in response to SIT can be explained, at least partly, by an enhanced metabolic flexibility of the IMTG pool.

In Chapter 4, a single SIT session required subjects to perform repeated Wingate tests interspersed with periods of active recovery, as established by Gibala and colleagues (Burgomaster *et al.*, 2005; Gibala *et al.*, 2006). However, this form of SIT requires specialised equipment, high levels of internal and external motivation, and may also induce feelings of light headedness and nausea, suggesting that Wingate-based SIT is not particularly tolerable or appealing for many individuals. To overcome these problems, Chapter 6 aimed to establish and investigate the effectiveness of a novel practical SIT protocol, using the 'constant-workload' approach, as a means to improve insulin sensitivity. Accordingly, Chapter 6 demonstrates that 4 weeks 'constant-workload' SIT in obese individuals improves insulin sensitivity. Furthermore, constant-workload SIT tended to increase IMTG content, and significantly enhanced skeletal muscle oxidative capacity and the protein expression of both PLIN2 and PLIN5. As with Wingate-based SIT, the magnitude of the changes in oxidative capacity, IMTG, PLIN2 and PLIN5 content and insulin sensitivity following 'constant-workload' SIT were similar to those observed following 4 weeks of traditional ET. Therefore, as with ET, the improvements in

insulin sensitivity induced by 'constant workload' SIT appear to be, in part, attributable to enhanced regulation of IMTG metabolism.

The results of Chapters 4 and 6 provide evidence to suggest that improved regulation of the IMTG pool is one mechanism that underlies the improvement in insulin sensitivity observed following SIT. In addition, as a "lack of time" represents the most commonly cited barrier to regular exercise participation (Stutts, 2002), the observation that SIT and ET induce similar physiological and metabolic adaptations provides a basis for using SIT as a means to maintain or improve metabolic health. Whether SIT remains a viable alternative to ET outside of the laboratory in a 'real world' environment should form the basis of future investigations.

7.3.4 General reflections on the role of PLINs in regulating IMTG utilization and insulin sensitivity in human skeletal muscle

In Chapters 4 and 5 of this thesis, it is reported that exercise training enhances IMTG breakdown during a bout of moderate-intensity exercise. These observations are consistent with reports demonstrating that the IMTG pool makes a significant contribution to total substrate oxidation during exercise in endurance-trained athletes (van Loon *et al.*, 2003; Stellingwerff *et al.*, 2007), and from cross-sectional comparisons between trained and untrained individuals (Klein *et al.*, 1994; Coggan *et al.*, 2000). In contrast, it appears that IMTG does not provide a readily available fuel source during exercise in sedentary, obese and/or type 2 diabetes patients (Blaak *et al.*, 2000; Schrauwen *et al.*, 2002). Consequently, it is suggested that the capacity to utilise IMTG during exercise (indicative of a metabolically flexible IMTG pool) is associated with insulin sensitivity.

A number of different methods of investigating IMTG utilisation during exercise are available, including the use of stable isotopic tracers to indirectly quantify the sum of IMTG plus lipoprotein-TG oxidation, as well as directly measuring IMTG content before and after exercise using both non-invasive (^{1}P-MRS) and invasive (biochemical extraction, immunofluorescence microscopy) techniques. Throughout this thesis IMTG content was measured using oil red O staining in combination with myosin heavy chain type I staining, in order to quantify fibre type-specific IMTG content and

breakdown during exercise. In Chapter 2, a ~50% reduction in IMTG content in type I fibres was observed during exercise at ~65% VO2 peak, whereas in Chapter 4 a ~16% decrease in IMTG content in type I fibres was observed using an identical bout of exercise as described in Chapter 2. Despite the differences in the magnitude of change in IMTG content from pre to post exercise between Chapter's 2 and 4, similar rates of fat oxidation (~0.41 $g.min^{-1}$), measured through indirect calorimetry, were observed. This suggests that the contribution of IMTG breakdown to total fat oxidation was different between the two groups, despite the exercise bout being of a similar intensity and duration. Whilst it is difficult to reconcile the differences in IMTG breakdown reported, it does highlight that a large variability in the capacity to use IMTG as a fuel source during exercise may exist in sedentary individuals. This is reflected in the large variation observed in IMTG breakdown during exercise in Chapter 4 (S.E. = ~11%), although a much smaller variation was apparent in Chapter 2 (S.E. = 7%). Therefore, it is also important to consider any methodological limitations which may partly explain the differences in IMTG breakdown between Chapter's 2 and 4. For example, the coefficient of variation for repeated measurements of IMTG content through oil red O staining is between 7 and 14% (Shepherd et al., unpublished observations; De Bock *et al.*, 2005; De Bock *et al.*, 2007). In addition, no estimation of the contribution of plasma FFA to total fat oxidation during exercise was made in Chapter's 2 and 4, making it impossible to conclude that differences in plasma FFA oxidation were apparent between the two studies. Given that plasma FFA availability likely regulates the contribution of IMTG utilisation to total fat oxidation, information regarding plasma FFA concentrations during exercise should be included in future studies, and estimates of plasma FFA oxidation using stable isotopic tracers would provide a more complete view.

An interesting finding of both Chapter's 2 and 4 was that PLIN2 and PLIN5 appear to be important in the breakdown of IMTG during exercise, since LDs containing either PLIN2 and/or PLIN5 are preferentially broken down (compared to those LDs that are not associated with either PLIN2 and/or PLIN5) during moderate-intensity exercise. This finding stemmed from the observation that ~70% of LDs contain either PLIN2 and/or PLIN5 at rest, and in response to exercise it is those PLIN-associated LDs that are reduced. Of course, it is possible, as acknowledged in Chapter 2, that PLIN2 and/or PLIN5 may be present at undetectable levels on those PLIN-null-LDs, rather than being completely

absent. In addition, images used for colocalisation analysis only provide a two-dimensional view, whereas LDs, by nature, are three-dimensional objects and are not at a uniform depth throughout a 5 µm muscle section (as used throughout this thesis). Consequently, the plane in which each LD is imaged and the proportion of each LD that is observed will likely impact the true 'colocalisation value'. Nevertheless, the inter-subject variation in colocalisation values for both PLIN2 and PLIN5 with IMTG before and after exercise was low, suggesting that the technical limitations of colocalisation analysis did not significantly impact the overall outcome. Furthermore, the observation that not all LDs contain PLIN2 and/or PLIN5 is consistent with several *in* vitro observations suggesting that the protein composition of the LD could influence its function (Ducharme & Bickel, 2008; Fujimoto *et al.*, 2008; Wolins *et al.*, 2006), which appears logical given that there are >300 proteins associated with the LD proteome (Zhang *et al.*, 2011).

As described in the previous sections of this discussion, the most consistent finding of this thesis is that exercise training (including HIT, ET or RT) enhances the protein expression of both PLIN2 and PLIN5 in skeletal muscle, and this appears to occur concomitant with an increase in IMTG content. Although Chapter's 4, 5 and 6 all suggest that the increase in PLIN2 and PLIN5 expression parallels the change in IMTG content, in line with several other reports (Minnaard *et al.*, 2009; Amati *et al.*, 2011; Peters *et al.*, 2012), the results of Chapter 3 provide evidence that this might not always be true. To this end, PLIN2 expression increased in type I fibres after 2 months of ET in type 2 diabetes patients, whereas IMTG content in type I fibres was not significantly enhanced (Chapter 3). In addition, between 2 and 6 months of training no change in PLIN2 protein expression was observed in type I fibres, whereas IMTG content was significantly increased, although it is important to acknowledge that this was a significant change from baseline (rather than an increase from 2 to 6 months). Taken together, these data provide evidence that PLIN2 expression is dissociated from IMTG content, contrary to the findings of Chapters 4, 5 and 6, and several other reports (Minnaard *et al.*, 2009; Amati *et al.*, 2011; Peters *et al.*, 2012). However, given that PLIN2 expression did not demonstrate the 'typical' fibre type difference at baseline in Chapter 3, it could be argued that the increase in PLIN2 (and not IMTG content) is a mechanism by which the fibre type difference is re-established. Furthermore, it is possible that the increase in PLIN2 expression from baseline to 2

months is required in order to facilitate an increase in IMTG content from baseline to 6 months. In support, a number of studies performed in cultured cells *in vitro* demonstrate that overexpression of PLIN2 enhances TAG concentration (Imamura *et al*., 2002; Fukushima *et al*., 2005; Listenberger *et al*., 2007; Bosma *et al*., 2012a), whereas knockdown of PLIN2 reduces TAG concentration (Chang *et al*., 2006; Bell *et al*., 2008; Bosma *et al*., 2012a). Therefore, the possibility should be considered that in type 2 diabetes patients there is a dissociation between PLIN2 expression and IMTG content, and that 6 months of ET re-establishes the relationship whereby PLIN2 expression mirrors IMTG content.

Although linearity exists between IMTG content and the expression of PLIN2 and PLIN5 (in type 2 diabetes following ET), the relationship between PLIN2 or PLIN5 expression and insulin sensitivity is less clear. Indeed, PLIN2 and PLIN5 expression were both significantly related to insulin sensitivity in Chapter 4; however, on closer inspection of the data only ~16% of the variance in insulin sensitivity was attributed to PLIN2 and PLIN 5 expression. Furthermore, no significant relationship between PLIN2 or PLIN5 expression and insulin sensitivity was observed in Chapters 5 and 6. Together, these data raise the question as to the importance of the PLIN proteins to insulin sensitivity. Data obtained in vitro support an important role for the PLIN proteins in maintaining insulin sensitivity. Specifically, knockdown of PLIN proteins in AML12 cells reduces insulin-stimulated Akt/PKB phosphorylation and increase IRS-1 ser^{307} phosphorylation (Bell *et al*., 2008). Furthermore, Bosma *et al.* (2012) recently reported that PLIN2 overexpression augments TAG storage and protects against palmitate-induced impairments in insulin-stimulated glucose uptake, whereas knockdown of PLIN2 increases palmitate incorporation into DAG. These studies provide clear evidence to support a key role for the PLIN proteins in maintaining insulin sensitivity. However, it should be noted that improvements in insulin sensitivity are observed following thiazolidinedione treatment concomitant with both an increase and a decrease in PLIN protein expression (Phillips *et al*., 2005; Minnaard *et al*., 2009), suggesting that a direct relationship between PLIN protein expression and insulin sensitivity is not apparent. Furthermore, it should be noted that improvements in insulin sensitivity are observed following exercise training when IMTG content remains unchanged, is reduced or increased (reviewed in van Loon & Goodpaster, 2006; Shaw *et al*., 2010), and therefore in many instances IMTG content is disassociated from insulin sensitivity (Bruce *et al*., 2003). Rather, it is well-appreciated that

greater flexibility of the IMTG pool to respond to changes in energy demand is linked to insulin sensitivity, and PLIN protein expression likely contributes to this relationship. Given that both PLIN2 and PLIN5 appear to be important for IMTG breakdown during exercise (Chapter 2 and 4), an increase in PLIN protein expression is likely to be important when an increase in IMTG content occurs following exercise training, in order to preserve lipolytic regulation of the expanded IMTG pool. Through this adaptation, the flexibility of the IMTG pool will be enhanced, providing a greater capacity for IMTG utilisation during exercise and more efficient storage of incoming FA into IMTG, thereby reducing the potential for the generation of FA metabolites which may otherwise reduce insulin sensitivity. However, to truly appreciate the importance of the increases in PLIN protein expression to insulin sensitivity, a model of exercise training that does not promote greater IMTG storage concomitant with enhanced PLIN expression would be required. In light of the observations regarding the regulation of IMTG metabolism and insulin sensitivity, it is important to note that the most robust correlate of insulin sensitivity is mitochondrial density (Chapter 4, 5 and 6), which is ultimately required in order to oxidise fatty acids generated from IMTG breakdown.

7.4 Summary

The results of this thesis firstly generate new evidence regarding the underlying mechanisms regulating IMTG breakdown during moderate-intensity exercise, by demonstrating the potential importance of PLIN2 and PLIN5 in this process. Furthermore, the results provide new insight into the underlying mechanisms by which endurance, resistance and high intensity interval training elicit positive metabolic adaptations in lean sedentary, obese and obese type 2 diabetes patients. In particular, the potential importance of upregulating PLIN2 and PLIN5 protein expression in response to exercise training in order to maintain metabolic regulation of the intramuscular lipid pool is demonstrated, and these adaptations likely contribute to the improvements in insulin sensitivity observed following exercise training.

7.5 Future research directions

7.5.1 High intensity interval training

The phrase 'high intensity interval training' or 'HIT' has, in this researcher's opinion, become an umbrella term that now encompasses a range of HIT protocols. For example, HIT could refer to Wingate-based or constant-workload SIT, as described in Chapters 2 and 4 of this thesis, or to other forms of low-volume HIT (Little *et al.*, 2010; Metcalfe *et al.*, 2012). Therefore, the term 'HIT' actually refers to exercise training undertaken at a number of different workloads and interval durations, and it is likely that each different protocol will induce very different metabolic adaptations. To this end, a series of studies are now required to evaluate the effectiveness of each HIT protocol with regards to physiological and clinical factors, such as aerobic capacity, insulin sensitivity and risk factors of cardiovascular disease.

It is of significance that a HIT session incorporating a total of 2-3 min 'all out' effort may actually be 30 min in duration when a warm-up, recovery intervals and cool down are included. With this in mind, a recent study has investigated whether HIT, in which an exercise session consists of up to two bouts of high intensity interval exercise and has a total duration of 10 minutes, remains sufficient to improve insulin sensitivity (Metcalfe *et al.*, 2012). Indeed, 6 weeks of HIT improved maximal exercise capacity and insulin sensitivity in healthy male subjects, and therefore this study highlights that even 10 to 20 seconds of HIT during a 10 minute bout of exercise is effective in improving insulin sensitivity. However, an interesting question arising from this and previous studies regarding the use of HIT is to determine the minimum amount of exercise required to improve insulin sensitivity. In order to answer this question, a series of studies which systematically compare the training impulse and resultant training adaptations of different forms of HIT are now required.

In addition to determining the optimum HIT protocol, future studies should focus on investigating the longer term benefits of performing regular HIT in comparison to more traditional ET. Previously published studies generally engaged participants in 2-6 weeks of HIT, and therefore whether the positive adaptations observed following short term HIT still remain after many months of training is unknown. In addition, it is apparent that HIT and ET induce similar whole body and cellular metabolic adaptations (Gibala *et al.*, 2006; Burgomaster *et al.*, 2008). However, it is speculated that the time course of physiological changes may differ between HIT and ET, such that the highly intense nature of

HIT may induce rapid adaptations, whereas the low intensity exercise performed in ET may result in a slower adaptive response, but which may ultimately progress further (Gibala & McGee, 2008). In addition, studies comparing the effects of HIT and ET have been performed with relatively small subject numbers ($n \leq 10$), and the studies presented in this thesis (Chapters 4 & 6) suggest that greater subject numbers are required to determine differences in key outcomes such as insulin sensitivity and aerobic capacity. Therefore, it is essential that long term studies are undertaken with a larger number of subjects before definitive conclusions can be drawn as to whether HIT and ET induce similar improvements in metabolic health and reduce the risk of developing cardiovascular disease.

To date, all studies examining the effectiveness of HIT have been conducted within a laboratory setting, where the exercise is prescribed by the investigators and subjects are encouraged to complete the bouts of high intensity exercise. However, if HIT is to become an accessible and feasible exercise mode for the general population, studies are required to explore ways that HIT can be performed by the general population in the setting of leisure facilities and/or exercise rehabilitation clinics. This issue has been partially addressed by ourselves (Chapter 6) and Gibala and colleagues (Little *et al*., 2010) using a 'constant-load' approach to HIT. In addition, a study recently started in Birmingham has employed HIT in an instructor-led class environment using spinning bikes. In this study, 90 subjects (aged 25-60 years) are recruited and split into two groups of 45 individuals who then undertake 10 weeks of either HIT or ET. Notably, the exercise intensity of the high intensity exercise bout is under each individual's own control, meaning that each individual is performing exercise that they perceive to be of a 'high intensity'. Using a multi-disciplinary approach to compare the physiological and psychological adaptations to HIT and ET, this study will ultimately provide evidence as to whether HIT is a feasible exercise training mode for the general population.

7.5.2 Current gaps in our understanding of the relationship between IMTG metabolism and insulin sensitivity

Section 1.5.3 of the introduction concluded that an imbalance between the processes that increase intramuscular FA concentration (FA uptake and IMTG lipolysis) and those processes that consume FA (IMTG synthesis and mitochondrial β-oxidation) may lead to an increase in the concentration of FA

metabolites, which are proposed to contribute to the development of skeletal muscle insulin resistance. Therefore, understanding the mechanisms regulating the balance between IMTG synthesis and IMTG hydrolysis remains an area of significant interest,

In this respect, Chapters 2 and 4 of this thesis provide evidence to suggest that PLIN2 and PLIN5 potentially play an important role in the use of IMTG during moderate-intensity exercise. Although these findings provide an insight into the potential roles of PLIN2 and PLIN5 in skeletal muscle, further research is required to fully elucidate the mechanism through which PLIN2 and PLIN5 mediate IMTG use. Clues are provided from cell culture studies performed *in vitro* showing that PLIN2 and/or PLIN5 interact with HSL and/or ATGL. However, whether PLIN2/PLIN5 interact with ATGL/HSL under lipolytic conditions in skeletal muscle remains to be determined. To this end, colocalisation analysis of fluorescently-labelled proteins provides one tool to investigate whether two specific proteins of interest are associated with each other. However, it is important to recognise that an accurate assessment of the co-distribution of two fluorophores is heavily dependent on the resolution of the microscope, and as such the colocalisation of two fluorescently-labelled probes does not necessarily confirm a physical apposition. Therefore, future studies should employ a combination of methods in order to determine whether PLIN2 and/or PLIN5 interact with ATGL and/or HSL, and how these interactions are associated with IMTG use during exercise. One such methodological option is to examine the co-immunoprecipitation of two proteins from a skeletal muscle sample obtained before and after a period of moderate-intensity exercise. Indeed, using this method it has recently been reported that PLIN2 co-precipitation with ATGL is reduced in response to intermittent titanic stimulation of isolated rat soleus muscle (MacPherson *et al.*, unpublished conference poster communication, IBEC, 2012). However, further work is clearly warranted to fully elucidate how PLIN2 and/or PLIN5 interact with ATGL and/or HSL, and how this relationship affects the capacity to use IMTG as a substrate source during exercise.

In mouse skeletal muscle it is reported that 324 proteins are associated with the intramuscular lipid pool (Zhang *et al.*, 2011), although the precise role of many of these proteins in cellular metabolism is unknown. Whilst this study particularly highlights the limitations of our understanding of LDs in skeletal muscle, it does provide one methodological option to further identify proteins which may be important in the regulation of IMTG metabolism. Isolation of the LD fraction of skeletal muscle

generally involves a series of homogenisation and centrifugation steps. Once the LD fraction is isolated, liquid chromatography coupled to mass spectrometry analysis is employed to determine the identity of all the proteins found in the LD fraction. Thus, analysis of the LD proteome provides one method to first identify proteins which may be of interest for further investigation. In particular, comparing the LD proteome of skeletal muscle samples obtained from lean, obese and obese type 2 diabetes patients, or samples obtained before and after an exercise training programme, may provide insight as to whether differences in the LD proteome exist between groups, and should form the basis of future studies.

In addition to PLIN2 and PLIN5, two other proteins of the PLIN family are expressed in skeletal muscle, known as PLIN3 and PLIN4 (originally termed TIP47 and S3-12, respectively). In rat skeletal muscle, PLIN3 colocalizes with intramuscular LDs and is also found in the cytosolic pool, and this distribution is not altered by either muscle contraction or adrenaline stimulation (Prats *et al.*, 2006). However, the distribution of PLIN3 has not been described in human skeletal muscle. Furthermore, from studies performed in adipocytes it is proposed that PLIN3 plays a role in LD synthesis (Wolins *et al.*, 2003; Wolins *et al.*, 2005; Bulankina *et al.*, 2009), suggesting that PLIN3 may be present at the endoplasmic reticulum, the proposed site of IMTG synthesis and the formation of new LDs (Brasaemle & Wolins, 2012). As the LD, endoplasmic reticulum and various other muscle organelles are visible in electron micrographs, use of electron microscopy coupled to nanogold labelling of the various PLINs (which provides a greater resolution than a light-powered microscope) would provide information as to the cellular location and potential role of the PLINs in skeletal muscle. This technique has recently been employed to demonstrate that PLIN5 is associated with both LDs and the mitochondria in rat soleus muscle (Bosma *et al.*, 2011).

Once developed, the use of LD proteomic analysis, co-immunoprecipitation assays and electron microscopy methods in combination with the immunofluorescence microscopy assays outlined in this thesis, should provide a powerful tool to further understand the mechanisms mediating IMTG lipolysis and utilisation and also the relationship between IMTG metabolism and insulin sensitivity. However, it is also important to further understand the mechanism by which dysregulation of IMTG metabolism

directly influences insulin sensitivity. In this respect, Chapters 3, 4, 5 and 6 of this thesis conclude that an increase in skeletal muscle oxidative capacity, IMTG content, IMTG breakdown and PLIN2 and PLIN5 protein expression all contribute to the mechanism underlying the improvement in insulin sensitivity observed following exercise training. The mechanism for this improvement is proposed to be related to a reduction in the concentration of fatty acid metabolites, although in this thesis these measurements were not made. Previous longitudinal studies have reported reduced concentrations of DAG and ceramide following a period of exercise training in obese individuals and obese type 2 diabetes patients (Bruce *et al.*, 2006; Dubé *et al.*, 2008; Dubé *et al.*, 2011). However, cross-sectional studies are inconsistent with regards to the relationship between increased concentrations of total DAG and ceramide and the development of insulin resistance (Bosma *et al.*, 2012b; Coen & Goodpaster, 2012). More recently, it has been suggested that the saturation of the FA metabolites is a more important determinant of insulin sensitivity. To this end, saturated DAG concentrations are higher in sedentary individuals compared to trained athletes (Bergman *et al.*, 2010)but in a separate study, athletes had higher saturated DAG concentrations than obese individuals (Amati *et al.*, 2011). Therefore, it may also be important to consider the subcellular location of the FA metabolites. Indeed obese individuals and type 2 diabetes patients demonstrate greater saturated DAG concentrations in the membrane fraction of skeletal muscle compared to endurance-trained athletes (Bergman *et al.*, 2012). Given that the inhibitory action of DAG on insulin signalling (DAG activation of PKCθ leading to IRS-1 ser^{307} phosphorylation) is thought to occur in close proximity to the plasma membrane and the site of insulin action, consideration of the subcellular location of the FA metabolites may be key to understanding their role in lipid-induced insulin resistance. It is also important to mention that different stereo-specific DAG isoforms exist, based on the position at which the FA bind to the glycerol backbone (1,2-DAG, 2,3-DAG and 1,3-DAG). However, only 1,2-DAG has so far been associated with defects in insulin signalling (Turinsky *et al.*, 1990a). Taken together, it is clear that a systematic evaluation of FA metabolite saturation, subcellular location and stereo-specific isoforms is required not only using sedentary lean and obese individuals and obese type 2 diabetes patients, but also habitually active lean and obese individuals and highly trained athletes. The effect of exercise training interventions on the FA metabolite saturation, subcellular location and stereo-specific isoforms are also warranted. A series of carefully controlled studies using such methodologies are likely to greatly

improve our understanding of the role that lipid metabolites play in the development of skeletal muscle insulin resistance.

7.6 Final conclusions

The work contained within this thesis has developed novel immunofluorescence microscopy methods that have been used to generate new knowledge concerning the regulation of IMTG breakdown during moderate-intensity exercise. In this respect, evidence demonstrating that the PLIN proteins are important for the use of IMTG during exercise is reported for the first time. In addition, the effect of various forms of exercise training on the protein expression of the PLIN proteins is reported. Together, these findings suggest that the PLIN proteins are important in the regulation of IMTG metabolism, and therefore may be a key determinant of skeletal muscle insulin sensitivity. Future studies should focus on determining the exact role of these proteins, and other LD-associated proteins, in skeletal muscle to fully appreciate their role in maintaining cellular lipid homeostasis. The work in this thesis also provides important evidence that improvements in IMTG metabolism occur in response to high intensity interval training. Therefore, this adds to the growing body of literature demonstrating that HIT is a time-efficient training strategy to induce positive metabolic outcomes. It is expected that the evidence obtained in this thesis will inform future research into interventions that aim to tackle the worldwide health threat associated with physical inactivity.

Chapter 8

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