Lipid droplet remodelling and reduced muscle ceramides following sprint interval and moderate-intensity continuous exercise training in obese males

Sam O Shepherd[1], Matthew Cocks[1], Peter J Meikle[2], Natalie A Mellett[2], Aaron M Ranasinghe[3], Thomas A Barker[3], Anton JM Wagenmakers[1], Christopher S Shaw[4]

[1]*Research Institute for Sport & Exercise Sciences, Liverpool John Moores University, UK*

[2]*Baker IDI Heart & Diabetes Institute, Melbourne, Australia*

[3]*School of Clinical and Experimental Medicine, Cardiovascular and Respiratory Sciences, University of Birmingham, UK*

[4]*Centre for Physical Activity and Nutrition Research, School of Exercise and Nutrition Sciences, Deakin University, Geelong, Australia*

Running title: Lipid droplet remodelling with exercise training in obese males

Key words: skeletal muscle, intramyocellular lipid, diacylglycerol

Word count: 3961

No. of figures & tables: 6

Corresponding author and person to whom reprint requests should be sent:

Dr Christopher S. Shaw
Centre for Physical Activity and Nutrition Research
School of Exercise and Nutrition Sciences
Deakin University
Geelong
Victoria
Australia
Tel: +61 3 5227 3394
Email: chris.shaw@deakin.edu.au

Key points summary

- Improvements in insulin sensitivity with moderate intensity, continuous exercise training may be related to improvements in skeletal muscle lipid handling and lowering of diacylglycerol (DAG) and/or ceramide concentrations.
- Sprint interval training (SIT) is a time efficient alternative to moderate-intensity continuous training (MICT), but its effect on muscle DAG and ceramide concentrations and the expression of the perilipin family of lipid droplet-associated proteins is yet to be studied in obese individuals.
- We show that SIT and MICT lead to comparable reductions in muscle ceramide, and this coincided with greater expression of the perilipin proteins and improved contact between lipid droplets and mitochondria.
- We conclude that SIT and MICT induce similar lipid droplet remodelling and reductions in muscle ceramide concentrations, and these adaptations may explain how SIT improves insulin sensitivity.

Abstract

In obesity, improved muscle insulin sensitivity following exercise training has been linked to the lowering of diacylglycerol (DAG) and ceramide concentrations. Little is known, however, about how improved insulin action with exercise training in obese individuals relates to adaptations to lipid droplets (LD) in skeletal muscle. In this study we investigated the hypothesis that improvements in insulin sensitivity that are seen following short-term sprint interval training (SIT) and moderate intensity continuous training (MICT) in obese individuals coincide with increased perilipin (PLIN) expression, a greater number of LD in contact with mitochondria and reduced muscle concentrations of DAGs and ceramides. Sixteen sedentary obese males performed 4 weeks of either SIT (4-7x 30s sprints at 200% W_{max}, 3 days.week^{-1}) or MICT (40-60 min cycling at ~65% VO_{2peak}, 5 days.week^{-1}). In muscle biopsies obtained pre- and post-training, PLIN2 (SIT 90%, MICT 68%) and PLIN5 (SIT 47%, MICT 34%) expression increased in type I fibres only, and PLIN3 increased in both type I (SIT 63%, MICT 67%) and type II fibres (SIT 70%, MICT 160%) (all $P<0.05$). Training did not change LD content but increased the number of LD in contact with mitochondria (SIT 12%, MICT 21%, $P<0.01$). Ceramides were reduced following training (SIT -10%, MICT -7%, $P<0.05$), but no changes in DAGs were detected. No training × group interactions were observed for any variables. These results confirm the hypothesis that exercise training, via the observed LD remodelling mechanisms, contributes to the lowering of muscle ceramide concentrations and improved insulin action with exercise training.

Abbreviations

DAG, diacylglycerol; DHC, dihexosylceramide; FA, fatty acid; GM, gangliomyelin; IMTG, intramuscular triglyceride; LD, lipid droplet; MICT, moderate intensity continuous training; MHC, monohexosylceramide; PLIN, perilipin; SIT, sprint interval training; TAG, triacylglycerol; THC, trihexosylceramide (THC).

Introduction

Intramuscular triglyceride (IMTG) accumulation in sedentary and obese individuals is associated with low skeletal muscle insulin sensitivity and an increased risk of developing type 2 diabetes (Kelley *et al.*, 1999; Goodpaster *et al.*, 2001). However, endurance-trained athletes store even larger quantities of IMTG while exhibiting high levels of insulin sensitivity. This phenomenon is termed the athlete's paradox (Goodpaster *et al.*, 2001; van Loon *et al.*, 2004). Subsequently, it has been proposed that lipid metabolites such as diacylglycerols (DAG) and ceramides play a key role in the development of insulin resistance rather than IMTG (van Loon & Goodpaster, 2006; Samuel & Shulman, 2012). Indeed, evidence from cell culture and animal models demonstrates that elevated DAG (Itani *et al.*, 2002) or ceramides (Adams *et al.*, 2004) directly impair insulin signalling. Evidence from human studies is less clear, with some (Adams *et al.*, 2004; Straczkowski *et al.*, 2007; Moro *et al.*, 2009; Thrush *et al.*, 2009; Bergman *et al.*, 2012), but not all (Skovbro *et al.*, 2008; Bergman *et al.*, 2010; Jocken *et al.*, 2010; Amati *et al.*, 2011) studies reporting higher DAG and ceramide concentrations in skeletal muscle of insulin resistant obese and/or type 2 diabetes patients compared to healthy, insulin-sensitive individuals. Investigations exploring the degree of saturation and specific fatty acid composition of these lipids may unravel their specific role in the development of insulin resistance.

To date, few studies have evaluated the effect of longitudinal exercise training interventions on muscle DAGs and ceramides. A number of studies have reported reductions in ceramide concentrations (Bruce *et al.*, 2006; Dubé *et al.*, 2008; Dubé *et al.*, 2011), whereas the concentration of DAGs has been shown to decrease (Dubé *et al.*, 2008; Dubé *et al.*, 2011) or remain unchanged (Bruce *et al.*, 2006; Louche *et al.*, 2013) following training. Importantly, these studies have only investigated the effect of moderate-intensity continuous training (MICT). Sprint interval training (SIT) has gained increased attention as it can increase skeletal muscle oxidative capacity and improve whole-body insulin sensitivity with substantial reductions in time commitment and total training workload, compared to MICT (Burgomaster *et al.*, 2008; Gibala *et al.*, 2012; Cocks *et al.*, 2013; Shepherd *et al.*, 2013). Therefore, SIT offers promise as an alternative to continuous moderate-intensity exercise training to prevent and

treat metabolic disease. This is the first study to investigate whether improvements in insulin sensitivity following SIT occur alongside reductions in DAG and ceramide concentrations.

IMTG are stored in lipid droplets (LD), the majority of which are in close spatial contact with the mitochondrial network in endurance-trained individuals (Tarnopolsky *et al.*, 2007; Shaw *et al.*, 2008). Over 300 proteins are associated with the LD phospholipid monolayer (Zhang *et al.*, 2011), the most abundant of which are the perilipin (PLIN) proteins. It is generally accepted that PLIN2 and PLIN5 promote IMTG storage (MacPherson & Peters, 2015), since overexpression of either protein augments IMTG content in skeletal muscle while keeping insulin sensitivity high (Bosma *et al.*, 2012a; Bosma *et al.*, 2013). PLIN5 knock-out on the other hand results in elevated ceramide concentrations and lower insulin-mediated glucose disposal rates (Mason *et al.*, 2014), highlighting the importance of PLIN5 for maintaining high levels of insulin sensitivity. We recently reported that improvements in insulin sensitivity in sedentary lean individuals following SIT occur alongside increases in IMTG storage and greater expression of PLIN2 and PLIN5 (Shepherd *et al.*, 2013). At present, however, it is not known whether exercise training-induced increases in the abundance of the PLIN proteins contributes to improvements in insulin sensitivity, via decreases in DAG and ceramide content, in obese individuals at risk of developing metabolic syndrome.

Less attention has been paid to PLIN3, despite this protein also being highly expressed in skeletal muscle (Wolins *et al.*, 2006). Fatty acid packaging in LD is reduced when PLIN3 is ablated in fibroblastic cells (Sztalryd *et al.*, 2006), suggesting that PLIN3 is important for triacylglycerol storage. PLIN3 may also play a role in triacylglycerol oxidation, since skeletal muscle PLIN3 content is positively associated with whole-body fat oxidation (Covington *et al.*, 2014) and *ex vivo* palmitate oxidation (Covington *et al.*, 2014; Covington *et al.*, 2015). Data on the effect of MICT on skeletal muscle PLIN3 content, measured through immunoblotting of whole muscle homogenates, are contradictory with both an increase (Louche *et al.*, 2013) and no change (Peters *et al.*, 2012) being reported in obese individuals. Part of this contradiction could be the consequence of muscle fibre type differences. Using quantitative confocal immunofluorescence microscopy methods in combination with

fibre type stains previously developed in our laboratory (Shepherd *et al.*, 2012) we previously reported that 6 months of MICT in type 2 diabetes patients increased PLIN2 expression in type 1 fibres only (Shaw *et al.*, 2012), highlighting the importance of considering fibre type when investigating exercise training adaptations.

In the current study, we used muscle fibre type-specific methods to investigate the hypothesis that SIT and MICT in obese individuals would augment protein expression of PLIN2, PLIN3, and PLIN5. We also employed transmission electron microscopy to assess mitochondrial density and the proportion of LD in direct contact with mitochondria within the muscle fibre. Finally, measurements of total and subspecies of DAGs and ceramides were made to test the hypothesis that SIT and MICT would lead to a reduction in the concentration of these lipid metabolites in muscle.

Materials and Methods

Participants and ethical approval

The samples collected in this study have been used in a previous publication which focused on the effects of SIT and MICT on microvascular adaptations in skeletal muscle (Cocks *et al.*, 2016). Readers are referred to this publication for a more detailed description of the characteristics of the volunteers as well as for the training-induced changes in aerobic capacity and insulin sensitivity. The latter variables are also relevant for the interpretation of the current study. In brief, 16 young, sedentary obese males, who were engaging in less than one hour of structured physical activity per week, and were free of known metabolic or cardiovascular disorders, as determined by a medical professional during a pre-study screening visit, were recruited. Two participants had impaired fasting glucose (fasting plasma glucose ≥6.1 mmol.L^{-1}; SIT n=1, MICT n=1) and four subjects had impaired fasting glucose and impaired glucose tolerance (2 h oral glucose tolerance concentration between 7.8 and 11.1 mmol.L^{-1}; SIT n=2, MICT n=2). The study protocol adhered to the Declaration of Helsinki and was approved by the South Birmingham NHS Research Ethics Committee.

Pre- and post-training experimental procedures

Experimental procedures, including measures of aerobic capacity (VO_{2peak}), body composition and insulin sensitivity, were undertaken before and after training (>48 h following the final exercise training session and identical in all respects to pre-training) as previously described (Cocks *et al.*, 2016). Muscle samples (~100 mg) were obtained from the *vastus lateralis* under local anaesthesia using the Bergström technique (Bergström, 1975) following an overnight fast. The biopsy was separated and preserved for immunofluorescence microscopy, transmission electron microscopy, and analysis of lipid species. A fasting blood sample was obtained as previously described (Cocks *et al.*, 2016). An ILab-600 semi-automatic spectrophotometer was used to determine plasma non-esterified fatty acid (NEFA), total triglyceride, total-, LDL- and HDL-cholesterol concentrations using compatible commercially available kits (all Instrumentation Laboratory Ltd UK, Warrington, UK, except NEFA: Randox Laboratories Ltd, Co. Antrim, UK). Following the pre-training experimental procedures 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 either the HIT or MICT group.

Training procedures

Sprint interval training (SIT): Subjects performed repeated 30 s sprints on a cycle ergometer against a constant load equivalent to 200% W_{max}, interspersed with 2 min recovery during which subjects cycled against a small load (30 W) maintaining a cadence of <50 rpm. Subjects trained three times per week for four weeks, and were excluded if they were absent from more than two sessions. Four sprints per training session were performed in week one, after which an additional sprint was included on each consecutive week, such that seven sprints were performed per training session in week four. Heart rate was recorded throughout each session.

Moderate-intensity continuous training (MICT): Subjects in the MICT 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 two training sessions. All subjects cycled at a workload equivalent to ~65% VO_{2peak} for 40 min during the first seven sessions, increasing to 50 min during sessions 8 to 14, and 60 minutes during sessions 15 to 20. VO_{2peak} was reassessed after two weeks of training and workload adjusted accordingly.

Immunofluorescence microscopy

Muscle tissue was prepared for immunohistochemical analysis by embedding in Tissue-Tek OCT Compound (Sakura Finetek Europe, The Netherlands) and freezing in liquid nitrogen-cooled isopentane. Serial 5 µm cryosections were cut at -30°C and transferred on to ethanol-cleaned glass slides. The neutral lipid dye oil red O was used to image and quantify IMTG (Koopman *et al.*, 2001). All primary and secondary antibodies for immunofluorescence microscopy have been used previously (Shaw *et al.*, 2008; Shepherd *et al.*, 2012, 2013) except for the PLIN3 primary antibody (guinea pig polyclonal anti-TIP47, Progen Biotechnik, Germany). Training-induced changes in fibre-specific PLIN2, PLIN3 and PLIN5 content, and COX expression (as a marker of muscle oxidative capacity) were assessed using established and validated widefield and confocal immunofluorescence microscopy

techniques (Shaw *et al.*, 2008; Shepherd *et al.*, 2012, 2013). Image processing was undertaken using Image-Pro Plus 5.1 software (Media Cybernetics, MD, USA). A total of 97±9 fibres per muscle cross-section (38±4 type I fibres, 59±6 type II fibres) were analysed for each variable. Fluorescence staining intensity was used to indicate training-induced changes in mitochondrial content. IMTG, PLIN2, PLIN3 and PLIN5 content were expressed as the positively stained area fraction relative to the total area of each muscle fibre. For all fibre type-specific analyses, fibres stained positively for myosin heavy chain type 1 were classified as type I muscle fibres, whereas all other fibres were assumed to be type II muscle fibres.

Transmission electron microscopy

Fresh muscle tissue was immediately fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.3) for 24 h, followed by four rinses in 0.1 M sodium cacodylate buffer. Secondary fixation was undertaken with 1% osmium tetroxide (Agar Scientific, Essex, UK) for 1 h, followed by two rinses in 0.1 M sodium cacodylate buffer. Muscle tissue was subsequently dehydrated using increasing concentrations of alcohol (50, 70, 90, and 100%), followed by exposure to propylene oxide (Agar Scientific, Essex, UK). Dehydrated tissue was incubated in a propylene oxide/resin (1:1) and then embedded in 100% Mollenhauer resin (polymerised for >16 h at 60°C) (Agar Scientific, Essex, UK). Resin blocks (with tissue embedded) were trimmed and 1 µm sections were prepared to check fibre orientation. Longitudinal orientated ultra-thin sections (100 nm) were obtained using an ultramicrotome (Reichert Jung Ultracut, Vienna, Austria) fitted with a diamond blade and collected on to a formvar coated microscope grid (200 copper mesh size), followed by staining with uranyl acetate and lead citrate.

Sections were viewed and photographed at x10,000 magnification using a Jeol 1200 Ex transmission electron microscope (TEM) (Jeol, Tokyo, Japan) with a Megaview III FW camera. Four micrographs of the intermyofibrillar region per fibre from four different muscle fibres per time point (i.e. 16 micrographs per time point per person, 32 micrographs in total) were obtained in a randomized manner. Micrograph analysis was undertaken using Image-Pro Plus 5.1 software (Media Cybernetics, MD,

247 USA). A grid with squares of 500x500 nm (0.25 μm^2) was superimposed on to each micrograph, and
248 the number of points (two intersecting grid lines) that were in contact with mitochondria or LD was
249 summed and expressed relative to the total number of points on the grid (corresponding to a total of 784
250 points). This grid size was selected in line with a recent study that aimed to standardise the grid size
251 used across studies (Broskey *et al.*, 2013). This process was undertaken for each micrograph and the
252 values averaged to calculate mitochondria or LD volume density (expressed as % area of muscle
253 occupied by mitochondria or LDs). Individual mitochondria and LDs were isolated using the 'area of
254 interest' tool in the Image-Pro Plus software and mean individual mitochondria and LD size (μm^2) was
255 subsequently determined. The total number of mitochondria or LDs was expressed per square
256 micrometer of tissue (#.μm^2 $tissue^{-1}$). The number of LDs in contact with mitochondria was manually
257 counted and expressed as a percentage of the total number of LDs per micrograph. Acquisition and
258 analysis of all micrographs was performed blinded to subject, condition and time-point. Examples of
259 LDs in contact and not in contact with mitochondria are provided in Fig. 3.
260
261 ***Lipid composition analysis***
262 Muscle lipids were extracted using a single-phase chloroform/methanol extraction. Triacylglycerol
263 (TAG), DAG, ceramide, monohexosylceramide (MHC) dihexosylceramide (DHC), trihexosylceramide
264 (THC), sphingomyelin (SM), gangliomyelin (GM) were analysed using electrospray ionization-tandem
265 mass spectrometry, as previously described (Weir *et al.*, 2013).
266
267 ***Statistics***
268 Training-induced changes in blood glucose, insulin and lipids, TEM LD and mitochondria measures
269 and muscle lipid species, were assessed using a two-factor repeated measures ANOVA. A three-factor
270 repeated measures ANOVA was used to examine fibre-specific training-induced changes in IMTG,
271 PLIN2, PLIN3, PLIN5 and COX content. Significant interactions were investigated using Bonferroni
272 adjustment post hoc analysis. All data are expressed as means ± S.E.M.
273
274

Results

Insulin sensitivity

As we have previously reported (Cocks *et al.*, 2016), insulin sensitivity, as measured by the Matsuda index, was increased by both SIT (11%) and MICT (24%), with no significant difference between groups.

Immunofluorescence microscopy analysis

Muscle fibre type-specific COX expression (expressed as mean fluorescence intensity) was greater in type I compared to type II fibres both pre and post-training ($P<0.01$, Fig. 1A, 1B & Supplementary Fig. S1). Training increased COX expression in both type I (SIT 29%, MICT 36%) and type II fibres (SIT 49%, MICT 36%; main training effect, $P<0.01$, Fig. 1A, 1B & Supplementary Fig. S1), with no difference between groups. IMTG content (expressed as % area stained) was higher in type I versus type II muscle fibres both pre and post-training ($P<0.01$, Fig. 1C, 1D & 2). The increase in IMTG content in response to training both in type I (SIT 26%, MICT 22%) and type II fibres (SIT 35%, MICT 26%; Fig. 1C, 1D & 2) did not reach statistical significance ($P=0.1$). PLIN2, PLIN3 and PLIN5 content (expressed as % area stained) was greater in type I compared to type II fibres ($P<0.01$, Fig. 1E-J & 2) across all time points. In response to training PLIN2 content increased, although this only reached significance in type I fibres (SIT 90%, MICT 68%; training × fibre interaction, $P<0.001$, Fig. 1E & 2). In contrast, PLIN3 content increased following training in both type I (SIT 63%, MICT 67%, Fig. 1G & 2) and type II fibres (SIT 70%, MICT 160%; main training effect, $P<0.01$, Fig. 1H & 2). Training led to an increase in PLIN5 content in type I fibres only (SIT 47%, MICT 34%; training × fibre interaction, $P<0.01$, Fig. 1I & 2). PLIN2, PLIN3 and PLIN5 expression was not different between the two training interventions at any time point.

Transmission electron microscopy analysis

Training induced a significant increase in mitochondrial volume density (expressed as % area of muscle) (SIT 46%, MICT 88%; main training effect, $P<0.01$, Table 1), with no difference between groups. This increase was attributed to a greater number of mitochondria (SIT 33%, MICT 20%; main

303 training effect, P=0.01, Table 1) and increased mitochondrial size (SIT 27%, MICT 51%; main training
304 effect, P<0.01, Table 1), with no difference between groups. In contrast, no significant changes were
305 observed in LD volume density, LD number or LD size in response to training (P>0.05, Table 1).
306 However, there was an increase in the proportion of LDs in contact with mitochondria (Fig. 3) following
307 training (SIT 12%, MICT 21%; main training effect, P<0.01, Table 1 & Fig. 3), with no difference
308 between groups.
309
310 ***Muscle TAG, DAG and sphingolipids***
311 Training did not change the concentration of total TAG (P>0.05, Fig. 4A) or specific TAG subspecies
312 (data not shown). There was also no change in total DAG concentrations with training (P>0.05, Fig.
313 4A), however there was a significant increase in DAG 18:1/18:2 (main training effect, P=0.02) and
314 DAG 18:0/18:2 (main training effect, P=0.04) with training (data not shown). Training induced a
315 significant decrease in total ceramide content (SIT -10%, MICT -7%; main training effect, P=0.03, Fig.
316 4A), with no difference between groups. Further examination of the specific ceramide species revealed
317 that training led to a significant decrease in Cer 18:0 (P=0.01, Fig. 4C). We also examined the effect
318 of training on other complex ceramides and sphingomyelin, and found that training led to an increase
319 in total trihexosylceramide (THC) concentration (SIT 32%, MICT 10%; main training effect, P=0.02,
320 Fig. 4B), with no difference between groups. The THC species THC 24:0 and THC 24:1 were the only
321 species that significantly increased in response to training (both P<0.01, Fig. 4D).

Discussion

In the present study we demonstrate for the first time that in previously sedentary obese males, a short-term SIT and MICT intervention both lead to 1) a reduction in muscle ceramide concentrations, 2) an increase in the number of LDs in contact with mitochondria, and 3) muscle fibre-specific increases in PLIN2, PLIN3 and PLIN5. Importantly, these adaptations were comparable in response to both SIT and MICT interventions and occurred alongside an improvement in whole-body insulin sensitivity (Cocks *et al.*, 2016).

The first novel finding of the present study was that 4 weeks of either SIT or MICT significantly reduced muscle ceramide concentrations in obese individuals. Reductions in muscle ceramide concentrations have previously been reported following 8-16 weeks endurance training in obese individuals (Bruce *et al.*, 2006; Dubé *et al.*, 2011), and we now extend the ceramide-lowering effect of exercise training to include SIT. Further analysis revealed that training specifically lowered Cer18:0, which was the most abundant of the ceramide species measured. This may be an important adaptation, since it has recently been reported in humans that the muscle concentration of Cer18:0 is inversely related to insulin sensitivity (Bergman *et al.*, 2016). Ceramides are elevated in the muscle of insulin resistant populations (Adams *et al.*, 2004; Straczkowski *et al.*, 2007; Moro *et al.*, 2009; Thrush *et al.*, 2009; Amati *et al.*, 2011) and are a potent inhibitor of muscle insulin action through the activation of the phosphatase PP2A and subsequent dephosphorylation and inhibition of Akt (Summers, 2006). Therefore, the reduction in ceramide concentrations, and particularly Cer18:0, observed following training likely contributes to the improved insulin sensitivity observed in response to SIT and MICT (Cocks *et al.*, 2016). We also observed a significant increase in total skeletal muscle THC concentration, with increases occurring in the THC 24:0 and THC 24:1 species. The relevance of THC to insulin sensitivity is not clear and warrants further investigation.

Contrary to our hypothesis, we did not detect a change in total DAG content but rather an increase in specific DAG species following training (DAG 18:1/18:2 and DAG 18:0/18:2). Although previous studies and reviews have heavily implicated DAG in the pathogenesis of insulin resistance (Itani *et al.*,

2002; Bergman *et al.*, 2012; Samuel & Shulman, 2012), the relationship between muscle DAG and insulin sensitivity has proved complex. Our results are in line with a number of studies that have demonstrated a disconnect between muscle DAG content and insulin sensitivity (reviewed by Amati, 2012). Most notably, Amati et al. (2011), demonstrated that trained individuals displayed higher DAG levels than sedentary lean and obese individuals. Therefore, it appears that changes in DAG concentrations may not be directly relevant for the improvements in insulin sensitivity following exercise training.

The second novel finding of the study is that short-term SIT led to an increase in the number of LDs that are in contact with mitochondria. In line with this observation, it has been reported previously that 6-12 weeks of MICT augments the number of LDs in contact with mitochondria in both lean (Tarnopolsky *et al.*, 2007) and obese (Devries *et al.*, 2013) individuals. This is likely to be an important adaptation, because a large proportion of extracellular FAs flux through the IMTG pool prior to oxidation in the mitochondria (Meex *et al.*, 2015), and therefore the contact of LDs with mitochondria is important to efficiently channel FA released by IMTG lipolysis into the mitochondrial reticulum for oxidation (Rambold *et al.*, 2015). Therefore, the greater number of LDs in contact with mitochondria will aid in the efficient transfer of LD-derived FAs to the mitochondria and contribute to the well described increase in IMTG-derived FA oxidation following exercise training. Such an improvement in the channelling of FA's towards oxidation rather than other lipid pathways, such as ceramide synthesis, may also provide an explanation behind the lowered concentration of ceramides observed post-training.

Previous investigations into the PLIN proteins in human skeletal muscle have predominantly focused on PLIN2 and PLIN5. Less attention, however, has been paid to PLIN3, which is also expressed in skeletal muscle (Wolins *et al.*, 2006). Studies report either an increase (Louche *et al.*, 2013) or no change (Peters *et al.*, 2012) in PLIN3 protein content in whole muscle homogenates following MICT in obese individuals. Here, we report for the first time that SIT and MICT leads to greater PLIN3 protein expression in both type I and type II fibres in obese individuals. Like PLIN2 and PLIN5, acute

endurance-type exercise in mice leads to an increase in PLIN3 mRNA abundance (Koves *et al.*, 2013), suggesting that repeated bouts of exercise during a training programme will subsequently lead to greater PLIN3 protein expression. A precise role for PLIN3 in skeletal muscle has not yet been identified, but evidence is accumulating to suggest a function for PLIN3 in IMTG oxidation. In support, human studies report a positive association between PLIN3 expression and both whole-body fat oxidation (Covington *et al.*, 2014) and ex vivo palmitate oxidation (Covington *et al.*, 2014; Covington *et al.*, 2015), and PLIN3 is reported to be present within the mitochondrial fraction of sedentary and endurance-trained rats (Ramos *et al.*, 2015), thereby highlighting a possible role for PLIN3 in IMTG oxidation.

We also observed greater expression of both PLIN2 and PLIN5 in response to SIT and MICT in obese individuals, but interestingly the elevated expression of these proteins was specific to type I fibres. With regards to PLIN2, it is notable that other studies investigating the effect of MICT specifically in obese individuals have failed to observe an increase in the expression of this protein (Peters *et al.*, 2012; Louche *et al.*, 2013). This may be explained by the methodology used, since immunoblotting of whole muscle homogenates (as used in previous studies) does not take into account fibre type differences. In line with the results of the present study, we previously reported that PLIN2 expression is upregulated specifically in type I fibres following MICT in obese type 2 diabetes patients (Shaw *et al.*, 2012). The increase in PLIN5 is consistent with other studies showing greater PLIN5 expression following MICT in obese individuals (Peters *et al.*, 2012; Louche *et al.*, 2013), and with our previous study demonstrating that SIT and MICT augment PLIN5 in lean individuals (Shepherd *et al.*, 2013). An abundance of data supports a role for PLIN5, and to a lesser extent PLIN2, under conditions of increased metabolic demand that favour high lipolytic rates. For example, protein kinase A activation promotes TAG hydrolysis in non-muscle cells expressing PLIN5 (Wang *et al.*, 2011b), an effect that is dependent upon PLIN5 phosphorylation (Pollak *et al.*, 2015). Further, we have previously shown that PLIN2 (Shepherd *et al.*, 2012) and PLIN5-containing LD (Shepherd *et al.*, 2013) are preferentially broken down during moderate-intensity exercise. The PLIN proteins may also play a mechanistic role in the functional linkage between LDs and the mitochondrial network, since PLIN5 recruits mitochondria to the LD surface in cultured non-muscle cells leading to increased rates of mitochondrial β-oxidation in response

406 to protein kinase A activation (Wang *et al.*, 2011b). In addition, both PLIN3 and PLIN5 have been
407 reported to be present within mitochondria (Bosma *et al.*, 2012b; Ramos *et al.*, 2015) and electrical
408 contraction of rat skeletal muscle increases the content of PLIN5 in the mitochondrial fraction (Ramos
409 *et al.*, 2015). The exact mechanisms involved in the functional linkage of LDs and mitochondria
410 following exercise training and the role of the PLIN proteins in this process remains unresolved.
411 Nevertheless, when taken together these results suggest that augmented PLIN protein expression
412 following exercise training is important to support IMTG-derived FA oxidation during periods of
413 elevated metabolic demand.
414
415 It has also been proposed that PLIN proteins may also facilitate IMTG storage when metabolic demand
416 is low. Indeed, under basal conditions the PLIN proteins restrict lipolytic rates by preventing the
417 binding of ATGL with its co-activator, CGI-58, on the LD surface (Bell *et al.*, 2008; Wang *et al.*,
418 2011a). As such, overexpression of PLIN2 or PLIN5 in skeletal muscle augments TAG storage (Bosma
419 *et al.*, 2012a; Bosma *et al.*, 2013), whereas knockdown or knockout of PLIN2 or PLIN5 results in
420 smaller and fewer LD, elevated lipolytic rates, and a reduction in FA-induced TAG accumulation
421 (Bosma *et al.*, 2012a; Kuramoto *et al.*, 2012; Mason *et al.*, 2014). Although less is known about PLIN3,
422 ablation of this protein reduces the ability to store FA's in LD in fibroblastic cells (Sztalryd *et al.*, 2006),
423 suggesting that PLIN3 also plays a role in TAG storage. Despite elevations in muscle PLIN protein
424 abundance post-training, IMTG content and LD number did not increase significantly in the present
425 study. It is possible that the short duration of the training period may explain the lack of a significant
426 increase in IMTG content, since increases in IMTG content in obese individuals do occur following 12
427 to 16 weeks of MICT (Pruchnic *et al.*, 2004; Dubé *et al.*, 2008; Dubé *et al.*, 2011). Importantly, the
428 fact that SIT and MICT augmented PLIN protein expression while IMTG content did not increase
429 significantly, suggests that the increased PLIN abundance may be an early event in the remodelling of
430 the intramuscular LD pool in obese individuals and type 2 diabetes patients (Shaw *et al.*, 2012).
431
432 Taken together, these results suggest that remodelling of the intramuscular lipid pool contributes to the
433 observed improvements in insulin sensitivity with short term exercise training (Cocks *et al.*, 2016). The

greater PLIN protein expression observed following training could be crucial here, because overexpression of PLIN2 or PLIN5 in rats improves (PLIN2) or maintains (PLIN5) insulin sensitivity in rats fed a high-fat diet (Bosma *et al.*, 2012a; Bosma *et al.*, 2013). Furthermore, when PLIN2 was overexpressed, IMTG content was increased, but importantly no changes in DAG concentrations were observed (Bosma *et al.*, 2012a). These data suggest that PLIN2 and PLIN5 are important for facilitating storage of FAs entering skeletal muscle in IMTG, rather than the FAs being used to synthesise DAG and/or ceramides. Indeed, the increase in PLIN5 expression may be particularly important, as recent work from Mason *et al.* (2014) demonstrated that whole-body PLIN5 knock-out in mice induces elevations in muscle ceramide. Together these data suggest that the increased expression of the PLIN proteins in the present study may be mechanistically linked to the lower ceramide concentrations and improved insulin sensitivity that we observe post-training.

In conclusion, this study has generated novel data that 4 weeks of exercise training induces remodelling of skeletal muscle LDs, evidenced by increased abundance of PLIN2, PLIN3 and PLIN5 and increased spatial contact between LDs and the mitochondrial network. These adaptations occur alongside a reduction in the muscle ceramide concentrations, which provide a possible mechanistic explanation behind the observed improvements in whole-body insulin sensitivity. These results add to the growing body of evidence that SIT and MICT counteract the metabolic impairments that result from obesity and a sedentary lifestyle, with SIT offering a time-efficient and effective alternative to MICT.

References

Adams JM, II, Pratipanawatr T, Berria R, Wang E, DeFronzo RA, Sullards MC & Mandarino LJ. (2004). Ceramide Content Is Increased in Skeletal Muscle From Obese Insulin-Resistant Humans. *Diabetes* **53,** 25-31.

Amati F. (2012). Revisiting the diacylglycerol-induced insulin resistance hypothesis. *Obes Rev* **13 Suppl 2,** 40-50.

Amati F, Dube JJ, Alvarez-Carnero E, Edreira MM, Chomentowski P, Coen PM, Switzer GE, Bickel PE, Stefanovic-Racic M, Toledo FG & Goodpaster BH. (2011). Skeletal muscle triglycerides, diacylglycerols, and ceramides in insulin resistance: another paradox in endurance-trained athletes? *Diabetes* **60,** 2588-2597.

Bell M, Wang H, Chen H, McLenithan JC, Gong DW, Yang RZ, Yu D, Fried SK, Quon MJ, Londos C & Sztalryd C. (2008). Consequences of lipid droplet coat protein downregulation in liver cells: abnormal lipid droplet metabolism and induction of insulin resistance. *Diabetes* **57,** 2037-2045.

Bergman BC, Brozinick JT, Strauss A, Bacon S, Kerege A, Bui HH, Sanders P, Siddall P, Wei T, Thomas MK, Kuo MS & Perreault L. (2016). Muscle sphingolipids during rest and exercise: a C18:0 signature for insulin resistance in humans. *Diabetologia* **59,** 785-798.

Bergman BC, Hunerdosse DM, Kerege A, Playdon MC & Perreault L. (2012). Localisation and composition of skeletal muscle diacylglycerol predicts insulin resistance in humans. *Diabetologia* **55,** 1140-1150.

Bergman BC, Perreault L, Hunerdosse DM, Koehler MC, Samek AM & Eckel RH. (2010). Increased intramuscular lipid synthesis and low saturation relate to insulin sensitivity in endurance-trained athletes. *J Appl Physiol* **108,** 1134-1141.

Bergström J. (1975). Percutaneous needle biopsy of skeletal muscle in physiological and clinical research. *Scand J Clin Lab Invest* **35,** 609-616.

Bosma M, Hesselink MK, Sparks LM, Timmers S, Ferraz MJ, Mattijssen F, van Beurden D, Schaart G, de Baets MH, Verheyen FK, Kersten S & Schrauwen P. (2012a). Perilipin 2 improves insulin sensitivity in skeletal muscle despite elevated intramuscular lipid levels. *Diabetes* **61,** 2679-2690.

Bosma M, Minnaard R, Sparks LM, Schaart G, Losen M, de Baets MH, Duimel H, Kersten S, Bickel PE, Schrauwen P & Hesselink MK. (2012b). The lipid droplet coat protein perilipin 5 also localizes to muscle mitochondria. *Histochem Cell Biol* **137,** 205-216.

Bosma M, Sparks LM, Hooiveld GJ, Jorgensen JA, Houten SM, Schrauwen P, Kersten S & Hesselink MK. (2013). Overexpression of PLIN5 in skeletal muscle promotes oxidative gene expression and intramyocellular lipid content without compromising insulin sensitivity. *Biochim Biophys Acta* **1831,** 844-852.

Broskey NT, Daraspe J, Humbel BM & Amati F. (2013). Skeletal muscle mitochondrial and lipid droplet content assessed with standardized grid sizes for stereology. *J Appl Physiol (1985)* **115,** 765-770.

Bruce CR, Thrush AB, Mertz VA, Bezaire V, Chabowski A, Heigenhauser GJ & Dyck DJ. (2006). Endurance training in obese humans improves glucose tolerance and mitochondrial fatty acid oxidation and alters muscle lipid content. *Am J Physiol Endocrinol Metab* **291,** E99-E107.

Burgomaster KA, Howarth KR, Phillips SM, Rakobowchuk M, MacDonald MJ, McGee SL & Gibala MJ. (2008). Similar metabolic adaptations during exercise after low volume sprint interval and traditional endurance training in humans. *J Physiol* **586,** 151-160.

Cocks M, Shaw CS, Shepherd SO, Fisher JP, Ranasinghe A, Barker TA & Wagenmakers AJ. (2016). Sprint interval and moderate-intensity continuous training have equal benefits on aerobic capacity, insulin sensitivity, muscle capillarisation and endothelial eNOS/NAD(P)Hoxidase protein ratio in obese men. *J Physiol* **594,** 2307-2321.

Cocks M, Shaw CS, Shepherd SO, Fisher JP, Ranasinghe AM, Barker TA, Tipton KD & Wagenmakers AJ. (2013). Sprint interval and endurance training are equally effective in increasing muscle microvascular density and eNOS content in sedentary males. *J Physiol* **591,** 641-656.

Covington JD, Galgani JE, Moro C, LaGrange JM, Zhang Z, Rustan AC, Ravussin E & Bajpeyi S. (2014). Skeletal muscle perilipin 3 and coatomer proteins are increased following exercise and are associated with fat oxidation. *PLoS One* **9,** e91675.

Covington JD, Noland RC, Hebert RC, Masinter BS, Smith SR, Rustan AC, Ravussin E & Bajpeyi S. (2015). Perilipin 3 Differentially Regulates Skeletal Muscle Lipid Oxidation in Active, Sedentary, and Type 2 Diabetic Males. *J Clin Endocrinol Metab* **100,** 3683-3692.

Devries MC, Samjoo IA, Hamadeh MJ, McCready C, Raha S, Watt MJ, Steinberg GR & Tarnopolsky MA. (2013). Endurance training modulates intramyocellular lipid compartmentalization and morphology in skeletal muscle of lean and obese women. *J Clin Endocrinol Metab* **98,** 4852-4862.

Dubé JJ, Amati F, Stefanovic-Racic M, Toledo FG, Sauers SE & Goodpaster BH. (2008). Exercise-induced alterations in intramyocellular lipids and insulin resistance: the athlete's paradox revisited. *Am J Physiol Endocrinol Metab* **294,** E882-888.

Dubé JJ, Amati F, Toledo FG, Stefanovic-Racic M, Rossi A, Coen P & Goodpaster BH. (2011). Effects of weight loss and exercise on insulin resistance, and intramyocellular triacylglycerol, diacylglycerol and ceramide. *Diabetologia* **54,** 1147-1156.

Gibala MJ, Little JP, Macdonald MJ & Hawley JA. (2012). Physiological adaptations to low-volume, high-intensity interval training in health and disease. *J Physiol* **590,** 1077-1084.

Goodpaster BH, He J, Watkins S & Kelley DE. (2001). Skeletal muscle lipid content and insulin resistance: evidence for a paradox in endurance-trained athletes. *J Clin Endocrinol Metab* **86,** 5755-5761.

Itani SI, Ruderman NB, Schmieder F & Boden G. (2002). Lipid-induced insulin resistance in human muscle is associated with changes in diacylglycerol, protein kinase C, and IkappaB-alpha. *Diabetes* **51,** 2005-2011.

Jocken JW, Moro C, Goossens GH, Hansen D, Mairal A, Hesselink MK, Langin D, van Loon LJ & Blaak EE. (2010). Skeletal muscle lipase content and activity in obesity and type 2 diabetes. *J Clin Endocrinol Metab* **95,** 5449-5453.

Kelley DE, Goodpaster B, Wing RR & Simoneau JA. (1999). Skeletal muscle fatty acid metabolism in association with insulin resistance, obesity, and weight loss. *Am J Physiol* **277,** E1130-1141.

Koopman R, Schaart G & Hesselink MK. (2001). Optimisation of oil red O staining permits combination with immunofluorescence and automated quantification of lipids. *Histochem Cell Biol* **116,** 63-68.

Koves TR, Sparks LM, Kovalik JP, Mosedale M, Arumugam R, DeBalsi KL, Everingham K, Thorne L, Phielix E, Meex RC, Kien CL, Hesselink MK, Schrauwen P & Muoio DM. (2013). PPARgamma coactivator-1alpha contributes to exercise-induced regulation of intramuscular lipid droplet programming in mice and humans. *J Lipid Res* **54,** 522-534.

Kuramoto K, Okamura T, Yamaguchi T, Nakamura TY, Wakabayashi S, Morinaga H, Nomura M, Yanase T, Otsu K, Usuda N, Matsumura S, Inoue K, Fushiki T, Kojima Y, Hashimoto T, Sakai F, Hirose F & Osumi T. (2012). Perilipin 5, a Lipid Droplet-binding Protein, Protects Heart from Oxidative Burden by Sequestering Fatty Acid from Excessive Oxidation. *J Biol Chem* **287,** 23852-23863.

Louche K, Badin PM, Montastier E, Laurens C, Bourlier V, de Glisezinski I, Thalamas C, Viguerie N, Langin D & Moro C. (2013). Endurance exercise training up-regulates lipolytic proteins and reduces triglyceride content in skeletal muscle of obese subjects. *J Clin Endocrinol Metab* **98,** 4863-4871.

MacPherson RE & Peters SJ. (2015). Piecing together the puzzle of perilipin proteins and skeletal muscle lipolysis. *Appl Physiol Nutr Metab* **40,** 641-651.

Mason RR, Mokhtar R, Matzaris M, Selathurai A, Kowalski GM, Mokbel N, Meikle PJ, Bruce CR & Watt MJ. (2014). PLIN5 deletion remodels intracellular lipid composition and causes insulin resistance in muscle. *Mol Metab* **3,** 652-663.

Meex RC, Hoy AJ, Mason RM, Martin SD, McGee SL, Bruce CR & Watt MJ. (2015). ATGL-mediated triglyceride turnover and the regulation of mitochondrial capacity in skeletal muscle. *Am J Physiol Endocrinol Metab* **308,** E960-970.

Moro C, Galgani JE, Luu L, Pasarica M, Mairal A, Bajpeyi S, Schmitz G, Langin D, Liebisch G & Smith SR. (2009). Influence of Gender, Obesity, and Muscle Lipase Activity on Intramyocellular Lipids in Sedentary Individuals. *J Clin Endocrinol Metab* **94,** 3440-3447.

Peters SJ, Samjoo IA, Devries MC, Stevic I, Robertshaw HA & Tarnopolsky MA. (2012). Perilipin family (PLIN) proteins in human skeletal muscle: the effect of sex, obesity, and endurance training. *Appl Physiol Nutr Metab* **37,** 724-735.

Pollak NM, Jaeger D, Kolleritsch S, Zimmermann R, Zechner R, Lass A & Haemmerle G. (2015). The interplay of protein kinase A and perilipin 5 regulates cardiac lipolysis. *J Biol Chem* **290,** 1295-1306.

Pruchnic R, Katsiaras A, He J, Kelley DE, Winters C & Goodpaster BH. (2004). Exercise training increases intramyocellular lipid and oxidative capacity in older adults. *Am J Physiol Endocrinol Metab* **287,** E857-862.

Rambold AS, Cohen S & Lippincott-Schwartz J. (2015). Fatty acid trafficking in starved cells: regulation by lipid droplet lipolysis, autophagy, and mitochondrial fusion dynamics. *Dev Cell* **32,** 678-692.

Ramos SV, Turnbull PC, MacPherson RE, LeBlanc PJ, Ward WE & Peters SJ. (2015). Changes in mitochondrial perilipin 3 and perilipin 5 protein content in rat skeletal muscle following endurance training and acute stimulated contraction. *Exp Physiol* **100,** 450-462.

Samuel VT & Shulman GI. (2012). Mechanisms for insulin resistance: common threads and missing links. *Cell* **148,** 852-871.

626 Shaw CS, Jones DA & Wagenmakers AJ. (2008). Network distribution of mitochondria and lipid
627 droplets in human muscle fibres. *Histochem Cell Biol* **129,** 65-72.
628
629 Shaw CS, Shepherd SO, Wagenmakers AJ, Hansen D, Dendale P & van Loon LJ. (2012). Prolonged
630 exercise training increases intramuscular lipid content and perilipin 2 expression in type I
631 muscle fibers of patients with type 2 diabetes. *Am J Physiol Endocrinol Metab* **303,** E1158-
632 1165.
633
634 Shepherd SO, Cocks M, Tipton KD, Ranasinghe AM, Barker TA, Burniston JG, Wagenmakers AJ &
635 Shaw CS. (2012). Preferential utilization of perilipin 2-associated intramuscular triglycerides
636 during 1 h of moderate-intensity endurance-type exercise. *Exp Physiol* **97,** 970-980.
637
638 Shepherd SO, Cocks M, Tipton KD, Ranasinghe AM, Barker TA, Burniston JG, Wagenmakers AJ &
639 Shaw CS. (2013). Sprint interval and traditional endurance training increase net intramuscular
640 triglyceride breakdown and expression of perilipin 2 and 5. *J Physiol* **591,** 657-675.
641
642 Skovbro M, Baranowski M, Skov-Jensen C, Flint A, Dela F, Gorski J & Helge JW. (2008). Human
643 skeletal muscle ceramide content is not a major factor in muscle insulin sensitivity.
644 *Diabetologia* **51,** 1253-1260.
645
646 Straczkowski M, Kowalska I, Baranowski M, Nikolajuk A, Otziomek E, Zabielski P, Adamska A,
647 Blachnio A, Gorski J & Gorska M. (2007). Increased skeletal muscle ceramide level in men at
648 risk of developing type 2 diabetes. *Diabetologia* **50,** 2366-2373.
649
650 Summers SA. (2006). Ceramides in insulin resistance and lipotoxicity. *Prog Lipid Res* **45,** 42-72.
651
652 Sztalryd C, Bell M, Lu X, Mertz P, Hickenbottom S, Chang BH, Chan L, Kimmel AR & Londos C.
653 (2006). Functional compensation for adipose differentiation-related protein (ADFP) by Tip47
654 in an ADFP null embryonic cell line. *J Biol Chem* **281,** 34341-34348.
655
656 Tarnopolsky MA, Rennie CD, Robertshaw HA, Fedak-Tarnopolsky SN, Devries MC & Hamadeh MJ.
657 (2007). Influence of endurance exercise training and sex on intramyocellular lipid and
658 mitochondrial ultrastructure, substrate use, and mitochondrial enzyme activity. *Am J Physiol*
659 *Regul Integr Comp Physiol* **292,** R1271-1278.
660
661 Thrush AB, Brindley DN, Chabowski A, Heigenhauser GJ & Dyck DJ. (2009). Skeletal muscle
662 lipogenic protein expression is not different between lean and obese individuals: a potential
663 factor in ceramide accumulation. *J Clin Endocrinol Metab* **94,** 5053-5061.
664
665 van Loon LJ & Goodpaster BH. (2006). Increased intramuscular lipid storage in the insulin-resistant
666 and endurance-trained state. *Pflugers Arch* **451,** 606-616.
667
668 van Loon LJC, Koopman R, Manders R, van der Weegen W, van Kranenburg GP & Keizer HA. (2004).
669 Intramyocellular lipid content in type 2 diabetes patients compared with overweight sedentary
670 men and highly trained endurance athletes. *Am J Physiol Endocrinol Metab* **287,** E558-565.
671
672 Wang H, Bell M, Sreenevasan U, Hu H, Liu J, Dalen K, Londos C, Yamaguchi T, Rizzo MA, Coleman
673 R, Gong D, Brasaemle D & Sztalryd C. (2011a). Unique Regulation of Adipose Triglyceride
674 Lipase (ATGL) by Perilipin 5, a Lipid Droplet-associated Protein. *J Biol Chem* **286,** 15707-
675 15715.
676
677 Wang H, Sreenevasan U, Hu H, Saladino A, Polster BM, Lund LM, Gong DW, Stanley WC & Sztalryd
678 C. (2011b). Perilipin 5, lipid droplet associated protein provides physical and metabolic linkage
679 to mitochondria. *J Lipid Res* **52,** 2159-2168.
680

Weir JM, Wong G, Barlow CK, Greeve MA, Kowalczyk A, Almasy L, Comuzzie AG, Mahaney MC, Jowett JB, Shaw J, Curran JE, Blangero J & Meikle PJ. (2013). Plasma lipid profiling in a large population-based cohort. *J Lipid Res* **54,** 2898-2908.

Wolins NE, Quaynor BK, Skinner JR, Tzekov A, Croce MA, Gropler MC, Varma V, Yao-Borengasser A, Rasouli N, Kern PA, Finck BN & Bickel PE. (2006). OXPAT/PAT-1 Is a PPAR-Induced Lipid Droplet Protein That Promotes Fatty Acid Utilization. *Diabetes* **55,** 3418-3428.

Zhang H, Wang Y, Li J, Yu J, Pu J, Li L, Zhang S, Peng G, Yang F & Liu P. (2011). Proteome of skeletal muscle lipid droplet reveals association with mitochondria and apolipoprotein a-I. *J Proteome Res* **10,** 4757-4768.

Additional information

Competing interests

The authors have no conflicts of interest to declare.

Author contributions

SOS, CSS and AJMW: conception and design of the experiments, collection, analysis and interpretation of data, drafting and final revisions of the manuscript; MC: conception and design of the experiments, collection, analysis and interpretation of data; PJM and NAM: analysis and interpretation of data, revisions of manuscript; AMR and TAB: collection of data, revisions of manuscript. All authors have read and approved the final submission.

Funding

This work was supported by a grant from the Insulin Dependent Diabetes Trust.

Acknowledgements

The antibody against myosin (human slow twitch fibres, A4.840) used in the study was developed by Dr. Blau and obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by the University of Iowa, Department of Biological Sciences, Iowa City, IA 52242.

743 **Tables**

744

Table 1. *Effect of 4 weeks SIT or MICT on mitochondria and lipid droplet characteristics assessed by transmission electron microscopy*

	SIT		MICT	
	Pre	Post	Pre	Post
Mitochondrial Vd (%)*	3.74±0.50	5.48±0.47	4.66±0.40	8.76±1.08
# Mitochondria (#.μm^{-2})*	0.28±0.02	0.37±0.03	0.36±0.03	0.40±0.02
Mean mitochondria area (μm^2)*	0.09±0.01	0.11±0.01	0.10±0.01	0.15±0.02
LD touching mitochondria (%)*	44.5±1.9	50.0±2.4	49.0±2.3	59.2±1.7
LD Vd (%)	2.76±0.74	3.50±0.57	2.24±0.39	2.62±0.20
# LD (#.μm^{-2})	0.12±0.03	0.12±0.02	0.07±0.01	0.11±0.01
Mean LD area (μm^2)	0.18±0.04	0.21±0.04	0.22±0.03	0.20±0.20

745

746 Mitochondria and lipid droplet physical characteristics, quantified from electron microscopy images

747 obtained before and after 4 weeks of SIT or MICT. Values are means ± S.E.M. (SIT *n*=6, MICT *n*=7).

748 *Main training effect ($P<0.05$ vs. pre-training)

749

750

751

752

753

754

755

756

757

758

759

760

761

762

763

764 **Figure legends**

765

766 **Figure 1. Training-induced changes in fibre-specific COX expression, IMTG, PLIN2, PLIN3 and**
767 **PLIN5 content**

768 Immunofluorescence images of muscle sections were used to quantify muscle fibre type-specific COX
769 expression (expressed as whole cell mean fluorescence intensity) (A, B). Fibre-type specific content of
770 IMTG (C, D), PLIN2 (E, F), PLIN3 (G, H) and PLIN5 content (expressed as % area stained) (I, J) was
771 quantified from immunofluorescence images of muscle sections following selection of a uniform
772 intensity threshold representative of positive signal. Fibres stained positively for myosin heavy chain
773 type I were classified as type I fibres, while those with no staining were classified as type II fibres.
774 Values are presented as means ± S.E.M. (n=8 per group). *Main fibre effect (P<0.05 vs. type I fibres).
775 †Main training effect (P<0.05 vs. pre-training). ‡Significant training effect within fibre (P<0.05 vs. pre-
776 training).

777

778 **Figure 2. Immunofluorescence images of IMTG, PLIN2, PLIN3 and PLIN5**

779 Representative images of IMTG, stained with oil red O, and PLIN2, PLIN3 and PLIN5 before and after
780 4 weeks of SIT. Right panels are corresponding images of myosin heavy chain I (MHC I) (stained
781 green or red) in combination with wheat germ agglutinin Alexa Fluor 350 (WGA) to identify the cell
782 border (stained blue) in skeletal muscle. Positively stained fibres (green or red) are type I fibres, all
783 other fibres are assumed to be type II fibres. White bars represent 50 μm.

784

785 **Figure 3. Transmission electron micrographs of human skeletal muscle**

786 A: Representative image from one muscle fibre at x10,000 magnification, with light arrows indicating
787 a lipid droplet and dark arrows indicating mitochondrial fragments. Scale bar represents 2 μm.
788 Representative image indicating a lipid droplet not in contact with a mitochondria fragment (B) and a
789 lipid droplet in contact with a mitochondria fragment (C). B and C are at x40,000 magnification. Scale
790 bar represents 0.5 μm.

791

Figure 4. Effect of 4 weeks SIT or MICT on muscle lipids

Changes in muscle total TAG, DAG and ceramide concentrations (A) and complex ceramides (*MHC* monohexosylceramide, *DHC* dihexosylceramide, *THC* trihexosylceramide, *GM* ganglioside) and sphingomyelin (SM) concentrations in muscle in response to 4 weeks of SIT or MICT (B). Changes in the composition of individual ceramide (C) and THC (D) species were also quantified in response to SIT or MICT. All data is expressed as fold change relative to the average MICT Pre value (n=7 per group). *Main training effect (P<0.05 vs. corresponding Pre-Training value). †Trend for training effect (P=0.06 vs. corresponding Pre-Training value).

Supplementary Figure 1. Effect of 4 weeks SIT or MICT on COX expression

Representative images of COX staining before and after 4 weeks of SIT. Right panels are corresponding images of myosin heavy chain I (MHC I) (stained green) in combination with wheat germ agglutinin Alexa Fluor 350 (WGA) to identify the cell border (stained blue) in skeletal muscle. Positively stained fibres (green or red) are type I fibres, all other fibres are assumed to be type II fibres. White bars represent 50 μm.

Figure 1

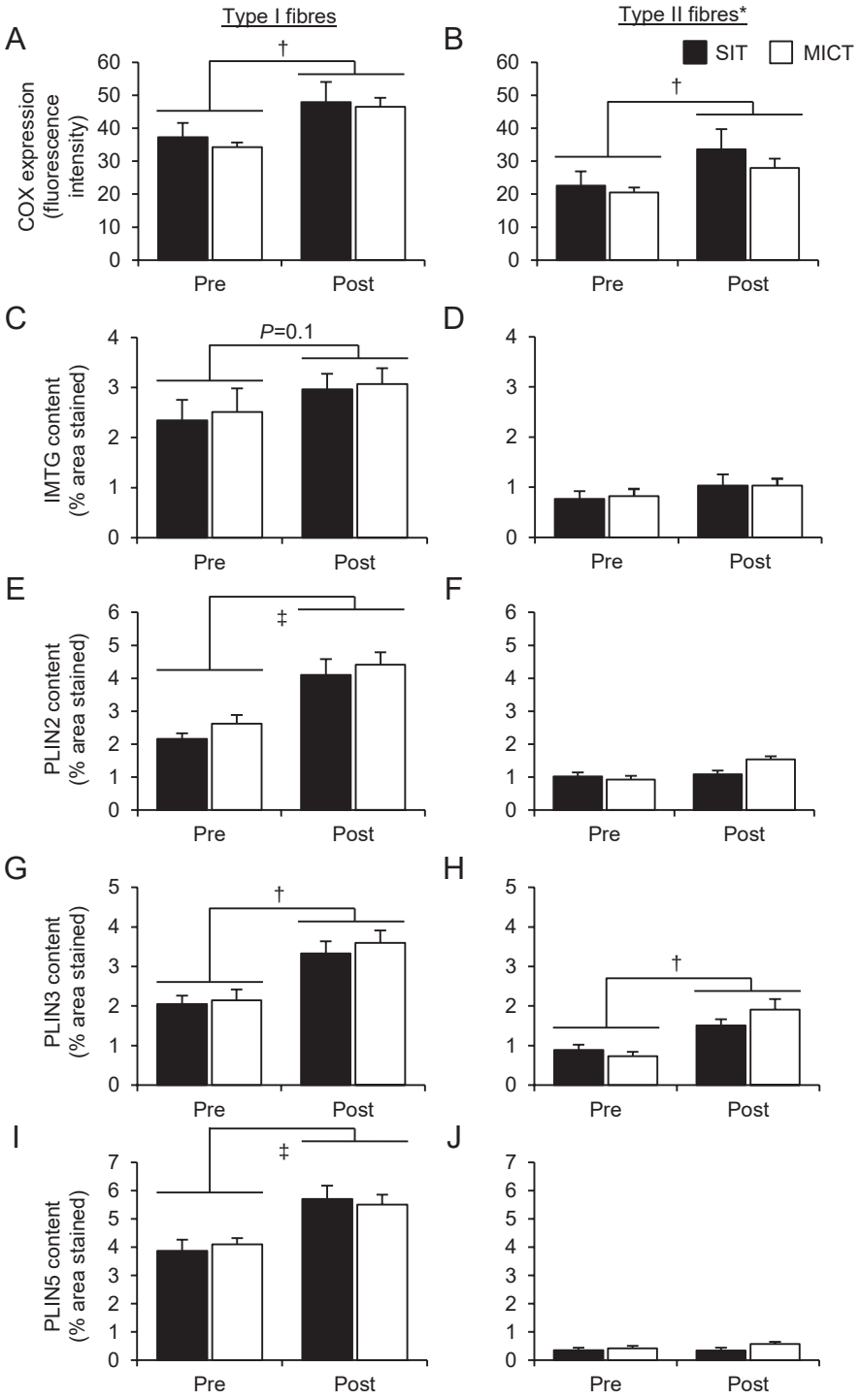

Type I fibres
Type II fibres*
SIT
MICT
A
B
C
D
E
F
G
H
I
J
COX expression (fluorescence intensity)
IMTG content (% area stained)
PLIN2 content (% area stained)
PLIN3 content (% area stained)
PLIN5 content (% area stained)
Pre
Post
†
‡
P=0.1

Figure 2

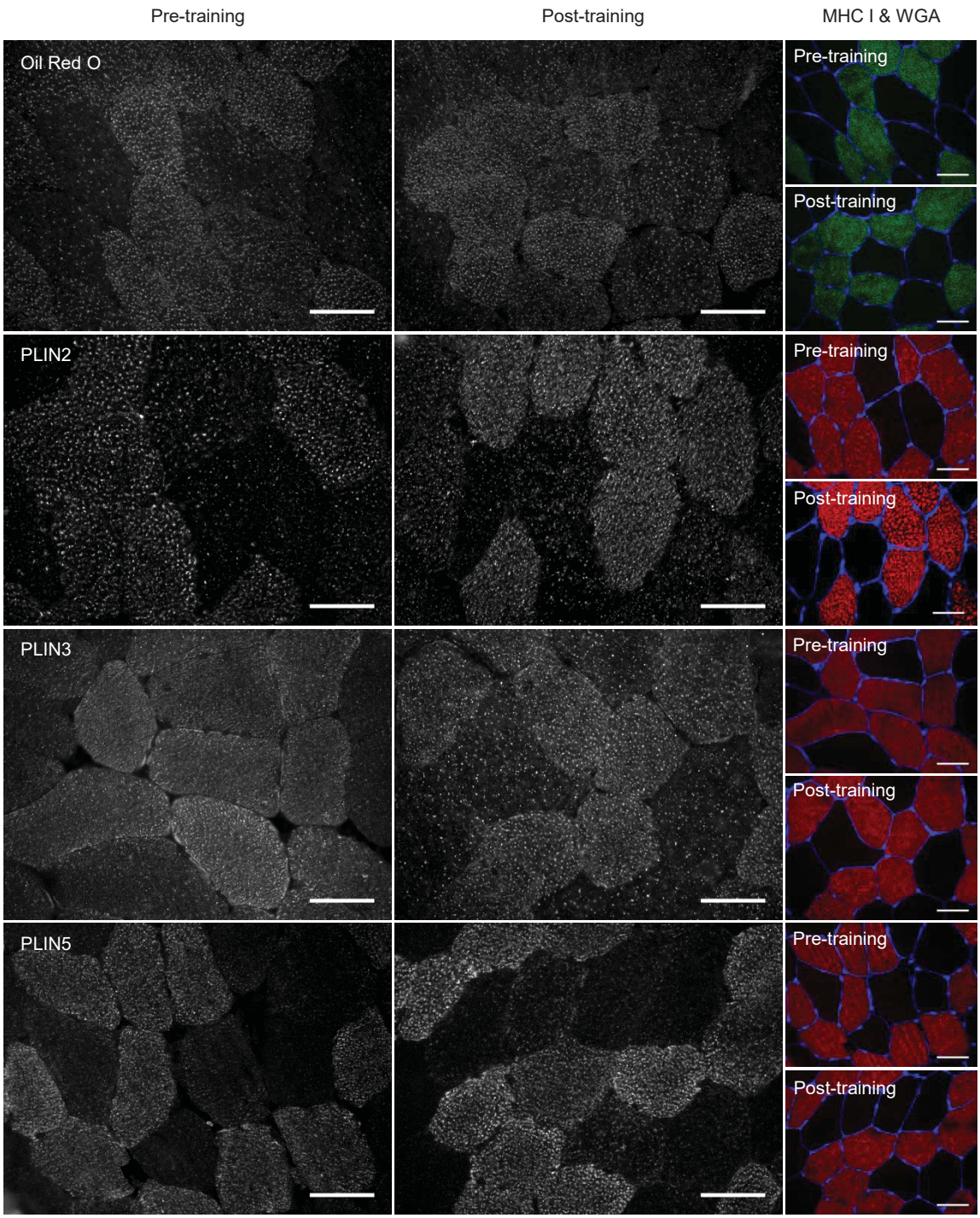

Pre-training
Post-training
MHC I & WGA
Oil Red O
Pre-training
Post-training
PLIN2
Pre-training
Post-training
PLIN3
Pre-training
Post-training
PLIN5
Pre-training
Post-training

Figure 3

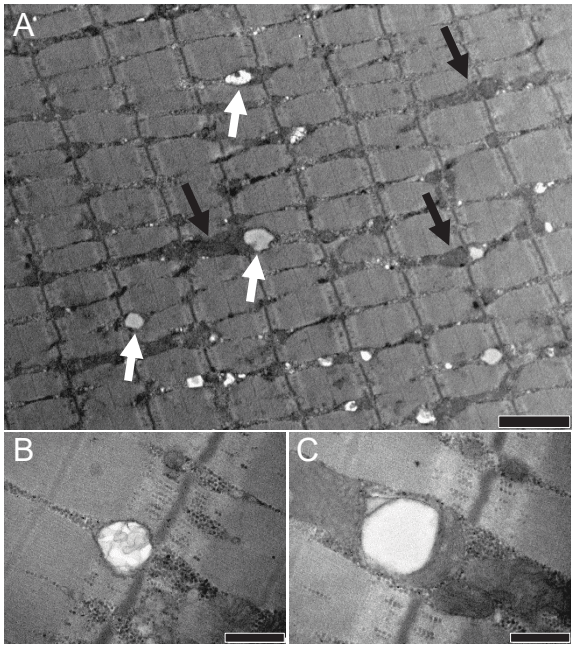
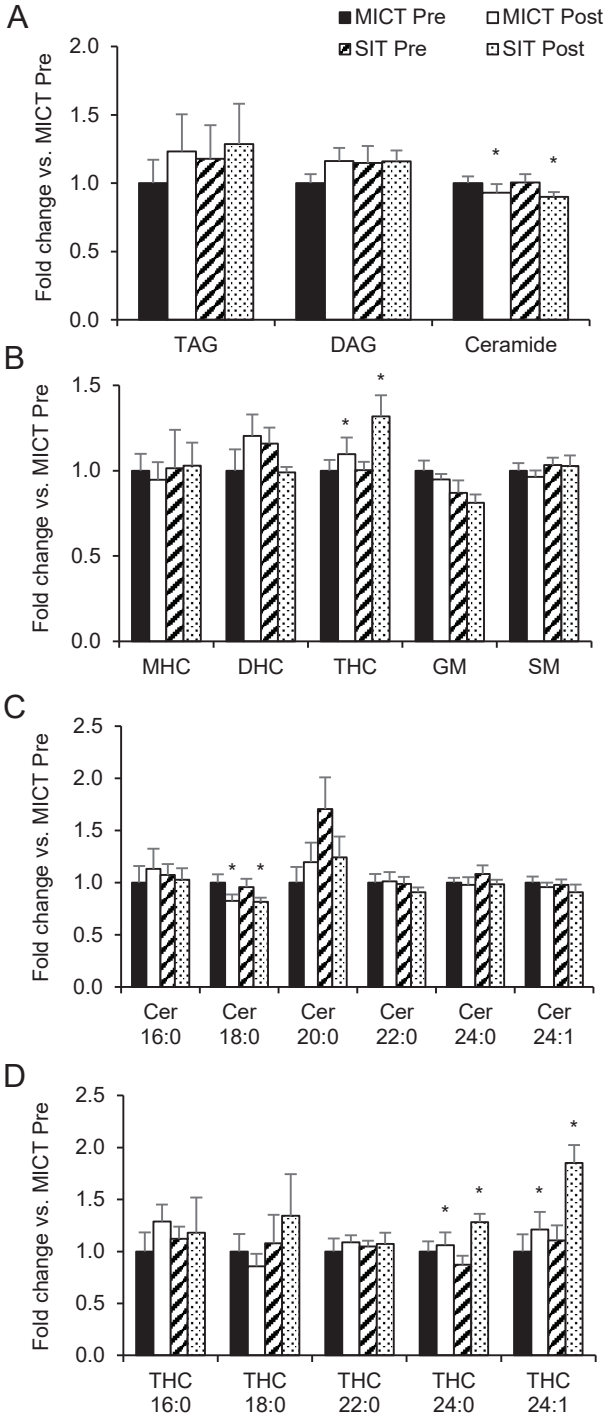


Figure 4


A
MICT Pre
MICT Post
SIT Pre
SIT Post
Fold change vs. MICT Pre
2.0
1.5
1.0
0.5
0.0
TAG
DAG
Ceramide
B
Fold change vs. MICT Pre
MHC
DHC
THC
GM
SM
C
Fold change vs. MICT Pre
2.5
Cer 16:0
Cer 18:0
Cer 20:0
Cer 22:0
Cer 24:0
Cer 24:1
D
Fold change vs. MICT Pre
THC 16:0
THC 18:0
THC 22:0
THC 24:0
THC 24:1

Supplementary Figure 1