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

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1School of Sport and Exercise Sciences, The University of Birmingham, Birmingham, United Kingdom; 2Institute of Sport, Exercise, and Active Living, Victoria University, Melbourne, Australia; 3Jessa Hospital, Heart Centre Hasselt, Hasselt, Belgium; 4Faculty of Medicine, Hasselt University, Diepenbeek, Belgium; and 5Department of Human Movement Sciences, Nutrition and Toxicology Research Institute, Maastricht University Medical Centre, Maastricht, The Netherlands

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Prolonged exercise training increases intramuscular lipid content and perilipin 2 expression in type I muscle fibers of patients with type 2 diabetes. Am J Physiol Endocrinol Metab 303: E1158–E1165, 2012. First published September 4, 2012; doi:10.1152/ajpendo.00272.2012.—The aim of the present study was to investigate changes in intramuscular triglyceride (IMTG) content and perilipin 2 expression in skeletal muscle tissue following 6 mo of endurance-type exercise training in type 2 diabetes patients. Ten obese male type 2 diabetes patients (age 62 ± 1 yr, body mass index BMI 31 ± 1 kg/m2) completed three exercise sessions/week consisting of 40 min of continuous endurance-type exercise at 75% VO2peak for a period of 6 mo. Muscle biopsies collected at baseline and after 2 and 6 mo of intervention were analyzed for IMTG content and perilipin 2 expression using fiber 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 twofold in response to the 6 mo of exercise training in both type I and type II muscle fibers (P < 0.05). A threefold increase in perilipin 2 expression was observed from baseline to 2 and 6 mo of intervention in the type I muscle fibers only (1.1 ± 0.3, 3.4 ± 0.6, and 3.6 ± 0.6% of fibers stained, respectively, P < 0.05). Exercise training induced a 1.6-fold increase in mitochondrial content after 6 mo of training in both type I and type II muscle fibers (P < 0.05). In conclusion, this is the first study to report that prolonged endurance-type exercise training increases the expression of perilipin 2 alongside increases in IMTG content in a type I muscle fiber-type specific manner in type 2 diabetes patients.

Address for reprint requests and other correspondence: L. J. C. van Loon, Dept. of Human Movement Sciences, Nutrition and Toxicology Research Institute, Maastricht University Medical Centre, Universiteitsingel 50, P. O. Box 616, 6200 MD Maastricht, The Netherlands (e-mail: lvanloon@maastrichtuniversity.nl).
23). Therefore, TG accumulation in cells expressing perilipin 2 has been attributed to the subsequent lowering of basal lipolytic rates, which also promotes tissue insulin sensitivity (1). In agreement, human studies demonstrate that perilipin 2 gene expression is higher in insulin-sensitive vs. insulin-resistant individuals (8) and improvements in insulin-mediated glucose disposal in response to weight loss, and the pharmacological treatment of type 2 diabetes alters the expression of perilipin 2 in skeletal muscle (27, 32). However, the impact of prolonged endurance-type exercise training on fiber-type specific perilipin 2 protein expression remains to be assessed.

We hypothesized that prolonged endurance-type exercise training increases muscle lipid storage and upregulates the expression of perilipin 2. Given the importance of considering muscle fiber type when investigating IMTG and related proteins, we applied immunofluorescence microscopy techniques to investigate muscle fiber type-specific changes in IMTG, perilipin 2, and cytochrome c oxidase (COX) content following 2 and 6 mo of endurance-type exercise training in type 2 diabetes patients.

MATERIALS AND METHODS

Participants

Ten type 2 diabetes patients participated in the current study [62 ± 1 yr, body mass index (BMI) 31.2 ± 0.9 kg/m²]. Participants had been diagnosed for ≥12 mo, 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 50 type 2 diabetes patients, as described in detail elsewhere (16).

Study Design

Participants completed a 6-mo endurance-type exercise training program. Prior to commencement of the study, and after 2 and 6 mo of the intervention, oxidative capacity, body composition, and oral glucose tolerance were assessed as described previously (16). Muscle biopsies were taken from the vastus lateralis in the morning and following an overnight fast and were analyzed for mitochondrial content, IMTG, and perilipin 2 expression. The measurements at 2 and 6 mo were performed ≥4 days after the last exercise session. Oral blood glucose and/or lipid-lowering medications were stopped 3 days prior to these measurements.

Training Intervention

Participants undertook three supervised training sessions per week in the rehabilitation center 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 VO2peak. The relationship between VO2peak and heart rate was reassessed after 2 mo, 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) and frozen in liquid nitrogen-cooled isopentane. Cryosections of 5-μm thickness were fixed in 3.7% formaldehyde and permeabilized for 5 min in 0.5% Triton X-100. Sections were then incubated for 1 h with a mouse monoclonal anti-ADRP/perilipin 2 antibody (Progen), as described previously (38, 39). As a key protein in the electron transport chain, identification of COX using a mouse monoclonal anti-OxPhos Complex IV (COX) antibody (Invitrogen) was also used as a marker of the mitochondrial network of skeletal muscle. Fiber type determination was achieved through incubation of muscle sections with mouse anti-myosin heavy chain type I (A4.840-c, developed by Dr. H. M. Blau, Developmental Studies Hybridoma Bank). Sections were then incubated with either an Alexa Fluor goat anti-mouse IgG2a 594 (for OxPhos Complex IV) or an Alexa Fluor goat anti-mouse IgG1 594 (for perilipin 2) in combination with an Alexa Fluor goat anti-mouse IgM 488 (for myosin heavy chain I) (Invitrogen) for 30 min. Coverslips were mounted with a glyceral and mowiol 4-88 solution in 0.2 M Tris buffer (pH 8.5) (including 0.1% DABCO anti-fade medium). When IMTG visualization was undertaken, the neutral lipid dye oil red O staining protocol, in combination with immunofluorescence, was used (47). In this respect, oil red O was applied to sections for 30 min following incubation with antibodies for fiber type determination.

Image Capture, Processing, and Data Analysis

Image capture was performed in a blinded fashion on a wide-field Nikon E600 microscope with a 40 × 0.75 NA objective coupled to a SPOT RT KE color 3-shot charge-coupled device camera (Diagnostic Instruments) for the fiber-type specific determination of IMTG and perilipin 2. 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 63 × 1.4 NA oil immersion objective was used to obtain digital images of mitochondria, IMTG, and perilipin 2. 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). Wide-field images were used to assess fiber type-specific content of IMTG and perilipin 2. Confocal images were used to assess COX content and LD size and to visualize the subcellular distribution of perilipin 2. Fibers positively stained for myosin heavy chain I were considered type I muscle fibers, and nonstained fibers were considered type II muscle fibers. Identification of COX, IMTG, and perilipin 2 was achieved through the selection of an intensity threshold that was used uniformly for all images in that series. COX, IMTG, and perilipin 2 content was expressed as the percentage fiber area positively stained. IMTG and perilipin 2 density were expressed as number of positively stained “spots” corrected for fiber area (μm²). 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 fibers per muscle cross-section were analyzed for COX, IMTG, and perilipin 2 analysis, respectively.

Statistics

All data are expressed as means ± SE. Significance was set at the 0.05 level of confidence. Changes in whole body characteristics, exercise capacity, body composition, and insulin sensitivity were analyzed using a one-way repeated measures ANOVA, with the within-subject factor as “time” (0 vs. 2 vs. 6 mo). Changes in COX, IMTG, and perilipin 2 were assessed using a two-way repeated-measures ANOVA with two within-subject factors: “fiber” (type I vs. type II fibers) and “time” (0 vs. 2 vs. 6 mo). Significant main effects or interactions were assessed using Bonferroni adjustment post hoc analysis.
RESULTS

Participants

Participant characteristics are displayed in Table 1. Significant reductions in body mass and BMI were observed with training (P < 0.05; Table 1) and were accompanied by a reduction in relative trunk fat and leg fat percentage of 6 ± 2 and 5 ± 2% posttraining, respectively (P < 0.05). Maximal oxygen uptake demonstrated a significant increase over time (P < 0.05) and was 16 ± 3% higher after 2 mo of training (from 23.4 ± 1.5 to 27.1 ± 1.7 ml·kg\(^{-1}\)·min\(^{-1}\), P < 0.01) and remained 13 ± 7% higher compared with baseline values after 6 mo 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 Hb A1c (16), in this small subcohort of 10 subjects the decrease in Hb A1c following training failed to reach significance (7.0 ± 0.4, 6.7 ± 0.3, and 6.5 ± 0.2% for 0, 2, and 6 mo, respectively, P = 0.20). No significant changes in fasting plasma glucose and insulin concentrations, 2-h post-oral glucose tolerance test glucose concentrations, or homeostasis model assessment index of insulin sensitivity were observed in response to training (P > 0.05).

Immunohistochemical Analysis

COX. Muscle fiber type-specific COX expression was significantly greater in type I compared with type II muscle fibers at all time points (P < 0.01; Fig. 1). Six months of endurance-type exercise training induced a time-dependent increase in COX expression in both type I and type II fibers (P < 0.01). Following 6 mo of exercise training, COX expression was higher than baseline in both type I (8.9 ± 2.1 vs. 14.0 ± 2.9% fiber stained) and type II muscle fibers (5.4 ± 1.8 vs. 8.8 ± 2.4% fiber stained). The confocal micrographs of COX-stained muscle fibers at baseline, 2 mo, and 6 mo are presented in Fig. 1B. These images demonstrate greater COX density in both intermyofibrillar and subsarcolemmal regions of the muscle fibers after 6 mo of training and are most prominent in the subsarcolemmal regions.

Table 1. Subject characteristics

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Time</th>
<th>Effect of Time (P Value)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>2 Mo</td>
</tr>
<tr>
<td>Age, yr</td>
<td>62 ± 1</td>
<td>91.7 ± 3.4</td>
</tr>
<tr>
<td>Height, m</td>
<td>1.72 ± 0.02</td>
<td>16.5 ± 1.6</td>
</tr>
<tr>
<td>Body weight, kg</td>
<td>92.9 ± 3.4</td>
<td>16.8 ± 2.3</td>
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<tr>
<td>Body mass index, kg/m²</td>
<td>31.2 ± 0.9</td>
<td>17.0 ± 1.5</td>
</tr>
<tr>
<td>Insulin sensitivity</td>
<td>9.2 ± 0.7</td>
<td>9.0 ± 0.7</td>
</tr>
<tr>
<td>Fasting glucose, mmol/l</td>
<td>17.9 ± 1.6</td>
<td>16.5 ± 1.6</td>
</tr>
<tr>
<td>2-h Glucose, mmol/l</td>
<td>17.8 ± 2.3</td>
<td>16.8 ± 2.3</td>
</tr>
<tr>
<td>Fasting insulin, μIU/ml</td>
<td>7.5 ± 1.4</td>
<td>7.0 ± 1.5</td>
</tr>
<tr>
<td>HOMA index</td>
<td>7.0 ± 0.4</td>
<td>6.7 ± 0.3</td>
</tr>
<tr>
<td>Exercise capacity</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VO₂peak, ml·kg(^{-1})·min(^{-1})</td>
<td>2.15 ± 0.14</td>
<td>2.46 ± 0.15*</td>
</tr>
<tr>
<td>VO₂peak, ml·kg(^{-1})·min(^{-1})</td>
<td>23.4 ± 1.5</td>
<td>27.1 ± 1.7*</td>
</tr>
<tr>
<td>W(_\text{max}) (W)</td>
<td>180 ± 12</td>
<td>189 ± 11</td>
</tr>
<tr>
<td>%Trunk fat</td>
<td>38.4 ± 1.4</td>
<td>37.3 ± 1.7</td>
</tr>
<tr>
<td>%Leg fat</td>
<td>23.2 ± 1.6</td>
<td>22.4 ± 1.5</td>
</tr>
</tbody>
</table>

Data provided represent means ± SE (n = 10). HOMA, homeostasis model assessment; VO₂peak, peak oxygen uptake; W\(_\text{max}\), maximal workload. *P < 0.05 vs. baseline.

IMTG. IMTG content, expressed as the area fraction stained, differed between type I and type II muscle fibers at each time point (P < 0.01; Fig. 2A). Six months of endurance-type exercise training induced a time-dependent increase in IMTG content in both type I and II muscle fibers. IMTG content had increased ~1.9-fold in type I (2.3 ± 0.4 vs. 4.3 ± 0.5%, P < 0.01) and type II fibers (0.9 ± 0.1 vs. 1.7 ± 0.3%, P < 0.01) following 6 mo of training. The increase in IMTG content was mirrored by significant increases in IMTG density after 6 mo of training in type I fibers (0.051 ± 0.007 and 0.079 ± 0.008 LD/μm² for 0 and 6 mo, respectively, P < 0.01) but not in type II muscle fibers (0.022 ± 0.002 and 0.031 ± 0.003 LD/μm² for 0 and 6 mo, respectively, P = 0.056). No significant changes in IMTG content or density were apparent after 2 mo of training in either fiber type (P > 0.05). There were no differences in LD size, as determined by confocal microscopy, between fiber types or in response to endurance-type exercise training (P > 0.05).

Perilipin 2. At baseline, perilipin 2 expression, calculated as the percentage of area stained, did not differ between type I and type II muscle fibers (P > 0.05; Fig. 2B). Six months of exercise training induced a time-dependent increase in perilipin 2 expression in type I muscle fibers only (P < 0.05). Compared with baseline, perilipin 2 expression in type I muscle fibers had increased approximately threefold after 2 mo of training (1.1 ± 0.3 vs. 3.6 ± 0.6%, P < 0.05), with no further increase observed after 6 mo of training. This fiber type-specific training response resulted in greater perilipin 2 expression in type I compared with type II muscle fibers after 2 and 6 mo of training (P < 0.05). Representative immunofluorescence images of perilipin 2 expression in type I and type II muscle fibers at baseline and after 2 and 6 mo of training are shown in Fig. 3. Compared with baseline, perilipin 2 density in type I fibers also increased approximately twofold after 2 and 6 mo (0.018 ± 0.003, 0.033 ± 0.004, and 0.035 ± 0.005 perilipin 2 objects/μm² for 0, 2, and 6 mo, respectively, P < 0.05), whereas perilipin 2 density in type II muscle fibers did not change (P > 0.05). Therefore, perilipin 2 density was higher in type I than type II muscle fibers after 2 and 6 mo of training only.
Higher-magnification images of perilipin 2 were obtained using confocal laser scanning microscopy and are shown in Fig. 3. These images show a clear increase in perilipin 2 expression after 6 mo of training. These images demonstrate that distinct rings of perilipin 2 can be observed frequently and are more abundant after prolonged exercise training.

**DISCUSSION**

Prolonged endurance-type exercise training is known to improve insulin-stimulated glucose uptake and glycemic control in type 2 diabetes patients (17). 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 fibers. In accord with this, we show for the first time that training induces a greater expression of perilipin 2 in type I muscle fibers.

Insulin sensitivity is enhanced by regular physical activity, which explains why significant improvements in glycemic control were observed in the previous study after 6 mo of exercise training in a large cohort of type 2 diabetes patients (16). In the subset of participants used in this study there was no significant change in glycemic control, as was evident from the Hb A1c levels after 6 mo of training (Table 1). Nevertheless, a decline from 7.0 ± 0.4 down to 6.5 ± 0.2% in Hb A1c is of great clinical significance because it would translate into a >10% reduction in the risk of premature death, a 5–10% reduction in the risk of myocardial infarction, and an ~20% reduction in the risk of microvascular disease (25).

Skeletal muscle oxidative capacity and whole body fatty acid oxidation are good predictors of muscle insulin sensitivity (5, 13, 14, 19). Obese individuals with insulin resistance and type 2 diabetes commonly display a reduced capacity for oxidative metabolism (2, 20, 21, 36). Thus, it is likely that increased oxidative capacity following exercise interventions is mechanistically linked to improvements in metabolic health in this population. Accordingly, we observed an ~1.6-fold increase in COX expression in skeletal muscle following 6 mo of endurance-type exercise training (Fig. 1). 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 (16).

In agreement with previous data investigating mitochondrial content following a 10-wk training intervention in type 2 diabetes patients using transmis-
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 (13, 46). 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 with 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 with a reduction in the concentration of diacylglycerol and ceramide (9). 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 protect against the development of insulin resistance during (intra)lipid infusion (37). The present study adds to this growing body of evidence by demonstrating greater IMTG storage and improved glycemic control in response to 6 mo of training in type 2 diabetes patients. Some studies employing a shorter training duration have failed to observe a significant increase in type 1 muscle fiber IMTG content following training in type 2 diabetic patients (26). Therefore, it is possible that a more prolonged intervention, such as the 6-mo endurance training program 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 fibers, 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, whereas LD size remained unchanged (44). IMTG expansion through an increase in the number of smaller LDs would be a metabolic advantage because 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 perilipin 2. In the current study, despite an approximately twofold higher IMTG concentration in the type I muscle fibers being observed (Fig. 2A), there was no difference in perilipin 2 expression between type I and type II muscle fibers (29). This is the first study to show a type I muscle fiber-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 wk of exercise training (10, 35). Although electron microscopy (29), we show that increased COX expression is prominent in subsarcomemmal regions of type I fibers after prolonged endurance-type exercise training.

The exercise training-induced increase in skeletal muscle oxidative capacity was accompanied by an approximately twofold elevation in skeletal muscle lipid deposition in both type I and type II muscle fibers (Fig. 2). This is the first study to show a type I muscle fiber-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 wk of exercise training (10, 35). Although
We show that when type 2 diabetes patients are physically active, type I muscle fibers exhibit a greater expression of perilipin 2 than type II muscle fibers. This is in agreement with our previous observations of a greater perilipin 2 expression in the type I muscle fibers of sedentary individuals and trained cyclists (39, 40). The increase in perilipin 2 expression in type I muscle fibers is likely to result in enhanced coverage of the LD surface with perilipin 2. This adaptation would limit rates of basal lipolysis and promote IMTG storage in the basal state. Furthermore, hormone-sensitive lipase translocates to perilipin 2-coated LDs during muscle contraction and adrenaline stimulation (34), and perilipin 2-associated LDs are depleted during exercise (40). We hypothesize that an increase in perilipin 2 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 toward 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 perilipin 2 expression and intramuscular lipid oxidation.

A nonexercise 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 nonexercising controls (7, 41). Therefore, we can be confident that the related changes in perilipin 2 expression and IMTG storage in the present study are specific adaptations to the exercise intervention. Because perilipin 2 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 of 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 fiber 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 perilipin 2 expression. The greater
perilipin 2 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 glycemic control.

ACKNOWLEDGMENTS

The antibody against myosin (human slow fibers, A4.840) used in this study was developed by Dr. H. M. Blau and was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the National Institute of Child Health and Human Development and maintained by The University of Iowa, Department of Biological Sciences, Iowa City, IA.

DISCLOSURES

No conflicts of interests, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS


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