Adipose triglyceride lipase in human skeletal muscle is upregulated by exercise training

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Alsted TJ, Nybo L, Schweiger M, Fledelius C, Jacobsen P, Zimmermann R, Zechner R, Kiens B. Adipose triglyceride lipase in human skeletal muscle is upregulated by exercise training. Am J Physiol Endocrinol Metab 296: E445–E453, 2009. First published December 23, 2008; doi:10.1152/ajpendo.90912.2008.—Mobilization of fatty acids from stored triacylglycerol (TG) in adipose tissue and skeletal muscle [intramyocellular triacylglycerol (IMTG)] requires activity of lipases. Although exercise training increases the lipolytic capacity of skeletal muscle, the expression of hormone-sensitive lipase (HSL) is not changed. Recently, adipose triglyceride lipase (ATGL) was identified as a TG-specific lipase in various rodent tissues. To investigate whether human skeletal muscle ATGL protein is regulated by endurance exercise training, 10 healthy young men completed 8 wk of supervised endurance exercise training. Western blotting analysis on lysates of skeletal muscle biopsy samples revealed that exercise training induced a twofold increase in skeletal muscle ATGL protein content. In contrast to ATGL, expression of comparative gene identification 58 (CGI-58), the activating protein of ATGL, and HSL protein was not significantly changed after the training period. The IMTG concentration was significantly decreased by 28% at termination of the training program compared with before. HSL-phosphorylation at Ser560 was increased, HSL-Ser659 phosphorylation was unchanged, and HSL-phosphorylation at Ser460 was decreased altogether, indicating an enhanced basal activity of this lipase. No change was found in the expression of diacylglycerol acyl transferase 1 (DGAT1) after training. Inhibition of HSL with a monospecific, small molecule inhibitor (76-0079) and stimulation of ATGL with CGI-58 revealed that significant ATGL activity is present in human skeletal muscle. These results suggest that ATGL in addition to HSL may be important for human skeletal muscle lipolysis.

The in vivo regulation of ATGL activity is not established yet, but it involves an activating protein named comparative gene identification 58 (CGI-58; also named α/β-hydrolase fold

Hormone-sensitive lipase (HSL) has generally been accepted to be the primary lipase responsible for hydrolysis of intramyocellular triacylglycerol (IMTG). This notion is supported by findings in both rat and human skeletal muscle demonstrating that immunoinhibition of HSL with an anti-HSL antibody completely abolished contraction-induced increase in triacylglycerol (TG)-lipase activity (29, 40, 52). On the other hand, dissociations between HSL activity and net change of IMTG content during skeletal muscle contractions in humans have been observed (40, 50, 51). Also, in resting human skeletal muscle, it was shown that 40–80% TG-hydrolase activity was still remaining after immunoinhibition of HSL (40, 52). In line with this, recent studies (17) revealed that basal TG-hydrolase (lipolytic) activity was not reduced in the skeletal muscle of HSL knockout mice compared with wild-type controls and that, in the wild-type mice, diacylglycerol (DAG) rather than TG was found to accumulate in skeletal muscle and in adipose tissue. These findings together indicate that TG lipases other than HSL may be of importance in skeletal muscle TG hydrolysis. Recently, a previously unknown TG lipase, named adipose triglyceride lipase (ATGL), was identified (22, 49, 55). This lipase was found to be expressed at the mRNA level in mouse and human adipose tissue (22, 31, 49, 55) and to a lesser extent in other mouse tissues including skeletal muscle (49, 55). ATGL has been shown to catalyze the initial step in TG catabolism and to possess high substrate specificity by almost exclusively hydrolyzing the first ester bond in TG (55). Furthermore, ATGL has recently been shown to be an important TG hydrolase in myotubes and rat skeletal muscle (53).

In human adipose tissue, ATGL was recently found to be expressed at the protein level (23, 41) and to be downregulated by energy restriction (23). Little is known about ATGL in human skeletal muscle, but with the use of Immunohistochemistry it was recently shown to be expressed exclusively in type I fibers (24). It is not known whether ATGL protein expression in skeletal muscle might be affected by physical activity, but a hypothesis could be that changes in muscle activity would induce changes in ATGL protein expression like many other proteins (19). An increased ATGL expression with endurance training might be expected since some (18, 20, 36) but not all (1, 26) endurance training studies have indicated that utilization of IMTG during exercise is enhanced in the endurance-trained state. This increased lipolytic capacity does not seem to relate to changes in HSL protein expression, since HSL protein levels are not changed by endurance training in rat skeletal muscle (10) and in human subjects HSL content is similar in untrained and trained skeletal muscle (18).

HSL activity is regulated by phosphorylation on at least five serine sites (47), four of which have been found to be phosphorylated on human skeletal muscle HSL (39, 40, 50). No longitudinal studies have investigated the effect of endurance exercise training on human skeletal muscle HSL protein content and serine phosphorylations. The in vivo regulation of ATGL activity is not established yet, but it involves an activating protein named comparative gene identification 58 (CGI-58; also named α/β-hydrolase fold

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domain-containing protein 5 (ABHD5); Ref. 32). CGI-58 was found to be widely expressed at the mRNA level in several tissues of mice, being highest in adipose tissue but also detectable in skeletal muscle (32, 48). Mutations in the CGI-58 gene have been identified as causative for Chanarin-Dorfman syndrome (33), a condition that is characterized by excessive TG accumulation in various tissues, including skeletal muscle (8). Furthermore, individuals with Chanarin-Dorfman syndrome also suffer from myopathy (8), indicating an important in vivo function of CGI-58 and ATGL in skeletal muscle tissue. However, no study has shown that ATGL activity is present in human skeletal muscle.

The IMTG content reflects a balance between lipolytic breakdown and synthesis through esterification. Diacylglycerol acyl transferase (DGAT) catalyzes the last reaction in mammalian TG synthesis using fatty acyl-CoAs and DAG as substrates (54). There are two DGAT isoforms, of which DGAT1 is the dominant isoform in skeletal muscle (6, 7). DGAT1 has been found to be increased in mouse and human skeletal muscle after an acute bout of exercise (21, 43) and to be more abundant at the mRNA level in endurance-trained compared with untrained human subjects (44). DGAT1 expression has not been examined in human skeletal muscle after weeks of endurance exercise training.

The purpose of the present study was to investigate whether regular exercise training of healthy male subjects with a sedentary lifestyle could affect muscle ATGL, CGI-58, and HSL protein expressions. Regulation of HSL was further investigated by measuring the phosphorylation status on three different serine sites involved in HSL activation. To verify the existence of functional ATGL in human skeletal muscle TG-hydrolase activity assays were performed using an inhibitor of HSL and recombinant CGI-58 (rCGI-58).

MATERIALS AND METHODS

Subjects

Ten healthy young males (age: 30 ± 1 yr; body wt: 91.7 ± 4.8 kg; and body mass index: 26.6 ± 1), untrained [maximal oxygen uptake (V\textsubscript{O2peak}): 36.7 ± 1.1 mL min\textsuperscript{-1} kg\textsuperscript{-1}] and nonsmokers, were recruited to participate in the study. Subjects did not participate in regular physical activity (<1 time per wk for the past 6 mo). Before volunteering, all subjects were given full oral and written information about the course of the study and possible risks associated with participation. Written consent was obtained from each subject. The study was approved by the Copenhagen Ethics Committee (KF-01-203/03) and conformed to the code of ethics of the World Medical Association (Declaration of Helsinki II).

Subjects participated in a controlled exercise training program for 8 wk. Initially and after the exercise training period, the fitness level of the subjects was measured and muscle biopsies from the vastus lateralis muscle were obtained for measurements of lipase proteins, enzyme activities, and substrate levels.

Experimental design

Exercise testing. Initially, to determine the physical fitness level of the subjects, V\textsubscript{O2peak} was measured. Thus subjects performed an incremental exercise test until exhaustion on a Monark Ergomedic 839E bicycle ergometer (Monark, Varberg, Sweden). Expired air was collected in Douglas bags for later analysis of \textit{O}\textsubscript{2} and \textit{CO}\textsubscript{2} content. The measured V\textsubscript{O2} was accepted as maximal when no further increase in \textit{V}\textsubscript{O2} in response to an increased workload was obtained and when the respiratory exchange ratio was at least 1.15 (42).

On another day after 1 day on a controlled standardized diet (65 E% carbohydrate, 20 E% fat, and 15 E% protein), subjects arrived in the morning in the laboratory after an overnight fast (12 h). After 60 min of rest in the supine position, a venous catheter was inserted into an antecubital vein for blood sampling. Thereafter, a needle biopsy was obtained, with the use of a Bergstrom needle with suction (2), from the vastus lateralis muscle under local anesthesia with 2% lidocaine. The muscle sample was quickly frozen (within 10 s) in liquid nitrogen and stored at −80°C until subsequent biochemical analyses. At the end of the training period, muscle biopsies were again obtained from the vastus lateralis muscle 48 h after the last exercise bout.

Training program. The subjects followed an 8-wk supervised training program. The training was performed on a bicycle ergometer. The first week included three exercise sessions, increased to four sessions per week during the following 4 wk and five sessions in weeks 7 and 3 sessions in week 8. Each session lasted 40–90 min and was initiated by a 10 min warm-up at 50% V\textsubscript{O2peak} followed by intermittent exercise of 2–6 min duration of varying high (60–85% V\textsubscript{O2peak}) and low (45–55% V\textsubscript{O2peak}) intensities. On three occasions during the training period, maximal oxygen uptake was measured on a bicycle ergometer and workload in the training sessions was adjusted accordingly.

Diet. Instructions were given not to make any dietary changes during the training period. Initially, as well as during the last week of the training period, a detailed 3-day food registration was completed. The subjects weighed and recorded all food and beverages to the accuracy of 1 g. Subsequently, energy intake and macronutrient composition of the diet were calculated (Dankost 2000; Danish Catering Center, Copenhagen, Denmark).

Dual-energy X-ray absorptiometry scanning. Body composition was measured using dual-energy X-ray absorptiometry (DEXA, LUNAR DPX IQ RBD, version 4.6b; Madison, WI) before and after the training period.

Analyses

Breath samples. Expired volumes of air was collected in Douglas bags and measured with a chain-suspended Collins spirometer. A small sample of mixed expired air was analyzed for contents of \textit{O}\textsubscript{2} (VacuMed 17518A; VacuMed, Ventura, CA) and \textit{CO}\textsubscript{2} (VacuMed 17515A).

Blood analysis. Plasma insulin concentrations were determined by radioimmunoassay (Pharmacia insulin radioimmunoassay 100; Pharmacia & Upjohn Diagnostics, Upsala, Sweden), and blood glucose levels were measured using an ABL 615 analyzer (Radiometer, Copenhagen, Denmark). Insulin sensitivity was assessed by the homeostasis model assessment index for insulin resistance (HOMA\textsubscript{IR}), which is calculated from the basal glucose and insulin concentrations (35).

Muscle analyses. Muscle tissue (~80 mg wet wt) was freeze-dried and dissected free of all visible exogenous adipose tissue, connective tissue, and blood under a stereo microscope (Stemi 2000-C, Zeiss, Oberkochen, Germany). The dissected muscle samples were then divided into pools for the respective biochemical analyses.

Muscle lysate preparation. Freeze-dried and dissected muscle tissue was homogenized, while kept at ~0°C in an ice slurry in a buffer (1:80 wt:vol) containing 50 mM HEPES pH 7.5, 150 mM NaCl, 20 mM Na pyrophosphate, 20 mM β-glycerophosphate, 10 mM Na fluoride, 2 mM Na orthovanadate, 2 mM EDTA, 1 mM EGTA, 1% Nonidet P-40, 10% glycerol, 2 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 3 mM benzamidine using a polytron homogenizer (PT 1200; Kinematica, Luzern, Switzerland) until no visible particles remained. Homogenates were then mixed thoroughly with 100 μl of kinase buffer and 20 μl of protein G-agarose (Protein G-50 Purification Kit, Roche Diagnostics, Mannheim, Germany) and incubated on ice for 30 min before centrifugation at 17,500 g at 4°C for 20 min. The supernatant was taken, and 5 μl were aliquoted for determination of protein content using the bicinchoninic acid method (Pierce, Rockford, IL) and BSA standards.
Protein content was measured in triplicate, and a maximal coefficient of variation of 5% between replicates was accepted.

Western blotting. Expression of ATGL, CGI-58, HSL, DGAT1, and perilipin as well as HSL serine phosphorylations was determined using Western blotting on the muscle lysates. For the various proteins, actin was blotted as a loading control. The lysates were boiled in Laemmli buffer before being subjected to SDS-PAGE and immunoblotting. Equal amounts of muscle lysate proteins were loaded onto 7.5% polyacrylamide self-casted gels (Bio-Rad, Herlev, Denmark) and transferred to polyvinylidene fluoride membranes (Millipore transmembranes; Millipore, Bedford, MA). The membrane was blocked for 2 h at room temperature with 2% skimmed milk or 5% BSA in Tris-buffered saline containing 0.1% Tween and thereafter incubated overnight at 4°C with a primary antibody. Primary antibodies were rabbit anti-ATGL (cat. no. 2138; Cell Signaling Technology, Beverly, MA), rabbit anti-CGI-58 (cat. no. NB110-4156; Novus Biologicals, Littleton, CO), rabbit anti-HSL (kindly donated by Dr. Cecilia Holm, Dept. of Cell and Molecular Biology, University of Lund, Lund, Sweden), sheep anti-phospho-HSL Ser659, sheep anti-phospho-HSL Ser660, goat anti-DGAT1 (cat. no. NB100-57086; Novus Biologicals), guinea pig anti-perilipin (cat. no. GP29; Progen Biotechnik, Heidelberg, Germany), and rabbit anti-Actin (cat. no. A2668; Sigma-Aldrich, St. Louis, MO). Secondary antibodies were horseradish peroxidase-conjugated anti-rabbit, antiguinea-pig (cat. no. P0448 and P0141; DAKO; Glostrup, Denmark), anti-sheep and anti-goat (cat. no. 81-1620 and 81-1620; Zymed, Carlsbad, CA). Antigen-antibody complexes were visualized using enhanced chemiluminescence (Immobilien Western; Millipore) and quantified using a Kodak Image station 2000MM (Kodak, Glostrup, Denmark). The specificity of the primary ATGL antibody was tested using skeletal muscle lysates from ATGL knockout and wild-type mice. The antibody that was used in the present study is highly specific in mouse skeletal muscle tissue (Fig. 1A).

HSL protein and ATGL mRNA are more highly expressed in adipocytes compared with skeletal muscle tissue, and TG is highly concentrated in adipocytes (15, 30, 55). To verify that the muscle tissue examined in the present study was not contaminated by adipocytes, the dissected muscle samples were analyzed for the presence of perilipin protein expression, which is present in adipocytes but not in skeletal muscle (14). This procedure has been conducted earlier in our laboratory (39).

TG-hydrolase activity assay. Freeze-dried and dissected muscle tissue from eight of the subjects was homogenized on ice in a buffer containing 0.25 M sucrose, 1 mM EDTA, 1 mM dithiothreitol, 40 mM β-glycerophosphate, 10 mM Na pyrophosphate, 20 µg/ml leupeptin, 20 µg/ml antipain, 6.25 µg/ml pepstatin A, and 0.31 µM okadaic acid. Thereafter, the homogenate was centrifuged at 18,500 × g at 4°C for 45 s, and the resulting supernatant was recovered and used for TG-hydrolase activity measurements. The substrate containing triolein and tri[9,10(n)-3H]olein (cat. no. TRA 191; GE Healthcare, Buckinghamshire, UK) was emulsified with phosphatidylcholine: phosphatidylcholine (3:1) in 3 ml of 0.1 M potassium phosphate buffer (pH 7.0) and 1 ml of 20% fatty acid free BSA in 0.1 M potassium buffer (pH 7) by sonication (Branson sonifier 250; Branson, Danbury, CT). For TG-hydrolase activities, 100 µg of a lysate pool of the eight subjects were incubated in a shaking water bath at 60 min at 37°C with 100 µl [3H]triolene substrate (167 nmol, 1.25 × 10^4 cpm) and an enzyme dilution buffer (20 mM potassium phosphate, pH 7.0, 1 mM EDTA, 1 mM diethylolethritol, and 0.02% fatty acid free BSA) in a total volume of 200 µl. All samples were run in triplicates. The reaction was interrupted by addition of 3.25 ml of methanol: chloroform:heptane (10:9:7) and 1 ml of 0.1 M potassium carbonate and 0.1 M boric acid pH 10.5. After the mixture was vortexed for 10 s and centrifuged at 1,100 g for 20 min, 1 ml of the upper phase was removed and the radioactivity of the released 3H-labeled fatty acids was determined by liquid scintillation counting (Tri-Carb 2500 TR; Packard Instruments). In some of the assay conditions, the muscle lysate was incubated with recombinant GST-tagged CGI-58 (rCGI-58) purified from yeast as previously described (32, 45) and/or with a nonspecific, small molecule inhibitor of HSL (76-0079; Nordisk). The specificity of this inhibitor has previously been tested in mouse white adipose tissue (45). Also, control assays were run with the respective vehicles: DMSO for the HSL inhibitor and dialysis buffer for rCGI-58 (150 mM KCl and 10 mM potassium phosphate pH 7.4 and 0.1% NP-40). As a positive control, TG-hydrolase activity with 76-0079 and/or rCGI-58 present was also performed on lysates of mouse white adipose tissue (data not shown).

IMTG. Approximately 80 mg of wet muscle tissue were freeze-dried and dissected free of all visible adipose tissue, connective tissue, and blood under a microscope. The dissected fibers were pooled, and hereof 2 mg were used for determination of IMTG as previously described by Kiens and Richter (27).

Activities of citrate synthase and 3-hydroxyacyl-CoA dehydrogenase. Maximal activity of citrate synthase (CS) and 3-hydroxyacyl-CoA dehydrogenase (HAD) was measured at 25°C in freeze-dried muscle tissue in accordance to Lowry and Passonneau (34). CS was measured on a spectrophotometer (Hitachi 912 automatic analyzer; Roche Diagnostics, Hvidovre, Denmark) and HAD by fluorometry (ratio-2 filter fluorometer; Optical Technology Devices, Bie & Berntsen, Roedovre, Denmark).

Fig. 1. Testing of adipose triglyceride lipase (ATGL) antibody specificity and representative blot against perilipin in freeze-dried and dissected human vastus lateralis muscle. A: skeletal muscle extract proteins of wild-type (WT) and ATGL knockout (KO) mice were immunostained for ATGL expression. Eight micrograms of protein extracts of white adipose tissue from wild-type mice were loaded as a control. Actin was detected as a loading control. B: skeletal muscle lysates corresponding to 40 µg of protein were loaded in each lane. Two micrograms of human white adipose tissue (WAT) were loaded as a positive control. MWAT, mouse white adipose tissue; MskM, mouse skeletal muscle.
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Table 1. General adaptations to training

<table>
<thead>
<tr>
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<th>Pretraining</th>
<th>Posttraining</th>
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<tbody>
<tr>
<td>Body mass, kg</td>
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<td>90.9±4.9</td>
</tr>
<tr>
<td>Body fat, %</td>
<td>31.4±1.5</td>
<td>29.3±1.8*</td>
</tr>
<tr>
<td>Lean body mass, kg</td>
<td>62.5±2.7</td>
<td>63.7±2.6</td>
</tr>
<tr>
<td>( V_{O2\text{peak}} ) l/min</td>
<td>3.4±0.2</td>
<td>4.0±0.2†</td>
</tr>
<tr>
<td>ml·kg body mass(^{-1})·min(^{-1})</td>
<td>36.7±1.1</td>
<td>44.0±1.5†</td>
</tr>
<tr>
<td>Insulin, µU/ml</td>
<td>5.48±1.01</td>
<td>4.86±0.99</td>
</tr>
<tr>
<td>Glucose, mM</td>
<td>5.01±0.17</td>
<td>4.74±0.10</td>
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<tr>
<td>HOMA_I(_A)</td>
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<td>1.02±0.21</td>
</tr>
<tr>
<td>HAD, µmol·g(^{-1})·min(^{-1})</td>
<td>22.6±1.7</td>
<td>32.1±0.8*</td>
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<tr>
<td>CS, µmol·g(^{-1})·min(^{-1})</td>
<td>46.9±3.4</td>
<td>76.9±4.3†</td>
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Data are means ± SE; \( n = 10 \). *\( P < 0.05 \), †\( P < 0.001 \) compared with pretraining value. HAD, 3-hydroxyacyl-CoA dehydrogenase; CS, citrate synthase. HOMA_I\(_A\), homeostasis model assessment index for insulin resistance.

Statistics. Statistical evaluations were performed using SPSS 16.0 for windows. Data are presented as means ± SE. To test for differences between pre- and posttraining samples, paired sampled t-tests were performed. Differences in TG-hydrolase activity was tested using a one-way ANOVA. When the ANOVA indicated significance, the Tukey’s post hoc test was used for further analysis. The strength of association between parameters was analyzed by Pearson product moment correlation analysis. Differences between samples and correlations between parameters were considered to be statistically significant when the \( P \) value was >0.05.

RESULTS

Diet

The average macronutrient composition of the diet was 53 E% carbohydrate, 30 E% fat, and 17 E% protein and did not change during the experimental period (data not shown).

Effects of training

Initially, total body weight averaged 91.7 ± 4.8 kg and no changes were found after the training period (Table 1). Also, lean body mass was not changed significantly, whereas a significant decrease in body fat percentage from 31 ± 1.5 to 29.3 ± 1.8% (\( P < 0.05; n = 10 \)) was observed (Table 1). The exercise training induced an increase in \( V_{O2\text{peak}} \) from 3.4 ± 0.2 to 4.0 ± 0.2 l/min (\( P < 0.001 \)) and expressed relative to body weight from 36.7 ± 1.1 to 44.0 ± 1.5 ml·kg body mass\(^{-1}\)·min\(^{-1}\) (\( P < 0.001 \)). The maximal activity of HAD was increased by 42% (\( P < 0.05 \)) and CS was increased 64% (\( P < 0.001 \); Table 1) after the training period. Altogether, these findings reveal that the 8-wk training program resulted in an increased aerobic and oxidative capacity. No changes were observed in the basal concentration of plasma insulin, blood glucose, or the HOMA_I\(_A\) index after the training period compared with before.

Perilipin protein expression

With the use of Western blotting, it was observed that loading 2 µg of total protein of human white adipose tissue extracts produced a strong band, whereas the perilipin protein content was below the detection limit in the skeletal muscle samples when loading 40 µg of total protein (Fig. 1B).

ATGL, CGI-58, and HSL protein expressions

To verify the specificity of the ATGL antibody used in the present study, muscle from ATGL knockout mice was immunoblotted for ATGL expression and the signal was absent (Fig. 1A).

After the 8-wk training program, a significant twofold increase in ATGL protein expression in the vastus lateralis muscles was observed (\( P < 0.001 \); Fig. 2A). CGI-58, the activating protein for ATGL, was not changed by the exercise training program (Fig. 2B). In contrast to muscle ATGL protein content, no effect was observed of exercise training on HSL protein content in skeletal muscle (Fig. 2C).

IMTG

IMTG content averaged 85.6 ± 12.3 mmol/kg dry wt in the untrained subjects. After the endurance exercise period, a significantly lower IMTG content (61.7 ± 14.3 mmol/kg dry wt) was observed (\( P < 0.01; n = 8 \); Fig. 2D). Due to the lack of adequate muscle sample material, only eight subjects were included in this analysis.

HSL phosphorylations

Phosphorylation at HSL Ser\(^{660}\) was significantly increased (\( P < 0.05 \)), HSL Ser\(^{559}\) phosphorylation was unchanged, and HSL Ser\(^{565}\) phosphorylation was decreased (\( P < 0.05 \); Fig. 3, A–C) after the training period. When expressed in relation to total HSL protein, only the decreased HSL Ser\(^{565}\) phosphorylation remained significant (\( P < 0.05 \); data not shown).

DGAT1

The protein expression of DGAT1 was not changed from before to after the training period (Fig. 3D).

TG-hydrolase activity

Inhibition of HSL using the monospecific inhibitor (76-0079) gradually reduced the TG-hydrolase activity (Fig. 4A). At maximal inhibition (100 µM), TG-hydrolase activity was still 58% of the activity without inhibitor (\( P < 0.05 \)), indicating that other TG lipases exist in human skeletal muscle. Incubating muscle lysates with various amounts of recombinant CGI-58 (rCGI-58) increased TG-hydrolase activity up to 42% above basal (\( P < 0.05 \); Fig. 4B). It was further investigated whether ATGL could be responsible for the remaining TG-hydrolase activity after maximal inhibition of HSL. For this purpose, HSL in muscle lysates was inhibited using 150 µM 76-0079 and 1.5 µg rCGI-58. As shown in Fig. 4C, there was still significant CGI-58-stimulated (ATGL) activity when HSL was maximally inhibited (\( P < 0.05 \)).

DISCUSSION

The major findings in the present study were as follows: 1) human skeletal muscle ATGL protein content was significantly increased after 8 wk of endurance exercise training; 2) significant ATGL activity stimulated by rCGI-58, the activating protein of ATGL, was present in human skeletal muscle; 3) CGI-58 was not changed by exercise training; 4) in contrast to ATGL, the protein level of HSL was not significantly changed by exercise training, whereas HSL-phosphorylation at Ser\(^{660}\) was increased, HSL-Ser\(^{659}\) phosphorylation was un-
changed, and HSL-Ser565 phosphorylation was decreased, indicating an increased activity of this lipase; and 5) IMTG content was decreased during the exercise training period. Importantly, perilipin, a specific marker of adipocytes, was not detected in muscle lysates, supporting that the measurements of ATGL, CGI-58, HSL protein, and IMTG were of intramuscular origin and that the tissue samples were not contaminated with adipocytes. Altogether, the present study revealed several new findings.

In the present study, it was demonstrated that ATGL activity, as assessed by TG-hydrolase activity in the presence of rCGI-58, a specific ATGL activator, is present in human skeletal muscle. After complete inhibition of HSL, with the use of a specific HSL inhibitor, TG-hydrolase activity was still 58% of control, confirming the presence of one or more additional TG lipases than HSL in human skeletal muscle. This level of inhibition is higher than previous findings from our laboratory (80% remaining activity; Ref. 40) and lower than what others have found (40% remaining activity; Ref. 52) using a HSL antibody to block HSL TG-hydrolase activity. Importantly, at maximal inhibition of HSL, TG-hydrolase activity could be increased by adding rCGI-58 to the assay, indicating ATGL activity. In mouse white adipose tissue, ATGL and HSL together accounts for 95% of TG-hydrolase activity, as shown with the same HSL inhibitor that was used in the present study, and ATGL is the only lipase activated by...
CGI-58 in this tissue (45). However, the data of the present study do not exclude the presence of other TG hydrolases than ATGL and HSL in human skeletal muscle. The functional importance of ATGL in skeletal muscle TG hydrolysis has been demonstrated by knockdown and gain-of-function experiments in myotubes (53). In a recent study (53), in vivo overexpression of ATGL in rat skeletal muscle resulted in an increased TG-hydrolase activity and decreased intracellular TG levels, whereas downregulation of ATGL produced the opposite responses. Furthermore, skeletal muscle of ATGL knockout mice exhibits severe TG accumulation and decreased TG-hydrolase activity (16). Finally, mutations of the human ATGL and CGI-58 genes have been found in patients with neutral lipid storage disease with myopathy, which is characterized by TG accumulation in various tissues including skeletal muscle (8, 11, 28, 33), suggesting a defect of ATGL function. Thus these data provide strong indications that ATGL and CGI-58 play important roles in skeletal muscle TG hydrolysis.

The increased ATGL protein content seen after the training period in the present study together with the observation of a basal ATGL activity points toward an increased lipolytic capacity in human skeletal muscle after exercise training. Accordingly, a decrease in basal IMTG content was observed after the exercise training intervention period in the present study, but an alteration in esterification of fatty acids to IMTG may also contribute to the lower basal IMTG content seen after the training intervention period. On the other hand, alterations in fatty acid esterification in skeletal muscle were not reflected in changes in DGAT1 protein expression, the enzyme responsible for synthesis of TG from DAG, as DGAT1 protein expression remained unchanged after the training intervention period.

Fig. 3. HSL Ser<sup>660</sup> (A), Ser<sup>659</sup> (B), and Ser<sup>655</sup> (C) phosphorylations, and diacylglycerol acyl transferase 1 (DGAT1; D) protein expression in human vastus lateralis muscle before and after 8 wk of endurance training. Human skeletal muscle protein extracts (32 µg) were loaded. Four micrograms of rat white adipose tissue (R) were loaded as positive control for HSL phosphorylations, and 10 µg of mouse liver (M) were loaded as control for DGAT1 expression. Actin was blotted as a loading control. Data are means ± SE; n = 10. *P < 0.05 compared with pretraining value.
HSL protein expression was not changed by exercise training in the present study, which was supported by findings in a study in rodents in which HSL protein expression was not different in skeletal muscle of swim-trained rats compared with sedentary controls (10). Furthermore, muscle HSL activity was similar at basal (rest) in untrained and trained male subjects and no differences were seen in IMTG between the two groups (18).

The present findings that skeletal muscle ATGL, but not HSL protein content, was enhanced by exercise training might lead to an accumulation of DAG due to an incomplete lipolysis. However, muscle DAG content does not appear to increase but rather decreases with exercise training (5, 9), suggesting that HSL protein is already present at adequate levels in untrained skeletal muscle. HSL activity is regulated by phosphorylation and translocation to the lipid droplets (3, 38). Increased phosphorylation of HSL at two PKA-targeted sites, Ser659 and Ser660, has been observed in exercising human skeletal muscle along with an increased in vitro HSL activity (39, 50), suggesting that these covalent regulations are important for HSL activation in this tissue. During exercise, one study (50) has shown a reduced HSL activity concomitant with an increased HSL-Ser565 phosphorylation. However, in another human study (40) where muscle glycogen was manipulated to induce different levels of AMP-activated protein kinase activation and consequently high and low HSL Ser565 phosphorylations during exercise, no effect of this phosphorylation on HSL activity was found. Thus the functional role of HSL-Ser565 phosphorylation during exercise is not clear. It has, however, not been studied whether changes in phosphorylation on these sites in resting skeletal muscle are followed by changes in activity. In the present study, we observed an unchanged phosphorylation on HSL-Ser659, an increased Ser660, and decreased Ser565 phosphorylation on HSL, indicating an increased HSL activity after exercise training, which complements the increase in ATGL protein expression.

Earlier findings (37) demonstrated that recovery from an acute bout of exercise was associated with transient increases in transcription of several metabolically related genes in human skeletal muscle. In the present study, muscle tissue for protein analysis was obtained 48 h after the last exercise session. However, it is not known how long of a time it takes to induce increases in the ATGL protein. Even though it seems unlikely that one single exercise bout would induce a twofold increase in protein expression, as seen in the present study, it cannot be excluded that part of the increases in ATGL protein content was a result of the last exercise session. Nevertheless, whether elicited by the last exercise bout or due to accumulating effects of the various training bouts, it is apparent that regular exercise training induces a marked increase in ATGL protein expression.

Interestingly, in the present study IMTG content was decreased after the training period. This is in contrast to findings in cross-sectional studies showing either a higher (13) or a similar (46) IMTG content in trained compared with untrained subjects. In studies (4, 5) performed with subjects of similar body composition and training status as in the present study or with obese subjects, changes in IMTG content after a comparable training period were not obtained. However, in type 2 diabetic patients, endurance training led to a decrease in IMTG content (4). In other longitudinal training studies, IMTG has been found to either decrease (1) or increase (9). The differ-

Fig. 4. Triacylglycerol (TG)-hydrolase activity in human skeletal muscle. A: dose-dependent effect of the specific HSL-inhibitor 76-0079 on TG-hydrolase activity in human skeletal muscle protein extracts. Data are percentage of control [without inhibitor or DMSO (vehicle)]. B: dose-dependent effect of recombinant CGI-58 on TG-hydrolase activity in human skeletal muscle protein extracts in the presence and absence of rCGI-58 (1.5 μM) and 76-0079 (150 μM). Data are means ± SE of triplicate determinations performed on a pool of 8 subjects. *P < 0.05 compared with 0; **P < 0.05 compared with 76-0079.
ence between these studies might relate to differences in muscle fiber type distribution, as type 1 fibers have a larger IMTG content than type 2 fibers (25). Also the diet consumed days before the muscle biopsy is obtained influences IMTG content (25). Moreover, total body fat mass is probably a major determinant for IMTG content. Indeed, in the present study, no changes were obtained in total body mass, but a significant reduction was found in the percentage of body fat and a correlation between IMTG and body fat percentage was found ($r^2 = 0.51$; $P < 0.05$; $n = 8$), which is in accordance with previous findings (12, 39).

In some previous studies, but not all, a larger net change in IMTG during submaximal exercise has been demonstrated in trained than in untrained subjects (1, 18, 20, 26, 36). However, this changed turnover of IMTG in the trained state has been difficult to explain. Therefore, it could be speculated that the increased ATGL protein expression that was seen in the present study may in part explain the changed turnover of IMTG during exercise in the trained state.

In summary, the present study demonstrated that ATGL protein expression was increased in human skeletal muscle after 8 wk of regular exercise training, whereas CGI-58 and HSL protein expression was increased in human skeletal muscle IMTG during exercise in the trained state.

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