Sex differences in hormone-sensitive lipase expression, activity, and phosphorylation in skeletal muscle at rest and during exercise

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Roepstorff, Carsten, Morten Donsmark, Maja Thiele, Bodil Vistisen, Greg Stewart, Kristian Vissing, Peter Schjerling, D. Grahame Hardie, Henrik Galbo, and Bente Kiens. Sex differences in hormone-sensitive lipase expression, activity, and phosphorylation in skeletal muscle at rest and during exercise. Am J Physiol Endocrinol Metab 291:E1106–E1114, 2006. First published July 5, 2006; doi:10.1152/ajpendo.00097.2006.—Women have been shown to use more intramuscular triacylglycerol (IMTG) during exercise than men. To investigate whether this could be due to sex-specific regulation of hormone-sensitive lipase (HSL) and to use sex comparison as a model to gain further insight into HSL regulation, nine women and eight men performed bicycle exercise (90 min, 60% V02peak), and skeletal muscle HSL expression, phosphorylation, and activity were determined. Supporting previous findings, basal IMTG content (P<0.001) and net IMTG decrease during exercise (P<0.01) were higher in women than in men and correlated significantly (r=0.72, P=0.001). Muscle HSL mRNA (80%, P=0.11) and protein content (50%, P<0.05) were higher in women than in men. HSL total activity increased during exercise (47%, P<0.05) but did not differ between sexes. Accordingly, HSL specific activity (HSL activity per HSL protein content) increased during exercise (62%, P<0.05) and was generally higher in men than in women (82%, P<0.05). A similar pattern was observed for HSL Ser659 phosphorylation, suggesting a role in regulation of HSL activity. Likewise, plasma epinephrine increased during exercise (P<0.05) and was higher in men than in women during the end of the exercise bout (P<0.05). We conclude that, although HSL expression and Ser659 phosphorylation in skeletal muscle during exercise is sex specific, total muscle HSL activity measured in vitro was similar between sexes. The higher basal IMTG content in women compared with men is therefore the best candidate to explain the higher IMTG net hydrolysis during exercise in women.

intramuscular triacylglycerol; epinephrine; extracellular signal-regulated kinase

TRIACYLGLYCEROL STORED IN skeletal muscle fibers (intramuscular triacylglycerol, IMTG) represents a large source of energy that may be used for muscle contraction during exercise. In men, it is still controversial to what extent IMTG is utilized during exercise (18, 29, 40). This is probably so because methodological limitations in measuring IMTG content have made it difficult to detect the relatively small net hydrolysis of IMTG that appears to occur during submaximal exercise in men (13, 29, 40, 46). On the other hand, in women it has been shown that IMTG content is reduced by ~25% during 90-min bicycle exercise at 60% peak oxygen uptake (V02peak) (33), and it can be estimated that IMTG covers a large fraction (~25%) of oxidative energy production in such an exercise bout in women (27). Therefore, the degree of IMTG hydrolysis during submaximal exercise appears to depend on sex, being higher in women than in men. It is presently unknown what may be the cause underlying this sex difference in IMTG hydrolysis during exercise.

Hormone-sensitive lipase (HSL) is thought to catalyze the hydrolysis of IMTG in skeletal muscle as it does in adipose tissue (14, 21). In rodent as well as in human skeletal muscle, neutral lipase activity increases during contraction (22, 28, 41), and it has been shown that the increase in neutral lipase activity elicited by contraction is completely accounted for by HSL activation both in rats and in man (22, 28, 45). Because HSL is probably the main lipase responsible for IMTG hydrolysis during exercise, it is possible that higher IMTG hydrolysis during exercise in women than in men could be due to higher HSL expression and/or higher exercise-induced HSL activation in women.

The mechanism(s) behind contraction-induced HSL activation in skeletal muscle is still poorly understood. HSL activity appears to be regulated by site-specific phosphorylation on several serine residues on HSL (14, 28, 45). From studies in different cell lines, HSL Ser563, Ser659, and Ser660 are all thought to be phosphorylated by cAMP-dependent protein kinase (PKA) with positive effect on HSL activity (1, 31). The existence of such a mechanism also in skeletal muscle is supported by studies showing that epinephrine increases skeletal muscle HSL activity both at rest and during contraction (23, 44). In consequence, the rise in circulating epinephrine levels seen during exercise may partly explain why skeletal muscle HSL is activated during exercise. However, it is still unclear which of the PKA sites that are important in mediating an effect of epinephrine on HSL activity in skeletal muscle.

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during exercise. Because HSL Ser<sup>563</sup> phosphorylation in skeletal muscle has been shown not to respond to submaximal exercise in humans, this site appears not to play any major role during exercise (28, 39). Conversely, HSL Ser<sup>600</sup> phosphorylation in skeletal muscle increased gradually during prolonged submaximal exercise in humans and was elevated by epinephrine in L6 myotubes, suggesting that this site responds to epinephrine and may be involved in regulation of HSL activity (45). HSL Ser<sup>659</sup> phosphorylation has not yet been investigated in human skeletal muscle during exercise. It has been shown that plasma epinephrine levels are higher in men than in women during prolonged submaximal exercise (27, 33, 35). Therefore, comparison between sexes of HSL activity and HSL phosphorylation on PKA sites in skeletal muscle could help elucidate any mechanistic relationship between plasma epinephrine, HSL site-specific phosphorylation, and HSL activity during exercise.

Based on studies in adipocytes, HSL Ser<sup>600</sup> is a presumed target of extracellular signal-regulated kinase (ERK), which has been shown to activate HSL (11). Studies on isolated rat soleus muscle demonstrated that the increase in HSL activity during contraction could be reduced by inhibition of ERK, suggesting that ERK is involved in HSL activation by contraction also in skeletal muscle (5). It follows that a possible sex difference in HSL activation during exercise could be due to sex differences in ERK activation by exercise.

In the present study, moderately trained women and men performed 90-min bicycle exercise at 60% VO<sub>2</sub>peak. Plasma epinephrine as well as IMTG content, ERK phosphorylation, and HSL expression, activity, and Ser<sup>563</sup> and Ser<sup>659</sup> phosphorylation in skeletal muscle were determined at rest and during exercise. Using this approach, we aimed at elucidating why IMTG hydrolysis during exercise is higher in women than in men; whether plasma epinephrine and, hence, HSL Ser<sup>563</sup> and Ser<sup>659</sup> phosphorylation are involved in regulation of HSL activity during exercise in women and men; and whether ERK activation is different between women and men and thus affects HSL activity differently during exercise.

**MATERIALS AND METHODS**

**Subjects.** Nine women and eight men, all moderately trained, were recruited to participate in the study (Table 1). 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-01-046/02) and conformed to the code of ethics of the World Medical Association (Declaration of Helsinki II). All subjects were young, healthy, and nonsmokers. The women had a VO<sub>2</sub>peak of 40–55 ml O<sub>2</sub>·min<sup>−1</sup>·kg body mass<sup>−1</sup>, and the men had a VO<sub>2</sub>peak of 50–65 ml O<sub>2</sub>·min<sup>−1</sup>·kg body mass<sup>−1</sup>. All subjects participated on a regular basis (3–4 h/wk) in leisure time activities such as running, cycling, strength training, and different gamesports. Body mass index was in the range of 20–25. The women were eumenorrheic with a menstrual cycle length between 28 and 35 days and did not take any oral contraception. All preexercise testing and the main experiments in women were carried out during the midfollicular phase of their menstrual cycle (days 7 to 10 from first day of menstrual cycle). The results presented in this study are part of a larger study on sex differences in intramuscular metabolic regulation during exercise, which has in minor part been published previously (30).

**Experimental protocol.** To determine VO<sub>2</sub>peak, all subjects performed an incremental exercise test on a Monark Ergomedic 839E bicycle ergometer (Monark, Varberg, Sweden). In addition, they filled out a questionnaire regarding habitual physical activity and exercise training. Body composition was determined by hydrostatic weighing (32) with a correction for residual lung volume measured by the oxygen dilution method (24). The determination of body composition was carried out after a 4-h fasting period during which subjects refrained from all food and liquids. Female and male groups had similar VO<sub>2</sub>peak/kg lean body mass, habitual physical activity level, and exercise training history.

**Diet.** For 8 days preceding the main trial, all subjects consumed an isoenergetic diet containing 65 energy percent (E%) carbohydrate, 20 E% fat, and 15 E% protein. The constituents of the diet were weighed out at the laboratory and delivered to the subjects. The amount of energy to be consumed was individually determined from body weight, sex, and habitual physical activity level based on guidelines from the World Health Organization (7). To ensure that subjects kept their body mass steady during the 8-day dietary regimen, energy consumption during the diet period was adjusted after 4 days based on daily monitoring of body mass.

**Experimental protocol.** The subjects arrived at the laboratory at 8:30 AM after an overnight fast. Subjects had abstained from exercise training for 36 h before the trial. The subjects rested in the supine position for ~30 min. Expired air was then sampled in a Douglas bag for determination of resting pulmonary oxygen uptake and carbon dioxide excretion. A venous catheter was inserted into the antecubital arm vein for blood sampling, and after an additional 15 min of rest, blood was drawn. A muscle biopsy was then obtained from the vastus lateralis muscle under local anesthesia of the skin and fascia. Immediately afterwards, the subjects initiated a 90-min bicycle exercise bout at 60% VO<sub>2</sub>peak on a Monark Ergomedic 839E bicycle ergometer. Breath and blood samples were obtained after 30, 60, and 90 min. After 90 min of bicycling, the subjects terminated exercise, and within 30 s another biopsy was obtained from the contralateral vastus lateralis muscle. During the experimental trial, subjects were offered water ad libitum.

**Breath samples.** Expired volumes of air in the Douglas bags were measured with a chain-suspended Collins spirometer, and a small sample of mixed expiratory air was analyzed for O<sub>2</sub> (Servomex S-3A) and CO<sub>2</sub> (Beckman LB2). The respiratory exchange ratio (RER) was calculated as the ratio between pulmonary CO<sub>2</sub> excretion and O<sub>2</sub> uptake.

**Blood samples.** Plasma fatty acid (FA) concentrations were measured using a colorimetric commercial assay kit (Wako Chemicals, Richmond, VA) using a COBAS FARA 2 autoanalyzer (Roche Diagnostic, Basel, Switzerland). Glycerol concentrations in plasma were measured spectrophotometrically on the COBAS FARA auto-

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**Table 1. Subject characteristics**

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<tr>
<th></th>
<th>Women</th>
<th>Men</th>
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<tbody>
<tr>
<td>n</td>
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<td>8</td>
</tr>
<tr>
<td>Age, yr</td>
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<td>25±1</td>
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<tr>
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<td>1.71±0.03&lt;sup&gt;*&lt;/sup&gt;</td>
<td>1.85±0.02</td>
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<td>Body mass, kg</td>
<td>65.0±2.3&lt;sup&gt;*&lt;/sup&gt;</td>
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<td>Body fat, %</td>
<td>24.7±1.5&lt;sup&gt;*&lt;/sup&gt;</td>
<td>12.1±2.3</td>
</tr>
<tr>
<td>Lean body mass, kg</td>
<td>48.9±1.9&lt;sup&gt;*&lt;/sup&gt;</td>
<td>69.9±3.1</td>
</tr>
<tr>
<td>VO&lt;sub&gt;2&lt;/sub&gt;peak, l/min</td>
<td>3.2±0.1&lt;sup&gt;*&lt;/sup&gt;</td>
<td>4.4±0.2</td>
</tr>
<tr>
<td>ml/kg BM·1·min&lt;sup&gt;−1&lt;/sup&gt;</td>
<td>48.8±1.3&lt;sup&gt;*&lt;/sup&gt;</td>
<td>55.6±1.2</td>
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<tr>
<td>ml/kg LBMM·1·min&lt;sup&gt;−1&lt;/sup&gt;</td>
<td>65.0±1.7</td>
<td>63.4±0.8</td>
</tr>
<tr>
<td>Maximal work load, W</td>
<td>264±13*</td>
<td>364±14</td>
</tr>
<tr>
<td>Training history</td>
<td>3.2±0.6</td>
<td>3.0±0.4</td>
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<tr>
<td>Duration, h/wk</td>
<td>3.5±0.6</td>
<td>3.2±0.5</td>
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Data are means ± SE. *P < 0.001, sex difference. BM, body mass; LBMM, lean body mass.
analyzer with the use of glycerol kinase, pyruvate kinase, and lactate dehydrogenase reactions. Concentrations of insulin (Pharmacia insulina radioimmunoassay 100; Pharmacia & Upjohn Diagnostics, Uppsala, Sweden) as well as epinephrine and norepinephrine (KatCombi radioimmunoassay; Immuno-Biological Laboratories, Hamburg, Germany) in plasma were determined by radioimmunoassay.

**Muscle biopsies.** The biopsies were quick-frozen (<10 s) in liquid nitrogen while still in the biopsy needle and stored at −80°C for subsequent biochemical analysis. Muscle tissue (80 mg wet weight) was freeze-dried and dissected free of all visible adipose tissue, connective tissue, and blood under a microscope. The dissected muscle fibers were pooled and then divided into subpools for the respective analyses.

**IMTG.** The concentration of IMTG was determined as described previously (17, 33). In short, freeze-dried and dissected tissue samples were incubated with tetraethylammoniumhydroxide to selectively hydrolyze triacylglycerol (TG). Samples were then assayed for glycerol by fluorometry.

**Muscle lysates.** Freeze-dried and dissected muscle tissue was homogenized (1:30 wt/vol) in a buffer containing 50 mM HEPES (pH 7.5), 150 mM NaCl, 20 mM sodium pyrophosphate, 20 mM β-glycerophosphate, 10 mM NaF, 2 mM sodium orthovanadate, 2 mM EDTA, 1% Nonidet P-40, 10% glycerc, 2 mM PMSE, 1 mM MgCl₂, 1 mM CaCl₂, 10 µg/ml leupeptin, 10 µg/ml aprotinin, and 3 mM benzamidine. Homogenates were rotated end over end for 1 h at 4°C and then cleared by centrifugation at 17,500 g at 4°C for 1 h. Protein content in the supernatant was measured using the bicinchoninic acid method (Pierce, Rockford, IL).

**Western blotting.** Expression of HSL, ERK1/2, and perilipin as well as phosphorylation of HSL Ser⁶⁶³, HSL Ser⁶⁵⁹, ERK1 Thr²⁰⁴, and ERK2 Thr¹⁸⁵/Tyr¹⁸⁷ were detected using Western blotting on the muscle lysates. The lysates were boiled in Laemmli buffer before being subjected to SDS-PAGE and immunoblotting. Primary antibodies were rabbit anti-HSL (kindly donated by Dr. Cecilia Holm, Dept. of Cell and Molecular Biology, Lund University, Sweden), sheep anti-HSL Ser⁶⁶³-phos (28), sheep anti-HSL Ser⁶⁵⁹-phos (see below for specifications), rabbit anti-ERK1/2 (catalog no. 9102; Cell Signaling Technology, Beverly, MA), rabbit anti-ERK1 Thr²⁰⁴/ Tyr²⁰⁵-phos/ERK2 Thr¹⁸⁵/Tyr¹⁸⁷-phos (catalog no. 9101; Cell Signaling Technology), and guinea pig anti-perilipin (catalog no. GP29; Progen Biotechnik, Heidelberg, Germany). Secondary antibodies were horseradish peroxidase-conjugated anti-rabbit, anti-guinea pig (catalog nos. P0448 and P0414; DAKO, Glostrup, Denmark), and anti-sheep (catalog no. 81-8620; Zymed, San Francisco, CA). Anti-guinea-antibody complexes were visualized using enhanced chemiluminescence (ECL+; Amersham Biosciences, Amersham, UK) and quantified using a Kodak Image Station E440CF (Kodak, Glostrup, Denmark). To generate the anti-HSL Ser⁶⁵⁹-phos antibody, two peptides were synthesized (CGFHPRRSSQGATQM and CGFHPRRSSQGATQM-KLH [residues 653–666 of rat HSL with either no phosphorylation or a single phosphate at Ser⁶⁵⁹ and with cysteine added at the NH₂ terminus for coupling purposes], coupled to keyhole limpet hemocyanin (KLH) were spotted onto a nitrocellulose membrane and probed using the anti-HSL Ser⁶⁵⁹-phos antibody.

![Fig. 1. Testing of phosphospecific hormone-sensitive lipase (HSL) Ser⁶⁵⁹ antibody. A: representative Western blots of HSL total protein and of HSL phosphorylated on Ser⁶⁵⁹ in human skeletal muscle (SM) and in human paraumbilical adipose tissue (AT) sampled at rest. B: sequential dilutions (1 µl each) of the two peptides CGFHPRRSSQGATQM-KLH and CGFHPRRSSQGATQM-KLH were incubated with tetraethylammoniumhydroxide to selectively hydrolyze triacylglycerol (TG). Samples were then assayed for glycerol by fluorometry.](http://ajpendo.physiology.org/)

where a single band was detected at ~84-kDa (Fig. 1A). Because HSL expression is severalfold higher in adipocytes than in skeletal muscle, we assumed that the ~84-kDa band detected by the anti-HSL Ser⁶⁵⁹-phos antibody indeed corresponded to HSL. The specificity of the anti-HSL Ser⁶⁵⁹-phos antibody against HSL when phosphorylated on Ser⁶⁵⁹ was investigated by testing whether the anti-HSL Ser⁶⁵⁹-phos antibody recognized the phosphopeptide and/or the dephosphopeptide (Fig. 1B). Samples of the phosphopeptide and the dephosphopeptide were prepared and their concentrations determined via absorbance at 205 nm. Seven sequential dilutions of each peptide were prepared, and 1 µl of each was spotted onto a nitrocellulose membrane at 1-cm intervals and left to dry. Next, the membrane was washed briefly with Tris-buffered saline containing 0.1% Tween and probed using the anti-HSL Ser⁶⁵⁹-phos antibody. The anti-HSL Ser⁶⁵⁹-phos antibody detected the phosphopeptide in a concentration-dependent manner, whereas the dephosphopeptide was not recognized to any major extent by the anti-HSL Ser⁶⁵⁹-phos antibody (Fig. 1B). Therefore, we assumed that the anti-HSL Ser⁶⁵⁹-phos antibody was indeed phosphospecific, recognizing HSL only when phosphorylated on Ser⁶⁵⁹.

**HSL activity.** Freeze-dried and dissected muscle tissue was homogenized (5), and the homogenate was centrifuged for 45 s (15,800 g, 4°C) in an Eppendorf microcentrifuge tube. The resulting supernatant containing ~2.5 mg protein/ml was used for subsequent measurement of lipase activity. Lipase activity against tri-[¹⁴C]olein was measured at pH 7.0, which is the pH optimum for HSL, with or without preincubation of the muscle supernatants with a chicken anti-HSL antibody (5, 22). HSL activity was calculated as neutral lipase activity measured in the presence of anti-HSL antibody (28). HSL activity was expressed in milliunits per milligram of protein, where 1 unit of enzyme activity is equivalent to 1 µmol of FAs released per minute at 37°C.

**Real-time RT-PCR.** Total RNA was isolated from ~25 mg of wet muscle tissue by phenol extraction (TriReagent; Molecular Research Center, Cincinnati, OH) as previously described (3). Integrity of RNA was confirmed by denaturing agarose gel electrophoresis. Total RNA (500 ng) was converted into cDNA in 20 µl using the OmniScript reverse transcriptase (Qiagen, Valencia, CA) according to the manufacturer’s protocol. For each target mRNA, 0.25 µl of cDNA were amplified in a 25-µl SYBR Green PCR reaction containing 1X
Quantitate SYBR Green Master Mix (Qiagen) and 100 nM of each primer (sense/antisense primers: HSL: GGAGGATCACAACAGGACCTGGGAC/AGCCAGGGCCTTACCTCAC; RPLP0: GGAAAATCTGATTTTCTCGGTCCT/CCAGGAATCGTGTGCTACCCGTTG; GAPDH: CCTCTGACCAACACAGTCCTT/GAGGGGCCATCCACAGTCTT). The amplification was monitored in real time using the MX3000P real-time PCR machine (Stratagene, La Jolla, CA). The Ct values were related to a standard curve made with the cloned PCR products. HSL mRNA was normalized to mRNA coding for the large ribosomal protein P0 (RPLP0). RPLP0 was chosen as internal control under the assumption that RPLP0 mRNA was constitutively expressed (4). To validate this assumption, we also normalized HSL mRNA to another unrelated “constitutive” RNA, GAPDH mRNA, with similar results (data not shown).

Statistics. Data are presented as means ± SE. For variables independent of time, a t-test was performed to test for differences between women and men. For variables measured before and after exercise as well as variables measured before and during exercise, a two-way analysis of variance, with repeated measures for the time factor, was performed to test for sex differences or changes due to time. When a significant main effect of time was found, significant pairwise differences were detected using Tukey’s post hoc test. Correlation analysis was performed using Pearson product moment. In all cases, a probability of 0.05 was used as the level of significance.

RESULTS

Workload. The average workload during the 90-min bicycle exercise trial was 132 ± 6 and 174 ± 7 W in women and men, respectively (P < 0.001). Pulmonary oxygen uptake (VO2) during exercise averaged 1.9 ± 0.1 and 2.6 ± 0.1 l O2/min in women and men, respectively (P < 0.001). Expressed per kilogram of lean body mass (LBM), the VO2 averaged 38.7 ± 1.1 and 37.7 ± 0.6 ml O2·kg LBM−1·min−1 during exercise in women and men, respectively (not significant, NS). Relative exercise intensity averaged 60 ± 1% VO2 peak in both women and men.

Respiratory exchange ratio. The RER at rest did not differ significantly between women and men (Table 2). From rest to 30 min of exercise, RER increased (P < 0.05) in both women and men, after which it remained unchanged (NS) throughout exercise. RER was significantly lower in women than in men at 60 and 90 min of exercise (P < 0.05).

Circulating metabolites. Concentrations of FA and glycerol in plasma are shown in Table 2. In both men and women, the plasma FA concentration remained unchanged from rest to 30 min of exercise (NS) but then increased continuously (P < 0.05). The plasma FA concentration tended to be higher in women than in men (P = 0.065). At rest, the plasma glycerol concentration did not differ significantly between women and men. An increase (P < 0.05) was observed from rest to 30 min of exercise in both sexes, and then a significant continuous increase was seen throughout exercise. During exercise the plasma glycerol concentration was higher in women than in men (P < 0.05).

Hormones. Plasma insulin, epinephrine, and norepinephrine concentrations are shown in Table 2. At rest, neither of these hormones differed significantly between women and men. The plasma insulin concentration decreased continuously (P < 0.05) from rest to 90 min of exercise and was higher in women than in men at the end of exercise (P < 0.05). The plasma epinephrine concentration increased continuously (P < 0.05) from rest to 90 min of exercise and was higher in men than in women at the end of exercise (P < 0.05). The plasma norepinephrine concentration increased (P < 0.05) from rest to 30 min of exercise and remained elevated throughout exercise. No significant sex differences were observed in the plasma norepinephrine concentration.

Perilipin. Because TG and HSL are highly concentrated in adipocytes, possible contamination of muscle samples with adipocytes is an important issue when measuring TG and HSL content in muscle (12, 46). To test whether our dissected muscle samples contained any adipocytes, we analyzed our samples for the presence of perilipin, a specific adipocyte marker protein (10). The perilipin content in the muscle samples (40 µg total protein) was below the detection limit of our assay, whereas loading of 3 µg of total protein from adipose tissue produced a strong band (Fig. 2).

Table 2. RER and plasma substrate and hormone concentrations at rest and during 90 min of bicycle exercise at 60% VO2 peak

<table>
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<th>60 min Exercise</th>
<th>90 min Exercise</th>
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<td>RER</td>
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<td>Women</td>
<td>0.74±0.03</td>
<td>0.87±0.01†</td>
<td>0.84±0.01†</td>
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<td>Men</td>
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<td>0.89±0.01*†</td>
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<tr>
<td>Women</td>
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<td>577±59</td>
<td>755±65‡</td>
<td>1,026±93†‡§</td>
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<tr>
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<td>Glycerol, µM</td>
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<tr>
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<td>153±27</td>
<td>344±30†</td>
<td>465±33‡</td>
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<tr>
<td>Men</td>
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<td>242±19*‡</td>
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<td>Men</td>
<td>1.25±0.33</td>
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<td>7.19±1.19†</td>
<td>8.43±1.22‡‡</td>
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Data are means ± SE. *P < 0.05, different from women. †P < 0.05, different from rest. ‡P < 0.05, different from 30 min. §P < 0.05, different from 60 min.
IMTG. At rest, IMTG concentration was 95% higher in women than in men ($P < 0.001$) (Fig. 3A). During exercise, a 23% net decrease in IMTG content occurred in women ($P < 0.01$), whereas no significant change in IMTG content was observed in men.

HSL. HSL mRNA expression in the vastus lateralis muscle was ~80% higher in women than in men, but this sex difference did not reach statistical significance ($P = 0.11$; Fig. 4A). HSL protein expression was ~50% higher in women than in men ($P < 0.05$; Fig. 4B). No significant changes occurred in HSL mRNA or protein expression from rest to 90 min of exercise.

Because HSL protein expression was higher in women than in men, HSL site-specific phosphorylation and HSL activity were both expressed relative to HSL protein content to promote direct sex comparison (Fig. 5). HSL Ser$^{563}$ phosphorylation per HSL arbitrary unit was 43% higher in men than in women ($P < 0.05$) and did not change significantly from rest to exercise (Fig. 5A). HSL Ser$^{659}$ phosphorylation per HSL arbitrary unit was also higher in men than in women ($59\%$, $P < 0.05$; Fig. 5B). Moreover, HSL Ser$^{659}$ phosphorylation increased ~27% from rest to exercise irrespective of sex ($P < 0.05$).

Total neutral lipase activity against tri-$[^3H]$olein increased 22% ($P < 0.01$) from rest ($0.59 \pm 0.07$ and $0.62 \pm 0.04$ mU/mg protein in women and men, respectively) to 90 min of exercise ($0.72 \pm 0.08$ and $0.75 \pm 0.08$ mU/mg protein in women and men, respectively) without any significant differences between women and men. When muscle supernatants were preincubated with anti-HSL, neutral lipase activity against tri-$[^3H]$olein was reduced by ~30% at rest. The increase in total neutral lipase activity against tri-$[^3H]$olein seen from rest to exercise was abolished by preincubation with anti-HSL. HSL activity, calculated as neutral lipase activity inhibited by preincubation with anti-HSL, did not differ between women ($0.17 \pm 0.03$ mU/mg protein) and men ($0.17 \pm 0.03$ mU/mg protein) at rest. Independent of sex, an ~47% increase was observed in HSL activity from rest to 90 min of exercise ($P < 0.05$). When expressed per HSL arbitrary unit (Fig. 5C), HSL activity was ~82% higher in men than in women, independent of exercise, and increased from rest to exercise in both sexes (62%, $P < 0.05$).

ERK1/2. ERK1 and ERK2 protein expression did not differ between women and men (NS), and no significant changes were observed from rest to 90 min of exercise (data not shown). ERK1 Thr$^{202}$/Tyr$^{204}$ phosphorylation increased ~25-fold ($P < 0.001$) from rest to 90 min of exercise and did not differ significantly between women and men (Fig. 6A). ERK2 Thr$^{185}$/Tyr$^{187}$ phosphorylation increased ~7-fold ($P < 0.001$) from rest to 90 min of exercise (Fig. 6B). ERK2 Thr$^{185}$/Tyr$^{187}$ phosphorylation tended to be higher in men than in women ($P = 0.08$).

Correlation analyses. In an attempt to find potential factors to explain the sex difference in basal IMTG concentration and in IMTG net breakdown during exercise, we tested whether basal IMTG concentration correlated with percent body fat and whether the net decrease in IMTG concentration during exercise correlated with basal IMTG concentration. We found that basal IMTG concentration correlated with percent body fat.
DISCUSSION

The present study revealed several interesting findings related to regulation of HSL activity and IMTG breakdown during exercise in skeletal muscle of women and men:

1) HSL expression, at the protein as well as the mRNA level, was higher in women than in men;
2) HSL activity increased in response to exercise but was similar in women and men;
3) HSL specific activity, i.e., the activity per HSL protein content as well as HSL Ser659 phosphorylation, increased in response to exercise, and both were higher in men than in women; and
4) the net decline in IMTG during exercise correlated positively with the basal IMTG concentration, which again correlated positively with percent body fat.

The higher IMTG net decrease in women than in men seen in the present study during prolonged submaximal exercise (Fig. 3) is in accordance with previous findings from our laboratory (27, 33). The lack of any reduction in IMTG content during exercise in men in the present study contradicts some but not all previous studies (reviewed in Refs. 18, 29, 38, 40). The controversy that exists in the literature regarding IMTG breakdown during exercise in men may be due to methodological limitations in measuring IMTG content relative to the, after all, not very large utilization of IMTG during most 60- to 90-min exercise bouts, but a thorough discussion of this issue is beyond the scope of the present report (for reviews, see Refs. 18, 29, 38, 40). However, regarding IMTG breakdown, the important observation in the present study was the significant net decrease in IMTG content during exercise only in women, a repeated finding in our laboratory. Until now, the mechanism(s) that causes this sex difference in IMTG breakdown during exercise has been largely unknown. HSL is thought to be the major rate-limiting enzyme in IMTG breakdown. Several previous studies have shown that in skeletal muscle, HSL activity is elevated by exercise (5, 22, 28, 45). In the present study, this was also the case in both women and men. However, HSL activity increased to the same extent during exercise in women and men, which, combined with the higher HSL protein expression in women (Fig. 4B), implied that HSL specific activity was higher in men than in women both at rest and during exercise (Fig. 5C). This finding contradicted our expectations, because part of our hypothesis was that HSL activity during exercise would be greater in women than in men, which then could have explained the higher IMTG breakdown during exercise in women.
higher HSL Ser659 phosphorylation in men than in women and to exercise and was higher in men than in women. The potential factors involved in regulation of IMTG hydrolysis in skeletal muscle during submaximal exercise.

HSL Ser563 phosphorylation was also higher in men than in women both at rest and during exercise (Fig. 5A). However, HSL Ser563 phosphorylation did not increase from rest to exercise, which is in accordance with previous studies (28, 39). The lack of increased HSL Ser563 phosphorylation by exercise despite increased plasma epinephrine suggests that HSL Ser563 is not an important site in human skeletal muscle in vivo. However, it cannot be excluded that HSL Ser563 is maximally phosphorylated already in the resting condition and that this is the reason why this site does not respond to increased epinephrine during exercise. In that case, it is still reasonable to conclude that HSL Ser563 does not play any role in regulation of HSL activity in skeletal muscle during exercise.

ERK is a potential candidate to explain the higher specific activity of HSL in men compared with women, because HSL activation by contraction was shown to depend on ERK in isolated rat soleus muscle (5). This is in line with studies in adipocytes, where ERK activation caused an increase in HSL activity and lipolysis (11). It was demonstrated that HSL activation by ERK in adipocytes involved phosphorylation of HSL on Ser659 (11). In the present study, ERK2 phosphorylation tended to be generally higher in men than in women (∼30–50%, \( P = 0.08 \); Fig. 6). It may be that the higher ERK phosphorylation in men than in women results in the higher HSL specific activity in men than in women both at rest and during exercise.

In the present study, HSL activity (Fig. 5C) could not explain the sex difference in IMTG net decrease seen during exercise (Fig. 3). Dissociation between HSL activity and IMTG net breakdown has been found in several previous studies (42, 43). Thus, during 180-min bicycle exercise at 60% \( \dot{V} \text{O}_2 \text{peak} \) with or without nicotinic acid ingestion, IMTG content decreased in both conditions, and more so with nicotinic acid ingestion, without any detectable activation of HSL (42). Furthermore, during 60-min bicycle exercise at 70% \( \dot{V} \text{O}_2 \text{peak} \) with low or normal muscle glycogen stores, IMTG content decreased only when muscle glycogen was low, whereas HSL was activated only when muscle glycogen was normal (43). Altogether, it appears that factors other than HSL activity are important in governing IMTG breakdown in skeletal muscle during exercise. This is in line with studies in primary rat adipocytes, where several \( \beta \)-adrenergic agents could markedly elevate lipolysis without affecting HSL activity (25). Other potential factors involved in regulation of IMTG hydrolysis in skeletal muscle during exercise could be HSL expression or in vivo allosteric regulation of HSL. Strikingly, HSL expression in skeletal muscle was markedly higher in women than in men in the present study at both the mRNA and protein level (Fig. 4, A and B). This finding suggests that the capacity for IMTG hydrolysis is higher in women than in men. Also, the higher IMTG net breakdown in women than in men during exercise could be a result of sex-specific allosteric regulation of HSL in vivo. Long-chain fatty acyl-CoA (LCFA-CoA) is the only known allosteric regulator of HSL activity. It has been shown

One factor to explain the higher specific activity of HSL during exercise in men than in women could be the higher plasma epinephrine concentrations seen in men during the last 30 min of exercise (Table 2). Higher plasma epinephrine concentrations in men than in women during prolonged submaximal exercise have been observed previously (33, 35). It also has been shown that high concentrations of epinephrine can lead to elevated HSL activity during muscle contraction in both rodents and humans (19, 20). It follows that a sex-specific catecholamine response to submaximal exercise could be the reason for higher HSL specific activity during exercise in men than in women. Still, this does not explain why HSL specific activity was higher in men than in women at rest when plasma epinephrine did not differ between sexes. Sex differences in sensitivity to epinephrine may exist. Alternatively, other factors than plasma epinephrine may regulate HSL activity at rest.

A very interesting finding in the present study was that skeletal muscle HSL Ser659 phosphorylation increased from rest to exercise and was higher in men than in women. The higher HSL Ser659 phosphorylation in men than in women during exercise was probably due to the higher plasma epinephrine in men at the end of exercise. However, the higher HSL Ser659 phosphorylation in men than in women at rest must have been due to other factors. In that regard, it is important to realize that protein phosphatases also are expected to act on HSL. The very similar patterns of HSL Ser659 phosphorylation (Fig. 5B) and HSL specific activity (Fig. 5C) in the present study indicate that HSL Ser659 serves an important role in regulation of HSL activity in human skeletal muscle, as it has been shown to do in adipocytes (1). Altogether, HSL Ser659 appears to be an important site in mediating the effect of epinephrine on HSL activity in human skeletal muscle during submaximal exercise.

HSL Ser563 phosphorylation was also higher in men than in women both at rest and during exercise (Fig. 5A). However, HSL Ser563 phosphorylation did not increase from rest to exercise, which is in accordance with previous studies (28, 39). The lack of increased HSL Ser563 phosphorylation by exercise despite increased plasma epinephrine suggests that HSL Ser563 is not an important site in human skeletal muscle in vivo. However, it cannot be excluded that HSL Ser563 is maximally phosphorylated already in the resting condition and that this is the reason why this site does not respond to increased epinephrine during exercise. In that case, it is still reasonable to conclude that HSL Ser563 does not play any role in regulation of HSL activity in skeletal muscle during exercise.

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In the present study, HSL activity (Fig. 5C) could not explain the sex difference in IMTG net decrease seen during exercise (Fig. 3). Dissociation between HSL activity and IMTG net breakdown has been found in several previous studies (42, 43). Thus, during 180-min bicycle exercise at 60% \( \dot{V} \text{O}_2 \text{peak} \) with or without nicotinic acid ingestion, IMTG content decreased in both conditions, and more so with nicotinic acid ingestion, without any detectable activation of HSL (42). Furthermore, during 60-min bicycle exercise at 70% \( \dot{V} \text{O}_2 \text{peak} \) with low or normal muscle glycogen stores, IMTG content decreased only when muscle glycogen was low, whereas HSL was activated only when muscle glycogen was normal (43). Altogether, it appears that factors other than HSL activity are important in governing IMTG breakdown in skeletal muscle during exercise. This is in line with studies in primary rat adipocytes, where several \( \beta \)-adrenergic agents could markedly elevate lipolysis without affecting HSL activity (25). Other potential factors involved in regulation of IMTG hydrolysis in skeletal muscle during exercise could be HSL expression or in vivo allosteric regulation of HSL. Strikingly, HSL expression in skeletal muscle was markedly higher in women than in men in the present study at both the mRNA and protein level (Fig. 4, A and B). This finding suggests that the capacity for IMTG hydrolysis is higher in women than in men. Also, the higher IMTG net breakdown in women than in men during exercise could be a result of sex-specific allosteric regulation of HSL in vivo. Long-chain fatty acyl-CoA (LCFA-CoA) is the only known allosteric regulator of HSL activity. It has been shown
that skeletal muscle HSL activity can be inhibited by physiological concentrations of palmitoyl-CoA in vitro (42). Higher intramuscular LCFA-CoA concentrations in men than in women during exercise could therefore explain why IMTG breakdown is lower in men than in women. However, to our knowledge, intramuscular LCFA-CoA concentrations during exercise have never been compared between men and women. Finally, basal IMTG content is higher in women than in men (27, 33, 47, and present study). The latter may improve the enzyme-substrate interaction between activated HSL and the IMTG-containing lipid droplets during exercise. Accordingly, in the present study there was a significant correlation between basal IMTG content and the IMTG net decline during exercise (Fig. 3C). Such correlation also has been shown in previous studies, where subjects have performed either resistance or endurance exercise (6, 33).

Women have a higher percentage of body fat compared with men. In sex-comparative studies with healthy, young subjects representing a broad range of training status, the average percentage of body fat was 20–30% in women and 9–20% in men (2, 8, 9, 15, 16, 26, 36, 37, 47). The percentages of body fat in women (24.7 ± 1.5%) and men (12.1 ± 2.3%) in the present study are in agreement with these previous studies. In an attempt to analyze whether higher percent body fat in women than in men could be an explanatory factor of the higher basal IMTG content in women, we performed a linear regression analysis on percent body fat and basal IMTG content. Interestingly, this analysis revealed that basal IMTG content correlated with percent body fat (Fig. 3B). This supports another recent study (47). Together with the association seen between basal IMTG content and IMTG net decline during exercise (Fig. 3C), it may be that a large part of the sex difference in IMTG net breakdown during exercise can be explained by the generally higher availability of body fat in women compared with men.

TG and HSL content are several-fold higher in adipocytes than in muscle cells (23). Therefore, we thought that it was appropriate to resolve whether the observed sex differences in IMTG and HSL content in skeletal muscle could simply be ascribed to higher adipose tissue contamination in women’s biopsies than in men’s. To test this, we used Western blotting against perilipin, a specific adipocyte marker protein (10). We observed that none of the muscle samples contained detectable amounts of perilipin (Fig. 2). It follows that, in the present study, the higher TG and HSL content in muscle biopsies from women than from men appeared to be of myocellular origin. This is also in accordance with preliminary findings from our laboratory with Oil Red O staining of intramyocellular lipids showing that intramyocellular lipid staining was higher in women than in men in biopsies obtained at rest (Roepstorff C, Nielsen CB, and Kiens B, unpublished observation).

In summary, the present study revealed that HSL specific activity during prolonged submaximal exercise is higher in men than in women, at least partly due to higher plasma epinephrine concentration in men resulting in higher HSL SeF phosphorylation. On the other hand, muscle HSL expression is higher in women than in men, which fully compensates the sex difference in HSL specific activity during exercise. Consequently, total HSL activity during exercise is similar in women and men and therefore cannot explain the higher IMTG net decrease during exercise in women compared with men. Rather, correlative analyses suggest that the higher IMTG hydrolysis during exercise in women than in men is at least partly explained by higher basal IMTG content in women. It is speculated that this could be due to improved enzyme-substrate interaction between IMTG and activated HSL in women.

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REFERENCES


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34. Sugden C, Crawford RM, Halford NG, and Hardie DG. Regulation of spinach SNF1-related (SnRK1) kinases by protein kinases and phosphatases is associated with phosphorylation of the T loop and is regulated by 5′-AMP. Plant J 19: 433–439, 1999.