Reduced plasma FFA availability increases net triacylglycerol degradation, but not GPAT or HSL activity, in human skeletal muscle

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Watt, Matthew J., Anna G. Holmes, Gregory R. Steinberg, Jose L. Mesa, Bruce E. Kemp, and Mark A. Febbraio. Reduced plasma FFA availability increases net triacylglycerol degradation, but not GPAT or HSL activity, in human skeletal muscle. Am J Physiol Endocrinol Metab 287: E120–E127, 2004. First published January 28, 2004; 10.1152/ajpendo.00542.2003.—Intramuscular triacylglycerols (IMTG) are proposed to be an important metabolic substrate for contracting muscle, although this remains controversial. To test the hypothesis that reduced plasma free fatty acid (FFA) availability would increase IMTG degradation during exercise, seven active men cycled for 180 min at 60% peak pulmonary O2 uptake either without (CON) or with (NA) prior ingestion of nicotinic acid to suppress adipose tissue lipolysis. Skeletal muscle and adipose tissue biopsy samples were obtained before and after each trial. NA ingestion resulted in decreased plasma FFA availability (CON: 13.9 ± 2.5, NA: 9.1 ± 3.0 nmol·min−1·mg protein−1) and decreased whole body fat oxidation and increased carbohydrate oxidation. Despite the decreased whole body fat oxidation, net IMTG degradation was greater in NA compared with CON at 180 min (NA, 0.10 ± 0.03; CON, 0.07 ± 0.01 mM). The decreased plasma FFA during NA was associated with decreased (P < 0.05) adipose tissue hormone-sensitive lipase (HSL) activity (CON: 13.9 ± 2.5, NA: 9.1 ± 3.0 nmol·min−1·mg protein−1). NA ingestion resulted in decreased whole body fat oxidation and increased carbohydrate oxidation. Despite the decreased whole body fat oxidation, net IMTG degradation was greater in NA compared with CON (net change: CON, 1.4 ± 0.77; NA, 0.10 ± 0.01 kg). The increased IMTG degradation did not appear to be due to reduced fatty acid esterification, because glycerol 3-phosphate activity was not different between trials and was unaffected by exercise (rest: 0.21 ± 0.07; 180 min: 0.17 ± 0.04 nmol·min−1·mg protein−1). HSL activity was not increased from resting rates during exercise in either trial despite elevated plasma epinephrine, decreased plasma insulin, and increased ERK1/2 phosphorylation. AMP-activated protein kinase (AMPKα)1 activity was not affected by exercise or NA, whereas AMPKα2 activity was increased (P < 0.05) from rest during exercise in NA and was greater (P < 0.05) than in CON at 180 min. These data suggest that plasma FFA availability is an important mediator of net IMTG degradation, and in the absence of plasma FFA, IMTG degradation cannot maintain total fat oxidation. These changes in IMTG degradation appear to dissociate, however, from the activity of the key enzymes responsible for synthesis and degradation of this substrate.

Free fatty acids; intramuscular triacylglycerols; hormone-sensitive lipase; glycerol-3-phosphate acyltransferase; enzymatic regulation; exercise; AMP-activated protein kinase

FATTY ACIDS derived primarily from the breakdown of adipose tissue triacylglycerols are a metabolic substrate for contracting skeletal muscle. The oxidation of plasma-derived free fatty acids (FFAs) coupled with other fat sources [presumably in-

tramuscular triacylglycerols (IMTG)] contributes ∼50% of the total energy expenditure during short-duration (∼30 min) moderate-intensity [∼60% peak pulmonary O2 uptake (VO2peak)] exercise (29, 33). There is, however, controversy regarding the degree of IMTG degradation during prolonged exercise and, consequently, their importance as a metabolic substrate. Some studies that have directly measured IMTG content by muscle biopsy and chemical extraction have observed no, or limited, net IMTG degradation after a prolonged exercise bout (2, 12, 16, 28, 31), whereas others using the same direct methods (10, 27) or nuclear magnetic resonance spectroscopy (6, 18) have reported biologically significant reductions. In this regard, we have previously demonstrated net IMTG degradation during the initial 2 h of a 4-h exercise bout and no decrease in the second 2 h (34). Prolonged exercise is characterized by a slow and progressive increase in both plasma FFA availability and fat oxidation (1, 34), suggesting that extracellular fatty acid availability may be important in regulating IMTG utilization.

IMTG degradation is catalyzed by the enzyme hormone-sensitive lipase (HSL). Skeletal muscle HSL is rapidly activated at the onset of muscle contraction (20, 37) and decreases late in prolonged moderate-intensity exercise (35). HSL, in rodent muscle, is partially activated during contractions by increased PKC activity and subsequent phosphorylation by extracellular regulated kinase (ERK1/2) (7). In contrast, studies in purified HSL (9) demonstrate decreased HSL activity when AMP-activated protein kinase (AMPK) is elevated. HSL is also activated by epinephrine (17), although the increase in epinephrine is not mandatory for increased HSL activity during contractions/exercise (20, 38). Thus the available evidence suggests that HSL activity is controlled by β-adrenergic and contraction-related mechanisms, which appear to function independently (19, 38). Whether the metabolic perturbations induced by elevated plasma FFA during prolonged exercise affect HSL activity and IMTG degradation is not known. In this regard, the accumulation of lipid intermediates allosterically decreases HSL activity isolated from adipose tissue (15), and long-chain acyl-CoA progressively increases during prolonged exercise (35), suggesting that this may be the mechanism underlying the small net IMTG utilization late in exercise.

We have previously suggested that during prolonged exercise IMTG degradation is not concomitantly matched by an increased HSL activity (35). This may be due to the fact that IMTG content is a measure of a balance between degradation
and fatty acid esterification into IMTG. Studies in rodents (8, 14) and humans (12, 30) demonstrate continuous turnover of the IMTG pool, although esterification rates are markedly less in humans compared with rodents. Glycerol-3-phosphate acyltransferase (GPAT) catalyzes the first and committed step of glycerolipid synthesis in numerous tissues, including adipose, liver, and skeletal muscle (4). Skeletal muscle GPAT activity is unaffected by short-duration exercise in rodents (23) or pharmacological AMPK activation (22) in C2C12 myocytes; however, there are currently no data pertaining to GPAT activity in human skeletal muscle at rest or during exercise. In the current study, we have performed such analyses with the hypothesis that increased GPAT activity may result in greater fatty acid esterification and preclude the detection of IMTG degradation during exercise.

In the present study, we used nicotinic acid to suppress adipose tissue lipolysis and the progressive increase in plasma FA availability and tested the hypothesis that reduced FA availability increases IMTG degradation during exercise. To further elucidate the role of plasma FFA availability on IMTG degradation, we measured activities of HSL and GPAT, enzymes central to IMTG degradation and repletion, respectively. In separate experiments, we also tested the effect of long-chain acyl-CoA availability on HSL activity. We hypothesized that decreased FFA availability would increase IMTG degradation secondary to increased HSL activity and decreased GPAT activity.

**METHODS**

**Human In Vivo Experiment**

**Subjects.** Seven recreationally active men (age 24 ± 3 yr; weight 80 ± 3 kg; body mass index 23.4 ± 0.9 kg/m²) provided their written informed consent and participated in the experiment after being provided with details of the experimental protocol and the associated risks. The Royal Melbourne Institute of Technology (RMIT) Human Ethics Committee approved the study, and experimental procedures comply with The Declaration of Helsinki. Subjects initially completed an incremental cycling test (Lode, Groningen, The Netherlands) to volitional exhaustion to determine their peak pulmonary oxygen uptake (\(\dot{V}O_2\text{peak}\)), which averaged 53.8 ± 3.1 ml·kg⁻¹·min⁻¹.Expired contents of oxygen and carbon dioxide were collected, and ventilation was analyzed online (Quark b2, COSMED, Rome, Italy). At least two days later, subjects completed a practice trial after an overnight fast. The purpose of this trial was to familiarize the subjects with the effects of nicotinic acid and confirm their ability to perform the exercise bout. Subjects ingested 10 mg/kg of nicotinic acid (NA) and rested for 60 min, after which they cycled for 120 min at 60% \(\dot{V}O_2\text{peak}\). Further NA doses (5 mg/kg) were ingested at 30-min intervals at rest (−30 and 0 min) and throughout exercise. For the day preceding each trial, subjects were provided with a food parcel [14 MJ, 80% carbohydrate (CHO)] and were required to abstain from exercise, caffeine, and alcohol. All trials were performed at an ambient temperature of 19–21°C.

**Experimental protocol.** Subjects visited the laboratory on two occasions and completed 180 min of cycle exercise at 60% \(\dot{V}O_2\text{peak}\). On one occasion, subjects ingested NA, as described above; the other occasion was a control trial (CON). Trials were randomized, counterbalanced, and conducted ≥1 wk apart. Subjects arrived at the laboratory (−0800) after an overnight fast, voided, and rested quietly on a bed. A Teflon catheter was introduced into a forearm vein, and a resting blood sample was obtained. The catheter was kept patent by infusing saline after sampling. Subjects ingested an initial NA dose or nothing, and they rested for 60 min. With subjects under local anesthesia (lidocaine), three incisions were made through the skin and underlying fascia of the vastus lateralis muscle for subsequent muscle sampling with the percutaneous biopsy technique. An incision was also made at a site ~10 cm lateral of the navel for percutaneous fat sampling. Resting blood, muscle, and fat samples were obtained before exercise while the subject remained supine on the bed. Subjects then moved to the cycle ergometer and commenced cycle exercise for 180 min. Respiratory gases and venous blood samples were collected at 30-min intervals. Muscle and fat samples were obtained at 90 and 180 min while subjects remained on the cycle ergometer. Muscle samples were excised and frozen in liquid nitrogen within 30 s of exercise cessation, and fat samples were obtained and frozen within 90 s. Subjects were instructed to ingest one liter of water per hour to ensure hydration.

**Analysis.** BLOOD METABOLITES AND HORMONES. Five milliliters of whole blood were mixed in a sodium-heparin collection tube, and the plasma was obtained after centrifugation at 5,000 g for 2 min. Glucose and lactate were immediately analyzed by an automated method (Yellow Springs Instruments 2300 STAT, Yellow Springs, OH). Plasma samples were frozen at −80°C for later analysis of FFAs by an enzymatic colorimetric method (NEFA C, Wako Chemicals, Richmond, VA), and insulin (Coat-a-Count, DPC, Los Angeles, CA) and epinephrine (LDN, Nordhorn, Germany) by radioimmunoassay.

**MUSCLE METABOLITES.** Muscle samples were divided into aliquots under liquid nitrogen. One piece of muscle (~80 mg wet muscle) was freeze-dried, dissected free of nonmuscle contaminants under magnification, and divided into four aliquots. Muscle for glycogen analysis (3 mg) was extracted in 2 M HCl and neutralized with 0.67 M NaOH, and glycogen content was determined as described (25). A second aliquot of muscle (2 mg) was extracted according to Harris et al. (13), and ATP, phosphocreatine, creatine, and lactate were determined by enzymatic fluorometric techniques (24). IMTG content was determined by extraction of the triacylglycerol from ~6 mg of tissue in chloroform-methanol (2:1), saponification of the triacylglycerol in an ethanol-KOH solution at 60°C, and fluorometric determination of glycerol (10).

**HSL ACTIVITY ASSAY.** An aliquot of freeze-dried muscle (6 mg) was used to determine HSL activity, as described previously (37). Briefly, the powdered muscle was homogenized, and after centrifugation the supernatant was removed and stored on ice for immediate analysis of HSL activity. A substrate consisting of 5 mM triolein, [9,10,12-^3^H]triol Fore, 0.6 mg phospholipid (phosphatidylinositol-phosphaticidinsolito, 3:1, wt/wt), 0.1 M potassium phosphate, and 20% BSA was emulsified by sonication. The muscle homogenate supernatant was incubated at 37°C with the triolein substrate and stopped after 20 min by the addition of 3.25 ml of a methanol-chloroform-heptane (10:9:7, vol/vol/vol) solution. Potassium carbonate (1.0 ml of 0.1 M) and boric acid (0.1 M) were added to facilitate the separation of the organic and aqueous phases. One milliliter of the upper phase containing the released fatty acids was removed for determination of radioactivity on a beta spectrometer (Tri-Carb 1500, Packard, Canberra, Australia).

**HSL was also determined in adipose tissue samples obtained at 0, 90, and 180 min. An aliquot of adipose tissue was homogenized for 2 × 10 s on ice in 20 volumes of homogenizing buffer with a Polytron homogenizer at full speed. After centrifugation, the fat cake was removed, and the supernatant was removed for immediate analysis of HSL activity, as described above.**

**GPAT ACTIVITY ASSAY.** Skeletal muscle GPAT activity was determined as described by Muoio et al. (22). After homogenization, total GPAT activity was measured with 900 µM \([^3^H]\)glycerol 3-phosphate (\([^3^H]G-3-P\), ARC. St. Louis, MO) and 90 µM palmitoyl-CoA. The reaction was run for 20 min at 37°C and stopped with 1% HClO₄ and chloroform-methanol (2:1). After a series of washes with HClO₄, 1 ml of the organic phase containing the labeled G-3-P incorporated into lysophosphatidic acid was dried, and 4 ml of scintillation fluid were
added. Radioactivity was determined on a beta spectrometer. All measurements were made in triplicate, and means of these values are reported. Mitochondrial GPAT activity could not be reliably measured and represented <10% of total activity. This is presumably due to its low abundance (22). Therefore, the results presented are GPAT activity from both microsomal and mitochondrial GPAT. The intra-assay coefficient of variation from nine aliquots, each measured in triplicate from one muscle sample, was 4.6 ± 2.0% (mean ± SD) at 0.22 ± 0.04 nmol·min⁻¹·mg protein⁻¹.

**MUSCLE AMPK ACTIVITY AND ACETYL-COA CARBOXYLASE PHOSPHORYLATION.** Frozen muscle samples (~30 mg) were homogenized in 200 ml of ice-cold lysis buffer, as previously described (3). Homogenates were centrifuged at 14,000 g for 25 min, and the supernatant was incubated with AMPKα1 and AMPKα2 antibody-bound protein A-agarose beads for 2 h. Immunocomplexes were washed with PBS and suspended in 50 mM Tris (pH 7.5) buffer for quantification of 10% glycerol (vol/vol), 2 mM sulfonyl fluoride, 10 mM NaCl, 10 mM NaF, 1 mM Na₃VO₄, 5 mM EDTA, 0.5% Triton X-100, 0.22 mg/ml aprotinin. Muscle lysates (80 mg protein) were solubilized in Laemmli sample buffer and boiled for 5 min, resolved by SDS-PAGE, and immunoblotted with horseradish peroxidase-conjugated secondary antibody (1:2,000; Amersham, Uppsala, Sweden) and horseradish peroxidase-conjugated streptavidin (DAKO, Carpinteria, CA). This antibody detects endogenous levels of ERK1/2 only when phosphorylated on the Thr202/Tyr204 residues. After incubation with horseradish peroxidase-conjugated secondary antibody (1:2,000; Amersham Biosciences, Castle Hill, NSW, Australia), the immunoreactive proteins were detected with enhanced chemiluminescence (Perkin Elmer, Rowville, Australia) and quantified by densitometry.

The expression and phosphorylation of acetyl-CoA carboxylase (ACC)β were measured via Western blotting of the same muscle homogenate used to measure AMPK activity. ACCβ was purified by binding to immobilized monomeric avidin agarose beads. The ACCβ fraction was subjected to SDS-PAGE. The phosphorylation of ACCβ was detected by immunoblot with anti-phospho-ACCβ-Ser²¹⁷ polyclonal antibody. The total ACCβ expression level was determined by horseradish peroxidase-conjugated streptavidin (DAKO, Carpinteria, CA).

**ERK1/2 PHOSPHORYLATION.** A final muscle sample (~15 mg wet muscle) was homogenized (Polytron; Brinkman Instruments, Westbury, NY) in ice-cold buffer containing 50 mM HEPES, 150 mM NaCl, 10 mM NaF, 1 mM Na₃VO₄, 5 mM EDTA, 0.5% Triton X-100, 10% glycerol (vol/vol), 2 μg/ml leupeptin, 100 μg/ml phenylmethylsulfonyl fluoride, and 2 μg/ml apro tinin. Muscle lysates (80 μg) were solubilized in Laemmli sample buffer and boiled for 5 min, resolved by SDS-PAGE on 12% polyacrylamide gels, transferred to a nitrocellulose membrane, blocked with 3% BSA, and immunoblotted with the phospho-ERK1/2 MAPK antibody (1:1,000; Cell Signaling, Beverly, MA). This antibody detects endogenous levels of ERK1/2 only when phosphorylated at Thr²⁰²/Tyr²⁰⁴. After incubation with horseradish peroxidase-conjugated secondary antibody (1:2,000; Amersham Biosciences, Castle Hill, NSW, Australia), the immunoreactive proteins were detected with enhanced chemiluminescence (Perkin Elmer, Rowville, Australia) and quantified by densitometry.

**Calculations and Statistics**

Whole body carbohydrate and fat oxidation rates were estimated using stoichiometric equations (26). Free ADP and AMP concentrations were calculated as described previously (37). Statistical analysis was performed by two-way analysis of variance with repeated measures (time × trial), and specific differences were located using a Student-Newman-Keuls post hoc test. For the isolated rat studies, comparisons were made by a one-way analysis of variance. Statistical significance was set at P < 0.05. Data are expressed as means ± SE.

**RESULTS**

**Respiratory Responses During Exercise**

Oxygen uptake (VO₂) and minute ventilation (Ve) increased (P < 0.05) late in exercise and were not different between trials (Table 1). Whole body CHO oxidation was greater (P < 0.05, main effect) during NA compared with CON (Table 1). Fat oxidation was lower (P < 0.05, main effect) throughout exercise in NA, and there were no significant changes over time (Table 1). The absence of change in CHO oxidation may have been the result of increased expired CO₂ due to subjects hyperventilating late in exercise (at 150 and 180 min).

### Table 1. Respiratory responses at rest and during 180 min of cycle exercise at 60% VO₂ peak with or without nicotinic acid ingestion

<table>
<thead>
<tr>
<th>Trial</th>
<th>Min</th>
<th>VO₂, ml·kg⁻¹·min⁻¹</th>
<th>Ve, l/min</th>
<th>CHO oxidation, kJ/min</th>
<th>Fat oxidation, kJ/min</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>30</td>
<td>60</td>
<td>90</td>
<td>120</td>
<td>180</td>
</tr>
<tr>
<td>CON</td>
<td>30.1±1.1</td>
<td>29.8±0.9</td>
<td>31.3±1.1</td>
<td>32.3±0.8</td>
<td>34.1±1.3</td>
</tr>
<tr>
<td>NA</td>
<td>29.6±1.0</td>
<td>30.7±0.6</td>
<td>31.8±0.7</td>
<td>32.7±1.0</td>
<td>33.2±1.1</td>
</tr>
<tr>
<td>RER</td>
<td>57±2</td>
<td>57±3</td>
<td>61±1</td>
<td>60±2</td>
<td>64±3</td>
</tr>
<tr>
<td>CON</td>
<td>60±2</td>
<td>60±2</td>
<td>63±2</td>
<td>66±4</td>
<td>72±2</td>
</tr>
<tr>
<td>NA</td>
<td>0.93±0.01</td>
<td>0.91±0.01</td>
<td>0.91±0.02</td>
<td>0.87±0.01</td>
<td>ND</td>
</tr>
<tr>
<td>CHO</td>
<td>0.94±0.01</td>
<td>0.92±0.01</td>
<td>0.92±0.01</td>
<td>0.91±0.02</td>
<td>ND</td>
</tr>
<tr>
<td>RER</td>
<td>40.3±2.4</td>
<td>37.4±2.6</td>
<td>37.4±2.2</td>
<td>32.6±2.3</td>
<td>ND</td>
</tr>
<tr>
<td>CON</td>
<td>41.7±2.0</td>
<td>39.3±1.3</td>
<td>42.6±2.9</td>
<td>40.3±5.8</td>
<td>ND</td>
</tr>
<tr>
<td>NA</td>
<td>13.1±1.7</td>
<td>14.9±2.2</td>
<td>16.3±3</td>
<td>22.1±2.1</td>
<td>ND</td>
</tr>
<tr>
<td>Fat</td>
<td>10.1±1.8</td>
<td>13.8±1.4</td>
<td>12.8±2.3</td>
<td>18.6±5.6</td>
<td>ND</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 7, VO₂ peak, peak O₂ uptake; NA, nicotinic acid trial; CON, control trial; Ve, minute ventilation; RER, respiratory exchange ratio; CHO, carbohydrate; ND, not determined. *Significant main effect (trial); †significant main effect (time), P < 0.05.
concentration was higher ($P < 0.05$, main effect) throughout exercise in both trials (Table 2). Plasma epinephrine increased ($P < 0.05$, main effect) during exercise in both trials and was higher ($P < 0.05$, main effect) in NA compared with CON (Table 3). Plasma insulin decreased ($P < 0.05$, main effect) during exercise in both trials.

### Muscle Metabolite Responses at Rest and During Exercise

IMTG content at rest averaged 27.2 ± 1.5 mmol/kg dry mass (dm) and was not different between trials (Fig. 2). IMTG content was decreased at 90 min in NA, and no such decrease was observed in CON. By 180 min, IMTG was decreased at 90 min during NA, and no such decrease was observed in CON. By 180 min, IMTG was the same for CON and NA, respectively. Muscle glycogen content progressively decreased ($P < 0.05$) during exercise and was not different ($P = 0.09$) between trials (Table 4). Muscle ATP and lactate were unaffected by exercise or NA administration (Table 4). Phosphocreatine was decreased ($P < 0.05$) during exercise in both trials and was not different between trials (Table 4). Creatine was increased ($P < 0.05$) during exercise and was not different between trials (Table 4). Free ADP tended ($P = 0.052$) to be higher in NA compared with CON. Free AMP was increased ($P < 0.05$) at 180 min in NA (Table 4).

### HSL and GPAT Activity

HSL activity averaged 2.10 ± 0.18 mmol·min$^{-1}$·kg$^{-1}$·dm at rest and was not different between trials (Fig. 3A). HSL activity was not affected by exercise in either trial. GPAT activity at rest averaged 0.21 ± 0.07 mmol·min$^{-1}$·mg protein$^{-1}$ and was unaffected by both NA and exercise (Fig. 3B).

### AMPK Activity and ACCβ Phosphorylation

AMPKα1 activity was not affected by exercise or NA ingestion (Fig. 4). AMPKα2 activity was increased ($P < 0.05$) in NA compared with CON.

### Table 2. Plasma metabolites at rest and during 180 min of cycle exercise at 60% $\dot{V}O_2$ peak in NA or CON trial

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Trial</th>
<th>0</th>
<th>30</th>
<th>60</th>
<th>90</th>
<th>120</th>
<th>180</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose, mM</td>
<td>CON</td>
<td>5.4 ± 0.1</td>
<td>5.3 ± 0.2</td>
<td>5.1 ± 0.1</td>
<td>5.1 ± 0.1</td>
<td>4.7 ± 0.1</td>
<td>4.5 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>NA</td>
<td>6.0 ± 0.1*</td>
<td>5.2 ± 0.1†</td>
<td>5.1 ± 0.1†</td>
<td>5.0 ± 0.1†</td>
<td>4.6 ± 0.2†</td>
<td>3.8 ± 0.1†</td>
</tr>
<tr>
<td>Lactate, mM</td>
<td>CON†</td>
<td>1.3 ± 0.2</td>
<td>2.4 ± 0.4</td>
<td>1.8 ± 0.3</td>
<td>1.8 ± 0.4</td>
<td>2.0 ± 0.2</td>
<td>2.5 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>NA</td>
<td>1.8 ± 0.3</td>
<td>2.6 ± 0.4</td>
<td>2.1 ± 0.5</td>
<td>2.0 ± 0.3</td>
<td>2.3 ± 0.3</td>
<td>3.0 ± 0.3</td>
</tr>
</tbody>
</table>

Values are means ± SE; $n = 7$. ND, not determined. Significant ($P < 0.05$) difference *from corresponding time point for CON or †from 0 min of the same trial. ‡Significant main effect (time), $P < 0.05$. 

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**Table 3. Plasma hormones at rest and during 180 min of cycle exercise at 60% $\dot{V}O_2$ peak in NA or CON trial**

<table>
<thead>
<tr>
<th>Hormone</th>
<th>Trial</th>
<th>0</th>
<th>90</th>
<th>180</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epinephrine, nM</td>
<td>CON</td>
<td>0.27 ± 0.06</td>
<td>1.16 ± 0.48</td>
<td>1.70 ± 0.35</td>
</tr>
<tr>
<td></td>
<td>NA</td>
<td>0.72 ± 0.11</td>
<td>2.45 ± 0.68</td>
<td>3.09 ± 0.70</td>
</tr>
<tr>
<td>Insulin, pM</td>
<td>CON</td>
<td>51.9 ± 4.8</td>
<td>16.3 ± 5.4</td>
<td>6.9 ± 1.5</td>
</tr>
<tr>
<td></td>
<td>NA</td>
<td>54.7 ± 4.3</td>
<td>17.7 ± 3.0</td>
<td>6.6 ± 1.7</td>
</tr>
</tbody>
</table>

Values are means ± SE; $n = 7$. *Significant main effect for time; †significant main effect for trial, $P < 0.05$. 

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**Fig. 1.** Plasma FFA concentration (A) and adipose tissue hormone-sensitive lipase (HSL) activity (B) at rest and during 180 min of cycle exercise at 60% peak $O_2$ pulmonary uptake ($\dot{V}O_2$-peak) with (NA) or without (CON) nicotinic acid ingestion. Values are expressed as means ± SE; $n = 7$. *Significant difference from CON at corresponding time point; †main effect for trial ($P < 0.05$).
from rest during exercise in NA and was greater ($P < 0.05$) than in CON at 180 min (Fig. 4). In contrast, no significant increases (32%) in AMPK were observed in CON during exercise. Exercise increased ($P < 0.05$) ACCβ phosphorylation at 180 min in NA, and no differences (26%, NS) were observed in CON (Fig. 5). ACCβ phosphorylation was greater ($P < 0.05$) in NA compared with CON at 180 min.

**ERK1/2 Phosphorylation**

Phospho-ERK1/2 was lower ($P < 0.05$) in NA at rest and after 90 min of exercise compared with CON (Fig. 6). During both trials, phospho-ERK1/2 was increased ($P < 0.05$) from resting values at 90 min, and by 180 min phospho-ERK1/2 had decreased ($P < 0.05$) to resting values in NA and to values lower than rest in CON.

**In Vitro Experiments**

In the separate in vitro experiments using isolated rat soleus, palmitoyl-CoA decreased basal HSL activity by ~20% at 10 μM. Further decreases in HSL activity were not observed at higher palmitoyl-CoA concentrations (Fig. 7).

**Table 4. Muscle metabolites and adenine nucleotides at rest and during 180 min of cycle exercise at 60% $V_O^2_{peak}$ in NA or CON trial**

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Trial</th>
<th>0</th>
<th>90</th>
<th>180</th>
<th>7. dm, dry mass</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycogen, mmol/kg dm</td>
<td>CON</td>
<td>458±35</td>
<td>267±46</td>
<td>173±29</td>
<td></td>
</tr>
<tr>
<td></td>
<td>NA*</td>
<td>466±46</td>
<td>247±45</td>
<td>101±7</td>
<td></td>
</tr>
<tr>
<td>Phosphocreatine, mmol/kg dm</td>
<td>CON</td>
<td>74±4</td>
<td>60±6</td>
<td>52±6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>NA*</td>
<td>74±5</td>
<td>54±5</td>
<td>43±2</td>
<td></td>
</tr>
<tr>
<td>Creatine, mmol/kg dm</td>
<td>CON</td>
<td>59±7</td>
<td>75±6</td>
<td>82±9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>NA*</td>
<td>61±1</td>
<td>90±9</td>
<td>94±5</td>
<td></td>
</tr>
<tr>
<td>Lactate, mmol/kg dm</td>
<td>CON</td>
<td>5.6±1.7</td>
<td>3.6±2.1</td>
<td>11.7±5.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>NA</td>
<td>7.1±3.8</td>
<td>4.3±1.9</td>
<td>4.2±2.1</td>
<td></td>
</tr>
<tr>
<td>ATP, mmol/kg dm</td>
<td>CON</td>
<td>22.7±1.8</td>
<td>23.5±2.3</td>
<td>23.8±2.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>NA</td>
<td>25.4±1.6</td>
<td>22.5±1.8</td>
<td>21.6±1.4</td>
<td></td>
</tr>
<tr>
<td>Free AMP, μmol/kg dm</td>
<td>CON</td>
<td>0.7±0.1</td>
<td>2.1±0.7</td>
<td>2.1±0.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>NA</td>
<td>0.8±0.1</td>
<td>2.7±0.7</td>
<td>4.6±0.7</td>
<td></td>
</tr>
<tr>
<td>Free ADP, μmol/kg dm</td>
<td>CON</td>
<td>119±13</td>
<td>216±31</td>
<td>200±24</td>
<td></td>
</tr>
<tr>
<td></td>
<td>NA</td>
<td>142±2.2</td>
<td>595±277</td>
<td>318±34</td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SE; $n = 7$. *Significant main effect for time; †significant difference from corresponding time point for CON, $P < 0.05$. ‡significant difference from 0 min of the same trial; §significant difference from 0 min of the same trial ($P < 0.05$).

**DISCUSSION**

The present study demonstrates that, in circumstances in which plasma FFA are not limited, net IMTG degradation and the FFA contribution to energy turnover during prolonged exercise are negligible. Conversely, when plasma FFA availability is limited, the increase in IMTG breakdown is augmented but cannot maintain normal fat oxidation rates. In addition, the increased net IMTG degradation was unlikely to be due to decreased esterification, because GPAT activity was not different between trials. These data also demonstrate that increased IMTG degradation is not due to covalent modification of HSL.

In the present study, subjects were fasted overnight to avoid the confounding effects of changes in the hormonal milieu.
induced by feeding. The most important of these changes is elevated insulin, which has persistent effects for several hours, even when circulating concentrations return to basal levels (5).

Although we acknowledge that the fasting of subjects may have exacerbated the difference in plasma FFA levels, the concentration during CON was nonetheless comparable with that seen during prolonged exercise, even with preexercise CHO ingestion (34).

In our previous study (34), the absence of change in IMTG from 2 to 4 h of exercise occurred concomitantly with augmented plasma FFA concentration, suggesting that the release of adipose tissue FA and their uptake by active tissues may regulate IMTG use. In the present study, plasma FFA was completely suppressed (0.1 mM) with NA ingestion, which resulted in a net IMTG degradation after 90 min and further decreases by 180 min. In contrast, IMTG was not reduced at 90
min and was decreased by only 9% at 180 min, when the characteristic exercise-induced rise in plasma FFA occurred. Thus these data provide further evidence that IMTG is not normally an important metabolic substrate during prolonged exercise when plasma FFAs are readily available, and they explain, at least partially, the absence of change observed in previous studies. Of note, during NA when plasma FFA availability was attenuated, total fat oxidation could not be maintained, even though IMTG were only reduced by ~25% from the preexercise content. Hence, these data also demonstrate that, whereas IMTG can contribute to fat oxidation, they cannot maintain “normal” fat oxidation when plasma FFAs are limited.

It is sometimes assumed that direct measures of net IMTG degradation are a poor reflection of IMTG utilization because of simultaneous hydrolysis and esterification in skeletal muscle. Studies in isolated rodent skeletal muscle demonstrate concomitant IMTG hydrolysis and exogenous FA esterification during contractions (8, 14), and these observations are supported by data in human skeletal muscle (30), although esterification rates are only ~10% of IMTG turnover rates during exercise (12). In the present study, we measured GPAT activity as a surrogate marker of FA esterification, because GPAT is the first and committed step of glycerolipid synthesis (4). We observed no change in GPAT activity with NA administration or during exercise in either trial, which is consistent with the GPAT response to exercise in rodents (23). It is possible that a substrate limitation during NA may have affected GPAT activity in vivo and that this would not be detected in our in vitro assay system, in which saturating palmitoyl-CoA is present. In the absence of tracer-derived measurements of FA esterification, however, the present findings of similar GPAT activity rates between trials suggests that the differences in net IMTG degradation between trials are most likely to be a function of increased breakdown and not of decreased esterification.

HSL is the rate-limiting enzyme for IMTG degradation and is primarily controlled by reversible phosphorylation. HSL activity is increased early in exercise (37), and peak activity rates are attained at ~60 min and decline at 120 min (36). In the present study, HSL activity was not elevated from resting rates at 90 and 180 min of exercise during either trial. At 90 min, HSL was not different from rest despite the expected stimulatory effects of increased plasma epinephrine and decreased plasma insulin (acting through PKA) and increased ERK1/2 phosphorylation. We previously suggested that the return of HSL to resting rates late in prolonged exercise was due to increased AMPK activity, although we made no direct measurements to support those claims (35). In the present study, AMPKα2 activity in both trials tended (~50%, not significant) to increase at 90 min. Consistent with the inhibitory AMPK effects in purified HSL (11), the tendency for increased AMPK observed in the present study may have prevented and/or overridden the other stimulatory hormonal and ERK effects, resulting in attenuation of the normal exercise-induced HSL increase. Such regulation would be consistent with the premise of mutually exclusive phosphorylation that was proposed in adipose tissue (11). In this regard, it seems likely that AMPK is the most influential among the hierarchy of the known covalent effectors of skeletal muscle HSL; however, studies using antibodies that target the known serine phosphorylation sites are required to confirm this. HSL activity was also unchanged from rest at 180 min in both trials despite marked increases in plasma epinephrine and further decreases in plasma insulin. The expected rise in HSL activity was presumably attenuated due to decreased ERK1/2 phosphorylation and increased AMPKα2 activity during NA. Thus, although hormonal regulators such as epinephrine and insulin can regulate HSL (17, 38), intramuscular factors clearly play a dominant role in the control of HSL activity during prolonged moderate-intensity exercise.

Aside from covalent effects, studies conducted in adipose tissue HSL demonstrate allosteric inhibition by intermediates of lipid metabolism (15). Long-chain fatty acid (LCFA)-CoA accumulates in skeletal muscle late in prolonged exercise at a time when no net IMTG degradation is observed, which is consistent with the potential for allosteric regulation of HSL by LCFA-CoA (34, 35). In this regard, we report an ~20% reduction in HSL activity when palmitoyl-CoA is added to homogenates from resting rat soleus muscle. The 20% reduction in HSL activity is significant, given that contractions and epinephrine, two powerful stimulators of HSL, independently increase activity by ~30–60% (19, 38). Although we did not measure LCFA-CoA in the human study, the relatively low rates of whole body fat oxidation, low adipose tissue HSL and GPAT activities, and reduced plasma FFA availability suggest that LCFA-CoA was unlikely to accumulate in NA. In contrast, previous studies have demonstrated marked LCFA-CoA accumulation during exercise when plasma FFA concentrations are high (36). Taken together, these data support an inhibitory role of LCFA-CoA on HSL activity and may explain the apparent mismatch between HSL activity and IMTG degradation between trials.

NA is proposed to decrease adipose tissue lipolysis by operating through a recently identified HM74 receptor (32). This receptor is coupled to an inhibitory G protein, which is thought to result in reduced PKA stimulation of HSL activity. To our knowledge, this is the first study to confirm that NA decreases HSL activity in vivo; however, HSL activity is not completely blunted, suggesting that factors such as ERK1/2 phosphorylation maintain permissive adipose tissue lipolysis. HM74 mRNA has been detected only in adipose tissue and the spleen, and not in skeletal muscle, suggesting that NA did not directly affect skeletal muscle HSL activity.

In conclusion, decreased plasma FFA concentrations and delivery to the muscle increase IMTG degradation during prolonged moderate-intensity exercise. Despite the increased IMTG degradation, the rate of fat oxidation cannot be maintained at the rates observed when plasma FFA are not limited. The increased IMTG degradation was not due to reduced GPAT or greater HSL activity, and the decreased HSL activity was most likely attenuated by elevated AMPKα2 activity, but it may have resulted from relieving the allosteric inhibition induced by LCFA-CoA.

GRANTS

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