Molecular Responses to Moderate Endurance Exercise in Skeletal Muscle

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This study examined alterations in skeletal-muscle growth and atrophy-related molecular events after a single bout of moderate-intensity endurance exercise. Muscle biopsies were obtained from 10 men (23 ± 1 yr, body mass 80 ± 2 kg, and VO$_{2peak}$ 45 ± 1 ml · kg$^{-1}$ · min$^{-1}$) immediately (0 hr) and 3 hr after a 60-min bout of cycle exercise (60% ± 5% VO$_{2peak}$). Corresponding muscle biopsies were also obtained under resting conditions. The phosphorylation status of insulin/IGF-PI3K molecular-signaling proteins, ubiquitin-proteasome-related gene expression, FOXO transcription factors, and myogenic regulatory factors in muscle samples was analyzed using multiplex analysis, Western blotting, and quantitative real-time polymerase chain reaction (qRT-PCR). A condition–time interaction was observed for Akt phosphorylation ($p < .05$) with multiplexing. Regardless of endurance exercise, Akt phosphorylation decreased and ERK phosphorylation increased at 3 hr compared with 0 hr ($p < .05$). Levels of p70S6K phosphorylation were 110% greater ($p < .05$) at 3 hr than at 0 hr using Western blots. MuRF mRNA expression postexercise increased; levels were 4.7- and 5.7-fold greater ($p < .05$) at 0 hr and 3 hr, respectively, than at rest with qRT-PCR. Atrogin mRNA expression was up-regulated 3.2-fold 3 hr postexercise compared with rest. These findings demonstrate modest changes in the molecular responses to moderate endurance exercise in the absence of nutrition. This study provides the groundwork for future investigations designed to optimize the metabolic conditions necessary to positively influence the cellular mechanisms specific to skeletal-muscle protein turnover during recovery from endurance exercise.

Keywords: protein synthesis, protein breakdown, muscle protein turnover, translation initiation, intracellular signaling

Over the past 2 decades, many studies using stable isotopes to assess protein kinetics have investigated the effects of resistance exercise on muscle protein synthesis (MPS) and breakdown (MPB) to characterize exercise-induced alterations in human skeletal-muscle protein turnover (SMPTO; Kumar, Atherton, Smith, & Rennie, 2009; Phillips, Tipton, Aarsland, Wolf, & Wolfe, 1997). It is generally accepted that resistance exercise induces primarily anabolic responses (Chesley, MacDougall, Tarnopsky, Atkinson, & Smith, 1992; MacDougall et al., 1995), with enhanced MPS lasting more than 48 hr measured under fasted conditions (Phillips et al., 1997). These findings are consistent with the typical skeletal-muscle phenotypic adaptations (i.e., myofibrillar protein accretion) seen after resistance training (Kumar et al., 2009). However, much less is known about skeletal-muscle protein metabolic response to endurance-type exercise. Two studies indicated an increase (Carraro, Stuart, Hartl, Rosenblatt, & Wolfe, 1990; Sheffield-Moore et al., 2004) and one observed no change (Tipton et al., 1996) in measures of post-endurance-exercise SMPTO. The discrepant observations regarding endurance-type exercise’s influence on SMPTO may be attributable to between-studies differences in exercise intensity and the training state of the study volunteers. Furthermore, the skeletal-muscle protein metabolic response to exercise type (resistance vs. endurance) and training state (untrained vs. trained) appeared to be different between particular skeletal-muscle subfractions, that is, myofibrillar and mitochondrial (Wilkinson et al., 2008). Although a recent study (Harber et al., 2009) assessed the acute and 24-hr fed-state skeletal-muscle protein synthetic response to endurance exercise, the influence of a single bout of endurance exercise on fasted postexercise skeletal-muscle protein synthesis and breakdown remains poorly understood.

Alterations in the activity of protein synthetic molecular-signaling proteins and components specific to the ubiquitin proteasome pathway (UPP) likely contribute to some of the observed exercise-induced changes in MPS.
and MPB (Drummond, Dreyer, Fry, Glynn, & Rasmussen, 2009; Rose & Richter, 2009; Lecker, Goldberg, & Mitch, 2006). Specifically, moderate endurance exercise has been shown to increase the phosphorylation of the mitogen-activated protein kinases (MAPK), including the extracellular signal-regulated protein kinase (ERK) 1/2 (Aronson et al., 1997). Other studies have demonstrated enhanced phosphorylation of Akt, as well as other components of the insulin–IGF-P13K molecular-signaling pathway, such as p70S6K and ribosomal protein S6 (rpS6), after endurance exercise (Benziane et al., 2008; Sakamoto, Arnolds, Ekberg, Thorell, & Goodyear, 2004). Limited studies have characterized UPP-related gene expression in response to resistance (Louis, Raue, Yang, Jemiolo, & Trappe, 2007; Yang, Jemiolo, & Trappe, 2006), endurance (Coffey et al., 2006a), or combined resistance and endurance exercise (Coffey et al., 2006b). Although enhanced proteolytic gene expression may not be representative of protein content, changes in UPP-related gene expression in response to resistance exercise are consistent with exercise-induced alterations in MPB typically observed when tracer methodologies were employed to determine SMPTO (Phillips et al., 1997). Although these findings would suggest a direct relationship, it is important to acknowledge that static “snapshots” of various cellular markers of MPS and MPB are not always indicative of dynamic measures of protein turnover (Greenhaff et al., 2008). Consequently, given the discrepancies between the limited studies in this area, no consensus has been reached regarding the role of these cellular mechanisms in mediating the protein metabolic response to endurance exercise.

As such, our objective was to further characterize the effect of a single bout of moderate endurance exercise on the skeletal-muscle growth and atrophy-related molecular response in healthy, physically active adult men. A combination of molecular methodologies was employed in an attempt to better understand the transcriptional and translational regulatory factors affecting MPS and MPB measured under fasted conditions during recovery from endurance exercise. We hypothesized that the phosphorylation of various proteins along the insulin/IGF-P13K molecular-signaling pathway would demonstrate the modest effect of endurance exercise on translation initiation. Likewise, we expected UPP-related gene expression together with other markers of MPB to reflect the proteolytic environment in the early stages of recovery from endurance exercise.

**Methods**

**Participants**

Volunteers (10 men) were free-living, active-duty military personnel who participated regularly in a combination of endurance- (i.e., running and marching) and resistance-type exercise (i.e., calisthenics and free weights) 3–4 days/week as part of their standard military physical-training regimens. Volunteers were required to be weight stable (± 2 kg) for a minimum of 2 months before initiating the study and healthy and active as indicated by baseline-study screening (VO2peak 40–50 ml · kg⁻¹ · min⁻¹). Prospective volunteers reporting metabolic or cardiovascular abnormalities, musculoskeletal injuries, or use of medications known to influence protein metabolism were excluded from participation. Volunteers provided informed consent after receiving a thorough explanation of study procedures. This study was approved by the Human Use Review Committee at the U.S. Army Research Institute of Environmental Medicine, Natick, MA, and the institutional review board at Tufts University, and all volunteers were medically cleared for participation. The investigators adhered to the policies for protection of human participants as prescribed in Army Regulation 70-25, and the research was conducted in adherence with the provisions of 32 CFR part 219.

**Experimental Design**

The study was part of a larger investigation designed to assess protein turnover that lasted 13 days, including two skeletal-muscle biopsy protocols to assess molecular regulators of SMPTO once at rest (Day 8) and again after a moderate endurance-exercise bout (Day 13). On the evening before each muscle biopsy protocol, volunteers received a standardized meal and stayed overnight in the Metabolic Research Unit of the Jean Mayer USDA Human Nutrition Research Center on Aging at Tufts University.

**Diet.** Individual energy requirements necessary to ensure volunteer weight maintenance were established based on self-reported 3-day diet and physical activity records before study initiation. Baseline diet records were reviewed and analyzed by a registered dietitian using Food Processor (version 8.5, 2005, ESHA Research, Salem, OR), and energy-expenditure estimations were based on recorded activities and corresponding metabolic equivalents (Ainsworth et al., 2000). Study dietitians provided volunteers instruction on the study diet and told them to maintain their usual training and physical activity patterns throughout the study. Physical activity and diet adherence were monitored every 48 hr with verbal physical activity questionnaires and 24-hr diet recalls, respectively. Volunteers were required to maintain a eucaloric diet throughout the study, composed of approximately 55–65% carbohydrate, 1.2 g · kg⁻¹ · day⁻¹ protein, and the remaining energy derived from fat.

**Additional Baseline Measurements.** Baseline anthropometrics were performed using standardized techniques and equipment. Height was measured in duplicate to the nearest 0.1 cm using an anthropometer (Item No. 101, Seritex, Inc., Carlstadt, NJ). After an overnight fast, body weight was measured using a calibrated digital scale (WB-110A, Tanita, Tokyo, Japan) to the nearest 0.1 kg. Body composition was assessed at baseline using dual-energy X-ray absorptiometry (DPX-IQ DEXA, Lunar Corp., Madison, WI).
Peak oxygen uptake (VO$_{2peak}$) was determined at baseline by analyzing expired gases during a progressive-intensity cycle-ergometer test via an open-circuit indirect respiratory system (TrueOne 2400 Metabolic Measurement System, ParvoMedics, Sandy, UT).

**Protocol**

**Resting Muscle Biopsy Protocol.** After 7 days of adaptation to the eucaloric diet, volunteers underwent the resting biopsy protocol on Study Day 8 subsequent to an overnight fast. They refrained from any physical activity for 48 hr before the muscle biopsy protocol. With each volunteer resting in a supine position, a percutaneous muscle biopsy (0 hr) was taken under sterile conditions from one incision in the lateral portion of the vastus lateralis muscle approximately 20 cm above the knee with a 5-mm Bergstrom biopsy needle. This study was originally designed to assess skeletal-muscle protein synthesis using stable-isotope methodology, so a second muscle biopsy was obtained from the same incision 3 hr after the initial biopsy. Approximately 100 mg of mixed muscle tissue was obtained with each biopsy. Each sample was blotted dry of blood, visible fat and connective tissue were removed, and the tissue was frozen in liquid nitrogen and stored at –80 °C until further analysis. Unfortunately, because of unforeseen analytical complications, skeletal-muscle protein kinetic data are not available.

Blood samples were taken at baseline and concurrent with each muscle biopsy to determine the potential influence of plasma substrates and hormones on the molecular regulatory mechanisms associated with protein turnover.

**Postexercise Muscle Biopsy Protocol.** On Study Day 13 volunteers underwent the postexercise muscle biopsy protocol after an overnight fast. Physical activity restrictions were the same as previously described for the resting protocol. After baseline blood sampling, volunteers completed 60 min of upright cycling at 60% ± 5% VO$_{2peak}$. Intensity was verified at three time points during exercise using an open-circuit respiratory system. Immediately after exercise, volunteers moved to a hospital bed and reclined, and the initial muscle biopsy (0 hr) was performed. Subsequent muscle-biopsy and blood-sampling procedures were performed as previously described for the resting protocol, ending with a muscle biopsy and blood sample taken 3 hr after the first muscle biopsy.

**Plasma Substrate and Hormone Analysis.** Amino acid concentrations were determined in duplicate from lithium-heparin-processed plasma samples using high-performance liquid chromatography and o-Phthalaldehyde postcolumn derivatization (Agilent 1100 Series HPLC, Agilent Technologies, Foster City, CA). Glucose, insulin, and cortisol concentrations were determined in duplicate from lithium-heparin-processed plasma samples using an advanced automated immunoassay instrument (Immulite 2000, Siemens Healthcare Diagnostic, Deerfield, IL).

**Multiplex Analysis of Muscle Samples.** Multiplexing was used to quantify phosphorylation of multiple proteins specific to the insulin–IGF-PI3K molecular-signaling pathway according to methods previously employed by McClung, Tarr, Barnes, Scrimgeour, and Young (2007). Briefly, muscle samples were homogenized, sonicated, and then centrifuged at 6,000 rpm for 4 min. Supernatants were analyzed for protein using the DC protein assay (Bio-Rad Laboratories, Hercules, CA). Samples were run in triplicate on a 96-well filter plate; filter plates were washed, and antibody-coupled beads for Akt (Ser 473), ERK 1/2 (Thr 202/Tyr 204), GSK-3α/β (Ser 21/Ser 9), IRS-1 (Tyr 989), p38 MAPK (Thr 180/Tyr 182), p90RSK (Ser 380), and p70S6K (Thr 421/Ser 424) were added to each well. We measured p70S6K (Ser 424/Thr 421) phosphorylation, because this site, not p70S6K (Thr 389), has been shown recently to be responsive to endurance exercise (Mascher, Andersson, Nilsson, Ekblom, & Blomstrand, 2007). Samples were added to each well and incubated overnight at room temperature. The following morning, plates were vacuum filtered and washed, and detection antibodies (Bio-Rad Laboratories) were added. After an incubation period and a series of washes, streptavidin-PE was added to each well and plates were incubated. Finally, beads were resuspended in buffer and the plate was read on a Luminex 100 (Luminex Co., Austin, TX), which detected fluorescence at the appropriate wavelength.

Raw data were processed using Bio-Plex Manager software (Version 5.0, Bio-Rad Laboratories), which provided phosphorylation states for each protein, with mean and standard deviation for each sample run in triplicate. Protein-phosphorylation data were then exported for statistical analysis and presented as fold change compared with resting at 0 hr.

**Western Blotting.** Original stock lysates prepared for multiplexing were also used for Western blotting. Briefly, samples were prepared using a 1:20 dilution of 2-mercaptoethanol and Laemmli sample buffer and stored at –80 °C before sodium dodecyl sulfate polyacrylamide gel electrophoresis. For determination of the relative abundance of proteins implicated in translation initiation and protein degradation and their phosphorylation states, equal amounts of protein from the tissue homogenates were subjected to the electrophoresis using precast Tris-HCl gels (Bio-Rad Laboratories). Proteins were then transferred to polyvinylidene fluoride membranes followed by exposure to the appropriate phosphospecific primary antibodies. Corresponding total proteins were also immunoblotted to confirm equal loading of gels. Phosphospecific and total protein-specific primary antibodies for Akt (Ser 473), 4E-BP1 (Thr 37/46), p70S6K (Ser 424/Thr 421), rpS6 (Ser 235/236), and ERK 1/2 (Thr 202/Tyr 204) were produced in rabbits and purchased commercially (Cell Signaling Technology Inc., Danvers, MA). Labeling was performed using
antirabbit immunoglobulin G conjugates with horseradish peroxidase (Bio-Rad Laboratories). Signals were detected using chemiluminescence (SuperSignal, West Pico Kit, Pierce Biotechnology, Rockford, IL) followed by exposure on imaging film (Kodak, Rochester, NY). Bands were quantified using the ChemiGenius® Bioimaging system (Syngene, Frederick, MD). Data are expressed as fold change compared with resting at 0 hr. Total protein expression did not differ between conditions (resting vs. postexercise) or times (data not shown).

**Quantitative Real-Time Polymerase Chain Reaction.** Total RNA was isolated from ~10-mg muscle samples that were homogenized and purified according to manufacturer’s instructions using TRI reagent (Sigma, St. Louis, MO). RNA quantity and 260:280 ratios were measured on a Nanodrop ND-1000 spectrophotometer (Nanodrop, Wilmington, DE). Equal amounts of total RNA were then synthesized into cDNA (Fermentas, Hanover, MD). ABgene Absolute qPCR SYBR Green Master Mix (ABgene, Surrey, UK) with ROX dye was used for all quantitative real-time polymerase chain-reaction protocols. Forward and reverse quantitative real-time polymerase chain-reaction primers for transcripts of interest were designed using NCBI gene sequences and the Primer Design platform provided by Integrated DNA Technologies (Coralville, IA). NCBI BLAST searches were performed on primer sequences to ensure specificity. The constitutively expressed gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a reference standard. Amplification reactions were performed on a Stratagene MX3000P (Agilent Technologies, La Jolla, CA) according to the manufacturer’s instructions: 15 min at 95 °C for enzyme activation, followed by 40 cycles of 30 s at 95 °C (denature), 1 min at 60 °C (anneal), and 1 min at 72 °C (extension; 40 cycles) with the designed primers (Table 1). A melting curve was performed at the end of each reaction to detect possible multiple polymerase chain-reaction products including primer dimers. The differences in mRNA expression between resting and postexercise time points were determined by the relative quantification method using the threshold cycle method and real-time polymerase chain-reaction efficiencies of the target gene normalized to the housekeeping gene GAPDH. Expression of GAPDH was not affected by resting or the postexercise protocol (data not shown).

**Statistical Analyses**

Baseline volunteer characteristics were described using common descriptive statistics. This study was a repeated-measure design with two within-participant factors (condition resting vs. postexercise, time 0 hr vs. 3 hr). A repeated-measures analysis of variance was used to evaluate the effects of moderate endurance exercise on the molecular regulation of protein turnover and remaining criterion measures. The alpha level for statistical significance was set at $p < .05$ and adjusted accordingly for multiple comparisons using a Bonferroni’s correction. All data were analyzed using SPSS (version 15.0, 2006, SPSS Inc., Chicago, IL).

**Results**

**Baseline Characteristics**

Baseline participant characteristics are provided in Table 2. Body weight was maintained throughout the 13-day intervention, and dietary carbohydrate and protein intake remained constant at the prescribed 55–65% of total energy intake and 1.2 g/kg body weight, respectively.

**Plasma Substrate and Hormone Analysis**

Overall, glucose concentrations were lower ($p < .05$) postexercise (88 ± 2 mg/dl) than resting (91 ± 1.5 mg/dl). Specifically, glucose concentrations were approximately 18% lower ($p < .05$) at 0 hr postexercise compared with resting (Table 3). Regardless of condition, glucose concentrations decreased ($p < .05$) over time. Total amino acid and nonessential amino acid concentrations were

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>MuRF</td>
<td>5′-TGA GCC AGA AGT TTG ACA CG-3′</td>
<td>5′-TGA TGA GTT GCT TGG CAG TC-3′</td>
</tr>
<tr>
<td>Atrogin</td>
<td>5′-GAA CAT CAT GCA GAG GCT GA-3′</td>
<td>5′-GCC AGA GTT TCT TCC ACA GC-3′</td>
</tr>
<tr>
<td>E3α</td>
<td>5′-ACT GCC TCC TGA ACT CCA GA-3′</td>
<td>5′-ACG ACC TTC TTT GTC AAT GG-3′</td>
</tr>
<tr>
<td>FOXO1</td>
<td>5′-GCA TTC ATG GAC AAC AGG-3′</td>
<td>5′-CAT CCC CTC CAA CAT-3′</td>
</tr>
<tr>
<td>FOXO3</td>
<td>5′-GCA AGC ACA GAG TTG GAT GA-3′</td>
<td>5′-CAG GTC GTC CAT GAG GTT TT-3′</td>
</tr>
<tr>
<td>FOXO4</td>
<td>5′-CCG TGA AGC AGA CTA ATG-3′</td>
<td>5′-ACC TCA GAC TGT GGC CAC AA-3′</td>
</tr>
<tr>
<td>PSMA1</td>
<td>5′-GGC TCA GGA GTT TTT GGA TT-3′</td>
<td>5′-GAC ATG GCT CTG CAC TCA AA-3′</td>
</tr>
<tr>
<td>Myostatin</td>
<td>5′-TTG GTG GAT GAC TCT CTC-3′</td>
<td>5′-CAT TTG GGT TTT CCA TCC AC-3′</td>
</tr>
<tr>
<td>GAPDH</td>
<td>5′-AT TGC CCT CAA CGA CCA CTT TGT-3′</td>
<td>5′-TCT CTC TCT TCC TGT GCT CTT GC-3′</td>
</tr>
</tbody>
</table>

*Note:* MuRF = muscle ring finger; atrogin = muscle-specific F-box protein; E3α = ubiquitin ligase; FOXO1 = forkhead box 01; FOXO3 = forkhead box 03; FOXO4 = forkhead box 04; PSMA1 = proteasome subunit 1; GAPDH = glyceraldehydes-3-phosphate dehydrogenase. Myostatin was formerly known as growth-differentiation factor 8.
lower at 3 hr and did not differ for condition (resting vs. postexercise). Essential amino acid and branched-chain amino acid concentrations were not influenced by moderate endurance exercise and remained stable over time. Plasma insulin concentrations tended to be lower over time ($p = .06$), but they were not different between conditions (resting vs. postexercise). Cortisol was not influenced by the exercise bout and remained stable over time. There were no interaction effects (condition–time) for plasma substrates or hormone concentrations.

**Multiplex Analysis of Muscle Samples**

No differences ($p > .05$) between postexercise and resting phosphorylation were noted for any protein after a 60-min, moderate endurance-exercise session (Table 4). However, a significant interaction (condition–time; $p = .05$) was noted for Akt (Ser 473) phosphorylation, as phosphorylation levels decreased over time for resting but were elevated immediately postexercise and decreased over time. Akt (Ser 473) phosphorylation was approximately 51% lower and ERK 1/2 (Thr 202/Tyr 204) phosphorylation was nearly 157% greater at 3 hr than at 0 hr ($p < .05$). GSK-3 $\alpha/\beta$ (Ser 21/Ser 9) phosphorylation,

### Table 2 Baseline Participant Characteristics, N = 10

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>M ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>23 ± 1</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>178 ± 1</td>
</tr>
<tr>
<td>Body mass (kg)</td>
<td>80 ± 2</td>
</tr>
<tr>
<td>Body fat (%) measured by dual-energy X-ray absorptionmetry</td>
<td>19 ± 1</td>
</tr>
<tr>
<td>VO$_2$peak (ml·kg$^{-1}$·min$^{-1}$) measured by an open-circuit indirect respiratory system</td>
<td>45 ± 1</td>
</tr>
</tbody>
</table>

### Table 3 The Effect of a 60-min, Moderate Endurance-Exercise Bout on Analyte Concentrations, M ± SE, N = 10

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Baseline</th>
<th>0 hr</th>
<th>3 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Rest</td>
<td>Postex</td>
<td>Rest</td>
</tr>
<tr>
<td>Glucose, mg/dl$^a$</td>
<td>94 ± 2</td>
<td>93 ± 1.6</td>
<td>91 ± 1.6</td>
</tr>
<tr>
<td>TAA, μmol/L$^b$</td>
<td>2,451 ± 50</td>
<td>2,459 ± 53</td>
<td>2,423 ± 106</td>
</tr>
<tr>
<td>NEAA, μmol/L$^b$</td>
<td>1,651 ± 40</td>
<td>1,651 ± 33</td>
<td>1,628 ± 71</td>
</tr>
<tr>
<td>EAA, μmol/L</td>
<td>800 ± 19</td>
<td>808 ± 24</td>
<td>795 ± 21</td>
</tr>
<tr>
<td>BCAA, μmol/L</td>
<td>396 ± 16</td>
<td>391 ± 18</td>
<td>380 ± 17</td>
</tr>
<tr>
<td>Insulin, μIU/ml</td>
<td>4.9 ± 1</td>
<td>4.4 ± 1</td>
<td>3.2 ± 1</td>
</tr>
<tr>
<td>Cortisol, nmol/L</td>
<td>261 ± 31</td>
<td>308 ± 25</td>
<td>256 ± 29</td>
</tr>
</tbody>
</table>

**Note.** Postex = postexercise; TAA = total amino acids; NEAA = nonessential amino acids; EAA = essential amino acids; BCAA = branched-chain amino acids; rest = Study Day 8; postex = Study Day 13.

$^a$Significant overall condition main effect (rest vs. postex) with $p < .05$. Differences detected between 0 hr postex and 0 hr rest with $p < .05$.

$^b$Significant overall (rest and postex) time main effect detected at 3 hr compared with 0 hr and baseline, $p < .05$.

### Table 4 The Effect of a 60-min, Moderate Endurance-Exercise Bout on Phosphorylation Status of Skeletal-Muscle-Specific Molecular-Signaling Proteins Using Multiplex Analysis

<table>
<thead>
<tr>
<th>Phosphoprotein</th>
<th>0 hr</th>
<th>3 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Rest</td>
<td>Postex</td>
</tr>
<tr>
<td>Akt$^a$</td>
<td>1.0 ± 0</td>
<td>1.26 ± 0.13</td>
</tr>
<tr>
<td>ERK 1/2$^b$</td>
<td>1.0 ± 0</td>
<td>1.19 ± 0.58</td>
</tr>
<tr>
<td>GSK-3α/β</td>
<td>1.0 ± 0</td>
<td>0.87 ± 0.08</td>
</tr>
<tr>
<td>IRS-1</td>
<td>1.0 ± 0</td>
<td>0.78 ± 0.24</td>
</tr>
<tr>
<td>p38 MAPK</td>
<td>1.0 ± 0</td>
<td>1.21 ± 0.32</td>
</tr>
<tr>
<td>p70$^{gsk}$</td>
<td>1.0 ± 0</td>
<td>0.84 ± 0.15</td>
</tr>
<tr>
<td>p90$^{rsk}$</td>
<td>1.0 ± 0</td>
<td>0.98 ± 0.05</td>
</tr>
</tbody>
</table>

**Note.** Postex = postexercise; Akt = protein kinase B (Ser 473); ERK 1/2 = extracellular regulated kinase (Thr 202/Tyr 204); GSK-3α/β = glycogen synthase kinase (Ser 21/Ser 9); IRS-1 = insulin receptor substrate (Tyr 989); p38 MAPK = p38 mitogen activated protein kinase (Thr 180/Tyr 182); p70$^{gsk}$ = 70 kDa S6 kinase (Thr 421/Ser 424); p90$^{rsk}$ = 90 kDa ribosomal S6 kinase (Ser 380); rest = Study Day 8; postex = Study Day 13. Values are mean fold change ± SE, N = 10 participants.

$^a$Significant overall (rest and postex) time main effect detected between 0 hr and 3 hr with $p < .05$.

$^b$Significant condition–time interaction with $p < .05$. 

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regardless of condition, tended ($p = .07$) to be lower at 3 hr after the initial muscle biopsy (0 hr).

**Western-Blot Analysis**

Akt (Ser 473) and 4E-BP1 (Thr 37/46) phosphorylation were not influenced by either exercise or time, and no interactions (condition–time) were noted. Time affected p70S6K (Thr 421/Ser 424) phosphorylation; levels were 110% greater ($p < .05$) at 3 hr than at 0 hr (Figure 1). No condition or condition–time interaction effects were noted for p70S6K (Thr 421/Ser 424) phosphorylation. However, phosphorylation appeared ($p = .07$) to be greater postexercise than at rest. Phosphorylation of rpS6 (Ser 235/236) was not different between rest and postexercise, with no observed time or interaction (condition–time) effects. ERK 1/2 (Thr 202/Tyr 204) phosphorylation was not influenced by moderate endurance exercise (resting vs. postexercise), and no interactions (condition–time) were noted. However, similar to multiplexing, ERK 1/2 phosphorylation tended ($p = .057$) to increase over time (0 hr vs. 3 hr).

**Quantitative Real-Time Polymerase Chain Reaction**

Moderate endurance exercise increased the expression of MuRF mRNA 4.7- and 5.7-fold 0 hr and 3 hr postexercise, respectively, compared with the respective 0-hr and 3-hr resting time points (Figure 2; $p < .001$). Similarly, atrogin expression increased as a result of moderate endurance exercise ($p < .001$). Post hoc analysis identified a 3.2-fold increase in atrogin expression ($p < .01$) 3 hr postexercise compared with rest at the same time point (3 hr).

The expression of E3 Alpha, FOXO1, FOXO3, FOXO4, PSMA-1, and myostatin was not influenced by moderate endurance exercise or different when measured over time during resting and postexercise protocols.

**Discussion**

Recent studies have shown that endurance exercise affects molecular-signaling pathways contributing to the cellular regulation of MPS (Benziane et al., 2008; Sakamoto et al., 2004). In the current study, moderate endurance exercise failed to produce significant alterations in the phosphorylation state of key protein synthetic intracellular signaling proteins. More specifically, Akt, 4E-BP1, p70S6K, and rpS6 phosphorylation were not influenced solely by moderate endurance exercise. Our findings are in agreement with some (Mascher et al., 2007; Wojtaszewski, Nielsen, Kiens, & Richter, 2001) but not all reports (Coffey, Zhong, et al., 2006; Sakamoto et al., 2004; Wilson, Hargreaves, & Howlett, 2006). Similar to our findings, cycling exercise (60 min at 50% VO$_{2\text{max}}$ and 90 min at 75% VO$_{2\text{max}}$) failed to elicit changes in Akt (Ser 473) phosphorylation in the absence of nutrition (Wojtaszewski et al., 2001). Mascher et al. (2007) also showed no increase in fasted Akt phosphorylation immediately and 3 hr after a 60-min cycling bout at 75% VO$_{2\text{max}}$. In that same study, exercise stimulated an increase in p70S6K (Ser 424/Thr 421) phosphorylation in early recovery, but phosphorylation returned to basal levels by 3 hr, with no concomitant effect on p70S6K (Thr 389) phosphorylation. We found that p70S6K (Ser 424/
Thr 421) phosphorylation tended to be greater in both experimental conditions, with no subsequent downstream effect on rpS6 (Ser 235/236) phosphorylation, indicating that p70s6k was not influenced solely by moderate exercise (Pearson et al., 1995). Other studies support the lack of an exercise-induced effect on p70s6k and rpS6 phosphorylation noted in the current study (Coffey, Zhong, et al., 2006). The rationale for our observation of increased p70s6k phosphorylation over time in both the resting and exercise conditions may be explained by the biopsy technique we used. Although the angle of the biopsy needle was altered with each subsequent biopsy, the same biopsy site was accessed. This likely caused an inflammatory response, which is supported by our observed increase in ERK 1/2 over time in both the resting and exercise conditions. ERK 1/2 is directly upstream from p70s6k, and ERK 1/2 controls protein kinase C-mediated p70s6k activation on the Ser 424/Thr421 sites (Boppart, Burkin, & Kaufman, 2006; Duchene et al., 2008).

Insulin and amino acid availability are potent regulators of MPS and associated growth-related cell-signaling processes (Bohe, Low, Wolfe, & Rennie, 2003; Fujita et al., 2007). In the current study, total amino acid and nonessential amino acid concentrations were reduced and insulin levels appeared to be lower as a consequence of reduced glucose concentrations after exercise. Studies have shown that nutrient provision (i.e., protein or protein + carbohydrate) during recovery from endurance exercise enhances substrate availability, amino acids in particular, resulting in marked increases in MPS and associated protein synthetic intracellular signaling (Howarth, Moreau, Phillips, & Gibala, 2009; Kammer et al., 2009). In light of these recent findings, the failure to elicit major changes in MPS-associated molecular events, considering the observed substrate and endocrine environment, is not surprising given that measurements were made in the fasted state (Wojtaszewski et al., 2001), an effect that would likely be reversed by nutrient provision.

Exercise intensity, as well as training state, may have contributed to the observed molecular protein metabolic response to endurance exercise. More specifically, phosphorylation MAPK intracellular signaling proteins, specifically ERK 1/2, p38 MAPK, and p90s6k, are up-regulated in response to endurance exercise (Aronson et al., 1997; Yu, Blomstrand, Chibal, Krook, & Zierath, 2001) in an intensity-dependent manner (Widegren, Wretman, Lionikas, Hedin, & Henriksson, 2000; Yu et al., 2003). Our study failed to show an exercise-induced change in the phosphorylation state of ERK 1/2, p38 MAPK, or p90s6k immediately and 3 hr after the 60-min, moderate-intensity endurance-exercise bout compared with the resting state. These findings are not surprising given that we found no change in rpS6 phosphorylation (Benziane et al., 2008). The exercise intensity used in the current study may have been too low to elicit major changes in MAPK signaling in this group of healthy, physically active men. Yu et al. (2003) reported that intense, repeated short-term cycling exercise bouts elicited lower MAPK-signaling responses in endurance-trained than in untrained adults.

Those authors suggested that endurance-exercise training induces an adaptation in MAPK signaling, in which a greater stimulus is required to activate signaling transduction through this pathway in trained individuals than in less fit volunteers. Although our volunteers were not trained cyclists per se, they performed routine aerobic exercise, which may have conferred a metabolic training effect that requires a greater intensity-dependent stimulus to affect protein synthetic intracellular signaling, as suggested by Yu et al. (2003).

Skeletal-muscle protein breakdown, a process tightly regulated through the UPP, was modestly affected by endurance exercise. Our findings indicate that the expression of two skeletal-muscle-specific E3 ligases, MuRF and atrogin, was up-regulated immediately after exercise, an effect that persisted 3 hr into recovery. Similar changes in proteolytic gene expression were reported by others after a single session of endurance exercise (Louis et al., 2007), a response that does not appear to be mediated by training history (Coffey, Shield, et al., 2006). Louis et al. found that after a 30-min run at 75% VO2max gene expression was increased 6.3-fold for MuRF and 1.6-fold for atrogin compared with rest when measured over the first 4 hr of recovery, a time period comparable to that of the current study. The noted up-regulation in proteolytic gene expression may in part be explained by the apparent decrease in insulin concentrations after exercise in the fasted state. Insulin has been shown to be a potent regulator of muscle proteolysis through the insulin–IGF–PI3K cell-signaling pathway by affecting the activity and expression of key proteolytic enzymes (Kandarian & Jackman, 2006). Our findings are consistent with the literature and suggest that moderate endurance exercise performed in a fasted state modifies UPP-specific gene expression. Therefore, it is likely that nutrient provision would not only enhance postexercise protein utilization by stimulating MPS-specific intracellular signaling but also serve to attenuate the observed proteolytic effect through the same insulin-dependent pathway.

The strengths of this study include the use of multiple molecular techniques to characterize the effects of moderate endurance exercise on cellular markers of MPS and MPB. To the best of our knowledge these techniques have not previously been used concurrently to study the molecular responses to endurance exercise. Other strengths include the tightly controlled repeated-measures design. The limitations of this study include the lack of stable-isotope data to quantify the effects of moderate endurance exercise on SMPTO. This limits our ability to compare our results with those in the literature and extend our findings to direct measures of skeletal-muscle protein turnover in the context of recovery from endurance exercise (Harber et al., 2009; Tipton, Ferrando, Williams, & Wolfe, 1996). Furthermore, the conditions under which the measurements occurred (i.e., fasting) and the moderate exercise intensity used in the experimental protocol resulted in only modest changes, especially in the phosphorylation of signaling proteins associated with translation initiation. Thus, future investigations
that account for these limitations would provide useful information necessary to promote the maintenance of skeletal-muscle integrity during recovery from endurance exercise.

In summary, this investigation demonstrated that moderate endurance exercise, performed while fasted by moderately trained adult men, enhanced MuRF and atrogin gene expression and produced modest changes in MPS-associated molecular-signaling proteins over the first 3 hr of recovery. These findings are consistent with others and suggest that the skeletal-muscle protein molecular response to endurance exercise is mediated by nutritional state, exercise intensity, and training state. Although recent studies have begun to characterize the growth- and atrophy-related molecular response to endurance exercise, there are still discrepancies. Therefore, future investigations combining molecular methodologies with direct assessment of protein turnover will serve to better define the effect of various nutritional and exercise interventions on the complex regulatory mechanisms associated with skeletal-muscle protein turnover.

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