Influence of divergent exercise contraction mode and whey protein supplementation on atrogin-1, MuRF1, and FOXO1/3A in human skeletal muscle

Renae J. Stefanetti,1 Séverine Lamon,1 Stine K. Rahbek,1 Jean Farup,2 Evelyn Zacharewicz,1 Marita A. Wallace,1 Mikkel H. Vendelbo,3 Aaron P. Russell,1 and Kristian Vissing2

1Centre for Physical Activity and Nutrition, School of Exercise and Nutrition Sciences, Deakin University, Burwood, Australia; 2Section of Sport Science, Department of Public Health, Aarhus University, Aarhus, Denmark; and 3Department of Internal Medicine and Endocrinology, Aarhus University Hospital, Aarhus, Denmark

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Stefanetti RJ, Lamon S, Rahbek SK, Farup J, Zacharewicz E, Wallace MA, Vendelbo MH, Russell AP, Vissing K. Influence of divergent exercise contraction mode and whey protein supplementation on atrogin-1, MuRF1, and FOXO1/3A in human skeletal muscle. J Appl Physiol 116: 1491–1502, 2014. First published January 23, 2014; doi:10.1152/japplphysiol.00136.2013.—Knowledge from human exercise studies on regulators of muscle atrophy is lacking, but it is important to understand the underlying mechanisms influencing skeletal muscle protein turnover and net protein gain. This study examined the regulation of muscle atrophy–related factors, including atrogin-1 and MuRF1, their upstream transcription factors FOXO1 and FOXO3A and the atrogin-1 substrate eIF3-f, in response to unilateral isolated eccentric (ECC) vs. concentric (CONC) exercise and training. Exercise was performed with whey protein hydrolysate (WPH) or isocaloric carbohydrate (CHO) supplementation. Twenty-four subjects were divided into WPH and CHO groups and completed both single-bout exercise and 12 wk of training. Single-bout ECC exercise decreased atrogin-1 and FOXO3A mRNA compared with basal and CONC exercise, while MuRF1 mRNA was upregulated compared with basal. ECC exercise downregulated FOXO1 and phospho-FOXO1 protein compared with basal, and phospho-FOXO3A was downregulated compared with CONC. CONC single-bout exercise mediated a greater increase in MuRF1 mRNA and increased FOXO1 mRNA compared with basal and ECC. CONC exercise downregulated FOXO1, FOXO3A, and eIF3-f protein compared with basal. Following training, an increase in basal phospho-FOXO1 was observed. While WPH supplementation with ECC and CONC training further increased muscle hypertrophy, it did not have an additional effect on mRNA or protein levels of the targets measured. In conclusion, atrogin-1, FOXO3A, and eIF3-f mRNA, and protein levels, are differentially regulated by exercise contraction mode but not WPH supplementation combined with hypertrophy-inducing training. This highlights the complexity in understanding the differing roles these factors play in healthy muscle adaptation to exercise. Strength training; atrophy signaling; exercise mode; whey protein supplementation

Muscle hypertrophy occurs as a product of the net muscle protein accumulation over time (45, 52) when the rate of muscle protein synthesis (MPS) is greater than muscle protein breakdown (MPB). A single-bout of resistance exercise (RE) performed in the fasted state increases MPS and to a smaller degree MPB (3, 4, 46). Ingestion of essential amino acids (EAA) combined with RE synergistically increases MPS (3, 4, 10, 46, 47, 50, 58) and inhibits (3, 10, 58) or downregulates MPB (3, 26), resulting in an enhanced net muscle protein accumulation (37, 46, 58). Activation of MPS is primarily mediated through mammalian target of rapamycin (mTOR) signaling (6, 7, 18, 19, 25, 30, 48); however, the regulation of the MPB pathways in response to anabolic stimulation has been less well defined. Atrogin-1 [also known as muscle atrophy F-box (MAFbx)] and muscle RING-finger-1 (MuRF1) are muscle-specific E3-ubiquitin ligases (5, 27) and transcriptional targets of the Forkhead box O (FOXO) family members, FOXO1 and FOXO3A (12, 55, 56). Their ablation in mice protects against muscle wasting (5, 27), suggesting that they may be primary mediators of MPB and muscle atrophy in disease conditions. Atrogin-1 and MuRF1 are elevated in numerous models of rodent and human muscle atrophy [reviewed in (23)]; however, their levels remain unchanged in some human wasting conditions (40, 53). Similarly, atrogin-1 and MuRF1 mRNA levels are elevated following muscle hypertrophy-inducing RE training (39). Known substrate targets of atrogin-1 include proteins involved in muscle protein synthesis and regeneration such as elongation initiation factor 3 subunit 5 (eIF3-f) (38), MyoD (57), and myogenin (33). MuRF1 targets proteins involved in muscle structure and contraction such as myosin heavy chain (MHC) (14, 16, 22) as well as several proteins involved in glycolysis and glycogen metabolism (29, 35, 36). Atrogin-1 and MuRF1 regulate distinct components involved in skeletal muscle function under basal, catabolic, and anabolic conditions. This highlights their complex and important role in skeletal muscle health.

Atrogin-1 and MuRF1 levels are differentially regulated by exercise contraction mode and amino acid availability. Following single-bout RE, comprising both eccentric (ECC) and concentric (CONC) contraction modes, atrogin-1 mRNA is downregulated 3–12 h postexercise (15, 41, 42), while MuRF1 mRNA is upregulated 1–4 h postexercise (26, 41, 42, 61). Isolated ECC RE decreases atrogin-1, while isolated CONC RE increases MuRF1 (34, 44). This supports the notion that they react to different exercise-induced stressors and target different substrates. The effect of essential amino acid supplementation combined with RE on the regulation of atrogin-1 and MuRF1 remains equivocal with reports showing either a decrease in atrogin-1 mRNA only (9) or no change in either target (17, 31, 51). Atrogin-1, MuRF1, as well as FOXO1 and FOXO3A, protein levels have not been thoroughly investigated...
following contraction mode-specific RE, but such investigation is required to understand the potential relevance of their mRNA changes.

The present study aimed to conduct a comparative study to investigate the influence of unilateral ECC vs. CONC RE on the mRNA and/or protein levels of atrogin-1, MuRF1, FOXO1, FOXO3A, and eIF3-f in human skeletal muscle. Measurements were made before and after 1) short-term exercise habituation, 2) single-bout RE, and 3) 12 wk of hypertrophy-inducing training. A second aim was to also examine the effect of leucine-enriched whey protein hydrolysate (WPH) supplementation combined with RE on the potential single-bout exercise and training-induced changes in mRNA and protein, compared with a control isocaloric carbohydrate supplement (CHO). We hypothesized that the mRNA and/or protein levels of atrogin-1, MuRF1, FOXO1, FOXO3A, and eIF3-f would 1) be regulated to a greater extent in the exercise-habituated fed state following acute unilateral ECC RE, compared with CONC RE; 2) be attenuated with WPH supplementation following both single-bout unilateral ECC and CONC RE; and 3) be regulated to a greater extent following unilateral ECC hypertrophy-inducing RE training, compared with other combinations of contraction mode and supplementation.

METHODS

Ethical approval. All subjects were informed about the purpose and risks of the study and provided written informed consent in accordance with the Declaration of Helsinki. The study was approved by the local ethical committee of Region Midtjylland (j. no. M-20110003).

Subjects. Twenty-four healthy untrained young men (mean ± SEM height 181.5 ± 1.5 cm; weight 78.1 ± 1.8 kg; age 23.9 ± 0.8 years; fat 16 ± 0.9%) volunteered to participate in the study. Subject criteria to be fulfilled to allow participation in the study comprised 1) no participation in systematic resistance or high-intensity training for lower-extremity muscles 6 mo prior to inclusion in the study, 2) no history of lower-extremity musculoskeletal injuries, and 3) no vegan diet or use of dietary supplements or prescription medicine that would potentially influence muscle size.

Experimental design. The complete study included the completion of a 7-day exercise habituation phase to accustom the subjects to...
unilateral ECC and CONC muscle contraction, a single-bout exercise trial to investigate acute exercise responses, and a 12-wk training study to investigate accumulated exercise responses. Among the 24 subjects completing the habituation and acute trials, 5 subjects dropped out before the training trial was initiated. Another 3 subjects were recruited and completed the training trial with the remaining 19 subjects.

To investigate the acute and accumulated effects of ECC contraction mode vs. CONC contraction mode, the two legs of each subject were randomly assigned to ECC or CONC contraction mode. This within-subject design was used to minimize the potential differences in the training response that are inherent with group designs (e.g., initial training status, habitual nutritional intake, and/or hormonal status). Furthermore, to investigate for acute and accumulated exercise-induced effects of WPH vs. a control isocaloric carbohydrate (CHO) supplement, subjects were randomly divided into two groups of 12 subjects and dietary supplements were provided in a double blinded fashion.

An overview of the pre-single-bout trial procedures and the single-bout trial is shown in Fig. 1A. Three days prior to commencing the study, the subjects were asked to abstain from any physical activity other than their normal daily activities. Thereafter, a basal muscle biopsy was obtained from one randomly chosen leg. Three days following the basal muscle biopsy the subjects commenced a period of exercise habituation. On three occasions during 7 days the subjects completed ECC and CONC exercise on alternate legs using a protocol similar to the single-bout exercise trial. Three days following the completion of the exercise habituation period, muscle biopsies were obtained from both legs after an overnight fast and after at least 30 min of supine rest. These biopsies provided contraction mode-specific habituated basal levels for ECC (Basal ECC) and CONC (Basal CONC) exercise. Three days after these biopsies were taken the subjects completed the single-bout exercise trial (see Fig. 1B). Approximately 7 days after the single-bout trial the subjects commenced a 12-wk training period. Three days after the last exercise session of the training period a final muscle biopsy was obtained from each leg.

Exercise habituation and single-bout exercise protocol. All exercise habituation and the single-bout exercise sessions were conducted using an isokinetic dynamometer (Humac Norm, CSMi Medical Solutions, Stoughton, MA). Exercise consisted of 6 sets of 10 maximal ECC or CONC repetitions at 30 deg s⁻¹ angular velocity with 1 min of recovery between sets. One leg completed all sets of either isolated ECC or CONC exercise. Following this, the alternate leg completed all sets of the alternate mode of contraction; contraction mode was completed in a randomized order. The overall protocol of the single-bout trial is represented in Fig. 1B. Subjects had been instructed to abstain from physical activity in the days prior to the single-bout trial and arrived after an overnight fast at 08:30 h on the day of the single-bout trial. Prior to the exercise session, subjects rested in the supine position for ~30 min. At 09:00 h subjects commenced the exercise session; the same protocol as used for the exercise habituation sessions. Immediately after completion of the exercise session the subjects ingested a WPH supplement or control isocaloric CHO supplement (see below). The subjects continued to fast for another 5 h postexercise (except water ad libitum) and biopsies were obtained from each leg at time points corresponding to 1, 3, and 5 h after exercise. To control for potential effects of circadian rhythm, the absolute daily time points and time resolution of the protocol were strictly adhered to for all subjects.

Supplementation. On the single-bout trial day, immediately after completion of the exercise session, subjects of the WPH group were given a single bolus solution consisting of 0.30 g whey protein + 0.30 g CHO per kilogram lean body mass, while subjects in the CHO supplement group received an isocaloric solution consisting of 0.60 g CHO per kilogram lean body mass. The supplements were diluted in artificially flavored water. The BCAA content of the WPH supplement (produced by Arla Foods Ingredients, Viby J., Denmark) was 27.7% (leucine 14.2%, isoleucine 6.6%, valine 6.9%), which is considered high compared with standard milk-based whey protein sources (32). On all training days, the subjects in both groups received a fixed amount of the respective supplements. Each intake consisted of an 8% solution (equal to 663 kJ), with the WPH drink consisting of 19.5 g whey protein + 19.5 g of carbohydrate and the CHO drink consisting of 39 g of carbohydrate. Half of the solution was ingested just before each training session and the other half was ingested immediately after each training session. The subjects were instructed not to eat or drink anything calorie-containing for 1½ h prior to and during the 1 h immediately after the exercise session.

Training protocol. The subjects completed 33 exercise sessions over a 12-wk training period. Training was aimed to promote muscle hypertrophy and was conducted as a progressive overload RE training program. Exercise training frequency was two to three times per week depending on the progression phase. All exercise sessions commenced with a standardized warm-up consisting of 5 min of light bicycling exercise. The load for the ECC leg was aimed at 120% of CONC loading with a training supervisor assisting to allow isolated exercise modality of the two legs. This corresponded to the approximate strength difference between slow ECC and CONC contractions during isokinetic strength testing (1). Both the ECC and CONC leg training programs consisted of isotonic knee extensions [repetition loading equal to repetition maximum (RM)] with the following set × repetitions; 6 × 10–15 RM (sessions 1–4), 8 × 10–15 RM (sessions 5–10), 10 × 10–15 RM (sessions 11–20), 12 × 6–10 RM (sessions 21–28), and 8 × 6–10 RM (sessions 29–33) (Technogym-Selection line, Technogym, Italy). Subjects were instructed to perform each repetition in a controlled manner (2-s tempo) during both the CONC and the ECC phase of the exercise with 2 min of recovery interspersed between sets. Training was supervised by qualified instructors to ensure proper execution and loading.

Preparation of muscle biopsies. Muscle biopsies were obtained from the middle section of the m. vastus lateralis muscle using the Bergström needle technique, with at least 3 cm between incision sites of each biopsy and care taken to reach the identical sampling depth between biopsies. The muscle samples were quickly dissected free of visible fat and connective tissue, weighed and divided into smaller parts for protein and RNA extraction, and stored at ~80°C until further investigation.

Western blot analysis. Frozen muscle biopsies (~30 mg) were minced between nitrogen cooled pistons and homogenized in an ice-cold buffer containing 20 mM Tris-HCl, 50 mM NaCl, 250 mM Table 1. Primer and probe sequences used for PCR analysis

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Sequences</th>
<th>Reverse Sequences</th>
<th>Probe Sequences</th>
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<tbody>
<tr>
<td>Atrogin-1</td>
<td>GCAGCTGGAACAAACATTTGAGATGAC</td>
<td>TGAGCCCTGTGATGATGGTGA</td>
<td>(BHQ-1)</td>
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<tr>
<td>MuRF1</td>
<td>CCTGAAGACCTTGAGCTTCGCTC</td>
<td>GTTGATGCTTCTGCTCCTGCTC</td>
<td>(Texas Red)</td>
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<tr>
<td>FOXO1</td>
<td>AAGAGGCGCGCTCTTCTAAA</td>
<td>CTGTGGTGGCTGCACTAGTGC</td>
<td>(BHQ-2)</td>
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<td>FOXO3A</td>
<td>CTTCAAGGATAGGCGGCACA</td>
<td>TCTGGCAGCACTGTACATATG</td>
<td>(BHQ-3)</td>
</tr>
<tr>
<td>RPLP0</td>
<td>TCTGACCACCCCTCTATT</td>
<td>CGACGACGAGAGACCTGGAGAAATT</td>
<td>(BHQ-4)</td>
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</tbody>
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Atrogin-1, muscle atrophy F-box; MuRF1, muscle ring finger-1; FOXO1, forkhead transcription factor-1; FOXO3A, forkhead transcription factor-3; RPLP0, ribosomal protein, large, PO.
Proteins from whole tissue lysates were separated by SDS-polyacrylamide gel (PAGE) in a buffer containing 12 mM Tris-HCl (pH 8.8), 200 mM glycine and 0.1% SDS. Proteins were transferred onto an Immobilon-FL PDVF membrane (Millipore, Billerica, MA) in a Bjerrum buffer containing 50 mM Tris, 17 mM glycine, and 10% methanol. Membranes were blocked with 5% BSA in PBS for 1 h at room temperature and were thereafter incubated at 4°C overnight with the following primary antibodies (diluted in 5% BSA in PBS): MuRF1 (MP3401, ECM Biosciences, Versailles, KY) at 1:1000; FOXO1 (C29H4, Cell Signaling Technology, Danvers, MA) at 1:500; FOXO3a (ab17026, Abcam, Cambridge, MA) at 1:500; and eIF3-f (Jomar Bioscience, Adelaide, Australia) at 1:500. Membranes blocked with 5% BSA in TBST for 1 h at room temperature were thereafter incubated at 4°C overnight with the following primary antibodies (diluted in 5% BSA in TBST): phospho-FOXO1 (Ser256, 9461, Cell Signaling Technology, Danvers, MA) at 1:500 and phospho-FOXO3a (Ser253, 9466, Cell Signaling Technology, Danvers, MA) at 1:400. Following overnight primary antibody incubation, membranes were washed with either PBS or TBST (4 × 5 min) and were subsequently incubated for 1 h with the following infrared-fluorescent conjugated secondary antibodies, diluted at 1:400 with the following infrared-fluorescent conjugated secondary antibodies, diluted at 1:5000 in PBS or TBST, containing 50% Odyssey Blocking Buffer (LI-COR Biosciences, Lincoln, NE) and 0.01% SDS: IRDye 800CW goat anti-rabbit IgG (LI-COR Biosciences, Lincoln, NE) for MuRF1, phospho-FOXO1, phospho-FOXO3A, FOXO1, and eIF3-f; IRDye 800CW donkey anti-mouse IgG (LI-COR Biosciences, Lincoln, NE) for FOXO3A; and Alexa Fluor® 680 rabbit anti-mouse IgG (Invitrogen, Carlsbad, CA) for GAPDH. Following another series of washing, proteins were exposed using the Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE), and individual protein band optical densities were quantified using Odyssey Infrared Imaging System software. To control for protein loading, all blots were normalized against GAPDH protein (G8795, Sigma-Aldrich, Sydney, Australia). The specificity of the MuRF1 antibody was established and results are shown in Supplementary Fig. 1.

RNA extraction and real-time PCR. Total RNA (from ~20 mg of muscle) was extracted using the guanidinium thiocyanate-phenol-chloroform extraction method, as previously described (13), and the concentration was determined spectrophotometrically using a Nanodrop 1000 (Thermo Fischer Scientific, Wilmington, DE). One microgram of RNA was reverse transcribed to cDNA using High Capacity RNA-to-cDNA kit (Applied Biosystems, Forster City, CA) according to manufacturer’s protocol. Prior to PCR, 1 μl ribonuclease H (RNase H) (Life Technologies, Mulgrave, VIC, Australia) was added to each sample and incubated at 37°C for 30 min. Real-time PCR was performed using the Stratagene MX3000 PCR system (Agilent Technologies, Santa Clara, CA). All PCR runs were performed in triplicate with reaction volumes of 20 μl, containing Fast SYBR Green Master Mix (Life Technologies), forward and reverse primers, and cDNA template. To compensate for variations in input RNA amounts and efficiency of the reverse transcription, data was normalized to large ribosomal protein PO (RPLPO, 36B4) mRNA. PCR primer sequences are provided in Table 1. PCR conditions for the amplified genes have been published previously (39).

Muscle cross-sectional area. Magnetic resonance imaging (MRI) of thigh muscle was performed with a 1.5-T scanner (Philips Achieva, Best, the Netherlands), as previously described (20). Subjects were seated in a resting position for 30–45 min before entering the scanner and were instructed not to move when lying in the scanner. A minimum of 48–72 h was interspaced between the last training session and MRI scanning to minimize the risk of fluids shift. The MRI scans were performed on both legs using the body coil to enable quantification of muscle cross-sectional area (CSA). The later offline analyses were conducted using a free software program (Osiris, 4.1.1, Osiris Foundation, Geneva, Switzerland). After an initial frontal survey scan, 50 transversal slices were acquired, of which only 3 were used for the present study. The first slice was 70 mm proximal to the distal part of the femur condyles and the other slices were acquired by 10.220.32.246 on April 28, 2017 http://jap.physiology.org/ Downloaded from J Appl Physiol • doi:10.1152/japplphysiol.00136.2013 • www.jappl.org
acquired proximally from this point. AT1-weighted, fast spin echo sequence with the following parameters was used: scan matrix = 576 × 576, field of view = 46 × 46 cm, number of slices = 50, slice thickness = 7 mm, slice gap = 3 mm, repetition time = 2 s, echo train length = 18, number of signal averages = 2, TR = 500 ms, TE = 6.2 ms, and pixel size = 0.8 × 0.8 mm. From the frontal and transverse scans, the femur length (from the femur condyles to the apex of the trochanter) was calculated. Following this, the knee extensor muscle CSA (mm. vastus lateralis, vastus medialis, vastus intermedius, and rectus femoris) was manually outlined at ⅓ of the femur length representing midthigh level. All analyses were conducted by an investigator blinded with regards to intervention (supplementation and contraction mode).

Isometric strength performance. Subsequent to a standardized warm-up consisting of 5 min low-intensity exercise on a stationary ergometer cycle (Monark, Varberg, Sweden), the subjects were seated in an isokinetic dynamometer (Humac Norm, CSMI, Stoughton, MA) with 90° hip flexion and restraining straps crossing the torso and tested leg. Both legs were tested since they were trained differently, and the test order was randomized between the ECC and CONC leg. The transverse axis of the subject’s knee was aligned with the axis of the dynamometer. The test leg was attached to the dynamometer arm while the other leg was placed behind a stabilization bar. The dynamometer was adjusted individually so the contact point between the subjects’ leg and the dynamometer arm was 3 cm proximal to the malleolus medialis.

Maximal voluntary contraction (MVC) was measured at 70 deg knee flexion (0 deg equals full extension). Before starting the test, the subject’s lower leg was weighed to enable gravity correction of the measured torque. The subject was allowed four trials (however, if a subject continued to improve, additional trials were provided), and all contractions were interspaced with 1 min of recovery time. MVC was determined as the highest peak torque from the best of the four trials.

Statistical analyses. Power analysis was calculated for the change in gene and protein expression, as these dependent variables tend to have a lower magnitude of change and higher variability following exercise interventions compared with other dependent variables measured. From our previous experience we expected a variation of about 20–30% of the mean for all groups. With an alpha level set at \( P < 0.05 \) and a required power set at 0.8, we calculated that 12 subjects per group would be required to detect a change of 20–25%.

Data are presented as mean fold change ± SEM. All statistical analyses were performed using IBM SPSS Statistics 20 (SPSS, Chicago, IL). For all statistical tests \( P < 0.05 \) was considered significant. The effect of time (pre vs. post), group (WPH vs. CHO) and contraction mode (ECC vs. CONC) and their interactions on quadriceps CSA.

Fig. 3. Effect of exercise habituation on protein expression. Contraction mode-specific effects (ECC vs. CONC) of exercise habituation (prehabilitation basal vs. posthabituation basal) are shown as fold change ± SEM from basal set to a fixed value of 1 for MuRF1 (A), eIF3-f (B), FOXO1 (C), phospho-FOXO1 (D), FOXO3A (E), and phospho-FOXO3A (F). Different from basal prehabilitation. \(*P < 0.05\), \(**P < 0.01\), \(****P < 0.0001\).
and MVC were assessed using a mixed-effect three-way ANOVA with repeated measures for time.

Posthabituation mRNA and protein levels were normalized to prehabituation basal levels (no supplement intervention during exercise habituation) and were analyzed for effect of contraction mode only using one-way repeated-measures ANOVA.

Post-single-bout mRNA and protein levels were normalized to posthabituation basal levels and analyzed for contraction mode (ECC vs. CONC) × supplement type (WPH vs. CHO) × time using the mixed-effect three-way ANOVA with repeated measures for time. As no effects of dietary supplementation type were observed during the single-bout exercise trial, data were merged by supplementation and the effects of contraction mode × time were assessed using two-way repeated-measures ANOVA.

Posttraining protein levels were normalized to prehabituation basal levels and were shown to be independent of dietary supplementation type. Accordingly, both exercise and training changes were analyzed for effect of contraction mode using a one-way repeated-measures ANOVA.

When a significant effect of contraction mode and/or time was observed during the single-bout exercise trial, data were merged by contraction mode only using one-way repeated-measures ANOVA.

Accordingly, both exercise and training changes were analyzed for effect of single-bout exercise following contraction mode using a one-way repeated-measures ANOVA.

Representative examples of Western blots for all protein targets measured during the single-bout exercise habituation and single-bout exercise are shown in Fig. 4. A summary of gene and protein results following exercise habituation are shown in Table 2.

**Effect of single-bout exercise following exercise habituation.** There were no effects of WPH supplementation on any of the mRNA or protein targets measured during the single-bout exercise trial (data not shown). Following ECC and CONC exercise habituation, the effects of single-bout ECC and CONC exercise was investigated. Compared with basal levels, ECC, but not CONC exercise, decreased atrogin-1 mRNA levels by 52% following CONC exercise only downregulated FOXO3A protein by 20% at 3 and 5 h postexercise (P = 0.0001; Fig. 6A). Both CONC and ECC habituation, the effects of single-bout ECC and CONC exercise was investigated. Compared with basal levels, ECC, but not CONC exercise, decreased atrogin-1 mRNA levels by 52% at 3 and 5 h postexercise (P = 0.0001; Fig. 6A). Both CONC and ECC exercise downregulated the total FOXO1 protein by 39% and 36% at 3 and 5 h postexercise (P = 0.0001; Fig. 5D). No change was observed in MuRF1 mRNA at 3 h (P = 0.0001) and 5 h postexercise (34% at 5 h postexercise (P = 0.0001)). In contrast, following ECC, but not CONC exercise, FOXO3A mRNA was decreased 3 and 5 h postexercise (41% at 5 h postexercise (P = 0.0001)).

No alterations in MuRF1 protein were observed following ECC or CONC exercise (Fig. 6A). Compared with basal, eIF3-f protein level was reduced by 30% at 5 h post-CONC-exercise only (Fig. 6B). Both CONC and ECC exercise downregulated the total FOXO1 protein by 39% and 36% at 3 and 5 h postexercise (P < 0.01; Fig. 6D). CONC exercise only downregulated FOXO3A protein by 20% at 5 h postexercise (P < 0.05; Fig. 6E), although the

<table>
<thead>
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<tr>
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<td>100</td>
</tr>
<tr>
<td>eIF3-f</td>
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<tr>
<td>phospho-FOXO1</td>
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**Effect of exercise habituation.** Compared with basal levels, habituation for isolated ECC and CONC exercise increased atrogin-1 mRNA by 50% and 70%, respectively (P < 0.05; Fig. 2A), while MuRF1 mRNA increased by 40% with CONC only (P < 0.05; Fig. 2B). Additionally, habituation exercise increased FOXO1 mRNA by 28% (P < 0.01) and 41% (P < 0.0001), respectively (Fig. 2C), and FOXO3A mRNA by 42% (P = 0.0001) following ECC and CONC exercise, respectively (P < 0.01; Fig. 2D).

No change was observed in MuRF1 protein levels (Fig. 3A). The protein levels of the atrogin-1 substrate, eIF3-f, increased by 34% following CONC exercise (P < 0.05; Fig. 3B). Total FOXO1 protein levels increased by 30% and 36% at 3 and 5 h postexercise (P < 0.05; Fig. 3C), while phospho-FOXO1 protein levels increased by 28% (P < 0.01) and 41% (P < 0.0001), respectively, following ECC and CONC habituation exercise (Fig. 3D). No change was observed for FOXO3A (Fig. 3E) or phospho-FOXO3A protein (Fig. 3F). Representative examples of Western blots for all protein targets are shown in Fig. 4. A summary of gene and protein results following exercise habituation are shown in Table 2.

**Effect of single-bout exercise following exercise habituation.** There were no effects of WPH supplementation on any of the mRNA or protein targets measured during the single-bout exercise trial (data not shown). Following ECC and CONC exercise habituation, the effects of single-bout ECC and CONC exercise was investigated. Compared with basal levels, ECC, but not CONC exercise, decreased atrogin-1 mRNA levels by 52% at both 3 and 5 h postexercise (P = 0.0001; Fig. 5A). Following both ECC and CONC exercise, MuRF1 mRNA levels were increased at 1 h by 49% (P < 0.001) and 54% (P < 0.01), respectively (Fig. 5B). MuRF1 mRNA expression was further increased at 3 h by 74% (P < 0.001; Fig. 5B) in the CONC group only. CONC, but not ECC exercise, increased FOXO1 mRNA at 3 h (P = 0.01) and 5 h postexercise (34% at 5 h postexercise (P < 0.01)). In contrast, following ECC, but not CONC exercise, FOXO3A mRNA was decreased at 3 and 5 h postexercise (22% at 5 h postexercise (P < 0.0001)).

No alterations in MuRF1 protein were observed following ECC or CONC exercise (Fig. 6A). Compared with basal, eIF3-f protein level was reduced by 30% at 5 h post-CONC-exercise only (Fig. 6B). Both CONC and ECC exercise downregulated the total FOXO1 protein by 39% and 36% at 3 and 5 h postexercise (P < 0.01). In contrast, following ECC, but not CONC exercise, FOXO3A mRNA was decreased 3 and 5 h postexercise (41% at 5 h postexercise (P < 0.0001)).

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response to ECC exercise was similar. A significant difference in phospho-FOXO3A protein was observed between ECC and CONC exercise at 5 h postexercise ($P < 0.01$; Fig. 6F). A summary of gene and protein results following single-bout exercise are shown in Table 2.

**Effect of training without and with whey protein supplementation.** We have previously reported in more detail the effect of contraction mode-specific training and WPH supplementation on total volume load of work (i.e., repetitions $\times$ sets $\times$ load accomplished during training), quadriceps CSA, and MVC in this cohort (20). Briefly, total volume load for the ECC trained leg was $11 \pm 0.8\%$ and $10.3 \pm 0.8\%$ higher than for the CONC-trained leg in the WPH and CHO groups respectively ($P < 0.001$). There was no difference between the groups in total volume load. A group $\times$ time interaction was observed ($P < 0.001$) for quadriceps CSA, whereby WPH supplementation combined with either ECC or CONC training had the greater effect on midlevel quadriceps CSA compared with CHO supplementation ($P < 0.001$). Consequently, atrogin-1, MuRF-1, FOXO1, and FOXO3A mRNA levels increased by 72 $\pm$ 13% ($P < 0.0001$) compared with basal and 62 $\pm$ 13% ($P < 0.001$) compared with CONC training (Fig. 7). No changes were observed on the levels of MuRF1, eIF3-f, FOXO1, FOXO3A, and phospho-FOXO3A protein levels following ECC and CONC training (Fig. 5). A summary of the protein results following RE training are shown in Table 2.

**DISCUSSION**

Atrogin-1 and MuRF1 are involved in MPB and believed to play an important role in muscle wasting in disease conditions (5, 27). Atrogin-1 and MuRF1, and their transcriptional regulators FOXO1 and FOXO3A, are expressed in nondiseased skeletal muscle suggesting they play a role in maintaining muscle homeostasis in healthy muscle. RE is a positive regulator of muscle health and regulates atrogin-1, MuRF1, FOXO1, and FOXO3A mRNA levels, although this appears to depend on the type of exercise and the contraction mode employed. To better understand the exercise-induced regulation of these atrophy targets we employed unilateral isolated ECC or CONC exercise tasks. This permitted us to investigate the effects of contraction mode on atrogin-1, MuRF1, FOXO1, FOXO3A, and eIF3-f following 1) exercise habituation, 2) single-bout exercise, and 3) prolonged training. Furthermore, we investigated the effect of WPH supplementation combined with both ECC and CONC exercise. Atrogin-1, MuRF-1, FOXO1,
FOXO3A, and eIF3-f mRNA and/or protein were sensitive to single-bout RE in the exercise-habituated state, in a contraction mode-dependent manner. An acute downregulation of phospho-FOXO1 and phospho-FOXO3A occurred independently of contraction mode. Prolonged hypertrophy-inducing ECC and CONC exercise training resulted in muscle hypertrophy; a response that was augmented with WPH supplementation. However, atrogin-1, MuRF-1, FOXO1, FOXO3A, and eIF3-f were not sensitive to WPH supplementation.

**Effect of exercise habituation.** Unaccustomed exercise, especially ECC RE, inflicts muscle damage (44, 59). In a non-exercise-habituated muscle it may be difficult to distinguish between the effects of muscle-damaging exercise and exercise-stimulated responses of our mRNA and protein targets. To reduce this potential confounding effect, the subjects completed three exercise sessions over a 7 day period followed by 3 days of recovery, prior to conducting the single-bout protocol. Exercise habituation increased basal level of atrogin-1, FOXO1, FOXO3A mRNA levels, independently of the mode of muscle contraction. MuRF1 mRNA levels and eIF3-f protein levels increased only following CONC exercise habituation. No changes in MuRF1 protein levels were observed. Total and phosphorylated FOXO1 protein increased in parallel with the mRNA levels. In contrast, total and phosphorylated FOXO3A protein levels did not change. This may be explained by insufficient accumulated stimulation or by autoubiquitination that can occur for ubiquitin ligases (54). Accordingly, as we have shown previously (60), short-term exercise habituation is able to regulate the basal level of many target mRNAs and proteins. It supports the need to consider appropriate familiarization procedures for human exercise study designs. This would increase the ability to distinguish between true exercise-induced responses and responses due to other stressors inherent to an exercise study protocol itself.

**Effect of single-bout resistance exercise and whey protein supplementation.** Following the habituation phase, the subjects completed a single-bout of unilateral ECC vs. CONC RE with WPH or CHO supplementation. No effects of WPH supplementation were observed on any of the mRNA and protein targets measured which supports several recent publications.

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Fig. 6. Effect of single-bout exercise on protein expression. Contraction mode-specific effects (ECC vs. CONC) of single-bout exercise (posthabituation basal vs. postexercise recovery time points) are shown as fold change ± SEM from basal set to a fixed value of 1 for MuRF1 (A), eIF3-f (B), FOXO1 (C), phospho-FOXO1 (D), FOXO3A (E), and phospho-FOXO3A (F). Different from basal posthabitation. *P < 0.05, **P < 0.01. Difference between contraction modes, *P < 0.05, **P < 0.01.
### Table 2. Summary of mRNA and protein results after exercise habituation, single-bout exercise, and training

<table>
<thead>
<tr>
<th>Gene</th>
<th>Contraction</th>
<th>Habituation, %</th>
<th>1 h</th>
<th>3 h</th>
<th>5 h</th>
<th>Training, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atrogin-1</td>
<td>ECC</td>
<td>↑ 50 ± 8</td>
<td>--</td>
<td>↓</td>
<td>↓</td>
<td>52 ± 8§</td>
</tr>
<tr>
<td></td>
<td>CONC</td>
<td>↑ 70 ± 9</td>
<td>--</td>
<td>◊</td>
<td>◊</td>
<td>52 ± 8§</td>
</tr>
<tr>
<td>MuRF1</td>
<td>ECC</td>
<td>↑ 40 ± 9</td>
<td>↑</td>
<td>49 ± 13</td>
<td>§</td>
<td>54 ± 15</td>
</tr>
<tr>
<td></td>
<td>CONC</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>↑ 74 ± 14§</td>
</tr>
<tr>
<td>FOXO1</td>
<td>ECC</td>
<td>↑ 39 ± 8</td>
<td>--</td>
<td>◊</td>
<td>◊</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>CONC</td>
<td>↑ 41 ± 8</td>
<td>--</td>
<td>◊</td>
<td>◊</td>
<td>--</td>
</tr>
<tr>
<td>FOXO3A</td>
<td>ECC</td>
<td>↑ 42 ± 13</td>
<td>--</td>
<td>◊</td>
<td>◊</td>
<td>53 ± 12†</td>
</tr>
<tr>
<td></td>
<td>CONC</td>
<td>↑ 48 ± 13</td>
<td>--</td>
<td>◊</td>
<td>◊</td>
<td>40 ± 12†</td>
</tr>
</tbody>
</table>

Protein

| MuRF1      | ECC         | --            | --  | --  | --  | --          |
|            | CONC        | --            | --  | --  | --  | --          |
| elf-3f     | ECC         | --            | --  | --  | --  | --          |
|            | CONC        | --            | --  | --  | --  | --          |
| FOXO1      | ECC         | ↑ 30 ± 14     | --  | --  | --  | 36 ± 11     |
|            | CONC        | ↑ 36 ± 14     | --  | --  | --  | 39 ± 11     |
| FOXO3A     | ECC         | ↑ 28 ± 8      | --  | --  | --  | 19 ± 6*     |
|            | CONC        | ↑ 41 ± 8      | --  | --  | --  | 72 ± 13±    |
| FOXO3A     | ECC         | --            | --  | *   | *   | --          |
|            | CONC        | --            | --  | †   | †   | --          |
| phospho-FOXO1| ECC    | --            | --  | N/A | N/A | --          |
|            | CONC        | --            | --  | N/A | N/A | --          |
| phospho-FOXO3A| ECC | --            | --  | N/A | N/A | --          |
|            | CONC        | --            | --  | N/A | N/A | --          |

Contraction mode-specific effects [eccentric (ECC) vs. concentric (CONC)] of exercise-habituation (prehabitation basal vs. posthabitation basal), single-bout exercise (posthabitation basal vs. postexercise recovery time points) and training (prehabitation basal vs. posttraining basal) are shown as % change (mean ± SE) from basal set to a fixed value of 100% for measured gene and protein targets. Different from specified basal levels, *P < 0.05, †P < 0.01, §§P < 0.001, ‡P < 0.0001. --, no change.

(17, 31, 51). The supplement groups were therefore combined and provided a very large sample size of 24 individual legs per exercise group. Atrogin-1 mRNA decreased with ECC exercise, while MuRF1 mRNA increased with CONC exercise. Interestingly, FOXO1 mRNA was increased following ECC exercise, while FOXO3A was decreased following CONC exercise. Our contraction mode-specific RE changes in atrogin-1, MuRF1, FOXO1, and FOXO3A support previous observations following conventional RE (44, 57). The regulation of atrogin-1 mRNA may be associated with the mechanical stress specifically characteristic of ECC exercise (e.g., stretch-induced damage of sarcomeric protein), whereas the regulation of MuRF1 mRNA may be related to the metabolic stress specifically characteristic of CONC exercise (e.g., increased turnover of metabolic enzymes).

MuRF1 protein levels did not change following single-bout exercise and were not influenced by WPH supplementation. This is in contrast to an observed increase in MuRF1 protein 3 h following conventional RE and an attenuation when combined with BCAA supplementation (9). This discordance in results may be an effect of our subjects completing the exercise habituation phase. Atrogin-1 protein levels were not shown, as we were not convinced of the specificity of the commercially available antibodies. Therefore, we measured the protein levels of an atrogin-1 substrate involved in protein synthesis, translation initiation factor, and elongation initiation factor 3 subunit 5 (eIF3-f) (38). eIF3-f protein levels were not influenced by ECC exercise but decreased with CONC exercise. Decreasing atrogin-1 may release basal suppression on muscle protein synthesis related processes, thereby contributing to a net gain in muscle protein accretion. However, this requires experimental validation. The downregulation of the eIF3-f protein 5 h following CONC exercise appears counterintuitive, considering this exercise stress increases muscle protein synthesis. Decreasing eIF3-f may be an indication that muscle protein synthesis had reached its peak and was beginning to return to basal levels. It is also possible that the atrogin-1/eIF3-f regulatory relationship does not exist in human muscle following single-bout exercise, and it is not within the scope of this study to establish such as possibility.

Following single-bout ECC exercise, phospho-FOXO1 and phospho-FOXO3A levels were decreased. Dephosphorylation of FOXO transcription factors allows their translocation to the nucleus (8) where they regulate MuRF1 and atrogin-1 gene expression in rodents (55). The decrease in atrogin-1 mRNA and associated decrease in phospho-FOXO1 and phospho-FOXO3A during recovery following CONC exercise appears counterintuitive. Considering this exercise stress increases muscle protein synthesis. Decreasing eIF3-f may be an indication that muscle protein synthesis had reached its peak and was beginning to return to basal levels. It is also possible that the atrogin-1/eIF3-f regulatory relationship does not exist in human muscle following single-bout exercise, and it is not within the scope of this study to establish such as possibility.

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FOXO3A levels following ECC exercise does not support this relationship in human muscle following exercise. It is possible that other transcription factors are involved in the exercise-induced regulation of atrogin-1 gene expression. For example, the JunB transcription factor can prevent FOXO3A binding to the atrogin-1 promoter (49). Although speculative, the exercise performed in the present study may have stimulated a JunB inhibition of the phospho-FOXO3A regulation of atrogin-1 gene expression. PGC1α can also inhibit the FOXO transcriptional activation of atrogin-1 (11, 21) and potentially may have played a role during this single-bout exercise trial.

Effect of resistance exercise training and whey protein supplementation. Following the single-bout exercise trials, participants completed 12 wk of unilateral ECC vs. CONC RE training. Additionally, subjects received a WPH supplement or an isocaloric CHO. In line with previous findings, our RE training program resulted in an increase in muscle hypertrophy that was associated with an increase in muscle strength (2, 28). In regards to WPH supplementation, we found that RE training-induced muscle hypertrophy was augmented with WPH supplementation; an observation previously found by some (2, 28) but not others (31). While WPH supplementation augmented muscle hypertrophy, there was no further increase in MVC. A lack of consistency between training-induced changes in hypertrophy and strength has been observed previously (2, 28, 31, 43) and is in part explained by the influence exerted by complex neuromuscular interactions also affecting muscle strength (24).

An increase in basal level phospho-FOXO1 was observed following ECC training only, suggesting a potential reduction in basal active FOXO1 protein posttraining. No ECC or CONC training-induced changes were observed for any of the other protein targets measured. Eight weeks of traditional hypertrophy-inducing RE training has been shown to increase atrogin-1 protein and decrease nuclear FOXO1 protein levels (39). Nuclear fractionation analysis in the present study may have provided us with an opportunity to more precisely evaluate the potential activity of the FOXO proteins.

Limitations. In the present study we investigated the effects of ECC vs. CONC RE training that was matched for equal relative intensity. As elegantly shown by Eliasson et al. (19), the maximal absolute intensity inherent to ECC, when compared with CONC exercise, may be the most important factor initiating intracellular signaling. Increasing the ECC training load and therefore its maximal absolute intensity might have allowed the detection of additional significant changes in some of our intracellular targets. With this in mind, the work by Eliasson et al. investigated Akt/mTOR signaling in the fasted state, while the current study investigated the FOXO, atrogin-1, and MuRF1 targets in the fed state. The sensitivity of dietary conditions, especially toward specific amino acids, on Akt/mTOR signaling may be very different than the sensitivity of the FOXO/atrogin-1/MuRF1 signaling components. Another limitation of this work is the lack of a noncaloric control group. This group was excluded for practical reasons. Finally, it could be argued that our protocol did not account for daily protein intake, an important parameter in any supplementation intervention. During the recovery phase following the single-bout exercise session, the subjects received nothing but the supplement to avoid any confounding effect of additional protein or carbohydrate intake. During the training period, the subjects were instructed to not ingest additional calories for 90 min prior to and during the 60 min following each exercise session, although total daily protein or carbohydrate intake was not controlled. However, previously published data from this study demonstrate that with the WPH supplementation, the subjects developed significantly larger muscle as well as interconnected tendon hypertrophy (20), which support the hypothesis that habitual daily protein intake did not overrule net hypertrophy.

CONCLUSION

This study investigated the effect of isolated ECC and CONC exercise on members of the FOXO/atrogin-1/MuRF1 signaling axis. Exercise was performed following exercise habituation as a single-bout trial or as training and combined with WPH supplementation. Our results suggest the following: 1) exercise habituation prior to investigating the acute responses on exercise-induced FOXO/atrogin-1/MuRF1 regulation should be employed; 2) atrogin-1 and MuRF1 mRNA levels are regulated in a contraction mode-dependent manner; 3) a discordance between patterns of FOXO phosphorylation and atrogin-1/MuRF1 mRNA levels, as well as discordance between acute and chronic responses, suggests other regulatory factors may influence the exercise-induced regulation of atrogin-1; and 4) FOXO1, FOXO3A, atrogin-1, and MuRF1 are not sensitive to WPH supplementation following single-bout exercise performed following habituation and during hypertrophy-inducing exercise.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


REFERENCES


