Proteolytic mRNA expression in response to acute resistance exercise in human single skeletal muscle fibers

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Yang, Yifan, Bozena Jemiolo, and Scott Trappe. Proteolytic mRNA expression in response to acute resistance exercise in human single skeletal muscle fibers. J Appl Physiol 101: 1442–1450, 2006. First published July 13, 2006; doi:10.1152/japplphysiol.00438.2006.—The purpose of this study was to characterize changes in mRNA expression of select proteolytic markers in human slow-twitch [myosin heavy chain (MHC) I] and fast-twitch (MHC IIA) single skeletal muscle fibers following a bout of resistance exercise (RE). Muscle biopsies were obtained from the vastus lateralis of eight young healthy sedentary men [23 ± 2 yr (mean ± SD), 93 ± 17 kg, 183 ± 6 cm] before and 4 and 24 h after 3 × 10 repetitions of bilateral knee extensions at 65% of one repetition maximum. The mRNA levels of TNF-α, calpains 1 and 2, muscle RING (really interesting novel gene) finger-1 (MuRF-1), atrogin-1, caspase-3, B-cell leukemia/lymphoma (Bcl)-2, and Bcl-2-associated X protein (Bax) were quantified using real-time RT-PCR. Generally, MHC I fibers had higher (1.6- to 5.0-fold, P < 0.05) mRNA expression pre- and post-RE. One exception was a higher (1.6- to 3.9-fold, P < 0.05) Bax-to-Bcl-2 mRNA ratio in MHC IIA fibers pre- and post-RE. RE increased (1.4- to 4.8-fold, P < 0.05) MuRF-1 and caspase-3 mRNA levels 4–24 h post-RE in both fiber types, whereas Bax-to-Bcl-2 mRNA ratio increased 2.2-fold (P < 0.05) at 4 h post-RE only in MHC I fibers. These results suggest that MHC I fibers have a greater proteolytic mRNA expression pre- and post-RE compared with MHC IIA fibers. The greatest mRNA induction following RE was in MuRF-1 and caspase-3 in both fiber types. This altered and specific proteolytic mRNA expression among slow- and fast-twitch muscle fibers indicates that the ubiquitin/proteasomal and caspase pathways may play an important role in muscle remodeling with RE.

gene expression; tumor necrosis factor-α; calpain; muscle really interesting novel gene finger-1; atrogin-1; muscle atrophy F-box; caspase-3; Bcl-2-associated X protein; B-cell leukemia/lymphoma-2

HUMAN SKELETAL MUSCLE HAS a high degree of plasticity that can undergo adaptive remodeling with increased or decreased activity. Understanding the regulation of both protein synthesis and degradation is important, since the outcome of these adaptive responses reflects net protein balance (i.e., proteolysis). However, most of the research emphasis has been on protein synthesis (8), with the regulation of proteolysis less understood, especially in humans (8), due to limitations in methodology (3, 45). Although in vitro muscle preparations with specific inhibitors or activators uniquely allow the quantification of the contribution by different proteolytic pathways, in vitro measurements of proteolysis do not necessarily reflect in vivo proteolysis (3). Various techniques, such as stable-isotope tracers and microdialysis, have been developed to better measure in vivo mixed or specific muscle protein degradation (45). While these methods have provided insight into muscle proteolysis in humans, a better understanding of the molecular coordination and regulation of proteolysis is warranted.

Proteolysis is necessary for normal day-to-day function and skeletal muscle remodeling. For a normal 70-kg adult man, ~280–340 g of protein are being turned over each day (26). Following resistance exercise (RE), both mixed-muscle protein synthesis and degradation (31) are increased. This increase in proteolysis following RE is not necessarily detrimental and may in fact be a requirement for the remodeling and regeneration processes following RE. It is well known that chronic RE can induce increases in strength and muscle mass (12). However, not much is known about the proteolytic pathways that contribute to the increase in proteolysis following RE in humans. To take this a step further, understanding muscle fiber-specific regulation is warranted, as slow- and fast-twitch muscle fibers have been shown to adapt differently in terms of metabolic, structural, and contractile properties to the same stimulus, such as RE (12, 42, 44, 47, 49), aging (41), bed rest (1, 43), and spaceflight (1, 11, 40, 46). Even the expression of genes that are in abundance and thought to be relatively stable [housekeeping genes (HKGs)] have been shown to differ among slow- and fast-twitch muscle fibers in response to a bout of run exercise (20). This differential acute response among muscle fiber types may play a critical role in the subsequent adaptations to chronic perturbations. In addition, basal gene expression has been shown to be variable among fiber types (19), which may influence the response and eventual adaptations to an exercise stimulus. To date, little is known about exercise-induced mRNA changes in human single skeletal muscle fibers.

Given the aforementioned reasons, the purpose of this study was to characterize the changes in mRNA expression of select proteolytic markers in human slow-twitch [myosin heavy chain (MHC) I] and fast-twitch (MHC IIA) single skeletal muscle fibers following an acute bout of RE. It is well established that chronic RE can lead to increases in muscle mass and strength (12). However, the molecular mechanisms for RE-induced adaptations, especially the regulation of proteolysis in hypertrophyng skeletal muscle, are less clear in humans (8). It has been suggested that training adaptations in skeletal muscle are the result of cumulative effects of transient changes in gene expression following each acute bout of exercise (12, 28). We have shown that an acute bout of RE was sufficient to elicit transient, but robust, increases in mRNA levels of myogenic and metabolic genes 2–12 h post-RE (51). However, the response of proteolytic genes to RE was not investigated.

The eight select markers of muscle proteolysis based on cell culture and animal and human studies were as follows: tumor
Subjects

MATERIALS AND METHODS

tumor necrosis factor (TNF)-α, calpains 1 and 2, muscle RING (really interesting novel gene) finger-1 (MuRF-1), atrogin-1, cysteine-dependent aspartate protease (caspase)-3, B-cell leukemia/lymphoma (Bcl)-2, and Bcl-2-associated X protein (Bax). In view of the general increase in proteolysis and gene expression in some of the select markers following unaccustomed or strenuous exercise, we hypothesized that the mRNA levels of TNF-α, calpains 1 and 2, MuRF-1, atrogin-1, caspase-3, and mRNA ratio of Bax/Bcl-2 in MHC I and IIa muscle fibers will increase within 4–24 h after an acute bout of RE. We also hypothesized that the MHC I muscle fibers will have greater mRNA levels of the select markers at rest and following exercise to reflect a greater proteolytic activity (14) due to the reliance on MHC I fibers during normal activities of daily living and the order of recruitment from slow to fast fibers during skeletal muscle activation.

MATERIALS AND METHODS

Subjects

Eight young, healthy, nonsmoking, sedentary men volunteered for this study (Table 1). Subjects did not participate in regular, structured physical activities for at least 5 mo before the study, except for occasional light exercise (approximately once per month or less), daily commuting to and from school or work (5–10 min of cycling or walking each way), and normal activities of daily living. All subjects were given oral and written information about the experimental procedures and potential risks before giving their informed consent to participate in this study, which was approved by the Institutional Review Board of Ball State University.

Experimental Design

A schematic overview of the experimental design is shown in Fig. 1. In this study, subjects performed an acute bout of RE. One week before the RE, subjects had their body mass, height, and skinfolds measured for subject profiling. Following which, subjects had their one repetition maximum (1 RM) assessed.

The RE consisted of three sets of 10 repetitions (3 × 10) of bilateral knee extensions at 65% of concentric 1 RM. This exercise protocol was chosen because our laboratory has documented increases in cross-sectional areas of MHC I and IIa fibers in sedentary young men (48), old men (44), and old women (42); strength and power of MHC I and IIa fibers in sedentary old men (44) and women (42); and whole muscle strength in sedentary young (48) and old men and women (42, 48) following 12 wk of resistance training using this exercise protocol (with a small variation in intensity). Recently, our laboratory had demonstrated mRNA increases in several myogenic and metabolic genes with an acute bout of 3 × 10 bilateral knee extensions at 70% of 1 RM (51). The reduced intensity (65 vs. 70%) used in this study was to allow the sedentary subjects in this study (vs. recreationally active) to complete 30 contractions while still providing a strenuous stimulus to the muscle.

A total of three muscle biopsies from the vastus lateralis and three blood samples were taken before (Pre) and 4 and 24 h after the RE. The muscle biopsies were taken from both thighs, alternating with each muscle biopsy, starting with either the left or right leg (randomly assigned).

Activity and food restriction. Subjects were instructed not to engage in any physical activities, except activities of daily living, the 1-RM assessment, and RE bout, at least 48 h before the 1-RM assessment, and thereafter until the end of the last muscle biopsy (24 h postexercise). No alcohol and caffeine consumption were allowed at least 48 h before the 1-RM assessment and the first (Pre) muscle biopsy. Subjects also abstained from food that they do not normally consume at least 24 h before the first muscle biopsy. They fasted with ad libitum water intake at least 10 h before the first muscle biopsy and thereafter until after the last muscle biopsy, except for the two standardized meals provided by our laboratory at 4 and 10–11 h postexercise, respectively. The meals were timed such that the subjects fasted at least 10 h before each muscle biopsy. The total amount of calories (50% carbohydrate, 30% fat, and 20% protein) provided by the two standardized meals was based on each individual estimated energy expenditure for that day, including the RE. The estimated energy expenditure was calculated as the sum of the basal metabolic rate and the energy expenditure for the bout of RE. The basal metabolic rate was calculated using the Harris Benedict equation (18) times an activity factor of 1.2 for sedentary subjects. The energy expenditure for 3 × 10 knee extensions was estimated to be approximately six metabolic equivalents (2, 24). The eucaloric meals were provided to minimize variations in gene expression due to caloric and energy composition (percentage of carbohydrate-fat-protein) influences.

Thirty minutes before each muscle biopsy, subjects rested quietly in a supine position. Subjects remained in the laboratory until after their first standardized meal. Thereafter, they were allowed normal ambulation and returned to the laboratory for the second standardized meal and the 24 h postexercise muscle biopsy, respectively.

Table 1. Subject characteristics

<table>
<thead>
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<th>Mean ± SD</th>
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<tr>
<td>Age, yr</td>
<td>23 ± 2</td>
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<td>Weight, kg</td>
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<tr>
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<tr>
<td>CHO, %</td>
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<tr>
<td>Fat, %</td>
<td>30 ± 1</td>
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<tr>
<td>Protein, %</td>
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1 RM, one repetition maximum; EEE, estimated daily energy expenditure; CHO, carbohydrate.

Activity and food restriction.

Fig. 1. Schematic overview of experimental design. 1 RM, one repetition maximum; RE, resistance exercise (3 × 10 bilateral knee extensions); Pre, pre-RE; MB, muscle biopsies; BD, blood draw; SM, standardized meals (50% carbohydrate, 30% fat, 20% protein).
The 1 RM of bilateral knee extension for each subject was assessed on a Cybex Eagle Knee Extensor (Cybex, New York, NY) 1 wk before the exercise bout. Subjects first warmed up 5–10 min on a cycle ergometer (Met 100, Cybex) at a self-selected pace and performed stretches of the involved muscle groups after the warm up. They were also familiarized with the equipment and procedures before the 1-RM assessment. The mass on the knee extensor device was progressively increased with each successful set until subjects could no longer maintain proper form and/or fully extend at the given mass. The 1 RM was defined as the maximum mass that the subject could lift in one repetition (both concentric and eccentric contractions) during the concentric phase of the bilateral knee extension.

**RE Bout**

To prevent the influence of warm up on the gene expression responses to RE, subjects performed three sets of 10 repetitions of bilateral knee extensions at 65% of 1 RM without warming up. The exercise was performed on the same machine as used for the 1-RM assessment. Two minutes of passive rest between each set were given.

**Muscle Biopsies**

A total of three muscle biopsies (6) were taken from the vastus lateralis of the participants before (Pre) and 4 and 24 h postexercise. The Pre and 4-h muscle biopsies were taken from opposing legs, whereas the 24-h muscle biopsy was taken proximal to the Pre muscle biopsy. Each muscle sample was divided into longitudinal sections and placed in 0.5 ml of RNAlater (Ambion, Austin, TX) and incubated overnight at 4°C and then transferred to −20°C until fiber separation.

**Blood Analyses**

A blood sample (~5 ml) was taken from an antecubital vein in conjunction with each muscle biopsy for a total of three blood samples per subject. Blood samples were analyzed for concentrations of serum total protein (BCA protein assay kit; Pierce, Rockford, IL), glucose (enzymatic assay; Pointe Scientific, Lincoln Park, MI), insulin (ELISA kit; Diagnostic Systems Laboratories, Webster, TX), and creatine kinase (enzymatic assay; Pointe Scientific) using commercially available kits, according to the manufacturers’ procedures.

**Muscle Fiber Separation**

A small muscle bundle from each muscle biopsy was placed in a petri dish filled with RNAlater. On average, 151 individual muscle fibers (~6 mm in length) per subject were separated under a light microscope. Approximately one-third of each isolated muscle fiber was clipped and placed in 40 μl of SDS sample buffer for fiber-type identification by SDS-PAGE. The remaining muscle fiber was placed in a tube containing 0.5 ml of RNA isolation reagent, TRI Reagent, and 2 μl of PolyAcryl Carrier (Molecular Research Center, Cincinnati, OH) and stored at −80°C for RNA extraction after fiber-type identification.

**MHC Isoform Identification**

SDS-PAGE analysis, as detailed elsewhere (49), was used to determine the MHC isoform of each isolated single muscle fiber. Briefly, one-third of each of the clipped fiber was solubilized in 40 μl of 1% SDS sample buffer (1% SDS, 6 mg/ml EDTA, 0.06 M Tris, pH 6.8, 2 mg/ml bromphenol blue, 15% glycerol, and 5% β-mercaptoethanol) and subjected to SDS-PAGE. The MHC isoforms were identified according to their migration distances against known molecular weight markers.

**RNA Extraction**

Following MHC isoform identification, 20 corresponding, clipped MHC I fibers and 20 MHC IIa fibers in RNA extraction buffers were randomly selected for RNA extraction for each subject at each time point. Each single fiber was extracted according to the manufacturer’s protocol (Molecular Research Center). The 20 RNA pellets (obtained at the end of each extraction) of the same fiber type were combined and dissolved in 40 μl of DNase- and RNase-free water and incubated overnight at 4°C before being transferred to −80°C before real-time RT-PCR assays.

**Muscle Fiber Recruitment**

To indirectly assess that the MHC I and IIa fibers studied were recruited during the RE bout, mRNA expression of pyruvate dehydrogenase kinase 4 (PDK4) using real-time RT-PCR (as described below) was also analyzed. PDK4 is a metabolic marker, whose mRNA expression has been shown to increase significantly 2–8 h following a similar bout of RE (51). We assumed that, if the fibers were metabolically stimulated during the bout of RE (an increase in PDK4 mRNA expression following RE), it was recruited during the bout of RE.

**Reverse Transcription**

Oligo(dT) primed first-strand cDNA were synthesized using SuperScript II RT (Invitrogen, Carlsbad, CA). This system was optimized for sensitive RT-PCR using low amounts of RNA. A first reaction mix of 10 μl for each sample, consisting of 5 μl of RNA extract, 1 μl of 10 mM dNTP, 1 μl of oligo(dT)12–18 (0.5 μg/μl), and 3 μl of DNase- and RNase-free water, was incubated at 65°C for 5 min and then placed on ice for 1 min. A second reaction mix of 9 μl, consisting of 2 μl of 10× RT buffer, 4 μl of 25 mM MgCl2, 2 μl of 0.1 M dithiothreitol, and 1 μl of RNaseOUT Recombinant RNase Inhibitor, was then added to the first reaction mix and incubated at 42°C for 2 min. Finally, 1 μl (50 units) of SuperScript II RT was added to each tube (giving a total volume of 20 μl), incubated at 42°C for 50 min, then 70°C for 15 min to terminate the reaction, and chilled on ice thereafter. Produced cDNA samples were diluted to a final volume of 60 μl (1:12 vol/vol RNA dilution). All incubations were done in the Peltier Thermal Cycler DNA engine (MJ Research, Waltham, MA).

**Real-Time PCR**

Quantification of mRNA transcription (in duplicates) was performed in a 72-well Rotor-Gene 3000 Centrifugal Real-Time Cycler (Corbett Research, Mortlake, NSW, Australia). GAPDH was used as a HKG for internal control, as it had been shown that its expression remained stable using a similar RE protocol (51), and its use as a HKG was validated in this study as well (see Validation of HKG below). The reaction mix consisted of 2.5 μl of 10× SYBRgreen Real-Time PCR Buffer (Biosource, Camarillo, CA), 0.625 μl of 10 mM dNTPs, 0.2 μl of 5 U/μl Platinum Taq DNA Polymerase (Invitrogen, Carlsbad, CA), 1 μl of 10 μM of each forward and reverse primers in the case of the nine genes of interest (GOI) or 2.5 μl of the “mRNA specific” primers in the case of GAPDH and PDK4 in the case of GAPDH, 2.5 μl of cDNA, and DNase- and RNase-free water to 25 μl. All primers used in this study were “mRNA specific” and designed for gene expression analysis using Vector NTI 9 software (Invitrogen) (Table 2). GAPDH primers were purchased from Biosource.

The PCR parameters were initial denaturing at 95°C for 2 min to activate the Platinum Taq DNA polymerase, followed by 40 cycles of 20 s at 95°C, 20 s at the corresponding annealing temperature (58–60°C), and 20 s at 72°C for acquisition of fluorescence signal. In the case of calpain 2, MurF-1, and Bax-a, a second acquisition temperature (86°C) for 15 s was included to eliminate a sporadic, nonspecific fluorescence signal. A melting curve analysis was gener-
Validation of GAPDH for use as a HKG was performed to ensure that its expression was unaffected by the RE. This was done by calculating the relative fold changes of GAPDH and time should be insignificant (23).

In this study, the relationship between the relative fold changes of GAPDH and time should be insignificant (23).

The mRNA expression of the nine GOIs at each time point were analyzed using one-way repeated-measures ANOVA. For each GOI, the relative mRNA expression at each time point for both MHC I and IIa fibers was compared using two-way repeated-measures ANOVA, with factors of time (Pre, and 4 and 24 h postexercise) and fiber type (MHC I and IIa). Significant interactions (time × fiber type) were followed up by one-way repeated-measures ANOVA within each fiber type (MHC I and IIa) and paired t-tests between fiber types at each corresponding time point. Where one-way repeated-measures ANOVA was significant, paired t-tests with Holm sequential Bonferroni correction were used to identify which time points differ in mRNA expression within the same fiber type. Similarly, paired t-tests with Holm correction were used to identify differences where main effect of time was significant but not interaction. For changes in mRNA expression of TNF-α, Friedman test and Sign test were used in place of repeated-measures ANOVA and paired t-tests, respectively. Significance was set at $P < 0.05$ for all analyses. Statistical analyses were performed using SPSS 10.0 for Windows software package. Data in Tables 1 and 3 are presented as means and SDs to provide an index of the variability of the data, while data in Figs. 2–5 are presented as means $\pm$ SE to characterize the uncertainty about the estimated values of population parameters.

RESULTS

RE Bout

All subjects completed the $3 \times 10$ repetitions of bilateral knee extensions at $65 \pm 1\%$ 1 RM except one, who completed 26 repetitions ($2 \times 9$ followed by $1 \times 8$).
MHC Composition

A total of 2,426 single skeletal muscle fibers were isolated from their respective muscle bundles from all eight subjects, of which 43% were MHC I fibers, 29% were MHC IIa fibers, 1% were MHC IIX fibers, 25% were MHC IIa/IIX fibers, and 2–3% were the remaining hybrid fibers (mixed MHC isoforms).

Serum Parameters

There were no differences in serum levels of total protein, glucose, and insulin before and 4 and 24 h after RE (Table 3).

Changes in mRNA Expressions with RE

TNF-α. TNF-α mRNA expression in human single skeletal muscle fibers was either low ($C_T$ of 29–32) or not reliably detectable ($C_T >32$) across all time points in both fiber types after RE. Although all subjects had at least one time point with detectable TNF-α mRNA expression in each fiber type, the lack of detectable TNF-α mRNA expression in some samples in both fiber types and across all time points resulted in a smaller sample size and hence power to detect a significant finding. TNF-α mRNA expression did not differ with time and between fiber types.  

Calpains 1 and 2. There was no significant time × fiber type interaction for calpains 1 and 2. MHC I fibers expressed more (2.0- to 5.0-fold, $P < 0.05$) mRNA of both calpains 1 and 2 than MHC IIa fibers at all three time points, as shown in Fig. 2. There were no changes in mRNA levels of calpains 1 and 2 at 4 and 24 h following RE.

MuRF-1 and atrogin-1. Time × fiber type interaction was significant for MuRF-1 ($P < 0.05$) but not for atrogin-1. MHC I fibers had a higher basal (Pre) mRNA expressions of MuRF-1 (2.0-fold) and atrogin-1 (2.7-fold) than MHC IIa fibers ($P < 0.05$), as shown in Fig. 3. This fiber-specific difference in mRNA expression was sustained at 4 h (1.7-fold) and 24 h postexercise (2.5-fold) only for atrogin-1 ($P < 0.05$). MuRF-1 mRNA levels increased at 4 h post-RE in both MHC I (2.2-fold) and IIa fibers (4.8-fold) ($P < 0.05$), but they returned to basal levels by 24 h postexercise in both fiber types. As for atrogin-1, its mRNA expression did not differ from basal level post-RE in both fiber types. However, mRNA level of atrogin-1 at 24 h post-RE decreased 1.6-fold ($P < 0.05$) compared with 4 h post-RE collectively for both fiber types.  

Caspase-3, Bax, and Bcl-2. Time × fiber type interaction was significant for Bax-to-Bcl-2 mRNA ratio ($P < 0.05$) but not for caspase-3 mRNA expression. MHC I fibers expressed more (1.6- to 2.9-fold, $P < 0.05$) mRNA of both caspase-3 (Fig. 4) and Bcl-2 than MHC IIa fibers at all three time points. There were no differences in mRNA expression of Bax between the fiber types across all three time points. As such, MHC I fibers consistently had a lower (1.6- to 3.9-fold, $P < 0.05$) Bax-to-Bcl-2 mRNA ratio at all three time points (Fig. 4). Caspase-3 mRNA level in both fiber types increased similarly at 4 h (1.4-fold, $P < 0.05$) and 24 h (1.8-fold, $P < 0.05$) post-RE. Bax mRNA level was 1.5-fold ($P < 0.05$) higher at 24 h post-RE than at 4 h post-RE collectively for both fiber types. There were no changes in Bcl-2 mRNA levels following RE. Taken together, Bax-to-Bcl-2 mRNA ratio increased 2.2-fold ($P < 0.05$) in MHC I fibers at 24 h postexercise, with no change in MHC IIa fibers.

PDK4. Time × fiber type interaction was not significant for PDK4. RE increased PDK4 mRNA expression similarly in both MHC I and MHC IIa fibers at 4 h post-RE, as shown in Fig. 5 ($P < 0.05$). There were no differences in PDK4 mRNA expression before and 4 h after RE between MHC I and IIa fibers.

DISCUSSION

The aim of this study was to characterize the changes in mRNA expression of select proteolytic genes in human slow-
twitch and fast-twitch muscle fibers before and after an acute bout of RE. The major findings of this study were that 1) MHC I muscle fibers have higher mRNA levels of most of the selected proteolytic markers at rest and after RE compared with MHC IIa muscle fibers; 2) an acute bout of RE induced transient increases in mRNA expression of the ubiquitin/proteasomal pathway (MuRF-1) in both MHC I and IIa muscle fibers; 3) apoptotic marker caspase-3 was higher after RE collectively for both muscle fiber types; and 4) isolated human single muscle fibers have low to undetectable mRNA levels of TNF-α.

In general, MHC I fibers had a greater mRNA expression of proteolytic genes before and after RE. This most likely reflects a higher protein degradation in MHC I fibers (14) due to the reliance on MHC I fibers during normal activities of daily living, and the order of recruitment from slow to fast fibers during skeletal muscle activation. Even with high-intensity exercise, which increases the recruitment of fast fibers, the slow fibers are still being recruited. One exception was a consistently lower Bax-to-Bcl-2 mRNA ratio in MHC I fibers compared with MHC IIa fibers, before and after RE. This indicates possible fiber-specific differences in mechanisms regulating basal protein degradation and skeletal muscle remodeling following exercise.

For the post-RE gene induction data, one aspect to consider was the recruitment of both MHC I and MHC IIa fibers during the bout of RE. To indirectly assess muscle fiber recruitment, we used PDK4 as a metabolic marker, given that its mRNA expression has been shown to increase significantly 2–8 h following a similar bout of RE (51). The present study found that the RE bout increased PDK4 mRNA expression similarly in both MHC I and MHC IIa fibers at 4 h post-RE with no difference in basal PDK4 mRNA level between the two fiber types. This indicates that both MHC I and IIa fibers were metabolically stimulated as a result of the RE bout, thus implying that both fiber types were recruited during the RE bout.

The high basal mRNA levels of MuRF-1 and atrogin-1 compared with the rest of the genes studied are in agreement with and highlight the major role of the ubiquitin/proteasomal pathway in basal protein degradation (13). To our knowledge, this is the second human study to date to examine the mRNA expression of MuRF-1 and atrogin-1 following exercise. Jones et al. (21) found no changes in mRNA expression of MuRF-1 and atrogin-1 immediately and 24 h after RE (4 h post-RE was not investigated) compared with basal levels, in agreement with the finding of this study. The early (4 h post-RE) increases in MuRF-1 transcription in this study suggest the need to produce more MuRF-1 proteins to meet the demands of increased mixed-muscle protein degradation following RE (31) via the ubiquitin/proteasomal pathway. The general activity of the ubiquitin/proteasomal pathway has been shown to increase following different modes of exercise in both humans and animals (34). Moreover, the magnitude of increases in mRNA levels of MuRF-1 in both fiber types also reflects the major contribution of the ubiquitin/proteasomal pathway in increased...
proteolysis following RE. This is in agreement with the notion that the ubiquitin/proteasomal pathway is the primary regulator for selective proteolysis that eventually leads to skeletal muscle remodeling and adaptation (13).

Both MuRF-1 and atrogin-1 are muscle-specific E3 ligases that were recently discovered as markers of multiple models of muscle atrophy (7, 16). While RE certainly does not lead to atrophy, it remains unclear if the two genes have distinct roles in proteolysis or other physiological functions. The protein substrates targeted by MuRF-1 and atrogin-1 are not well established. However, MuRF-1 has been implicated in the regulation of myofibrillar degradation and/or myofibrillogenesis (assembly of skeletal muscle proteins into myofibrils) (25) through its binding to titin (9). MuRF-1 also binds to glucocorticoid modulatory element binding protein-1 (25), which is a nuclear transcription factor, and thus MuRF-1 may also regulate muscle gene expression. Given the increases in activity of the ubiquitin/proteasomal pathway and gene expression of various components in this pathway following exercise (34), the increase in MuRF-1 mRNA expression following RE in this study possibly indicates an increase in proteolysis via the ubiquitin/proteasomal pathway and/or gene regulation as part of the remodeling process (34).

Atrogin-1 has been implicated in protein-protein interaction (22) and has recently been shown to bind to MyoD and target it for degradation (39). Therefore, atrogin-1 may directly influence proteolysis by possibly targeting myofibrillar proteins for degradation by proteasomes or decrease protein synthesis by targeting specific nuclear transcription factors for degradation. In contrast to MuRF-1 and a novel finding in this study, atrogin-1 mRNA expression did not change from basal level following RE. However, the consistently high expression of atrogin-1 mRNA across all three time points in both fiber types suggests a role in general regulation of protein degradation or synthesis.

Exercise-induced cell death is often attributed to necrosis, and little is known about exercise-induced apoptosis or the effects of exercise on apoptotic markers. An increase in apoptosis following acute exercise had been demonstrated in animals and humans using prolonged, unaccustomed, or eccentric exercise (32, 35, 50). Although sedentary subjects were used in this study, each set of knee extensions only lasted ~1 min for a total of ~3 min, with 2 min of rest in between each set. Hence, in comparison, the RE bout used in this study was less strenuous or traumatic than those used in the studies that demonstrated exercise-induced apoptosis. Furthermore, in this study, there was no increase in serum creatine kinase (indirect marker of muscle damage) and mRNA levels of calpains, which have been upregulated in myotrauma following unaccustomed or strenuous exercise (4, 10). Thus, taken together, the increases in caspase-3 mRNA expression collectively for both fiber types at both 4 and 24 h post-RE with a nonparallel increase in Bax-to-Bcl-2 mRNA ratio only in MHC I fibers at 24 h post-RE suggest an increase in proteolytic activity of the caspases, independent of apoptosis. Recently, Belizario et al. (5) found 54–177% increases in the proteolytic activities of caspases-1, -3, -6, -8, and -9 in the skeletal muscle of mice undergoing cancer cachexia in the absence of apoptosis. Importantly, there is increasing evidence suggesting the involvement of caspases in several nonapoptotic processes, including the regulation of skeletal muscle differentiation (36).

The low or undetectable TNF-α mRNA expression in isolated human single skeletal muscle fibers before and after RE in this study is in contrast to the detected levels of TNF-α mRNA in human skeletal muscle at rest and following exercise, as reported in other studies (17, 29, 30, 33). One possible reason might be the elimination of TNF-α mRNA production from other sources, such as macrophages and inflammatory cells within the skeletal muscle using single skeletal muscle fibers, as opposed to skeletal muscle homogenate. The timing of the muscle biopsy and/or the magnitude of stimulus provided by the RE in this study could also contribute to the low or undetectable mRNA expression of TNF-α following exercise. Nieman et al. reported approximately twofold increases in TNF-α mRNA immediately after a 2-h RE bout (29) and a 3-h run (30). On the other hand, Rabinovich et al. (33) reported no change in TNF-α mRNA level immediately after 11 min of cycling at 40% peak work rate.

Calpains have been implicated in the proteolysis of cytosolic proteins during normal protein turnover, muscle cachexia, tissue injury, necrosis, and apoptosis (15, 52). Specifically, calpain 1 has been suggested to play a role in isofrom transformation and basal proteolytic conditions, whereas calpain 2 is suggested to be involved in myoblast fusion and fiber regeneration with necrosis and injury (4, 27, 37, 38). Given the acute nature of this study and the apparent insignificant muscle trauma following RE, the lack of increases in calpains 1 and 2 mRNA expressions at 4 and 24 h following RE is in agreement with their proposed roles.

In summary, to our knowledge, this is the first study to investigate fiber-specific proteolytic gene expression in human single skeletal muscle fibers. The data from the present study show that MHC I fibers have a greater proteolytic mRNA expression compared with MHC IIa fibers before and after RE. Furthermore, RE induced an increase in proteolytic mRNA expression of the ubiquitin/proteasomal pathway and caspases independent of apoptosis. This altered and specific proteolytic activity among slow- and fast-twitch muscle fibers indicates that these pathways may play an important role in the muscle remodeling process with RE.
ACKNOWLEDGMENTS

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