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HSP-72 AND UBIQUITIN EXPRESSION AND CASPASE-3 ACTIVITY AFTER A SINGLE BOUT OF ECCENTRIC EXERCISE

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ABSTRACT

HSP-72 AND UBIQUITIN EXPRESSION AND CASPASE-3 ACTIVITY AFTER A SINGLE BOUT OF ECCENTRIC EXERCISE. **Darryn S. Willoughby, John Rosene, And Jay Myers.** *JEPonline* 2003;6(2):96-104. This study determined the effect of a single bout of eccentric exercise on muscle strength and soreness, the mRNA and protein expression of heat shock protein-72 (HSP-72) and the ubiquitin proteolytic pathway, myofibrillar protein content, and caspase-3 apoptosis activity. Nine males were randomly assigned to either an eccentric exercise (ECC) or control group (CON). ECC underwent one eccentric exercise bout involving 7 sets of 10 repetitions at 150% 1-RM of the knee extensors. Blood samples and muscle strength and soreness values were obtained before and at 12, 24, and 48 hr post-exercise. Muscle biopsies were obtained 48 hr post-exercise. Data were analyzed with 2X4 factorial ANOVAs and independent groups t-tests ($P < 0.05$). Compared to CON, peak decreases in strength of 117% and increases in soreness of 224% for ECC occurred at 24 hr post-exercise ($P < 0.05$). ECC underwent increases in caspase-3 activity of 766%, along with respective increases of 97%, 118%, 98%, and 82% in HSP-72, UBI, E2, and 20S mRNA at 48 hr post-exercise ($P < 0.05$). ECC also underwent increases in HSP-72, UBI, E2, and 20S protein expression of 128%, 194%, 238%, and 313%, respectively ($p < 0.05$). These results indicate that a single bout of eccentric exercise increases the activity of caspase-3 and up-regulates the expression of HSP-72 UBI, E2, and 20S.

Key words: muscle injury, gene expression, proteolysis

INTRODUCTION

Exercise involving eccentric contractions is known to induce muscle injury, thereby, disrupting the sarcolemma and allowing myofibrillar protein leakage (e.g., creatine kinase, myosin heavy chain fragments, and troponin-I) from skeletal muscle fibers (1). Eccentric exercise also results in decrements in dynamic and static strength and muscle soreness (2).

Eccentric exercise is known to induce apoptosis in skeletal muscle by increasing the activity of the apoptotic protease caspase-3 (3). In addition, eccentric exercise is known to facilitate myofibrillar proteolysis by way of the ATP-dependent ubiquitin (UBI) proteolytic pathway. This pathway is responsible for conjugating damaged proteins into poly-ubiquitinated proteins through activity of a ubiquitin conjugating enzyme (E2). Poly-ubiquitinated proteins are then channeled into the catalytic 20S proteasome where proteolysis occurs (4). A single bout of eccentric exercise has been shown to increase the level of UBI 48 hr post-exercise (5).

Conversely, eccentric exercise has also been shown to increase heat shock protein-72 (HSP-72). Heat shock protein-72 is a molecular chaperone expressed in response to physiological stressors such as eccentric exercise and seems to have an anti-proteolytic and anti-apoptotic effect by preventing protein degradation and/or instigating the repair of damaged proteins by facilitating re-folding and assembly (6). A single bout of eccentric exercise has been shown to increase skeletal muscle HSP-72 48 hr post-exercise (7).

Up to this point, it appears that no attempts have been made to determine the pre- and post-translational events involved with HSP-72 expression and the ubiquitin proteolytic pathway, as well as caspase-3 mediated apoptosis, in response to a single bout of eccentric exercise in humans. Therefore, the purpose of this study was to determine the mRNA and protein expression of HSP-72, UBI, ubiquitin conjugating enzyme (E2), ubiquitin 20S proteasome (20S), caspase-3 activity, myofibrillar protein concentration, plasma skeletal muscle troponin-I [(sTnI) a marker of muscle injury] concentration, and muscle strength and soreness in response to a single bout of eccentric exercise.

METHODS

Subjects

Nine untrained (no consistent, structured weight training six months prior to beginning the study) males with an average (\pm SD) age of 20.55 ± 1.33 yr, height of 176.95 ± 3.75 cm, and body mass of 73.07 ± 8.31 kg volunteered to participate in the study. Subjects with contraindications to exercise as outlined by the American College of Sports Medicine (ACSM) were not allowed to participate. Subjects signed informed consent documents and were then randomly assigned to either a control group involving no eccentric exercise [CON (n = 4)] or an eccentric exercise group [ECC (n=5)]. Approval to conduct the study was granted by the Institutional Review Board for Human Subjects and all experimental procedures involved in the study conformed to the ethical considerations of the Helsinki Code.

Dynamic Strength Testing and Muscle Soreness Assessment

Maximum dynamic knee extensor strength (MDS) of the dominant leg (Universal, Cedar Rapids, IA) was assessed using the standard one-repetition maximum (1-RM) protocol prior to the eccentric exercise bout, and at 12, 24, and 48 hr after the bout. To help prevent fatigue, a goal of only five trials was set for all 1-RM testing sessions (8,9). As a result, the average (\pm SD) number of trials for all subjects over the four 1-RM testing sessions was $3.67 (\pm 0.54)$.

Perceived muscle soreness (SORE) was assessed prior to the exercise bout and at 12, 24, and 48 hr after the exercise bout by each subject placing a mark along a 10-inch continuum with zero indicating no soreness and 10 indicating very, very sore (2).

Eccentric Exercise Bout

All subjects were instructed to continue ingesting a normal mixed diet during the course of the study and to refrain from strenuous physical exercise 5 days prior to the exercise bout. All subjects underwent a warm-up consisting of one set of 10 repetitions (rep) at 50% 1-RM of the dynamic knee extension exercise. The ECC group performed 7 sets of 10 reps at 150% of the 1-RM employing eccentric (forced-lengthening) contractions of the dominant knee extensors. Each rep lasted ~2-3 sec, with 15 sec of rest between reps, and the 7 sets were each separated by 3 min of rest (2).

Blood Sampling and Muscle Biopsies

Blood was obtained from the antecubital vein prior to the warm-up for the exercise bout and at 12, 24, and 48 hr post-exercise. Blood was allowed to stand for 10 min, centrifuged, and then frozen at -20°C . Based on the guidelines of previous studies (5,7), percutaneous needle biopsies (30.02 ± 4.08 mg) were obtained at 48 hr post-exercise from the exercised vastus lateralis muscle at a depth of ~ 1 cm and immediately frozen at -70°C for later analysis.

Total RNA Isolation

Total cellular RNA was extracted from biopsy samples with a monophasic solution of phenol and guanidine isothiocyanate (8,9,10,11) contained within the TRI-reagent (Sigma Chemical Co., St. Louis, MO). The RNA concentration was determined by optical density (OD) at 260 nm (by using an OD_{260} equivalent to $40 \mu\text{g}/\mu\text{l}$), and the final concentration was adjusted to $1 \mu\text{g}/\mu\text{L}$. The RNA integrity was verified by an $\text{OD}_{260}/\text{OD}_{280}$ ratio of approximately 2.0 (8,9,11,12). The RNA samples were stored at -70°C until later analyses.

Reverse Transcription and cDNA Synthesis

Two μg of total skeletal muscle RNA were reverse transcribed to synthesize cDNA using $25 \text{ u}/\mu\text{g}$ of AMV reverse transcriptase enzyme (Promega, Madison, WI). Starting PCR template concentration was then standardized by adjusting the cDNA reactions for all samples to 200 ng prior to amplification (8,9,11).

Oligonucleotide Primers for PCR

The following 5' sense and 3' antisense oligonucleotide primers were used to isolate HSP-72 mRNA (5' primer: bases 1801-1821, 3' primer: bases 2302-2282, GenEMBL AC M11717), UBI mRNA (5' primer: bases 435-455, 3' primer: bases 638-618, GenEMBL AC M26880), E2 mRNA (5' primer: bases 74-94, 3' primer: bases 555-575, GenEMBL AC U39318), and 20S mRNA (5' primer: bases 305-325, 3' primer: bases 778-798, GenEMBL AC D00759). We have recently shown these primers to amplify PCR fragments of 500, 204, 494, and 507 bp for HSP-72, UBI, E2, and 20S, respectively (11,13).

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an external reference standard for detecting the relative change in the quantity of HSP-72, UBI, E2, and 20S mRNA using PCR. For GAPDH mRNA (5' primer: bases 616-636, 3' primer: bases 1189-1169, GenEMBL AC NM 002046), we have previously shown these primers to amplify a PCR fragment of 574 bp (8,9,11).

PCR Amplification

Two hundred ng of cDNA were added to each of the PCR reactions for GAPDH, HSP-72, UBI, E2, and 20S and each PCR reaction was amplified with a thermal cycler (Bio Rad, Hercules, CA). The amplification profile involved a denaturation step at 95°C for 30 sec, primer annealing at 55°C for 30 sec, and extension at 72°C for 60 sec (8,9,11). To help control for differences in amplification efficiency during thermocycling, all PCR reactions were prepared from the same stock solution. Also, the number of cycles was optimized at 30 so that the amplified signal was still on the linear portion of a plot with the yield expressed as a function of the absorbance at OD_{260} and the number of PCR cycles. The specificity of the PCR was demonstrated with an absolute negative control using a separate reaction containing no cDNA (8,9,11). The two PCR runs for all subjects resulted in coefficients of variation of 1.63%, 2.55%, 2.81%, 3.67%, and 3.05%, respectively, for GAPDH, HSP-72, UBI, E2, and 20S mRNA.

HSP-72, Ubiquitin, E2, and 20S mRNA Quantitation

Each amplified PCR reaction was purified of contaminants such as primer dimers and amplification primers using the Wizard PCR Preps DNA Purification System (Promega, Madison, WI). The concentration of mRNA was determined spectrophotometrically at a wavelength of OD_{260} (8,9,11,12). The HSP-72, UBI, E2, and 20S mRNA concentration was calculated and normalized relative to GAPDH (ng of target mRNA/ng of GAPDH mRNA) based on a statistical method previously described (14). It should be noted, however, that this method of PCR quantitation determines relative mRNA concentration only and should not be interpreted as absolute concentration values.

HSP-72, Ubiquitin, E2, 20S, and sTnI Quantitation

The protein content of skeletal muscle HSP-72, UBI, E2, 20S, and sTnI (a marker of muscle injury) was determined with an antibody-sandwich enzyme-linked immunoabsorbent assay (ELISA)(8,11) using primary antibodies for anti-HSP-72, anti-UBI, anti-sTnI (Stressgen Biotechnologies, Victoria, BC, Canada), anti-E2, and anti-20S (Affiniti Research Products, Mamhead, UK). The anti-UBI antibody has a high affinity for poly-ubiquitinated proteins. The anti-20S antibody has a high affinity for the $-\alpha$ and $-\beta$ subunits comprising the 20S proteasome. The secondary antibody (ICN Biomedical, Aurora, OH) involved immunoglobulin-G (IgG) conjugated to the enzyme horseradish peroxidase. The assays were run in duplicate and the protein concentrations were determined at an optical density of 450 nm with a microplate reader (Bio Rad, Hercules, CA). Protein concentrations for HSP-72, UBI, E2, and 20S were expressed relative to muscle wet weight. Intra-assay coefficients of variation were 2.95%, 2.74%, 3.07%, 2.98%, and 3.18%, respectively, for HSP-72, UBI, E2, 20S, and sTnI.

Myofibrillar Protein Quantitation

Total protein remaining from the total RNA isolation procedure was isolated with isopropanol, ethanol, and 0.3 M guanidine hydrochloride (8,9,11,15). Myofibrillar protein was further isolated with 1.0% SDS (16). Based on our previous work (8,9,11), myofibrillar protein content was determined spectrophotometrically based on the Bradford method (17) at a wavelength of 595 nm using bovine serum albumin as the standard and quantified relative to muscle wet weight. All assays were performed in duplicate with a coefficient of variation of 2.54%.

Caspase-3 Activity

As an indicator of apoptosis, caspase-3 content (pmol), the relative change in fluorescence units [Δ FU (change in fluorescence units/min)], and caspase-3 enzyme specific activity [ESA (pmol AMC liberated/min/ μ g protein)] were determined with the CaspACETM fluorometric assay system (Promega, Madison, WI) based on the manufacturer's guidelines. The assay was performed in duplicate with a fluorometer (VersaFluor, BioRad, Hercules, CA) using an excitation wavelength of 360 nm and an emission wavelength of 460 nm and produced coefficients of variation of 3.55%, 5.48%, and 6.87%, respectively, for caspase-3 content, Δ FU, and ESA.

Statistical Analyses

Statistical analyses for MDS, SORE, and sTnI were performed by utilizing separate 2 x 4 [Group (ECC, CON)] x [Test (pre-exercise and 12, 24, and 48 hr post-exercise)] factorial analyses of variance (ANOVA). Further analysis of the main effects for Group and Test were performed with separate one-way ANOVAs. Significant between-group differences were determined involving the Neuman-Keuls Post Hoc Test and statistical power determined accordingly. However, to protect against Type I error, the conservative Hunyh-Feldt Epsilon correction factor was used to evaluate observed within-group F-ratios. The effects of sample size were determined using the Partial Eta² (H^2) statistic. In an attempt to determine significant differences between CON and ECC for the selected criterion variables occurring in muscle 48 hr post-exercise, separate independent groups t-tests were performed for HSP-72, UBI, E2 and 20S mRNA and protein, myofibrillar protein, and caspase-3 activity. A probability level of ≤ 0.05 was adopted throughout.

RESULTS

Maximum Dynamic Strength

A significant Group x Test interaction was observed for MDS ($F(3,7)=6.85$, $p=0.001$, power=0.91, $H^2=0.31$). In addition, significant main effects for Group ($F(1,7)=9.27$, $p=0.005$, power=0.84, $H^2=0.25$) and Test ($F(3,7)=2.98$, $p=0.048$, power=0.64, $H^2=0.24$) were detected that indicated ECC and CON to be significantly different from one another. Post-hoc analyses indicated that ECC experienced significantly greater ($p<0.05$) decrements in MDS at 12, 24, and 48 hr post-exercise when compared to CON. In addition, ECC underwent significant decrements ($p<0.05$) in MDS at 12, 24, and 48 hr post-exercise when compared to pre-test values (Figure 1).

Muscle Soreness

A significant Group x Test interaction was observed for SORE ($F(3,7)=5.03$, $p=0.007$, $\text{power}=0.87$, $H^2=0.35$). In addition, significant main effects for Group ($F(1,7)=33.18$, $p=0.000$, $\text{power}=1.00$, $H^2=0.54$) and Test ($F(3,7)=12.06$, $p=0.000$, $\text{power}=0.99$, $H^2=0.57$) were detected that indicated ECC and CON to be significantly different from one another. Post-hoc analyses indicated that ECC experienced significantly greater ($p<0.05$) increases in SORE at 12, 24, and 48 hr post-exercise when compared to CON. In addition, ECC underwent significant increases ($p<0.05$) in SORE at 12, 24, and 48 hr post-exercise when compared to pre-test values (Figure 2).

Plasma sTnI Content

A significant Group x Test interaction was observed for sTnI ($F(3,7)=5.14$, $p=0.002$, $\text{power}=0.95$, $H^2=0.37$). In addition, significant main effects for Group ($F(1,7)=35.95$, $p=0.000$, $\text{power}=1.00$, $H^2=0.51$) and Test ($F(3,7)=5.36$, $p=0.002$, $\text{power}=0.95$, $H^2=0.38$) were detected that indicated ECC and CON to be significantly different from one another. Post-hoc analyses indicated that ECC experienced significantly greater ($P < 0.05$) increases in sTnI at 12, 24, and 48 hr post-exercise when compared to CON. In addition, ECC underwent significant increases ($p<0.05$) in sTnI at 12, 24, and 48 hr post-exercise when compared to pre-test values (Figure 3).

HSP-72, Ubiquitin, E2, 20S, and Myofibrillar Protein Levels

Group ECC was shown to be significantly greater than CON for HSP-72 ($t(7)=7.14$, $p=0.001$), UBI ($t(7)=5.85$, $p=0.001$), E2 ($t(7)=4.89$, $p=0.035$), and 20S ($t(7)=4.46$, $p=0.042$) mRNA levels (Figure 4). Also, the protein levels of ECC for HSP-72 ($t(7)=7.47$, $p=0.001$), UBI ($t(7)=15.06$, $p=0.001$), E2 ($t(7)=6.67$, $p=0.001$), 20S ($t(7)=23.22$, $p=0.000$) were significantly greater than CON. The myofibrillar protein content ($t(7)=-7.68$, $p=0.001$) of ECC was significantly less than CON (Figure 5).

Caspase-3 Activity

In regard to caspase-3 activity (Table 1), ECC was shown to be significantly greater than CON for caspase-3 content ($t(7)=4.31$, $p=0.004$), Δ FU ($t(7)=2.79$, $p=0.027$), and ESA ($t(7)=.54$, $p=0.042$).

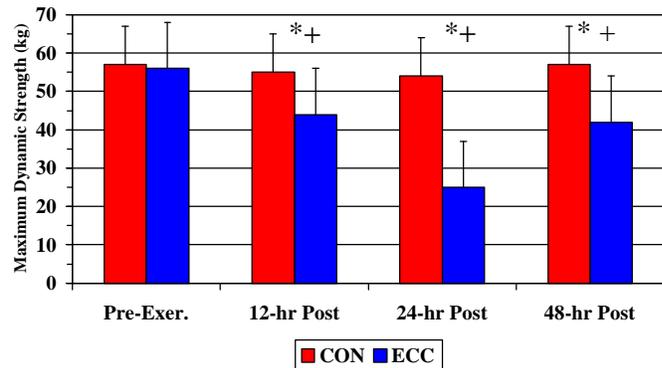


Figure 1. Maximum dynamic strength before and 12, 24, and 48 hr post-exercise. Data are means \pm SD, $n=9$. * ECC significantly different from CON, + ECC significantly different from pre-exercise values ($p<0.05$).

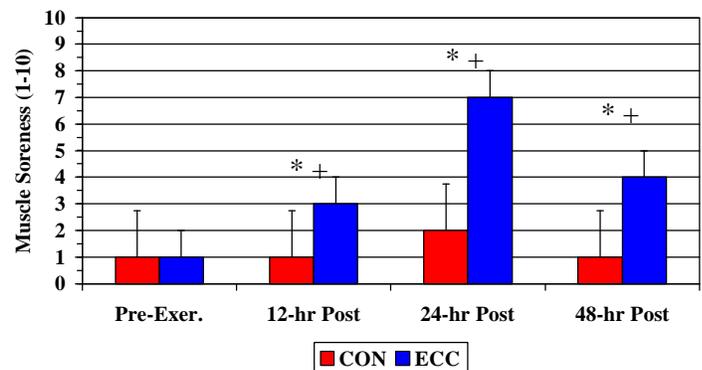


Figure 2. Perceived muscle soreness before and 12, 24, and 48 hr post-exercise. Data are means \pm SD, $n=9$. * ECC significantly different from CON, + ECC significantly different from pre-exercise values ($p<0.05$).

Table 1. Group means (\pm SD) and percent differences from control for caspase-3 activity at 48 hr post-exercise.

Variable	CON	ECC	%D from CON
Caspase-3 (content)	79.85 (13.27)	141.27 (25.65)*	77
Caspase-3 (Δ FU)	0.52 (0.008)	2.03 (1.05)*	290
Caspase-3 (ESA)	1.35 ⁻⁷ (7.9 ⁻⁸)	1.17 ⁻⁶ (1.05 ⁻⁶)*	766

caspase-3 content (pmol); caspase-3 Δ FU (change in fluorescence units/min); caspase-3 enzyme specific activity (pmol AMC liberated/min/ μ g protein); * significantly different from CON ($p<0.05$)

DISCUSSION

The present study demonstrates that a single bout of eccentric exercise was effective in producing muscle injury. When compared to CON, ECC displayed peak increases in plasma sTnI of 773% and muscle soreness of 224%, along with decrements in MDS of 117%, all occurring at 24 hr post-exercise. Also, when compared to CON the ECC group displayed significant increases of 766% for caspase-3 ESA at 48 hr post-exercise, indicative of apoptosis.

Eccentric exercise in rodents has been shown to elevate the activity of caspase-3 (3). Caspase-3 mediated apoptosis is likely elevated in response to eccentric exercise due to the loss of critical sarcoplasmic proteins upon disruption of the sarcolemma (18). In addition to caspase-3 activity, the present results also show that the mRNA and protein expression of HSP-72, UBI, E2, and 20S were significantly elevated at 48 hr post-exercise. Two previous studies have shown both HSP-72 (7) and UBI (5) protein to be elevated at 48 hr after eccentric exercise of the elbow flexors. However, these two studies did not determine the mRNA expression for HSP-72, UBI, E2, and 20S or the protein expression for E2 and 20S. Since eccentric exercise produces a large susceptibility to protein damage (19), in the present study the fact that UBI, E2, and 20S mRNA were elevated at 48 hr post-exercise may have resulted in decreases in myofibrillar protein content due to an increase in ubiquitin-mediated proteolysis. The majority of myofibrillar protein breakdown that occurs in skeletal muscle is a result of up-regulation of the ubiquitin-proteolytic pathway (20).

The observed significant increases in the mRNA and protein expression of UBI, E2, and 20S likely contributed to the 40% decrease in myofibrillar protein content, compared to control, at 48 hr post-exercise. This decrease in myofibrillar protein content is also likely a result of losses in various myofibrillar proteins which is illustrated, at least in part, by the

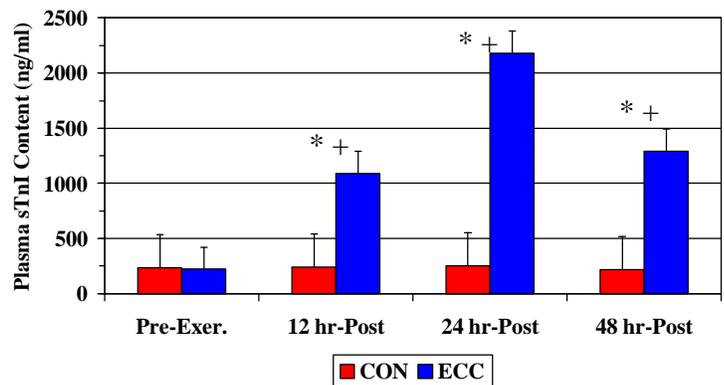


Figure 3. Skeletal muscle troponin-I concentrations (ng/ml) before and 12, 24, and 48 hr post-exercise. Data are means \pm SD, n=9. * ECC significantly different from CON, + ECC significantly different from pre exercise values (p<0.05).

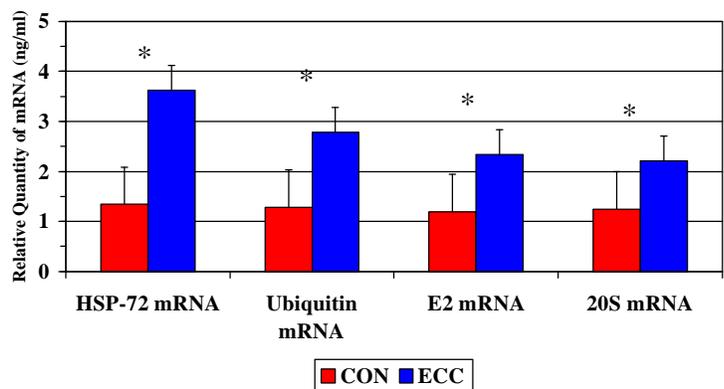


Figure 4. Relative skeletal muscle HSP-72, UBI, E2, and 20S mRNA concentrations 48 hr post-exercise. Data are means \pm SD, n=9. * ECC significantly different from CON (p<0.05).

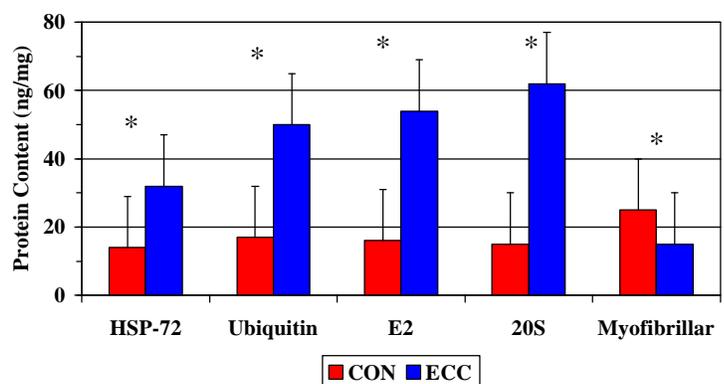


Figure 5. Relative skeletal muscle HSP-72, UBI, E2, 20S (ng/mg wet weight), and myofibrillar protein (μ g/mg wet weight) concentrations 48 hr post-exercise. Data are means \pm SD, n=9. * ECC significantly different from CON (p<0.05).

observed increases in plasma sTnI beginning at 12 hr post-exercise and continuing through 48 hr post-exercise.

Previous research has shown eccentric exercise in rodents to be associated with increases in UBI protein content and DNA fragmentation (21). A previous study with humans has shown 64% increases in UBI protein 48 hr after a single bout of eccentric exercise of the elbow flexors (5). In addition, poly-ubiquitinated protein was significantly elevated 24 hr after eccentric exercise of the knee extensors (22). In the present study, when compared to CON, eccentric exercise resulted in increases of 118%, 98%, and 82% for UBI, E2, and 20S mRNA, respectively, along with increases of 194%, 238%, and 313% in UBI, E2, and 20S protein, respectively, at 48 hr post-exercise. Due to the up-regulation in both mRNA and protein expression, our results seem to suggest that the muscle injury arising from eccentric exercise increases the activity of the ubiquitin-proteolytic pathway through both pre- and post-translational mechanisms.

It has previously been shown in humans that a single bout of eccentric exercise of the elbow flexors resulted in increases in the protein levels of HSP-72 at 48 hr post-exercise (7). In the present study we observed the ECC group to undergo significant increases of 97% for HSP-72 mRNA and 128% for HSP-72 protein at 48 hr post-exercise when compared to CON. Elevated levels of HSP-72 are known to protect cells from stress-induced apoptosis and from the proteolytic effects of UBI degradation (23). Due to its role as a stress protein, the mRNA and protein expression of HSP-72 was likely elevated in response to elevations in the activity of caspase-3 and the ubiquitin-proteolytic pathway since apoptosis and increased proteolytic activity were conceivably occurring in the muscle due to the eccentric exercise. Therefore, the expression of both HSP-72 mRNA and protein may have occurred in an attempt to carry out the role of a molecular chaperone by binding to denatured proteins and facilitating the re-assembly of protein complexes (24).

It has been shown, however, that dynamic exercise, primarily composed of concentric contractions is capable of increasing the expression of HSP-72 in humans. Specifically, HSP-72 mRNA levels have been shown to be elevated 3 hr after treadmill exercise while HSP-72 protein was essentially unchanged (25). In addition, HSP-72 mRNA levels have also been shown to be significantly increased 40 minutes prior to fatigue and at the point of fatigue (~186 min) during bicycle exercise (19). There are also data from 14 days of training in rowers demonstrating increases in HSP-72 protein that seem to be dependent upon exercise intensity (26).

It should be noted that our present results occurred in response to an exercise protocol employing only eccentric contractions, thereby subjecting the knee extensor muscles to a considerable amount of muscle injury. It has been shown that eccentric exercise is more effective in manifesting the symptoms of muscle injury than exercise utilizing primarily concentric contractions, with the greatest amount of muscle injury occurring between 24-48 hr post-exercise (27). Therefore, it is conceivable that the mRNA expression of both HSP-72 and UBI, E2, and 20S in the present study is likely so pronounced with eccentric exercise that it is elevated at 48 hr post-exercise.

After eccentric exercise, muscle proteolysis is elevated and the quantity of abnormal proteins increase; this is a stimulus which instigates the need for the anti-proteolytic chaperone effect of HSP-72. Ubiquitin and HSP-72 are both considered stress proteins. Therefore, it is conceivable that HSP-72, UBI, E2, 20S were elevated in the present study in response to the muscle injury, decreased myofibrillar protein content, and apoptosis that seemed to be occurring within the exercise muscle. In light of these effects, we conclude that a single bout of eccentric exercise in humans, and the resultant muscle injury and decreased muscle strength, is effective in increasing the activity of the apoptotic protease caspase-3 and the ubiquitin proteolytic pathway, as well as increasing the mRNA and protein expression of HSP-72.

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