HSP72 expression is specific to skeletal muscle contraction type

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Abstract

Exercise is capable of inducing the cellular stress response and increasing skeletal muscle heat shock protein (HSP) content. HSPs function as molecular chaperones and play roles in facilitating protein folding thereby contributing to muscle proteostasis. To determine the relationship between muscle contraction types, muscle damage, and HSP content, one tibialis anterior (TA) muscle from male Sprague-Dawley rats ($n = 5/\text{group}$) was electrically stimulated while actively lengthening (LC), shortening (SC), or remaining to stagnate (IC) for 15 repetitions (3 sets of five). Two additional LC groups underwent 5 and 10 repetitions. Maximal tetanic tension (MTT) was recorded prior to (pre) and at 5 min after (post) the last contraction. Twenty-four hours after stimulation, TA muscles were removed, processed, and assessed for damage and for HSP25 and HSP72 content. Post-MTT was significantly decreased following 15 LCs, (24%; $p < 0.05$) but not following 15 SCs or 15 ICs. Post-MTT was also decreased by 8% ($p < 0.05$), and 18% ($p < 0.05$) for muscles subjected to 5 and 10 LCs, respectively. HSP72 content increased after all LCs conditions but not following ICs or SCs. HSP25 content remained unchanged following all contractions. Similarly, muscle damage was observed only after LCs and not after other contraction types. In conclusion, muscle HSP72 content can be increased with as few as 5 maximal lengthening contractions and appears to be related to muscle damage. This may have important implications for muscle rehabilitation and exercise training programs.

Keywords Heat shock proteins · Skeletal muscle · Lengthening contraction · Eccentric · Muscle damage

Introduction

The cellular stress response (CSR) involves the rapid synthesis of the cyto-protective heat shock proteins (HSPs) in an attempt to restore proteostasis and minimize the damage caused by the imposed stressor(s) (Morimoto 1998). A variety of stressors such as increased temperature (Kim et al. 2004) (heat stress), oxidative stress (Benjamin et al. 1990; Dimauro et al. 2016), alterations in pH (Petronini et al. 1995), and reductions in adenosine triphosphate (ATP; Benjamin et al. 1992) are all capable of activating the CSR and increasing cellular HSP content. Given that these stressors are known to occur in skeletal muscle during exercise, it is perhaps not surprising that a number of studies (Locke et al. 1991, 1995; Thompson et al. 2001; Milne and Noble 2002; Thompson et al. 2003), though not all (Puntschart et al. 1996; Kim et al. 2004), have shown an increased muscle HSP content

following exercise. Given that skeletal muscle is comprised of ~20% protein by mass and often functions under extreme conditions, minimizing proteostasis perturbations would be expected to be an important exercise-induced adaptation. Since all of the exercise-related cellular stressors ultimately originate from muscle contraction, it follows that different types of muscle contraction may differentially influence the CSR and thus HSP content.

The function of skeletal muscles is to generate force and overcome resistance. According to the sliding filament theory, the contractile proteins, actin and myosin, use the energy from ATP to slide past each other allowing the muscle to undergo a shortening contraction (SC). Thus, if the force or torque generated by a muscle exceeds the resistance, a SC occurs. However, if the resistance is equal to or exceeds the maximal force generated, the contractile filaments will be unable to shorten and will either remain static (isometric, IC) or undergo a lengthening contraction (LC). It should be noted that during LCs, the muscle is still generating force against the resistance but the muscle is forcibly lengthened. Not surprisingly, LCs are known to result in greater muscle damage than other contraction types (McCully and Faulkner 1985; Fridén and Lieber 2001). In addition, LCs are extensively used in certain types of

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muscle rehabilitation and exercise training programs, although exactly why LCs are better suited than other contractions remains unknown. Using a rodent model, our previous work has demonstrated an increased muscle damage coupled with an elevated HSP content following 100 LCs but not following 100 SCs (Holwerda and Locke 2014). A subsequent study (Pollock-Tahiri and Locke 2017) examined 60, 40, and 20 LCs and showed that as few as 20 LCs still increased muscle HSP72 content. With perhaps the exception of 20 LCs, the 40–100 LCs would be considered supra-physiological and not surprisingly, muscle damage was observed. In view of this, the purpose of this study was to examine a more physiologically relevant range (5–15 LCs) of muscle contractions, determining the minimum number of LCs required to elevate muscle HSP content while minimizing the effect of muscle damage.

Methods and materials

Animals

Male Sprague-Dawley rats ($n = 5/\text{group}$; $\sim 390\text{--}430$ g) were obtained from Charles River Laboratories (Quebec, Canada). Animals were housed in pairs and underwent a 2-week 12-h light/dark cycle acclimatization period with food and water provided ad libitum. All procedures were approved by the University of Toronto Animal Care Committee and in accordance with the guidelines of the Canadian Council on Animal Care. All experiments were conducted under an isoflurane/oxygen mixture anesthesia (1 L/min). Animals were monitored following stimulations and euthanized 24 h later.

Contraction protocol

An unconscious rodent-isolated hindlimb model similar to that previously described was used (Holwerda and Locke 2014; Pollock-Tahiri and Locke 2017). Anesthetized rats were placed supine on a 37°C warming plate (806D, Aurora Scientific Inc., Aurora, Canada) connected to a continuous-flow anesthesia machine via a fitted mask (isoflurane/oxygen mixture; 1 L/min; Harvard Apparatus, St. Laurent, Quebec) throughout the experiment. The left hind leg was secured between two posts using a $25\text{G} \times 1.5$ in. needle inserted at the patellar tendon. Two $28\text{G} \times 0.5$ needle electrodes were inserted subcutaneously, adjacent and parallel to the TA and the foot secured to a pedal connected to a computer-controlled servomotor (301C, Aurora Scientific Inc., Aurora, Canada). Foot position was optimized and stimulation testing was conducted to determine optimal stimulation parameters such as the minimal voltage (V) needed to attain tetanus. Using a biphasic stimulator (701C Aurora Scientific Inc., Aurora, Canada), an IC was induced for 0.5 s at increasing voltages. The optimal parameters ranged between 8 and 12 V at 150 Hz.

Values were recorded using Dynamic Muscle Control software (DMC; 610A, Aurora Scientific Inc., Aurora, Canada) and assessed using Dynamic Muscle Analysis software (DMA; 611A, Aurora Scientific Inc., Aurora, Canada). The tetanic force was measured both prior to and at 5 min following the last contraction. The IC group maintained optimal foot position and stimulated isometrically for 0.8 s. Both the LC and SC groups were isometrically stimulated for 0.2 s to reach tetanus then actively lengthened or shortened for 0.6 s. The three contraction type groups (SC, IC, and LC) underwent three sets of 5 (15 contractions total). Each contraction was spaced apart by 2 s and a rest time of 2 min between each set. Two additional LC groups underwent one and two sets totaling 5 and 10 repetitions, respectively.

Tissue collection

All rats were euthanized 24-h post-stimulation by means of cardiac puncture/exsanguination while under anesthesia. The TA muscles of both hind limbs were excised (stimulated and contra-lateral), weighed, divided, and processed accordingly.

Protein determination and western blotting

A portion of each TA (~ 70 mg) was homogenized in 15 volumes of RIPA buffer containing protease inhibitor cocktail solution using a microtube homogenizer (Benchmark Scientific, USA) set to 4000 RPM. Following 30-s homogenization cycles, samples were centrifuged at 3000 rpm for 10 s; the supernatant was removed and protein concentration was determined using the method described by Lowry et al. (1951) with bovine serum albumin as a standard. Muscle homogenates ($100\text{ }\mu\text{g}$) were loaded into a gradient gel (5–15%) and separated by one-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis as previously described (Holwerda and Locke 2014; Pollock-Tahiri and Locke 2017). Separated proteins were transferred from the gel to the nitrocellulose membranes ($0.22\text{-}\mu\text{m}$ pore size; Bio-Rad, Mississauga, Canada) as described by Towbin et al. (1979) and adapted to the Bio-Rad mini-protein II gel transfer system (Pollock-Tahiri and Locke 2017). Following a similar methodology outlined by Pollock-Tahiri and Locke (2017), transferred membranes were blocked in 5% BSA (5% BSA powder in Tris-buffered saline (TBS) 500 mM NaCl, 20 mM Tris, pH 7.5) for 1 h. Blocked membranes underwent two 5-min wash cycles in Tween-Tris-buffer saline (TTBS; TBS plus 0.05% Tween 20) then incubated in a polyclonal antibody specific to HSP25 (ADI-SPA-801, Enzo, USA) and/or HSP72 (ADI-SPA-812-F, Enzo, USA) diluted 1:1000 in TTBS with 2% Blotto. Primary incubated membranes were washed twice (2×5 min) before being incubated for 1 h at room temperature in a 1:1000 dilution with goat anti-rabbit

secondary antibody conjugated to horseradish peroxidase (Cell Signaling #7074S) or 1:500 dilution of GAPDH (Abcam #181602).

A final incubation in 4 mL of horseradish peroxidase (HRP) substrate (Luminata Forte) developing solution was performed in low light for 3 min and protein bands visualized using a chemiluminescence western blot scanner (LI-COR C-DiGit 3600, USA). Membranes were scanned for 12 min and bands quantified using Image Studio Digits 2.0 software. Data from bands for HSP25 or 72 were compared between stimulated and contra-lateral limbs. Fold change was calculated by dividing the stimulated values by the contra-lateral with values greater than 1.0 suggesting an increase.

Muscle fiber morphology

To assess fiber morphology, 8–12- μ m transverse sections of the TA mid-belly were cut using an American Optical cryostat at -20°C , air dried, stained with hematoxylin and eosin, and viewed via a Zeiss Axioskop light microscope. A Canon 70D digital camera attached to the microscope using a Zarf Enterprise Microscope Adaptor (Spokane, WA) was used to photograph stained cross-sections. Morphological characteristics and evidence of muscle fiber damage were evaluated using a qualitative method similar to that outlined by Pollock-Tahiri and Locke (2017). Each muscle was examined for fiber damage at $\times 10$ magnification and distinguished by the presence of intramuscular or extracellular inflammatory cells, membrane disruptions, and necrosis (rounded/swollen fibers, eosinophilic fibers, and degraded fibers which stain light). Damage was considered mild when necrosis was localized to few fibers within a single fascicle partnered with a low level of inflammatory infiltration.

Electron microscopy

Transmission electron microscopy (TEM) was performed as previously described (Pollock-Tahiri and Locke 2017). Briefly, small pieces of the TA muscle (2–3 mm) were fixed in 3% glutaraldehyde in phosphate buffer (pH 7.4) at 4°C . Muscle pieces were washed in 0.1 M Sorenson's phosphate buffer (3×10 min each) and placed in 1% OsO_4 for an hour. After three additional washes, a series of ethanol incubations dehydrated the muscle pieces (50% 10 min, 70% 10 min, 80% 15 min, 90% 2×10 min, 100% 2×10 min). Muscle pieces were placed in ethanol: Spurr's resin mixture (3:1) for 30 min, 1:1 for 1 h, and 1:3 for the last hour before being left to rotate overnight in 100% Spurr's resin. Samples were left in fresh Spurr's resin for approximately 6 h the next day then embedded over night at 65°C . Fixed blocks were sectioned and stained with 3% uranyl acetate and Reynolds lead citrate. Grids were examined and photographed with a Hitachi HT7700 transmission electron microscope.

Statistical analysis

Muscle mass and protein content from western blots for HSPs were expressed as both absolute and specific values (normalized to body mass). These data points from stimulated TA muscles were compared with contra-lateral (CL) TA values through a one-way ANOVA with Tukey's post hoc testing and Bartlett's variance testing. Force values were converted into percent of the first contraction or as a percent of the pre-tetanic force. These data points were also analyzed using a two-way ANOVA with multiple comparisons to show the decrements in force against repetitions. Data are reported as the mean $\pm$ SEM. For all analyses, the level of significance was set at $p < 0.05$.

Results

Physical characteristics

There were no significant differences in the body mass between any groups (Table 1). To determine if contraction type or number altered muscle mass, excised CL and stimulated TA muscles were compared. Given that no difference in the body or muscle mass was observed, data were expressed as a ratio of stimulated/CL. When performed, no significant differences were detected in muscle mass within or between groups (Table 1). Thus, the animals and likely the muscles from the animals used for the present experiments were similar in size prior to experimentation.

Muscle force

To determine if the three contraction types differed in their ability to generate force, peak muscle torque was measured. When presented as a percent of the torque generated from the first contraction, TA muscles subjected to ICs and SCs (Fig. 1) showed significant decreases in peak torque at the second ($p < 0.001$) or fourth ($p < 0.05$) contraction of each set, respectively. As expected, the IC and SC groups recovered between each set demonstrating comparable peak torque values on the subsequent contractions. In contrast, the TA muscles subjected to 15 LCs (Figs. 1 and 2) demonstrated a significant decrease ($p < 0.001$) in peak torque at the eighth contraction and remained decreased thereafter. Additionally, as the number of repetitions progressed, a continual decline in peak torque measures was observed in LC groups (Fig. 2).

The force-generating ability of each TA muscle was assessed pre- and post-tetanus for all contraction types as well as for the two additional LC groups. When compared with pre-tetanus (Fig. 3), no significant loss of force was observed for the IC and SC groups at 5-min post-stimulation. However, after 15 LCs, TA muscle force-generating ability remained

Table 1 Body mass and muscle mass following all contraction protocols and prior to extraction

Animals (number/group)	Contraction type	Number of contractions	Body mass (g)	Muscle mass (stimulated/CL)
5	SC	15	422.5 ± 3.56	0.99 ± 0.02
5	IC	15	420.5 ± 3.83	0.97 ± 0.05
5	LC	15	416 ± 4.41	1.05 ± 0.03
5	LC	10	410.4 ± 4.89	1.02 ± 0.01
5	LC	5	411.9 ± 6.57	1.01 ± 0.02

Both body mass and muscle mass are similar between all groups

significantly (24%) reduced post-exercise ($76.3 \pm 3.1\%$; $p < 0.001$). When subjected to 5 LCs or 10 LCs, a significant 8% ($p < 0.01$) and 18% ($p < 0.05$) decline in force was also observed. Post-tetanic force following 15 LCs was also significantly lower than the resulting force of 5 LCs ($p < 0.01$). Taken together, the observed pattern of force shown by the TA muscles subjected to LCs shows clear separations in force generation recovery/damage between contraction types as well as with the number of LCs.

Damaged fiber morphology following LCs

To determine the general morphology of TA muscles at 24 h after each contraction type, light microscopy was used to qualitatively assess muscle damage. Hematoxylin and eosin-stained TA cross-sections from each contraction group were assessed and compared to the muscles from the contra-lateral control limb. CL TA muscles were characterized by homogeneous staining patterns and showed no evidence of damage or immune cell infiltration (Fig. 4 panel a). TA muscles following ICs or SCs also showed no morphological damage (Fig. 4 panels b and c). In contrast, some evidence of muscle fiber damage was observed following 15 LCs and also after 10 LCs (Fig. 4 panels e and f). Both images showed evidence of muscle damage in the form of increased fiber size (swelling), infiltration, and necrotic fibers. It should be noted that fiber

damage was infrequent and only a very few areas of damage were observed. These areas were surrounded by large areas of ostensibly healthy fibers. Similarly, the apparent muscle damage observed was less following 10 LCs than 15 LCs and no evidence of damage was detected in TA muscles subjected to only 5 LCs (Fig. 4 panel d).

Electron microscopy

Given the scarcity of muscle fiber damage detected by light microscopy, TEM was used to conduct a qualitative analysis of ultra-structural changes. TEM assessment of ultra-structural integrity showed sarcomeric components such as z-lines, myofilaments, and mitochondria which were, for the most part, normal in appearance; however, some evidence of damage was detectable. Figure 5 shows electromicrographs within a muscle fiber from contra-lateral (Fig. 5 panel a) and stimulated TA muscles following ICs and SCs (Fig. 5 panels b and c). No evidence of damage (z-line streaming, disruption to sarcomeres, or mitochondrial irregularities) was observed in fibers from IC- or SC-treated muscles (Fig. 5 panels d–f). However, following 5, 10, and 15 LCs, evidence of damage in regions of fibers from the stimulated TA was observed. It is important to note that similar to light microscopy, the damage observed in the muscle fibers following LCs was infrequent. Finally, as the number of repetitions increased to 10 and 15, damage markers such as z-line streaming and sarcomeric disruption were observed more frequently.

Fig. 1 Torque loss in TA muscles subjected to 15 lengthenings (LCs), isometric contractions (IMs), or shortening contractions (SCs). Shortening contractions: solid line with squares. Lengthening contractions: dotted line with circles. Isometric contractions: dashed line with triangles. Data are presented as the mean $\pm$ SEM and level of significance was set at $p < 0.05$. ** $p < 0.05$, *** $p < 0.01$, **** $p < 0.001$

Fig. 2 Torque loss in TA muscles subjected to 5, 10, or 15 LCs. Five lengthening contractions: dotted line with triangles. Ten lengthening contractions: solid line with squares. Fifteen lengthening contractions: dashed line with circles. Data are presented as the mean $\pm$ SEM and level of significance was set at $p < 0.05$. **** $p < 0.001$

Fig. 3 Decreased maximal tetanic tension post-stimulation in TAs subjected to LCs but not ICs or SCs. Pre-tetanic values set to 100 with post-tetanic values expressed as a percentage of pre-tetanic value. **a** Shows the pre- and post-tetanic values for each contraction type group

matched to the number of repetitions (15). **b** Shows the pre- and post-tetanic values for LC groups differing in the number of repetitions. Data are presented as the mean $\pm$ SEM and level of significance was set at $p < 0.05$. ** $p < 0.05$, *** $p < 0.01$, **** $p < 0.001$

Fig. 4 LCs increase muscle damage. Hematoxylin and eosin-stained cross-sections of TAs subjected to ICs (**b**) and SCs (**c**) appear undamaged with similar uniformity to control (**a**). Ten or 15 LCs (panels e and f) exhibit few areas of damage while fibers subjected to 5 LCs (**d**) appear undamaged. White arrows indicate damaged muscle fibers. All panels are presented at a $\times 10$ magnification. The scale shown is set to 100 μm

Fig. 5 Cross-sections of TAs subjected to 5, 10, and 15 LCs (panels **d–f**) exhibit areas of ultra-structural damage visible by electron microscopy. Cross-sections of TAs subject to IC (**b**) or SC (**c**) appear undamaged with similar integrity of that of control (**a**). White arrows indicate damaged z-lines. Panel **a** presents a control muscle while panels **b–f** present a representative region of corresponding muscle cross-sections. The scale shown is set to 1 μ m

Intramuscular HSP25 and 72 content

HSP25 (correctly identified as HSPB1; Kampinga et al. 2009) was detected in muscle homogenates by western blotting and quantified by densitometry. Despite a trend towards an increased HSP25 content, when bands from blots were quantified and the data presented as a ratio of stimulated over CL, no significant differences were detected in between LC, IC, and SC groups (Fig. 6 panels a and b). In addition, no significant difference in muscle HSP25 content was detected between the three LC stimulated groups (Fig. 7 panels a and b). Thus, muscle HSP25 content does not appear to be elevated following 15 contractions of any type.

HSP72, the inducible isoform of the HSP70 family (correctly identified as HSPA1A; Kampinga et al. 2009), was also assessed in muscle homogenates following the three

contraction types and additional LCs. A representative western blot showing HSP72 content after the three contraction types is presented in Fig. 6 panel a. When expressed as a fold change relative to CL, no significant increase in HSP72 content was observed in TA muscles subjected to ICs or SCs (Fig. 6 panel C). However, TA muscles subjected to 15 LCs showed a marked significant increase (17.7 ± 3.4 fold; $p < 0.01$) that differed from both SC and IC ($p < 0.001$) groups. Further investigation showed HSP72 content was significantly increased in TA muscles subjected to 5 (4.4 ± 1.0 fold; $p < 0.05$) and 10 LCs (8.4 ± 2.68 fold; $p < 0.05$) (Fig. 7 panels a and c). The HSP72 content following 15 LCs was significantly greater than 5 LCs ($p < 0.01$) but not 10 LCs ($p > 0.05$). These data show that LCs but not SCs or ICs result in increased muscle HSP72 content.

Fig. 6 Intramuscular HSP72 content, but not HSP25, is significantly increased in TA muscles subjected to 15 LCs but not 15 ICs or 15 SCs. **a** Shows a representative western blot bands for HSP25, HSP72, and GAPDH for each contraction type. **b, c** Present HSP content as a fold change from contra-lateral control limb. Data are presented as the mean $\pm$ SEM and level of significance was set at $p < 0.05$

Discussion

As demonstrated by this study and others (Locke et al. 1991, 1995; Skidmore et al. 1995; Thompson et al. 2001; Milne and Noble 2002; Thompson et al. 2002; Thompson et al. 2003; Kim et al. 2004; Touchberry et al. 2012), exercise is capable of increasing muscle HSP content. Since muscle contraction is the basis for any type of exercise, we examined the relationship between three contraction types and two HSPs in rat skeletal muscle. There are several novel features of the present study. First, 24 h following muscle contractions, 15 LCs resulted in a marked increase in TA muscle HSP72 content (but not HSP25 content), while 15 ICs or 15 SCs showed no increase in either HSP content. Secondly, as few as 5 LCs was sufficient to elevate HSP72 content in the rat TA muscle.

Given that studies examining exercise have been inconsistent in showing increases in muscle HSP content (Puntschart et al. 1996), these findings may explain why certain types of exercise elicit greater HSP responses. Furthermore, since cells/tissues with an increased HSP content demonstrate cellular protection (Morimoto 1998), it follows that exercises composed of specific contractions types may allow for increased muscle HSP72 content that may confer protection.

In the present study, the pattern of TA muscle torque observed during the 15 contractions (all types) generally showed a reduction in muscle torque as contraction number increased. This was especially evident towards the latter contractions of each set (repetitions 4 and 5). Muscles stimulated with 15 ICs and 15 SCs recovered during the 2 min of rest between each set, such that there were no differences in muscle torque

Fig. 7 Intramuscular HSP72 content, but not HSP25, is significantly increased in TA muscles subjected to 5, 10, or 15 LCs. **a** Shows a representative western blot band for HSP25, HSP72, and GAPDH for each contraction type. **b, c** Present HSP content as a fold change from contra-lateral control limb. Data are presented as the mean $\pm$ SEM and level of significance was set at $p < 0.05$

between the 1st, 6th, and 11th contractions for these types of contractions. In contrast, muscles stimulated with 15 LCs showed a continuous reduction in muscle torque after the 1st LC and no recovery was conferred by the 2 min of rest provided between sets. While no direct metabolic measures were performed herein, some insights into metabolic status can be gleaned by examining the recovery of muscle function. When maximal tetanic tension (MTT) was assessed for all groups 5 min after the 15th contraction, TA muscles stimulated with 15 ICs or 15 SCs showed a full recovery of MTT suggesting the observed decreases in torque were likely due to transient metabolic perturbations. Since LCs are known to be less metabolically demanding than other contractions (Abbott et al. 1952; Peñailillo et al. 2017), the decrease in torque observed in the LC conditions was likely due to muscle damage and not merely metabolic perturbations. In support of this, a significant 24% reduction in MTT was observed in muscles following stimulation with 15 LCs. There was also a significant reduction in MTT following 5 and 10 LCs. The idea that the loss of TA muscle torque was related to the damage imparted by LCs is further supported by the morphological muscle fiber damage observed following both light and electron microscopy analyses. Indeed, necrotic muscle fibers were observed in all muscles subjected to 15 LCs; though few in number, these fibers showed damage in the form of ruptured cell membranes, immune cell infiltration, and z-line streaming. In contrast, no morphological evidence of damage was observed in muscles subjected to 15 IC or 15 SCs. These data support the established concept that LCs damage muscle fibers resulting in an inability to generate force, while IC and SC contractions are generally less damaging to muscle fibers.

As previously mentioned, exercise is capable of increasing muscle HSP content. However, the exact stimulus within exercise responsible for activating the cellular stress response remains unclear. To date, a number of exercise-related stressors including increased temperature (Milne and Noble 2002; Kim et al. 2004), reactive oxygen species (Benjamin et al. 1990; Dimauro et al. 2016), alterations in pH (Petronini et al. 1995), reduced fuel sources (Benjamin et al. 1992), and calcium alterations (Tupling et al. 2004) have been associated with increased cellular HSP content. Since “heat shock” or increased temperature was the original stressor used to increase cell HSP content, it is not surprising that Milne and Noble (2002) showed that increases in rodent skeletal muscle HSP72 content were strongly related to exercise intensity. However, Skidmore et al. (1995) exercised rats in a cold (14 °C) environment and showed that the increased muscle HSP72 content was independent of core body temperature. For all contraction types used in the present study, the 15 contractions would be unlikely to cause a significant increase in core or muscle temperature. Since it is known that the common feature of HSP inducers is denatured proteins, any stressor that perturbs proteostasis might be expected to increase

HSP content. Since only LCs resulted in an increased muscle HSP72 content, it suggests that the accumulation of damaged muscle proteins from LCs may be the key factor influencing increases in muscle HSP72 content. However, a single protein-damaging stressor or combination of protein-damaging stressors would also be capable of increasing muscle HSP72 content, provided enough damaged proteins have accumulated. The exact contribution of each stressor when acting in a cumulative or synergistic manner remains to be determined.

HSP72 appears to provide protection to cells and tissues including skeletal muscle (McArdle et al. 2003; Touchberry et al. 2012). Thus, an increase in skeletal muscle HSP content should provide protection to stressors such as those imposed by contraction. Indeed, McArdle et al. (2003) demonstrated a blunted decrease in contractile force in the extensor digitorum longus muscle from transgenic mice overexpressing HSP70. Given that as few as 5 LCs increased muscle HSP72 content, it suggests a possible mechanism to help explain a phenomenon known as the “repeated bout effect” (RBE; Thompson et al. 2002; Paulsen et al. 2009). The RBE occurs when a lesser number of lengthening contractions are imposed on a muscle prior to the same muscle experiencing a higher number of lengthening contractions. The prior, less stressful bout, appears to reduce the extent of the damage and performance decrements that would ordinarily occur had the prior bout been omitted. It is tempting to speculate that the induction of HSP72 and possibly other HSPs following a mild stress (a few LCs) might protect muscle fibers against more severe subsequent stressors. Although HSPs have been suggested to play a role in the RBE (Thompson et al. 2002; Paulsen et al. 2009), they have not been adequately investigated.

Skeletal muscles are comprised of fibers that differ in their contractile, metabolic, and biochemical characteristics. The rat TA muscle is comprised predominantly of the three type II (fast) muscle fibers and contains a very low (~3%) percentage of type I (slow) muscle fibers (Armstrong and Phelps 1984). Compared with type I muscle fibers, type II muscle fibers have been shown to be more susceptible to lengthening muscle contractions (Vijayan et al. 2001). While the exact reason(s) for this remains unclear, type I muscle fibers are known to express elevated constitutive levels of HSP72 (Locke et al. 1991). Given that a high level of HSPs confers cellular protection, the elevated constitutive HSP content in type I fibers may also confer protection from the damage associated with lengthening. In contrast, type II muscle fibers, with their low constitutive level of HSPs, may be less well protected and therefore more susceptible to the damage associated with lengthening. According to the Henneman size principle, smaller type I muscle fibers would be recruited prior to the larger type II muscle fibers. Thus, any daily lengthening muscle contractions, such as those opposing gravity, may be capable of increasing muscle HSP content and thereby confer

protection. In other words, normal daily muscle usage involving LCs might be adaptive towards subsequent contractions. Since no evaluation of fiber types was performed in the present study, it remains unclear whether the LC-induced increase in muscle fiber HSP72 content (and damage) was more predominant in specific fiber types (damaged or otherwise), or whether HSP72 was expressed homogeneously in all fibers regardless of type or damage.

In humans, both HSP25 and HSP72 have been reported to increase following LCs and suggested to play a key role in providing protection to cellular structures within skeletal muscle fibers (Thompson et al. 2002; Bozaykut et al. 2014). In the present study, muscles subjected to LCs showed an elevation in only HSP72 content and not HSP25 content. While the exact reason for the lack of an increased HSP25 content remains unclear, a number of reasons such as exercise intensity and/or duration, the number of contractions, the extent of damage, fiber type composition, or even muscle species differences may account for differences observed between our model and others (Paulsen et al. 2009; Thompson et al. 2002).

From an applied perspective, LCs are known to improve muscle function and are commonly used in muscle rehabilitation programs (Alfredson et al. 1998). However, the exact reason for their effectiveness remains unclear (Kingma et al. 2007). It is tempting to speculate that an elevation of HSPs following LCs may explain, at least in part, why LCs are employed over other types of muscle contractions. In addition, rehabilitation protocols (Alfredson et al. 1998) commonly use 3 sets of 15 contractions each. While caution should be used in directly transferring our results to humans, it follows that if an increase in HSP content underlies the use of LC, then our work suggests a lesser number of repetitions would be just as efficient. This may have important implications for muscle rehabilitation programs in that they may be easier to administer and possibly increase adherence.

In conclusion, when matched for the number of contractions, LCs induced a greater HSP72 response compared with both ICs and SCs. At 24 h after a single bout of LCs, rat TA muscle HSP72 content was significantly increased, while there were no significant changes in HSP25 content. Most importantly, this study demonstrated that as few as 5 LCs were capable of significantly increasing muscle HSP72 content with minimal observable muscle damage. Whether increased HSP72 content affords muscle protection remains to be determined.

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Compliance with ethical standards

All procedures were approved by the University of Toronto Animal Care Committee and in accordance with the guidelines of the Canadian Council on Animal Care.

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