Subcellular fractionation reveals HSP72 does not associate with SERCA in human skeletal muscle following damaging eccentric and concentric exercise

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Submitted 4 February 2013; accepted in final form 19 April 2013

Skeletal muscle unaccustomed to exercise is susceptible to muscle damage. This is more evident during eccentric contractions, where the muscle is exposed to greater strain during lengthening contractions, compared with concentric contractions, where the muscle shortens during contractions. Hallmarks of exercise-induced eccentric muscle damage are myofibrillar disorganization, low-frequency and long-duration fatigue, and membrane disruption, in addition to small increases in intracellular Ca2+ concentration and a reduced force-generating capacity (1, 11). Interestingly, if the same type of eccentric contraction is repeated by an individual, the amount of damage seen is reduced compared with the original bout performed (39).

Heat shock proteins are a family of ubiquitously expressed stress proteins that enable cells to survive physiological stress. Heat shock proteins (HSPs) act as molecular chaperones assisting the folding, function, and localization of newly synthesized and partially denatured proteins (24). One of the most notable HSPs is heat shock protein 72 (interchangeably referred to as inducible HSP70 or HSPA1A, to be called HSP72 herein). HSP72 has significant roles in cellular protection and adaptation in skeletal muscle, including as an inhibitor of inflammatory factors (3, 26, 30) and being able to bind to and preserve the function of the sarcoplasmic reticulum Ca2+-ATPase (SERCA)(37). HSP72 mRNA and protein contents can be readily upregulated by several environmental and intracellular stresses, and the overexpression of HSP72 in skeletal muscle confers cytoprotection and muscle function preservation in aged mice (2). There are a multitude of events during and after eccentric exercise that likely contribute to the hallmark of damage described above, and of interest to the current study is the reduced activity of the SERCA reported by others (35) and the necessity for this protein to be repaired and protected to resume normal muscle function. In addition to conferring protection of membrane proteins including Na+-K+-ATPase and K+-channels (7), HSP72 has been shown to stabilize both the structure and function of SERCA after thermal stress (9, 37). In nonstressed muscle, over 80% of the total HSP72 protein pool is readily diffusible and so not already associated with SERCA (16, 28).

Several studies have reported that exercise can induce HSP72 protein expression in both rodent (12, 14, 31, 32) and human (6, 15, 19, 27) skeletal muscle. The effect of exercise on HSP72 protein has also been assessed using the less quantitative immunohistochemical methodology (36). Studies that have examined HSP72 protein translocation have only done so in purified fractions or using immunohistochemistry, where there is no possibility to relate any movement of HSP72 protein in purified fractions or using immunohistochemistry, where there is no possibility to relate any movement of HSP72 protein to its primary membrane-bound target proteins, in particular SERCA.

HSP72; eccentric exercise; absolute quantification; single fibers; repeated bout

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(16). Comparison of HSP72 protein between different muscle fibers in skeletal muscle from untrained individuals has been reported (36), although the very low signal at rest makes it difficult to interpret those data quantitatively. That study, nevertheless, was able to demonstrate that after isometric exercise, a greater increase in HSP72 protein was apparent in Type I compared with Type II fibers, possibly as a consequence of the differing oxidative capacity of Type I compared with Type II fibers. Whether the exercise-induced increases in HSP72 protein are sufficient to protect the numerous potential target proteins and binding sites is unknown because the absolute concentration of HSP72 protein in human skeletal muscle is unknown. Further complicating this is that the stoichiometry of HSP72 and SERCA association is not known.

In this study, to investigate the actions and limitations of HSP72 protein in human skeletal muscle we quantified the fiber type-specific amount of HSP72 protein in skeletal muscle fibers obtained from vastus lateralis biopsies at rest. Furthermore, we measured the response of HSP72 protein in skeletal muscle from healthy individuals prior to, 3 h, 24 h, and 7 days postexercise. Biopsies were taken under local anesthetic, and at least 3 cm separated each sampling site. Biopsies were taken from both the concentrically and eccentrically exercised leg after the first bout of exercise, whereas a biopsy was taken only from the eccentrically exercised leg after the second bout. A control group of subjects (n = 5) had biopsies taken from one predetermined leg using the same time protocol, allowing the isolated effects of repeated biopsy sampling to be evaluated. Samples were immediately frozen in liquid N2.

MATERIALS AND METHODS

Subjects

The present study is an extension of a larger study that is described in previous papers (21, 22, 38, 39). For this study, tissue from a subsample of subjects from the previous study were used (11 subjects for whom full data sets were available and 5 control subjects; mean ± SD: 24 ± 3 yr old, 183 ± 9 cm height, 79 ± 8 kg mass). All participants were male, healthy, and physically active. Informed written consent was given by all the subjects, and all experimental protocols were approved by the Danish Ethical Committee of Aarhus (J. ne. 20040159) and carried out in accordance with Declaration of Helsinki. In addition, individual fibers were collected from freeze-dried human vastus lateralis muscle collected from resting healthy, young, active, untrained male volunteers (n = 3) from a separate study (20), which was spare tissue originally collected for other completed research projects approved by the Human Research Ethics Committee at the University of Melbourne.

Exercise Protocol

The exercise protocol is described in detail elsewhere (22, 39). Briefly, subjects (n = 11) performed a single bout of ~30 min of bench-stepping at 60 steps/min at a predetermined step height of 110% of the lower leg length. Thus stepping up with one leg causes the muscles involved in knee extension to work concentrically (CONC), and stepping down with the same leg first instigates the same muscles in the opposite leg to work eccentrically (ECC). After 8 wk, the same subjects repeated the exercise bout using the same protocol and performed the exercise using the same legs for CONC and ECC as the first exercise bout, with biopsies being taken from the eccentrically contracted leg only (ECC2).

Muscle Biopsies

For the exercise biopsies, samples were obtained from the vastus lateralis 1 wk prior to each exercise bout (pre) and at 3 h, 24 h, and 7 days postexercise. Biopsies were taken under local anesthetic, and at least 3 cm separated each sampling site. Biopsies were taken from both the concentrically and eccentrically exercised leg after the first bout of exercise, whereas a biopsy was taken only from the eccentrically exercised leg after the second bout. A control group of subjects (n = 5) had biopsies taken from one predetermined leg using the same time protocol, allowing the isolated effects of repeated biopsy sampling to be evaluated. Samples were immediately frozen in liquid N2.

Collection of Single-Muscle Fibers

As described in an earlier study (20), segments of individual fibers were dissected from freeze-dried portions of human muscle (n = 3) using a dissection microscope and fine jewelers forceps. By use of a television connected to the microscope, fiber length was measured (10 ± 2.9 mm, mean ± SD, n = 50). By use of the forceps, fibers were placed in 10 μl 3× SDS loading buffer (0.125 M Tris-Cl, pH 6.8, 4% SDS 10% glycerol, 4 M urea, 10% mercaptoethanol, 0.001% bromophenol blue) diluted 1:2 (vol:vol) with Na+ homogenization solution (see below) and stored at −20°C until analyzed by Western blotting.

Whole Human Muscle Preparation

To determine the level of HSP72 protein in muscle samples with all constituents present (i.e., no centrifugation), muscle samples were cut into 10-μm sections (model CM1950 cryostat, Leica, Melbourne, Australia). Samples collected from each individual (n = 11) for each intervention (ECC1, ECC2, CONC) and from each time point (n = 4), as well as the each time point for the control individuals (n = 5) were used (152 samples in total). Depending on the diameter of the muscle sample, eight to ten sections were placed immediately in 200 μl of cold Na+ homogenization solution containing in mM: 165 Na+, 1 free Mg2+ (10.3 total Mg2+) 90 HEPES, 50 EGTA, 8 ATP, and 10 creatine phosphate (pH 7.10) with phosphatase inhibitor (phosphostop, Roche Diagnostics), 295 μmol/kgH2O, where the strong EGTA buffering kept the free [Ca2+] very low (<10 nM) at all times. Samples were kept on ice (~10 min) until homogenized. After homogenization, 50 μl of the sample were then added to 3× SDS loading buffer (2:1 vol:vol), and the remainder of the sample was used for crude fractionation. Samples were then stored at −20°C until Western blot analysis.

Crude Fractions for Determination of Distribution of HSP72 in Muscle

To determine the amount of HSP72 protein in muscle samples with all constituents present (i.e., no centrifugation), muscle samples were cut into 10-μm sections (model CM1950 cryostat, Leica, Melbourne, Australia). Samples collected from each individual (n = 11) for each intervention (ECC1, ECC2, CONC) and from each time point (n = 4), as well as the each time point for the control individuals (n = 5) were used (152 samples in total). Depending on the diameter of the muscle sample, eight to ten sections were placed immediately in 200 μl of cold Na+ homogenization solution containing in mM: 165 Na+, 1 free Mg2+ (10.3 total Mg2+) 90 HEPES, 50 EGTA, 8 ATP, and 10 creatine phosphate (pH 7.10) with phosphatase inhibitor (phosphostop, Roche Diagnostics), 295 μmol/kgH2O, where the strong EGTA buffering kept the free [Ca2+] very low (<10 nM) at all times. Samples were kept on ice (~10 min) until homogenized. After homogenization, 50 μl of the sample were then added to 3× SDS loading buffer (2:1 vol:vol), and the remainder of the sample was used for crude fractionation. Samples were then stored at −20°C until Western blot analysis.
Table 1. Antibody details

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MHC, myosin heavy chain; HRP, horseradish peroxidase; DSHB, Developmental Studies Hybridoma Bank.

therefore these fractions contain the total distribution of HSP72 protein with enrichment but no loss of sample.

**Western Blotting**

**Protein distribution.** Protein from total homogenate samples, purified HSP72 protein, and individual fiber segments were separated on 4–15% Criterion Stain Free gels (BioRad, Hercules, CA). Gels were imaged (BioRad Stain Free imager), and then protein was transferred from the gel onto nitrocellulose membrane and blocked with blocking buffer (5% skim milk in Tris-buffered saline with Tween 20) for 2 h. After blocking, membranes were generally cut between the 72- and 55-kDa markers into two sections, and the separate membranes were probed with the required primary antibody (Table 1) overnight at 4°C and for at least 2 h at room temperature (RT) with constant rocking. Subsequent to washes, secondary antibodies (Table 1; both diluted 1 in 20,000 in blocking buffer) were applied for 1 h (RT). Images of the membrane were collected after exposure to chemiluminescence substrate (Thermo Scientific SuperSignal West Femto) using a CCD camera attached to Chemi Doc MP (BioRad), and Image Lab 4 software was used for the collection of images and Quantity One software used for densitometry (both from BioRad). The positions of molecular mass markers were captured under white light prior to chemiluminescent imaging without moving the membrane. Each Western blot had a calibration curve consisting of the same human whole homogenate mixture on each blot, allowing values obtained from different gels to be compared. All samples from one subject for a given exercise protocol were run on the same gel.

**Absolute quantification of HSP72 protein.** For analyses of the absolute amount of HSP72 protein, total muscle homogenates (4–25 μg wet weight muscle) obtained from the resting biopsy for each exercised (n = 11) or nonexercised (n = 3) subject were loaded onto a gel together with known amounts of purified human recombinant HSP72 protein expressed in Escherichia coli [range 2.5–30 ng (Stressmarq cat.SPR-103)], the latter allowing a calibration curve to be generated (see Fig. 1). On a given gel, three different amounts of a sample were run (~2–20 μg tissue), and those that fell within the range of the pure HSP72 protein calibration curve were used for quantification. Samples were run on up to three gels with similar results for repeats of the same sample on the same or different gels, which were then averaged to give the final HSP72 concentration for an individual. The mass of human muscle in each lane was determined using the sum of the density of all the bands on the Stain Free gel andclass="ref" data-ref-type="fn" data-ref-number="1">a</class> calibration curve derived from known amounts of rat SOL muscle run on the same gel (not shown). A calibration curve was generated for each gel by plotting the density of the Western blot signal for HSP72 for each purified HSP band against the amount of HSP72 protein loaded (Fig. 1B). When quantifying absolute amounts of HSP72, the density of the relevant band was converted to an equivalent protein amount, according to the calibration curve derived from the pure protein sample run on the same Western blot. The amount of HSP72 protein in a given sample was expressed relative to the mass of muscle loaded in that lane, and the average was calculated for all repeated samples run on the same gel. Once the absolute amount of HSP72 protein was determined for an individual then the relative amounts between conditions could be expressed as absolute amounts of HSP72 protein. This was done by taking the absolute amount of HSP72 protein in the resting sample for an individual and then expressing the HSP72 content in a given sample based on this resting amount and the relative changes in HSP72 protein observed between samples for the given individual.

**Relative quantification of HSP72 protein.** For comparison of the amount of HSP72 protein in different fiber types, the sum of the density of all the bands on the Stain Free gel was taken as a measure of the relative total protein in a given fiber. The density of HSP72 protein in a given fiber was expressed relative to this total amount of sample and then expressed relative to the mean of all Type I fibers ran on that same gel (generally 6–11 fibers) from the same subject. Fiber types were identified by the presence of myosin heavy chain type I (MHCII) and SERCA2 for Type I (slow twitch) fibers or the presence of MHCII and SERCA1 for Type II (fast twitch) fibers [as seen in Fig. 2 and see (20)]. There were two fibers that had both types of MHC and SERCA proteins present that were deemed to be either hybrid fibers or presented a situation where two fibers had been simultaneously collected. These two fibers were excluded from further comparisons (see fiber labeled H in Fig. 2A).

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**Fig. 1. Quantification of heat shock protein (HSP) 72 shown for 2 subjects.** A: total protein from human muscle homogenates (4–25 μg muscle wet weight, n = 2) and 2.5–20 ng purified HSP72 were separated on a 4–15% Criterion Stain Free gel and HSP72 detected by Western blotting. B: calibration curve derived by plotting the band density of each purified HSP72 against the known amount of HSP72 protein content and then HSP72 content in each subject determined from the calibration curve (H1: 4.6 ng in 6 μg of muscle; H2: 14 ng in 17 μg of muscle), equating to 10.7 and 11.4 μmol/kg wet weight, respectively. AU, arbitrary units.
Exercise and Muscle Damage

Eccentric-induced muscle damage was measured by a number of indicators, as described in detail elsewhere (22, 39). Briefly, there was a 17% decrease in muscle force 1–2 days after eccentric exercise, and there was no significant force reduction in the concentrically exercised leg. Eccentric exercise also resulted in an ~40% increase in perceived muscle soreness over that of the concentrically exercised leg 2 days after exercise. Other indicators measured 3 and 4 days post ECC1 included ~100-fold increase in plasma myoglobin and ~10–30-fold increase in plasma creatine kinase, also suggesting muscle damage, likely from the eccentrically exercised leg.

Absolute and Relative Amounts of HSP72 Protein in Single Fibers and Whole Skeletal Muscle

The absolute amount of HSP72 protein present in resting human vastus lateralis muscle was 11.4 ± 0.7 μmol HSP72/kg wet weight muscle mass (n = 14, see examples in Fig. 1). This amount of HSP72 protein was measured in whole muscle homogenates consisting of both Type I and Type II fibers. Analyses of individual fiber segments revealed no difference in the amount of HSP72 between Type I and Type II fibers (Fig. 2), although for all three subjects there was a large variability (3–10 fold) in the relative amounts of HSP72 protein between the same fiber types obtained from the same individual (Fig. 2B).

Exercise Effects of HSP72 Protein Expression in Whole Muscle

Analyses of the HSP72 protein in control (nonexercised) subjects showed no change in HSP72 protein content between the different biopsy times (pre: 1.0 ± 0.2; 3 h: 1.0 ± 0.3; 24 h: 1.4 ± 0.6; and 7 days: 1.2 ± 0.3; means ± SE, amounts were normalized to pre levels; n = 5, P > 0.05). In the exercised subjects, there was no significant difference in the amount of HSP72 initially present in the contralateral legs, that is, between the pre levels for the ECC1 and CONC cases. With ECC1, compared with before exercise (pre), the amount of HSP72 protein was ~1.8-fold higher 24 h after the exercise, but was not significantly different at the other time points (3 h or 7 days; Fig. 3B). In contrast, there was no significant change in the amount of HSP72 protein at any of the time points after the CONC exercise. With ECC2, the second bout of eccentric exercise performed 8 wk later, the pre level of HSP72 was not significantly different from that present before the original ECC1 bout, and there was no significant change at any time point after ECC2 (Fig. 3B). The HSP72 content (when expressed relative to pre) for a given individual did not significantly correlate with their percent decline in force observed after any type of exercise (ECC1: y = 0.02x + 1.4, r² = 0.022, P = 0.7, n = 11; ECC2: y = 0.08x + 0.6, r² = 0.33, P = 0.065, n = 11; CONC: y = 0.006x + 1.2, r² = 0.006, P = 0.8, n = 11). To get the full spectrum of force changes, all exercise types were grouped (i.e., ECC1, ECC2, and CONC) and there was also no significant correlation between force decline for a given individual and their HSP72 protein content (y = 0.08x + 1.1, r² = 0.09, P = 0.09, n = 33).

Statistical Analyses

HSP72 protein data from both whole muscle and crude fractionation studies were normalized to a given sample’s bands on the Stain Free gel images (myosin band for whole muscle or all bands, i.e., total protein, for fractionation data) and normalized to the calibration curve. Data were standardized to average value for Pre samples (ECC1 pre for both sets of ECC data and CONC pre for CONC data) and expressed as means ± SE. Data were not normally distributed and so were subjected to the Friedman nonparametric repeated-measures test for pre vs. all combinations of times and bouts followed by post hoc analysis (Dunn’s multiple comparison test). All statistics were performed using GraphPad Prism version 4. Significance was set at P < 0.05.
Distribution of HSP72 Protein after Exercise

Crude fractionation into cytosolic, membrane, and cytoskeletal fractions was used to ascertain the amount of HSP72 in different skeletal muscle compartments (Fig. 4). The presence of the protein GAPDH and lack of myofibrillar or membrane proteins in the cytosolic fraction is shown (Fig. 4). As expected cytoskeletal proteins such as MHCIIa, actin, and tropomyosin are present in the cytoskeletal fraction. The membrane/nuclear fraction contained SERCA2 proteins and also contained lamin A/C, an associated nuclear protein (not shown). For a subset of exercised samples (n = 5) we also examined SERCA1 and found that it, as well as SERCA2, was not seen in the cytosol in appreciable amounts at any time (not shown). The Stain Free image of the gel also indicates the distribution of prominent proteins in the various fractions.

Almost all of the HSP72 protein was found in the cytosolic fraction, and in ECC1 this did not change either with time after exercise (Figs. 4 and 5) or with the type of exercise (not shown, P > 0.05). If any HSP72 protein was present in either the membrane or cytoskeletal fraction it was typically below the resolution limit, and clearly the great majority of HSP72 still remained in the cytosolic fraction (Fig. 5). There was no significant change in the amount of HSP72 in either the membrane or cytoskeletal fraction for any time point (see Figs. 4A and 5) or type of exercise (not shown).

Cytosolic HSP72 and HSP72 Distribution

The amount of HSP72 protein in the cytosolic fraction was examined before (pre), 3 h, 24 h, and 7 days after ECC1, CONC, and ECC2 as well as in control samples. There was ~3- and 1.7- fold increases in the amount of HSP72 in the cytosolic fraction 24 h and 7 days after ECC1, respectively (Fig. 4C). There was no change in the amount of HSP72 protein in the cytosolic fraction compared with pre for either CONC or ECC2 samples (P > 0.05, not shown).

DISCUSSION

The five major findings of this study are 1) the amount of HSP72 protein is similar between Type I and Type II fibers obtained from resting human muscle; 2) that ~2-fold upregulation of HSP72 protein in human skeletal muscle occurs with an initial bout of eccentric exercise; 3) there is no detectable translocation of HSP72 protein and HSP72 protein remains in the cytosol at 3 h, 24 h, and 7 days after exercise, despite the damaging nature of the eccentric contractions performed; 4) HSP72 and SERCA1/2 never appear in the same crude fractionation, indicating they could not associate either at rest or after eccentric damage; and 5) in resting human skeletal muscle the absolute quantification of HSP72 protein in human skeletal muscle was determined to be ~11 μmol/kg wet mass.

Upregulation of HSP72 with Eccentric Exercise

The step exercise protocol used for the current study enabled direct comparison of the effects of concentric (CONC) and eccentric (ECC1) exercise, as well as a bout of repeated eccentric exercise (ECC2) performed 8 wk after ECC1 in the same leg of the same subject. By use of muscle force and muscle soreness as a measure of muscle damage, the eccentrically contracted leg showed a higher magnitude of muscle damage than the concentrically contracted leg, as previously reported for the subjects used in the present study (39).

We hypothesized that an increase in HSP72 protein would occur after ECC1 as a consequence of the damage-inducing contractions. We observed only a modest increase (~2-fold) in HSP72 protein 24 h after ECC1 and notably, this did not correlate with the amount of force decline seen 24 h after the exercise bout. This remained the case when data points for all exercise types were used (n = 33). In the present study a realistic exercise protocol was chosen to mimic a regime that might be undertaken under normal exercising conditions. The ~17% force decline, relative to the force produced before exercise and used as a marker of muscle damage, was also
modest compared with much greater effects with more intense bouts of eccentric exercise (27). By use of Western blotting, previous studies reported variable responses of HSP72 protein content to exercise from 2-fold to 30-fold increases, seemingly dependent on exercise intensity, duration, or timing of muscle biopsy collection (33, 34). Although a two-fold increase in HSP72 protein was observed 3 h after exhaustive (4–5 h) knee extensor exercise in a glycogen-depleted leg, with no difference in the control leg (6), a 30-fold increase in HSP72 protein 6 days after single-legged cycling was reported (15). Neither of those studies performed isolated eccentric contractions and the differences in nutritional status between most studies and that by Febbraio and colleagues (6) make it difficult to compare these studies. Other investigations on exercise-induced HSP72 protein have employed immunohistochemical methodologies. In one such study, eccentric exercise increased the number of muscle fibers expressing HSP72 (28). Another study showed minimal HSP72 protein staining in resting muscle, but clear increases in HSP72 protein in at least some of the Type I fibers, with seemingly less response in Type II fibers (36). The major advance of our study is that we analyzed muscle in its entirety, with no loss of sample and as such provide quantitative measures for HSP72 protein.

The repeated bout effect is well documented, whereby an individual quickly adapts to even a single bout of eccentric exercise after which there is less damage seen in the muscle fibers (8, 38). We did not observe any significant change in HSP72 protein after ECC2; however, we cannot rule out that the peak response was missed because of the timing of the muscle biopsies (i.e., peak might have occurred between 24 h and 7 days) or that the intersubject variability masked differences. Our data suggest that a stimulus other than the damage occurring as a result of eccentric exercise caused the increase in HSP72 protein, and further work is required to elucidate the mechanism.

No translocation of HSP72 with Eccentric Exercise

A major finding of the present work is that despite HSP72 protein increasing after ECC1, the protein remains almost exclusively within the cytosolic environment. By use of the crude fractionation methodology where no constituent is dis-
carded and samples are analyzed side by side, we are able to discuss the total cellular pool of HSP72 protein and quantitatively compare between the crude fractions obtained. Samples from the current study had previously been analyzed using a fractionation technique that created enriched purified cytosolic and cytoskeletal fractions (28). By use of that technique it is necessary to discard portions of the sample during the enrichment steps to obtain purified fractions and hence obtain crucial data about whether HSP72 protein was indeed present in a given fraction; however, it did not provide information about the total muscle pool of HSP72 (27, 38). Prior to exercise, there was little detectable HSP72 protein in either the crude membrane or crude cytoskeletal fractions, with the large majority of the total pool being seen in the cytosolic fraction (Fig. 5). The pattern was the same at all time points after all exercise regimes (ECC1 seen in Figs. 4 and 5). Validation of the crude fractionation process revealed only a small amount of contamination between fractions, notably with the SERCA 1/2 appearing in both the membrane and cytoskeletal fractions and a small amount of the cytosolic protein GAPDH in the cytoskeletal fraction. Perhaps surprisingly, the HSP72 and the SERCA1/2 proteins were not seen in the same fraction at any time points examined, which implies that they cannot be associated to any great extent or that any association must be only transient in nature. This also suggests that previous work reporting translocation of HSP72 proteins after eccentric exercise was detecting only a very small absolute amount of the total HSP72 pool redistributing (28) and the significance of such small movements is unclear.

**HSP72 Protein Content is Similar in Type I and Type II Fibers from Resting Human Muscle**

Single fiber Western blotting was used to demonstrate that HSP72 protein is similarly expressed in Type I and Type II fibers obtained from resting vastus lateralis in humans. A previous study also stated that HSP72 protein in resting muscle was not fiber type dependent; however, in that work the less quantitative approach of immunohistochemistry was used, and of note, the HSP72 staining was very low and any nonlinearities in the detection could not have been taken into account (36). Importantly, our study used calibration curves on all blots, and the amount of HSP72 protein in all single fiber samples fell within this calibration curve. Although the different fibers appear as a broadly homogenous group, the amount of HSP72 protein in individual fibers of a given type obtained from a given individual is highly variable (Fig. 2B). The amount of HSP72 protein in separate fibers from the same individual sometimes showed a 10-fold difference in abundance, perhaps indicating that individual fibers undergo different cellular stress levels or perhaps cell cycle transitions at a given time. This fiber variability may also explain why there is a general trend for HSP72 protein levels to increase with any type of exercise and some subjects respond to a greater extent to a given exercise stimulus (6, 15, 19, 27). Other work has identified that HSP72 protein is fiber type dependent in rodent and rabbit (13, 16, 18, 23). The species difference identified likely reflects the extreme nature of Type IIB fibers in rodents, being almost exclusively glycolytic and with very fast contractile properties, which predominant in the fast-twitch muscles examined in those studies. Type IIB fibers are not present in human skeletal muscle, and the presence of Type IIX fibers is quite variable between individuals, with some individuals having zero% or up to 30% Type IIX fibers (4) and studies typically reporting between ~3 and 8% Type IIX fibers in a study cohort (4, 29).

A limitation of our study is that although we measured HSP72 in single fibers in resting muscle, it was not possible to measure the fiber type specific HSP72 response after exercise because the muscle samples from this study were embedded for cryosectioning and not optimal for single fiber dissection. As mentioned, previous work has shown an augmented HSP72 protein response in human Type I compared with Type II fibers after isometric exercise (36). Given that 1) there is more damage in Type II compared with Type I fibers after eccentric contractions (8), 2) there is a higher concentration of SERCA pumps in Type II compared with Type I fibers (5), and 3) that HSP72 protein levels are not different between fiber types at rest (Fig. 2), we hypothesize that HSP72 protein would need to increase to a greater extent in Type II fibers to provide the necessary cytoprotection, concurring with the amount of damage likely present after eccentric exercise. This hypothesis warrants further investigation.

**Absolute HSP72 Protein Content in Human Skeletal Muscle**

We quantitatively determined the absolute amount of HSP72 protein in resting human skeletal muscle to be ~11 μmol/kg wet weight muscle mass. This was achieved by comparing amounts of HSP72 in samples with a standard curve generated using purified recombinant human HSP72 protein. The determined amount is substantially higher than that found in rat EDL and SOL muscles (1.1 and 4.6 μmol/kg wet weight, respectively) (16), possibly reflecting species differences. From other findings here (Fig. 3B) it can be inferred that the amount of HSP72 present in human vastus lateralis muscle increased to ~20 μmol/kg wet weight tissue 24 h after ECC1. Upon appropriate stress, HSP72 protein can bind to both SERCA1 and SERCA2 (9, 37), as well as many other proteins including Na+-K+-ATPase (7). Quantitatively, the concentration of SERCA in muscle is much higher than other target proteins and as such is the most important binding site of HSP72. The total amount of SERCA is ~110 and 20 μmol/kg wet weight muscle in rat EDL and SOL muscles, respectively (40), with similar amounts also found in mouse (25). These relative amounts of SERCA protein fit well with phosphate incorporation assays that determined an ~5-fold difference between rat EDL and SOL muscles (5). The latter study further found the levels to be ~10-fold lower in human vastus lateralis muscle compared with rat EDL muscle. Together, these data suggest that mixed human vastus lateralis muscle contains ~10–20 μmol SERCA/kg wet weight muscle. Given our findings here that the amount of HSP72 is ~11 and ~20 μmol/kg wet weight muscle at rest and after ECC1, respectively, we can now state that there is sufficient HSP72 protein to bind to and protect most if not all of the SERCA proteins present in human muscle fibers, assuming a single binding site per SERCA. We note here that although there are other membrane targets of HSP72 protein, such as Na+-K+-ATPase, the latter is present at ~15-fold lower concentration than SERCA in human skeletal muscle (5). In summary, our study showed that the ECC1 bout was sufficient to upregulate the
Consideration of HSP72 Content in Muscle

Our findings could be interpreted to suggest that HSP72 protein might not need to bind tightly to sites such as SERCA to confer its cytoprotection, although there are data that would suggest the opposite (9, 37). We previously showed using skinned rat fibers that binding of a high proportion of the HSP72 protein pool can be instigated if the muscle is subjected to enough of a particular type of stress (17). In that study, HSP72 remained diffusible in muscle heated to 40°C, but almost the entire pool was found to be bound at 45°C, with a 50% response in muscle exposed to above 44°C (17). This binding was likely to SERCA, as it has been shown that HSP72 binds tightly to SERCA with heat stress (9, 37). Overall, our findings show that HSP72 binding is not triggered by stressful stimuli per se and that tight binding instead seemingly depends on the specific type of stress (e.g., heat vs. damaging eccentric exercise). It is nevertheless possible that the instigation of HSP72 activity is very transient and that the HSP72 only remains bound to a substrate if given a high enough level of overall stress. Unraveling these modes of action is vital to HSP72 biology given the role that HSP72 plays in conferring cytoprotection, highlighted by the recent identification of a role for HSP72 in the pathogenesis of Duchenne muscular dystrophy using a mouse model (10).

ACKNOWLEDGMENTS

We thank Prof. Glenn McConell (current address Victoria University, Australia) for providing the human muscle samples for freeze-dried fiber collection. The monoclonal antibodies directed against adult human MHC isoforms (A4.840 and A4.74) used in the present study were developed by Dr. Blau, those directed against SERCA1 were developed by Dr. D. Fambrough. All were obtained from the Development Studies Hybridoma Bank, under the auspices of the NICHD and maintained by the University of Iowa, Department of Biological Sciences, Iowa City, IA 52242.

GRANTS

This study was supported by National Health and Medical Research Council of Australia (1051460).

DISCLOSURES

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

AUTHOR CONTRIBUTIONS


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