Muscle fiber type-specific response of Hsp70 expression in human quadriceps following acute isometric exercise

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Tupling AR, Bombardier E, Stewart RD, Vigna C, Aqui AE. Muscle fiber type-specific response of Hsp70 expression in human quadriceps following acute isometric exercise. J Appl Physiol 103: 2105–2111, 2007.—To investigate the time course of muscle fiber type-specific heat shock protein 70 (Hsp70) expression in human skeletal muscle after acute exercise, 10 untrained male volunteers performed single-legged isometric knee extensor exercise at 60% of their maximal voluntary contraction (MVC) with a 50% duty cycle (5-s contraction and 5-s relaxation) for 30 min. Muscle biopsies were collected from the vastus lateralis before (Pre) exercise in the rested control leg (C) and immediately after exercise (Post) in the exercised leg (E) only and on recovery days 1 (R1), 2 (R2), 3 (R3), and 6 (R6) from both legs. As demonstrated by Western blot analysis, whole muscle Hsp70 content was unchanged (P > 0.05) immediately after exercise (Pre vs. Post), was increased (P < 0.05) by ~43% at R1, and remained elevated throughout the entire recovery period in E only. Hsp70 expression was also assessed in individual muscle fiber types I, IIA, and IIX/IIB by immunohistochemistry. There were no fiber type differences (P > 0.05) in basal Hsp70 expression. Immediately after exercise, Hsp70 expression was increased (P < 0.05) in type I fibers by ~87% but was unchanged (P > 0.05) in type II fibers (Pre vs. Post). At R1 and throughout recovery, Hsp70 content in E was increased above basal levels (P < 0.05) in all fiber types, but Hsp70 expression was always highest (P < 0.05) in type I fibers. Hsp70 content in C was not different from Pre at any time throughout recovery. Glycogen depletion was observed at Post in all type II, but not type I, fibers, suggesting that the fiber type differences in exercise-induced Hsp70 expression were not related to glycogen availability. These results demonstrate that the time course of exercise-induced Hsp70 expression in human skeletal muscle is fiber type specific.

HEAT SHOCK PROTEINS (HSPs) are families of highly conserved stress proteins that are induced by several environmental and intracellular stresses (16). In mammalian cells, the members of the 70-kDa family (HSP70s) may be the most highly induced proteins of the cellular stress response (17, 43). The HSP70 family is composed of constitutively expressed proteins (or cognate) designated Hsc70 and highly inducible members designated Hsp70, which are rapidly upregulated under conditions of oxidative stress (13, 43). After the demonstration by Locke et al. (18) that a single bout of exercise can induce Hsp70 expression in rat skeletal muscle, several other studies reported exercise-induced increases in Hsp70 content in rodent (11, 24, 31, 33, 34, 38) and human (6, 14, 25, 36) skeletal muscle.

Skeletal muscle is a very heterogeneous tissue composed of a large variety of functionally diverse muscle fibers that possess unique patterns of gene expression. Muscle fiber type differences in the basal expression of Hsp70 (i.e., unstressed muscle) have been noted in various animal species, with the highest levels found in muscles that are composed predominantly of type I fibers (i.e., soleus) compared with muscles that are composed predominantly of type II fibers (i.e., extensor digitorum longus and white gastrocnemius) (10, 11, 18, 19, 24). Comparisons between different muscle fiber types within a given muscle by immunohistochemical techniques have revealed that basal expression of Hsp70 is largely restricted to type I and IIA muscle fibers in rabbit tibialis anterior (27) and rat plantaris (30), with the highest levels found in type IIA fibers.

In response to exercise, increased Hsp70 expression in rat skeletal muscle has been observed in all muscle fiber types that are recruited during exercise (24), but some animal studies have shown that the time course of changes in Hsp70 content and the relative increase in Hsp70 in response to acute (11) and chronic (10, 27) activity may be fiber type specific. In these studies, the relative increase in Hsp70 content is highest in muscles composed predominantly of type II muscle fibers, which is likely related to the lower basal Hsp70 levels in these muscles. With chronic exercise, two studies have shown that induction of Hsp70 occurs more rapidly in slower oxidative (type I and IIA) muscle fibers than in fast (type IIX and IIB) muscle fibers (10, 27), which could largely be explained by differences in fiber type recruitment patterns. Nevertheless, there may be inherent fiber type differences in the Hsp70 response to exercise that are independent of differences in motor unit recruitment. Moreover, no study has investigated fiber type-specific expression of Hsp70 in human skeletal muscle under basal conditions or in response to exercise.

In this study, immunohistochemistry coupled with myosin ATPase staining was performed on serial cross sections of muscle biopsy samples from the vastus lateralis of young healthy men at rest (before exercise) and at selected times for up to 6 days following a single bout of intermittent isometric knee extensor exercise. It was hypothesized that Hsp70 expression would be higher at rest and would increase more rapidly in response to exercise in type I than type II fibers but that the relative peak increase in Hsp70 content after exercise would be higher in type II fibers.

METHODS

Participants. Ten untrained but healthy male university students participated in the study. Their mean age, height, and mass were 18.0 ± 0.47 yr, 173.8 ± 2.7 cm, and 70.5 ± 3.43 kg, respectively. As

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a condition of entry into the study, participants could not be engaged in vigorous exercise on a regular basis and were asked to avoid vigorous exercise completely for a 2-wk period spanning 1 wk before starting the study until completing the study. All participants were fully informed of all experimental procedures and all associated risks before written consent was obtained. Written approval for the research was granted by the Office of Research Ethics at the University of Waterloo.

Experimental protocol. The participants were asked to perform a single-legged isometric knee extension protocol with the exercised leg randomized among participants. The experimental setup and exercise protocol are described elsewhere (8, 37). Briefly, the exercise protocol consisted of 30 min of contractions at 60% maximal voluntary contraction (MVC) and 50% duty cycle with a contraction and relaxation schedule set at 5 s of contraction-5 s of relaxation. MVC torque was determined in a familiarization session that was held 4–6 days before the beginning of the experiment. On average, MVC torque of the participants was 550 ± 36 N·m. The target force was maintained throughout most of the protocol, although seven participants were unable to maintain a target of 60% MVC in the last minutes of the exercise session (data not shown). All participants completed 30 min of exercise, despite the neuromuscular fatigue.

Muscle tissue samples (~50 mg) were obtained from the vastus lateralis with use of the needle biopsy technique under suction immediately before (Pre) exercise in the rested control leg (C) and after (Post) the exercise protocol in the exercised leg (E) only and at 1 (R1), 2 (R2), 3 (R3), and 6 (R6) days after exercise in C and E. The biopsies were taken from each leg from five separate tissue-sampling sites under local anesthesia (1% xylocaine). The control leg was included in our design so that we could distinguish between the effects of exercise per se and the effects of repeated muscle biopsies on fiber type-specific Hsp70 expression, because it has been shown that Hsp70 mRNA levels increase in the absence of exercise because of the sampling itself (40). One portion of the biopsy samples was oriented under a dissecting microscope, mounted with optimal cutting temperature medium, rapidly frozen in isopentane that was precooled with liquid nitrogen, and stored at −80°C. These samples were used for histochemical determination of fiber type-specific Hsp70 expression and glycogen content. The remaining portion of the biopsy samples was diluted in a sample buffer and homogenized as described previously (37). Small aliquots of homogenate were then quick frozen in liquid nitrogen and stored at −80°C for later analysis of protein content and determination of Hsp70 expression by Western blotting.

Immunohistochemistry and histochemistry. Immunohistochemistry and histochemical analyses were performed on serial cross sections of tissue (8–10 µm) that were cut in a cryostat maintained at −20°C. Hsp70 immunohistochemistry was carried out according to the procedures described by Neufet al. (27) with minor modifications. Briefly, frozen muscle sections were fixed to microscope slides in a 100% cold acetone solution for 10 min, washed (once for 5 min) in PBS (10 mM, pH 7.2), and permeabilized in 0.5% Triton X-100 in PBS for 5 min. After another wash (3 times for 5 min each) in PBS, sections were blocked with 5% horse serum solution for 30 min at 22°C in a humidified chamber. The primary monoclonal antibody specific to the inducible form of Hsp70 (SPA-810, Stressgen Biotechnologies) was applied to the sections (1:200 dilution in PBS) for 1 h at room temperature. After the sections were washed (3 times for 5 min each) in PBS, biotinylated horse anti-mouse immunoglobulin was diluted in a sample buffer and homogenized as described previously (37). Small aliquots of homogenate were then quick frozen in liquid nitrogen and stored at −80°C for later analysis of protein content and determination of Hsp70 expression by Western blotting.

Western blot analysis. Western blotting was performed to determine the relative expression levels of Hsp70 in whole muscle homogenates prepared from muscle biopsy samples. After linearity of band density was ensured, samples were applied to 10% polyacrylamide gels, and proteins were separated using standard SDS-PAGE protocols (15) and then transferred to polyvinylidene difluoride membranes (Roche Diagnostics, Mannheim, Germany). After they were blocked with a 10% skim milk suspension, the membranes were incubated for 16 h at 4°C with anti-Hsp70 monoclonal antibody (SPA-810). Then, after the membranes were washed in Tris-buffered saline-0.1% Tween, they were treated with horseradish peroxidase-conjugated anti-mouse secondary antibody (Santa Cruz Biotechnology). The membranes were washed again, and the signals were detected with an enhanced chemiluminescence kit (Amersham Pharmacia Biotech) using a bioimaging system, and densitometric analysis was performed using GeneSnap software (Syngene). All samples were run in duplicate on separate gels, and Hsp70 content was expressed relative to Pre levels.

Statistical analyses. A repeated-measures two-way ANOVA was employed to analyze the fiber type-specific (type I vs. type IIA vs. type IIX/IIAX) changes in Hsp70 and glycogen content in response to exercise and during recovery (Pre vs. Post vs. R1 vs. R2 vs. R3 vs. R6). To determine the effects of the exercise protocol and recovery on changes in whole muscle Hsp70 content and fiber type-specific peak changes in Hsp70 expression, a one-way ANOVA with repeated measures was used. The significance level was set at 0.05, and, when appropriate, a Newman-Keuls post hoc test was used to compare specific means. Values are means ± SE.

RESULTS

MVC force. At Post, MVC torque was reduced (P < 0.05) by 54 ± 4.2% in E compared with Pre. By R1, MVC torque partially recovered (P < 0.05) to within 32 ± 5.2% of Pre values and did not fully recover until R6 in E (data not shown). MVC torque was also reduced by 12 ± 3.4% in C at Post, since participants could not avoid contracting the quadriceps of the contralateral control leg during the exercise protocol. Therefore, samples that were taken during the recovery period following exercise from the control leg are not representative of rested control samples; however, for the purposes of this study, it is useful to compare the fiber type-specific Hsp70 responses between the control and exercised legs, since the responses were very different (see Fig. 4).

Myosin ATPase staining. Representative sections that were stained for myosin ATPase (preincubation pH 10.3) are shown in Figs. 1 (A and D) and 2 (A–C). These slides only distinguish between type I and II fibers; however, analyses were performed separately on type IIA and IIX/IIAX fibers (preincubation pH 4.55 and 10.3 (3) and for relative glycogen content by the periodic acid-Schiff reaction.

Fibers were randomly chosen from the myosin ATPase stains (n = 10 for fiber types I, IIA, and IIX/IIAX) and identified with the aid of a microscope linked to computer-based imaging analysis software (Image-Pro PLUS). No attempt was made to distinguish between a hybrid type IIX fiber and a pure type IIX fiber; rather, immediately stained fibers (preincubation pH 4.55) were classified type IIX/IIAX. After fiber type determination, corresponding Hsp70 sections were analyzed for staining intensity using the above-mentioned software. Intensity was calculated by subtracting the negative background (no primary antibody) and the background of the slide that was incubated with the primary antibody from the corresponding Hsp70-positive serial section and expressed in arbitrary linear (red-scale) units. All cross sections from a single participant were always stained and analyzed on the same day.
4.55). On average, the muscle samples contained 37.4 ± 2.3% type I fibers, 49.2 ± 2.1% type IIA fibers, and 13.4 ± 1.4% type IIAX/IIX fibers.

Fiber type-specific basal Hsp70 content. A representative sample showing fiber type-specific basal Hsp70 content from one participant is shown in Fig. 1C, and the mean results from all participants are summarized in Fig. 3. Overall, basal Hsp70 content varied between individual participants (data not shown), but there were no ($P > 0.05$) fiber type differences (Fig. 3).

Fiber type-specific Hsp70 response to exercise. Fiber typespecific changes in Hsp70 content measured immediately following the exercise are shown for one participant in Fig. 1, and representative samples showing the fiber type-specific changes in Hsp70 expression at R1 and R6 in E only are shown in Fig. 2. The mean results for all participants and all time points are summarized in Fig. 3. Using immunohistochemistry, we were able to detect an increase in Hsp70 content immediately after exercise, but only in type I fibers ($P < 0.05$), with no differences ($P > 0.05$) in Hsp70 content in type IIA or IIAX/X fibers.
compared with Pre. The average increase in Hsp70 content in type I fibers at Post was \(97\%\). At R1, we observed a further \(57\%\) increase \((P < 0.05)\) in Hsp70 content in type I fibers that corresponded to a nearly threefold increase compared with Pre. Hsp70 content was also increased \((P < 0.05)\) in type IIA and IIA/IIX fibers at R1 compared with Pre and Post; however, Hsp70 content was \(71\%\) higher \((P < 0.05)\) in type I than type II fibers at R1. There were no differences \((P > 0.05)\) in Hsp70 expression between type IIA and IIA/IIX fibers at R1 or any other time throughout the study. Compared with R1, no further changes \((P > 0.05)\) in Hsp70 content were observed at R2, R3, or R6 in any of the fiber types; therefore, the differences in Hsp70 content between type I and II fibers that were noted at Post and R1 persisted throughout the recovery period \((P < 0.05)\). There was a difference between R3 and R6, but only in type I fibers, where Hsp70 content was lower \((P < 0.05)\) at R6 than at R3.

Although mean Hsp70 levels were similar at all recovery times, the time corresponding with peak expression levels in individual participants varied. Generally, within an individual, the time course for the peak Hsp70 response was the same for each fiber type, so if we group the fiber type response for individual participants, we see that peak Hsp70 expression occurred at R1 \((n = 2)\), R2 \((n = 3)\), R3 \((n = 4)\), or R6 \((n = 1)\). Compared with Pre, the average peak Hsp70 expression level in type I, IIA, and IIA/IIX fibers was \(396 \pm 61\%\), \(236 \pm 28\%\), and \(214 \pm 28\%\), respectively.

Figure 4 shows representative Hsp70 sections from biopsy samples that were taken from C and E at matched time points during recovery from the same participant. Importantly, the fiber type-specific Hsp70 response that was evident in E at all recovery time points did not occur in C, even though there was significant contractile activity-induced fatigue in C at Post. In fact, there were no changes in Hsp70 content in C compared with Pre (data not shown).

Global changes in Hsp70 expression in response to exercise.

The whole muscle Hsp70 response was assessed by Western blotting (Fig. 5). The results were similar to the immunohistochemistry results, but some important differences were noted, particularly with respect to the magnitude and time course of the response. First, muscle Hsp70 content was not different \((P > 0.05)\) at Post compared with Pre. Thereafter, Hsp70 content was increased \((P < 0.05)\) in E by \(43\%\) at R1 and remained elevated throughout the recovery period compared with Pre and Post. By R6, Hsp70 content was lower \((P < 0.05)\) than R1 levels.

Glycogen depletion patterns of individual muscle fibers with exercise. A representative sample showing the glycogen depletion of individual muscle fiber types immediately after exercise is shown in Fig. 1 \((B \text{ and } E)\). Glycogen depletion was observed in all type II, but not type I, fibers at Post. Specifically, in type
different from R1 (P < 0.05). The glycogen depletion pattern that we observed in type I fibers, a nonsignificant 9% reduction (P = 0.07) in glycogen content was observed immediately following exercise, whereas glycogen content was reduced (P < 0.05) by 67.5 ± 1.8% and 73.4 ± 8.1% in type IIA and IIX fibers, respectively. Glycogen content was completely restored in all fiber types by R2 (data not shown).

DISCUSSION

In the present study, we have used immunohistochemistry to investigate the skeletal muscle fiber type-specific Hsp70 response to intermittent isometric exercise in humans. Several novel findings have emerged. 1) In contrast to rats (10, 11, 18, 19, 24, 30) and rabbits (27), where fiber type differences in the basal expression of Hsp70 exist, we found no fiber type differences in basal Hsp70 content in human vastus lateralis. 2) Hsp70 protein in human skeletal muscle could be rapidly (i.e., within 30 min) increased in response to exercise, a response that is specific to type I fibers. 3) Hsp70 expression is increased in all fiber types within 24 h after exercise and remains elevated for up to 6 days after exercise. 4) Peak Hsp70 expression following exercise is higher in type I than in type II fibers. 5) The glycogen depletion pattern that we observed in individual fibers indicates that glycogen depletion is not obligatory for increasing Hsp70 expression in response to exercise, at least in type I fibers.

Previous studies in humans have indicated that Hsp70 mRNA in skeletal muscle increases rapidly in response to exercise (5, 7, 32, 42), whereas the Hsp70 protein response, as determined by Western blotting, is more delayed (i.e., >24 h) (14, 25). In contrast, using immunohistochemistry, we were able to detect increases in Hsp70 protein immediately follow-}

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\caption{Relative changes in Hsp70 content in vastus lateralis before (Pre) and after (Post, R1, R2, R3, and R6) exercise determined by Western blot analysis of whole muscle homogenates. A: representative Western blots of 2 participants who showed varying Hsp70 responses. In Std lane, 50 ng of recombinant rat Hsp70 protein (SPP-758, Stressgen Biotechnologies) was used as a positive control and molecular weight standard. B: mean data for all participants normalized relative to α-actin content (not shown). Values are means ± SE (n = 10). *Significantly different from Pre and Post (P < 0.05). ‡Significantly different from R1 (P < 0.05).
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are used to encourage rats to exercise, was the primary factor responsible for increased muscle Hsp70 content after treadmill running in rats. Khassaf et al. (14) showed that serial muscle biopsies can be taken from human vastus lateralis without causing an increase in muscle Hsp70 content. Finally, Morton et al. (26) showed that elevated core and muscle temperature with passive heating to levels comparable to exercise do not increase Hsp70 content in human vastus lateralis.

It is possible that the fiber type differences in the exercise-induced Hsp70 response we observed could reflect differences in fiber type recruitment patterns and, therefore, the stress intensity experienced by individual muscle fibers. Although we cannot determine the total level of activation of individual fibers throughout the 30-min exercise protocol, we can draw some conclusions on the basis of our glycogen depletion data and other findings from the literature. Initially, during the first several contractions at 60% MVC, type I and IIA, but not IIAX/IIX, fibers would be recruited (1); however, as fatigue develops throughout the protocol, force would be maintained by recruiting additional type II motor units (2) and, probably, by increasing firing rates of the activated type I and IIA units (1). In support of this view, glycogen depletion in individual fibers shows clearly that type IIA and IIAX/IIX fibers were recruited with this exercise protocol. The lack of glycogen depletion in type I fibers is a normal response during intermittent intense isometric exercise that is not reflective of motor unit recruitment patterns during this type of exercise (1, 9). Therefore, we would conclude that the different Hsp70 response, at least in type I and IIA fibers, is not due to differences in recruitment.

It is believed that glycogen depletion could be a primary stimulus associated with prolonged concentric exercise for increased Hsp70 protein expression in skeletal muscle (5, 6). Surprisingly, we found that the peak Hsp70 response to fatiguing isometric exercise was higher and the time course was more rapid in muscle fibers with the lowest level of glycogen depletion. From our results, it is clear that glycogen depletion was not a stimulus for increasing Hsp70 expression in type I fibers; however, we cannot rule out the possibility that the nearly twofold increase in Hsp70 in type IIA and IIA/IIAX fibers in the present study could be related to the glycogen depletion that occurred in those fibers.

It is well known that oxidative stress is involved in the induction of Hsp70 under a number of conditions including exercise (7, 14, 17, 21). Thus the differential Hsp70 response in different muscle fiber types could reflect differences in the level of oxidative stress that occurs with exercise. This might involve differences in the rate of production of reactive oxygen species (ROS) and/or reactive nitrogen species (RNS) or in the types of ROS and RNS that accumulate during exercise. Moreover, if there are fiber type differences in the susceptibility of various protein isoforms to oxidation by ROS and/or RNS, this could also explain the different fiber type-specific Hsp70 responses we observed, since the downstream effects of oxidative stress, namely, protein oxidation and destabilization, may act as the signal for increased expression of Hsp70 with exercise (21, 23). Interestingly, there is evidence that the Ca2+ pump isoform that is preferentially expressed in type I fibers [i.e., sarco(endo)plasmic reticulum Ca2+-ATPase (SERCA2a)] is more susceptible to oxidation than the Ca2+ pump isoform expressed in type II fibers (i.e., SERCA1a) (35, 39).

High levels of Hsp70 expression in skeletal muscle cells are associated with protection against hypoxic injury (41), Ca2+ overload-induced muscle damage (20), and contraction-induced muscle damage and loss of function (4, 22). Moreover, the degree of myocardial protection against ischemia-reperfusion injury in rats has been shown to be directly correlated with the amount of Hsp70 induced by prior heat shock treatment (12). Thus our results suggest that adaptation from acute exercise may provide greater protection to type I than type II muscle fibers against similar types of stress.

In summary, our results provide new insights into the exercise-induced stress response in human skeletal muscle and raise several interesting questions for further examination regarding the mechanisms responsible for the different Hsp70 response in type I vs. type II fibers. Future studies should employ male and female participants to examine the influence of sex on the fiber type-specific Hsp70 response to exercise, and different exercise protocols should be used to determine whether fiber type differences in Hsp70 expression are a general response to exercise or specific to certain types of exercise.

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REFERENCES


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