Time course and differential responses of the major heat shock protein families in human skeletal muscle following acute nondamaging treadmill exercise

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The stress of both acute (13, 21, 27, 31, 37, 40) and chronic exercise (12, 30, 35, 39) has been consistently shown to induce increases in HSP content in the skeletal muscle of various animal species. The stress response in rodent models is now relatively well defined and is typically observed several hours postexercise (25). Skeletal muscle also adapts to increased contractile activity via upregulation of the antioxidant defense network (14). The adaptation of such defense systems offers a potential mechanism for the increased tolerance to exercise and protection from contraction-induced damage associated with exercise training.

Acute exercise also stimulates increased HSP production in human skeletal muscle. An increase in HSP70 content has been observed in the vastus lateralis following acute one-legged cycling (17, 18) and exhaustive knee extensor exercise (11) and in the biceps brachii following damaging contractions (42–44). An increased content of HSP27 and αB-crystallin has also been observed in the vastus lateralis following downhill running (8). Two studies have investigated the heat shock response (limited to HSP70) of human skeletal muscle following running exercise protocols (36, 47). These authors failed to detect an increase in HSP70 expression at 3 h (36) and 24 h (47) following periods of moderately demanding running exercise. Previous data from our laboratory (17), however, demonstrated that biopsy samples beyond 24 h postexercise are likely needed to detect exercise-induced changes in muscle HSP levels. A comprehensive time course study of the stress response following running exercise has yet to be performed. This is particularly important given the relevance of running to the general population (i.e., as a keep-fit activity) and in an array of sporting activities.

Despite these initial descriptions, the exercise-induced stress response of human skeletal muscle remains poorly characterized and understood. Interpretation of data from human studies is often limited to the response of one particular HSP family (most notably HSP70) and is complicated by the variations in timing of tissue sampling, differing subject characteristics (e.g., age, training status, gender, nutritional status), and the disparate exercise protocols utilized by investigators. This is particularly important where there is a damaging component to the exercise protocol (where damage is defined as gross necrosis and a significant reduction in the force-generating capability...
of the muscle) whereby phagocytic cells that migrate to the site of injury contain relatively high levels of HSPs (18). The use of a nondamaging exercise protocol, however, provides a more controlled methodological approach whereby the increased expression of HSPs is likely to have arisen from skeletal muscle cells rather than changes in phagocytic cell content.

The aim of the present study was to characterize the time course and pattern of response of the major HSP families in human skeletal muscle following acute, nondamaging treadmill running exercise. These include the HSP70 family (HSP70 and heat shock cognate (HSC) 70), mitochondrial HSP60, and two members of the small HSP family (HSP27 and αB-crystallin). Given recent evidence that baseline HSP levels display marked individual variation (17), we also examined the extent of individual variation of baseline HSPs and antioxidant protein levels.

MATERIALS AND METHODS

Development of a nondamaging exercise protocol. In a preliminary study, eight active male subjects [mean ± SD: age, 24 ± 3 yr; weight, 77 ± 6 kg; height, 1.79 ± 0.07 m; maximum O2 uptake (V\textsubscript{O2 max}), 56.3 ± 5 ml kg\textsuperscript{-1} min\textsuperscript{-1}; lactate threshold, 70.1 ± 3.4% V\textsubscript{O2 max}] performed a 45-min treadmill running protocol at a speed corresponding to their lactate threshold (11.5 ± 0.9 km/h) on a motorized-driven treadmill (Woodway, Auf-Schrauben, Germany). Measurements of maximal quadriceps isometric force and voluntary activation were assessed immediately before and at 2 h, 6 h, 24 h, 48 h, 72 h, and 7 days postexercise, according to Morton et al. (33). Venous blood samples were also obtained at these time points and analyzed for serum creatine kinase activity, according to a modification of the spectrophotometric method of Jones et al. (15). Data deemed this protocol to be nondamaging in nature in that it resulted in no significant increases in serum creatine kinase levels (Fig. 1A) and no significant reductions in the force-generating capability of the quadriceps muscles (Fig. 1, B and C).

Subjects. Eight active but untrained men volunteered to participate in the study (mean ± SD: age, 24 ± 4 yr; weight, 78.9 ± 7.4 kg; height, 1.8 ± 0.05 m; V\textsubscript{O2 max}, 54.9 ± 4 ml kg\textsuperscript{-1} min\textsuperscript{-1}; lactate threshold, 69.8 ± 4.8% V\textsubscript{O2 max}). All subjects gave written, informed consent to participate after details and procedures of the study had been fully explained. Subjects refrained from any exercise (outside of the study) throughout the testing period and from alcohol and caffeine intake for at least 24 h before any of the testing sessions or muscle biopsy sampling. No subjects had a history of neurological disease or musculoskeletal abnormality, and none was under any pharmacological treatment during the course of the study. The study was approved by the Ethics Committee of Liverpool John Moores University.

Design and exercise protocol. Four to five days after having initially been assessed for V\textsubscript{O2 max} (6) and lactate threshold (4), subjects completed the 45-min nondamaging treadmill running protocol (outlined above) at a speed corresponding to their lactate threshold on a motorized-driven treadmill (Woodway). Muscle biopsies were obtained from the vastus lateralis immediately before the exercise protocol and at 24 h, 48 h, 72 h, and 7 days postexercise. This intensity of exercise (as opposed to percentage of V\textsubscript{O2 max}) was chosen so as to normalize the exercise intensity between individuals with different aerobic capacities (2). This exercise intensity also compares well with similar intensities previously used in treadmill protocols examining the heat shock response to exercise (36, 47).

Ratings of perceived exertion (RPE) (3), thermal comfort scale (TCS) (45), and heart rate (Polar 610i, Kempele, Finland) were recorded at 5-min intervals throughout exercise. Oxygen uptake (V\textsubscript{O2}) was also measured (MetaLyzer 3B, Cortex Biophysics, Leipzig, Germany) for 5-min periods between 5–10, 20–25, and 35–40 min of exercise. Fingertip capillary blood samples were obtained at 15, 30, and 45 min and analyzed immediately in duplicate for whole blood lactate concentration (Lactate Pro, Arkray, Kyoto, Japan) to verify the intensity of exercise. Core temperature was measured using a rectal probe placed 10 cm beyond the anal sphincter (ELLAB) and was monitored continuously during exercise (CTF9004, ELLAB). Muscle temperature was measured in the vastus lateralis immediately pre- and postexercise using a needle thermistor (CTF9004, ELLAB) inserted to a depth of 3 cm (38). When the needle thermistor was removed, the injection site was cleaned with a sterile alcohol injection swab and covered with waterproof dressing. The ambient temperature of the laboratory during each exercise session was 18 ± 0.8°C. Fluid intake was not permitted at any time during exercise.

Muscle biopsies. Muscle biopsies were taken from the vastus lateralis under local anesthesia (0.5% marcaine) using a Pro-Mag 2.2 biopsy gun (MD-TECH, Manan Medical Products, Northbrook, IL). Biopsies on consecutive time points were taken from alternate legs, and samples obtained (~50 mg) were immediately frozen in liquid nitrogen and stored at −80°C for later analysis. Previous data have shown that the process of serial muscle biopsies per se is insufficient to induce the expression of HSPs (17), indicating that serial biopsies could be taken without inducing the production of stress proteins in the remaining tissue.

Biochemical procedures. Samples were homogenized in a 1% solution of SDS containing protease inhibitors (27). Each sample was centrifuged at 4°C, and the total protein content of the supernatant was measured using bicinchoninic acid (Sigma Chemical, Dorset, UK). Total protein was separated by SDS-PAGE using a 12% polyacrylamide gel and 4% stacking gel (National Diagnostics). Proteins were transferred onto a nitrocellulose membrane, as previously described (27). The muscle content of HSP70, HSC70, HSP60, HSP27, and αB-crystallin was analyzed using a panel of mouse monoclonal
(HSP60 and HSP70), rat (HSC70), or rabbit polyclonal antibodies (HSP27, αB-crystallin, and MnSOD) (Stressgen, Victoria, Canada). Bands were visualized using an enhanced chemiluminescence detection system (Pierce) and Chemi-doc image capture system with Quantity One software (Bio-Rad). The content of HSPs was expressed as a percentage of the preexercise content for each subject. For analysis of antioxidant enzyme activity, samples were homogenized in 100 mM phosphate buffer (pH 7.0). Total SOD activity was measured according to the method of Crapo et al. (7). Catalase activity was measured by following the kinetic decomposition of hydrogen peroxide at 240 nm using a method derived from Claiborne (5).

**Statistical analyses.** Changes in exercise-related variables during the exercise protocol (i.e., heart rate, RPE/TCS, blood lactate, \( \dot{V}O_2 \), and core temperature) and changes in muscle HSP content and antioxidant enzyme activity following exercise were analyzed using repeated-measurements general linear models (GLM). Differences in muscle temperature between pre- and postexercise was assessed using a Student’s t-test for paired samples. Where there was a significant main effect for time, paired t-tests with Bonferroni corrections were used for post hoc analysis. In a “summary of statistics” approach (1), Student’s t-test for paired samples was also used to examine pre-to peak changes in HSP expression. Peak changes were taken as the time point at which subjects demonstrated their maximal change in muscle HSP content. Correlations between baseline protein levels were assessed using Pearson’s correlation coefficient. All data are presented as means ± SD, with \( P \) values of <0.05 indicating statistical significance.

**RESULTS**

**Physiological responses to the exercise protocol.** The exercise protocol was performed at a running speed of 11.7 ± 0.5 km/h. Heart rate, RPE, and TCS during exercise are displayed in Table 1. All of these variables displayed a significant (\( P < 0.05 \)) and progressive linear increase during exercise. \( \dot{V}O_2 \) and blood lactate values showed no significant change during exercise. Specifically, \( \dot{V}O_2 \) corresponded to 68.8 ± 4.8, 70.5 ± 3.8, and 69.5 ± 3.7% of \( \dot{V}O_2 \) max at 5–10, 20–25, and 35–40 min of exercise, respectively. Blood lactate values were 3 ± 0.4, 3.3 ± 0.6, and 3.5 ± 0.7 mmol/l after 15, 30, and 45 min of exercise, respectively. Core and muscle temperature changes during the exercise protocol are presented in Fig. 2. Exercise induced a significant rise (\( P < 0.05 \)) in core temperature, increasing from 37.5 ± 0.2°C at rest to 39.2 ± 0.3°C immediately postexercise. Muscle temperature also exhibited a significant increase (\( P < 0.05 \)) during exercise, increasing from 36.2 ± 0.7°C at rest to 40 ± 0.3°C immediately postexercise.

**Changes in HSP content following exercise.** Muscle HSP70 content showed a significant and variable increase following exercise (Fig. 3A). This response achieved significance (\( P < 0.05 \)) at 48 h (179% of preexercise content) and 7 days postexercise (178% of preexercise content). When considering peak responses (Fig. 3B), which typically occurred at 48 h postexercise, HSP70 increased to 210 ± 7% of preexercise levels (range, 135–366%). The HSP70 response to exercise showed a marked individual variation in both magnitude and time course of the response. Representative Western blots highlighting an example of such individual variation are presented in Fig. 3, C–E.

The pattern of changes in muscle HSC70 content mirrored the response of HSP70, although this change was not statistically significant when assessed using a repeated-measures GLM (Fig. 4A). However, HSC70 increased significantly (\( P < 0.05 \)) to 170 ± 75% of preexercise levels (range, 116–340%) at the time of peak expression, typically occurring at 48–72 h postexercise (Fig. 4B).

The muscle content of HSP60 showed no significant statistical change when assessed using a repeated-measures GLM (Fig. 5A). When examining peak responses (Fig. 5B), which again occurred at 48 and 72 h postexercise in individual

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**Table 1. Heart rate and ratings of perceived exertion and thermal comfort during the exercise protocol**

<table>
<thead>
<tr>
<th>Time, min</th>
<th>Preexercise</th>
<th>10</th>
<th>20</th>
<th>30</th>
<th>40</th>
<th>Postexercise</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart rate, beats/min</td>
<td>76 ± 6</td>
<td>164 ± 6*</td>
<td>169 ± 6*</td>
<td>174 ± 5*</td>
<td>179 ± 6*</td>
<td>179 ± 6*</td>
</tr>
<tr>
<td>RPE</td>
<td>6</td>
<td>12 ± 1*</td>
<td>13 ± 1*</td>
<td>14 ± 1*</td>
<td>16 ± 2*</td>
<td>16 ± 2*</td>
</tr>
<tr>
<td>TCS</td>
<td>5 ± 1</td>
<td>6 ± 1*</td>
<td>7 ± 1*</td>
<td>7 ± 1*</td>
<td>8 ± 1*</td>
<td>8 ± 1*</td>
</tr>
</tbody>
</table>

Values are means ± SD. RPE, ratings of perceived exertion; TCS, thermal comfort scale. *Significant difference from preexercise values, \( P < 0.05 \).
subjects, HSP60 increased significantly ($P < 0.05$) to $139 \pm 23\%$ of preexercise values (9–73%)

HSP27, αB-crystallin, and MnSOD protein content showed no significant change following exercise when assessed by either repeated-measures GLM or evaluating pre- to peak changes (data not shown).

Antioxidant enzyme activity. Muscle total SOD and catalase activity showed no significant change following exercise (Figs. 6 and 7, respectively).

Baseline HSP levels. A comparison of preexercise levels of muscle HSP content is shown in Fig. 8. Western blots from only seven subjects are presented due to insufficient availability from one subject. HSC70, HSP27, and αB-crystallin were constitutively expressed and showed little individual variation between subjects. In contrast, HSP70 and MnSOD expression displayed marked variation, exhibiting up to a 3- and 1.5-fold difference between subjects, respectively. Of all baseline proteins, correlations were only evident between HSP70 and MnSOD ($r = 0.81$).

Fig. 3. A: changes in content of heat shock protein (HSP) 70 in the vastus lateralis before and after exercise. B: peak changes in HSP70 content. *Significant difference from preexercise values, $P < 0.05$. C–E: representative Western blots of individual subjects who showed varying HSP70 responses.

DISCUSSION

The present study has characterized the time course and magnitude of response of the major HSP families in the skeletal muscle of an active young male population following an acute bout of moderately demanding and nondamaging treadmill exercise. Our data provide novel data for the literature and have demonstrated that running exercise is a sufficient stimulus to upregulate the expression of several HSPs (most notably HSP70). Although examination of individual data reveals that 48 h postexercise appears to be an appropriate time point for which to detect maximal exercise-induced increases in HSP expression, we advocate the use of multiple postexercise biopsy samples in future studies. This is because the stress response appears highly variable between subjects in that some individuals may not display peak responses to 72 h or 7 days following exercise. Data also demonstrate a differential effect of aerobic exercise on specific HSPs.

HSP70 and HSP60 response. Muscle HSP70 and HSP60 content exhibited a peak significant increase of 2-fold and 1.4-fold, respectively. The discrepancy between the present study and those who previously failed to observe a significant change in HSP70 content following running exercise may be related to restrictions of postexercise biopsy samples to within 1 day following exercise (36, 47). This is also a likely explanation for the absence of an increased HSP60 content immediately after 2-h cycling exercise (10). Whereas transcription of

Fig. 4. A: changes in content of heat shock cognate 70 (HSC70) in the vastus lateralis before and after exercise. B: peak changes in HSC70 content. *Significant difference from preexercise values, $P < 0.05$.

Fig. 6. Total superoxide dismutase (SOD) activity of the vastus lateralis muscle before and after exercise.
The increase in muscle HSP70 and HSP60 content in the present study is somewhat smaller than that previously observed following one-legged cycling (17, 18). Of note, however, is the difference in training status of the subject groups between studies. Although the present subjects were not specifically trained athletes, they were involved in an average of 2 h of physical activity per week (e.g., recreational sport), significantly more activity than the sedentary lifestyles led by the subjects involved in the previous investigations. It may therefore be speculated that sedentary subjects may mount a greater stress response than that of active or trained subjects (whose muscles are somewhat more preconditioned to exercise stresses) so as to combat any homeostatic disruption evoked by the exercise stress. This is true in the muscles of rodents, whereby the exercise-induced increase in HSP70 in rat soleus muscle is higher in untrained rats than endurance-trained rats (41). In a similar manner to the HSP70 response, the lower HSP60 response observed here may also be due to the differing characteristics of active and sedentary subjects. For example, the mitochondria of active subjects are likely to be more equipped with other endogenous defense mechanisms to cope with the changes in oxygen flux that occur during exercise. In such cases, a dramatic HSP60 response may therefore not need to be mounted. In line with this is the increased baseline activity of total SOD activity in the present subjects compared with the sedentary subjects studied by Khassaf et al. (17, 18).

Although our data displayed individual variation in both the time course and magnitude of HSP70 responses (see individual Western blots, Fig. 2, C–E), this variation was substantially lower than that previously demonstrated by Khassaf et al. (17). We believe this to be attributable to the more “tightly controlled” exercise protocol utilized in the present study. When exercising at the lactate threshold (as opposed to percentage of $V_{O2\text{max}}$), relatively homogenous physiological (both cardiac and metabolic) responses are observed (2). This is true even when comparisons are made between trained and untrained subjects (2). These authors speculated that such homogenous responses may be due to similar fiber-type recruitment patterns. The latter appears relevant in an evaluation of the stress response considering data from rodent studies suggesting that HSP70 expression in the skeletal muscle of rodents following treadmill running displays an intensity-dependent relationship that is partly reflective of muscle recruitment patterns (31).

Small-HSP response. The small HSPs are thought to play an important role in the remodeling of myofibrillar structure following stressful and damaging insults (34). It has therefore been suggested that the small HSPs may be particularly active in the recovery process following exercise-induced muscle damage. In agreement with this, an increased expression of HSP27 has been consistently observed at 48 h after lengthening contractions of the biceps (42–44). Feasson et al. (8) also observed an approximate twofold increase in both HSP27 and αB-crystallin at 24 h following a 30-min downhill running protocol. In contrast to the above studies, the present data revealed no increases in muscle content of the small HSPs. This may be due, in part, to the nondamaging nature of our exercise protocol that does not appear to cause any overt structural or functional damage in active young male populations. It is possible, therefore, that whereas the HSP70 and HSP60 proteins may be upregulated during exercise by oxidative, thermal, metabolic or cytokine signals, the small HSPs are more responsive to contractile-induced mechanical stresses. It may also be that the relatively high baseline levels of both small HSPs were already sufficient to counteract any stresses that the contractile and structural proteins encountered during exercise. At present, data concerning the exercise-induced expression of small HSPs in human skeletal muscle are extremely limited, and thus further descriptive studies characterizing a response to various exercise protocols appear warranted.

Baseline stress protein levels. Of all baseline protein levels examined, only HSP70 and MnSOD showed large individual variation. This interindividual variation in resting muscle HSP70 levels is not novel and has been demonstrated previously (17). Data from rodent studies have shown that HSP70 is preferentially expressed in muscles with a high proportion of oxidative fibers (13, 16, 22, 23). It is possible, therefore, that the variations in baseline HSP70 levels observed here may be due to differing fiber-type characteristics between subjects. We were unable to quantify muscle fiber types due to the small tissue samples obtained by our chosen biopsy technique. Nevertheless, the observation of moderate correlations between
HSP70 and MnSOD protein levels suggests that differences in baseline expression of HSP70 between individuals may be related to differences in fiber-type content.

**Signals inducing increased HSP expression.** It was not the intent of the present study to provide a comprehensive analysis as to the possible signals initiating the stress response during exercise. Nevertheless, previous data from our laboratory in both mice (27) and humans (17) have suggested that such a response may be mediated by an oxidative stress and an accompanying transient and reversible oxidation of protein sulfydryl groups. Alternate activators during exercise include (but are not limited to) elevated temperature or metabolic stress. Only one study has previously reported on the extent of muscle temperature change with their chosen exercise protocol (9). In the present study, we observed a large increase in both muscle (4°C) and core (2°C) temperature, demonstrating a local and systemic hyperthermia effect. At present, we therefore feel it difficult to conclusively dismiss the role of increased muscle and core temperature in contributing to the exercise-induced production of HSPs in human skeletal muscle.

**Biological significance of increased HSP expression.** An increased muscle content of HSPs following exercise is thought to restore cellular homeostasis, promote cellular remodeling, and provide cytoprotection against further insults (25). Data from our laboratory in rodent models using preconditioning stresses (26, 28) or transgenic approaches (24) have demonstrated that HSPs provide increased protection against contraction-induced damage. A diminished HSP response to contractile activity has also been observed in muscles of old rats (46) suggesting that stress proteins may, in part, play an important role in maintaining muscle function during the ageing process. Indeed, muscles of old transgenic mice overexpressing HSP70 displayed an enhanced recovery of muscle function following a period of damaging contractions compared with muscles of old wild-type mice (24). Further studies are required to examine whether such a biological role of increased muscle HSP levels exists in a human model.

In summary, the present data demonstrate that the skeletal muscle of healthy active young male subjects responds to a period of moderately demanding and nondamaging running exercise via an upregulation of several HSPs (predominantly HSP70). Given the well-documented cytoprotective role of these proteins, it is possible that their increased expression following exercise functions to restore cellular homeostasis and to offer increased protection against further stressful insults. Further studies are required to examine the differing HSP responses to varying types of exercise protocols in specific subject populations (i.e., age, gender, and training status specific), to investigate possible mechanisms of activation, and to determine the precise biological significance of this increased expression.

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