Prolonged exercise training induces long-term enhancement of HSP70 expression in rat plantaris muscle

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Ogata T, Oishi Y, Higashida K, Higuchi M, Muraoka I. Prolonged exercise training induces long-term enhancement of HSP70 expression in rat plantaris muscle. Am J Physiol Regul Integr Comp Physiol 296: R1557–R1563, 2009. First published February 25, 2009; doi:10.1152/ajpregu.90911.2008.—Skeletal muscle may develop adaptive molecular chaperone enhancements as a potential defense system through repeated daily exercise stimulation. The present study investigated whether prolonged exercise training alters the expression of molecular chaperone proteins for the long term in skeletal muscle. Mature male Wistar rats were subjected for 8 wk to either a single bout of acute intermittent treadmill running (30 m/min, 5 min × 4, 5° grade) or prolonged treadmill running training (15–40 m/min, 5 min × 4, 5–7° grade). Levels of five molecular chaperone proteins [heat shock protein (HSP)25, HSP60, glucose-regulated protein (GRP)78, HSP70, and heat shock cognate (HSC)70] were measured in response to acute exercise and prolonged training. HSP70 levels were increased 6 and 24 h after acute exercise, but expression returned to control level within 2 days. In contrast, prolonged training had a long-term effect on HSP70 expression. Levels of HSP70 were notably increased by 4.5-fold over control 2 days after prolonged training; the enhancement was maintained for at least 14 days after training ended. However, other molecular chaperone proteins did not show adaptive changes in response to prolonged training. In addition, HSP70 enhancement by prolonged exercise training was not accompanied by transcription of HSP70 mRNA. These findings demonstrate that prolonged training can induce long-term enhancement of HSP70 expression without change at the mRNA level in skeletal muscle.

MOLECULAR CHAPERONES such as heat shock proteins (HSPs) are known to contribute to reducing cellular damage (6, 12). HSPs have multiple functions in maintaining intracellular integrity via protection, repair, and even control of cell death signaling (10, 31). HSP70, a stress-inducible isoform, has been well studied in mammalian skeletal muscle, and the expression of HSP70 has been found to be increased in stress stimulations. For example, the expression of HSP70 is reported to be enhanced by thermal stress and exercise (7, 13, 25). In rat hindlimb studies, HSP70 expression is upregulated immediately after thermal stress in soleus muscle and within 24 h in plantaris muscle (25). In human skeletal muscle, both exhaustive endurance exercise by cycling (5) and resistance exercise with maximal eccentric repetitions (32) markedly increase HSP70 expression. Furthermore, it is reported that transgenic mice with overexpressed HSP70 show a reduced number of damaged myofibers and a smaller deficit of force generation in skeletal muscle following severe lengthening contractions induced by electrical stimulation (17). Together, these studies suggest that the expression of molecular chaperone proteins including HSP70 may play important roles in protection and repair of skeletal muscle from exercise-induced stresses.

High-intensity exercise, such as repeated eccentric contraction and exhaustive running, is known to induce skeletal muscle damage via mechanical and oxidative stresses and subsequent activation of proteases (1, 23). In this regard, prior exercise training attenuates contraction-induced injury in skeletal muscle following an acute single bout of exercise. For example, rats previously trained with either downhill or uphill running show reduced myofiber injury after 90 min of downhill running as well as an attenuation of the induced increment of glucose-6-phosphate dehydrogenase in muscle and creatine phosphokinase (CPK) activity in plasma (30).

The acquisition of muscle tolerance to contraction-induced muscle damage through exercise training appears to be partially associated with molecular mechanisms including chaperone functions in addition to neuromuscular and morphological adaptations. Several studies have reported that prolonged exercise training increases several molecular chaperone proteins in skeletal muscle, such as HSP25, HSP70, and glucose-regulated protein (GRP)78 (7, 21). However, it is not understood whether the upregulation of molecular chaperone proteins accompanying training is adaptive and persistent because most studies have examined protein levels only 1 or 2 days after exercise training.

The purpose of the present study, therefore, was to determine whether prolonged high-intensity exercise training provides persistent adaptive effects on a molecular level. We hypothesized that repeated daily exercise enhances the expression of molecular chaperone proteins for extended periods after training. In this study, therefore, we examined the time course of changes in molecular chaperone expression during detraining periods after prolonged exercise training using intermittent high-intensity treadmill running for 8 wk. We also examined the time courses of molecular chaperone levels following a single bout of acute exercise with treadmill running and compared differences in the effects between acute exercise and prolonged training. Several types of chaperone proteins were examined in the present study: HSP25 (a small HSP), HSP60 (a mitochondrial HSP), GRP78 (an endoplasmic reticulum HSP), HSP70, and heat shock cognate (HSC)70.

MATERIALS AND METHODS

Animals

Four-month-old male Wistar rats were used in this study. All procedures were reviewed and approved by the Committee on Animal
Care and Use at Waseda University and followed the Guiding Principles for the Care and Use of Animals in the Field of Physical Sciences established by the Physiological Society of Japan. Rats were housed in a temperature-controlled (22 ± 2°C) room with a 12:12-h light-dark cycle and were provided rat chow and water ad libitum.

Experimental Designs and Tissue Preparations

We performed two different experimental manipulations. One group of rats performed a single bout of acute exercise; the second group had prolonged exercise training. Sedentary control groups were used for each manipulation.

Single bout of acute exercise. Thirty rats were randomly assigned into sedentary control (n = 7) and single bout of acute exercise (n = 23) groups. The acute exercise protocol involved rats running on a motorized treadmill under an intermittent exercise regimen (30 m/min, 5° grade, 5 min × 4, pause 2 min). After the single bout of acute exercise, rats were maintained under sedentary conditions. Experimental animals were assigned to groups at four different time intervals after acute exercise (PE): 6 h (PE-6 h; n = 6), 2 days (PE-2 days; n = 6), and 4 days (PE-4 days; n = 6).

Prolonged exercise training. Thirty-seven rats were randomly assigned to control (n = 8) and prolonged exercise training (n = 29) groups. As in the acute exercise experiment, control rats were kept sedentary. The rats in the exercise training group performed intermittent treadmill exercise training (15–40 min/m, 5–7° grade, 5 min × 4, pause 2 min, 8 wk) as described in Table 1. After the 8-wk training period, the experimental rats were maintained under sedentary conditions and assigned into detraining (DT) groups for 2 days (DT-2 days; n = 6), 7 days (DT-7 days; n = 6), 10 days (DT-10 days; n = 5), 14 days (DT-14 days; n = 6), and 28 days (DT-28 days; n = 6) after the final training session.

All rats were anesthetized with pentobarbital sodium (50 mg/kg ip), and the plantaris muscles were removed bilaterally at the time points defining each group after either acute exercise or prolonged training. The muscles were cleaned of excess fat and connective tissue and wet weighed. The right plantaris muscle was used for biochemical analysis, and the left was used for histochemical analysis.

Muscle Fiber Type Composition

Serial cross sections (10 µm thick) were cut at the midbelly region of the left plantaris muscle in a cryostat (−20°C) at rest length and mounted on glass slides. For assessment of the fiber type composition of each plantaris muscle sample, sections were stained immunohistochemically as described previously (24). The sections were incubated for 60 min with phosphate-buffered saline (PBS) containing 10% normal goat serum and 0.3% Triton X-100 at room temperature to block nonspecific reactions and reacted for 60 min at room temperature with anti-skeletal myosin [SLOW (M8421) and FAST (M4276), Sigma, St. Louis, MO] antibodies as primary antibodies in PBS containing 5% normal goat serum and 0.1% Triton X-100. After incubation, the sections were rinsed and reacted for 60 min with Alexa Fluor 488 goat anti-mouse IgG (A-11001, Molecular Probes, Eugene, OR) as a secondary antibody in PBS containing 5% normal goat serum and 0.1% Triton X-100. The sections were rinsed thoroughly and mounted in Aqua-Poly/Mount medium (Polysciences, War- rington, PA). All fibers on the cross section of each plantaris muscle were classified as slow, slow + fast (hybrid), or fast fibers based on their reaction to the primary antibodies; fiber type composition was calculated with fluorescence microscopy.

Homogenization Procedure

Muscle samples from the right plantaris were homogenized with 15 vol of homogenizing buffer (1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate in PBS). Total protein per plantaris muscle mass was measured with the method of Bradford (2). The homogenized sample was centrifuged at 10,000 g for 20 min. The extracted supernatant protein content was quantified at a final protein concentration of 2 µg/µl and then boiled in sample buffer for 2 min.

Western Blotting

Sample buffer containing extracted protein from plantaris muscle was separated with 10% acrylamide gels and 4% stacking wide gels by standard electrophoretic methods (11). A total of 80 µg of protein was applied to each lane of the gels. Electrophoresis was continued at 40 mA (constant current/gel) for ~60 min until the dye front migrated to the bottom of the gel. At that time, the gels were subjected immediately to transfer blotting.

The gels were transferred to polyvinylidene difluoride (PVDF) membranes with a semidyblotting unit (FSD-300B, TGK, Tokyo) at 120 mA (constant current/gel) for 40 min. After transfer, the blots were blocked with 5% nonfat-skim milk in PBS containing 0.05% Tween 20 (PBS-T) for 1 h at room temperature. After being washed in PBS-T, the blots were incubated overnight at 4°C with the primary antibodies diluted 1:250 to 1:2,000 in PBS-T containing 5% bovine serum albumin. Primary antibodies used in this study were as follows: rabbit polyclonal anti-HSP25 (SPA-801, Assay Designs, Ann Arbor, MI), mouse monoclonal anti-HSP60 (SPA-806), mouse monoclonal anti-KDEL (SPA-827), mouse monoclonal anti-HSP70 (SPA-810), rat monoclonal anti-HSC70 (SPA-815), rabbit polyclonal anti-heat shock factor (HSF)-1 (SPA-901), and rabbit polyclonal anti-HSF-2 (sc-13056, Santa Cruz Biotechnology, Santa Cruz, CA). The membranes were washed in PBS-T and then reacted with horseradish peroxidase-conjugated goat anti-mouse IgG (074-1806, KPL, Gaithersburg, MD), goat anti-rabbit IgG (074-1506, KPL), or goat anti-rat IgG (14-16-06, KPL) in PBS-T containing 5% bovine serum albumin for 1 h at room temperature. The membranes were then washed again in PBS-T and processed with an enhanced chemiluminescence (ECL) procedure (RPN2106, GE Healthcare, Little Chalfont, England). The ECL signals on the immunoblots were detected and quantified with a Fuji LAS3000 luminescent image system (Las-3000, Fuji Film, Tokyo, Japan) with Multi Gauge software (version 3.0, Fuji Film).

Real-Time PCR

Total RNA was isolated from plantaris muscle with the RNeasy Fibrous Tissue Mini Kit (Qiagen, Hilden, Germany). Single-strand cDNA synthesis was performed on 2 µg of total RNA extract with random primers [high-capacity cDNA Reverse Transcription Kit, Applied Biosystems (ABI), Foster City, CA]. Real-time PCR was performed with the Applied Biosystems 7500 Fast Real-Time PCR system. A predeveloped TaqMan primers and probes set (Pre-Developed Assay Reagents) for HSP70 was designed at ABI based on gene sequence information obtained from GenBank (Hspa1a: HSP70–1, accession no. L16764.1). Eukaryotic 18S ribosomal RNA (also designed from GenBank sequence information: accession no. X03025) was used as an internal control measure of mRNA content. DNA amplification was performed with the TaqMan Universal PCR Master

Table 1. Treadmill protocol for prolonged exercise training

<table>
<thead>
<tr>
<th>Training Week</th>
<th>Running Speed, m/min</th>
<th>Grade</th>
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<tbody>
<tr>
<td>1</td>
<td>15</td>
<td>5°</td>
</tr>
<tr>
<td>2</td>
<td>10</td>
<td>5°</td>
</tr>
<tr>
<td>3</td>
<td>25</td>
<td>7°</td>
</tr>
<tr>
<td>4</td>
<td>30</td>
<td>7°</td>
</tr>
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<td>5</td>
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<td>7°</td>
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<td>6</td>
<td>35</td>
<td>7°</td>
</tr>
<tr>
<td>7</td>
<td>40</td>
<td>7°</td>
</tr>
<tr>
<td>8</td>
<td>40</td>
<td>7°</td>
</tr>
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Animals trained 5 days per week for four 5-min durations (with 2-min pause) per day.
Mix (ABI) and a thermal cycling protocol of 50°C, 2 min; 95°C, 10 min; and 50 cycles of 95°C, 15 s followed by 60°C, 1 min.

Data were analyzed by ABI software with the threshold cycle (Ct), a value determined as the time, measured as cycle number, at which the reporter fluorescent emission increased beyond a threshold level that was defined as the background number at which cDNA amplification was first detected. Fluorescent emission data were captured and mRNA levels quantified for each gene with the Ct value. Change in (ΔΔCt) was calculated by subtracting the Ct for 18S rRNA from the Ct for the gene of interest. The relative expression of the HSP70 mRNA was then calculated with the expression 2^-ΔΔCt, with the results expressed as arbitrary units. Data reliability was also established by use of the internal control gene GAPDH (GenBank sequence information: accession no. M17701.1).

Statistical Analyses

All data are presented as means ± SE. Significant differences among groups were determined by one-way ANOVA and Tukey-Kramer post hoc tests. The relation between given variables is described by the Pearson product-moment correlation coefficient r. All differences were determined to be significant at the P < 0.05 level.

RESULTS

Plantaris Muscle Features During Detraining Periods

The ratio of plantaris muscle weight to overall body weight was significantly increased in DT-2 days, DT-7 days, DT-10 days, and DT-14 days groups compared with sedentary control rats despite a lack of significant change in the absolute weights of the plantaris muscle (Table 2). Total proteins in plantaris muscle were also unchanged during detraining periods. Differences in fiber type compositions were analyzed by immunohistochemistry. The proportions of slow, slow+fast, and fast fibers in plantaris muscle were not changed during detraining periods. There were no significant effects of training on fiber type distribution; the sole morphological finding during detraining was the relative increase in plantaris muscle weight. For PE groups, which were only exposed to a single acute exercise session, no significant changes were found in body weight, plantaris muscle weight, total protein, and fiber type composition (data not shown).

Changes of HSP25, HSP60, and GRP78 as Function of Time After Acute Exercise or Prolonged Training

Acute exercise increased HSP25 levels by 1.6 times control for the PE-24 h group (Fig. 1A, left), but no changes were seen for other PE groups. HSP25 contents after prolonged training were not different among the detraining time points (Fig. 1A, right). HSP60 (Fig. 1B) and GRP78 (Fig. 1C) showed no effects from either acute exercise or prolonged training; levels of both proteins were not different from those in the control groups.

Changes of HSP70 and HSC70 Expression After Acute Exercise or Prolonged Training

In contrast, HSP70 levels were increased under both protocols. Acute exercise raised HSP70 expression by a factor of 2.4 for PE-6 h rats and a factor of 2.1 for PE-24 h rats relative to control. The content then decreased and returned to control levels in the PE-2 days group (Fig. 2A, left). Prolonged training had a long-term enhancement on the protein expression level of HSP70. HSP70 levels were notably increased in the DT-2 days group (4.49 ± 1.05-fold; Fig. 2B, right). Although HSP70 content gradually diminished from DT-2 days to DT-28 days, HSP70 was still significantly elevated in the DT-14 days group (2.57 ± 0.25-fold) compared with control level. This indicates that the enhancement of HSP70 by prolonged training was maintained for >2 wk in plantaris muscle. In contrast, expression of HSC70 remained near control levels in all experimental groups (Fig. 2B), indicating that exercise has little effect on HSC70.

Relationship of Relative Muscle Weight per Body Weight and HSP70 Levels

Together, the findings of increased relative plantaris weight and elevated expression of HSP70 suggest that the effects of training might be related. We analyzed the relationship between HSP70 protein content and relative plantaris muscle weight (plantaris/body weight) by examining the corresponding Pearson’s correlation coefficient (r). When the plantaris muscles of control and detraining groups were collapsed and treated as a single pooled group, HSP70 protein content was positively correlated with relative plantaris muscle weight (r = 0.459, P = 0.0063, Fig. 3).

Time Courses of HSF Proteins and HSP70 mRNA After Prolonged Training

To investigate whether the increments of HSP70 during detraining periods were regulated on the transcription level, we examined the expression of transcriptional factors of HSP70 and HSP70 mRNA at each detraining time point (Fig. 4). Two types of transcriptional factors, HSF-1 and HSF-2, were not enhanced during detraining periods (Fig. 4, A and B). In addition, HSP70 mRNA levels in plantaris muscle were not enhanced during detraining periods (Fig. 4, C and D). For the HSF-1, a transcriptional factor of the HSP70 gene, the expression was significantly increased by 10.22 ± 0.33.4 on June 26, 2017 http://ajpregu.physiology.org/ Downloaded from

Table 2. Animal morphological characteristics during detraining

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<thead>
<tr>
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<th>Body Weight, g</th>
<th>Fiber Type Composition, %</th>
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<tbody>
<tr>
<td></td>
<td>Pre</td>
<td>IA</td>
</tr>
<tr>
<td>Cont</td>
<td>348±9</td>
<td>548±13</td>
</tr>
<tr>
<td>DT-2 days</td>
<td>371±8</td>
<td>478±15</td>
</tr>
<tr>
<td>DT-7 days</td>
<td>370±8</td>
<td>475±23</td>
</tr>
<tr>
<td>DT-10 days</td>
<td>352±6</td>
<td>483±27</td>
</tr>
<tr>
<td>DT-14 days</td>
<td>374±7</td>
<td>478±10</td>
</tr>
<tr>
<td>DT-28 days</td>
<td>362±13</td>
<td>484±18</td>
</tr>
</tbody>
</table>

Values are means ± SE. Control (Cont) rats were killed on same day as detraining (DT)-2 day rats were dissected. Pre, preexercise training; IA, immediately after exercise training; Post, postexercise training periods. *P < 0.05 compared with Cont rats.
altered from DT-2 days to DT-28 days (Fig. 4C), although HSP70 protein level was notably enhanced until 14 days after training. These findings indicate that the long-term enhancement of HSP70 content through the prolonged exercise protocol was not regulated on the transcriptional level.

**DISCUSSION**

It is well known that prior exercise training attenuates subsequent contraction-induced injury by acute high-intensity exercise in skeletal muscle (14, 27, 30). Cellular tolerance to stresses impacting cellular function is partially mediated by molecular chaperone proteins such as HSPs (6). To understand the beneficial effects of prolonged exercise training, it is important to elucidate whether exercise training has adaptive effects on the expression of HSPs. In the present study we demonstrated, for the first time, that prolonged exercise training results in a long-term enhancement of HSP70 for >2 wk after the last training session. This persistent effect may be one of the adaptive cellular responses to repeated daily exercise in skeletal muscle.

The chronic enhancement of HSP70 potentially plays an important role in maintaining cellular homeostasis in skeletal muscle. In mammals, HSP70 is associated with the protection of striated muscle from contraction-related injury (3, 17) and the attenuation of skeletal muscle atrophy during hindlimb unloading (22). For example, genetic overexpression of HSP70 in mouse skeletal muscle both reduces the number of damaged muscle fibers and produces a smaller than usual deficit of force generation in muscle after severe lengthening contractions (17). Overexpression of HSP70 also prevents the age-related accumulation of oxidation products in skeletal muscle (3). Furthermore, Naito et al. (22) demonstrated that applying heat

![Fig. 1. Changes of heat shock protein (HSP)25 (A), HSP60 (B), and glucose-regulated protein (GRP)78 (C) levels after acute exercise (left) and prolonged training (right). Protein expression in plantaris muscle was analyzed by Western blotting during periods after a single bout of acute treadmill running and treadmill running training for 8 wk. Cont, control; PE, postacute exercise; DT, detraining; d, days. Values are means ± SE. *P < 0.05 compared with control.](http://ajpregu.physiology.org/)
stress, which could increase HSP70, before hindlimb unloading reduced the degree of disuse muscle atrophy. Therefore, persistent enhancement of HSP70 in skeletal muscle potentially contributes to the protection of myofibers and the maintenance of muscle mass. In fact, a positive relationship was observed between HSP70 protein content and relative plantaris muscle weight in this study. These findings suggest that HSP70 might function as a molecular chaperone to maintain muscle mass at a higher level.

Interestingly, HSP70 mRNA was not changed during detraining periods. This phenomenon leads us to hypothesize that long-term enhancement of HSP70 by prolonged training is not accompanied by transcriptional regulation and regulated at a posttranscriptional level. In addition, the fact that HSF-1 and HSF-2 proteins are unchanged during detraining periods also supports this hypothesis. On the other hand, it was reported that HSF was activated by increments of muscle contraction (33). Collectively, it was suggested that the activation of HSF and subsequent transcription of HSP70 mRNA was a short transient after prolonged exercise training, although the activity of HSF was not measured in this study. Several previous studies have similarly reported inconsistencies between HSP70 protein and mRNA levels (8, 15, 21). Murlasits et al. (21) showed that, although HSP70 content was notably increased 24 h after the last exercise session of resistance training in skeletal muscle from young and old rats, the level of HSP70 mRNA was not changed.

There are two possible reasons why the elevation of HSP70 content is unaccompanied by an increase in mRNA levels during detraining periods after prolonged training. The first is an improvement of translation efficiency from mRNA to protein after transcription. It is thought that posttranscriptional...
A second possible reason for the HSP70 observation is a decline in the degradation rate of HSP70 that prolonged the half-life. The duration of the HSP70 increase caused by transient stress stimulation is short in mammalian cells (19, 25). Mizzen and Welch (19) indicated that the half-life of HSP70 induced by elevated temperature in rat embryo fibroblast cells was \(48\) h. In fact, we demonstrated that HSP70 content returned to control levels within \(48\) h after acute exercise. If the half-life of HSP70 is extended by prolonged exercise training, the notable increase of HSP70 during detraining periods might result from accumulation of HSP70 in plantaris muscle. Because protein degradation rate is strongly affected by changes of cellular circumstance in skeletal muscle (4), the half-life of HSP70 may change in skeletal muscle in response to altered cellular conditions created by stress. Several studies suggest that exercise training attenuates the activation of the ubiquitin-proteasome pathway (29). Prolonged exercise training may affect the half-life of HSP70, because degradation of HSP70 is mediated by ubiquitination (9). However, there is no direct evidence regarding whether repeated stress stimulation prolongs the half-life of HSP70. Future studies are needed to determine the relation between the half-life of HSP70 and exercise stimulation. Together, a long-term enhancement of HSP70 content may be associated with improvement of either or both translational efficiency and protein half-life.

In contrast, other molecular chaperones did not show the long-term effects of exercise training: HSP25 was transiently elevated by acute exercise but not by prolonged training, and HSP60, GRP78, and HSC70 were not responsive to either acute exercise or prolonged exercise training. Several studies previously reported that HSP60 and GRP78 were increased by exercise stimulation, but no increase was observed in constitutive HSC70 (7, 16, 21). The difference in findings between the present and previous studies may be associated with differences in the intensity and duration of the exercise protocols. In the present study, we used intermittent high-intensity exercise for prolonged training. This protocol may have induced an HSP70-specific response.

**Perspectives and Significance**

The present study demonstrated that prolonged exercise training provides long-term enhancement of HSP70 for >14 days after the last exercise session. HSP70 is known to have multiple cytoprotective functions including antioxidation, antiapoptosis, and helping protein formation (12, 31). These functions are critical for maintaining cellular integrity and preventing the development of age-related diseases.
functions of HSP70 are important in the prevention of and recovery from subsequent exercise-induced injury and may potentially contribute to acquisition of a muscle defense system with prolonged training.

GRANTS

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