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Heat stress attenuates skeletal muscle atrophy in hindlimb-unweighted rats

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Naito, Hisashi, Scott K. Powers, Haydar A. Demirel, Takao Sugiura, Stephen L. Dodd, and Junichiro Aoki.
Heat stress attenuates skeletal muscle atrophy in hindlimb-unweighted rats. *J. Appl. Physiol.* 88: 359–363, 2000.—This study tested the hypothesis that elevation of heat stress proteins by whole body hyperthermia is associated with a decrease in skeletal muscle atrophy induced by reduced contractile activity (i.e., hindlimb unweighting). Female adult rats (6 mo old) were assigned to one of four experimental groups ($n = 10$/group): *1*) sedentary control (Con), *2*) heat stress (Heat), *3*) hindlimb unweighting (HLU), or *4*) heat stress before hindlimb unweighting (Heat+HLU). Animals in the Heat and Heat+HLU groups were exposed to 60 min of hyperthermia (colonic temperature ~41.6°C). Six hours after heat stress, both the HLU and Heat+HLU groups were subjected to hindlimb unweighting for 8 days. After hindlimb unweighting, the animals were anesthetized, and the soleus muscles were removed, weighed, and analyzed for protein content and the relative levels of heat shock protein 72 (HSP72). Compared with control and HLU animals, the relative content of HSP72 in the soleus muscle was significantly elevated ($P < 0.05$) in both the Heat and Heat+HLU animals. Although hindlimb unweighting resulted in muscle atrophy in both the HLU and Heat+HLU animals, the loss of muscle weight and protein content was significantly less ($P < 0.05$) in the Heat+HLU animals. These data demonstrate that heat stress before hindlimb unweighting can reduce the rate of disuse muscle atrophy. We postulate that HSP70 and/or other stress proteins play a role in the control of muscle atrophy induced by reduced contractile activity.

non-weight bearing; hindlimb suspension; hyperthermia; heat shock protein 70; protein synthesis; soleus muscle

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IT IS WELL KNOWN THAT DECREASING the load on a skeletal muscle results in muscle atrophy (1, 8, 14–16). Studies using a rodent model of muscle disuse atrophy (i.e., hindlimb unweighting via tail suspension) indicate that the initial loss of muscle protein is primarily due to a decrease in the rate of protein synthesis (14). Subsequent atrophy then occurs by increased rates of protein degradation (14). It has been suggested that the initial decrease in protein synthesis during non-weight-bearing activity is a result of a reduced rate of nascent polypeptide chain elongation at the ribosomal level (7). Because the inducible form of the 70-kDa heat shock protein (HSP72) plays an important role in chaperoning nascent peptides during translation, it has been postulated that a decrease in cellular HSP72 levels in myocytes is a potential mechanism to explain the decreased translation observed during muscle disuse (8). Hence, it is conceivable that elevation of cellular HSP72 levels could serve as a countermeasure to attenuate the disuse-induced reduction in protein synthesis.

It also seems possible that HSP72 can play a protective role in the prevention of muscle protein degradation during periods of reduced contractile activity. A conceivable link between HSP72 and reduced protein degradation in muscle is as follows. Recent evidence demonstrates that muscle atrophy induced by immobilization is associated with oxidative injury in myocytes (6). This increase in oxidative stress may accelerate muscle protein breakdown because oxidatively modified proteins are more susceptible to proteolytic attack. Indeed, numerous oxidative modifications of proteins are concomitant with elevated proteolysis (5). In this regard, a function of HSP72 is to bind to nonnative or misfolded proteins and prevent their aggregation by promotion of refolding or renaturation (9). Hence, it seems conceivable that high relative levels of HSP72 could reduce the rate of proteolysis in cells during oxidative stress by repair of damaged proteins. Therefore, on the basis of the collective links between HSP72 and protein synthesis/degradation, it is conceivable that elevating HSP72 in skeletal muscle before unloading could be a countermeasure to retard disuse-induced muscle atrophy. Therefore, the present study was per-

formed to test the hypothesis that exposure to whole body heat stress before unloading of skeletal muscle would elevate muscle levels of HSP72 and attenuate the muscle atrophy associated with short-term, hindlimb-unloading in rats.

METHODS

Animals. This experiment was approved by the Juntendo University Animal Care and Use Committee. Forty specific-pathogen-free adult female Sprague-Dawley rats (6 mo old) were obtained from a licensed laboratory animal vendor (Hamamatsu, Shizuoka, Japan). On arrival from the vendor, animals were individually housed in a climate-controlled room (22 ± 1°C, 50 ± 5% relative humidity, and 12:12-h light-dark photoperiod) and were fed standard rat chow and water ad libitum. Three weeks after arrival from the vendor the rats were randomly assigned to one of four experimental groups: *1)* sedentary control [Con; 297.0 ± 2.0 (SE) g, *n* = 10], *2)* heat stress (Heat; 296.6 ± 3.3 g, *n* = 10), *3)* hindlimb unweighting (HLU; 297.6 ± 3.2 g, *n* = 10);, and *4)* heat stress plus hindlimb unweighting (Heat+HLU; 297.5 ± 3.1 g, *n* = 10).

Experimental protocol. Before hindlimb unweighting, animals in both the Heat and Heat+HLU groups were individually placed in an environmentally controlled heat chamber for 60 min (ambient temperature of 41.0 ± 0.1°C). Heat exposure has been shown to significantly elevate heat stress proteins in rat tissues (4). During this heat exposure, colonic temperatures were recorded by a calibrated thermistor probe (Shibaura Electronics, Tokyo, Japan) inserted 6–7 cm past the anal sphincter into the colon. Immediately after heat exposure, animals were quickly returned to a cage in a 22°C climate-controlled room and provided water and rat chow ad libitum.

Six hours after heat stress, animals in the HLU and Heat+HLU groups were exposed to hindlimb unweighting for 8 days by using the method described by Thomason et al. (16). Briefly, the tail of the rat was wrapped with an elastic tape (5-cm width), and the tail was suspended by a wire attached to a swivel mounted at the top of the cage; this arrangement permits each animal to perform free 360° rotation. The height of each animal was adjusted to allow the rat to support its weight and to move about freely on its forelimbs and to eat and drink freely while the hindlimbs were elevated to prevent contact with the floor or the side of the cage. The animals were checked daily for signs of tail lesions, discoloring, or unusual breathing patterns. Note that, during the 8-day experimental period, both Con and Heat animals remained in their cages and therefore were not exposed to hindlimb unweighting.

Muscle preparation. At the end of 8 day-hindlimb-suspension period, the animals were anesthetized with pentobarbital sodium (50 mg/kg). After a surgical plane of anesthesia was reached, the soleus muscles were quickly removed, carefully weighed, and then rapidly frozen in liquid nitrogen. Muscles were stored at −80°C until analysis of water content, the relative content of HSP72, and muscle protein content.

HSP72 analysis. To determine the levels of HSP72 in the soleus muscles of all experimental animals, we performed polyacrylamide gel electrophoresis and immunoblotting by using the techniques described by Locke et al. (10) and modified by Powers et al. (12). Briefly, samples were minced and homogenized in ice-cold homogenization buffer (10 mM Tris base, 10 mM NaCl, 0.1 mM EDTA, and 15 mM 2-mercaptoethanol, pH 7.6). Homogenates were centrifuged at 12,000 *g* for 15 min, and the total protein concentrations of the supernatants were then determined by using the Bradford technique (2). It is noteworthy that this process of centrifuga-
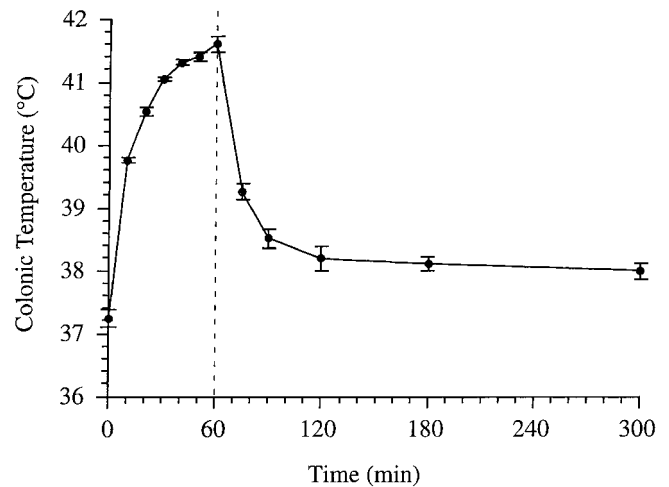

Fig. 1. Colonic temperature of rats exposed to heat stress in an environmental chamber for 60 min. Values are means ± SE; *n* = 10 rats.

tion would have removed contractile proteins, mitochondria, and nuclei from the sample; hence, a small fraction of the HSPs contained within the muscle fiber would have been eliminated from our analysis.

One-dimensional SDS (12% SDS) polyacrylamide gel electrophoresis (20 µg total protein) was performed to separate proteins by molecular weight. After separation, proteins were transferred to nitrocellulose membranes (pore size 0.45 µm) by using a Bio-Rad (Hercules, CA) mini trans-blot cell at a constant voltage of 100 V for 60 min. After protein transfer, the nitrocellulose membranes were blocked for 1 h by using a blocking buffer (3% gelatin and Tris-buffered saline, pH 7.5). The membranes were incubated for 2 h with a monoclonal antibody specific for HSP72 (StressGen, Victoria, BC) and then reacted with a secondary antibody (goat anti-mouse immunogloblin G conjugated to alkaline phosphatase; Bio-Rad) for 2 h. The membranes were subsequently reacted with bromochloroindolyl phosphate-nitro blue tetrazolium substrate. Quantification of the bands from the immunoblots was performed by using computerized densitometry. Standard curves were constructed during preliminary experiments to ensure linearity.

Measurement of muscle protein and water content. Segments of a soleus muscle from each experimental animal were fractionated into soluble (noncontractile proteins) and contractile (myofibrillar) protein on the basis of their differential salt solubilities and centrifugation characteristics by using methods described by Solaro et al. (13) and modified by Criswell et al. (3). Total muscle protein was taken as the sum of the soluble and myofibrillar protein. Protein concentrations were determined by using the biuret technique of Watters (18).

Table 1. *Body masses of the experimental groups before and after the 8-day experimental period*

Group	Body Mass Day 0, g	Body Mass Day 8, g	%Difference, %
Control	297.0 ± 2.0	296.8 ± 2.8	−0.07
Heat	296.5 ± 3.3	297.8 ± 3.2	+0.4
Hindlimb unweighting	297.6 ± 3.2	280.1 ± 3.3*	−5.9
Heat + hindlimb unweighting	297.5 ± 3.1	284.6 ± 4.6*	−4.3

Values are means ± SE. *Day 0* is before any experimental treatment. *Day 8* is after 8 days of normal housing with no experimental treatment (control and heat groups) or after 8 days of hindlimb unweighting (hindlimb and heat + hindlimb unweighting groups). *Significantly different from control and heat groups, *P* < 0.05.

Measurements of total water content in portions of the soleus muscle were made by using a freeze-drying technique incorporating a vacuum pump with a negative pressure of ~1 mmHg. The frozen samples were placed in the vacuum chamber and dried for 48 h before the measurement of dry mass. We have demonstrated previously that this 48 h is sufficient time to complete the drying process (3). Relative muscle water content was computed from the difference between the wet weight of the muscle section (before freeze-drying) and the dry weight of the same muscle section.

Statistical analysis. Group differences were analyzed by using ANOVA. When a significant F ratio occurred, a Fisher's (least significant difference) test was performed post hoc. Statistical significance was established at $P < 0.05$.

RESULTS

All animals exposed to heat stress were able to complete the experiment safely. Before the exposure to heat stress, the colonic temperature of the animals was
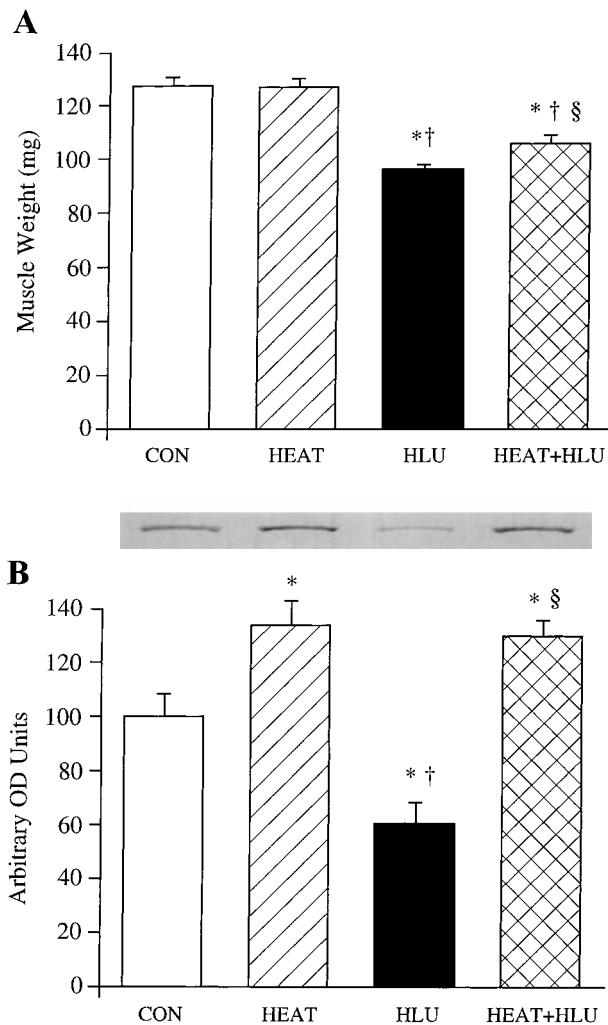
A

B

Fig. 2. *A*: muscle weight in soleus muscle in sedentary control (Con), heat stress (Heat), hindlimb unweighting (HLU), or heat stress plus hindlimb unweighting (Heat+HLU) groups. *B*: representative Western blotting to determine heat shock protein 72 levels and mean heat shock protein 72 content in soleus muscles of all experimental groups. Note that optical density (OD) values are expressed as a percentage of heat shock protein 72 levels in Con group. Values are means ± SE; $n = 10$ rats for each group. Significantly different ($P < 0.05$) from *Con; †Heat; §HLU.

Table 2. *Muscle protein concentration and dry mass-to-wet mass ratio in the soleus muscle of all experimental groups*

Group	Soleus Muscle Dry Mass-to-Wet Mass Ratio, mg/g	Total Protein, mg/g	Myofibrillar Protein, mg/g	Soluble Protein, mg/g
Control	265.4 ± 3.3	232.2 ± 5.3	136.9 ± 4.9	95.3 ± 1.1
Heat	267.4 ± 2.6	229.6 ± 5.3	134.1 ± 5.5	95.5 ± 1.0
Hindlimb unweighting	268.3 ± 2.0	220.4 ± 5.9	123.3 ± 5.1	97.1 ± 1.5
Heat + hindlimb unweighting	270.4 ± 3.03	227.2 ± 4.7	128.9 ± 4.4	98.2 ± 1.2

Values are means ± SE. Dry mass is expressed as mg of dry substance per g (wet wt) of muscle. Protein concentration is expressed as mg of protein per g (wet wt) of soleus muscle. Note that no significant differences ($P < 0.05$) existed between experimental groups in any of these measures.

37.2 ± 0.1 (SE)°C. On exposure to heat stress, colonic temperature gradually increased over time, reaching a peak temperature of 41.6 ± 0.1°C at the end of heating period (Fig. 1).

Table 1 contains the body weights of the four experimental groups before and after the 8-day experimental period. Note that no group differences existed in animal body weights at the beginning of the experiment. After 8 days of hindlimb unweighting, body weights of suspended rats in both the HLU and Heat+HLU groups were significantly decreased.

Soleus muscle weight did not differ between the Con and Heat groups. However, compared with Con, soleus muscle weights in Heat+HLU and HLU groups were significantly reduced by 17 and 25%, respectively. Importantly, the hindlimb-unweighting-induced loss of soleus muscle weight in the Heat+HLU animals was 32% less ($P < 0.05$) than in the HLU animals (Fig. 2*A*).

Muscle protein concentrations (mg protein/g muscle) and the dry mass-to-wet mass ratio did not differ ($P > 0.05$) among experimental groups (Table 2). Nonetheless, total, myofibrillar, and soluble protein content of the soleus muscle differed ($P < 0.05$) among the experimental groups and followed the same pattern as did total muscle mass (Table 3). Specifically, whereas hindlimb unweighting resulted in a significant loss of muscle protein in both the HLU and Heat+HLU groups,

Table 3. *Water and protein content in the soleus muscle of all experimental groups*

Group	Water Content, %	Total Protein, mg/muscle	Myofibrillar Protein, mg/muscle	Soluble Protein, mg/muscle
Control	73.5 ± 0.3	29.7 ± 1.0	17.5 ± 0.8	12.2 ± 0.3
Heat	73.2 ± 0.3	28.9 ± 0.8	16.8 ± 0.7	12.0 ± 0.3
Hindlimb unweighting	73.2 ± 0.2	21.1 ± 0.8*	11.8 ± 0.6*	9.3 ± 0.3*
Heat + hindlimb unweighting	73.0 ± 0.3	24.4 ± 0.9*†	14.0 ± 0.6*†	10.4 ± 0.3*†

Values are means ± SE. Water content is expressed as percentage of total muscle mass. Protein content is expressed as mg of protein per soleus muscle. *Significantly different from control and heat groups, $P < 0.05$. †Significantly different from hindlimb unweighting group, $P < 0.05$.

this effect was significantly ($P < 0.05$) retarded by prior heat stress. Finally, soleus water content (expressed as a percentage of total muscle mass) did not differ between the experimental groups (Table 3).

Figure 2B contains a representative Western blot of our HSP72 analysis and the means ± SE of the relative HSP72 levels in the soleus muscle of all experimental groups. Compared with control, HSP72 levels in the soleus muscle were significantly lower in HLU after 8 days of hindlimb unweighting. In contrast, HSP72 levels were significantly greater in both the Heat and Heat+HLU groups compared with Con. Finally, soleus HSP72 levels did not differ between the Heat+HLU and Heat animals.

DISCUSSION

Overview of principal findings. The key finding of this study was that exposure of rats to whole body heat stress results in a subsequent retardation of disuse muscle atrophy. Indeed, loss of protein in the soleus muscle induced by hindlimb unweighting was significantly reduced in animals exposed to heat stress before unweighting. Although the present experiments do not provide a mechanistic explanation for this observation, it seems possible that HSP72 and/or other stress proteins play a role in the control of muscle atrophy induced by reduced contractile activity.

Potential mechanism(s) for heat stress-induced protection against disuse atrophy. A previous investigation using the rodent hindlimb-unweighting model of disuse atrophy indicates that the loss of muscle protein during muscle unloading is due to both decreased protein synthesis and increased protein degradation (14). Therefore, an intervention that reduces the rate of muscle atrophy during unloading could do so by altering the rate of either muscle protein synthesis or degradation or some combination of the two. The present experiments did not measure the rate of protein synthesis or degradation during hindlimb unweighting. Therefore, we cannot reach a firm conclusion as to the mechanism responsible for the observed protection against muscular atrophy. Nonetheless, we postulate that heat stress-induced expression of heat shock proteins (e.g., HSP72) could have retarded muscle atrophy during unloading by both maintaining protein synthesis and decreasing the rate of muscle protein breakdown. A brief discussion of this postulate follows.

HSP72 and protein synthesis. Previous reports indicate that, after the initiation of muscle unloading by hindlimb unweighting, the rate of protein synthesis declines rapidly, whereas the rate of protein degradation follows a slower time course (reviewed in Ref. 1). For example, the rate of myofibrillar protein synthesis in the unloaded soleus muscle of adult female rats begins to decrease within the first 5 h of unloading and a maximal reduction (−59%) in protein synthesis is acheived at ∼7 days of unloading (14). In contrast, the rate of protein degradation after muscle unloading follows a slower time course. Specifically, after the initiation of muscle unloading, the rate constant of myofibrillar protein breakdown is unaltered during the first 48 h of unloading. After this 48-h lag time, the rate

of protein degradation begins to increase and reaches a peak on the 15th day of unloading (15).

Hence, muscle atrophy induced by 8 days of hindlimb unweighting in adult rats is likely to be largely due to a decreased rate of protein synthesis. We postulate that heat stress-induced expression of HSP72 and/or other stress proteins in skeletal muscle before unloading could assist in maintaining protein synthesis in these conditions. In this regard, a decrease in the nascent polypeptide elongation rate has been reported to be a significant contributor to the decline in protein synthesis rates in the unloaded soleus muscle (7, 8). Specifically, Ku et al. (8) reported a 28–44% decrease in polysomal HSP72 levels after 12–18 h of unloading of the soleus muscle. These investigators argued that the decrease in polysomal HSP72 is a potential mechanism to explain the reduced elongation rate of nascent polypeptides in atrophying muscle because HSP72 binds to the nascent polysome and guides it through the ribosome channel (8, 11). Furthermore, HSP72 has also been shown to affect the initiation of translation (17). In the present experiments, HSP72 levels in the soleus muscles of hindlimb-suspended animals were significantly (−40%) below control values. By contrast, HSP72 levels in soleus muscles of animals in the Heat and Heat+HLU groups were significantly (35%) above control. Therefore, it seems possible that elevating HSP72 levels in the soleus muscle before hindlimb unweighting could have maintained protein synthesis by sustaining polypeptide elongation rate.

HSP72 and protein degradation during oxidative stress. It is also conceivable that HSP72 can play a protective role in the prevention of muscle protein degradation during periods of reduced contractile activity. The potential mechanistic link between HSP72 and protein degradation is that muscle atrophy induced by immobilization is accompanied by oxidative injury in myocytes (6). This increase in oxidative stress accelerates muscle protein breakdown because oxidatively modified proteins are very susceptible to proteolytic attack (5). HSP72 could prevent this proteolytic activity by binding to oxidatively modified proteins and assist in their refolding (10). Hence, in the present experiments, it seems possible that high cellular levels of HSP72 induced by heat stress could reduce the rate of proteolysis during hindlimb unweighting by repair of oxidatively modified proteins. This is a testable hypothesis and is worthy of further investigation.

Summary and conclusions. To our knowledge, these are the first experiments to demonstrate that whole body heat stress provides protection against skeletal muscle atrophy induced by hindlimb unweighting. From a practical perspective, these experiments suggest that hyperthermia by heat stress alone or in combination with other countermeasures could assist in reducing the rate of muscle atrophy during periods of disuse. Potential applications of this simple countermeasure to reduce muscle atrophy include spaceflight and numerous clinical applications (immobilization of limbs due to bone injury).

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