Heat Stress Facilitates the Recovery of Atrophied Soleus Muscle in Rat

K. GOTO*, M. HONDA*, T. KOBAYASHI*, K. UEHARA*, A. KOJIMA*, T. AKEMA*, T. SUGIURA†, S. YAMADA‡, Y. OHIRO§, and T. YOSHIOKA*,¶

*Department of Physiology, St. Marianna University School of Medicine, Kawasaki, 216-8511 Japan; †Faculty of Education, Yamaguchi University, Yamaguchi, 753-8513 Japan; ‡Department of Life Science, Graduate School of Arts and Science, University of Tokyo, Tokyo, 153-8902 Japan; §School of Health and Sports Sciences, Osaka University, Osaka, 560-0043 Japan; and ¶Aomori University of Health and Welfare, Aomori, 030-8505 Japan

Abstract: Effects of heat stress on the recovery of atrophied soleus muscle were studied in rats. Ten-week-old male Wistar rats were randomly divided into cage control (CC) and 5-day hindlimb suspension group (HS). The half of the rats in group HS was exposed to heat stress (41°C for 60 min) in an incubator immediately after the hindlimb suspension (HS-H) and the other group of rats was not heat stressed (HS-C) prior to 10 days of ambulation recovery. One group of cage control rats (CH) was also exposed to heat similarly. The soleus muscles were dissected at four time points, i.e., immediately after the suspension (or heat stress), and 3, 5, and 10 days after the recovery (n = 8 per group at each time point). The absolute wet weight and water and protein content of whole soleus muscle in group HS-C were ~36, 27, and 8 mg less than CC (p < 0.05).

Thus, the percentage contribution of water and protein loss to the decrease in muscle weight was 75 and 22%, respectively. Although water content, as well as muscle weight, was elevated within 3 days, the increase of protein was delayed. Heat exposure prior to recovery accelerated the increase in protein content even in the control group. These phenomena were closely associated with 72-kD heat shock protein (HSP72) content. It is suggested that heat stress applied at the end of hindlimb unloading facilitated the recovery of atrophied soleus muscle of rat, through possibly HSP72-related events of protein metabolism. The data also indicated that the combination of heat and mechanical stress evoked larger and long lasting HSP72 response than does heat or mechanical stress alone. [The Japanese Journal of Physiology 54: 285–293, 2004]

Key words: atrophy, soleus, rat, recovery, heat stress, HSP72.

Skeletal muscle atrophy is induced by gravitational unloading [1–3]. It was reported that the major cause of the decrease in muscle weight following hindlimb suspension of rats was loss of water and protein [4]. However, the percentage protein content in the atrophied soleus muscle was even increased because the magnitude of the absolute water loss (41 mg) was greater than that of protein (6 mg) [4]. On the contrary, protein synthesis in atrophied skeletal muscles is stimulated by reloading (or increased loading) resulting in recovery of muscular mass including protein and water contents [1]. It is generally accepted that exercise induces the increment in synthesis and contents of protein in skeletal muscles. Therefore, exercise, as well as loading, is a useful tool for stimulation of recovery from muscular atrophy, i.e. rehabilitation. However, the precise mechanism responsible for exercise- and loading-induced increment in muscular protein contents is still unclear.

Exercise causes an increase of core body temperature [5] and of the expression of 72-kD heat shock protein (HSP72) [6–11]. The HSP72 content is also increased by elevation of temperature [12], ischemia [13], hypoxia [14, 15], acidosis [16], oxidants [17], and/or metabolic stress [15]. The HSP72 may have some protective roles against cellular stress as mo-
The protein translation would be more active during synthesis than that during the resting phase, because progressing phase may be more important in protein recovery from atrophy, as well as the atrophy-muscle after unloading.

It has been shown, on the other hand, that higher cellular levels of HSP72 induced by heat stress could play a role in the prevention of muscle protein degradation by repair of damaged protein caused by oxidative stress in living muscle cells [1]. The preconditioning by heat stress to increase the HSP72 content could attenuate muscle atrophy induced by hindlimb unloading [1]. It is considered that the prevention of muscular atrophy by heat stress would be related to the inhibition of protein degradations and the promotion of protein synthesis. These observations suggest that the exercise-induced increment in protein content may be, in part, due to the increment in HSP72 content caused by elevated core body temperature. Further, the application of heat stress to muscle cells might stimulate the synthesis of muscular proteins. Recently, we showed that heat stress stimulated protein synthesis in cultured skeletal muscle cells (L6) [22]. If heat stress has such effects on muscle cells, the recovery of atrophied muscle could be facilitated by heating of muscle after unloading.

The chaperoning effects of HSP72 during the recovery phase from atrophy, as well as the atrophy-progressing phase, may be more important in protein synthesis than that during the resting phase, because the protein translation would be more active during the recovery phase [23]. Therefore, the current study was carried out to test our hypothesis that application of heat stress to muscle cells during recovery from atrophy stimulates the synthesis of muscular proteins in vivo.

MATERIALS AND METHODS

Animals and treatments. All experimental procedures were conducted following the Guiding Principles for the Care and Use of Animals Approved by the Council of the Physiological Society of Japan. This study was also approved by the Committee on the Animal Care and Use at the university. Male Wistar rats, aged 10-week-old, were used. Rats were randomly divided into 4 groups: (1) cage control (CC), (2) cage control with heat stress (CH), (3) hindlimb suspension followed by normal recovery (HS-C), and (4) hindlimb suspension followed by heat stress before recovery (HS-H) (n = 32 in each group, respectively).

The rats in the HS-C and HS-H groups were subjected to continuous hindlimb suspension for 5 days [4, 24]. Briefly, the tails of the hindlimb-suspended rats were cleaned and strips of a padded adhesive tape (~5 mm width and 3 cm length) were placed longitudinally on the dorsal and ventral sides of the mid-tail (~5–10 cm from the base of the tail) and one strip was wrapped around the tail and longitudinal strips. The tape was wrapped loosely to maintain an intact blood flow and the distal third of the tail was untouched to allow for thermoregulation. A string was inserted through the gap between the tail and tape and fastened to the roof of the cage at a height allowing the forelimbs to support the weight, yet preventing the hindlimbs from touching the floor or the sides of the cage (55 × 38 cm and 34 cm height). The rats could reach food and water freely by using their forelimbs. Each cage control rat was kept individually in a cage with the same size as for hindlimb-suspended groups. All rats were housed in a vivarium room with a 12 hr light:12 hr dark cycle and with the temperature and humidity maintained at approximately 23°C and 55%, respectively. A commercial solid diet (CE-2, Nihon CLEA, Tokyo) and water were supplied ad libitum.

Immediately after the 5-day hindlimb suspension, ambulation recovery was allowed to the rats in the HS-C group. But the rats in the HS-H group were first exposed to heat stress (41°C for 60 min) in an incubator without anesthesia before the ambulation recovery. This heat stress caused an increase in a colonic temperature of rats up to 41°C. In a pilot study, the time course changes of colonic temperature was determined by using a digital thermometer (PTC-201, Unique Medical, Tokyo) equipped with a wired thermosensor (PTW-300, Unique Medical). The colonic temperature of each rat was measured at either 15th, 30th, 45th, or 60th min during heating. Approximately 30 min after the initiation of heat stress, the colonic temperature increased up to 41°C. At the end of heating, the colonic temperature ranged from 41 – 42°C. Therefore, the core temperature of rats was maintained at ~41°C at least for 30 min. Rats in the CH group were also exposed to heat stress similarly. Then, all rats were allowed to recover in the cages. Soleus muscles were dissected from both hindlimbs under anesthesia with i.p. injection of sodium pentobarbital (5 mg/100 g body weight) immediately after the termination of suspension with or without heat stress, and 3, 5, and 10 days after recovery (n = 8 per group at each stage). The samples were trimmed of excess fat and connective tissues, weighted, frozen in liquid nitrogen, and stored at −80°C.

The left soleus muscles were homogenized in 10
vol. of an isolation buffer (10 mM Tris-HCl, 10 mM NaCl, 0.1 mM EDTA, pH 7.6) using a glass-
homogenizer, and completely solubilized by alkali treat-
ment with one vol. of 2 N NaOH at 37°C for 30 min. Protein concentrations in the homogenates were
determined by using protein assay kit (Bio-Rad, Hercules,
CA). Total protein content in whole muscle was also
calculated. The right whole soleus muscle was used for the measurement of water content. The freeze-dried
muscles were placed in a freeze-dryer (–45°C) under vacuum for ~48–72 hr as was reported elsewhere [1].
Muscle tissues were then re-weighted after ~48 and
72 hr. The dry weights of all muscles, which were
measured after ~48 and 72 hr, were identical, suggest-
ing that water was lost completely within ~48 hr. Water
contents (percentage relative to muscle wet weight
and total content in whole muscle) were calculated.

**SDS-PAGE, Western blotting, and immunodetection.** The remaining portions of the
homogenate samples were solubilized in sodium-
dodecylsulfate (SDS) sample buffer [30% (v/v) gly-
cerol, 5% (v/v) 2-mercaptoethanol, 2.3% (w/v) SDS,
62.5 mM Tris-HCl, 0.05% (w/v) bromophenol blue,
pH 6.8] at a concentration of 1 mg protein per mL and
boiled for 3 min. The SDS- polyacrylamide gel elec-
trohoresis (PAGE) was carried out on 12.5% poly-
acrylamide [biascrylicamide/acrylamide, 1: 20 (w/w)]
slab gel (60 × 85 × 1 mm) containing 0.5% SDS at a
constant current of 20 mA for 90 min as was reported
previously [22]. Equal amounts of protein (20 µg) were
loaded on each gel.

The contents of HSP72 (HSP70 inducible) were
then analyzed [22]. Following SDS- PAGE, proteins
were transferred to polyvinylidene difluoride (PVDF)
membranes (0.2-µm pore size, Bio-Rad) by using the
Bio-Rad mini trans-blot cell at a constant voltage of
100 V for 60 min at 4°C. After the transfer, the mem-
branes were blocked for 1 hr by using a blocking buffer
[5% skim milk with 0.1% Tween 20 in Tris-buffered
saline (TTBS), pH 7.5]. Then, the membranes were
incubated for 1 hr with a polyclonal antibody for HSP72
(SPA-812, StressGen, Victoria, BC) and then reacted
with a secondary antibody (goat anti-rabbit
immunogloblin G conjugated to alkaline phosphatase;
Sigma Chemical, St. Louis, MO) for 2 hr. The mem-
branes were subsequently reacted with bromo-
chloroindolyl phosphate-nitroblue tetrazolium substrate.
Quantification of the bands from the immunoblots was
performed by using computerized densitometry [22].
Standard curves were constructed during the prelimi-
nary experiments to ensure linearity.

**Statistical analysis.** All values were expressed
as means ± SEM. Statistical significance was analyzed
by using analysis of variance followed by Scheffé’s
post hoc test. The significance was accepted at p <
0.05.

**RESULTS**

**Body and muscle weight.** Figure 1A shows
the response of the body weight to hindlimb suspen-
sion and ambulation recovery with or without heat
stress. No significant effects of hindlimb suspension
or heat exposure were noted in the body weight, al-
though the mean weight at end of suspension and 3rd
day after recovery tended to be less than controls (p >
0.05). Although the weight in the suspended group,
especially, tended to be increased during recovery, sta-
tistical significance was not obtained in any groups.

Atrophy of soleus muscle was induced following
hindlimb suspension (Fig. 1B). The absolute weight
in group HS-C was ~36 mg less than the age-matched
control, CC (p < 0.05). However, the suspension-
related decrease in muscle weight was normalized within
3 days (p < 0.05) and gradually increased further there-
after. No significant effect of heat exposure was noted
in the absolute muscle weight.

The soleus weight relative to body weight was also
lower in the hindlimb-suspended group (Fig. 1C). The mean relative wet weights in group HS-C and HS-H
were 12–14% less than group CC (p < 0.05). The relative
weight of muscle in the suspended group (HS-
C) insignificantly increased within 3 days of ambula-
tion recovery without heat stress (10%, p > 0.05), and
the weight remained unchanged thereafter. Further, the
relative weight in group HS-C was still lower than the
control level during the 10 days of recovery period (p
> 0.05).

The relative wet weight measured immediately af-
after heat exposure was not influenced. But the heat
stimulated the increase of muscle weight. The relative
mean weight of group HS-H was significantly
increased by 22% within 3 days (p < 0.05) and was
maintained stable during the rest of the recovery pe-
riod. The relative weights at 3rd and 5th day were also
~13% greater than group HS-C (p < 0.05).

**Water content.** The absolute water content of
soleus muscle was lowered by hindlimb suspension
(Fig. 2A). The mean level in group HS-C and HS-H
were 27.7 mg (27.4%) and 23.7 mg (23.3%) less than
that in the age-matched CC, respectively (p < 0.05).
However, the water content in whole muscle recov-
Significant effects of heat stress were not noted throughout the experimental period. Generally, the patterns of responses in the absolute water content were similar to those in the wet weight of muscle (Fig. 1B).

The mean percentage water content in the hindlimb-suspended group, without heat stress, was approximately 0.6% less than the control level (Fig. 2B, \( p > 0.05 \)). The water content was not influenced significantly by an acute heat exposure, either. However, the water content in group HS-C was significantly elevated 3 days after recovery (3.3%, \( p < 0.05 \)). They were also greater than the age-matched control levels (CC, \( p < 0.05 \)). The water content in HS-C group was maintained at a higher level until day 5 in the recovery period. Significant effects of heat stress were not noted throughout the experimental period. Generally, the patterns of responses in the absolute water content were similar to those in the wet weight of muscle (Fig. 1B).

The mean percentage water content in the hindlimb-suspended group, without heat stress, was approximately 0.6% less than the control level (Fig. 2B, \( p > 0.05 \)). The water content was not influenced significantly by an acute heat exposure, either. However, the water content in group HS-C was significantly elevated 3 days after recovery (3.3%, \( p < 0.05 \)). They were also greater than the age-matched control levels (CC, \( p < 0.05 \)). The water content in HS-C group was maintained at a higher level until day 5 in the recovery period. Significant effects of heat stress were not noted throughout the experimental period. Generally, the patterns of responses in the absolute water content were similar to those in the wet weight of muscle (Fig. 1B).
period compared to that observed immediately after the termination of suspension ($p < 0.05$), although it was decreased after 10 days to the control level. The heat stress-related increase of the percentage water content at day 0 in group HS-H (vs. HS-C) was not significant statistically ($p > 0.05$). But the water content was increased by 2.9% 3 days after heat exposure ($p < 0.05$). It was then decreased toward the control level at 5th day ($p > 0.05$) and was normalized at 10th day ($p < 0.05$).

**Protein content.** Figure 3-A shows the changes in the absolute protein contents of soleus. The total protein contents in group HS-C and HS-H were 7.8 mg (26.9%) and 7.0 mg (24.0%) less, respectively, than the age-matched CC at the end of 5-day suspension ($p < 0.05$). Even though the protein content remained unchanged during the first 3 days, it was gradually increased thereafter. It was significantly increased during the 10 days of recovery without heat stress (21%, $p < 0.05$), even though the mean level was still less than that in the age-matched controls ($p > 0.05$).

Heat stress applied prior to the ambulation recovery, stimulated the elevation of total protein content in the muscle. The protein content in hindlimb-suspended group was 23% increased within 5 days ($p < 0.05$), even though the effect of heat exposure at the 3rd day was not significant. It was further increased and reached the control level 10 days later (35%, $p < 0.05$). The levels at 5th (22%) and 10th day (17%) were also greater than those of the age-matched HS-C ($p < 0.05$), suggesting that heat exposure stimulated the post-suspension recovery of protein content. It was further indicated that heat stress was also stimulated the protein synthesis in control group. The protein content 5 days after heat exposure was 12% elevated relative to the level at day 0 ($p < 0.05$).

![Fig. 3](image-url) Changes in the absolute (A) and the percent protein content relative to the total wet weight (B) of soleus muscle in response to hindlimb suspension followed by ambulation recovery with or without heat stress. Mean ± SEM. $n = 8$ in each group. Abbreviations are the same as in Fig. 1. a, d, and f: $p < 0.05$ vs. day 0 (the end of suspension) in each group, the age-matched CC, and the age-matched HS-C, respectively.
The relative protein content normalized by total muscle weight at the end of suspension was similar to that in cage controls (Fig. 3B). However, the levels in both HS-C and HS-H groups were significantly decreased 3 days after recovery ($p < 0.05$). The mean content in group HS-C was less than that in the age-matched control also ($p < 0.05$). However, these levels were gradually increased during 10 days of recovery.

**HSP72.** Figure 4A illustrates the representative patterns of the responses in HSP72 content to heat stress and/or hindlimb suspension. The mean level of HSP72 content in the hindlimb-suspended group without heat stress was insignificantly less than the age-matched control (Fig. 4B, $p > 0.05$). However, it was 86% increased within 3 days of ambulation recovery ($p < 0.05$), although the mean level gradually decreased again. The level at 10th day was significantly lowered than that observed at 3rd day ($p < 0.05$).

Heat exposure resulted in an immediate increase of HSP72 content. Significant effect was noted even in the control group. The level of HSP72 content of control rats (CH) 3 days after heat stress was significantly higher than that in the age-matched CC ($p < 0.05$). But the level of expression was gradually decreased during the rest of the recovery period. The heat exposure increased the HSP72 content in the suspended group (HS-H) acutely (day 0, 59%, $p < 0.05$). The level of HSP72 content was further elevated 5 and 10 days later by 45 and 59%, respectively ($p < 0.05$). Although it was decreased at 10th day, relative to the level seen at 5th day, ($p < 0.05$), the mean level remained higher than other groups.

**DISCUSSION**

The effects of heat stress applied immediately after 5-day hindlimb suspension on the wet weight, water and protein contents, and HSP72 content in rat soleus muscle during the 10-day recovery period were studied. The results indicated that the application of heat stress stimulated the protein synthesis even in the control animals and caused a beneficial effect in the recovery of atrophied muscles. The findings in the present study support our hypothesis that heat stress stimulates the synthesis of muscular proteins and promote the recovery from atrophy.

**Muscle wet weight.** It is generally accepted that approximately 75–80% of the wet weight of skeletal muscle tissues is water. Atrophy of soleus muscle (~36 mg) was induced following the 5-day hindlimb suspension due to the loss of absolute water and protein contents. The magnitude of the mean absolute water loss (~27 mg) was greater than that of protein (~8 mg). Therefore, the percentage water content in the hindlimb-suspended group (HS-C) was slightly less than that in controls (CC, $p > 0.05$). This phenomenon agrees with the result of our previous study which reported that the percentage protein level was even increased due to the greater loss of water than protein [4].

**Fig. 4.** (A) Representative expression patterns of 72-kD heat shock protein (HSP72) in response to hindlimb suspension followed by ambulation recovery with or without heat stress. (B) Changes in the mean levels of HSP72 content. Mean ± SEM. $n = 8$ in each group. OD: optical density. Other abbreviations are the same as in Fig. 1. a, b, c, d, e, and f: $p < 0.05$ vs. day 0, day 3, day 5 in each group, the age-matched CC, the age-matched CH, and the age-matched HS-C, respectively.
The percentage contribution of water loss to the decrease in muscle wet weight of group HS-C and HS-H was 75% and 77%, relative to group CC, and that of protein loss was 22% and 23%, respectively. These results were also consistent with our previous study [4]. Therefore, it is indicated that the suspension-related muscle atrophy is caused by decreased content of both water and protein and that the decrease in muscle wet weight is influenced by the loss of water content mainly. This phenomenon may be closely related to the fluid shift from hindlimbs toward head, because the head-down tilt position is maintained during hindlimb suspension.

Both absolute and relative muscle weights were increased within 3 days. These recoveries were due to the increment of water content (Fig. 2), but not of protein (Fig. 3). Therefore, the percentage of water content was significantly elevated at the 3rd day of recovery (Fig. 2). Increased water content could be the cause of the whitish muscle color (data not shown). Water content in hindlimb muscles may be increased due to the redistribution of fluid, because the rat maintains the horizontal body position on the floor. Another possibility is edema-related increase in water content. Reloading of soleus muscle after a certain period of gravitational unloading causes muscle fiber damage [25]. Although the absolute water content remained unchanged during 3–10 days of recovery (Fig. 2), the absolute protein content gradually increased (Fig. 3). Thus, the percentage of water content in whole muscle declined toward the control level gradually (Fig. 2). These data strongly suggest that the termination of head-down tilt hindlimb suspension and reloading of muscle cause an acute increase of water content prior to increment of muscular protein content. But the recovery of protein content was also progressed gradually and the muscle atrophy was recovered.

Such a water-protein associated recovery from atrophy was further stimulated by application of heat stress immediately after the termination of suspension. The results in the present study clearly suggested that reloading-associated protein synthesis was further stimulated by initial heat stress. The total protein content in the heated muscle completely recovered to the age-matched control level within 10 days, even though that in the muscle without heat stress did not recover fully.

**HSP72.** It has been generally known that the content of HSP72 is up-regulated by not only hyperthermia but also numerous cellular stresses [16, 17, 21, 22, 26, 27]. In the present study, the content of HSP72 tended to decrease after hindlimb suspension (Fig. 4, p > 0.05). This phenomenon agrees with the previous reports [1, 2]. On the contrary, heat stress caused an increased content of HSP72. Elevation of the HSP72 content was noted in the muscles sampled from both control and hindlimb-suspended groups immediately after 60-min exposure. The level was further increased 3 days later. Such a heat exposure-related increase of HSP72 content was prominent in the previously suspended group and the effect was maintained longer.

The HSP72 content in the suspended group was also increased during the early recovery period even without heat stress. At the 3rd day of recovery, HSP72 content in HS-C group was significantly elevated compared with the age-matched control level (p < 0.05). As heat stress was not applied to the animals in group HS-C, the increments in HSP72 content may be induced by other cellular stresses. Reloading itself could be the strong stress for soleus muscle, which may stimulate the content of HSP72. It is reported that the HSP72 content in skeletal muscle of rodents is also increased in response to exercise [7–11, 28] or mechanical stretch [22]. The HSP72 levels in group HS-H were maintained higher during the 10 days of recovery period than those in other group (Fig. 4). The elevation of HSP72 content in both CH and HS-C groups was observed only at 3rd day. These results suggest that the combination of heat and mechanical stress evokes larger and long lasting HSP72 response than does heat or mechanical stress alone. The HSP72, the inducible form of HSP70, plays an important role in chaperoning nascent peptides during translation, in cellular protein transport, and in stability [19, 20]. Increased content of HSP72 may also stimulate the synthesis of cellular proteins, although the precise role of HSP72 in skeletal muscle cells is still unclear.

In the present study, the application of heat stress immediately after the suspension caused an elevation of HSP72 content in soleus muscle, and the higher level was maintained during the recovery period. Heat stress on the sedentary control (CH) group also increased the HSP72 content, muscle wet weight, and protein content. A faster recovery of wet weight and protein content was also observed in atrophied muscle, in which the heat stress was applied. However, the level of HSP72 content was not necessarily correlated with muscular protein level following heat stress and during recovery period. The patterns of responses in protein content (Fig. 3) and HSP72 content (Fig. 4) were different, even though both of them were increased during the recovery period. The elevation of HSP72 content preceded that of protein.

These observations may suggest that the increase in
protein synthesis could be caused by chaperoning effects of HSP72 and also by other mechanism. The content of HSP72 in cultured skeletal muscle fibers was stimulated by heat stress, mechanical stretch, or combination of heating and stretch [22]. Recently, it is also reported that activation of the signal pathway, in which phosphatidylinositol 3-kinase (PI3K), protein kinase B (Akt), and glycogen synthase kinase-3β (GSK-3β) are involved, plays an important role in the recovery from muscle atrophy [23]. Furthermore, our study indicated that the phosphorylation and activation of Akt and GSK-3β in cultured skeletal muscle cells were induced, when HSP72 content and protein content were increased in response to heat stress [29]. These observations strongly suggest that both HSP72 content and protein synthesis are stimulated by heat stress.

In conclusion, heat stress applied at the end of hindlimb unloading facilitated the recovery of atrophied soleus muscle of rat. It is suggested that heat-stress-associated increase in the content of HSP72 might be one of the key factors in the stimulation of protein synthesis during recovery period.

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Heat-Stress-Associated Recovery of Atrophied Muscle