Preconditioning With Whole-Body or Regional Hyperthermia Attenuates Exercise-Induced Muscle Damage in Rodents

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Summary
Our aim was to investigate whether hyperthermia before exercise protects against exercise-induced skeletal muscle damage. Two hyperthermia protocols were evaluated. In the first, male ICR mice were exposed to 30 min of whole-body heat in an environmental chamber at an ambient temperature of 42 °C. Heat-exposed and non-heat-exposed mice subsequently completed 60 min of downhill running on a treadmill, 24 h after exposure. Heat exposure significantly increased HSP70 and HSP25 content in the soleus muscle compared to controls. Plasma creatine kinase, muscle β-glucuronidase, and histochemical (hematoxylin and eosin stain) analysis demonstrated that muscle damage was lower in the heat-exposed mice than in the non-heat-exposed mice. In the second, the effect of regional heating of the legs, by microwave diathermy, on the prevention of exercise-induced muscle damage was evaluated in male Wistar rats. Microwave-treated and non-microwave-treated rats again completed the running protocol 24 h after exposure. Microwave diathermy increased the muscle temperature to 40 °C, significantly increased HSP70 and HSP25 content in the soleus muscle, and significantly attenuated exercise-induced muscle damage. Therefore, hyperthermia before exercise increases skeletal muscle HSPs and attenuates the risk of exercise-induced muscle injury.

Key words
Heat shock protein • Hyperthermia • Microwave treatment • Muscle damage • Creatine kinase • β-glucuronidase • Eccentric exercise

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Introduction
Heat shock proteins (HSPs) are a family of highly conserved proteins induced in response to a wide array of physiological and environmental stresses, including heat, cold, ischemia, hypoxia, and energy depletion. The inducible form of 70-kDa HSP (HSP70) has been intensively studied with its induction via preconditioning by heat shock, producing a state of resistance to subsequent stresses [1]. This protective function is attributed to the molecular chaperone function of HSPs, which prevents protein aggregation and promotes protein disaggregation by catalyzing the folding of damaged or denatured proteins [2]. HSP70 is also essential for the breakdown of non-foldable proteins [3].

Increasing the expression of HSP70 through hyperthermia exposure also protects against stress-induced organ damage. For example, hyperthermia exposure in rats prior to an 8-day period of hindlimb suspension significantly elevated HSP70 content in the soleus muscle and reduced suspension-induced muscle atrophy [4]. Similarly, hyperthermia before ischemia increased the level of HSP70 in the heart and suppressed deterioration of heart function after reperfusion [1]. Therefore, hyperthermia induced HSP70 expression has an overall protective effect on muscles.

HSP70 expression might be important within the
context of exercise-induced muscle damage and delayed onset of muscular soreness (DOMS), which typically develop several days after an intense bout of exercise in individuals unaccustomed to exercise [5]. Muscle damage and DOMS are generally reduced when a second bout of exercise is performed ≥2 weeks after the first [6,7]. It has been suggested that HSPs induced by the first bout of intense exercise exert a protective effect on the muscle during the subsequent bout of exercise [2]. Considering that exposure of myocytes to hyperthermia results in significant protection against 2,4-dinitrophenol- or A23187-induced cell damage, which mimics muscle damage during exercise [8], increasing muscular HSP70 by exposure to hyperthermia before exercise might protect against exercise-induced muscle damage.

In animals, exposure to a high-temperature environment is commonly used to increase whole-body HSP expression. This approach, however, is not suitable to specifically increase HSP in leg muscles to protect against muscle damage associated with running. Microwave diathermy might provide a solution to achieve a sufficient regional increase in temperature [9] or blood flow [10,11] to induce HSP expression in the lower limb muscles.

To test our hypothesis, mice and rats were subjected to whole-body heating in a hot chamber or regional leg heating using a microwave diathermy, followed by a 60-min bout of downhill running on a treadmill the next day. HSP expression and markers of exercise-induced muscle damage were evaluated between heat exposed and non-exposed animals. Our goal was to provide the evidence for the practical use of pre-exercise skeletal muscle heating for the prevention of exercise-induced muscle damage.

Methods

Ethics statement

The Animal Care and Use Committee of Nippon Medical School approved all experimental and animal care procedures.

Whole-body heat exposure and exercise protocol

Male outbred ICR mice (Nippon Curea Industry; age, 10 weeks) were used because they have greater running ability than inbred mice (C57BL/6, BALB/c). The mice were maintained in a controlled environment: 22 °C, 50 % humidity, and a 12/12 h day/night cycle (lights on 08:00-20:00 h). To ensure familiarity with the downhill running task, mice practiced the task 10 min/day, 5 days/week, at a treadmill speed of 10 m/min (0 % gradient) for 1 week before testing. We confirmed that this training did not enhance the level of muscle HSP70. After the familiarization period, the mice were randomly divided into 2 groups: control (n=32) and heat-exposed (n=32). Heat-exposed mice were anaesthetized using three mixed anesthetics via intraperitoneal injection and placed in an environmental chamber at an ambient temperature of 42 °C for 30 min. Before and after heat exposure, rectal temperature was measured by inserting a probe (Yellow Springs Instruments) 1 cm into the rectum. After heat exposure, the mice were fed a regular diet (Oriental Yeast Co.) and water ad libitum. The control mice were only subjected to the anesthesia.

Based on a previous study [12], heat-induced HSP72 expression was evaluated 24 h after heat exposure in 8 animals from the heat-exposed and control groups (Fig. 1A). Animals were sacrificed by decapitation and the soleus muscles of both hind limbs were immediately excised and rinsed with saline. The right soleus was frozen in liquid nitrogen and stored at -80 °C until enzymatic analysis. The left soleus was immersed in an embedding medium, frozen in isopentane cooled in liquid nitrogen, and stored at -80 °C until histochemical analysis. Blood was centrifuged at 1000×g for 20 min and the resultant plasma stored at -80 °C until analysis. The remaining control and heat-exposed mice completed the 60 min downhill running protocol (grade, -20°), at a treadmill speed of 25 m/min, as previously described [13]. Downhill running was selected based on previous reports of higher serum creatine kinase (CK), an indicator of muscle damage, after eccentric exercise (downhill running) than uphill running [5]. Once the exercise protocol was completed, the mice were fed their usual diet, with water ad libitum.

Nosaka et al. [14] reported that plasma CK activity does not increase immediately but 24 h after high-intensity exercise. Besides, Komulainen et al. [15] reported that there were no observed increase in β-glucuronidase activity and histochemical muscle damage in the skeletal muscles immediately after downhill running. Based on these previous results, we decided to sacrifice the mice as previously described, and collect samples at 12, 24, or 48 h after the running protocol (Fig. 2A). Muscles were harvested and stored along with blood samples, as described above.
Regional heat exposure and exercise

Microwave diathermy was used for regional heating of the leg muscles. As mice were too small for the apparatus, we carried out this protocol with 40 male Wistar rats (age, 8 weeks; weight, 280 g). Again, after 1 week of familiarization with treadmill running, the rats were randomly divided into two groups: control and microwave-exposed (ME) groups. For regional heating, the rats were anaesthetized using three mixed anesthetics, via intraperitoneal injection, and depilatory cream applied to the hind limbs for hair removal. With the rats in the couchant position, the probe of an N550 thermometer (Nikkiso YSI Co., Ltd., Tokyo, Japan) was inserted into the right gastrocnemius muscle to measure intramuscular temperature. Hindlimbs were then sandwiched between two pads connected to an SW-101 microwave apparatus (Ito Microwave Co., Japan) with an output frequency of 27 MHz, and 100-watt microwaves applied for 15 min (Fig. 4A), under continuous temperature monitoring to 40 °C (Fig. 4B). After exposure, the rats were returned to their cages and fed their usual diet, with water ad libitum.

The control rats were only subjected to anesthesia. Eight rats in each group were sacrificed 24 h after exposure as previously described and the soleus muscle harvested bilaterally for HSP quantification (Fig. 4C). The remaining rats completed the 60 min of downhill running protocol and subsequently returned to their cage with fed and water ad libitum. Rats in each group were sacrificed at 24 or 48 h after the protocol.

Polyacrylamide gel electrophoresis and immunoblotting

HSP70 and HSP25 content was evaluated in the soleus muscle using immunoblotting. Portions of the soleus muscles were homogenized in 15 mM Tris-HCl (pH 7.5) and 600 mM NaCl and centrifuged. The protein concentration of the supernatant was measured using the Coomassie Pulse Protein Assay Reagent Kit (Pierce, USA). The supernatant from each muscle sample was applied to a 1-mm-thick 12 % SDS-polyacrylamide gel as previously described [12]. After electrophoresis, the proteins were blotted onto a polyvinylidene difluoride (PVDF) membrane using a BioRad mini protein gel system (Bio-Rad). After blotting, the membrane was washed in Tris-buffered saline containing Tween-20 (TBS-T): 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 0.9 % Tween-20. The reaction was blocked using 5 % skimmed milk at room temperature for 2 h. Washing was repeated 3×. Subsequently, the membrane was incubated overnight at 4 °C with mouse monoclonal anti-HSP70 antibody (Stressgen SPA-810), rabbit polyclonal anti-HSP25 antibody (Stressgen, SPA-801), and mouse monoclonal anti-β-actin antibody (Santa Cruz Biotechnology, sc-32251) in TBS-T containing 3 % SK. After repeat washing in TBS-T 3×, the membrane was incubated at room temperature for 2 h with a secondary antibody coupled with alkaline phosphatase (Amersham ECL kit) in TBS-T containing 3 % SK. Finally, the membrane was washed in TBS-T 3× and developed in AttoPhos reagent (BioRad), according to the manufacturer's instructions. The secondary antibody was detected using enhanced fluorescence (FLA-2000 Fluoro image analyzer, Fuji Film). Densitometric analysis was performed using the Image Gauge (version 4.0, Fuji Film Co.). Relative protein expression was calculated as a ratio to α-actin protein as the control for equal protein loading.

Measurement of creatine kinase and β-glucuronidase activity

Plasma CK activity was measured using a commercial kit (CPK Test Wako Kit, Wako Pure Chemical). Muscle β-glucuronidase activity was determined as previously described [16]. The protein concentration of the supernatant was measured using a commercial kit (Coomassie Plus Protein Assay Reagent Kit, Pierce, US). The results were calculated per soluble protein and reaction time (h).

Histological analysis

Serial frozen cross-sections (10 mm) were obtained from the middle of the soleus muscle (10 mm thickness) using a cryostat microtome at -20 °C (Bright; OTF/AS) and mounted on 3- amino-propylethoxysilane-coated slides. For qualitative analysis of cellular concentration and changes in muscle fiber morphology, some frozen serial cross-sections were stained with Harris's hematoxylin-eosin (HE). The remaining serial frozen cross-sections were used for immunohistochemical analysis as follows. Plasma membranes and HSP70 were visualized using goat polyclonal anti-dystrophin (Santa Cruz Biotechnology sc-746) and a rabbit polyclonal anti-HSP70 antibody (Stressgen SPA-812), respectively. The cross-sections were air-dried and fixed in 4 % paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 15 min. The sections were then washed in 0.1 M PBS and incubated with 0.1 M PBS containing 10 % normal donkey serum and 1 % Triton X-100 at room temperature. For double-immunofluorescence staining, the sections were incubated simultaneously with the
primary antibodies diluted with 0.1 M PBS containing 5% normal donkey serum and 0.3% Triton X-100, overnight at 4 °C. The sections were washed in 0.1 M PBS and incubated with the secondary antibodies diluted with 0.1 M PBS containing 5% normal donkey serum and 0.1% Triton X-100 overnight at 4 °C. Fluorescein-conjugated donkey anti-rabbit IgG was used for the rabbit polyclonal primary antibody and rhodamine-conjugated donkey anti-goat IgG for the goat polyclonal antibody (Jackson Immunoresearch). The sections were then washed in 0.1 M PBS and mounted in Vectashield mounting medium (Vector Labs). Immunofluorescence-stained sections were viewed using an Olympus microscope with epifluorescence.

Statistics

All data are expressed as mean ± SE and were analyzed using GraphPad Prism 8 (MDF Co., Ltd, Tokyo, Japan). Group comparisons were performed using a two-way ANOVA followed by Dunnett's and Tukey's post hoc tests. Time-course changes were compared to baseline using a one-way ANOVA followed by t-test comparisons. Differences between groups were considered significant at p<0.05.

Results

Effects of whole-body heat exposure

Whole-body heat exposure increased rectal temperature from 36.1±1.0 °C to 41.1±1.0 °C. Twenty-four hours after exposure, there was a 2.2-fold increase in HSP70 and a 1.4-fold increase in HSP25 in the soleus muscle, compared to the control group (Fig. 1B-D). HE staining confirmed the absence of heat-induced muscle damage (Fig. 1E, F). All mice completed the 60 min downhill running protocol, indicating that heat exposure did not negatively impact running capacity. There was no effect of heat exposure on plasma CK activity. Plasma CK was significantly higher at 12 h after the running protocol, with this increase being lower in the heat-exposed group compared to the control group. At 24 h after downhill running, the level of plasma CK remained significantly higher than at baseline in the control group, with levels having returned to baseline in the heat-exposed group. The level of plasma CK in all the mice had returned to near-baseline values 48 h after downhill running in all mice (Fig. 2B).

Differences in soleus β-glucuronidase activity between the control and heat-exposed are shown in Fig. 2C. There was no effect of heat exposure on soleus β-glucuronidase activity. In the control group, β-glucuronidase activity was unchanged at 12 h and 24 h after running, with a significant increase at 48 h. In comparison, the β-glucuronidase activity remained unchanged in the heat-exposed group, even at 48 h after running.

Histological analysis using HE staining revealed muscle damage in the control group at 24 h and 48 h after running, including cell invasion and enlarged extracellular spaces (Fig. 2D, F). The extent of muscle damage was lower in the heat-exposed group than control group (Fig. 2F, G). Therefore, hyperthermia exposure appears to protect against muscle damage induced by downhill running.

On immunofluorescence analysis, some myofibers in the control group showed no fluorescence (red) with an anti-dystrophin antibody at 24 h after exercise (Fig. 3A). In the heat-exposed group, most myofibers showed fluorescence with the anti-dystrophin antibody (Fig. 3B). Therefore, myofibers were lost after downhill running in the control group but not in the heat-exposed group. On double immunofluorescence using antibodies for dystrophin and HSP70 (Fig. 3C), mononuclear cells observed in the spaces from which the myofibers had disappeared demonstrated strong signals for HSP70. Figures 3E and F shows HE-stained photos of the areas shown in Figures A-D.

Regional heat exposure and exercise

Regional heat exposure increased gastrocnemius muscle's temperature from 29.8±2.0 °C to 40.5±1.0 °C (Fig. 4B). Levels of HSP70 and HSP25 in the soleus muscles were 2.7- and 1.2-fold higher, respectively, for the ME group, compared to the control group (Fig. 4D-F), with no evidence of muscle damage in the ME group (Fig. 4G, H). Therefore, microwave diathermy increased muscular HSP70 content without inducing muscle damage. All rats completed the 60 min downhill running protocol, indicating that heat exposure did not negatively impact running capacity.

The plasma CK activity increased significantly in the control group at 24 h after downhill running, with no change in the ME group (Fig. 5B). There was also an increase in soleus β-glucuronidase activity in the control group at 24 h and 48 h after downhill running, with no significant increase in the ME group (Fig. 5C). As observed after whole-body exposure, there was no change in β-glucuronidase activity at 12 h after exercise in neither the control nor ME group. HE staining revealed
Fig. 1. Effect of preconditioning with whole-body hyperthermia on HSP70 and HSP25 content and histological assessment of muscular damage in the soleus. (A) Experimental protocol. (B) Representative photographs of western blotting for HSP70, HSP25, and β-actin; three mice/group are shown. (C, D) HSP70 and HSP25 content in the soleus muscle between control and heat-exposed groups. (E, F) Histological assessment of muscular damage by HE staining for the control (E) and heat-exposed (F) groups. Data are presented as mean ± SE (n=8); * p<0.05 vs. control group.

Fig. 2. Effect of preconditioning with whole-body hyperthermia on muscle damage after downhill running. (A) Experimental protocol. (B) Plasma CK activity. (C) Soleus β-glucuronidase activity. (D-G) Histological assessment of muscular damage by HE staining. (D) control group at 24 h; (E) heat-exposed group at 24 h; (F) control group at 48 h; and (G) heat-exposed group at 48 h. Scale bar, 100 μm. Data are presented as means ± SE (n=8); * p<0.05 vs. control mice without heat and exercise; # significant difference between control and heat-exposed group at the same time points.
Fig. 3. Immunohistochemical analysis of the soleus muscle for the control (A, C) and heat-exposed mice (B, D) 24 h after downhill running. Shown is immunohistochemical staining using anti-dystrophin antibody (red) and propidium iodide (blue) (A, B), and anti-dystrophin antibody (red) and anti-HSP70 antibody (green) (C, D). Simultaneously, the sections of the soleus of the control (E) and heat-exposed mice (F) were stained by HE. Scale bar, 100 μm.

Fig. 4. Effect of regional hyperthermia on HSP70 and HSP25 content and histological assessment of muscular damage in the soleus. (A) Representative photographs for a rat in the microwave-exposed group. (B) Change in muscle temperature for the 8 rats with microwave exposure. (C) Experimental protocol. (D) Representative photographs of Western blotting for HSP70, HSP25, and β-actin (3 rats per group). (E, F) HSP70 and HSP25 content in the soleus muscle. Cont, control rats; ME, microwave-exposed rats. (G, H) Histological assessment showing that preconditioning with microwave exposure did not cause cellular damage, with no evidence of mononuclear cell invasion. Transverse sections, from the mid-portion of the soleus, were stained with hematoxylin-eosin (HE); (G) control group and (H) microwave-exposed group; 8 rats per group. Data are presented as mean ± SE; *p<0.05 vs. control group.
muscle damage, including invading cells and enlarged extracellular spaces in the control but not in the ME groups at 48 h after exercise (Fig. 5D, E); note there was no evidence of muscle damage in either groups at 24 h post-exercise. Therefore, microwave exposure prevented exercise-induced muscle damage.

Discussion

The effectiveness of downhill running in inducing muscle damage has been reported in humans, with a 5-fold increase in plasma CK activity after a 45 min downhill run [2] and in mice, with a 4-fold increase at 24 h after running [5]. Our findings are consistent with these reports, with 4- to 5-fold increase in plasma CK activity in the control group at 12 and 24 h after exercise (Fig. 2B). Our findings of a positive effect of heat in attenuating an exercise-induced increase in plasma CK activity are consistent with the report by Touchberry et al. [2]. The mechanism by which intense exercise elevates plasma CK activity is unclear. However, our findings of a peak in plasma CK activity preceding the increase in β-glucuronidase activity is consistent with Maglara et al. [8] report of a release of CK from myocytes subjected to 2,4-dinitrophenol or A23187, which mimics the exercise-induced process, preceding the loss of integrity of the plasma membrane. Giombini et al. [1] suggested that damaged muscle cells attempt to maintain their viability by extruding the cytoplasm (including CK and other proteins) via an exocytotic type of mechanism. In addition, other investigators showed that the increase in plasma CK activity would signal the subsequent appearance of muscle damage [17,18,2]. Our results show that hyperthermia attenuates exercise-induced muscle damage.

β-glucuronidase is a lysosomal acid hydrolase, with its level providing an indirect global assessment of muscle damage [15]. While plasma CK activity increased early after exercise, muscle β-glucuronidase increased at 24-48 h after exercise as previously reported after
downhill running [13]. In addition, the transverse sections from the soleus in the Ex groups (mice and rats) showed focal necrosis and infiltration of inflammatory phagocytes at 24-48 h after downhill running; this necrosis was attenuated by heat exposure (whole-body and microwave, Fig. 2F, G and 5E). Our finding of a positive effect of hyperthermia pre-conditioning in attenuating exercise-induced muscle damage is consistent with previous findings of a significant protective effect of hyperthermia against DNP- and A23187-induced myotube damage [8].

In this study, the muscle temperature before the microwave diathermy was lower, approximately 30 °C, than as we expected. We searched for a previous paper describing rat muscle temperature, but unfortunately, we could not find one. Since anesthetized animals’ body temperature decreases, we suppose that muscle temperature before heating with the microwave also became 30 °C or less because of anesthesia. On the other hand, systemic or regional hyperthermia increased rectal or muscle temperature, respectively, to about 41 °C, and muscle HSP contents increased the next day following hyperthermia. These results indicated that this increase in temperature was adequate for the induction of HSPs.

Heat-induced increase in HSP70 and HSP25 expression likely resulted in attenuation of exercise-induced muscle damage. During intense exercise, reactive oxygen species (ROS) are produced in skeletal muscle via several pathways, including an increase in body temperature and energy depletion. ROS oxidize proteins, lipids, and DNA. Oxidized proteins are unfolded and tend to aggregate. HSP70 binds to unfolded proteins, refolds them, and maintains protein function [2], which might protect myofibers from cell death. This is consistent with previous reports that heat exposure before lethal stress increases stress resistance in cells, with continued functioning of organs [1,4]. Additionally, several studies have shown an effect of acute heating in increasing antioxidant enzyme activities, including superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px) [19,20,21]. This heat-induced increase in antioxidant activity might also attenuate exercise-induced muscle damage. Further research is warranted to fully clarify the mechanisms underlying the protective effect of heat preconditioning against exercise-induced muscle damage.

Microwave diathermy is widely used for the short-term management of musculoskeletal injuries [1]. Our findings further demonstrate the benefit of microwave diathermy in attenuating exercise-induced muscle damage in rats (Fig. 5B-E). Microwave diathermy has also been shown to increase HSP70 in humans [7]. Microwave diathermy provided 1 day before eccentric exercise was associated with faster recovery of muscle strength after exercise, smaller decreases in range of motion, and less development of muscle soreness [22]. Overall, our findings show that elevation of muscle temperature using microwave diathermy before exercise could play an essential role in the prevention and relapse of muscle injury through the enhancement of HSP70 in the skeletal muscle.

Like HSP70, HSP25 also increases in skeletal muscle due to heat exposure [23]. It has been reported that HSP25 knockout mice show a lower amount of voluntary wheel running and more fatigability when exercising than normal mice [24]. In experiments that use C2C12 myotubes, it has been reported that an increase in HSP25 suppresses LDH release from the cells into the medium under oxidative stress, suggesting that HSP25 suppresses oxidative stress-induced myocyte damage [25]. In the present study as well, the whole-body or regional heat exposure before exercise increased HSP25, that similar to HSP70, the increase in HSP25 contributed to the suppression of skeletal muscle damage during.

The increase in plasma CK activity after a 1-hour downhill run was suppressed in regional heating than in whole-body heating. Moreover, concerning the degree of muscle damage confirmed by HE staining, regional heating tended to have less damage than whole-body heating. Further, the increase in HSP70 by heating was also higher in regional heating than in whole-body heating. These results suggest that regional heating would effectively prevent muscle damage caused by exercise because regional heating increases more HSP70s in the skeletal muscle than systemic heating. However, since the species of animals used in both experiments are different (mice and rats), there would be possible that these differences were attributed to animal species' differences. To clarify this point, we think that further studies using the same animal species are needed. When considering the human application, whole-body heating exposes the cells to high temperatures other than the target organ (for example, the brain). This unnecessary high-temperature exposure may cause unexpected damage to organs other than the target organ. Therefore, regional heating with a microwave apparatus that can heat only the target skeletal muscle would be a more practical method when considering the human application in preventing exercise-
induced muscle damage.

Previous studies have shown that pre-exercise warm-up increases HSPs in the body and enhances stress tolerance after the warm-up. The present study demonstrated that heating to increase HSPs is not only possible with whole-body heating but also by microwave diathermy. The possibility of preconditioning with regional hyperthermia, using microwave diathermy, may help prevent skeletal muscle injury during exercise training in humans. In future, a detailed study in humans that will examine the relationship among microwave diathermy before exercise, HSPs expression in muscle, and DOMS is necessary.

**Conflict of Interest**
There is no conflict of interest.

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