TITLE
Post-exercise whole-body heat stress additively enhances endurance training-induced mitochondrial adaptations in mouse skeletal muscle

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RUNNING TITLE
Additive effects of heat stress on mitochondrial adaptations
ABSTRACT

A recent study demonstrated that heat stress induces mitochondrial biogenesis in C2C12 myotubes, thereby implying that heat stress may be an effective treatment to enhance endurance training-induced mitochondrial adaptations in skeletal muscle. However, whether heat stress actually induces mitochondrial adaptations in skeletal muscle in vivo is unclear. We herein report the novel findings that 1) whole-body heat stress produced by exposure of ICR mice to a hot environment (40 ºC, 30 min/day, 5 days/week, 3 weeks) induced mitochondrial adaptations such as increased mitochondrial enzyme activity (citrate synthase and 3-hydroxyacyl CoA dehydrogenase) and respiratory chain protein content (complexes I–V) in skeletal muscle in vivo and 2) post-exercise whole-body heat stress additively enhanced endurance training-induced mitochondrial adaptations (treadmill running, 25 m/min, 30 min/day, 5 days/week, 3 weeks). Moreover, to determine candidate mechanisms underlying mitochondrial adaptations, we investigated the acute effects of post-exercise whole-body heat stress on the phosphorylation status of cellular signaling cascades that subsequently induce mitochondrial gene transcription. We found that whole-body heat stress boosted the endurance exercise-induced phosphorylation of p38 MAPK; increased the phosphorylation status of p70S6K, a biomarker of mTORC1 activity; and unexpectedly dephosphorylated AMPK and its downstream target ACC in skeletal muscle. Our current observations suggest that heat stress can act as an effective post-exercise treatment. Heat stress treatment appeared to be clinically beneficial for people who have difficulty participating in sufficient exercise training, such as the elderly, injured athletes, and patients.

Keywords: Mitochondria, Heat stress, Exercise, Training, Skeletal muscle
INTRODUCTION

Mitochondrial adaptations in skeletal muscle, including mitochondrial biogenesis, increased mitochondrial oxidative enzyme activity, and an increased capacity to oxidize carbohydrates and fatty acids, contribute to the improvement of exercise capacity and prevention of disease associated with mitochondrial dysfunction (3, 11, 19, 20, 36). It is well accepted that endurance training is the best way to induce mitochondrial adaptations in skeletal muscle. However, considering the various social problems in today’s busy world, “not enough time” is one of the most commonly cited barriers to the establishment of a regular exercise habit (16, 45). Furthermore, it is difficult for people with low physical fitness, such as the elderly, injured athletes, and patients, to participate in sufficient exercise training. Hence, the establishment of an effective exercise training strategy is needed.

In the last decade, the effects of heat stress on protein synthesis in skeletal muscle have been well investigated. Heat stress activates cellular signaling associated with protein synthesis and subsequently induces skeletal muscle hypertrophy (26, 35, 38, 46). Moreover, such investigations have begun to reveal the benefit of heat stress on energy metabolism. For example, hot tub therapy improves insulin sensitivity and fasting blood glucose levels in patients with type 2 diabetes mellitus, and heat stress prevents high-fat diet-induced insulin resistance in rats (17, 21). In particular, Liu and Brooks (32) recently demonstrated that heat stress induces mitochondrial biogenesis in C2C12 myotubes. Because of the study by Liu and Brooks, we focused on heat stress as a novel treatment candidate to effectively enhance endurance training-induced mitochondrial adaptations in skeletal muscle. However, whether heat stress actually induces mitochondrial biogenesis in skeletal muscle in vivo remains unclear. Therefore, we hypothesized that heat stress induces mitochondrial adaptations in skeletal muscle in vivo and that heat stress additively or synergistically enhances endurance training-induced mitochondrial adaptations in skeletal muscle.

The primary purpose of this study was to investigate the effects of post-exercise heat stress on endurance training-induced mitochondrial adaptations such as the activity of mitochondrial enzymes (citrate synthase [CS] and 3-hydroxyacyl CoA dehydrogenase [3-HAD]), biomarkers for mitochondrial oxidative capacity, and content of respiratory chain proteins (complexes I–V), biomarkers for mitochondrial content, in skeletal muscle. We also investigated the effects of a single bout of heat stress on cellular signaling...
cascades (AMPK, p38 MAPK, CaMKII, and mTORC1) that subsequently induce the transcription of mitochondrial genes in skeletal muscle (1, 10, 24, 44, 50). The heat stress method we employed in this study was whole-body heat stress (placement of mice in a hot environmental chamber without anesthesia) because a previous study showed that anesthesia during heat stress impairs normal thermoregulation and leaves mice in a non-physiological condition (29).
MATERIALS AND METHODS

Ethical approval

All experimental protocols were approved by the Animal Experimental Committee of The University of Tokyo.

Experimental animals and procedures

Scheme describing experimental procedure is shown in Figure 1.

Experimental animals

Six-week-old male ICR mice (CLEA Japan Inc., Tokyo, Japan) were used throughout this study. Three to four mice were housed per cage (30 × 20 × 13 cm) on a 12/12-h light–dark cycle (dark: 7:00 AM to 7:00 PM) in an air-conditioned room (22 °C). All mice were provided with standard chow (MF; Oriental Yeast Co. Ltd., Tokyo, Japan) and water ad libitum throughout the experimental period.

Assessment of the effects of whole-body heat stress on spontaneous physical activity

We employed whole-body heat stress in this study. Mice in the bleeding cage were placed in a hot environmental chamber without anesthesia. To validate the heat stress method in this study, we investigated the effect of whole-body heat stress without anesthesia on the spontaneous physical activity level. Following a 3-day acclimation period, the mice were randomly divided into the following two groups: the control group (CON, n = 4) and the whole-body heat stress group (HS, n = 4). Mice in the CON group in the bleeding cage were placed in a normal-temperature environmental chamber (22 °C) for 60 min. Mice in the HS group were placed in a hot environmental chamber (40 °C) for 60 min. Details of the hot environmental chamber are described below. Two days before exposure, we marked the mid-portion of the backs of the mice for tracking. We recorded the mice’s spontaneous activity during heat exposure and non-heat exposure on a video camera (HDR-SR11, 30 fps; SONY, Tokyo, Japan), then analyzed the movement distance of the tracking marker using motion analysis software (Kinovea, ver. 0.8.15; Windows, Germany). Experiments were performed in the dark phase.

Single experiment 1: Investigation for the physiological responses
Following a 3-day acclimation period, the mice were randomly divided into the following four groups: the control group (CON, n = 6), the endurance exercise group (Ex, n = 6), the whole-body heat stress group (HS, n = 6), and the endurance exercise and post-exercise whole-body heat stress group (Ex+HS, n = 6). Details of the endurance training and whole-body heat stress protocols are described below. Immediately after exercise or whole-body heat stress induction, the mice were anesthetized using isoflurane (4% induction, 3% maintenance, 0.5 l/min). To evaluate physiological responses to treatments, body weight, rectal temperature, and plasma corticosterone level were measured.

Long-term experiment: Investigation of mitochondrial adaptations

Following a 3-day acclimation period, the mice were randomly divided into the following four groups: the control group (CON, n = 6), the endurance training group (ET, n = 6), the whole-body heat stress group (HS, n = 6), and the endurance training and post-exercise whole-body heat stress group (ET+HS, n = 7). Details of the endurance training and whole-body heat stress protocols are described below. Forty hours after the final exercise session or whole-body heat stress induction, the mice were sacrificed by cervical dislocation. The plantaris muscle (fast-twitch fiber-dominant) and soleus muscle (slow-twitch fiber-dominant) were dissected out, rapidly frozen in liquid nitrogen, and stored at −80 °C until further analysis of mitochondrial enzyme activity and performance of western blot.

Single experiment 2: Investigation for acute responses of cellular signaling cascades associated with mitochondrial adaptations

Following a 3-day acclimation period, the mice were randomly divided into the following four groups: the control group (CON, n = 6), the endurance exercise group (Ex, n = 7), the whole-body heat stress group (HS, n = 7), and the endurance exercise and post-exercise whole-body heat stress group (Ex+HS, n = 7). Immediately after exercise or whole-body heat stress induction, the mice were sacrificed by cervical dislocation. The plantaris and soleus muscles were dissected out, rapidly frozen in liquid nitrogen, and stored at −80 °C until further analysis of western blot.
Endurance exercise training protocol

The mice were subjected to endurance running (velocity: 25 m/min, duration: 30 min) using a motor-driven treadmill. In the long-term experiment, mice in the ET and ET+HS groups performed treadmill running 5 days/week for 3 weeks.

Heat stress protocol

The hot environmental chamber is shown in Figure 2. Rubber heaters (MRHSF; MiSuMi, Tokyo, Japan) were attached to the inner walls and bottom of a plastic case. The ambient temperature in the chamber was regulated by a thermocouple (E52-CA20AY D=3.2 4M; Omron Corporation, Tokyo, Japan) connected to a proportional integral derivative controller (E5EC-CX2ASM-000; Omron Corporation, Tokyo, Japan). The mice were exposed to the hot environmental chamber (temperature: 40 ºC, duration: 30 min) without anesthesia. In the long-term experiment, the mice underwent induction of whole-body heat stress 5 days/week for 3 weeks. Mice in the ET+HS and Ex+HS groups were exposed to the hot environmental chamber immediately after treadmill running. Mice in the non-whole-body heat stress group were exposed to a normal rearing environment chamber (22 ºC). The mice had no access to chow or water during the exposure.

Rectal temperature measurement

Rectal temperature was measured using thermocouple (E52-CA20AY D=3.2 4M; Omron Corporation, Tokyo, Japan) connected to data logger (GL200, GRAPHTEC Corporation, Yokohama, Japan).

Plasma corticosterone level analysis

To evaluate systemic stress responses, plasma corticosterone level was measured in single experiment 1. Approximately 50 μl of blood samples were collected from lateral tail vein, by using heparinized micro-hematocrit capillary tube (22-362-566, Thermo Fisher Scientific, MA, USA). Collected blood samples were immediately separated into plasma and hematocrit by centrifuge (10000 rpm, 5 min). Plasma samples were stored -80 ºC until further analysis. Plasma corticosterone levels were analyzed using commercial ELISA kit (Assaymax Corticosterone ELISA Kit, Assaypro LLC, MO, USA) following manufacturer's instruction.
Analytic methods for mitochondrial enzyme activity

The maximal activities of CS and 3-HAD were determined in whole muscle homogenates. Specifically, whole plantaris and soleus muscle specimens were homogenized in 100 vol/wt of 100-mM potassium phosphate buffer. Maximal CS and 3-HAD activities were measured spectrophotometrically using the method of Srere (43) and Bass (6).

Western blot analysis

Muscle samples were homogenized as previously described (22) using lysis buffer (1% Triton X-100, 50-mM Tris·HCl, 1-mM EDTA, 1-mM EGTA, 50-mM sodium fluoride, 10-mM sodium beta-glycerol phosphate, 5-mM sodium pyrophosphate, and 2-mM dithiothreitol; pH 7.5) containing 10 μg/mL of pepstatin A, aprotinin, and leupeptin; 1-mM Na orthovanadate; and 0.177 mg/mL of phenylmethylsulfonyl fluoride. The sample proteins were measured by the Bradford method. We loaded an equal amount (5–15 µg) of protein to detect the same protein, separated them using standard SDS-PAGE procedures (7.5–12% polyacrylamide gels), and transferred them to a polyvinylidene difluoride membrane (Hybond-P; GE Healthcare Japan, Tokyo, Japan). Membranes were blocked with 3% to 7.5% BSA in Tris-buffered saline containing 0.1% Tween-20 (TTBS) for 1 h and incubated overnight with the following primary antibodies: glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (ab8245; Abcam, OR, USA), 60-kD heat shock protein (HSP60) (ADI-SPA-806; Enzo Life Sciences, Tokyo, Japan), 72-kD heat shock protein (HSP72) (ADI-SPA-810-D; Enzo Life Sciences, Tokyo, Japan), cytochrome c oxidase subunit IV (COX IV) (ab14744; Abcam, OR, USA), MitoProfile Total OXPHOS Rodent WB Antibody Cocktail (NDUFB8, SDHB, UQCRC2, ATP5A, ab110413; Abcam), phos-AMPKa (Thr172) (#2513; Cell Signaling Technology [CST] Japan, Tokyo, Japan), AMPKa (#2532; CST Japan), phos-ACC (Ser79) (#3661; CST Japan), ACC (#3662; CST Japan), phos-p38 MAPK (Thr170/Tyr182) (#9211; CST Japan), p38 MAPK (#9212; CST Japan), p-CaMKII (Thr286) (# 3361; CST Japan), CaMK II (611292; BD Biosciences Japan, Tokyo, Japan), phos-Akt (Ser473) (#9271; CST Japan), phos-Akt (Thr308) (#9275; CST Japan), Akt (#9272; CST Japan), phos-mTOR (Ser2448) (#2481; CST Japan), mTOR (#2983; CST Japan), phos-p70S6K (Thr389) (#9205; CST Japan), and p70S6K (#9202; CST Japan). After incubation, the
membranes were washed in TTBS, incubated for 1 h at room temperature with secondary antibodies (A106PU or A102PT; American Qualex, CA, USA), and again washed in TTBS. Chemiluminescent reagents (SuperSignal West Pico Chemiluminescent Substrate; Thermo Fisher Scientific, IL, USA) were used to facilitate blot detection. The blots were scanned and quantified using ChemiDoc XRS (170-8071; Bio-Rad, CA, USA) and Quantity One (170-9600, Ver. 4.5.2, Windows; Bio-Rad). Quantified band intensity was normalized using GAPDH as a loading control.

**Statistical analysis**

All data are expressed as mean ± SE. To assess the effects of whole-body heat stress on spontaneous physical activity, two-way ANOVA was performed to examine the interaction between exposure duration and whole-body heat stress or the main effects of exposure duration and whole-body heat stress. In the long-term and single experiments, two-way ANOVA was performed to examine the synergistic effect (*i.e.* positive interaction) and antagonistic effect (*i.e.* negative interaction) between endurance training (exercise) and post-exercise whole-body heat stress. If significant interactions were observed, the Tukey–Kramer multiple-comparison test was performed to examine the differences among groups. If no significant interaction was observed, we examined the main effects of endurance training (exercise) and whole-body heat stress. Statistical significance was defined as P < 0.05. Statistical analysis was performed using JMP (Ver. 9.0.1, Macintosh; SAS Institute, NC, USA).
RESULTS

Assessment of the effects of whole-body heat stress on spontaneous physical activity

Whole-body heat stress without anesthesia causes a risk of an increased spontaneous physical activity level, which potentially induces mitochondrial adaptations. First, we investigated the effects of whole-body heat stress on spontaneous physical activity. A significant main effect of exposure time was observed (Fig. 3). In contrast, no significant main effect of whole-body heat stress was observed (Fig. 3). These results show that whole-body heat stress does not affect spontaneous physical activity for at least 60 min.

Single experiment 1: Investigation of the physiological responses

Rectal temperature immediately after treatments was significantly different among groups (Fig. 4). Negative main effect of whole-body heat stress on body weight was observed immediately after treatments but not 6-hour after treatments (Fig. 4). A synergistic effect (i.e. positive interaction) between endurance exercise and whole-body heat stress for plasma corticosterone level was observed. Subsequent post-hoc test showed that plasma corticosterone level in Ex+HS group was significantly higher than that in other groups (Fig. 4).

Long-term experiment: Investigation of mitochondrial adaptations

HSP60 and HSP72 protein content were significantly increased in response to whole-body heat stress.

It has been well described that the HSP60 and HSP72 protein content in rodent skeletal muscle are significantly increased by heat stress (35, 39). We investigated the HSP60 and HSP72 protein content as biochemical indicators of cellular heat shock response in our long-term experiment. Positive main effects of whole-body heat stress but not endurance training on HSP60 and HSP72 protein content were observed in the plantaris and soleus muscles (Fig. 5).

Animal characteristics in long-term experiment

We measured the final body weight, the plantaris and soleus muscle mass and epididymal adipose tissue mass as animal characteristics in long-term experiment (Table 1). Tendency and significance of the negative main effects of endurance training and
whole-body heat stress on body weight were observed, respectively (Table 1). Although
the average plantaris and soleus muscle mass in the ET, HS, and ET+HS groups was
higher than that in the CON group, no statistically significant main effect was observed.
Significant negative main effects of endurance training and whole-body heat stress on
epididymal adipose tissue mass were observed (Table 1).

**Whole-body heat stress induced mitochondrial adaptations and additively enhanced
endurance training-induced mitochondrial adaptations in skeletal muscle**

To evaluate mitochondrial adaptations, we measured the maximal activity of
mitochondrial enzyme (CS and 3-HAD) and the content of mitochondrial respiratory
chain proteins (complex I: NDUFB8, II: SDHB, III: UQCRC2, IV: COX IV, and V:
ATP5A) in the plantaris and soleus muscles. The positive main effects of not only
endurance training, but also whole-body heat stress, on the maximal activity of CS and
3-HAD and the protein content of respiratory chain complexes were observed in the
plantaris and soleus muscle (Figs. 6 and 7). No synergistic (*i.e.* no positive interaction) or
antagonistic (*i.e.* no negative interaction) effects between endurance training and
post-exercise whole-body heat stress on any measurements of mitochondrial adaptations
were observed in both the plantaris and soleus muscle. Therefore, these observations
support our hypothesis that post-exercise whole-body heat stress “additively” enhances
endurance training-induced mitochondrial adaptations in mouse skeletal muscle.

**Single experiment 2: Investigate the responses of cellular signaling cascades
associated with mitochondrial adaptations**

Whole-body heat stress enhanced exercise-induced p38 MAPK activation, but
inactivated AMPK

To seek candidate mechanisms underlying long-term mitochondrial adaptations,
we investigated the acute responses of the following signaling cascades associated with
exercise training-induced mitochondrial adaptations: AMPK, p38 MAPK, and CaMK II
(Fig. 8).

No significant interaction between endurance exercise and whole-body heat stress
for the phosphorylation status of AMPK was observed in the plantaris (*P = 0.11*) or soleus
muscles. A significant positive main effect of endurance exercise on the phosphorylation
status of AMPK was observed in the plantaris muscle. Similarly, the phosphorylation
status of ACC, a downstream target of AMPK, tended to increase with endurance exercise in the plantaris muscle. In the soleus muscle, although the average value of the phosphorylation status of AMPK and ACC in the Ex group was higher than that in the CON group, the differences did not reach statistical significance. In contrast, the negative main effects of whole-body heat stress on the phosphorylation status of AMPK and ACC were observed in the plantaris and soleus muscle. These results demonstrate that whole-body heat stress down-regulates AMPK activity in skeletal muscle.

A synergistic effect (i.e. positive interaction) between endurance exercise and post-exercise whole-body heat stress on the phosphorylation status of p38 MAPK was observed in the plantaris muscle. The positive main effects of endurance exercise or whole-body heat stress on the phosphorylation status of p38 MAPK was observed in the soleus muscle. No antagonistic effects between endurance exercise and post-exercise whole-body heat stress were observed in the soleus muscle. These observations indicate that post-exercise whole-body heat stress synergistically or additively boosts endurance exercise-induced p38 MAPK activation in the plantaris and soleus muscles, respectively.

There was no significant effect of endurance exercise or whole-body heat stress on the phosphorylation status of CaMK II.

Whole-body heat stress activated the mTORC1 pathway

Maximal Akt activation is required for phosphorylation at both Ser473 and Thr308 (2) and subsequently phosphorylates mTOR. However, recent studies demonstrated that the phosphorylation status of mTOR does not necessarily reflect mTORC1 activity (14, 15, 34). Many recent studies have investigated the phosphorylation status of p70S6K, a downstream target of mTORC1, as a biomarker of mTORC1 activity (23, 42). Therefore, to investigate the responses of the mTORC1 pathway activity to whole-body heat stress, we measured the phosphorylation status of Akt (Ser473 and Thr308), mTOR, and p70S6K (Fig. 9).

Significant positive main effects of whole-body heat stress, but not endurance exercise, on the phosphorylation status of Akt (Ser473 and Thr308) and p70S6K were found in the plantaris and soleus muscles. These results indicate that whole-body heat stress activates the mTORC1 pathway in skeletal muscle.
DISCUSSION

Key findings and clinical significance

We have provided evidence that whole-body heat stress induces mitochondrial adaptations, such as increased mitochondrial enzyme activities and respiratory chain protein content, in mouse skeletal muscle (Figs. 6 and 7). To our knowledge, this is the first study to show that heat stress induces mitochondrial adaptations in skeletal muscle in vivo similarly to endurance training. Because mitochondrial adaptations in the plantaris and soleus muscles were similar, whole-body heat stress-induced mitochondrial adaptations were not fiber type specific. Furthermore, we revealed no antagonistic effects (i.e. no negative interactions) between endurance training and post-exercise whole-body heat stress on any measurements of mitochondrial adaptations (Figs. 6 and 7). These observations indicate that post-exercise whole-body heat stress additively enhances endurance training-induced mitochondrial adaptations in mouse skeletal muscle, supporting our hypothesis. Incidentally, a previous study reported that high-fat diet also induces mitochondrial biogenesis in skeletal muscle (18). However, in another study, heat stress canceled mitochondrial biogenesis by high-fat diet in rat skeletal muscle (17), indicating that additive effects of heat stress on mitochondrial adaptations are not universally observed. Thus, it is notable that additive effects of heat stress on increased CS activity (a biomarker for oxidative capacity) and protein contents of NDUFB8 and ATP5A (biomarkers for mitochondrial content) by endurance training were especially observed in both plantaris and soleus muscle.

Our current findings suggest that heat stress can act as an effective post-exercise treatment to enhance mitochondrial adaptations in skeletal muscle. Therefore, heat stress treatment may be clinically beneficial for people who have difficulty participating in sufficient exercise training, such as the elderly, injured athletes, and patients.

Validation and profile of our heat stress protocol

A previous study showed that anesthesia during heat stress impairs normal thermoregulation and leaves mice in a non-physiological condition (29). Therefore, we employed whole-body heat stress without anesthesia. First, we examined the effects of whole-body heat stress on the spontaneous physical activity level. Regardless of whole-body heat stress, spontaneous physical activity in the first half was higher than that
in the latter half. This observation might have been due to expression of the mice’s exploratory behavior. Contrary to the effects of exposure time, whole-body heat stress without anesthesia did not increase spontaneous physical activity for at least 60 min (Fig. 3). This result indicates that whole-body heat stress-induced mitochondrial adaptations were not due to increased physical activity.

In the long-term experiment, the HSP60 and HSP72 protein content, common biomarkers for cellular heat shock response, were significantly increased by whole-body heat stress in the plantaris and soleus muscles (Fig. 5). These results show that whole-body heat stress in this study induced sufficient cellular stress response. Incidentally, we also evaluated acute physiological responses in Single experiment 1. Rectal temperature was significantly increased by whole-body heat stress and reached 39.4±0.1 ºC in HS group and 40.4±0.1 ºC in Ex+HS group, respectively (Fig. 4). Previous studies showed that minimal lethal core temperature is approximately 42 ºC or higher in mice (29), indicating our heat stress protocol in this study did not absolutely reach the fatal heat stroke. Plasma corticosterone level, a biomarker for systemic stress response, was synergistically increased by post-exercise whole-body heat stress. These observations can be interpreted that post-exercise whole-body heat stress further initiated systemic stress response in addition to local cellular stress responses in skeletal muscle.

Collectively, whole-body heat stress in this study was within physiological range and induced sufficient both physiological and biochemical responses with no changes in behavior.

**Determination of candidate mechanisms underlying whole-body heat stress-induced mitochondrial adaptations**

AMPK, p38 MAPK, and CaMK II are well-characterized cellular signaling cascades that subsequently induce mitochondrial gene transcription. A recent study demonstrated that heat stress-induced mitochondrial biogenesis was possibly mediated by AMPK activation in C2C12 myotubes (32). Moreover, heat stress increased the phosphorylation status of AMPK in isolated rat skeletal muscle (28). Therefore, we hypothesized that post-exercise whole-body heat stress additively or synergistically enhances exercise-induced AMPK phosphorylation and activation. However, whole-body heat stress unexpectedly down-regulated the phosphorylation status of AMPK and its downstream target ACC (Fig. 8). To our knowledge, this is the first study
to investigate the effects of heat stress on AMPK activity \textit{in vivo}. The AMPK response to
heat stress has been controversial. Previous study in Hep G2, a human liver carcinoma
cell line, reported that heat stress decreases AMPK activity without energy status change.
In accordance with this previous report, our current observation 1) may be explained by
rather heat stress condition (e.g. temperature, and/or duration) than differences
between \textit{in vitro} and \textit{in vivo} such as humoral factor involvement, and 2) may not be
caused by changes in cellular energy status. To cultivate better understanding of AMPK
activity in response to heat stress, further studies in various experimental conditions are
needed.

Interestingly, post-exercise whole-body heat stress dramatically boosted
exercise-induced p38 MAPK phosphorylation in skeletal muscle (Fig. 8). p38 MAPK is
phosphorylated and activated by various cellular stresses. Heat stress not only directly
affects cell temperature, but also indirectly affects cellular and humoral factors. Actually,
we and/or others demonstrated that exposing rodents to hot environment increases the
production of reactive oxygen species and levels of circulating corticosterone (Fig. 2)
which are known to activators of p38 MAPK (9, 25, 30, 31). These observations suggest
that increased circulating corticosterone and cellular oxidative stress can be candidates
for upstream of p38 MAPK activation. Further investigations from the cell and tissue
level to the whole-body level will shed light on upstream causes of p38 MAPK activation.

Endurance exercise in this study did not increase the phosphorylation status of
CaMKII in the plantaris and soleus muscles (Fig. 8). In accordance with previous studies,
CaMKII was significantly phosphorylated or activated by high-intensity exercise or at the
onset of moderate-intensity exercise (13, 40). Therefore, the reason why endurance
exercise in this study did not increase the phosphorylation status of CaMKII may be
explained by the low to moderate exercise intensity (25 m/min) and the sampling time
point after the onset of exercise (30 min). Similar responses to endurance exercise and
whole-body heat stress did not increase the phosphorylation status of CaMKII in the
plantaris and soleus muscles (Fig. 8). A previous study demonstrated that CaMKII is one
of the upstream of p38 MAPK (49). Considering our results and those of previous reports,
whole-body heat stress-induced p38 MAPK activation may not occur through the
CaMKII pathway.

Recent studies have revealed that mTORC1 is responsible for not only muscle
protein synthesis, but also mitochondrial gene transcription (4, 10, 37, 51). Whole-body
heat stress significantly increased the phosphorylation status of Akt (Ser473 and Thr308) and p70S6K in the plantaris and soleus muscles, indicating that whole-body heat stress activated the mTORC1 pathway (Fig. 9). A recent study showed that partial heat stress in the rat hind limb, but not the whole-body, also increased the phosphorylation status of Akt (Ser473) and p70S6K, but not mTOR, in the plantaris and soleus muscles (52). According to our results and those of previous reports, mTORC1 activation by heat stress is a general response. On the other hand, it has been regarded that AMPK is a negative upstream regulator of mTORC1 (27, 41). As described above, whole-body heat stress inactivated AMPK in this study. Therefore, heat stress-induced mTORC1 activation may be mediated by both Akt activation and AMPK inactivation. In addition, activation of mTORC1 can contribute to the enhancement of mitochondrial gene translation efficiency (12). Hence, post-exercise whole-body heat stress possibly enhances both mitochondrial gene transcription and translation efficiency via mTORC1 activation.

Nuclear-encoded mitochondrial proteins and mitochondrial transcription factors are translated and synthesized in the cytosol as precursor proteins. It has been clarified that newly synthesized mitochondrial precursor proteins are transported into the mitochondria and refolded by HSP60 and HSP72 (33). As mentioned above, whole-body heat stress significantly increased the HSP60 and HSP72 protein content in the plantaris and soleus muscles (Fig. 5). Therefore, one could speculate that an increase in HSP60 and HSP72 by whole-body heat stress may promote transportation efficiency into the mitochondria and folding process then enhance mitochondrial adaptations.

Taken together, our current observations suggest that activation of p38 MAPK and mTORC1 and increased HSP60 and HSP72 may be candidate mechanisms underlying whole-body heat stress-induced mitochondrial adaptations, which probably differ from those of exercise training.

Physiological significance of whole-body heat stress-induced acute cellular responses and long-term mitochondrial adaptations

HSP72 protein especially contributes to the repair of protein and cellular damage. Previous in vitro studies demonstrated that heat stress-induced HSP72 synthesis is required for AMPK inactivation, p38 MAPK activation, and mTORC1 activation (5, 8, 47). Therefore, the whole-body heat stress-induced acute cellular responses in this study may have contributed to increased HSP72 synthesis. On the other hand, maintenance of
mitochondrial ATP production is necessary to support cellular and tissue function not only under normal conditions, but also in response to cellular stress (48). To defend against heat stress, cell protective mechanisms including HSP72 synthesis, restoration of damaged proteins, and re-synthesis of irreversibly damaged proteins are induced, and are considered to be processes associated with high ATP demand (32). Therefore, whole-body heat stress-induced mitochondrial adaptations can contribute to meeting the cellular energy demand. Collectively, the physiological significance of acute cellular responses and chronic mitochondrial adaptations could be that both contribute to cellular protection.

**Perspectives and Significance**

The key findings of the present study are that 1) whole-body heat stress-induced mitochondrial adaptations are similar to those of endurance training and that 2) post-exercise whole-body heat stress additively enhances endurance training-induced mitochondrial adaptations in mouse skeletal muscle. Furthermore, we also provided the potential mechanisms underlying whole-body heat stress-induced mitochondrial adaptations. Our current findings suggest that heat stress can act as an effective post-exercise treatment. Heat stress treatment may be clinically beneficial for people who have difficulty participating in sufficient exercise training, such as the elderly, injured athletes, and patients.


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**Figure legends**

**Fig. 1. Experimental procedure**

**Fig. 2. Hot environmental chamber for whole-body heat stress.**
Mice in the bleeding cage were placed in a hot environmental chamber. A: Thermocontroller. B: Thermosensor. C: Rubber heater attached to inner walls and bottom of chamber. D: Mice in bleeding cage.

**Fig. 3. Effects of whole-body heat stress on physical activity level.**
Whole-body heat stress did not affect the level of spontaneous physical activity. Values are expressed as mean ± SE. Two-way ANOVA was performed.

**Fig. 4. Acute physiological responses after whole-body heat stress.**
Rectal temperature was increased after treatments. Plasma corticosterone level was synergistically increased by post-exercise whole-body heat stress. Although whole-body heat stress decreased body weight, decreased body weight was recovered within 6 hours after treatment. Values are expressed as mean±SE. B: main effect of whole-body heat stress. ****vs CON (P<0.0001), ###vs Ex (P<0.0001), ††† vs HS (P<0.001). Numerical annotation shows the relative changes compared with CON group.

**Fig. 5. Effects of whole-body heat stress on heat shock protein expression in skeletal muscle.**
Whole-body heat stress increased the HSP60 and HSP72 protein content in both the plantaris and soleus muscles. Values are expressed as mean ± SE. Two-way ANOVA was performed. B: Significant main effect of whole-body heat stress. Numerical annotation shows the relative changes compared with CON group.
Fig. 6. Long-term adaptations to whole-body heat stress on mitochondrial enzyme activity in skeletal muscle.
Whole-body heat stress increased mitochondrial enzyme activity. No antagonistic effects between endurance training and post-exercise whole-body heat stress were observed. Values are expressed as mean ± SE. Two-way ANOVA was performed. A: Significant main effect of endurance training. B: Significant main effect of whole-body heat stress. Numerical annotation shows the relative changes compared with CON group.

Fig. 7. Long-term adaptations to whole-body heat stress on mitochondrial respiratory chain protein content in skeletal muscle.
Whole-body heat stress increased mitochondrial respiratory chain protein content. No antagonistic effects between endurance training and post-exercise whole-body heat stress were observed. Values are expressed as mean ± SE. Two-way ANOVA was performed. A: Significant main effect of endurance training. B: Significant main effect of whole-body heat stress. (A): Tendency of main effect of endurance training. (B): Tendency of main effect of whole-body heat stress. Numerical annotation shows the relative changes compared with CON group.

Fig. 8. Acute responses to whole-body heat stress on cellular signaling cascades that subsequently induce mitochondrial gene transcription. Whole-body heat stress enhanced endurance exercise-induced p38 MAPK phosphorylation, but unexpectedly decreased the phosphorylation status of AMPK and its downstream target ACC in skeletal muscle. Values are expressed as mean ± SE. Two-way ANOVA was performed. A: Significant main effect of endurance training. B: Significant main effect of whole-body heat stress. (A): Tendency of main effect of endurance training. (B): Tendency of main effect of whole-body heat stress. Numerical annotation shows the relative changes compared with CON group.

Fig. 9. Acute responses to whole-body heat stress on mTORC1 pathway.
Whole-body heat stress increased the phosphorylation status of Akt (Ser473 and Thr308)
and p70S6K in skeletal muscle. Values are expressed as mean ± SE. Two-way ANOVA was performed. B: Significant main effect of whole-body heat stress. Numerical annotation shows the relative changes compared with CON group.
Table 1. Animal characteristics in long-term experiment

<table>
<thead>
<tr>
<th></th>
<th>CON</th>
<th>ET</th>
<th>HS</th>
<th>ET+HS</th>
<th>TWO WAY-ANOVA</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>ET x HS</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>42.37±0.82</td>
<td>41.13±0.63</td>
<td>40.90±0.85</td>
<td>39.72±0.45</td>
<td>n.s.</td>
</tr>
<tr>
<td>Plantaris muscle mass (mg)</td>
<td>40.81±0.93</td>
<td>42.90±2.62</td>
<td>44.73±2.19</td>
<td>42.60±1.79</td>
<td>n.s.</td>
</tr>
<tr>
<td>Plantaris muscle mass/body weight (mg/g 10^-4)</td>
<td>9.71±0.06</td>
<td>10.44±0.71</td>
<td>10.90±0.62</td>
<td>10.51±0.24</td>
<td>n.s.</td>
</tr>
<tr>
<td>Soleus muscle mass (mg)</td>
<td>20.24±0.32</td>
<td>23.25±1.07</td>
<td>22.98±1.29</td>
<td>22.81±0.85</td>
<td>n.s.</td>
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<tr>
<td>Soleus muscle mass/body weight (mg/g 10^-4)</td>
<td>4.88±0.10</td>
<td>5.64±0.26</td>
<td>5.58±0.31</td>
<td>5.57±0.22</td>
<td>n.s.</td>
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<tr>
<td>Epididymal adipose tissue mass (mg)</td>
<td>702.61±87.93</td>
<td>410.65±36.70</td>
<td>417.96±49.84</td>
<td>191.41±28.50</td>
<td>n.s.</td>
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<tr>
<td>Epididymal adipose tissue mass/body weight (mg/g 10^-4)</td>
<td>166.89±20.61</td>
<td>99.36±7.91</td>
<td>101.28±11.56</td>
<td>47.55±7.07</td>
<td>n.s.</td>
</tr>
</tbody>
</table>

Values are expressed as mean±SEM. Two-way ANOVA was performed.
Assessment of the effects of whole-body heat stress on spontaneous physical activity

**Single experiment 1: Investigation of the physiological responses**

**Acclimation 3 days**

<table>
<thead>
<tr>
<th>0</th>
<th>30</th>
<th>60</th>
<th>390</th>
<th>420 (min)</th>
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<tbody>
<tr>
<td>CON</td>
<td>Control</td>
<td>Sedentary for 6 hours</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ex</td>
<td>Treadmill running 25 m/min, 30 min</td>
<td>Sedentary for 6 hours</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HS</td>
<td>Whole-body heat stress 40 °C, 30 min</td>
<td>Sedentary for 6 hours</td>
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</tr>
<tr>
<td>Ex+HS</td>
<td>Treadmill running 25 m/min, 30 min</td>
<td>Whole-body heat stress 40 °C, 30 min</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Measurement immediately after treatments
- Body weight
- Core temperature
- Plasma corticosterone level

**Measurement 6 hours after treatments**
- Body weight

**Long-term experiment: Investigation of mitochondrial adaptations**

**Acclimation 3 days**

<table>
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<tr>
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<td>HS</td>
<td>Whole-body heat stress 40 °C, 30 min</td>
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</tr>
<tr>
<td>Ex+HS</td>
<td>Treadmill running 25 m/min, 30 min</td>
<td>Whole-body heat stress 40 °C, 30 min</td>
</tr>
</tbody>
</table>

Measurement
- Body composition
- Protein content of HSPs
- Mitochondrial adaptations

**Single experiment 2: Investigation of acute responses of cellular signalling cascades associated with mitochondrial adaptations**

**Acclimation 3 days**

<table>
<thead>
<tr>
<th>0</th>
<th>30</th>
<th>60 (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CON</td>
<td>Control</td>
<td></td>
</tr>
<tr>
<td>Ex</td>
<td>Treadmill running 25 m/min, 30 min</td>
<td></td>
</tr>
<tr>
<td>HS</td>
<td>Whole-body heat stress 40 °C, 30 min</td>
<td></td>
</tr>
<tr>
<td>Ex+HS</td>
<td>Treadmill running 25 m/min, 30 min</td>
<td>Whole-body heat stress 40 °C, 30 min</td>
</tr>
</tbody>
</table>

Measurement
- Responses of cellular signalling cascades

**Experiment day**

- 3 weeks (5 days/week)
- Sedentary for 40 hours
- 60 (min)
FIGURE 2

A
B
C
D
HS x Time: n.s.
HS: n.s.
Time: P<0.0001
Rectal temperature

![Rectal temperature graph](image)

**Body weight immediately after treatments**

![Body weight graph](image)

**Body weight 6 hours after treatments**

![Body weight graph](image)

**Plasma corticosterone level**

![Plasma corticosterone graph](image)
**Plantaris muscle**

Representatives of western blot

![Western Blot Images](Plantaris_HSP60_HSP72_GAPDH.png)

**HSP60**

- **CON**
- **ET**
- **HS**
- **ET+HS**

**HSP72**

- **CON**
- **ET**
- **HS**
- **ET+HS**

**GAPDH**

- **CON**
- **ET**
- **HS**
- **ET+HS**

![Graph](Plantaris_HSP60_HSP72_GAPDH.png)

**HSP60/GAPDH (Relative to CON)**

- **CON**
- **ET**
- **HS**
- **ET+HS**

**HSP72/GAPDH (Relative to CON)**

- **CON**
- **ET**
- **HS**
- **ET+HS**

**Soleus muscle**

Representatives of western blot

![Western Blot Images](Soleus_HSP60_HSP72_GAPDH.png)

**HSP60**

- **CON**
- **ET**
- **HS**
- **ET+HS**

**HSP72**

- **CON**
- **ET**
- **HS**
- **ET+HS**

**GAPDH**

- **CON**
- **ET**
- **HS**
- **ET+HS**

![Graph](Soleus_HSP60_HSP72_GAPDH.png)

**HSP60/GAPDH (Relative to CON)**

- **CON**
- **ET**
- **HS**
- **ET+HS**

**HSP72/GAPDH (Relative to CON)**

- **CON**
- **ET**
- **HS**
- **ET+HS**

**FIGURE 5**
Plantaris muscle

Maximal CS activity

Maximal β-HAD activity

Soleus muscle

Maximal CS activity

Maximal β-HAD activity

FIGURE 6
**Plantaris muscle**

Representatives of western blot

Complex I

- ET x HS: n.s.
- ET: P<0.01
- HS: P<0.01

Complex II

- ET x HS: n.s.
- ET: n.s. (P=0.05)
- HS: P<0.05

Complex III

- ET x HS: n.s.
- ET: P<0.05
- HS: n.s. (P=0.07)

Complex IV

- ET x HS: n.s.
- ET: P<0.05
- HS: P<0.01

Complex V

- ET x HS: n.s.
- ET: P<0.001
- HS: P<0.01

**Soleus muscle**

Representatives of western blot

Complex I

- ET x HS: n.s.
- ET: P<0.01
- HS: P<0.01

Complex II

- ET x HS: n.s.
- ET: n.s.
- HS: n.s.

Complex III

- ET x HS: n.s.
- ET: P<0.05
- HS: P<0.01

Complex IV

- ET x HS: n.s.
- ET: P<0.05
- HS: P<0.04

Complex V

- ET x HS: n.s.
- ET: P<0.05
- HS: P<0.01
**Plantaris muscle**

Representatives of western blot

<table>
<thead>
<tr>
<th></th>
<th>CON</th>
<th>Ex</th>
<th>HS</th>
<th>Ex+HS</th>
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<td>p-AMPK</td>
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**p-AMPK/AMPK**

![Bar chart](chart1.png)

**p-ACC/ACC**

![Bar chart](chart2.png)

**p-p38 MAPK/p38 MAPK**

![Bar chart](chart3.png)

**p-CaMKII/CaMKII**

![Bar chart](chart4.png)

---

**Soleus muscle**

Representatives of western blot

<table>
<thead>
<tr>
<th></th>
<th>CON</th>
<th>Ex</th>
<th>HS</th>
<th>Ex+HS</th>
</tr>
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**p-AMPK/AMPK**

![Bar chart](chart5.png)

**p-ACC/ACC**

![Bar chart](chart6.png)

**p-38 MAPK/p38 MAPK**

![Bar chart](chart7.png)

**p-CaMKII/CaMKII**

![Bar chart](chart8.png)
**Plantaris muscle**

Representatives of western blot

<table>
<thead>
<tr>
<th></th>
<th>CON</th>
<th>Ex</th>
<th>HS</th>
<th>Ex+HS</th>
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<tbody>
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</table>

**p-Akt (Ser473)/Akt**

- Ex x HS: n.s.
- Ex: n.s.
- HS: n.s.

**p-Akt (Thr308)/Akt**

- Ex x HS: n.s.
- Ex: n.s.
- HS: P<0.0001

**p-p70S6K/p70S6K**

- Ex x HS: n.s.
- Ex: n.s.
- HS: P<0.0001

**FIGURE 9**

**Plantaris muscle**

**Soleus muscle**

Representatives of western blot

<table>
<thead>
<tr>
<th></th>
<th>CON</th>
<th>Ex</th>
<th>HS</th>
<th>Ex+HS</th>
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**p-Akt (Ser473)/Akt**

- Ex x HS: n.s.
- Ex: n.s.
- HS: n.s.

**p-Akt (Thr308)/Akt**

- Ex x HS: n.s.
- Ex: n.s.
- HS: P<0.0001

**p-p70S6K/p70S6K**

- Ex x HS: n.s.
- Ex: n.s.
- HS: P<0.0001

**FIGURE 9**