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

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Tamura Y, Matsunaga Y, Masuda H, Takahashi Y, Takahashi Y, Terada S, Hoshino D, Hatta H. Postexercise whole body heat stress additively enhances endurance training-induced mitochondrial adaptations in mouse skeletal muscle. Am J Physiol Regul Integr Comp Physiol 307: R931–R943, 2014. First published July 30, 2014; doi:10.1152/ajpregu.00525.2013.—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. In the present study, we report the novel findings that I) whole body heat stress produced by exposure of ICR mice to a hot environment (40°C, 30 min/day, 5 days/wk, 3 wk) induced mitochondrial adaptations such as increased mitochondrial enzyme activity (citrate synthase and 3-hydroxacyl CoA dehydrogenase) and respiratory chain protein content (complexes I–V) in skeletal muscle in vivo and 2) postexercise whole body heat stress additively enhanced endurance training-induced mitochondrial adaptations (treadmill running, 25 m/min, 30 min/day, 5 days/wk, 3 wk). Moreover, to determine the candidate mechanisms underlying mitochondrial adaptations, we investigated the acute effects of postexercise 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 mammalian target of rapamycin complex 1 activity, and unexpectedly dephosphorylated AMP-activated protein kinase and its downstream target acetyl-CoA carboxylase in skeletal muscle. Our present observations suggest that heat stress can act as an effective postexercise 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.

mitochondria; heat stress; exercise; training; skeletal muscle

MITOCNDRIAL 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 the present study was to investigate the effects of postexercise heat stress on endurance training-induced mitochondrial adaptations such as the activity of mitochondrial enzymes [citrate synthase (CS) and 3-hydroxacyl 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 [AMP-activated protein kinase (AMPK), p38 MAPK, Ca$^{2+}$/calmodulin-dependent kinase II (CaMKII), and mammalian target of rapamycin (mTOR) complex (mTORC1)] that subsequently induce the transcription of mitochondrial genes in skeletal muscle (1, 10, 24, 44, 50). The heat stress method we used in the present study was whole body heat stress (placement of mice in a hot environmental chamber without anesthesia) because a previous study (29) has shown that anesthesia during heat stress impairs normal thermoregulation and leaves mice in a nonphysiological condition.

MATERIALS AND METHODS

Ethical Approval

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

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Experimental Animals and Procedures

The scheme describing experimental procedures is shown in Fig. 1. Experimental animals. Six-week-old male ICR mice (CLEA Japan, 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, Tokyo, Japan) and water ad libitum throughout the experimental period.

Assessment of the effects of whole-body heat stress on spontaneous physical activity. We used whole-body heat stress in the present 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. After a 3-day acclimation period, mice were randomly divided into the following two groups: the control group (CON group; n = 4) and the whole-body heat stress group (HS group; 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 midportion of the backs of the mice for tracking. We recorded spontaneous activity of the mice during heat exposure and

Fig. 1. Experimental procedure. Four experiments were performed: 1) assessment of the effects of whole-body heat stress on spontaneous physical activity, 2) single experiment 1: investigation of physiological responses, 3) long-term experiment: investigation of mitochondrial adaptations, and 4) single experiment 2: investigation of acute responses of cellular signaling cascades associated with mitochondrial adaptations. CON, control group; HS, whole body heat stress group; Ex, the endurance exercise group; Ex + HS, whole body heat stress + endurance exercise group.
nonheat exposure on a video camera (HDR-SR11, 30 frames/s, SONY, Tokyo, Japan) and then analyzed the movement distance of the tracking marker using motion analysis software (Kinovea, version 0.8.15, Windows). Experiments were performed in the dark phase.

**Single experiment 1: investigation of physiological responses.**
After a 3-day acclimation period, mice were randomly divided into the following four groups: the CON group (n = 6), the endurance exercise group (Ex group; n = 6), the HS group (n = 6), and the endurance exercise and postexercise whole body heat stress group (Ex + HS group; 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, mice were anesthetized using isoflurane (% induction, % maintenance, 0.5 l/min). To evaluate physiological responses to treatments, body weight, rectal temperature, and plasma corticosterone levels were measured.

**Long-term experiment: investigation of mitochondrial adaptations.**
After a 3-day acclimation period, mice were randomly divided into the following four groups: the CON group (n = 6), the endurance training group (ET group, n = 6), the HS group (n = 6), and the endurance training and postexercise whole body heat stress group (ET + HS group; 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, mice were euthanized 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 by Western blot analysis.

**Single experiment 2: investigation for acute responses of cellular signaling cascades associated with mitochondrial adaptations.**
After a 3-day acclimation period, mice were randomly divided into the following four groups: the CON group (n = 6), the Ex group (n = 7), the HS group (n = 7), and the EX + HS group (n = 7). Immediately after exercise or whole body heat stress induction, mice were euthanized by cervical dislocation. The plantaris and soleus muscles were dissected out, rapidly frozen in liquid nitrogen, and stored at −80°C until further analyzed by Western blot analysis.

**Heat Stress Protocol**

The hot environmental chamber is shown in Fig. 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, Tokyo, Japan) connected to a proportional integral derivative controller (ESEC-CX2ASM-000, Omron). Mice were exposed to the hot environmental chamber (temperature: 40°C, duration: 30 min) without anesthesia. In the long-term experiment, mice underwent induction of whole body heat stress 5 days/wk for 3 wk. Mice in the ET and ET + HS groups performed treadmill running 5 days/wk for 3 wk.

**Endurance Exercise Training Protocol**

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/wk for 3 wk.

**Rectal Temperature Measurements**

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

**Plasma Corticosterone Level Analysis**
To evaluate systemic stress responses, plasma corticosterone levels were measured in **single experiment 1**. Approximately 50-μl blood samples were collected from lateral tail vein using heparinized microhematocrit capillary tube (22-362-566, Thermo Fisher Scientific). Collected blood samples were immediately separated into plasma and hematocrit by centrifuge (10,000 rpm, 5 min). Plasma samples were stored −80°C until further analysis. Plasma corticosterone levels were analyzed using a commercial ELISA kit (Assaymax Corticosterone ELISA Kit, Assaypro) following the manufacturer's instructions.

**Analytic Methods for Mitochondrial Enzyme Activity**

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 et al. (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 β-glycerol phosphate, 5 mM sodium pyrophosphate, and 2 mM DTT; pH 7.5) containing 10 μg/ml of pepstatin A, aprotinin, and leupeptin, 1 mM Na orthovanadate, and 0.177 mg/ml PMSF. 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 3–7.5% BSA in Tris-buffered saline containing 0.1% Tween 20 (TBST) for 1 h and incubated overnight with the following primary antibodies: GAPDH (ab8245, Abcam), 60-kDa heat shock protein (HSP60; ADI-SPA-806, Enzo Life Sciences, Tokyo, Japan), 72-kDa heat shock protein (HSP72; ADI-SPA-810-D, Enzo Life Sciences), cytochrome c oxidase subunit IV (COX IV; ab14744, Abcam), MiToProfile Total OXPHOS Rodent WB Antibody Cocktail (NADH dehydrogenase (ubiquinone) 1B subcomplex 8 (NDUFB8), succinate dehydrogenase complex subunit B (SDHB), ubiquinol-cytochrome c reductase core protein II (UQCRC2), ATP synthase, H+ transporting, mitochondrial F1 complex, α-subunit (ATP5A), ab110413, Abcam), phosphorylated (p-)AMPKa [Thr172, no. 2513, Cell Signaling Tech-
technology (CST Japan, Tokyo, Japan), AMPKa (no. 2532, CST Japan), p-acetyl-CoA carboxylase (ACC; Ser79, no. 3661, CST Japan), ACC (no. 3662, CST Japan), p-p38 MAPK (Thr170/Tyr182, no. 9211, CST Japan), p38 MAPK (no. 9212, CST Japan), p-CaMKII (Thr286, no. 3361, CST Japan), CaMKII (no. 611292, BD Biosciences Japan, Tokyo, Japan), p-Akt (Ser473, no. 9271, CST Japan), p-Akt (Thr308, no. 9275, CST Japan), Akt (no. 9272, CST Japan), p-mTOR (Ser2448, no. 2481, CST Japan), mTOR (no. 2983, CST Japan), p-p70S6K (Thr389, no. 9205, CST Japan), and p70S6K (no. 9202, CST Japan). After incubation, membranes were washed in TBST, incubated for 1 h at room temperature with secondary antibodies (A106PU or A102PT, American Qualex), and washed again in TBST. Chemiluminescent reagents (SuperSignal West Pico Chemiluminescent Substrate, Thermo Fisher Scientific) were used to facilitate blot detection. Blots were scanned and quantified using ChemiDoc XRS (170-8071, Bio-Rad) and Quantity One (170-9600, version 4.5.2, Windows, Bio-Rad). Quantified band intensity was normalized using GAPDH as a loading control.

Statistical Analysis

All data are expressed as means ± 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 postexercise 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 (version 9.0.1, Macintosh, SAS Institute).

Fig. 3. Effects of whole body heat stress on the physical activity level. Whole body heat stress did not affect the level of spontaneous physical activity. Values are expressed as means ± SE. Two-way ANOVA was performed. n.s., Not significant.

Fig. 4. Acute physiological responses after whole body heat stress. Rectal temperatures were increased after treatments. Plasma corticosterone levels were synergistically increased by postexercise whole body heat stress. Although whole body heat stress decreased body weight, the decreased body weight was recovered within 6 h after treatment. Values are expressed as means ± SE. *Main effects of whole body heat stress. ****P < 0.0001 vs. the CON group; *****P < 0.0001 vs. CON group; (*)P = 0.09 vs. CON group; #P < 0.0001 vs. the Ex group; †††P < 0.001 vs. the HS group; ††††P < 0.0001 vs. HS group. Numbers above the bars are relative changes compared with the CON group.
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 Physiological Responses

Rectal temperature immediately after treatments was significantly different among groups (Fig. 4). A negative main effect of whole body heat stress on body weight was observed immediately after treatments but not 6 h after treatments (Fig. 4). A synergistic effect (i.e., positive interaction) between endurance exercise and whole body heat stress on plasma corticosterone level was observed. A subsequent post hoc test showed that the plasma corticosterone level in the Ex + HS group was significantly higher than those 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 HSP60 and HSP72 protein contents in rodent skeletal muscle are significantly increased by heat stress (35, 39). We investigated HSP60 and HSP72 protein contents as biochemical indicators of the 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 contents were observed in plantaris and soleus muscles (Fig. 5).

Animal characteristics in the long-term experiment. We measured the final body weight, plantaris and soleus muscle masses, and epididymal adipose tissue mass as animal characteristics in the 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

Fig. 5. Effects of whole body heat stress on heat shock protein (HSP) expression in skeletal muscle. Whole body heat stress increased HSP60 and HSP72 protein contents in both plantaris (top) and soleus (bottom) muscles. ET, endurance training group. Values are expressed as means ± SE. Two-way ANOVA was performed. *Significant main effect of whole body heat stress. Numbers above bars are relative changes compared with the CON group.
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...
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 postexercise whole body heat stress were observed. Values are expressed as means ± SE. Two-way ANOVA was performed. *Significant main effect of endurance training; †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. Numbers above bars are relative changes compared with the CON group.
mitochondrial respiratory chain proteins (complex I: NDUFB8, complex II: SDHB, complex III: UQCRC2, complex IV: COX IV, and complex V: ATP5A) in plantaris and soleus muscles. 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 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 postexercise whole body heat stress on any measurements of mitochondrial adaptations were observed in both plantaris and soleus muscles. Therefore, these observations support our hypothesis that postexercise whole body heat stress “additively” enhances endurance training-induced mitochondrial adaptations in mouse skeletal muscle.

Single Experiment 2: Investigation of Responses of Cellular Signaling Cascades Associated With Mitochondrial Adaptations

Whole body heat stress enhanced exercise-induced p38 MAPK activation but inactivated AMPK. To seek the 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 CaMKII (Fig. 8).

No significant interaction between endurance exercise and whole body heat stress for the phosphorylation status of AMPK was observed in 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, negative main effects of whole body heat stress on the phosphorylation status of AMPK and ACC were observed in plantaris and soleus muscles. These results demonstrate that whole body heat stress downregulates AMPK activity in skeletal muscle.

A synergistic effect (i.e., positive interaction) between endurance exercise and postexercise whole body heat stress on the phosphorylation status of p38 MAPK was observed in the plantaris muscle. 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 postexercise whole body heat stress were observed in the soleus muscle. These observations indicate that postexercise whole body heat stress synergistically or additively boosts endurance exercise-induced p38 MAPK activation in plantaris and soleus muscles, respectively.

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

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 (14, 15, 34) have demonstrated that the phosphorylation status of mTOR does not necessarily reflect mTORC1 activity. Many recent studies (23, 42) have investigated the phosphorylation status of p70S6K, a downstream target of mTORC1, as a biomarker of mTORC1 activity. 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 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 plantaris and soleus muscles were similar, whole body heat stress-enhanced mitochondrial adaptations were not fiber type specific. Furthermore, we revealed no antagonistic effects (i.e., no negative interactions) between endurance training and postexercise whole body heat stress on any measurements of mitochondrial adaptation (Figs. 6 and 7). These observations indicate that postexercise whole body heat stress additively enhances endurance training-induced mitochondrial adaptations in mouse skeletal muscle, supporting our hypothesis. Incidentally, a previous study (18) has reported that a high-fat diet also induces mitochondrial biogenesis in skeletal muscle. However, in another study (17), heat stress canceled mitochondrial biogenesis by high-fat diet in rat skeletal muscle, indicating that additive effects of heat stress on mitochon- drial 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 muscles.

Our present findings suggest that heat stress can act as an effective postexercise 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.
Fig. 9. Acute responses to whole body heat stress on the mammalian target of rapamycin (mTOR) complex 1 (mTORC1) pathway. Whole body heat stress increased the phosphorylation status of Akt (Ser473 and Thr308) and p70S6K in skeletal muscle. Values are expressed as means ± SE. Two-way ANOVA was performed. BSignificant main effect of whole body heat stress. Numbers above bars show relative changes compared with the CON group.
Validation and Profile of Our Heat Stress Protocol

A previous study (29) has shown that anesthesia during heat stress impairs normal thermoregulation and leaves mice in a nonphysiological condition. Therefore, we used whole body heat stress without anesthesia. First, we examined the effects of whole body heat stress on spontaneous physical activity levels. 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 mouse’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, HSP60 and HSP72 protein contents, common biomarkers for the cellular heat shock response, were significantly increased by whole body heat stress in plantaris and soleus muscles (Fig. 5). These results show that whole body heat stress in this study induced a 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°C in the HS group and 40.4 ± 0.1°C in the Ex + HS group, respectively (Fig. 4). A previous study (29) has shown that the minimal lethal core temperature is ~42°C or higher in mice, indicating that our heat stress protocol in this study did not absolutely reach the fatal heat stroke. The plasma corticosterone level, a biomarker for the systemic stress response, was synergistically increased by postexercise whole body heat stress. These observations can be interpreted that postexercise whole body heat stress further initiated a 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 the Candidate Mechanisms Underlying Whole Body Heat Stress-Induced Mitochondrial Adaptations

AMPK, p38 MAPK, and CaMKII are well-characterized cellular signaling cascades that subsequently induce mitochondrial gene transcription. A recent study (32) has demonstrated that heat stress-induced mitochondrial biogenesis was possibly mediated by AMPK activation in C2C12 myotubes. Moreover, heat stress increased the phosphorylation status of AMPK in isolated rat skeletal muscle (28). Therefore, we hypothesized that postexercise whole body heat stress additively or synergistically enhances exercise-induced AMPK phosphorylation and activation. However, whole body heat stress unexpectedly downregulated 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 in vivo. The AMPK response to heat stress has been controversial. A previous study in Hep G2 cells, a human liver carcinoma cell line, reported that heat stress decreases AMPK activity without an energy status change. In accordance with this previous report, our present observation (J) may be explained rather by heat stress condition (e.g., temperature and/or duration) than differences between in vitro and in vivo, such as humoral factor involvement, and 2) may not be caused by changes in cellular energy status. To cultivate a better understanding of AMPK activity in response to heat stress, further studies in various experimental conditions are needed.

Interestingly, postexercise 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 have demonstrated that exposing rodents to hot environment increases the production of ROS and levels of circulating corticosterone (Fig. 2), which are known to be 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 the upstream causes of p38 MAPK activation.

Endurance exercise in this study did not increase the phosphorylation status of CaMKII in plantaris and soleus muscles (Fig. 8). In accordance with previous studies (13, 40), CaMKII was significantly phosphorylated or activated by high-intensity exercise or at the onset of moderate-intensity exercise. 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 plantaris and soleus muscles (Fig. 8). A previous study (49) has demonstrated that CaMKII is upstream of p38 MAPK. 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 (4, 10, 37, 51) have revealed that mTORC1 is responsible for not only muscle protein synthesis but also mitochondrial gene transcription. Whole body heat stress significantly increased the phosphorylation status of Akt (Ser473 and Thr408) and p70S6K in plantaris and soleus muscles, indicating that whole body heat stress activated the mTORC1 pathway (Fig. 9). A recent study (52) has shown that partial heat stress in the rat hindlimb but not the whole body also increased the phosphorylation status of Akt (Ser473) and p70S6K but not mTOR in plantaris and soleus muscles. 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, postexercise 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 HSP60 and HSP72 protein contents in 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 the folding process and then enhance mitochondrial adaptations.

Taken together, our present 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 (5, 8, 47) have demonstrated that heat stress-induced HSP72 synthesis is required for AMPK inactivation, p38 MAPK activation, and mTORC1 activation. 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 resynthesis 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, given the physiological significance of acute cellular responses and chronic mitochondrial adaptations, it 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 2) postexercise 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 present findings suggest that heat stress can act as an effective postexercise 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.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


REFERENCES

ADDITIVE EFFECTS OF HEAT STRESS ON MITOCHONDRIAL ADAPTATIONS


