Mild heat stress induces mitochondrial biogenesis in C2C12 myotubes

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Liu CT, Brooks GA. Mild heat stress induces mitochondrial biogenesis in C2C12 myotubes. J Appl Physiol 112: 354–361, 2012. First published November 3, 2011; doi:10.1152/japplphysiol.00989.2011.—During endurance exercise, most (~75%) of the energy derived from the oxidation of metabolic fuels and ATP hydrolysis of muscle contraction is liberated as heat, the accumulation of which leads to an increase in body temperature. For example, the temperature of exercising muscles can rise to 40°C. Although severe heat injury can be deleterious, several beneficial effects of mild heat stress (HS), such as the improvement of insulin sensitivity in patients with type 2 diabetes, have been reported. However, among all cellular events induced by mild HS from physical activities, the direct effects and mechanisms of mild HS on mitochondrial biogenesis in skeletal muscle are least characterized. AMP-activated protein kinase (AMPK) and sirtuin 1 (SIRT1) are key energy-sensing molecules regulating mitochondrial biogenesis. In C2C12 myotubes, we found that 1 h mild HS at 40°C upregulated both AMPK activity and SIRT1 expression, as well as increased the expression of several mitochondrial biogenesis regulatory genes including peroxisome proliferator-activated receptor gamma coactivator-1α (PGC-1α) and transcription factors involved in mitochondrial biogenesis. In particular, PGC-1α expression was found to be transcriptionally regulated by mild HS. Additionally, after repeated mild HS for 5 days, protein levels of PGC-1α and several mitochondrial oxidative phosphorylation subunits were also upregulated. Repeated mild HS also significantly increased mitochondrial DNA copy number. In conclusion, these data show that mild HS is sufficient to induce mitochondrial biogenesis in C2C12 myotubes. Temperature-induced mitochondrial biogenesis correlates with activation of the AMPK-SIRT1-PGC-1α pathway. Therefore, it is possible that muscle heat production during exercise plays a role in mitochondrial biogenesis.

sirtuin; SIRT1; AMPK; PGC-1α; temperature; heat shock proteins; exertion; exercise; mitochondria; mitochondrial reticulum

TEMPERATURE DIRECTLY OR INDIRECTLY affects essentially every aspect of an organism’s physiology. It is well known that most species perform best in a fairly narrow temperature range, which is also associated with optimal enzyme functions (40). Mammals produce heat by thermogenesis that involves mechanisms such as shivering and uncoupling protein-induced mitochondrial proton leak (22, 42). Excess heat in mammals is dissipated by evaporative and nonevaporative means (4, 10).

Endurance exercise imposes a combination of stresses such as changes in calcium concentration, mechanical strain, alterations in the ADP-to-ATP ratio, hypoxic stress, and loosening of mitochondrial respiratory control on skeletal muscle (5, 33). Numerous studies have focused on how these stresses contribute to exercise-induced adaptations. Increased heat production is also a byproduct of endurance exercise and can also be counted among the stresses imposed on skeletal muscle by physical exercise. However, the direct effects of mild heat stress (HS)—heating within physiological limits—on mitochondrial biogenesis have not been well characterized in past studies.

Muscle tissue is capable of directly transforming the chemical energy derived from nourishment into mechanical work. Measured by the ratio of work performed to energy cost as calculated from oxygen uptake, the energy conversion efficiency of skeletal muscle is only 20–30% (14, 54). This is because the efficiency of ATP generation is ~60%, and the efficiency of ATP use is ~50% (14, 54). During vigorous exercise, the energy cost can increase by 10- to 20-fold compared with the resting metabolic rate (35), and the temperature of exercising muscles can rise to values as high as 40°C, which can negatively affect mitochondrial respiratory control and coupling efficiency (6, 48, 49). The heat generated during endurance exercise must be effectively transported to the skin and dissipated to the environment to prevent heat injury. Although heat stroke is currently the third leading cause of death in athletes in the United States (25), several beneficial effects of mild HS have been reported among several different species. For example, hot tub therapy can improve insulin sensitivity and lower blood glucose levels in patients with type 2 diabetes (24). Both heat treatment at 41 to 41.5°C and overexpression of heat shock protein 72 (HSP72) have been shown to prevent high-fat diet-induced insulin resistance in rodents (11, 17). Finally, mild HS was also observed to extend life span in yeast, worms, and flies (30, 37, 51).

Mild HS raises cellular ATP flux that raises AMP level, which, in turn, activates AMPK (12). AMPK is a key energy sensing molecule that maintains whole body energy homeostasis in response to reduced cellular energy status (19). Activated AMPK switches off biosynthetic pathways to reserve ATP for more essential processes and switches on catabolic pathways to boost ATP production by upregulating oxidative metabolism and mitochondrial biogenesis (19). Recently, an emerging body of evidence suggests that another energy-sensing molecule, SIRT1, works closely with AMPK to control cellular energy expenditure (47). SIRT1, a mammalian class III protein deacetylase, is an important regulator of metabolism, cancer, and life span (34). AMPK and SIRT1 act in cooperation with the master regulator of mitochondrial biogenesis, PGC-1α, to regulate energy homeostasis in response to environmental stimuli and nutritional signals (8, 9, 55). AMPK and SIRT1 directly activate PGC-1α through phosphorylation and deacetylation, respectively (27, 46). AMPK and SIRT1 also increase the expression of mRNAs encoding PGC-1α (2, 27).

Mitochondrial biogenesis entails mitochondrial DNA replication and transcription, as well as the synthesis and
import of proteins into the mitochondrial reticulum (29, 31). Because the oxidative phosphorylation system is located in the mitochondrial inner membrane (50), the physiological significance of mitochondrial biogenesis lies in the enhancement of oxidative phosphorylation, which ultimately results in a net ATP gain of more than 15 times the amount of ATP produced by glycolysis (1). We propose that the beneficial effects of mild HS, such as improved insulin sensitivity, may be associated with mitochondrial biogenesis. We hypothesize that the reduced level of cellular energy induced by mild HS can activate AMPK and SIRT1, both of which further upregulate PGC-1α and the downstream mitochondrial biogenesis program. To test this hypothesis, we used C2C12 myotubes as a model system to study the effect of acute and repeated mild HS on mitochondrial biogenesis by measuring activation of the AMPK-SIRT1-PGC-1α pathway, as well as the levels of transcription factors involved in mitochondrial biogenesis and mitochondrial components such as mitochondrial DNA copy number.

MATERIALS AND METHODS

Cell culture. Mouse C2C12 myoblast cell line was maintained in DMEM (4.5 g glucose/l) in the presence of 10% fetal bovine serum. Differentiation of C2C12 myoblasts into myotubes was induced with media containing 2% horse serum for 5 days. The cells were grown in a humidified 37°C incubator with 5% CO2 in air.

Mild heat stress exposure. For mild HS, the culture temperature was set at 40°C. The duration of mild HS was maintained for 1 h for each bout. The frequency was either a single bout on the 5th day of cultures being treated with mild HS for 1 h. After a 24-h recovery period, the cell lysates were used for determining Renilla and firefly luciferase activities with the Promega kit mentioned above.

Table 1. Primer sequences used for real-time quantitative PCR

<table>
<thead>
<tr>
<th>Primers</th>
<th>Strand</th>
<th>Sequence 5’-3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hspa1a</td>
<td>Forward</td>
<td>AAGATGGCTTGCCCGCCTTGAAGAGTACCTTTG</td>
</tr>
<tr>
<td>Hspa1b</td>
<td>Reverse</td>
<td>CAAATGGACATGCTGGGACCTTCGATGGAAGAGAGG</td>
</tr>
<tr>
<td>Cox4</td>
<td>Forward</td>
<td>GGGCGGAGAAGCCCTGAA</td>
</tr>
<tr>
<td>Nrf1</td>
<td>Forward</td>
<td>CCTGAGCTGCTTCTGAGCCCTT</td>
</tr>
<tr>
<td>Nrf2a</td>
<td>Reverse</td>
<td>CTGAGAGCTTTCTCCTTCTCAGCT</td>
</tr>
<tr>
<td>Tfam</td>
<td>Forward</td>
<td>CATTTATCTAGTCAGAAGAGATTCTC</td>
</tr>
<tr>
<td>Tbj1m</td>
<td>Reverse</td>
<td>GTATGTCGCGTTCCTTCGCGT</td>
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<tr>
<td>Tbj2m</td>
<td>Forward</td>
<td>CAAAGAGCATCCTGCATCTC</td>
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<tr>
<td>Cycs</td>
<td>Reverse</td>
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<tr>
<td>Cox2</td>
<td>Forward</td>
<td>CGAACCTCCGACTTTCGCTG</td>
</tr>
<tr>
<td>Cox4</td>
<td>Reverse</td>
<td>CGGGGAGAAGCCCTGAA</td>
</tr>
<tr>
<td>Glut4</td>
<td>Forward</td>
<td>TGCGGCTTCTTCCGACTGTTCT</td>
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<tr>
<td>Ldhα</td>
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<td>TGGCTAGGAGGTAGTCAAGGTT</td>
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<tr>
<td>Rn18 s</td>
<td>Forward</td>
<td>GCCGCTAGAGTGAGAAAT</td>
</tr>
<tr>
<td>Reverse</td>
<td>CAAACCTCGGACTTTTGTTT</td>
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Mild heat stress exposure. The frequency was either a single bout on the 5th day of cultures being treated with mild HS for 1 h. After a 24-h recovery period, the cell lysates were used for determining Renilla and firefly luciferase activities with the Promega kit mentioned above.

Mitochondrial DNA quantification. Total cellular DNA in C2C12 myotubes was extracted using Blood & Cell Culture DNA Mini Kit (Qiagen) according to the manufacturer’s instructions. DNA pellets were resuspended in TE (1 mM EDTA, 10 mM Tris, pH 8.0) prior to qPCR analysis. Real-time qPCR was performed for mitochondrial DNA-encoded cytochrome c oxidase subunit II (Cox2) (primers: forward 5'-GCCGCACTAATACACAGAGGAAATCAG-3', reverse 5'-CAATGTGCGCTTTCGTTTCTCG-3') and nucleus-encoded 18S ribosomal RNA genes (primers: forward 5'-TAGAGGGCAAACTAAGGGCCGCTT-3', reverse 5'-GCCGCACTAATACACAGAGGAAATCAG-3'). The ratio of Cox2 DNA copies to 18S rRNA represents the relative mitochondrial copy number.

Statistical analysis. Statistical significance of temperature-induced differences was evaluated by Student’s t-test assuming equal variance,

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and a value of \( P < 0.05 \) was considered significant. Results are presented as the mean ± SE of the mean. Statistical analyses were performed using Excel 2007 software (Microsoft).

RESULTS

**Mild HS induces cytoprotective heat shock response.** To investigate the direct cellular effects of mild HS on muscle cells, we incubated the C2C12 myotubes at 40°C for 1 h. HSPA1a and HSPA1b are the major stress-inducible genes of the heat shock protein 70-kDa family. They encode proteins with identical amino acid sequences known as HSP72 or HSP70. Although no significant change in HSP72 protein levels was observed immediately after mild HS (Fig. 1A), mRNA levels of HSPA1a and HSPA1b increased by more than 100-fold when measured 1 h after mild HS (data not shown). Two hours and 24 h after mild HS, the mRNA levels were still significantly higher for HSPA1a (3.41-fold, \( P < 0.05 \) and 1.82-fold, \( P < 0.05 \); Fig. 1C) and for HSPA1b (4.98-fold, \( P < 0.05 \) and 1.67-fold, \( P < 0.05 \); Fig. 1D). Furthermore, the increase in HSP72 protein levels by 2.55-fold (\( P < 0.05 \)) was a cytoprotective heat shock response.

**Mild HS upregulates AMPK activity and SIRT1 expression.** AMPK is a key energy-sensing molecule and is activated when cells undergo energy-depleting stresses. The degree of AMPK T172 phosphorylation (p-AMPK) was used as a measure of AMPK activation (19). It is evident from Fig. 2A that, immediately after mild HS, a significant increase in AMPK T172 phosphorylation (1.6-fold; \( P < 0.05 \)) was observed while the total AMPK (t-AMPK) protein levels remained unchanged. These results indicate that mild HS rapidly enhances AMPK activity.

AMPK is known to regulate SIRT1 expression (56). Therefore, we measured SIRT1 expression after mild HS. Our data showed that the SIRT1 protein levels remained unchanged immediately after mild HS (Fig. 2A). However, 24 h after mild HS, the SIRT1 protein levels were upregulated by 2.04-fold (\( P < 0.05 \); Fig. 2B). We also found that the SIRT1 mRNA level was upregulated 24 h after mild HS (1.51-fold, \( P < 0.05 \); Fig. 3A).

Nicotinamide adenine dinucleotide (NAD\(^+\)) is required in several important biological functions. In particular, NAD\(^+\) is an obligate cofactor for the deacetylase activity of SIRT1 (26). Nicotinamide phosphoribosyltransferase (Nampt) is responsible for the rate-limiting step in the NAD\(^+\)-biosynthetic pathway in mammals (45). Therefore, we analyzed Nampt expression after mild HS. Our data showed that 24 h after mild HS, Nampt protein levels were significantly upregulated (1.50-fold, \( P < 0.05 \); Fig. 2C). In summary, a single bout of mild heat stress results in a rapid upregulation of AMPK activity followed by an increase of Nampt and SIRT1 expression.

**Mild heat stress increases PGC-1α transcription.** PGC-1α is a well-studied transcriptional target of both SIRT1 and AMPK (2, 27). In our studies of its expression level after mild HS, no significant change in the mRNA level of PGC-1α was observed 2 h after mild HS while an increase of 1.36-fold (\( P < 0.05 \)) was seen 24 h later (Fig. 3A). In contrast, the mRNA levels of PGC-1β and PRC, the other two members of PGC-1 family transcription coactivators, remained constant at both 2 h and 24 h after mild HS (Fig. 3A).

To examine whether mild HS upregulates PGC-1α expression at the transcription level, the luciferase assay was used to monitor PGC-1α promoter activity. As shown in Fig. 3B, luciferase activity increased by 3.46-fold (\( P < 0.05 \)) 24 h after mild HS. In summary, our data indicate that mild HS is sufficient to increase PGC-1α promoter activity and modulate PGC-1α transcription in C2C12 myotubes.

**Mild heat stress activates mitochondrial biogenesis program.** To determine whether mitochondrial biogenesis program is upregu-

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Fig. 1. Mild heat stress (HS) activates heat shock response. 

**A:** C2C12 myotubes were exposed to 1-h mild HS (40°C) and harvested immediately for Western blotting to determine the heat shock protein (HSP) 72 protein levels. 

**B:** C2C12 myotubes were exposed to 1-h mild HS and then returned to the normal culture environment (37°C). After 24 h, cells were harvested for Western blotting to measure the HSP72 protein levels. Results were normalized to Histone H3. Quantification of band intensity is shown below the Western blot. Results are expressed as means ± SE from three independent trials. *\( P < 0.05 \) compared with the control. 

**C and D:** C2C12 myotubes were harvested 2 and 24 h after the above-mentioned mild HS. The mRNA levels of Hspa1a and Hspa1b were determined by qPCR and normalized to 18S ribosomal RNA. Results are expressed as means ± SE of triplicate samples in 1 representative experiment from 3 independent trials. *\( P < 0.05 \) compared with control.
lated in response to mild heat stress, a series of real-time qPCR assays was conducted to compare the expression of downstream genes of AMPK-SIRT1-PGC-1α pathway. We first analyzed the expression of nuclear respiratory factors 1 and 2 (NRF1 and NRF2), the transcription factors necessary for the coordination of nuclear-mitochondrial gene expression required for mitochondrial biogenesis. The effects of PGC-1α on mitochondrial biogenesis are mediated through its ability to both upregulate expression of NRF1 and NRF2 as well as coactivate both nuclear respiratory factors (55). Similar to PGC-1α, the mRNA levels of both Nrf1 and Nrf2/α remained constant 2 h after mild HS while a significant increase was observed after 24 h (1.35-fold, \( P < 0.05 \) and 1.46-fold, \( P < 0.05 \), respectively; Fig. 4, A and B).

Mitochondrial DNA replication and transcription are coupled events executed by unique enzyme systems. This process is regulated by mitochondrial transcription factors, including TFAM, TFB1M, and TFB2M (13). After nuclear translocation and coactivation by PGC-1α, NRF1 and NRF2 bind and activate the promoters of Tfam, Tfb1m, and Tfb2m (15). Because the mRNA levels of both Nrf1 and Nrf2/α were upregulated by mild HS, we subsequently measured the mRNA levels of Tfam, Tfb1m, and Tfb2m. Two hours after mild HS, the mRNA levels of these three mitochondrial transcription factors

Fig. 2. Mild HS upregulates AMPK activity and SIRT1 expression. A: C2C12 myotubes were exposed to 1-h mild HS (40°C) and harvested immediately for Western blotting to determine levels of total and phosphorylated AMPK (t-AMPK and p-AMPK) as well as SIRT1. B and C. C2C12 myotubes were exposed to 1-h mild HS and then returned to the normal culture environment (37°C). After a 24-h recovery period, cells were harvested for Western blotting to measure the expression of abovementioned proteins and Nampt, the rate-limiting enzyme in the NAD⁺ biosynthesis pathway. Results were normalized to Histone H3. Quantification of band intensity is shown below the Western blot. Results are given as the means ± SE from 4 independent trials.* \( P < 0.05 \) compared with the control.

Fig. 3. Mild HS increases PGC-1α transcription. A: C2C12 myotubes were exposed to mild HS for 1 h and then returned to the normal culture environment. The mRNA levels of Sirt1, PGC-1α, PGC-1β, and PRC were determined by qPCR and normalized to 18S ribosomal RNA. Results are expressed as means ± SE of triplicate samples in one representative experiment from 3 independent trials. \( * \) \( P < 0.05 \) compared with control. B: C2C12 myoblasts were transfected with PGL3-PGC-1α 2-kb promoter-firefly luciferase reporter and a CMV Renilla luciferase plasmid. After differentiation, the cells were treated with 1-h mild HS and then returned to the normal culture environment. After a 24-h recovery period, cells were harvested for luciferase assay. The data show the ratio of firefly to Renilla luciferase luminescence, which represents the normalized promoter activity of PGC-1α. Results are given as the means ± SE from 5 independent trials. \( * \) \( P < 0.05 \) compared with the control.
were not altered. However, 24 h after treatment, a significant increase was observed in the levels of all three transcription factors (1.55-fold, \( P < 0.05 \), 1.27-fold, \( P < 0.05 \), and 1.77-fold, \( P < 0.05 \), respectively; Figs. 4, C–E). In summary, these data demonstrate that the mitochondrial biogenesis program is upregulated by mild heat stress.

Mild heat stress induces gene expression for mitochondrial components. Because activation of the mitochondrial biogenesis transcriptional cascade should lead to increased expression of mitochondrial components, we analyzed the mRNA levels of mitochondrial oxidative proteins including cytochrome c (\( \text{Cycs} \)), nuclear-encoded cytochrome c oxidase subunit IV (\( \text{Cox4} \)), and mitochondrial DNA-encoded respiratory subunit \( \text{Cox2} \). No significant changes in the mRNA levels of \( \text{Cycs} \), \( \text{Cox2} \), and \( \text{Cox4} \) were observed 2 h after mild HS while significant increases were seen in all of them 24 h after treatment (1.40-fold, \( P < 0.05 \), 1.77-fold, \( P < 0.05 \), and 1.74-fold, \( P < 0.05 \), respectively; Fig. 4, F–H). In summary, not only the regulatory genes, but also genes of mitochondrial components were upregulated after mild HS.

In addition to the genes involved in mitochondrial biogenesis, we also measured the transcription of glucose transporter \( \text{Glut4} \), another target regulated by PGC-1\( \alpha \). No significant change in the mRNA level of \( \text{Glut4} \) was observed 2 h after mild HS while an increase of 1.45-fold (\( P < 0.05 \)) was seen after 24 h (Fig. 4I). At the same time, there was no difference observed in mRNA levels of lactate dehydrogenase isoform A (\( \text{Ldha} \)) after mild HS and 24 h (data not shown).

Repeated mild HS upregulates the expression of PGC-1\( \alpha \), oxidative phosphorylation subunits, and mitochondrial DNA copy number. Our initial studies demonstrated that a single bout of mild HS is capable of activating the expression of a diverse group of genes. To further characterize the adaptive response to mild HS in myotubes, repeated mild HS was used to mimic the repeated HS that occurs in muscle as the result of regular exercise.

C2C12 myotubes were exposed to 1 h of mild HS per day for 5 days and then harvested for subsequent assays. There was no significant difference in SIRT1 protein expression observed between control and HS groups (data not show). However, a significant increase in PGC-1\( \alpha \) protein expression was seen (1.58-fold, \( P < 0.05 \); Fig. 5). We were also interested in the effect of repeated mild HS on mitochondrial protein levels. As expected, repeated mild HS upregulated the protein levels of four representative oxidative phosphorylation subunits including Complex I subunit \( \text{NDUFB8} \) (1.94-fold, \( P < 0.05 \)), Complex II subunit \( 30 \text kDa} \) (1.76-fold, \( P < 0.05 \)), Complex III subunit \( \text{Core 2} \) (1.78-fold, \( P < 0.05 \)), and Complex V, or ATP synthase subunit-\( \alpha \) (1.61-fold, \( P < 0.05 \); Fig. 5).

Because mitochondria contain their own DNA encoding 13 proteins, measurements of mitochondria DNA copy number was used to verify changes in mitochondrial biogenesis levels (38). Real-time qPCR analysis showed that repeated mild HS for 5 days upregulated the mitochondrial DNA copy number by 1.68-fold (\( P < 0.05 \); Fig. 6). Taken together, repeated mild HS for 5 days resulted in a significant increase in PGC-1\( \alpha \) protein.

Fig. 4. Mild HS activates mitochondrial biogenesis program. C2C12 myotubes were exposed to mild HS for 1 h and then returned to the normal culture environment. Cells were harvested after 2- and 24-h recovery periods. The mRNA levels of nuclear regulatory factors \( \text{Nrf1} \) (A), \( \text{Nrf2} \) (B), mitochondrial transcription factors \( \text{Tfam} \) (C), \( \text{Tfb1m} \) (D), \( \text{Tfb2m} \) (E), mitochondrial components \( \text{Cycs} \) (F), \( \text{Cox2} \) (G), \( \text{Cox4} \) (H), and glucose transporter \( \text{Glut4} \) (I) were determined by qPCR and normalized to 18S ribosomal RNA. Results are expressed as means \( \pm \) SE of triplicate samples in 1 representative experiment from 3 independent trials. *\( P < 0.05 \) compared with the control.
expression as well as in mitochondrial components including oxidative phosphorylation proteins and mitochondrial DNA.

DISCUSSION

This study provides evidence in support of the hypothesis that mild HS is sufficient to induce mitochondrial biogenesis in C2C12 myotubes. Two types of evidence were obtained. First, key molecules of the mitochondrial biogenesis pathway, including energy-sensing AMPK and SIRT1 as well as their downstream targets such as PGC-1α, are activated by mild HS (Figs. 2 and 3). Second, levels of mitochondrial components, including several oxidative phosphorylation subunits and mitochondrial DNA, increase after mild HS (Figs. 4–6).

One of the most prominent mechanisms to rapidly cope with stresses is the heat shock response (21, 36). The heat shock response is almost ubiquitous across all life forms and is induced at temperatures a few degrees above the normal physiological range for most species (42). To cope with stresses, cells need to synthesize several critical heat shock proteins that can be used to restore structures of reversibly damaged proteins while degrading irreversibly damaged proteins as well as synthesizing new proteins (21, 36). All of these events are highly ATP costly processes and force cells into a reduced energy status. Investigators have shown that nondamaging running exercise for 45 min increases muscle temperature from 36.2 to 40°C (41). The authors also showed that the protein level of HSP72 significantly increased 48 h postexercise in muscle. Exercise-induced expression of HSPs in the heart is also one of the mechanisms responsible for the cardioprotective effects of exercise (16, 20). Our results showed that mild HS at 40°C increases immediately the mRNA levels and subsequently the protein levels of HSPα1 and HSPα2—the major stress-inducible members of the heat shock protein 70-kDa family (Fig. 1). In summary, temperature increase within the physiological range is sufficient to induce the cytoprotective heat shock response.

SIRT1 is well-known for its role in modulating the life span of a number of organisms including yeast, worms, flies, and mice with restricted caloric intake (34). Similarly, mild HS has also long been reported to extend life span in different species (30, 37, 51), although the mechanism is not yet clear. Recently, Westerheide et al. (53) showed that SIRT1 directly deacetylates heat shock factor 1 and thereby regulates the acute heat shock response in mammalian cells. That result may mean that SIRT1 plays a role in heat-induced life span extension. Our results show that SIRT1 protein level significantly increased 24 h after mild HS. To our knowledge, this is the first report showing the direct upregulation of SIRT1 protein expression by heat. Our results may mean that SIRT1 not only functions in the early stages of the heat shock response, but SIRT1 may also provide important long-term functions after HS is removed. Accordingly, upregulation of SIRT1 may play a crucial role in two renowned environmental stressors that extend life span in animals: calorie restriction and mild heat stress.

Based on the evidence of the increase in PGC-1α promoter activity and mRNA level our data show that PGC-1α is transcriptionally regulated by mild HS. Interestingly, PGC-1α levels have been found to be greatly induced in muscle and brown fat by cold exposure (44). The common effect of cold exposure and excess heat on cells is an increase in cellular energy requirement. Based on previous findings and those of the current study, there is clear evidence that PGC-1α plays a crucial role in response to temperature fluctuations in cells, and PGC-1α is widely considered to be the master regulator of mitochondrial biogenesis. As well, it has been shown that another member of the sirtuin family of protein deacetylases—SIRT3 is a downstream target of PGC-1α (32). SIRT3 is...
localized in mitochondria and regulates mitochondrial respiration capacity, oxidative stress, and insulin resistance in skeletal muscle (28). It will be interesting to test whether SIRT3 expression level and activity are also upregulated by mild HS in the future.

Glucose uptake is a rate-limiting step in muscle glucose metabolism (52), and both gain-of-function and loss-of-function mutations have shown that GLUT4 is essential in the maintenance of normal glucose homeostasis (7, 58). As well, in skeletal muscle, Glut4 is one of the targets regulated by PGC-1α (39). In our studies, Glut4 transcription was significantly upregulated by mild HS, possibly due to an increase in PGC-1α expression. Thus results of our study may reveal an additional factor that, among others (11, 17), contributes to the heat-induced improvement of glucose tolerance and insulin sensitivity.

Multiple lines of evidence have shown that endurance exercise training results in an increase in skeletal muscle mitochondrial biogenesis (23). The physiological significance of mitochondrial biogenesis is the enhancement of cellular oxidative phosphorylation, which improves endurance performance at the organismal level. We found that the levels of several oxidative phosphorylation proteins and mitochondrial DNA copy number significantly increased after repeated mild HS. Because we show that in C2C12 myotubes mild HS increases markers of mitochondrial biogenesis similar to what occurs in regular endurance training in vivo, it is possible that muscle heat production during exercise plays a role in mitochondrial biogenesis.

Mitochondrial biogenesis is a vital and exciting area of cell biology. Lack of normal mitochondrial function has a negative impact in insulin resistance and aging, among others (43). In this report, we show that mitochondrial biogenesis is upregulated in response to mild HS in C2C12 myotubes. The activation of AMPK-SIRT1-PGC-1α pathway and its downstream targets is correlated with the temperature-induced changes in mitochondrial biogenesis. One limitation of our report is that C2C12 myotube culture system may not entirely reflect what occurs in vivo. Our findings need to be confirmed in animal models to see whether the temperature-induced effects are reproducible in vivo. Future studies are also needed to test the hypothesis of a causal link between AMPK-SIRT1-PGC1α activation and temperature-induced mitochondrial biogenesis in muscle. If so, then, exercise-induced changes in muscle temperature may have the effect of raising the background levels of metabolic gene expression, upon which exercise-induced changes in metabolites, ions, and other known affectors of mitochondrial biogenesis operate. The results presented here may suggest the possibility of developing treatments for conditions caused by mitochondrial deficit using heat modalities when the ability for exercise is impaired.

GRANTS

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DISCLOSURES

GAB has a financial interest in CytoSport, Inc. Otherwise, no conflicts of interest, financial or otherwise are declared by the authors.

AUTHOR CONTRIBUTIONS

Author contributions: C-T.L. and G.A.B. conception and design of research; C-T.L. performed experiments; C-T.L. and G.A.B. analyzed data; C-T.L. and G.A.B. interpreted results of experiments; C-T.L. prepared Figs.; C-T.L. drafted manuscript; C-T.L. and G.A.B. edited and revised manuscript; C-T.L. and G.A.B. approved final version of manuscript.

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