Effect of body temperature during exercise on skeletal muscle cytochrome c oxidase content

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Mitchell, Christopher R., M. Brennan Harris, Anthony R. Cordaro, and Joseph W. Starnes. Effect of body temperature during exercise on skeletal muscle cytochrome c oxidase content. J Appl Physiol 93: 526–530, 2002.—This study determined the role of body temperature during exercise on cytochrome-c oxidase (CytOx) activity, a marker of mitochondrial content, and mitochondrial heat shock protein 70 (mtHSP70), which is required for import of nuclear-coded preproteins. Male, 10-wk-old, Sprague-Dawley rats exercised identically for 9 wk in ambient temperatures of 23°C (n = 10), 8°C with wetted fur (n = 8), and 4°C with wetted fur and fan (n = 7). These conditions maintained exercising core temperature (Tc) at 40.4, 39.2, or 38.0°C (resting temperature), respectively. During weeks 3–9, exercisers ran 5 days/wk up a 6% grade at 20 m/min for 60 min. Animals were housed at 23°C. Gastrocnemius CytOx activity in Tc = 38.0°C (83.5 ± 5.5 μatoms O₂·min⁻¹·g wet wt⁻¹) was greater than all other groups (P < 0.05), exceeding sedentary (n = 7) by 73.2%. Tc of 40.4 and 39.2°C also were higher than sedentary by 22.4 and 37.4%, respectively (P < 0.05). Quantification of CytOx content verified that the increased activity was due to an increase in protein content. In extensor digitorum longus, a nonactive muscle, CytOx was not elevated in Tc = 38.0°C. mtHSP70 was significantly elevated in gastrocnemius of Tc = 38.0°C compared with sedentary (P < 0.05) but was not elevated in extensor digitorum longus (P > 0.05). The data indicate that decreasing exercise Tc may enhance mitochondrial biogenesis and that mtHSP70 expression is not dependent on temperature.

endurance exercise; mitochondrial biogenesis; mitochondrial heat shock protein 70; glucose-regulated protein 75; rat

Temperature of the body and muscles is known to increase during exercise and to acutely affect muscle metabolism and function. In fish cold acclimation results in dramatic increases in mitochondrial content of muscle cells (see Ref. 34 for review). However, the role of temperature on mitochondrial adaptations to chronic exercise training has received little attention. Theoretically, changes in temperature could influence mitochondrial content in many ways. For example, changing body temperature during exercise has been reported to cause changes in the following: oxygen kinetics from blood to muscle mitochondria (28, 33), substrate utilization (11, 14, 19), plasma epinephrine concentration (27), blood pressure (6), peripheral vasconstriction (37), and coupling of ATP production to oxygen utilization (5, 39). In addition, temperature affects muscle viscosity, which could alter mechanical efficiency and increase the energy cost of exercise at low temperatures (14, 28).

The purpose of this experiment was to test the hypothesis that body temperature during a running exercise influences mitochondria biogenesis during an endurance-training program. Rats were trained for 9 wk on a motor-driven treadmill by using the same absolute exercise protocol in three different environments. Environmental conditions were established that kept core temperature (Tc) at resting value throughout 60 min of exercise or elevated Tc ~1.2 or 2.4°C within 15 min after beginning the exercise. The effect of these training programs on mitochondrial content in muscle was estimated by measuring the content of cytochrome-c oxidase (CytOx), which is the terminal member of the electron transport chain and plays a key role in the regulation of oxidative phosphorylation (see Refs. 20 and 40 for reviews). The use of a cytochrome or key enzyme within the Krebs cycle as an indicator of mitochondrial content is a standard practice because it is known that exercise-related changes in the electron transport chain and the Krebs cycle are positively correlated (see Ref. 17 for review).

Most mitochondrial proteins are composed of polypeptide subunits encoded by nuclear DNA and mitochondrian...
drial DNA. For example, 10 of the 13 subunits that make up mammalian CytOx are encoded in the nucleus (7, 18). Mitochondrial polypeptides encoded in the nucleus are imported into the mitochondria by translo-
cases in the outer membrane and inner membrane (TOM and TIM, respectively) (see Refs. 12, 18, 25, 29 for reviews). Import into the matrix requires the ATP-
dependent action of the 70-kDa mitochondrial heat shock protein (mtHSP70), which is bound to TIM on the matrix side. The amount of mtHSP70 within the organelle is typically considerably in excess of that required by TIM, and the excess remains free within the matrix to carry out chaperone duties (30). Ornatski et al. (26) and Mattson et al. (23) have reported that mtHSP70 is increased along with CytOx after chronic electrical stimulation and endurance exercise, respectively. In contrast, Samelson et al. (32) did not find a change in mtHSP70 content after endurance training, even though CytOx increased 84%. Interestingly, Samelson et al. also did not observe a change in cytosolic HSP70, which is known to be regulated by temperature (16, 35) and is typically found to increase after exercise training (16, 21). If mtHSP70 is also temperature sensitive, a critical exercise temperature may be necessary to achieve optimal mitochondrial biogenesis. Thus we also sought to determine whether body temperature during exercise affected mtHSP70 content.

METHODS

Animals and their training. Male, 10-wk-old, Sprague-Dawley rats were obtained from the breeding colony main-
tained by the University of Texas Animal Resource Center. Animals were kept on a 12:12-h light-dark cycle and fed ad libitum. Rats were randomly divided into one of the following four treatment groups: sedentary control (n = 7), exercised in a 23°C room (n = 10), exercised with wetted fur in an 8°C room (n = 8), and exercised with wetted fur and a fan in a 4°C room (n = 7). These conditions were designed to maintain exercising Tc at 40.4 or 39.2°C, or a normal resting temper-
ate of 38.0°C, respectively. All exercising groups were initially habituated to a motor-driven treadmill by running for 1 wk at 20 m/min at a 6% grade for 10 min/day. After habitation, the duration of exercise was gradually increased to 60 min/day for 5 days/wk by the end of 3 wk. The training protocol was then maintained at this duration and frequency for an additional 6 wk. We have previously determined that this exercise intensity is ~70% of maximum oxygen con-
sumption (VO2 max) in the untrained state (4). All animals were housed at 23°C when not exercising. Animals that ran in the cold were returned to a 23°C room immediately after exercise and dried with a towel. Preliminary studies were carried out to establish environmental conditions that would result in a Tc that remained equal to resting temperature during exercise or was elevated to a temperature midway between resting and normal room-temperature exercise. Throughout the exercise programs, Tc was monitored period-
ically with a digital thermocouple thermometer to assure consistency. Tc was measured by inserting the probe 5 cm into the rectum during brief rest periods at 15-min intervals. Trained animals were killed 24 h after the last exercise bout. This investigation was approved by the University’s Animal Care and Use Committee and conforms with the “Guide for the Care and Use of Laboratory Animals” published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1985).

Tissue preparation and assay methods. Animals were euthanized by opening the chest cavity under anesthesia with rodent anesthesia cocktail (obtained from the University of Texas Animal Resources Center) at a dosage of 0.7 ml/kg administered intraperitoneally. The composition of the cock-
tail was 100 mg/ml ketamine, 20 mg/ml xylazine, and 10 mg/ml Acepromazine. Entire gastrocnemius and extensor digitorum longus (EDL) muscles were then excised, wrapped in aluminum foil, and stored at −100°C for later analysis. CytOx, the final protein complex in the mitochondrial electron transport chain, was used as an indicator of the number of electron transport chains present in the muscle. The tissue was thawed, weighed, and homogenized in a Potter Elvehjem homogenizer in 20 vol of 100 mM KPO4, pH 7.4, and the homogenate was poured through a single layer of cheese-
cloth. An aliquot was treated with Triton X-100 (0.1% vol/vol, final concentration) before measuring CytOx activity polaro-
graphically at 25°C with a Clark-type oxygen electrode, as described by Runyan et al. (31). Ten ml of X-100-treated homog-
enate (30 μl) was added to 1.47 ml of assay medium contain-
ing (in mM) 50 K2HPO4, 0.1 EDTA, 0.62 tetramethylpenta-
decane, 12.5 sodium ascorbate, and 0.04 cytochrome c, pH 7.4. Another aliquot was treated with 1.7% Triton X-100 (final concentration) centrifuged at 600 g for 10 min, and the supernatant was used to determine CytOx content spectro-
photometrically, as described by Balaban et al. (2). The reduced (2 mM cyanide)-oxidized spectrum at 605–630 nm with an extinction coefficient of 10.8 mM/cm was used to calculate the concentration. All assays were performed in triplicate, and the mean value was used.

For the measurement of mtHSP70 [also known as glucose-regulated protein 75 (GRP-75)], the other gastrocnemius and EDL muscles were homogenized (1:20 wt/vol) in 50 mM K2HPO4 and 1 mM EDTA, pH 7.4. Samples were diluted 1:1 with Laemmi sample buffer containing 125 mM Tris (pH 6.8), 20% vol/vol glycerol, 2% wt/vol SDS, 0.0068% wt/vol bromophenol blue, and 200 mM DTT. Protein concentration of each sample was determined by the method of Lowry et al. (22), and 80 μg of protein were subjected to SDS-PAGE on a 10% resolving gel by using the Mini-Protean II system (Bio-
Rad, Richmond, CA). The proteins were then transferred to a polyvinylidene difluoride sheet (Bio-Rad) with a Bio-Rad semi-dry transfer unit, as described previously (36). Polyvinylidene difluoride membranes were blotted with GRP-75 mouse polyclonal (no. SPA-825, StressGen Biotechnologies), then with anti-mouse Ig horseradish peroxidase-linked whole antibody from sheep (NHA-931, Amersham Life Sci-
cence), and detected with Super Signal chemiluminescent substrate luminol/enhancer (Pierce, Rockford, IL). The re-
sulting labeled bands were quantified by using a Macintosh Ilvi computer (Apple Computer, Cupertino, CA). The scans were created by using an image scanner (600 dpi transpar-
cy module, Mirror Technologies) connected to the com-
puter. The scans were subsequently digitized and imported into an image analysis software program (Scion Image Beta 2, Scion, Frederick, MD), and the density of each individual band sample was calculated.

Statistical analysis. The reported values represent means ± SE. A one-way ANOVA was used to determine whether there was a significant difference among groups after 9 wk of training at three temperatures. Duncan’s mul-
tiple range post hoc test was used to determine differences between means. An independent t-test was used to compare CytOx activity and mtHSP70 content in the EDL. A P value of <0.05 was used as a limit for statistical significance.
Table 1. Final body and gastrocnemius weights

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Body Weight, g</th>
<th>Gastrocnemius Weight, g</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sedentary</td>
<td>7</td>
<td>396 ± 11</td>
<td>2.18 ± 0.09</td>
</tr>
<tr>
<td>Exercise, Tc = 38°C</td>
<td>7</td>
<td>394 ± 3</td>
<td>2.13 ± 0.06</td>
</tr>
<tr>
<td>Exercise, Tc = 39.2°C</td>
<td>8</td>
<td>379 ± 6</td>
<td>1.99 ± 0.06</td>
</tr>
<tr>
<td>Exercise, Tc = 40.4°C</td>
<td>10</td>
<td>379 ± 7</td>
<td>2.05 ± 0.03</td>
</tr>
</tbody>
</table>

Values are means ± SE. Tc, core temperature.

RESULTS

Animal characteristics. Animal body weight and muscle weight are summarized in Table 1. ANOVA indicated that there were no differences among groups for body weight or gastrocnemius mass.

CytOx activity in the gastrocnemius muscles of animals exercised for 9 wk at various Tc is displayed in Fig. 1. All three exercising groups had activities that were higher than the sedentary group mean of 48.2 ± 2.8 μatoms O·min⁻¹·g wet wt⁻¹ (P < 0.05), and the magnitude of the increases was related to exercise Tc. Animals exercising in a typical room temperature of 23°C had an exercising Tc of 40.4°C and increased CytOx activity 22.4% above sedentary. When conditions were altered to decrease exercise Tc to 39.2°C, activity was increased 37.4% above sedentary. When conditions were further altered to keep Tc clamped at resting temperature during exercise (38°C), CytOx activity was elevated 73.2%. Duncan’s multiple range post hoc test revealed that the activity in the 38°C Tc exercise group was significantly higher than all other groups. CytOx content followed the same trend as activity indicating that the above differences were due to differences in protein expression, not to a change in specific activity of the enzyme. Values for CytOx content (nmoles/g wet wt) in sedentary, Tc = 40.4°C, and Tc = 38.0°C were 1.95 ± 0.11, 2.36 ± 0.14, and 3.24 ± 0.09, respectively (P < 0.05 for any two groups).

A similar temperature-related effect was observed for mitochondrial HSP70 (Fig. 1B). The content of this mitochondrial stress protein was significantly greater than sedentary after chronic exercise at 38°C (P < 0.05).

The finding that rats run in the coldest environment had the highest gastrocnemius CytOx activity and mtHSP70 content prompted us to determine whether the cold environment caused changes unrelated to physical activity. For this purpose, we trained another group at 4°C and evaluated the EDL. Both the gastrocnemius and EDL are composed of 95% fast-twitch fibers (IIA + IIB) and 5% slow-twitch fibers (1), but the EDL is a non-weight-bearing, ankle plantiflexor that does not display an increase in CytOx when rats are exercise trained by running up an incline (8, 38). Although exercise Tc was maintained at 38°C, enzyme activity and mtHSP70 content in the EDL was unchanged compared with sedentary (Fig. 2), indicating that the cold exercise condition does not produce increases in muscle mitochondria content without increases in mechanical activity. Gastrocnemius CytOx activity increased 55% in this companion group (P < 0.05).

Fig. 1. Interaction of exercise and temperature on cytochrome-c oxidase (A) and mitochondrial heat shock protein 70 (mtHSP70; B) in gastrocnemius muscle. mtHSP70 was not measured in the intermediate temperature group (core temperature (Tc) = 39.2°C) because measuring it in the warmest and coldest group is sufficient to determine whether it is sensitive to temperature. Values are means ± SE. *Significantly different (P < 0.05) from all other groups. †Significantly different (P < 0.05) from sedentary group.

Fig. 2. Effect of chronic exercise at 38°C Tc on cytochrome c oxidase (A) and mtHSP70 (B) in extensor digitorum longus muscle. Values are means ± SE.
DISCUSSION

It is well known that the magnitude of the increase in skeletal muscle mitochondria content associated with exercise training is dependent on intensity and duration of training (10, 17). In the present study, we report that body temperature is another factor affecting mitochondrial biogenesis. Specifically, animals exercising in an environment that prevented an elevation of Tc had greater increases in CytOx and mtHSP70 contents compared with animals trained identically, except at a higher body temperature (Fig. 1).

Our data appear to conflict with the only other study to date exploring the role of thermal factors on mitochondrial adaptation during exercise training. Young et al. (42) trained young men for 8 wk at 60% \( \dot{V}_O_2 \max \) on cycle ergometers while submerged to the neck in water maintained at 20 or 35°C. The resulting difference in body and muscle temperatures during exercise did not appear to affect mitochondrial adaptation since citrate synthase activity in the vastus lateralis muscle increased by 38% over pretraining values at both temperatures. Although the average difference in rectal temperature at the end of the 60-min session was about 1.3°C, the rise in temperature was reported to be very gradual. As reported in a companion paper (41), the difference at 20 min of exercise was ~0.3°C and at 40 min ~0.7°C. In our study, decreasing temperature 1.2°C below normal exercise temperature (40.4°C compared with 39.2°C) for at least 45 min did not result in differences at the 95% level of statistical confidence. However, adjusting environmental conditions to keep exercise Tc from increasing above resting temperature (38°C) resulted in a dramatic increase in CytOx content. Although Young et al. used the Krebs cycle enzyme citrate synthase as a marker of mitochondrial content and we used CytOx, a member of the electron transport chain, it seems doubtful that the selection of mitochondrial markers is responsible for the differing results because several studies have reported that the two proteins increase the same relative amount in response to endurance training (9, 17, 31, 38). Therefore, it seems possible that the temperature difference in the study by Young et al. was not great enough over a long enough period to produce differences in mitochondria levels between the two exercise protocols.

Subjecting animals to cold environments that decrease body temperature results in elevated mitochondrial content unrelated to mechanical activity (15) via elevations of systemic factors, such as thyroid hormone (13, 24). However, in the present study, body temperature was not lowered; instead, the normal exercise-induced increase in body temperature was prevented or attenuated. Direct evidence that the environmental conditions used herein did not stimulate mitochondrial biogenesis in the absence of mechanical activity is the lack of an increase in CytOx in the EDL of exercising animals at all temperatures tested (Fig. 2). Another possibility to consider to explain the observed differences in CytOx is that the application of water to wet the fur of the cold-room runners could increase muscle overload by increasing body weight and/or causing slipping during the run. However, this possibility can be ruled out because CytOx in the group running with wetted fur in the 8°C room was significantly less than the group running with wetted fur in the 4°C room.

mtHSP70 (also known as GRP-75) plays a role in mitochondrial biogenesis by helping inward transport and subsequent folding of proteins synthesized in the cytosol (12, 18). Our finding that mtHSP70 was elevated along with CytOx in the cold runners indicates that, despite its name, mtHSP70 is not dependent on an increase in muscle temperature. Although some investigators have found mtHSP70 to be correlated to mitochondrial content (23), others have not (32). A possible explanation for the lack of correlation was proposed by Samelman et al. (32). They rationalized that mtHSP70 serves two functions: a small fraction is involved with transporting nuclear encoded subunits into the mitochondria, whereas most serve as an unfoldase within the matrix space (30). Because only a relatively small amount is directly involved in mitochondrial biogenesis at any one time and the large matrix pool can be used to support import functions, a direct correlation between mitochondrial content and mtHSP70 expression is not required to assure adequate biogenesis.

Likely reasons for the temperature-related differences in skeletal muscle mitochondrial content among the matched exercise programs are differences in muscle compliance, oxygen diffusion, and/or enzyme-specific activity. There is considerable evidence suggesting that one or more of these three factors is responsible for temperature-related differences in exercise performance. Galloway and Maughan (14) reported that exercising oxygen consumption progressively increased in humans as ambient temperature decreased from 21 to 11 to 4°C, which could be due to less compliant, more viscous muscle tissue at the lower temperatures. \( \dot{V}_O_2 \max \) is reported to be reduced ~10–30% for a decrease in Tc of 0.5–2.0°C, and the accumulation of lactic acid in the blood occurs at lower workloads at lower temperatures (see Ref. 28 for review). These observations could be at least partially due to a temperature-related decline in \( O_2 \) diffusion coefficient (3), mitochondrial state 3 respiration (5), as well as all other metabolic reactions according to the Q10 effect. Fish swimming in cold water are also known to have a greater energy cost at the same swimming speed and reduced oxygen diffusivity compared with warmer water (see Ref. 34 for review). To compensate, mitochondrial content increases in fish as they acclimate to the colder water (see Ref. 34 for review). We propose that a similar compensation for temperature-related physical constraints occurred within active muscles of the coldest exercising group in the present study. Greater mitochondrial density in the colder muscles would increase the concentration of enzymes associated with aerobic metabolism, thus compensating for the lower catalytic activity of the individual enzymes. In addition, a greater number of mitochondria would enhance
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oxygen and substrate delivery from capillaries to mitochondria by decreasing mean diffusion distances.

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