Exercise training decreases rat heart mitochondria free radical generation but does not prevent Ca$^{2+}$-induced dysfunction

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Submitted 1 August 2006; accepted in final form 7 February 2007

Starnes JW, Barnes BD, Olsen ME. Exercise training decreases rat heart mitochondria free radical generation but does not prevent Ca$^{2+}$-induced dysfunction. J Appl Physiol 102: 1793–1798, 2007. First published February 15, 2007; doi:10.1152/japplphysiol.00849.2006.—Exercise provides cardioprotection against ischemia-reperfusion injury, a process involving mitochondrial reactive oxygen species (ROS) generation and calcium overload. This study tested the hypotheses that isolated mitochondria from hearts of endurance-trained rats have decreased ROS production and improved tolerance against Ca$^{2+}$-induced dysfunction. Male Fischer 344 rats were either sedentary (Sed, n = 8) or endurance exercise trained (ET, n = 11) by running on a treadmill for 16 wk (5 days/wk, 60 min/day, 25 m/min, 6° grade). Mitochondrial oxidative phosphorylation measures were determined with glutamate-malate or succinate as substrates, and H$_2$O$_2$ production and permeability transition pore (PTP) opening were determined with succinate. All assays were carried out in the absence and presence of calcium. In response to 25 and 50 μM CaCl$_2$, Sed and ET displayed similar decreases in state 3 respiration, respiratory control ratio, and ADP:O ratio. Ca$^{2+}$-induced PTP opening was also similar. However, H$_2$O$_2$ production by ET was lower than Sed (P < 0.05) in the absence of calcium (323 ± 12 vs. 362 ± 11 pmol-min$^{-1}$·mg protein$^{-1}$) and the presence of 50 μM CaCl$_2$ (154 ± 3 vs. 197 ± 7 pmol-min$^{-1}$·mg protein$^{-1}$). Rotenone, which blocks electron flow from succinate to complex I, reduced H$_2$O$_2$ production and eliminated differences between ET and Sed. Mitochondrial superoxide dismutase and glutathione peroxidase were not affected by exercise. Catalase activity was extremely low but increased 49% in ET (P < 0.05). In conclusion, exercise reduces ROS production in myocardial mitochondria through adaptations specific to complex I but does not improve mitochondrial tolerance to calcium overload.

reactive oxygen species (ROS) generated by mitochondria are believed to play key roles in myocardial ischemia-reperfusion (I/R) injury (11, 27 for reviews) and myocardial dysfunction accompanying normal aging (17, 33 for reviews). During I/R, mitochondrial ROS generation can lead to general oxidative stress and calcium overload (8, 27 for reviews). The mitochondria then take up the calcium, which results in decreased ATP production and may cause the mitochondrial permeability transition pore (PTP) to open, further decreasing ATP production and releasing cytochrome c (8, 27). Age-related increases in mitochondrial oxidant production are generally accepted as a cause of myocardial cell loss via apoptosis and necrosis (28, 31). It is now well accepted that exercise participation provides intrinsic protection to the heart against I/R injury (2, 6, 7, 9, 10, 15, 25, 34, 37, 42) and delays age-related dysfunction and myocyte loss (31, 46). Although it has been reported that exercise-trained hearts regain ATP levels better than their sedentary counterparts following I/R (7), the specific adaptations responsible have not been adequately investigated.

There are at least two adaptive strategies within mitochondria that could contribute to cardioprotection; they could decrease ROS production or increase their ability to tolerate high calcium levels. Judge et al. (28) recently reported that lifelong voluntary wheel activity decreased H$_2$O$_2$ production by ∼10% (P < 0.05) in myocardial mitochondria of 24-mo-old Fischer 344 rats compared with their sedentary peers. They found similar reductions in both interfibrillar and subsarcolemmal mitochondrial populations. However, since younger animals were not included, it is uncertain whether the exercise program decreased H$_2$O$_2$ production independent of aging or whether it acted primarily to attenuate a possible age-related increase (4). Contributing to the uncertainty, Marcil et al. (36) reported that a 10-wk exercise program in young female Sprague-Dawley rats does not alter H$_2$O$_2$ production by myocardial mitochondria. In addition, the ability of myocardial mitochondria from endurance-trained animals to carry out oxidative phosphorylation in the presence of high calcium levels has not been evaluated. Thus the purpose of this study was to test the hypotheses that isolated mitochondria from hearts of endurance-trained rats have decreased ROS production and increased tolerance to calcium overload.

METHODS

Animals. Male 4-mo-old Fischer 344 rats were obtained from Harlan Sprague-Dawley (Indianapolis, IN). On arrival they were housed in a 22°C room with a 12:12-h light-dark cycle and fed ad libitum with Harlan Teklad 7013, NIH-31 rat chow. Rats were randomly assigned to either a sedentary control (Sed) (n = 8) or exercised trained (ET) (n = 11) group. ET rats were exercised on a motor-driven treadmill 5 days/wk for a total of 16 wk. After 1 wk of acclimation, the running speed and duration were gradually increased over the next 5 wk until the animals were running 60 min/day at a speed of 25 m/min up a 6° grade. They were maintained on this exercise protocol for 10 wk. This investigation was approved by the University’s Animal Care and Use Committee and conforms to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Publication No. 85–23, Revised 1996).

Mitochondrial isolation. Mitochondria were isolated 24 h following the last exercise bout. Animals were anesthetized with an intraperitoneal injection of 40 mg/kg body wt of pentobarbital sodium. Hearts were rapidly excised and briefly perfused through the aorta with oxygenated Krebs-Henseleit buffer to remove blood. Mitochondria were isolated from ventricular tissue (500–600 mg) by differential centrifugation as previously described (19). Briefly, the tissue was
homogenized with a Potter-Elvehjem homogenizer in 10 ml MSE buffer [225 mM mannitol, 75 mM sucrose, 1 mM EGTA, 10 mM 3-(N-morpholino)propanesulfonic acid (MOPS), pH 7.4] containing 3 mg Nagarse bacterial proteinase (P8038, Sigma-Aldrich, St. Louis, MO) to optimize release of all mitochondrial populations. Approximately 30 ml of MSEA (MSEA) was added to dilute the proteinase before centrifugation at 7,740 g for 7 min. The supernatant was discarded, and the pellet was rehomogenized in MSEA. After low-speed centrifugation at 600 g for 5 min, the supernatant was filtered through cheesecloth and centrifuged at 7,740 g for 10 min to obtain a mitochondria-rich pellet. The pellet was washed twice by resuspending in 225 mM mannitol, 75 mM sucrose, 0.1% BSA, 10 mM MOPS, pH 7.4 (MSA), and centrifuging at 7,740 g for 7 min. The final mitochondrial pellet was suspended in ~0.4 ml of MSA buffer and contained 31.3 ± 1.6 mg mitochondrial protein/ml as determined by the Lowry method (35a). EGTA was omitted during the wash steps and final mitochondrial suspension to prevent calcium buffering from this chelator during subsequent evaluations. Preliminary experiments indicated that removing EGTA during the final stages of the isolation procedure did not affect mitochondrial oxidative phosphorylation parameters in the absence of added calcium.

Mitochondrial swelling. Mitochondrial swelling, which is the result of loss of mitochondrial membrane potential and opening of OPT (1, 3, 13, 18), was recorded as a decrease in light absorbance (Abs) at 520 nm. As described by Baines et al. (3), mitochondria were suspended at 0.25 mg protein/ml in 120 mM KCl, 10 mM Tris, 20 mM MOPS, 5 mM KH2PO4, pH 7.4, and Abs was measured at 20-s intervals for 10 min in the absence and presence of 50 μM CaCl2 (200 nmol Ca2+/mg mitochondrial protein) (Fig. 1). When the OPT opening was prevented by adding 40 mM cyclosporin A to the mitochondria 5 min before adding CaCl2, Abs did not decline, confirming that Abs change was due to OPT opening. Preliminary titration experiments indicated that 25 μM CaCl2 (100 nmol Ca2+/mg mitochondrial protein) did not result in decreased Abs.

H2O2 production. Oxidant production from intact mitochondria was estimated by measuring the production of H2O2 formed by the dismutation of the superoxide anion (12, 24), which is imperative to the inner mitochondrial membrane (24). H2O2 freely diffuses out of the mitochondria and was detected by increasing fluorescence of amplex red in the presence of horseradish peroxidase (12) using a Varian Cary Eclipse fluorescence spectrophotometer with 570-nm excitation and 585-nm emission wavelengths. Mitochondria were suspended at 0.25 mg protein/ml in the swelling buffer supplemented with 5 mM succinate, 0.1 mM amplex red, and 0.1 U/ml horseradish peroxidase. After an initial rate of H2O2 production was determined, the buffer was further supplemented with 50 μM CaCl2 or 2.5 μM rotenone, or both. Rotenone reduces H2O2 production with succinate as substrate by preventing electron transport from complex II to complex I (24, 35). The rate of H2O2 production was linear under all conditions. Standard concentration curves were obtained by adding known amounts of H2O2 to the reaction medium.

FAD-linked succinate at a supraphysiological concentration was used in this study, as in many others, because it results in readily detectable ROS production without the need for electron transport chain inhibitors (24, 35, 45). An NAD-linked substrate was not used because they have generally been found to produce almost undetectable rates of H2O2 production in isolated rat heart mitochondria in the absence of inhibitors (23, 24, 47). Heart mitochondria produce superoxide at complex I and complex III (35, 48). Although succinate provides reducing equivalents to the electron transport chain at complex II, it reduces the entire chain because electrons readily flow up the chain to complex I (24, 35, 39, 45). Miwa and Brand (39) have stated that this reverse electron flow seems to be the most important pathway for physiologically relevant ROS production. Using high succinate concentrations ensures high membrane potential and a high state of NAD reduction (low NAD-to-NADH ratio) (24), which are key stimulators of ROS generation (23, 41) and are the conditions that exist at the end of ischemia, causing the burst of ROS production during the early phase of reperfusion. Succinate accumulates in the heart during ischemia (51), and its excessive oxidation on reoxygenation is considered a major source of ROS and tissue damage following ischemia (30, 41, 49). Furthermore, a considerable amount of FADH2 enters the electron transport chain at complex II on reperfusion because of a substrate shift toward fatty acid oxidation (see Ref. 29 for review).

Oxidative phosphorylation. Oxygen utilization was measured polarographically with a Clark-type oxygen electrode in a stirred reaction chamber maintained at 25°C as previously described (19). Mitochondria (~0.9 mg protein) were incubated in 1.45 ml of air-saturated medium containing 250 mM sucrose, 10 mM KH2PO4, 10 mM Tris, pH 7.5, and one of the following substrates: 10.6 mM glutamate plus 1.1 mM malate or 10.6 mM succinate plus 1.3 mM rotenone. All assays were carried out in duplicate in the presence of 0, 25, and 50 μM CaCl2. These concentrations correspond to 0, 40.3, and 80.6 nmol Ca2+/mg mitochondrial protein, respectively, which are below the threshold for OPT opening. After a 3-min incubation period to allow Ca2+ uptake, maximal respiration rate (state 3) was initiated with 500 nmol ADP. State 3 respiration, state 4 (basal) respiration, respiratory control ratio, and ADP/O ratio were determined.

Antioxidant enzymes and cytochrome oxidase. Catalase activity was determined on freshly isolated mitochondria according to the polarographic method described by Del Rio et al. (14). Glutathione peroxidase activity was determined using spectrophotometric analysis at 340 nm according to Gunzler and Flohe (22) and total superoxide dismutase in the mitochondria was determined by spectrophotometric analysis at 550 nm according to McCord and Fridovich (38). Skeletal muscle cytochrome oxidase activity in whole plantaris muscle was determined as described previously by Rumsey et al. (44) and served as a marker of exercise training status. All assays were performed in duplicate at 25°C, and the mean value was used.

Statistical analysis. Descriptive data (means ± SE) were calculated for each dependent variable. Overall group differences were analyzed...
using a one-way ANOVA. When appropriate, post hoc analyses were made using a Tukey’s honestly significant difference test. In all tests, a probability level of $P < 0.05$ was used as the decision rule for significance testing.

**RESULTS**

**Animal characteristics.** Animal body weights, heart weights, heart-to-body weight ratios, and plantaris cytochrome oxidase measurements are presented in Table 1. The decreased body weight accounted for the higher ($P < 0.05$) heart weight-to-body weight ratio of ET compared with Sed. Cytochrome oxidase in the plantaris muscle increased 100% after exercise, indicating that the exercise program was very effective in producing training adaptations in active skeletal muscle.

**Mitochondrial yield and antioxidant enzymes.** The yields of mitochondria isolated from the hearts of the sedentary and exercised animals were similar: 14.7 ± 1.2 and 15.0 ± 1.0 mg protein/g wet weight in Sed and ET, respectively. Antioxidant enzyme activities are reported in Table 2. Chronic endurance training increased mitochondrial catalase activity by 49% compared with Sed ($P < 0.05$). However, the activity of catalase was very low compared with the other antioxidant enzymes. The exercise program did not alter the activities of glutathione peroxidase or superoxide dismutase in the mitochondria.

**Oxidative phosphorylation.** States 3 and 4 respiratory rates and ADP:O ratios are reported in Fig. 2. State 3 rates and ADP:O ratios progressively declined in both sedentary and exercise-trained animals with increasing calcium concentration. Regardless of calcium concentration, all parameters were similar in sedentary and exercised groups when either glutamate-malate or succinate was supplied as substrate. The overall magnitude of the decline in state 3 rate from 0 to 50 μM CaCl$_2$ for glutamate-malate was 48% and for succinate was 38%. State 4 respiration for both substrates was not significantly altered at Ca$^{2+}$ additions up to 50 μM ($P > 0.05$).

**Mitochondrial swelling.** Changes in Abs of mitochondria following the addition of Ca$^{2+}$ are reported in Table 3. There were no differences between Sed and ET for total change in Abs occurring from initial baseline to 10 min after CaCl$_2$ addition, or maximum rate of Abs reduction, or the start of swelling following the addition of CaCl$_2$ ($P > 0.05$ for all determinations). These results indicate that PTP opening was similar between the two groups in response to the same Ca$^{2+}$ challenge.

**H$_2$O$_2$ production.** The values for H$_2$O$_2$ production in isolated cardiac mitochondria are reported in Fig. 3. The highest production occurred in the presence of succinate without calcium or rotenone, which is the condition that provides a high membrane potential and NADH reduction and allows superoxide to be produced at all superoxide-generating sites along the electron transport chain. Under this condition, H$_2$O$_2$ production was 11% ($P < 0.05$) less in ET compared with Sed (Fig. 3A, left). H$_2$O$_2$ production decreased in both groups on adding enough calcium to induce PTP opening, which decreases membrane potential and state of NADH reduction, but the amount produced by ET was still 22% ($P < 0.05$) less than Sed (Fig. 3A, right). A calcium-induced decrease in H$_2$O$_2$ production was 49% ($P < 0.05$) heart weight-to-body weight ratio of ET compared with Sed. Cytochrome oxidase in the plantaris muscle increased 100% after exercise, indicating that the exercise program was very effective in producing training adaptations in active skeletal muscle.

**Antioxidant enzymes**

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<thead>
<tr>
<th>Enzyme</th>
<th>Sed</th>
<th>ET</th>
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<tbody>
<tr>
<td>GPx</td>
<td>114±3</td>
<td>103±6</td>
</tr>
<tr>
<td>Catalase</td>
<td>0.59±0.03</td>
<td>0.88±0.11*</td>
</tr>
<tr>
<td>SOD</td>
<td>1,153±84</td>
<td>1,003±84</td>
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</table>

Values are mean units/mg protein ± SE. Sed, n = 8; ET, n = 11. Catalase unit = 1 μmol H$_2$O$_2$/min; superoxide dismutase (SOD) unit = 50% inhibition of cytochrome c reduction; glutathione peroxidase (GPx) unit = 1 μmol GSH/min. *Significantly different from Sed ($P < 0.05$).

![Fig. 2. Effects of exercise training and calcium overload on cardiac mitochondria oxidative phosphorylation. Substrates provided were succinate or glutamate plus malate (GM). Sed, sedentary ($n = 8$); ET, exercise trained ($n = 11$).](http://jap.physiology.org)
production under this set of conditions is in agreement with previous studies (45). Addition of rotenone, which blocks electron flow to the complex I superoxide-generating site, eliminated the differences between ET and Sed both in the absence and presence of calcium (Fig. 3B).

**DISCUSSION**

In the present study we sought to determine the interaction of endurance exercise training and calcium overload on selected functions of myocardial mitochondria. Calcium overload decreases mitochondria ATP production and causes the PTP to open (8, 27). We found that endurance exercise does not attenuate detrimental changes in these functions when challenged with a calcium overload. However, we also found that endurance exercise does reduce myocardia H2O2 production at complex I of the electron transport chain. The implications of these findings will be discussed below.

The finding in the present study that exercise reduced H2O2 production by myocardial mitochondria of Fischer 344 rats extends the previous findings by Venditti et al. (50) and Judge et al. (28). Venditti et al. (50) determined that chronic endurance training reduces H2O2 production from skeletal muscle mitochondria isolated from gastrocnemius muscles of Wistar rats; they did not evaluate myocardial mitochondria. Judge et al. (28) were actually the first to show that exercise could decrease H2O2 production in myocardial mitochondria of male Fischer 344 rats. They found similar reductions in both interfibrillar and subsarcolemmal mitochondrial populations. However, they evaluated H2O2 production only at old age (24 mo) after lifelong voluntary wheel activity. Since younger animals were not included, it is uncertain whether the exercise program decreased H2O2 production independent of aging or whether it acted primarily to attenuate a likely age-related increase (4). Our study, using the same rat strain and gender as Judge et al. (28), confirms that exercise can decrease myocardial mitochondria H2O2 production independent of old age. The magnitude of the decrease appears to be relatively modest (range of 10–22% in the two studies), suggesting that other adaptations may also contribute to the total exercise-induced cardioprotective phenotype.

In apparent conflict with the above studies, Marcil et al. (36) reported that a 10-wk exercise program similar to that used herein does not alter H2O2 production by myocardial mitochondria of female Sprague-Dawley rats. These investigators isolated mitochondria with the aid of Nagarse digestion as in the present study and evaluated H2O2 production using both succinate and glutamate-malate as substrates. It is possible that differences in sex or rat strain could have been responsible for the different results. ROS production was measured only in the absence of calcium, and the authors acknowledge the possibility that ROS production may be lower in trained mitochondria in the presence of calcium. Another possibility is that potential differences were masked because Marcil et al. (36) did not attempt to separate mitochondria into subsarcolemmal and interfibrillar populations; however, we feel this is unlikely because we also used a mixed mitochondrial preparation, and Judge et al. (28) reported comparable declines for both populations.

Theoretically, a decrease in ROS generation could be due to increased antioxidant enzyme activity or to less superoxide production. Previous studies by Venditti et al. (50) and by Judge et al. (28) speculated that the decrease in ROS is due to less superoxide production because antioxidant enzymes did not increase. The antioxidant enzyme results of the present study are in general agreement with these previous studies. Although exercise training resulted in a 49% increase in catalase activity (Table 2), it is still very low compared with superoxide dismutase and glutathione peroxidase (43, Table 2) and therefore would not be expected to play a significant role.

### Table 3. Mitochondrial swelling results

<table>
<thead>
<tr>
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<th>Sed</th>
<th>ET</th>
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<tbody>
<tr>
<td>Percent change in Abs without Ca**+** (baseline)</td>
<td>-1.4±0.5</td>
<td>-1.4±0.4</td>
</tr>
<tr>
<td>Percent change in Abs with Ca**+**</td>
<td>-14.7±1.3</td>
<td>-15.7±0.9</td>
</tr>
<tr>
<td>Maximum rate of Abs reduction, Abs/min</td>
<td>-0.0282±0.0010</td>
<td>-0.0285±0.0007</td>
</tr>
<tr>
<td>Start of swelling after adding Ca**+**, s</td>
<td>111.4±14.3</td>
<td>114.5±11.4</td>
</tr>
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</table>

Values are means ± SE. Sed, n = 8; ET, n = 11; Abs, absorbance. Percent change in Abs represents change in Abs after 10 min in the absence (baseline) or presence of 50 μM CaCl₂. See Fig. 1 for shape of curves.
in ROS detoxification unless glutathione peroxidase were to become ineffective by decreased GSH or NADPH. Perhaps the strongest evidence against a role for antioxidant enzymes in the exercise-induced decrease in ROS production is that the effect was abolished when rotenone prevented electrons from flowing to complex I (Fig. 3). If catalase, or any other antioxidant enzyme, had a significant role in the exercise-induced decrease of \( \text{H}_2\text{O}_2 \) production in intact mitochondria, the decrease should have been apparent regardless of the presence or absence of rotenone.

Furthermore, the finding that \( \text{H}_2\text{O}_2 \) production by Sed and ET was similar when complex I was blocked indicates that the ROS-generator site responsible for the exercise-induced decrease must be located there. The mechanism of superoxide production by complex I has not been established (21, 32). This is partially due to the fact that there is limited knowledge about the exact structure of this very large enzyme, consisting of over 40 subunits. Consistent with our conclusion regarding adaptations at complex I, decreased superoxide production at complex I in heart mitochondria has also been reported following long-term caloric restriction (20) and in mitochondria from long-lived species compared with short-lived species (26).

To our knowledge the present study is the first to investigate the ability of mitochondria from endurance-trained animals to carry out oxidative phosphorylation during direct calcium-induced stress. Calcium is a double-edged sword to mitochondrial respiratory function. Moderate calcium uptake stimulates pyruvate dehydrogenase and Krebs cycle enzymes, resulting in increased NADH redox potential and enhanced ATP synthesis (8). Higher amounts of mitochondrial calcium levels result in a progressive decrease oxidative phosphorylation, in part because both calcium maintenance and ATP production are both dependent on the membrane potential (16 for review). This gradual decline continues until the calcium load gets to a level that opens the PTP resulting in a sudden collapse of membrane potential, loss of ATP production, and massive release of Ca\(^{2+}\) (8, 16). Using well-established procedures for isolating mitochondria and measuring oxidative phosphorylation that we previously used to reveal that mitochondrial function is altered by brief hypoxia exposure (19), we found herein that endurance training has no impact on mitochondrial oxidative phosphorylation. In the absence of added calcium, all oxidative phosphorylation parameters were similar in ET and Sed regardless of substrate. Also, when challenged with identical Ca\(^{2+}\) concentrations, mitochondria from ET and Sed displayed similar declines in ATP production as indicated by progressive declines on state 3 respiration rates and ADP:O ratios (Fig. 2). Comparable declines were observed when either complex I- or complex II-linked substrates were being utilized. Resting respiration (state 4) did not significantly change in response to increased Ca\(^{2+}\) because the highest calcium load was below the PTP opening threshold of all mitochondria in our preparation. Palmer et al. (40) reported that respiration in undamaged, intact mitochondria increases while Ca\(^{2+}\) influx occurs (5), contributing to membrane damage and a large influx of Ca\(^{2+}\), ultimately resulting in apoptosis and necrosis (11, 27). The present study suggests that the cardioprotective effects of exercise training may be at least partially due to a reduction from mitochondrial ROS production, resulting in less Ca\(^{2+}\) influx on reperfusion. The reduction in calcium influx would attenuate PTP opening and loss of ATP production. This mechanism is consistent with the previous finding that isolated perfused hearts of endurance-trained rats exhibit less cytosolic Ca\(^{2+}\) uptake and better mitochondrial ATP production following an ischemic bout than hearts of sedentary rats (7). Finally, decreased ROS generation could also explain why chronic exercise programs do not lead to premature aging as would be predicted by the free radical theory of aging (17, 33).

**GRANTS**

This work was supported by National Institutes of Health Grant AG-02220 (J. W. Starnes).

**REFERENCES**


