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Original Contribution

Regulation of mitochondrial uncoupling respiration during exercise in rat heart: Role of reactive oxygen species (ROS) and uncoupling protein 2

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Abstract

The physiological significance of cardiac mitochondrial uncoupling protein 2 (UCP2)-mediated uncoupling respiration in exercise is unknown. In the current study, mitochondrial respiratory function, UCP2 mRNA level, UCP2-mediated respiration (UCR), and reactive oxygen species (ROS) generation, as well as manganese superoxide dismutase (MnSOD) activity were determined in rat heart with or without endurance training after an acute bout of exercise of different duration. In the untrained rats, state 4 respiration and UCR-independent respiration rates were progressively increased with exercise time and were 64 and 70% higher, respectively, than resting rate at 150 min, whereas UCR was elevated by 86% with no significant change in state 3 respiration. UCP2 mRNA level showed a 5- and 4-fold increase, respectively, after 45 and 90 min of exercise, but returned to resting level at 120 and 150 min. Mitochondrial ROS production and membrane potential ($\Delta\psi$) increased progressively until 120 min, followed by a decrease to the resting level at 150 min. MnSOD mRNA abundance showed a 2-fold increase at 120 min but MnSOD activity did not change with exercise. Training significantly increased mitochondrial ATP synthetase activity, ADP to oxygen consumption (P/O) ratio, respiratory control ratio, and MnSOD activity, whereas exercise-induced state 4 respiration, UCR, ROS production, and $\Delta\psi$ were attenuated in the trained rats. We conclude that (1) UCP2 mRNA expression and activity in rat heart can be upregulated during prolonged exercise, which may reduce cross-membrane $\Delta\psi$ and thus ROS production; and (2) endurance training can blunt exercise-induced UCP2 and UCR, and improve mitochondrial efficiency of oxidative phosphorylation due to increased removal of ROS.

Keywords: Exercise; Heart; Mitochondria; Reactive oxygen species; Training; Uncoupling protein 2

Introduction

Mitochondria are the primary energy-generating organelles in all of the aerobic eukaryotic cells and produce ATP through a process coupled with oxygen consumption termed oxidative phosphorylation [1]. Previous studies have widely reported structural and functional alterations of heart mitochondria after an acute bout of fatiguing exercise or several days of repeated exhaustive exercise [2,3]. Parallel to these observations are increased oxidative damage to myocardial lipid, protein, and DNA, as found in skeletal muscle following strenuous exercise [4–6]. These detrimental effects are thought to be elicited by increased oxygen flux and subsequent electron "leakage" from the electron transport chain (ETC) forming superoxide radicals ($O_2^{\bullet -}$) and other reactive oxygen species (ROS) during exercise [7]. Myocardium has been thought to have a lesser ability to adapt to prolonged exercise compared to skeletal muscle; however, recent advances in research have challenged this traditional view by revealing some unique potentials of this vital organ.

Abbreviations: BSA, bovine serum albumin; DCF, dichlorofluorescein; DCFH-DA, dichlorofluorescin acetate; ETC, electron transport chain; 4-HNE, 4-hydroxy-2-nonenal; MnSOD, manganese superoxide dismutase; NO, nitric oxide; ROS, reactive oxygen species; RT-PCR, real-time quantitative polymerase chain reaction; UCP, uncoupling protein 2; UCR, UCP2-mediated respiration.

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Located in the mitochondrial inner membrane, uncoupling proteins (UCP) are a heterogeneous family of proteins that may play an important role in partially dissipating the proton electrochemical gradient across the membrane [8]. The best characterized UCP1 is exclusively expressed in the brown adipose tissue of rodents with a key function of adaptive thermogenesis, whereas UCP3 is predominantly expressed in skeletal muscle. Ubiquitously expressed, UCP2 shares 59 and 73% of amino acid sequence identity with that of UCP1 and UCP3, respectively [9]. The electrochemical actions of UCP2 make it a plausible regulator of transmembrane proton potential and thus efficient in oxidative phosphorylation in the mitochondrial of a variety of organs and tissues including the myocardium [10]. Several studies have reported that alteration of UCP2 level could have important implications for the heart to deal with metabolic and oxidative stress [11,12]. However, so far there is little understanding about the role of UCP2 in exercise-induced oxidative stress and adaptation in the myocardium.

Physical training is known to have profound effects on myocardial anatomical, physiological, and metabolic functions [13]. Heart mitochondrial oxidative capacity, respiratory properties, and antioxidant defense have been reported to increase after endurance exercise, resulting in improved contractile performance and resistance to oxidative stress under a number of experimental conditions such as anoxia/hypoxia, ischemia–reperfusion and acute exercise [14–17]. However, we are not aware of any study regarding the influence of endurance training on UCP2 expression in the heart.

Thus, we have conducted the current study with the following aims: (i) to investigate the effects of an acute bout of exercise on UCP2 gene expression and UCP2-mediated respiration in rat heart mitochondria; (ii) to examine the role of UCP2 upregulation in mitochondrial respiratory function and oxidative phosphorylation; (iii) to evaluate how exercise-induced UCP2 would influence myocardial ROS production and MnSOD gene expression; and (iv) to assess how endurance training influences the interactions of UCP2 expression, mitochondrial function, and oxidant–antioxidant homeostasis during exercise in rat heart.

Methods

Animals and endurance training

One hundred male Sprague-Dawley rats (age 6–8 weeks, body weight 200 g) were used in the study. The animals were housed in double cages in a temperature-controlled room (21–22°C; 50–60% humidity) with a 12 h light/12 h dark cycle and provided free access to food and tap water. All experiments were approved by the Institutional Review Board of the Tianjin Sport University under the guidelines of the Chinese Academy of Sciences.

Five days after arrival and acclimation, the rats were randomly divided into two groups, trained (T, $N=50$) and untrained (U, $N=50$). The T rats were exercised on a motor-driven rodent treadmill for 5 days a week for a total of 6 weeks. The rats initially ran at 10 m/min and 0% grade for 30 min/day during the first week. Treadmill speed and grade as well as running time were increased to, respectively, 12 m/min and 3% for 60 min for the second week, and 15 m/min and 5% for 60 min for the third week. Thereafter, exercise intensity and duration were maintained until the end of the sixth week. This protocol was shown to induce mild heart hypertrophy and improve cardiac performance in rats [13]. The U rats were placed on a slow-moving treadmill for 30 min, five times per week, such that they were exposed to potential handing and environment stresses without producing a training effect.

Acute exercise

At the end of the training period, both T and U rats were subjected to an acute bout of treadmill running prior to being killed. Exercise was performed on a treadmill, beginning at 8.2 m/min at 0% grade corresponding to ~53% of maximal oxygen uptake ($\dot{V}O_{2peak}$) [18] for 15 min. After another 15 min of running at 15 m/min and 5% grade, (~64% $\dot{V}O_{2peak}$), treadmill speed and grade were increased to 19.3 m/min and 10% grade and rats ran continuously on this intensity (~76% $\dot{V}O_{2peak}$) for 0, 45, 90, 120, or 150 min (referred to as E_0, E_{45}, E_{90}, E_{120}, and E_{150}, respectively). Rats were randomly taken off the treadmill at the five time points ($N=10$) and sacrificed immediately.

Preparation of heart mitochondria

The animals were sacrificed by cervical dislocation. After the abdominal cavity was quickly opened, the heart was immediately excised, rinsed, blotted, and weighed. A portion of approximately 20–25 mg of the left ventricular tissue was dissected and finely minced in an ice-cold medium containing 0.25 M sucrose, 3.0 mM Hepes, 0.5 mM EDTA (pH 7.4) with a weight/volume ratio of 1/10. The remaining ventricular tissues were immediately frozen in liquid nitrogen and stored at −80°C for real-time PCR.

Rat heart mitochondria were prepared using differential centrifugation according to Ji et al. [19]. Briefly the minced blood-free tissue was homogenized in the above-noted buffer medium using a motor-driven Ultra-Turrax T8 glass homogenizer with a Teflon pestle (0–4°C) at a speed of 800 RPM with three passes. The homogenate was centrifuged at 800*g* for 10 min to eliminate nuclei and cell debris, and the resulting supernatant was centrifuged at 10,000*g* for 10 min. The supernatant fluid was discarded and the pellets were gently resuspended in the original buffer medium (40 ml). The suspension was centrifuged again at 10,000*g* for 10 min and the final pellets were suspended in 1 ml of isolation medium. The mitochondrial isolation procedures were completed within 1 h after the rat was killed. Mitochondrial protein content was assayed according to Lowry [20], using bovine serum albumin (BSA) as standard.

Mitochondrial respiration

Mitochondrial respiratory function was measured polarographically at 25°C using a Clark-type oxygen electrode (YSI, USA). Reactions were conducted in a 3-ml closed thermostatic and magnetically stirred glass chamber containing 130 mM KCl, 3.0 mM Hepes, 0.5 mM EDTA, 2.0 mM KH_2PO_4, and 1 mg/ml BSA as respiration medium (pH 7.4) and 2 mg of mitochondrial

protein. The medium was saturated with ambient oxygen to reach a concentration of 258 μM. After a 3-min equilibration period, mitochondrial respiration was initiated by adding 1 mM glutamate and 0.1 mM malate. After a stable state 4 respiration was established, state 3 respiration was initiated by the addition of 200 nM ADP. When all of the ADP added had been phosphorylated to ATP, the respiratory rate returned to state 4. The respiratory control ratio (RCR) was calculated as the ratio of the respiratory rate in state 3 to that in state 4. The ratio between phosphorylated ADP and oxygen consumed (P/O ratio) during state 3 respiration was calculated to reflect the efficiency of mitochondrial oxidative phosphorylation, according to Estabrook [21]. Fig. 1 illustrates a typical polarographic oxygen electrode trace obtained in the present study.

UCP-mediated respiration (UCR)

In an independent trial performed with glutamate (1 mM), malate (0.1 mM), and ADP (200 nM), when ADP was depleted and respiration returned to state 4, cardiac mitochondria were exposed to the addition of GDP (1 mM), a classical purine nucleotide inhibitor of UCP2, to assess the contribution of UCP2-mediated respiration [22]. The addition of this inhibitor which binds with high affinity to the nucleotide-binding site of UCP2 at a given concentration has been shown to inhibit UCP2 activity completely [10,23]. The difference between uninhibited sate 4 respiration and GDP-inhibited state 4 respiration was defined as UCR.

Mitochondrial ROS production

Cardiac mitochondrial ROS generation was determined in fresh mitochondrial suspensions using dichlorofluorescein (DCF) as a probe according to LeBel and Bondy [24] as modified by Bejma et al. [25]. This assay measures not only ROS but also nitric oxide (NO) and its derivatives [26]. Under the defined experimental conditions the majority of oxidants measured were ROS due to the presence of MnSOD and limited presence of NO synthase and its substrates. Briefly, the dichlorofluorescin acetate (DCFH-DA) stock solution was dissolved in 1.25 mM methanol and kept in a dark room at 0°C. To initiate the experiment, 1 mg heart mitochondria was added to a quartz cuvette containing 2 ml of 0.1 M phosphate buffer (pH 7.4), and 2 μl of 2.5 mM DCFH-DA (total volume 0.3 ml). The assay mixture was incubated at 37°C for 15 min to allow the DCFH-DA probe to enter the mitochondria. DCF formation was determined fluorometrically at the excitation wavelength of 499 nm and emission wavelength of 521 nm at 37°C for 2 min, using a Cary Eclipse fluorescence spectrophotometer (Varian, USA). A blank consisting of the appropriate buffer and 5.0 μM DCFH-DA without mitochondria was used to correct the autooxidation rate of DCFH-DA. The units were expressed as picomoles DCF formed per minute per milligram of protein.

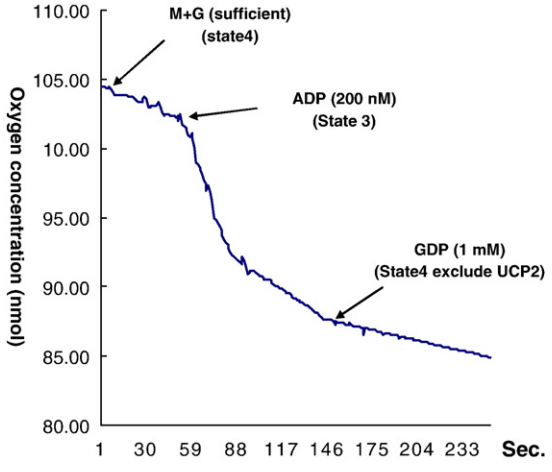

Fig. 1. A typical polarographic oxygen electrode trace obtained in the present study. Oxygen consumption rates of energized (1 mM glutamate+0.1 mM malate) rat heart mitochondria isolated from untrained and trained rats were determined at 25°C with the presence of 1 mg of mitochondrial protein. State 3 and state 4 respiration, respiratory control ratio (RCR), ADP/O ratio, and UCP-mediated basal respiration (UCR) were obtained according to Methods.

Mitochondrial membrane potential and ATP synthase activity

The isolated mitochondrial membrane potential ($\Delta\psi$) of permeabilized cells was measured by monitoring the fluorescence spectrum of rhodamine 123 at the excitation–emission wavelength of 500–525 nm [27]. The experiments were performed at 25°C in 2 ml of incubation medium containing 0.25 M sucrose, 3.0 mM Hepes, and 0.5 mM EDTA (pH 7.4) with 0.5 mg mitochondrial protein.

ATP synthase activity was determined using a bioluminescence technique [28]. Mitochondrial suspensions were added to a cuvette containing 0.1 M luciferase (Sigma Co.), 0.25 M sucrose, 3.0 mM Hepes, 0.5 mM EDTA, and 0.1 mM pyruvate+1 mM malate as substrate. After a background bioluminescence was established for correction, 4 μM ADP was added to initiate the action. ATP production was monitored at 25°C with a BioOrbit $20/20^{n}$ luminometer (Turku, Finland) and expressed as nanomoles per second per milligram protein.

UCP2 and MnSOD mRNA

UCP2 and MnSOD mRNA expression was determined in cardiac muscles by real-time quantitative PCR (RT-PCR). Total RNA was extracted from 0.1 g of tissue using the TRIzol reagent (Mrcgene), according to the manufacturer's instruction. RNA concentrations were estimated by measuring the absorbance at 260 nm, and purity was assessed by 260/280-nm absorbance ratio (Eppendorf, Hamburg, Germany). Total RNA (5 μg) was denatured at 70°C for 5 min, cooled immediately, and reverse-transcribed using 200 units of Moloney murine leukemia virus reverse transcriptase (RevertAid), 0.5 μg poly(dT) primer, and 20 nmol dNTP in a total volume of 20 μl. The reaction was assessed at 42°C for 60 min and at 70°C for 10 min. The RT-PCR was performed in a fluorescence temperature cycler (LightCycler TM; Roche Diagnostics, Mannheim, Germany) containing 4 pmol of each primer, 4 mmol/L $MgCl_2$, 2.0 μl DNA Master SYBR Green I (contains *Taq* DNA polymerase, reaction buffer, dNTP mix, SYBR Green I dye, and 10 mmol/L $MgCl_2$), and

2.0 μl template with a total volume of 20 μl. Amplification occurred in a three-cycle procedure (denaturation at 95°C for 5 s; annealing at 59°C for 30 s; and extension at 72°C for 60 s) for 40 cycles. The fluorescence signal was plotted against cycle number for all samples and external standards. Primer pairs for the real-time PCR were as follows: UCP2, 5′-TTCTACAAGGGGTTCATGCC-3′ and 5′-AGAAGGGTAAAGGGTGTGAG-3′; MnSOD, 5′-GCGACCTACGTGAACAATCTGAACG-3′ and 5′-TCAATCCCCAGCAGTGGAATAAGGC-3′; β-actin, 5′-TGGTGGGTATGGGTCAGAAGGACTC-3′ and 5′-CATGGCTGGGGTGTTGAAGGTCTCA-3′.

UCP2 and MnSOD mRNA abundance was normalized with that of β-actin.

MnSOD activity

SOD activities was measured using the method of Sun and Zigman [29], where one unit of SOD activity was defined as the amount of enzyme that causes a 50% inhibition of epinephrine autooxidation to adrenochrome. For Mn SOD activity, 1 mM KCN^- was added to the reaction mixture to inhibit CuZn SOD activity.

Statistical analysis

Data were analyzed with two-way ANOVA with the two main effects being exercise time and training. When a significant main effect was detected, the Bonferroni post hoc test was used to compare differences between means. Statistical Package for the Social Sciences (SPSS Inc., version 12.0) was used for all analyses. The significance level was set at $P<0.05$.

Results

Mitochondrial respiratory function

An acute bout of exercise increased state 4 respiration of the heart mitochondria in a time-dependent manner (Table 1). State 4 respiration rate was 42 and 64% ($P<0.05$) higher at 120 and 150 min, respectively, compared to the resting rate in U rats. Training significantly decreased exercise-induced state 4 respiration, as T rats showed only 26 ($P>0.05$) and 47% ($P<0.05$) increase in state 4 respiration at the respective time points. State 3 respiration was not affected either by an acute bout of exercise or by training. As a result, mitochondrial RCR declined over exercise time, showing a 21 and 33% reduction ($P<0.05$) at 120 and 150 min, respectively, in U rats. In contrast, RCR was well maintained in T rats with no change observed in any of the exercise groups. The P/O ratio of heart mitochondria was unchanged in all exercise groups except E_{150} which showed a mild reduction (9%, $P<0.05$). Overall, T rats demonstrated a higher P/O ratio than U rats and the differences were significant in E_{120} and E_{150} ($P<0.05$).

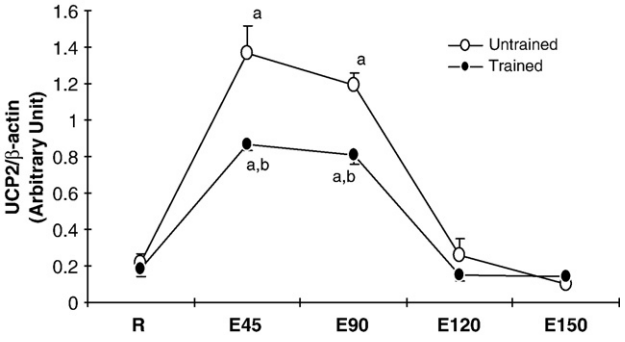

Fig. 2. Myocardium UCP2 mRNA abundance normalized with β-actin in the heart of untrained and trained rats. R, sedentary; E45, E90, E120, and E150, exercise for 45, 90, 120, and 150 min, respectively. [a] $P<0.01$, E vs R; [b] $P<0.05$, trained vs untrained at respective time points.

UCP mRNA and UCR

An acute bout of exercise dramatically increased UCP2 mRNA levels in both U and T rats (Fig. 2). For U rats, UCP2 mRNA was elevated by 7- and 6-fold after 45 and 90 min of exercise, respectively ($P<0.01$). The exercise-induced UCP2

Table 1
Mitochondrial respiratory functions and ATP synthase activity in rat heart

	Group	R	E_{45}	E_{90}	E_{120}	E_{150}
State 3	Untrained	68.2±7.0	70.2±5.2	73.9±6.9	74.9±4.6	71.4±5.9
	Trained	67.3±4.1	69.1±3.5	75.0±3.8	75.3±8.1	73.2±6.0
State 4	Untrained	13.0±7.5	14.0±4.2	15.3±1.5	18.5±2.3[a]	21.4±0.76[a]
	Trained	12.5±2.3	12.8±3.1	13.2±2.8	14.2±2.2[b]	16.4±2.5[a,b]
RCR	Untrained	5.09±0.52	4.99±0.49	4.75±0.41	4.01±0.32[a]	3.37±0.21[a]
	Trained	5.18±1.47	5.28±2.59	5.53±2.00	5.22±1.47[b]	4.51±1.96[b]
P/O	Untrained	1.65±0.28	1.66±0.42	1.76±0.20	1.68±0.11	1.50±0.22[a]
	Trained	1.78±0.42	1.76±0.36	1.82±0.58	2.03±0.41[a,b]	1.77±0.23[b]
UCR	Untrained	1.74±0.41	1.93±0.27	2.31±0.45	2.79±0.72	5.41±1.14[a]
	Trained	1.55±0.26	1.67±0.21	1.86±0.43	2.27±0.41	3.28±0.77[a,b]
UCR-independent State 4	Untrained	11.3±6.1	12.0±5.2	12. 9±4.1	16.9±5.9[a]	15.9±4.5[a]
	Trained	10.9±2.9	11.2±3.1	11.3±3.3	12.0±2.9	13.2±3.2[a,b]
ATP synthase	Untrained	36.4±2.1	37.7±2.0	39.8±3.5	42.9±2.6	40.6±4.9
	Trained	45.9±2.8[b]	50.0±2.5[b]	53.2±3.6[b]	51.4±3.5[b]	50.2±5.0[b]

Data are mean±SE ($N=10$). Unit: nmolO_2/min · mg protein, except ATP synthase (nmol/min · mg protein). R, sedentary; E_{45}, E_{90}, E_{120}, exercise for 45, 90, 120, and 150 min, respectively.
[a] $P<0.05$, E vs R; [b] $P<0.05$, trained vs untrained at respective time point.

mRNA was significantly less in T rats ($P<0.05$), albeit was still elevated by ~4-fold above resting level at the respective times. UCP2 mRNA abundance returned to resting levels at 120 and 150 min of exercise for both U and T rats.

Mitochondrial UCR showed progressive increases in response to the duration of the exercise bout (Table 1). In U rats, a 3-fold increase ($P<0.05$) in UCR was observed at 150 min, whereas in T rats the increase was only 2-fold ($P<0.05$). When expressed as the percentage of state 4 respiration, UCR of E_{45}, E_{90}, and E_{120} was not different from that of R in both U and T rats (data not shown). However, E_{150} of U rats showed an 86% ($P<0.05$) increase over the resting level, and E_{150} of T rats showed a 43% ($P<0.05$) increase. Thus, endurance training attenuated exercise-induced UCR in rat heart mitochondria.

UCR-independent state 4 respiration

Mitochondrial state 4 respiration could be attributed to two components: a basal component due to proton leak and the UCP-mediated respiration (i.e., UCR). Exercised U rats showed progressively increased UCR-independent state 4 respiration over time ($P<0.05$) (Table 1). This respiration in exercised T rats did not show a significant change until 150 min and was much lower than that in U rats ($P<0.05$).

ROS Generation and cross-membrane potential

In order to gain some insights into the exercise-induced UCP2 expression, we examine three additional properties of the isolated heart mitochondria, i.e., generation of ROS during state 4 respiration, cross-membrane potential $\Delta\psi$, and ATP synthase activity. As shown in Fig. 3, ROS production was unchanged after 45 min of exercise, but elevated at 90 and 120 min ($P<0.05$) in U rats. Interestingly, ROS showed a marked decrease at 150 min, returning to the resting levels. ROS generation was lower in T vs U rats overall with significant elevation above resting levels found only at 120 min ($P<0.05$). ROS production in T rats also showed a dramatic plunge at 150 min ($P<0.05$).

Mitochondrial cross-membrane potential was increased in U rats after 90 and 120 min of exercise ($P<0.05$), followed by a decrease at 150 min ($P<0.05$) (Fig. 4). Trained rats had lower $\Delta\psi$ compared to U rats ($P<0.05$), though a significant increase in $\Delta\psi$ was also observed at 120 min of exercise ($P<0.05$), followed by a decline at 150 min ($P<0.05$).

ATP synthase activity was not affected by an acute bout of exercise in either U or T rats (Table 1). However, the enzyme activity was significantly higher in T vs U regardless of exercise status ($P<0.05$).

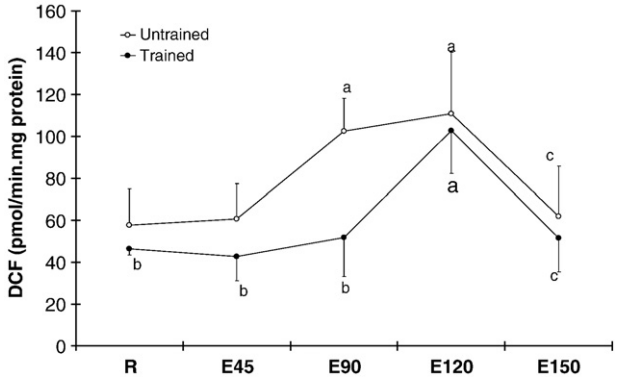

Fig. 3. Rate of DCF formation in the heart of untrained and trained rats. R, sedentary; E45, E90, E120, and E150, exercised for 45, 90, 120, and 150 min, respectively. [a] $P<0.05$, E vs R; [b] $P<0.05$, trained vs untrained at respective time points; [c] $P<0.05$, E150 vs E120.

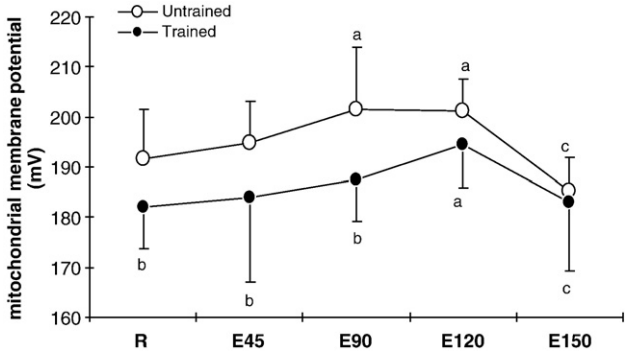

Fig. 4. Mitochondrial cross-inner membrane potential ($\Delta\psi$) in the heart of untrained and trained rats. R, sedentary; E45, E90, E120, and E150, exercised for 45, 90, 120, and 150 min, respectively. [a] $P<0.05$, vs R; [b] $P<0.05$, trained vs untrained at respective time point.

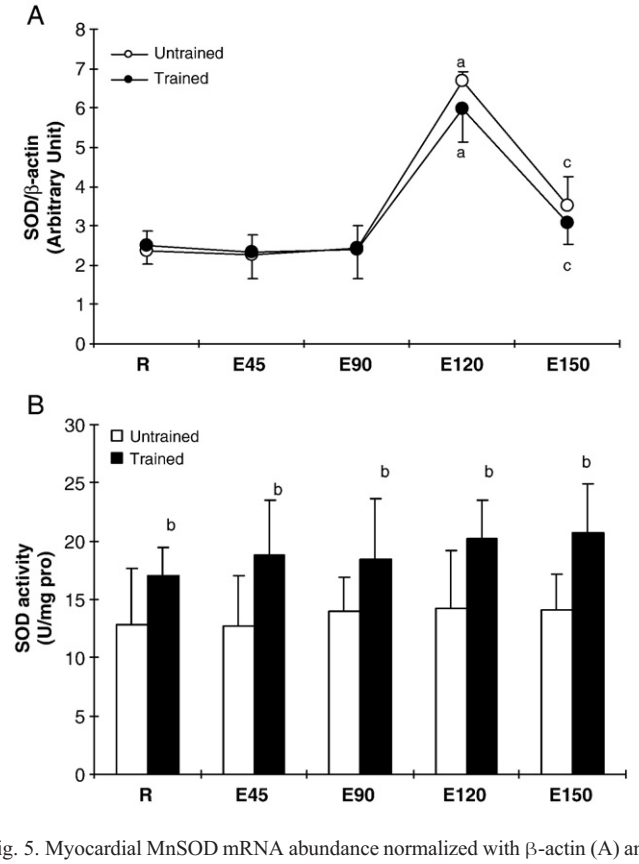

Fig. 5. Myocardial MnSOD mRNA abundance normalized with β-actin (A) and MnSOD activity (B) in the heart of untrained and trained rats. R, sedentary; E45, E90, E120, and E150, exercised for 45, 90, 120, and 150 min, respectively. [a] $P<0.05$, vs R; [b] $P<0.05$, trained vs untrained at respective time point. [c] $P<0.05$, E150 vs E120.

MnSOD mRNA and activity

An acute bout of exercise for 120 min increased MnSOD mRNA abundance by more than 2-fold in the heart of both U and T rats ($P<0.05$, Fig. 5A). However, this increase was not observed in rats exercised for 45, 90, or 150 min, whether or not they are trained. MnSOD activity was not affected by an acute bout of exercise, but was significantly higher in T vs U rats at rest and after an acute exercise bout ($P<0.05$, Fig. 5B).

Discussion

Previous studies suggest that hyper-β-adrenergic activity stimulates UCP2 expression accompanied by increased oxidative stress in the myocardium, whereas UCP2 overexpression protects the cardiomyocytes from ROS-induced apoptosis [10–12]. However, the physiological significance of UCP2 in heart mitochondria remains elusive. Since intense physical work is known to increase sympathetic activity and ROS production in the rodent heart, we hypothesized that an acute bout of exercise would upregulate UCP2 gene expression.

Data from the current study supported our hypothesis and provided new insights into UCP2 regulation during exercise in several aspects. First, this is the first report that a single bout of treadmill running could increase UCP2 mRNA expression in rat heart. Although the increase in UCP2 mRNA appeared transient lasting only for 90 min, UCP2-controlled mitochondrial respiration (UCR) showed a remarkable 3-fold increase after 150 min when UCP2 mRNA declined to the resting levels. We did not measure UCP2 protein content in the current study; however, the delayed increase in UCR most likely reflected a corresponding increase in UCP2 protein synthesis as shown by other authors [12], as well as our previous work on exercise-induced UCP3 in rat skeletal muscle [30]. Second, the UCP2 upregulation was associated with mitochondrial state 4 respiration, cross-membrane potential ($\Delta\psi$), and the formation of ROS (mostly H_2O_2), all of which showed time-dependent increases as a result of exercise. According to the chemiosmotic theory of oxidative phosphorylation, the electron transfer in ETC during State 4 respiration creates a proton gradient ($\Delta\mu H^+$) across the inner mitochondrial membrane, which may be used either to drive ATP synthesis through F_0F_1-ATPase or to generate heat through the so-called "proton leak" [21,31]. However, proton backflow to the matrix side of mitochondria inner membrane can be either passive through the lipid bilayer (i.e., UCR-independent state 4 respiration), or facilitated by UCPs [10]. An upregulation of UCP2 could alleviate cross-membrane pressure caused by $\Delta\mu H^+$, thus reducing the chance of $O_2^{\bullet -}$ generation due to "electron spill" [32]. This notion was supported by the findings that UCR rate was progressively increased over exercise time, which contributed to the bulk of the increased state 4 respiration. Furthermore, when UCR reaches the highest level at 150 min, both $\Delta\psi$ and ROS production showed a dramatic decrease (Figs. 3 and 4). Regarding the potential mechanism of the respiratory uncoupling, it is noteworthy that rising organ and core temperature during heavy exercise has been shown to directly increase mitochondrial state 4 respiration, along with elevated gene expression of heat shock protein (HSP) 70 peaking at 30–60 min postexercise in the skeletal muscle, heart, and liver of rats [33]. Exercise hyperthermia was hypothesized to directly cause oxidative stress since the above authors demonstrated that mitochondrial uncoupling (decreasing RCR) correlated lineally with increased $O_2^{\bullet -}$ generation with increased muscle temperature. Interestingly, we have shown previously that hyperthyroidism-induced $O_2^{\bullet -}$ production and proton leak during elevated state 4 respiration could lead to a higher heat production in the mitochondria, hence further increasing organ temperature [31]. Thus, it is possible that the increased UCP2 expression observed in the current study was part of the synergetic responses to elevated temperature and oxidative stress in the cardiac muscle during heavy exercise, the precise cause and effect of which requires further investigation.

Our data also suggest that there was a coordinated regulation in rat heart to reduce ROS generation and to preserve mitochondrial respiratory function during prolonged exercise. Noticeably, MnSOD mRNA levels were elevated after 120 min when ROS production was increased; however, when UCR was increased at 150 min, upregulation of MnSOD mRNA was severely attenuated (Fig. 5a). These findings suggest that UCP2 induction may be part of an "early response" of antioxidant defense aimed to reduce mitochondrial oxidative stress and preserve respiratory function. Inasmuch as heart is a highly aerobic organ with great oxidative potential, myocardial endogenous antioxidant reserve is relatively low compared to other tissues such as liver, lung, and kidney [7]. The upregulation of UCP2 provides an important mechanism to reduce ROS generation through ETC during the early stage of exercise, when oxygen flux increases several-fold but ROS removal by MnSOD and other antioxidants (e.g., glutathione) is still latent. Indeed, despite the decrease of RCR and P/O ratio, state 3 respiration and ATP synthesis were largely normal throughout the two and half hours of exercise period regardless of rats' training status.

While the primary function of UCP1 is to catalyze a proton leak across the mitochondrial inner membrane so as to dissipate the proton motive force used by the ATP synthase in favor of heat production, the physiological significance of UCP2 upreguation during exercise is unclear [8,9]. An increased UCP2 protein expression and UCR would seem to compromise the efficiency of mitochondrial oxidative phosphorylation in the heart. For example, Murakami et al. [34] provided evidence that in the chronic phase of heart failure, the expression of UCP2 was significantly increased in the myocardium, whereas the high-energy creatine phosphate and ATP production was decreased. However, several studies showed that mice knocked out of UCP2 revealed normal proton conductance, unchanged thermoregulation, thermogenesis, energy expenditure, and body weight, which seems to argue against UCP2's role as a critical metabolic regulator [35]. Furthermore, overexpression of UCP2 in pancreatic islet cells in obese Zucker rats was shown to increase oxidative capacity and ATP/ADP ratio and to improve beta-cell functions [36]. It is generally agreed that one of the important effects of increased UCP2 expression is to decrease ROS production in the mitochondria. During rigorous myocardial contraction, oxygen flux and electron flow through the ETC are markedly increased, resulting in a more negative inner

membrane potential ($\Delta\psi$), which favors $O_2^{\bullet -}$ generation through the ETC [37]. Thus, a modest reduction in $\Delta\psi$ (mitochondrial depolarization) facilitates a tighter association between the electrons and the ETC complexes, thereby limiting random electron disassociation and $O_2^{\bullet -}$ production, as first proposed by Liu et al. [38] and later confirmed by others [39,40]. Indeed, overexpression of UCP2 in cardiomyocytes has been shown to decrease ROS production and oxidative damage [41], whereas bone marrow transplant from UCP2 knockout donor mice enhanced nitrotyrosine formation, macrophage accumulation, and lesion size in the thoracic aorta of atherosclerotic-prone mice [42]. Our current findings that increased ROS production rate was closely associated with $\Delta\psi$ up to 120 min of exercise but both decreased markedly (−56 and −9%, respectively) when UCR was elevated 3-fold at 150 min support the role of UCP2 as a means of antioxidant defense. To our knowledge, this was the first demonstration of such reciprocal relationship between UCP2 and ROS production in the mitochondria of exercised heart.

The mechanisms that induce UCP2 activity and protein expression in the mitochondria have been investigated extensively under various experimental conditions. Echtay et al. [22] demonstrated that $O_2^{\bullet -}$ could activate inducible proton conductance of mitochondria from the matrix side through effects on UCPs and that this activation was sensitive to inhibition of purine nucleoside di- and triphosphates, although adenine nucleotide translocase (ANT) may also play a role. Our findings that UCP2 mRNA upregulation coincided with increased UCR, state 4 respiration (UCR plus UCR independent), and ROS generation were consistent with this scenario. Reid et al. [43] have shown that $O_2^{\bullet -}$ could be formed either in the matrix or outside mitochondria and enter the matrix in response to contractile activity in rat diaphragm muscle. While the time course of UCP2 elevation (at 45 and 90 min) preceding peak ROS production at 120 min seems perplexing, this is not necessarily in conflict with the superoxide theory of UCP activation. Potential explanations are: (1) $O_2^{\bullet -}$ might be produced in sufficient amounts during the early stage of exercise and activated UCP2, but was subsequently converted to H_2O_2 by MnSOD, because the DCFH assay used in our study measures primarily H_2O_2 instead of $O_2^{\bullet -}$ generation [26]. (2) Extramitochondrial production of $O_2^{\bullet -}$ not accounted for by the current assay conducted in isolated mitochondria might have contributed to the activation of UCP2. Bejma et al. [25] showed that an acute bout of treadmill running for 55 min in rats increased myocardial total ROS production by 47%, whereas the mitochondrial ROS level was not significantly changed. Xanthine oxidase, NADPH oxidase, and catecholamine autooxidation are among the main extramitochondrial sources to produce $O_2^{\bullet -}$ in the heart during heavy exercise [7]. In exploring the exact molecular mechanism of UCP2 activation, Echtay et al. [44] reported that 4-hydroxy-2-nonenal (4-HNE), a by-product of lipid peroxidation, and structurally related compounds (such as *trans*-retinoic acid, *trans*-retinal, and other 2-alkenals) were strong inducers of mitochondrial uncoupling through the UCPs. Thus, 4-HNE could be a potential activator of exercise-induced UCP2 in rat heart, since increased lipid peroxidation has been widely reported in skeletal muscle, liver, and heart in exercise studies [6,7]. A limitation of this scenario is that lipid peroxidation products usually remain in the membrane lipid phase. While quite capable of stimulating UCR through uncoupling, they are unlikely to travel to the nucleus and affect UCP2 mRNA transactivation seen in our study. We noted a report showing that fatty acids (linoleic and oleic acids) could activate UCP2 protein expression in rat hepatocytes [45]. These lipids increased the DNA-binding activity of nuclear factor (NF) κB and resulted in a dose- and time-dependent expression of UCP2 mRNA up to 4.5-fold, as well as UCP2 protein content after 24 h. While it is uncertain whether a similar mechanism exists in the myocardium, these data could shed some light on the molecular signaling of UCP2 upregulation during exercise.

Our study demonstrated that endurance training attenuated acute exercise-induced UCP2 mRNA and UCR in rat heart. This finding raised some interesting questions regarding the relationship among mitochondrial respiratory uncoupling, ROS generation, antioxidant defense, and ATP synthesis. An ameliorated exercise response of UCP2 after training could be viewed as a compensatory mechanism to avoid excessive sacrifice of oxidative phosphorylation efficiency and heat production. ATP synthesis is the top priority of the heart under metabolic stress; therefore, the organism cannot afford to have excessive amounts of proton shunt through UCP2 rather than though F_0-F_1 complex to produce ATP. However, reduction of proton gradient and $\Delta\psi$ via UCP upregulation is necessary for reducing ROS generation, which eventually could damage mitochondrial enzymes and affect ATP production [46]. To understand the complexity of the systems one would have to appreciate the role of MnSOD as an important regulator of ROS production in the mitochondria. MnSOD activity in rat heart was significantly (~30%) higher in the trained than untrained rats. This could explain why T rats had lesser an upregulation of UPC2 mRNA compared to U rats (4- vs 7-fold) after an acute bout of exercise, because MnSOD had removed a portion of $O_2^{\bullet -}$ as a potential stimulator of UCP2 (see previous). T rats also had displayed lower mitochondrial ROS generation in response to exercise up to 120 min possibly due to a training-induced glutathione peroxidase activity [47]. This would reduce the production of lipid peroxidation by- products such as 4-HNE as UCP2 and UCR activators in the T rats. McLeod et al. [48] reported that in the heart mitochondria from $Sod2^{-/-}$ mice, ATP production was markedly reduced due to a higher $O_2^{\bullet -}$ concentration which activated UCP2 excessively. Taken together, training adaptation of myocardial MnSOD may enhance mitochondrial tolerance to ROS production and hence a smaller UCP2 activation during intensive exercise, thus protecting the efficiency of oxidative phosphorylation. Indeed, in T rats mitochondrial respiratory control (RCR) and P/O ration were maintained relatively constant despite heavy prolonged exercise, whereas in U rats RCR and P/O were significantly decreased.

An interesting finding was that T rats showed lower cross-membrane potential (ψ) after exercising for every duration up to 120 min, compared to U rats. According to Nicholls [49], at constant P_i concentration the maximum ATP/ADP ratio that can be maintained by the mitochondria decreases by 10-fold with every 14 mV reduction of proton motive force (Δp). It seems paradoxical that training would undermine mitochondrial efficiency for ATP production. However, T rats had much higher (30%) higher ATP synthase activity than U rats, which could

compensate for a lower $\Delta\psi$. Furthermore, it is well-known that training changes not only mitochondrial composition but also mitochondrial number in the skeletal muscle and heart [13]. It is possible that an increase in mitochondrial population could also compensate for a moderate reduction of $\Delta\psi$, while enjoying the advantage of preventing excessive ROS production. As pointed out by Kadenbach [46], the most efficient rate of ATP synthesis may occur at a mitochondrial membrane potential low enough before significant generation of ROS occurs.

In summary, ATP depletion and ROS production are two major concerns during prolonged exercise. The present study demonstrated that UCP2 played a key role in decreasing cross-membrane potential $\Delta\psi$ and thus ROS production within heart mitochondria during an acute bout of prolonged exercise in rats. While acting as an uncoupler UCP2 slightly lowered the efficiency of oxidative phosphorylation but reduced ROS production. Thus, UCP2 may be viewed as part of the myocardial antioxidant defense system. Endurance training attenuated, but did not abolish, UCP2 activation due to the upregulation of MnSOD and ATP synthase. These adaptations optimize the heart to reach maximal efficiency of energy metabolism while minimizing exercise-induced oxidative stress.

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