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Original Contribution

Upregulation of uncoupling protein-3 in skeletal muscle during exercise: a potential antioxidant function

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ABSTRACT

Uncoupling protein-3 (UCP3) expression has been shown to increase dramatically in response to muscular contraction, but the physiological significance of UCP3 upregulation is still elusive. In this study, UCP3 mRNA and protein expression were investigated along with mitochondrial respiratory function, reactive oxygen species (ROS) generation, and antioxidant defense in rat skeletal muscle during and after an acute bout of prolonged exercise. UCP3 mRNA expression was elevated sharply at 45 min of exercise, reaching 7- to 8-fold above resting level at 150 min. The increase in UCP3 protein content showed a latent response but was elevated ~1.9-fold at 120 min of exercise. Both UCP3 mRNA and UCP3 protein gradually returned to resting levels 24 h postexercise. Mitochondrial ROS production was progressively increased during exercise. However, ROS showed a dramatic drop at 150 min although their levels remained severalfold higher during the recovery. Mitochondrial State 4 respiration rate was increased by 46 and 58% ($p<0.05$) at 90 and 120 min, respectively, but returned to resting rate at 150 min, when State 3 respiration and respiratory control index (RCI) were suppressed. ADP-to-oxygen consumption (P/O) ratio and ATP synthase activity were lowered at 3 h postexercise, whereas proton motive force and mitochondrial malondialdehyde content were unchanged. Manganese superoxide dismutase gene expression was not affected by exercise except for an increase in mRNA abundance at 3 h postexercise. These data demonstrate that UCP3 expression in rat skeletal muscle can be rapidly upregulated during prolonged exercise, possibly owing to increased ROS generation. Increased UCP3 may partially alleviate the proton gradient across the inner membrane, thereby reducing further ROS production by the electron transport chain. However, prolonged exercise caused a decrease in energy coupling efficiency in muscle mitochondria revealed by an increased respiration rate due to proton leak (State 4/State 3 ratio) and decreased RCI. We thus propose that the compromise of the oxidative phosphorylation efficiency due to UCP3 upregulation may serve an antioxidant function to protect the muscle mitochondria from exercise-induced oxidative stress.

Heavy physical exercise imposes a great challenge to the mitochondria, especially in the skeletal muscle. On one hand the mitochondrial electron transport chain (ETC) is responsible for providing almost all the energy (in the form of ATP) required for muscular contraction; therefore a tight coupling and high efficiency of oxidative phosphorylation are a top priority for the organelle [1]. On the other hand, the high flux of oxygen through the ETC has the potential of generating superoxide radicals and subsequently other reactive oxygen species (ROS) if adequate adjustments are not made, which could impose a serious threat to the integrity and functionality of mitochondria [2,3]. Thus, a mild uncoupling of the respiration can alleviate the membrane potential ($\Delta\psi$) across the inner membrane generated by the proton pump associated with the electron transfer thereby reducing ROS generation at the expense of oxidative phosphorylation efficiency [1]. This dilemma puts evolutionary pressure on the mitochondria to accommodate the competition between metabolic demand and antioxidant protection.

Located in the mitochondrial inner membrane, uncoupling proteins (UCP) are a heterogeneous family of proteins that play an important role in partially dissipating the proton electrochemical gradient across the membrane [4]. The best characterized UCP1 is expressed exclusively in the brown adipose tissue of rodents, with a key function of adaptive thermogenesis, whereas UCP2 is expressed ubiquitously, with a physiological role yet to be understood [5]. UCP3, expressed primarily in the skeletal muscle, shares 59% homology with UCP1 and is regarded as a plausible regulator of transmembrane

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proton potential and hence efficiency of oxidative phosphorylation [6]. Several previous studies have shown that UCP3 expression was increased in response to an acute bout of exercise or contractile activity in mammalian skeletal muscle, which was proposed to be linked to some important cell functions [6–8]. The exact physiological significance of this upregulation, however, is still elusive. Recently, Goglia et al. [9] postulated that by translocating fatty acid peroxides from the inner to the outer membrane leaflet, UCP may fulfill a role in antioxidant defense of the mitochondria. This intriguing view of UCP3 function prompted us to hypothesize that UCP3 may be induced in skeletal muscle during exercise in part to shunt protons back to the matrix and maintain a modest $\Delta\psi$, thus reducing superoxide radical production and regulating the balance between ATP production and antioxidant function.

This study was conducted with the following aims: (i) to investigate the effects of an acute bout of exercise on UCP3 gene expression and its time course, (ii) to examine the role of UCP3 upregulation in conjunction with mitochondrial respiratory function and oxidative phosphorylation, (iii) to evaluate the potential role of ROS production and its relationship with exercise-induced UCP3 upregulation, and (iv) to assess the functional and temporal relationship between UCP3 and the mitochondrial antioxidant enzyme manganese superoxide dismutase (MnSOD). Our data revealed that UCP3 upregulation during exercise may serve as an important "early response" antioxidant protection to muscle mitochondria.

Methods

Animals

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 University of Sport under the guidelines of the Chinese Academy of Sciences.

Acute exercise

Five days after arrival, all rats were accustomed to treadmill running at 8.2 m/min at 0% grade for 15 min for 1–2 weeks. At the end of the acclimation period, the rats were subjected to an acute bout of treadmill running before being killed according to a previously described protocol [10]. Exercise was performed on a motor-driven treadmill, beginning at 8.2 m/min at 0% grade corresponding to ~53% of maximal oxygen uptake (VO_{2peak}) for 15 min. After another 15 min of running at 15 m/min and 5% grade (~64% VO_{2peak}), treadmill speed and grade were increased to 19.3 m/min and 10% grade and rats ran continuously at this intensity (~76% VO_{2peak}) for 0, 45, 90, 120, or 150 min (referred to as R, 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. Four additional groups of rats were subjected to an acute bout of treadmill running at 19.3 m/min and 10% grade (~76% VO_{2peak}) for 150 min and killed after 3, 6, 12, or 24 h of rest postexercise. These groups of rats were referred to as PE_3, PE_6, PE_{12}, and PE_{24}, respectively.

Preparation of skeletal muscle mitochondria

The animals were sacrificed by cervical dislocation at rest or immediately after an acute bout of exercise at various time points. A portion of approximately 50–70 mg of the gastrocnemius muscle was quickly dissected and finely minced in an ice-cold isolation medium (see below) for mitochondrial preparation. The remaining muscle samples were immediately frozen in liquid nitrogen and stored at −80 °C. Muscle mitochondria were prepared using differential centrifugation as previously described [11]. Briefly, the muscle sample was quickly dissected and placed in an ice-cold isolation buffer (0.12 M KCl, 20 mM Hepes, 5 mM $MgCl_2$, 1 mM EDTA, pH 7.4) at weight/volume ratio of 1:10. The minced blood-free tissue was homogenized using a motor-driven Ultra-Turrax T8 glass homogenizer with a Teflon pestle at a speed of 800 rpm with three passes (0–4 °C). The homogenate was centrifuged at 17,000 *g* for 10 min and the resultant pellet was resuspended in 20 ml of the initial solution with 5 mg/ml fatty acid-free bovine serum albumin (BSA) and centrifuged again at 7000 *g* for 10 min. The mitochondrial pellet was suspended in 20 ml solution containing 0.3 M sucrose, 2.0 mM Hepes, and 0.1 mM EDTA (pH 7.4) and centrifuged at 3500 *g* for 10 min. The final mitochondrial pellets were suspended in 1 ml of the above-mentioned solution (with 5 mg/ml fatty acid-free BSA). The mitochondrial isolation procedures were completed within 1 h after the rat was killed.

Mitochondrial protein content was assayed using BSA as standard according to Lowry et al. [12].

Mitochondrial respiration

Mitochondrial respiratory function was measured polarographically at 25 °C using a Clark-type oxygen electrode (YSI, Yellow Springs, OH, 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 [13].

Mitochondrial ROS production

Muscle mitochondrial ROS generation was determined in fresh mitochondrial suspensions using dichlorofluoroscein (DCF) as a probe according to LeBel and Bondy [14] as modified by Bejma and Ji [15]. This assay measures not only ROS but also nitric oxide (NO) and its derivatives [16]. Under the defined experimental conditions the majority of oxidants measured were H_2O_2 owing 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 of 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, Palo Alto, CA, USA). A blank consisting of the appropriate buffer and 5.0 μM DCFH-DA without mitochondria was used to correct the autoxidation rate of DCFH-DA. The units were expressed as picomoles of DCF formed per minute per milligram of protein.

Mitochondrial membrane potential

The isolated mitochondrial $\Delta\psi$ was measured by monitoring the fluorescence spectrum of rhodamine 123 at the excitation–emission

wavelength of 500–525 nm [17]. 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 [18]. Mitochondrial suspensions were added to a cuvette containing 0.1 M luciferase (Sigma, St. Louis, MO, USA), 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 nmol/s per milligram of protein.

UCP3 and MnSOD mRNA

UCP3 and MnSOD mRNA expression was determined in skeletal muscles by real-time quantitative reverse-transcription PCR (RT-PCR). Total RNA was extracted from 0.1 g of tissue using the TRIzol reagent (Molecular Research Center, Cincinnati, OH, USA), according to the manufacturer's instruction. RNA concentrations were estimated by measuring the absorbance at 260 nm, and purity was assessed by 260 nm/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 (Light-Cycler; 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 in a total volume of 20 μl. Amplification occurred in a three-step cycle (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, for the UCP3 gene (179 bp) [19], 5′-GGAGCCATGGCAGTGACCTGT-3′ and 5′-TGTGATGTTGGGC-CAAGTCCC-3′; for the SOD gene (383 bp) [20], 5′-GCGACCTACGT-GAACAATCTGAACG-3′ and 5′-TCAATCCCCAGCAGTGGAATAAGGC-3′; and for the β-actin gene, 5′-TGGTGGGTATGGGTCAGAAGGACTC-3′ and 5′-CATGGCTGGGGTGTTGAAGGTCTCA-3′. UCP3 and MnSOD mRNA abundance was normalized to that of β-actin.

Expression of UCP3 and MnSOD protein

Mitochondria were lysed in SDS loading buffer and heated for 5 min at 100 °C. Ten-microgram protein aliquots from the mitochondrial lysates were loaded in each lane and separated on a 15% SDS–PAGE gel. Proteins were transferred to polyvinylidene difluoride membranes and immunological detection was performed by using a rabbit polyclonal antibody against UCP3 (ADI, USA) and a rabbit polyclonal antibody against MnSOD (Santa Cruz Biotechnology, Santa Cruz, CA, USA), respectively. The detection was achieved by using the enhanced chemiluminescence detection system with an anti-rabbit antibody as secondary antibody. Expression of the protein was normalized to that of cytochrome *c* oxidase (COX IV) detected in the same membrane.

MnSOD activity

SOD activity was measured using the method of Sun and Zigman [21], in which 1 unit of SOD activity was defined as the amount of enzyme that causes a 50% inhibition in epinephrine autoxidation to adrenochrome. For MnSOD activity, 1 mM KCN^- was added to the reaction mixture to inhibit CuZnSOD activity.

Thiobarbituric acid-reactive substance (TBARS) content

TBARS content was measured in muscle mitochondria as a marker of lipid peroxidation. Malondialdehyde, lipid peroxide, and other aldehydes reacted with thiobarbituric acid to produce a colored complex when the assay mixture was heated for 40 min at 95 °C followed by centrifugation at 750 *g* for 10 min. The organic layer was extracted in butanol and its absorbance was measured at 532 nm [22].

Statistical analysis

Data were analyzed with one-way ANOVA. When a significant main effect was detected, the Bonferroni post hoc test was used to compare differences between means. The 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

UCP3 mRNA and UCP3 protein

An acute bout of exercise dramatically increased the UCP3 mRNA levels in rat skeletal muscle (Fig. 1A). UCP3 mRNA level was elevated by four- and ninefold ($p<0.01$) after 45 and 90 min of exercise, respectively, and remained at seven- to eightfold higher than resting levels at PE_{120} and PE_{150}. UCP3 mRNA levels gradually declined over the 24-h recovery period but the PE_3 level was still significantly higher than R ($p<0.05$).

UCP3 protein content in the muscle showed a latent response compared to mRNA, with a significant 70–90% increase ($p<0.01$) in E_{120} and E_{150}, respectively, compared to R (Fig. 1B). UCP3 protein contents returned to basal levels during the postexercise period and were not different from that of R.

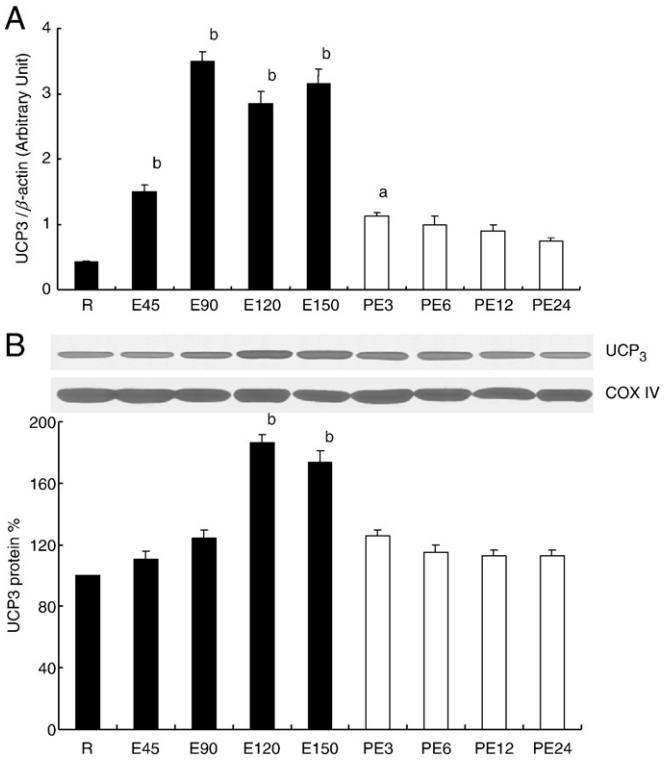

Fig. 1. Relative abundance of UCP3 (A) mRNA and (B) protein in rat skeletal muscle in response to an acute bout of exercise for 45 (E45), 90 (E90), 120 (E120), or 150 (E150) min and after 3 (PE3), 6 (PE6), 12 (PE12), or 24 (PE24) h recovery. R, rest. Each bar represents the mean±SEM ($N=10$). $^a p<0.05$, $^b p<0.01$ vs the R group.

Table 1
Mitochondrial respiratory functions in rat skeletal muscle

Group	State 4 (nmol O_2/min·mg)	State 3 (nmol O_2/min·mg)	RCI	State 4/State 3 (%)	*P*/*O*
R	9.61±0.57	27.91±2.28	2.90±0.13	34.84±1.58	2.10±0.14
E45	10.15±0.84	29.07±3.13	2.61±0.23	42.37±16.61	2.31±0.13
E90	14.04±0.62*	33.77±1.77	2.55±0.17	40.95±3.18	2.13±0.09
E120	15.14±1.36*	28.67±2.23	1.91±0.15**	50.95±3.42**	2.03±0.10
E150	8.91±0.67	19.24±1.42*	2.17±0.16**	48.15±3.23*	2.24±0.10
PE3	15.73±1.51*	24.58±2.78	1.69±0.09**	60.72±3.25**	1.79±0.09*
PE6	12.61±0.96	20.75±1.58	1.77±0.09**	57.86±2.91**	2.11±0.09
PE12	14.79±1.38*	32.93±1.34	2.25±0.19**	50.11±7.17*	1.99±0.13
PE24	13.91±1.06*	27.67±1.61	1.96±0.18**	54.48±4.76**	2.07±0.18

Data are means±SEM (N=10). R, sedentary; E45, E90, E120, and E150, exercise for 45, 90, 120, and 150 min, respectively; PE3, PE6, PE12, and PE24, 3, 6, 12, and 24 h postexercise, respectively. RCI, respiratory control index; *P*/*O*, ADP to oxygen consumption ratio.
* $p<0.05$ vs R.
** $p<0.01$ vs R.

Mitochondrial respiratory function

An acute bout of exercise increased the State 4 respiration of skeletal muscle mitochondria in a time-dependent manner (Table 1). State 4 respiration rate was 46 and 58% ($p<0.05$) higher at 90 and 120 min, respectively, compared to the resting rate. However, it returned to the resting level at 150 min. State 4 respiration was elevated during the postexercise period and the increase was significant at PE_3, PE_{12}, and PE_{24} ($p<0.05$).

State 3 respiration rate was not altered until 150 min after exercise, when a 31% reduction ($p<0.05$) was observed. State 3 respiration was not significantly different during postexercise recovery. Mitochondrial respiratory control index (RCI) showed a decline at 120 and 150 min of exercise and during the entire recovery period ($p<0.01$), mainly due to increased State 4 respiration. Conversely, the proton leak rate, i.e., the portion of respiration driven by proton leak, and not for ATP synthesis (State 4 respiration), of the total respiration (State 3 respiration), was increased by exercise during the same period of time ($p<0.05$ or 0.01, Table 1).

The *P*/*O* ratio of the skeletal muscle mitochondria was unchanged in the various treatment groups, except in PE_3, which showed a 15% reduction ($p<0.05$).

Mitochondrial ROS production

To gain some insights into the exercise-induced UCP3 expression, we examined three additional properties of the isolated muscle mitochondria, i.e., generation of ROS during State 4 respiration; cross-membrane potential, $\Delta\psi$; and ATP synthase activity. As shown in Fig. 2, ROS production showed a progressive increase ($p<0.05$) from 0 to

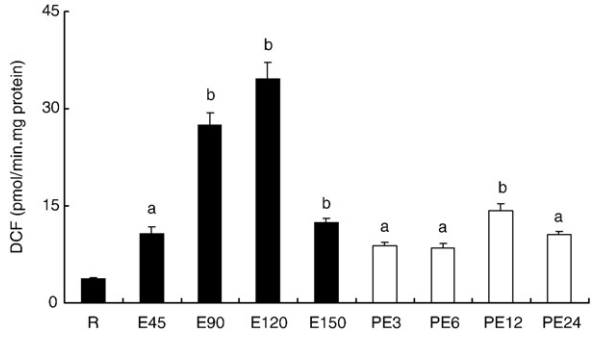

Fig. 2. Rate of DCF formation in rat skeletal muscle mitochondria in response to an acute bout of exercise for 45 (E45), 90 (E90), 120 (E120), and 150 (E150) min and after 3 (PE3), 6 (PE6), 12 (PE12), and 24 (PE24) h recovery. R, rest. Each bar represents the mean±SEM (N=10). [a]$p<0.05$, [b]$p<0.01$ vs the R group.

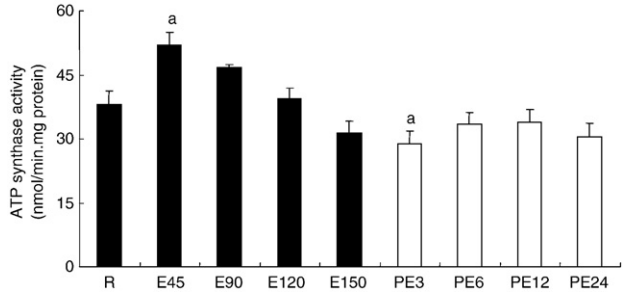

Fig. 3. Mitochondrial ATP synthase activity in rat skeletal muscle in response to an acute bout of exercise for 45 (E45), 90 (E90), 120 (E120), or 150 (E150) min and after 3 (PE3), 6 (PE6), 12 (PE12), or 24 (PE24) h recovery. R, rest. Each bar represents the mean±SEM (N=10).

120 min of exercise and reached ninefold higher ($p<0.01$) at 120 min compared to the resting level. Interestingly, ROS production plunged dramatically at 150 min but was still threefold higher ($p<0.05$) than the rest level. ROS production remained significantly elevated during the 24-h postexercise period ($p<0.05$).

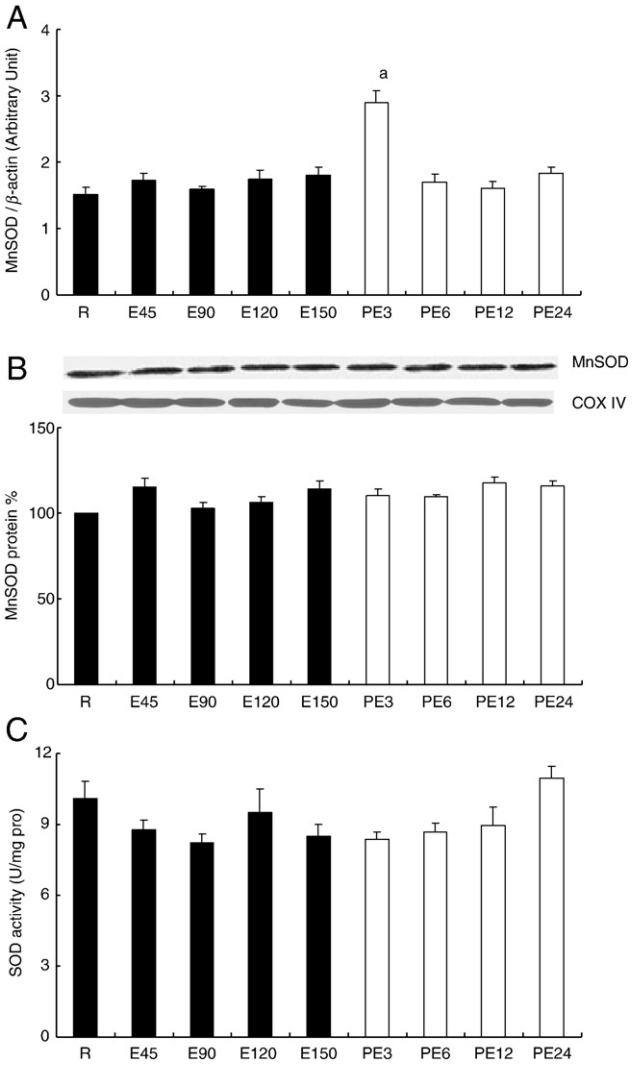

Fig. 4. (A) MnSOD mRNA relative abundance, (B) MnSOD protein content, and (C) MnSOD activity in rat skeletal muscle in response to an acute bout of exercise for 45 (E45), 90 (E90), 120 (E120), or 150 (E150) min and after 3 (PE3), 6 (PE6), 12 (PE12), or 24 (PE24) h recovery. R, rest. Each bar represents the mean±SEM (N=10). [a]$p<0.05$ vs the R group.

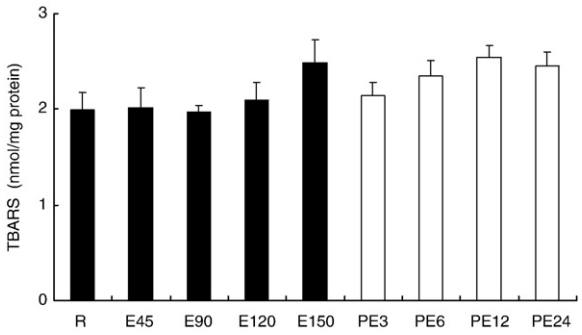

Fig. 5. Thiobarbituric acid-reactive substance (TBARS) levels in rat skeletal muscle mitochondria in response to an acute bout of exercise for 45 (E45), 90 (E90), 120 (E120), or 150 (E150) min and after 3 (PE3), 6 (PE6), 12 (PE12), or 24 (PE24) h recovery. R, rest. Each bar represents the mean ± SEM ($N = 10$).

Mitochondrial membrane potential and ATP synthase activity

Mitochondrial $\Delta\psi$ was measured at 173.0 ± 5.28 mV in the resting state and this level was not altered significantly by exercise or during recovery (data not shown). As shown in Fig. 3, ATP synthase activity was transiently increased ($p < 0.05$) at 45 min of exercise, possibly owing to a rapid increase in ADP formation, followed by a gradual return to basal levels. ATP synthase activity was significantly lower in the PE_3 group ($p < 0.05$) but remained constant thereafter.

MnSOD mRNA, MnSOD protein, and MnSOD activity

MnSOD mRNA concentration was not altered significantly during the acute exercise bout; however, it was increased twofold in the PE_3 group ($p < 0.05$, Fig. 4A). MnSOD protein (Fig. 4B) and activity (Fig. 4C) were not affected during or after the acute bout of exercise.

Mitochondrial TBARS levels

Mitochondrial TBARS content was not significantly affected by an acute bout of exercise or during recovery (Fig. 5).

Discussion

UCP3 is a member of the mitochondrial anion-carrier superfamily found only in the skeletal muscle [4,5]. It has a molecular structure highly homologous to that of UCP1, present primarily in the brown adipose tissue, and UCP2, present more ubiquitously. UCP3 has been proposed to play an important role in regulating energy expenditure, body weight, and thermoregulation; however, the exact physiological role of UCP3 remains elusive. The concept of increasing mitochondrial uncoupling respiration as an antioxidant mechanism is not new and was originally postulated by Papa and Skulachev [23]. In their view, mitochondria utilize multiple strategies to reduce ROS production during electron transfer, including but not limited to (1) mild uncoupling of State 4 respiration to keep the cross-membrane proton potential ($\Delta\mu H^+$) low, (2) opening of inner membrane pores to increase H^+ permeability and "backflow," and (3) ROS-induced inhibition of Krebs cycle enzymes to decrease the electron supply to the ETC. The role of UCPs, however, was not mentioned as a potential means to enhance uncoupling. Upon its discovery in 1997, UCP3 attracted immediate attention as a potential regulator of mitochondrial respiration and energy production in skeletal muscle [24,25]. Several subsequent studies showed that UCP3 expression could be induced by physical exercise and stimulated muscle contraction, raising renewed interest in the significance of the upregulation of this important mitochondrial protein [1–3]. Until now, there have been no data elucidating the antioxidant function of UCP3 during exercise.

The main finding of this study was that UCP3 may participate in mitochondrial antioxidant defense and serve as an "early response" to elevated ROS production and potential oxidative stress by increasing uncoupling respiration during prolonged exercise in rat skeletal muscle. Our data provided several lines of evidence that UCP3 expression may be tightly regulated to match both energy demand and antioxidant defense and play a pivot role in mitochondrial oxidative phosphorylation. First, we found that an acute bout of prolonged exercise dramatically stimulated the gene expression of UCP3, as shown by a seven- to ninefold increase in mRNA level and nearly doubled protein content in a slightly latent fashion. The time-course study suggests that UCP3 upregulation could occur as early as after 45 min of exercise, when a fourfold increase in mRNA level was observed. Interestingly, a significant increase in State 4 respiration did not take place until 90 min after exercise, suggesting that the uncoupled respiration may be dependent upon UCP3 protein expression. At rest, the bulk of State 4 respiration can be accounted for by proton leak and, to a small extent, ATP synthesis, whereas UCP-mediated respiration (UCR) is negligible. Under metabolic demand (i.e., increased substrate utilization and oxygen consumption), the contribution of UCR could be increased in the presence of uncouplers, such as fatty acids and/or UCPs (see below). Supporting this notion was the finding by Ljubicic et al. [6] showing that, in rat, the intermyofibrillar mitochondria uncoupling function of UCP3 could account for 50% of oleic acid-induced elevation of State 4 respiration. The above findings were also consistent with our previous study that, in rat heart, State 4 respiration and UCR were elevated after 120 min of exercise, when UCP2 expression was significantly increased [26].

Second, our data showed that ROS production was increased in a time-dependent fashion during prolonged exercise. However, there was a dramatic drop in ROS from 120 to 150 min of exercise. The reduced ROS production coincided with the highest level of UCP3 protein expression during the same period of time, whereas State 4 respiration markedly plunged. These findings, never reported before, strongly suggest that UCP3 had changed the pattern of electron flow along the ETC, consistent with the proposed role of UCP3 to enhance proton backflow across the mitochondrial inner membrane. During rigorous muscle contraction, oxygen flux and electron flow through the ETC are marked increased, resulting in a more negative $\Delta\psi$, which favors $O_2^{\cdot-}$ generation through the ETC [27]. A modest reduction in $\Delta\psi$ (mitochondrial depolarization) facilitates a tighter association between electrons and the ETC complex, thereby limiting random electron disassociation and $O_2^{\cdot-}$ production [5,28]. We expected to detect a decrease in $\Delta\psi$ as a measurement of $\Delta\mu H^+$ when UCP3 was elevated (120–150 min), but did not see a significant effect owing to the large variability. However, State 3 respiration was decreased at 150 min and RCI was lowered at 120–150 min, indicating a reduced efficiency of oxidative phosphorylation as a result of UCP3 upregulation.

Third, we investigated the gene expression of another important antioxidant in muscle mitochondria, MnSOD, in response to prolonged exercise and found it did not play an important role under the current experimental conditions. MnSOD mRNA was not upregulated until after exercise, whereas MnSOD protein content and enzyme activity were unaltered. Thus, our data suggest that during an acute bout of demanding, but not highly intense, exercise (e.g., 75% VO_2 peak), the primary strategy of mitochondria to reduce oxidative stress is decreasing the production of $O_2^{\cdot-}$ by overexpressing UCP3, instead of enhancing the removal of $O_2^{\cdot-}$ by overexpressing MnSOD. The physiological implication of this preferential regulation is not entirely clear. However, it is conceivable that an upregulation of MnSOD activity will lead to increased H_2O_2 production, which, unless quickly removed, can react with $O_2^{\cdot-}$ or Fe^{2+} to form hydroxyl radical. Also, increasing MnSOD gene expression is a much slower process compared to that of UCP3. Our previous studies have shown that despite a rather rapid rise in MnSOD mRNA levels, MnSOD protein

expression did not increase until 24–48 h postexercise [29,30]. Thus, although not mutually excluded, MnSOD upregulation may represent a long-term cellular strategy to cope with chronic oxidative challenge, such as endurance training, whereas UCP3 regulation serves as an immediate antioxidant response.

An increase in UCP3 gene expression to reduce $\Delta\psi$ and hence O_2^- production is thermodynamically unfavorable, because it decreases the efficiency of oxidative phosphorylation. According to the chemiosmotic theory, the electron transfer in the ETC 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 proton leak [13]. Proton backflow to the matrix side of the mitochondrial inner membrane can be either passive, through the intrinsic pores on the lipid bilayer, or active, facilitated by UCPs [1]. During prolonged muscle contraction, mitochondrial oxygen flux increases by several- up to 20-fold, making the ETC more reduced and $\Delta\mu H^+$ more elevated than at the resting state. It has been demonstrated by electron spin resonance that O_2^- formation resulting from semiquinone and the iron–sulfur protein of NADH reductase (complex I) is greatly increased after intense exercise in rat muscle and liver [2]. UCP3 upregulation could alleviate $\Delta\mu H^+$ and make the ETC slightly uncoupled [31], which inevitably decreases ATP production due to the "waste" of proton motive force. Thus, our work suggests that during prolonged muscular contraction, reducing ROS formation is more vital compared to the efficiency of oxidative phosphorylation, as failure of the former could permanently impair the mitochondria and the long-term welfare of the organism. Of the two "devils," evolution has chosen to accept the lesser devil by overexpressing UCP3 at the expense of energy production. Indeed, skeletal muscle mitochondria isolated from UCP3$^{-/-}$ mice showed higher ROS levels and increased markers of oxidative damage than wild type [32,33]. For example, O_2^--sensitive aconitase activity was decreased by 20–25% in UCP3 knockout mice, indicating an increase in O_2^- production in the mitochondria. No difference between UCP3$^{-/-}$ and wild-type mice was observed in liver mitochondria (liver mitochondria do not express UCP3 protein), suggesting that the effect observed in skeletal muscle was specifically mediated by deficiency of UCP3. According to the above scenario, one may expect the animals with intact UCP3-upregulating capacity to demonstrate less efficiency in producing ATP owing to increased proton shunt. Although the *P*/*O* ratio was kept relatively constant and ATP synthase activity was elevated initially during exercise, we did see a gradual decline in ATP synthase activity during exercise and a significantly lower *P*/*O* ratio 3 h postexercise (Table 1). These findings indicate that elevated UCP3 content produced a progressive effect over time to affect mitochondrial energy metabolism.

The mechanism of UCP3 induction in the mitochondria has been investigated extensively under various experimental conditions. Zhou et al. [7] postulated that activation of AMPK during exercise due to fuel depletion might stimulate UCP3 gene expression in

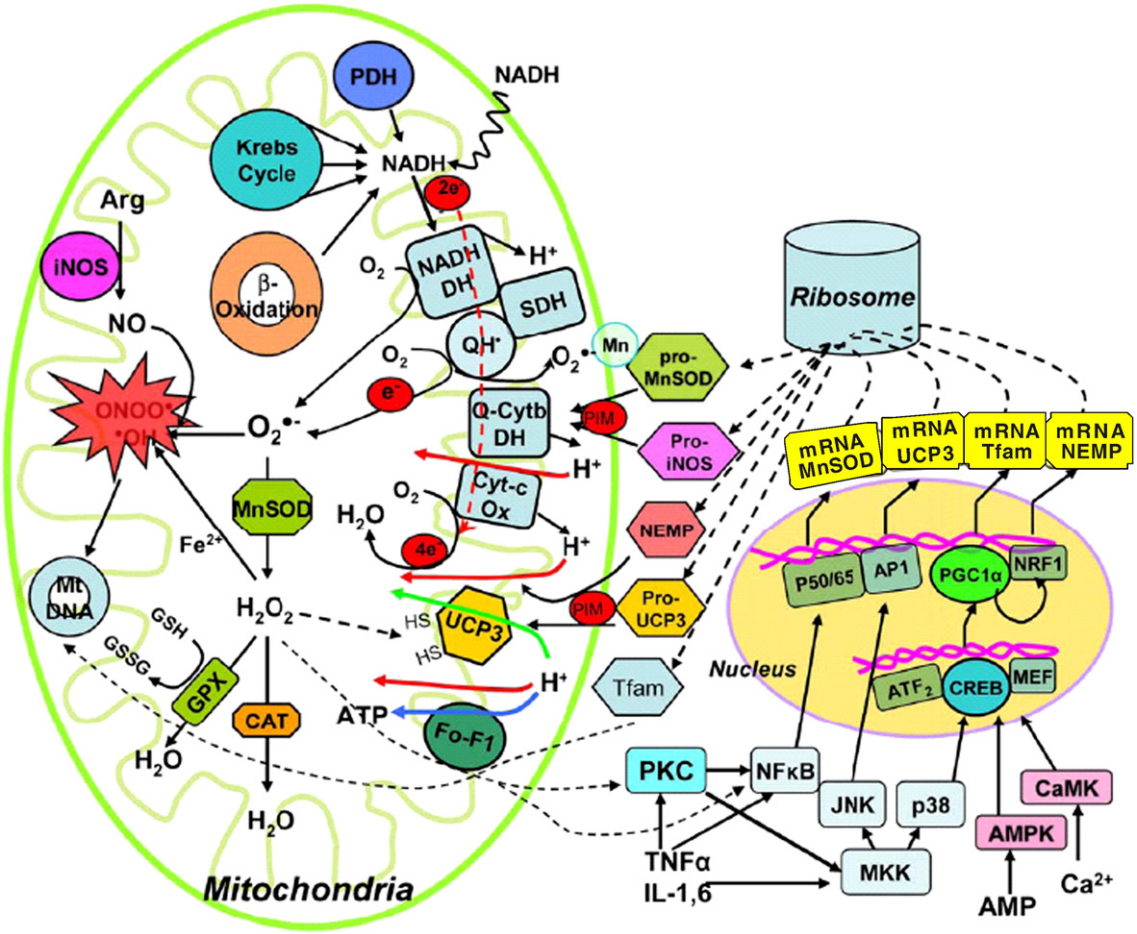

Fig. 6. Hypothetical illustration of UCP3 function and expression in the skeletal muscle. Note that H^+ can flow back to the matrix via (1) ATP synthase (F_0-F_1), (2) membrane pores independent of UCP3, and (3) pump activated by UCP3. Abbreviations used: AP1, activator protein-1; ATF_1, activating transcription factor-1; CAT, catalase; CREB, cAMP response element binding protein; Cyt-c Ox, cytochrome *c* oxidase; GPX, glutathione peroxidase; GSH, reduced glutathione; GSSG, glutathione disulfide; IL-1,6, interleukin-1 or -6; HSF, heat shock factors; MAPK, mitogen-activated protein kinase; MEF, myocyte enhancing factor; MnSOD, manganese superoxide dismutase; NEMP, nuclear-encoded mitochondrial protein; NFκB, nuclear factor κB; NRF1, nuclear respiratory factor-1; PDH, pyruvate dehydrogenase; PGC-1α, peroxisome proliferator-activated receptor γ coactivator 1α; SRF, serum response factor; TNFα, tumor necrosis factor-α.

skeletal muscle. Busquets et al. [34] and Masaki et al. [35] provided evidence that tumor necrosis factor α administration can increase skeletal muscle UCP3 expression, but the mechanism was unrevealed. Echtay et al. [36] reported that 4-hydroxy-2-nonenal (4-HNE), a by-product of lipid peroxidation, as well as structurally related compounds (such as *trans*-retinoic acid, *trans*-retinal, and other 2-alkenals), was a strong inducer of mitochondrial uncoupling through the UCPs. Furthermore, increasing muscle 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 70, peaking at 30–60 min postexercise in the skeletal muscle, heart, and liver of rats [37]. Exercise hyperthermia could be an important factor causing mitochondrial uncoupling (decreasing RCI) and increased $O_2^{\cdot-}$ generation. Recently, Anderson et al. [38] demonstrated that UCP3 gene expression was increased by an acute bout of exercise in mouse gastrocnemius muscle and this upregulation was dependent on mitochondrial H_2O_2 production. In the UCP3$^{-/-}$ mice, however, exercise failed to increase mitochondrial uncoupling respiration despite significantly elevated H_2O_2 production compared to that in the wild-type mice. Our findings that UCP3 mRNA was elevated sharply during the first 90 min of exercise and paralleled ROS production are consistent with the role of H_2O_2. Because the majority of the ROS detected by DCF oxidation could be accounted for by H_2O_2 under the assay condition [39], we postulate that H_2O_2 could be a key species stimulating UCP3 gene expression in the present study. Converted from $O_2^{\cdot-}$ by MnSOD in the mitochondria, H_2O_2 could diffuse out to the cytosol when it is not efficiently removed by glutathione peroxidase and catalase. How H_2O_2 induces UCP gene expression is currently unknown. St-Pierre et al. [40] recently demonstrated that peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1α) plays an important role in the concerted expression of mitochondrial proteins involved in metabolic as well as antioxidant functions in 10 T1/2 cells. UCP3 and UCP2 were among a wide range of genes transactivated by H_2O_2 challenge, such as MnSOD, catalase, and glutathione peroxidase. In the PGC-1α knockout cell line, however, H_2O_2 did not elicit these effects. Because an acute bout of exercise has been shown to activate the PGC-1α signaling pathway by a number of authors [41–45] and by us [46], PGC-1α could be an important mediator in the upregulation of UCP3 in the current study. Fig. 6 is a hypothetical illustration of the function and regulation of UCP3, as well as its relationship with MnSOD and other mitochondrial antioxidant systems. Note that two regulatory cycles may be operational simultaneously during metabolic stress, i.e., the ROS (especially $O_2^{\cdot-}$)-activated proton leak, which elevates State 4 respiration while decreasing RCI and *P*/*O* ratio [47], and the ROS–UCP "double uncoupling cycle," [48] which requires de novo expression of UCP3 with nuclear participation. Both cycles can effectively decrease ROS generation in the mitochondria with a sacrifice of efficiency in producing ATP.

In this study we examined the mitochondrial respiratory properties and UCP3 expression during the postexercise period up to 24 h. Our data show that State 4 respiration and ROS production were elevated significantly above resting levels even a day after exercise, whereas RCI and ATP synthase activity remained low. Our data may also provide a potential partial explanation for a well-studied phenomenon termed "excess postexercise oxygen consumption" (EPOC). For example, Bahr et al. [49] reported that EPOC was proportional to exercise intensity conducted 24 h earlier in human subjects. Two potential mechanisms might be in action despite UCP3 protein having returned to resting levels. First, prior exercise might have caused lipid peroxidation in mitochondria, giving rise to increased levels of 4-HNE, *trans*-retinoic acid, *trans*-retinal, and other 2-alkenals, which are strong stimulants of uncoupled respiration [36]. Second, exercise might have resulted in some structural damage to the mitochondrial membrane, which is known to increase State 4 respiration and further ROS production [31]. There findings were consistent with the results reported by Anderson et al. [38] in mouse gastrocnemius muscle during the 18-h recovery after a single bout of exercise.

In summary, we demonstrated that UCP3 gene expression can be upregulated in response to an acute bout of prolonged exercise in rat skeletal muscle, and the induction could be the result of increased mitochondrial ROS production. The physiological significance of increased UCP3 remains to be verified, but an increased proton backflow to the matrix facilitated by UCP3 may decrease superoxide production and thus serve as an early response of antioxidant protection. Our data suggest that this adaptation precedes MnSOD upregulation temporally and has some functional advantage, and it could be an important molecular mechanism to maintain the integrity of mitochondria at the expense of ATP production and the efficiency of oxidative phosphorylation under potential oxidative stress.

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