Endurance training attenuates the bioenergetics alterations of rat skeletal muscle mitochondria submitted to acute hypoxia: Role of ROS and UCP3

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Abstract: The physiological significance of skeletal muscle mitochondrial uncoupling protein 3 (UCP3) in hypoxia is elusive. In the current study, UCP3 mRNA and protein expressions were investigated along with mitochondrial respiratory function, reactive oxygen species (ROS) generation, as well as manganese superoxide dismutase (MnSOD) expression in rat skeletal muscle with or without endurance training after an acute and severe hypobaric hypoxia exposure for different time. Acute hypoxia induced a series of impairments in skeletal muscle mitochondrial bioenergetics. In untrained rats, UCP3 protein content increased by 60% above resting level at 4 h hypoxia, whereas MnSOD protein content and activity were unaltered. UCP3 upregulation increased mitochondrial uncoupling respiration thus reducing \( \text{O}_2 \cdot^- \) generation, but inevitably decreased ATP production. Training decreased acute hypoxia-induced upregulation of UCP3 protein (67% vs 42%) in rat skeletal muscle. ROS production in trained rats also showed a dramatic decrease at 2 h, 4 h and 6 h, respectively, compared with that in untrained rats. MnSOD protein contents and activities were significantly (50% and 34%) higher in trained than those in untrained rats. Training adaptation of MnSOD may enhance the mitochondrial tolerance to ROS production, and improve mitochondrial efficiency of oxidative phosphorylation. In trained rats, mitochondrial respiratory control (RCR) and P/O ratios were maintained relatively constant despite severe hypoxia, whereas in untrained rats RCR and P/O ratios were significantly decreased. These results indicate that (1) UCP3 mRNA and protein expression in rat skeletal muscle are upregulated during acute and severe hypobaric hypoxia, which may reduce the increased cross-membrane potential (\( \Delta \psi \)) and thus ROS production; (2) Endurance training can blunt hypoxia-induced UCP3 upregulation, and improve mitochondrial efficiency of oxidative phosphorylation due to increased removal of ROS.

Key words: muscle mitochondria; training; hypoxia; uncoupling protein 3; reactive oxygen species

耐力训练抑制急性低氧时骨骼肌线粒体生物能学变化: ROS 和 UCP3 的作用

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摘要: 骨骼肌线粒体解耦联蛋白 3 (uncoupling protein 3, UCP3)在低氧时的生理作用尚不清楚。本研究观察了大鼠在耐力训练前后, 模拟急性高原低氧各时间点的骨骼肌线粒体UCP3 mRNA和蛋白表达、线粒体呼吸功能、活性氧（reactive oxygen species, ROS）产生速率以及锰超氧化物歧化酶(manganese superoxide dismutase, MnSOD)表达和活性的变化。急性低氧导致线粒体一系列生物能学功能障碍。未训练大鼠UCP3 蛋白在 4 h 比静息时升高了 60%, 而 MnSOD 蛋白含量及活性在低氧暴露过程中无显著变化; UCP3 蛋白上调通过降低电子传递链耦联程度抑制 \( \text{O}_2 \cdot^- \) 产生，但同时降低了 ATP 合成效率。耐力训练显著抑制急性低氧诱导的骨骼肌UCP3蛋白上调(67% vs 42%)。训练组大鼠的 ROS 产生速率在低氧 2 h、4 h 和 6 h 时显著低于未训练组。
High-altitude exposure is an aggressive physiological stress inducing wide cellular deleterious effects. Mitochondria are the primary energy-generating organelle in all of the aerobic eukaryotic cells and produce ATP through a process coupled with oxygen consumption termed oxidative phosphorylation. Ultrastrutural data obtained from rat[1] and human[2] skeletal muscle exposed to hypobaric hypoxia revealed significant mitochondrial morphological changes, namely considerable swelling and cristae degeneration. Severe high-altitude hypoxia can cause increased cellular oxidative stress with consequent damage to lipids, proteins, and DNA[3]. Mitochondria reductive stress has been suggested as one possible source of free radicals involved in this apparently paradoxical phenomenon of hypoxia induced oxidative stress. The reduction in oxygen availability that is normally associated with hypoxia may result in the accumulation of reducing equivalents and subsequent electron “leakage” from the electron transport chain (ETC) forming superoxide radicals (O2•−) and other reactive oxygen species (ROS)[4].

Uncoupling proteins (UCPs) are a heterogeneous family of proteins that play an important role in partially dissipating the proton electrochemical gradient across the membrane. UCP3, expressing primarily in the skeletal muscle, is regarded a plausible regulator of trans-membrane proton potential and hence efficiency of oxidative phosphorylation[5]. Several previous studies have shown that UCP3 expression was increased in response to acute hypoxia in mammalian skeletal muscle, which was proposed to be linked to some important cell functions[6,7]. The exact physiological significance of this upregulation, however, is still elusive. Goglia et al.[8] postulated that by translocating fatty acid peroxides from the inner membrane leaflets to the outer ones, UCP may fulfill a role in antioxidant defense of the mitochondria. This intriguing view on UCP3 function prompted us to hypothesize that UCP3 may be induced in skeletal muscle during hypoxia in part to shunt protons back to the matrix and maintain a modest cross-membrane potential (Δψ), thus reducing superoxide radical production and regulating the balance between ATP production and antioxidant function.

Physical training is known to have profound effects on skeletal muscle anatomical structure, physiological and metabolic functions. Skeletal muscle mitochondrial oxidative capacities, 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[9]. This phenomenon usually referred to as cross-tolerance. However, we are not aware of the relation between UCP3 and training-induced muscle cross-tolerance effect after acute hypoxia.

Thus, we have conducted the current study with the following aims: (1) to investigate the effects of acute hypoxia on UCP3 gene expression in rat skeletal muscle mitochondria; (2) to examine the role of UCP3 upregulation in mitochondrial respiratory function and oxidative phosphorylation; (3) to evaluate how hypoxia-induced UCP3 would influence skeletal muscle ROS production and MnSOD gene expression; and (4) to assess how endurance training influences the interactions of UCP3 expression, mitochondrial function and oxidant-antioxidant homeostasis during hypoxia in skeletal muscle.

1 MATERIALS AND METHODS

1.1 Animals and endurance training

Eighty male Sprague-Dawley rats (~2 months, 200-230 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 free access to food and tap water.

Five days after arrival and acclimation, the rats were randomly divided into two groups, trained group (T, n=40) and untrained group (U, n=40). 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, 15 m/min and 5% for 60 min for the 2nd week. Thereafter, exercise intensity and duration were maintained until the end of the 6th week. This protocol was shown to induce mild skeletal muscle hypertrophy and improve the skeletal muscle maximal isometric tension in rats.[9] 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 a training effect.

1.2 Acute hypoxia
At the end of the training period, both T and U rats were randomly divided into four subgroups (n=10) and acutely exposed inside a hypobaric chamber to a simulated atmospheric pressure of 35.9 kPa (270 mmHg), which was equivalent to an altitude of 8,000 m, for 0, 2, 4, or 6 h (referred to as H0, H2, H4, and H6, respectively). The depressurization period reached the simulated altitude of 8,000 m and the pressurization period back to sea-level conditions took 30 min each. Rats were sacrificed immediately after hypoxia stimulation.

1.3 Preparation of skeletal muscle mitochondria
The animals were sacrificed by cervical dislocation at rest or immediately after acute hypoxia 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 (0.12 mol/L KCl, 20 mmol/L HEPES, 5 mmol/L MgCl2, 1 mmol/L EDTA, pH 7.4) with a tissue/buffer ratio of 100 mg/mL. 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.[8] The muscle sample was quickly dissected and placed in ice-cold isolation buffer solution (0.12 mol/L KCl, 20 mmol/L HEPES, 5 mmol/L MgCl2, 1 mmol/L EDTA, pH 7.4) with a tissue/buffer ratio of 100 mg/mL. The remaining muscle samples were immediately frozen in liquid nitrogen and stored at -80 °C.

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 mmol/L KCl, 10 mmol/L HEPES, 1 mmol/L EDTA, 2.5 mmol/L KH2PO4, and 1.5 mg/mL BSA as respiration medium (pH 7.4) and 2 mg of mitochondrial protein. The medium was saturated with ambient oxygen to reach an oxygenic concentration of 258 μmol/L. After 3 min equilibration period, mitochondrial respiration was initiated by adding 2 mmol/L succinate and 1 μmol/L rotenone. After stable state 4 respiration was established, state 3 respiration was initiated by the addition of 200 mmol/L 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.[12]

1.5 Mitochondrial ROS production
Skeletal muscle mitochondrial ROS generation was determined in fresh mitochondrial suspensions using dichlorofluoroscein (DCF) as a probe according to Bejma et al. Under the defined experimental conditions the majority of oxidants measured were H2O2. Briefly, 1 mg mitochondria was added to a quartz cuvette containing 2 mL of 0.1 mol/L phosphate buffer (pH 7.4), and 2 μL of 2.5 mmol/L dichlorofluoroscin acetate (DCFH-DA). 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 2.5 mmol/L DCFH-DA without mitochondria was used to correct autooxidation rate of DCFH-DA. The units were expressed as pmol DCF formed per minute per milligram of protein.

1.6 Mitochondrial membrane potential and ATP synthase activity
The isolated mitochondrial membrane potential (ΔΨ) of permeabized cells was measured by monitoring the fluorescence spectrum of Rhodamine 123 at the excitation-emission wavelength of 500-525 nm[14]. The experiments
were performed at 25 °C in 2 mL of incubation medium containing 0.25 mol/L sucrose, 3.0 mmol/L HEPES, and 0.5 mmol/L EDTA (pH 7.4) with 0.5 mg mitochondrial protein.

ATP synthase activity was determined using a bioluminescence technique[15]. Mitochondrial suspensions were added to a cuvette containing 0.1 mol/L luciferase (Sigma Co.), 0.25 mol/L sucrose, 3.0 mmol/L HEPES, 0.5 mmol/L EDTA, and 2 mmol/L succinate + 1 μmol/L rotenone as substrate. After a background bioluminescence was established for correction, 4 μmol/L of ADP was added to initiate the action. ATP production was monitored at 25 °C with a BioOrbit 20/20n luminometer (Turku, Finland) and expressed as nmol/s per milligram of protein.

1.7 UCP3 and MnSOD mRNA
UCP3 and MnSOD mRNA expression was determined in skeletal muscle by real-time quantitative PCR (RT-PCR). Total RNA was extracted from 0.1 g tissue using the TRIzol reagent (Mrcgene), according to the manufacturer’s instruction. Total RNA (5 μg) was denatured at 70 °C for 5 min and reversely transcribed using 200 units of Moloney murine leukaemia virus reverse transcriptase (RevertAid), 0.5 μg Oligo (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 MgCl₂, 2.0 μL DNA Master SYBR Green I 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 and annealing at 72 °C for 60 s) for 40 cycles. Primer pairs for the real-time PCR were[16]: UCP3 gene (179 bp): 5'-GGAGCCATGGCAGCTGTG-3', 5'-TGATGGTTGGGCAAAGTCCC-3'; SOD gene (383 bp): 5'-GCCAAGCTCTCAACAACTGGAACG-3', 5'-TCAAATCCCAGCAGTGAAAGGC-3'; β-actin gene: 5'-TGTTGCGGTATGGGTCAGAGGAAGC-3', 5'-CATGGGCTGGGCTGTGGAAGGCTCA-3'.

The specificity of the SYBR Green PCR signal was confirmed by melting curve analysis to demonstrate that only a single amplified product for each PCR reaction was obtained. mRNA expression was quantified using the comparative Ct (threshold cycle, numbers of the PCR cycle that overpassed the signal threshold that could be detected by the LightCycler 2.0) method[17]. The Ct of the internal control gene β-actin was subtracted from Ct of the target gene to obtain ΔCt (ΔCt =Ct_target - Ct_β-actin). The normalized fold changes of each target mRNA expression were expressed as the 2^ΔCt.

1.8 Expression of UCP3 and MnSOD protein
Mitochondria were lysated by resuspending them in SDS loading buffer, and then heated for 5 min at 100 °C. Ten-microgram protein aliquots from the mitochondrial lysates were loaded in each lane and separated in a 15% SDS-PAGE gel. Proteins were transferred to polyvinylidene difluoride membranes and immunological detection was performed by using a polyclonal antibodies against UCP3 (a rabbit affinity-pure UCP3 antiserum ADI, USA), a polyclonal antibodies against MnSOD (a rabbit affinity-pure MnSOD antiserum, Sant Cruz, USA), respectively, at: 1: 1 000 dilution, and an anti-rabbit antibody as secondary antibody. The detection was achieved by using the enhanced chemiluminescence (ECL) detection system. Because hypoxia can induce changes in mitochondrial composition[19], we used cytochrome C oxidase IV (COX IV), a protein marker of mitochondria, the confidential reference items for correction, in order to avoid the disturbance of hypothesis that UCP3/MnSOD protein concentration changes as a component of the changes in skeletal muscle mitochondria induced by hypoxia. The data that were normalized to COX IV expression were used for statistical analysis of target protein expression.

1.9 MnSOD activity
SOD activity was measured using xanthine peroxidase method[19], where one unit of SOD activity was defined as the amount of enzyme that causes a 50% inhibition of epinephrine autooxidation to adrenochrome. For MnSOD activity, 1 mmol/L KCN was added to the reaction mixture to inhibit Cu/Zn SOD activity.

1.10 Statistical analysis
The data were analyzed by Statistical Package for the Social Sciences (SPSS Inc., version 12.0) to assess the statistical difference and expressed as mean±SD. Data were analyzed with two-way ANOVA with the two main factors being hypoxia time and training. When a significant main effect was detected, the Bonferroni post-hoc test was used to compare differences between means. The significance level was set at P<0.05.

2 RESULTS

2.1 Mitochondrial respiratory function
As illustrated in Table 1, the acute and severe hypobaric hypoxia induced a significant impairment in the respiratory rates of isolated skeletal muscle mitochondria energized with succinate and rotenone in a time-dependent manner.
Acute hypoxia significantly decreased state 3 respiration by 14% \((P<0.05)\), 21% \((P<0.05)\) and 26% \((P<0.01)\) at 2 h, 4 h and 6 h, respectively, compared with resting level in U rats. Training induced a significant protective effect in state 3 respiration, as T rats showed only 9% \((P<0.05)\), 17% \((P<0.05)\) and 19% \((P<0.05)\) decrease in state 3 respiration at the respective time points. On the other hand, State 4 respiration rate was 17% \((P<0.05)\) and 15% \((P<0.05)\) higher at 4 h and 6 h, respectively, compared to the resting level in U rats. Training significantly decreased hypoxia-induced state 4 respiration, as T rats showed only 5% \((P<0.05)\) and 7% \((P<0.05)\) increase in state 4 respiration at the respective time points. As a result, mitochondrial RCR declined over hypoxia time, showing 27% \((P<0.05)\) and 32% \((P<0.05)\) reduction at 4 h and 6 h, respectively, in U rats. In contrast, RCR was well-maintained in T rats and showed only 17% \((P<0.05)\) and 19% \((P<0.05)\) decrease at the respective time points. The P/O ratio of skeletal muscle mitochondria significantly decreased by 31% \((P<0.05)\) and 28% \((P<0.05)\) at 4 h and 6 h, respectively, in U rats. Overall, T rats demonstrated a higher P/O ratio than U rats and the differences were significant at 4 h and 6 h \((P<0.05)\).

### 2.2 UCP3 mRNA and UCP3 protein

An acute and severe hypobaric hypoxia dramatically increased UCP3 mRNA levels in both U and T rats (Fig. 1A). For U rats, UCP3 mRNA was 7-fold \((P<0.01)\) and 2.5-fold \((P<0.05)\) of resting level after 2 h and 4 h of hypoxia, respectively. The hypoxia-induced increased UCP3 mRNA was significantly less in T rats \((P<0.05)\), although it was still elevated by ~4-fold \((P<0.05)\) and

![Fig. 1](https://via.placeholder.com/150)

**Fig. 1.** Relative abundance of UCP3 mRNA (A) and protein (B) in response to an acute and severe hypobaric hypoxia in skeletal muscle of untrained and trained rats. H₀, H₂, H₄, H₆: Acute hypoxia (simulated altitude of 8 000 m) for 0, 2, 4, and 6 h, respectively. *P<0.05, **P<0.01 vs H₀; #P<0.05, Trained vs Untrained at respective time point.

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### Table 1. Mitochondrial respiratory functions and ATP synthase activity in rat skeletal muscle

<table>
<thead>
<tr>
<th>Group</th>
<th>H₀</th>
<th>H₂</th>
<th>H₄</th>
<th>H₆</th>
</tr>
</thead>
<tbody>
<tr>
<td>State 3 respiration rate</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Untrained</td>
<td>56.15±8.66</td>
<td>48.29±7.98*</td>
<td>44.43±7.61*</td>
<td>41.79±8.75**</td>
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<td>Trained</td>
<td>55.17±10.03</td>
<td>52.29±9.15*</td>
<td>47.62±8.52*</td>
<td>46.85±7.59**</td>
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<tr>
<td>State 4 respiration rate</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Untrained</td>
<td>16.63±3.18</td>
<td>16.28±3.06</td>
<td>19.17±3.15*</td>
<td>19.09±3.13*</td>
</tr>
<tr>
<td>Trained</td>
<td>16.16±3.16</td>
<td>16.37±3.24</td>
<td>17.63±2.95*</td>
<td>17.25±3.11*</td>
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<tr>
<td>RCR</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Untrained</td>
<td>3.27±0.75</td>
<td>3.04±0.66</td>
<td>2.39±0.71*</td>
<td>2.23±0.63*</td>
</tr>
<tr>
<td>Trained</td>
<td>3.35±0.59</td>
<td>3.21±0.73</td>
<td>2.78±0.77*</td>
<td>2.73±0.69*</td>
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<tr>
<td>P/O</td>
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<td></td>
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<tr>
<td>Untrained</td>
<td>2.19±0.27</td>
<td>1.99±0.23</td>
<td>1.51±0.30*</td>
<td>1.57±0.27*</td>
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<tr>
<td>Trained</td>
<td>2.25±0.31</td>
<td>2.05±0.25</td>
<td>1.84±0.22*</td>
<td>1.71±0.29*</td>
</tr>
<tr>
<td>ATP synthase</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Untrained</td>
<td>27.15±2.17</td>
<td>28.41±3.56</td>
<td>25.69±2.93</td>
<td>26.52±2.85</td>
</tr>
<tr>
<td>Trained</td>
<td>36.42±3.08*</td>
<td>34.29±2.49*</td>
<td>35.17±3.25*</td>
<td>34.28±2.99*</td>
</tr>
</tbody>
</table>

Data are mean±SD \((n=10)\). Unit: nmol O₂/min per mg protein, except ATP synthase (nmol/min per mg protein). H₀, H₂, H₄, H₆: Acute hypoxia for 0, 2, 4, and 6 h, respectively. *P<0.05 vs H₀; **P<0.01 vs H₀; *P<0.05, Trained vs Untrained at respective time point. RCR, respiratory control ratio; P/O, ratio between phosphorylated ADP and oxygen consumed; ATP synthase, ATP synthase activity.
−1-fold ($P<0.05$) above resting level at the respective times. UCP3 mRNA abundance returned to resting levels at 6 h of hypoxia in both U and T rats.

UCP3 protein content in the muscle showed a latent response compared with its mRNA (Fig. 1B). In U rats, a 67% increase ($P<0.05$) in UCP3 protein content was observed at 4 h, whereas in T rats the increase was only 42% ($P<0.05$). Thus, endurance training decreased the hypoxia-induced UCP3 protein expression in rat skeletal muscle mitochondria.

2.3 ROS generation, $\Delta \psi$ and ATP synthase activity

In order to gain some insights into the hypoxia-induced UCP3 expression, we examined three additional properties of the isolated skeletal muscle mitochondria, i.e., generation of ROS during state 4 respiration, $\Delta \psi$ and ATP synthase activity. As shown in Fig. 2A, ROS production elevated by 99% at 2 h ($P<0.01$) in U rats, followed by a dramatic drop at 4 h and 6 h, although its level remained to be 68% ($P<0.05$) and 73% ($P<0.05$) higher, respectively, than resting level. ROS generation was lower in T rats than that in U rats overall ($P<0.05$) with maximum elevation above resting levels found at 2 h. ROS production in T rats also showed a dramatic decrease at 4 h ($P<0.05$) and 6 h ($P<0.05$).
Mitochondrial $\Delta\psi$ was increased in U rats after 2 h of hypoxia, followed by a decrease at 4 h ($P<0.05$) and 6 h ($P<0.05$), respectively (Fig. 2B). T rats had lower $\Delta\psi$ compared with U rats ($P<0.05$), though a significant increase in $\Delta\psi$ was also observed at 2 h of hypoxia ($P<0.05$), followed by a decline at 4 h ($P<0.05$) and 6 h ($P<0.05$), respectively.

ATP synthase activity was unaffected by acute hypoxia in either U or T rats (Table 1). However, the enzyme activity was significantly higher in T rats than that in U rats regardless of hypoxia status ($P<0.05$).

2.4 MnSOD mRNA, protein and activity
Acute hypoxia for 4 h increased MnSOD mRNA abundance (Fig. 3A) by more than 1.6 folds in the skeletal muscle of both U and T rats ($P<0.05$). MnSOD protein content (Fig. 3B) and activity (Fig. 3C) were not affected by hypoxia, but was significantly higher in T rats than that in U rats at resting state and after acute hypoxia ($P<0.05$).

3 DISCUSSION
UCP3 is a member of the mitochondrial anion carrier super family found only in the skeletal muscle$^{[5]}$. It has a molecular structure highly homologous to that of UCP1 found primarily in the brown adipose tissue, and UCP2 found more ubiquitously, and 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 a means of antioxidant is not new and was originally postulated by Papa and Skulachev$^{[20]}$. 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 cross-membrane proton potential ($\Delta\mu H^+$) low; (2) opening of inner membrane pores to increase $H^+$ permeability and “back flow”; (3) ROS-induced inhibition of Kreb Cycle enzymes to decrease electron supply to ETC. The role of UCPs, however, was not mentioned as a potential means to enhance uncoupling. Upon discovery in 1997, UCP3 attracted immediate attention as a potential regulator of mitochondrial respiration and energy production in skeletal muscle$^{[21]}$. Until now, there has been no data elucidating the physiological significance of UCP3 during hypoxia.

Data from the present study supported our hypothesis and provided new insights into UCP3 regulation during hypoxia in several aspects. First, this is the novel report that acute and severe hypobaric hypoxia could increase UCP3 expression in rat skeletal muscle. UCP3 mRNA showed a remarkable 6-fold increase and UCP3 protein content showed a remarkable 1.6-fold increase. Second, the UCP3 upregulation was associated with increases in mitochondrial state 4 respiration, $\Delta\psi$ and the generation of ROS (mostly $H_2O_2$), as a result of hypoxia. 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 either be used to drive ATP synthesis through $F_0F_1$-ATPase, or to generate heat through the so-called “proton leak”$^{[22]}$. However, proton back flow to the matrix side of inner membrane can be either passive through the lipid bilayer or facilitated by UCPs. An upregulation of UCP3 could alleviate cross-membrane pressure caused by $\Delta\mu H^+$, thus reducing the chance of $O_2^-$ generation due to “electron spill”$^{[20]}$. This notion was supported by the findings that UCP3 mRNA and protein expression were increased at certain hypoxia exposure time, which contributed to the bulk of the increased state 4 respiration. Furthermore, when UCP3 protein expression reached the highest level at 4 h, both $\Delta\psi$ and ROS production showed a dramatic decrease (Fig. 2A and Fig. 2B). As described by Amara et al., a small decrease in $\Delta\psi$ (10 mV) was enough to abolish around 55% of the ROS production$^{[23]}$. In the present study, although $\Delta\psi$ was decreased by only 7% at 4 h compared with 2 h, it was enough to decrease ROS production (decreased by 40% at 4 h compared with 2 h).

We also investigated the gene expression of another important antioxidant in muscle mitochondria, MnSOD, in response to acute and severe hypoxia and found it did not play an important role at the present experimental condition. MnSOD mRNA was not upregulated until 4 h, whereas MnSOD protein content and enzyme activity were also unaltered. Thus, our data suggest that during an acute and severe hypoxia, the primary strategy of mitochondria to reduce oxidative stress is decreasing the production of $O_2^-$ by over-expressing UCP3, instead of enhancing the removal of $O_2^-$ by over-expressing MnSOD. The physiological implication of this preferential regulation is not entirely clear. However, it is conceivable that increasing MnSOD gene expression takes considerable energy and is a much slower process compared to UCP3 upregulation. Our previous studies have shown that despite a rather rapid rise of MnSOD mRNA level, MnSOD protein expression did not increase until 30 h post exercise$^{[10]}$. Thus, MnSOD upregulation may represent a long term cellular strategy to cope with chronic oxidative challenge, such as long term...
hypoxia exposure, whereas UCP3 regulation serves as an immediate antioxidative response.

An increase in UCP3 gene expression to reduce Δψ and hence O$_2^-$ production is thermodynamically unfavorable, because it decreases the efficiency of oxidative phosphorylation. In mitochondrial, the potential energy of the proton gradient is not used to phosphorylate ADP to ATP, and UCPs represent a mechanism by which protons can re-enter the mitochondrial matrix bypassing ATP synthesis. Cellular hypoxia is a state that is generally characterized by being in a more cellular reductive state and in some cases has been described as a form of “reductive stress.” This reflects the elevations in reducing equivalents that build up, particularly in the mitochondria, when insufficient O$_2$ is available for reduction by the ETC. This buildup of reducing equivalents also makes electrons more available for reduction reactions such as the reduction of O$_2$ to superoxide. UCP3 upregulation could alleviate Δψ and make ETC more tightly coupled thus reducing O$_2^-$ generation, inevitably decreasing ATP production due to the “waste” of proton motive force. Thus, our work suggest that during acute and severe hypoxia, reducing ROS generation is more vital compared to maintaining 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 over-expressing UCP3 at the expense of energy production. The coupled respiratory control and the phosphorylation efficiency of mitochondria from mice previously submitted to acute hypoxia were clearly affected (Table 1), as inferred from the result that both RCR and P/O decreased dramatically, when UCP3 protein expression reached the highest level at 4 h. These findings suggest that the hypoxia induced UCP3 upregulation, resulting in a lower P/O ratio and Δψ due to increased proton shunt.

The mechanism of UCP3 induction in the mitochondria has been investigated extensively under various experimental conditions. Zhou et al. postulated that activation of AMPK during hypoxia due to fuel depletion might stimulate UCP3 gene expression in skeletal muscle. Echtay et al. reported that 4-hydroxy-2-nonenal (4-HNE), a by-product of lipid peroxidation, was strong inducer of mitochondrial uncoupling through the UCPEs. A limitation of this scenario is that lipid peroxidation products usually remain in the membrane lipid phase and are unlikely to travel to the nucleus and affect UCP3 mRNA transacitvation seen in our study. Our findings showed that UCP3 mRNA was elevated during the first 2 h of hypoxia and paralleled with H$_2$O$_2$ production. We postulate that H$_2$O$_2$ could be a key species to stimulate UCP3 gene expression in the present study. In cultured hepatocytes lipid emulsions have been shown to increase the DNA-binding activity of nuclear factor (NF) κB leading to a dose- and time-dependent induction of UCP2 transcripts. Since H$_2$O$_2$ can enhance NFκB nuclear binding, this signaling pathway could serve as a potential mediating way of UCP3 gene expression.

Our study demonstrated that endurance training decreased the acute hypoxia-induced UCP3 mRNA and protein expressions in rat skeletal muscle. This finding raised some interesting questions regarding the relationship between mitochondrial respiratory uncoupling, ROS generation, antioxidant defense and ATP synthesis. An ameliorated hypoxia response of UCP3 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 skeletal muscle under metabolic stress, therefore the organism cannot afford to have excessive amount of proton shunt through UCP3 rather than though F$_o$-F$_1$ complex to produce ATP. 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 protein content and activity in rat skeletal muscle were significantly (~50% and ~34%) higher in T rats than that in U ones. This could explain why T rats had lesser upregulation of UCP3 protein compared to U rats (67% vs 42%) after an acute and severe hypobaric hypoxia, because MnSOD had removed a portion of O$_2^-$ as a potential stimulator of UCP3 (see previous). T rats also had displayed lower mitochondrial ROS generation in response to hypoxia possibly due to a training-induced glutathione peroxidase activity. This would reduce the production of lipid peroxidation by-products, such as 4-HNE as an UCP3 activator in the T rats. McLeod et al. reported that in the myocardial mitochondria from Sod2 mice, ATP production was markedly reduced due to a higher O$_2^-$ concentration which activated UCP3 excessively. Taken together, training adaptation of skeletal muscle MnSOD may enhance mitochondrial tolerance to ROS production and hence a smaller UCP3 activation during severe hypoxia, thus protecting the efficiency of oxidative phosphorylation.

The antioxidation of UCP3 is important in forepart of acute hypoxia, though may not maintain the energetic capacity of mitochondria concurrently. Indeed, in T rats mitochondrial RCR and P/O ratio were maintained relatively constant despite severe hypoxia, whereas in U rats RCR and P/O ratio were significantly decreased.
What factor(s) compensate the depression of mitochondrial oxidative phosphorylation which was the by-product of UCP3 antioxidation? Present data showed that, in T rats, ATP synthase activity was higher (34%) than that in U rats at corresponding point. The concept proposed by Kadenbach may explain these phenomenon: 1) a relatively minor alterations of the inner mitochondrial membrane potential at high physiologic inner mitochondrial membrane potential significantly alter ETC ROS generation and 2) the maximal rate of ATP synthase energy generating capacity that occurs at low physiologic mitochondrial membrane potential can not be augmented by further increasing membrane potential.

In summary, we demonstrated that UCP3 gene expression is upregulated in response to an acute and severe hypobaric hypoxia in rat skeletal muscle, and the induction could be the result of increased mitochondrial ROS production. The physiological significance of increased UCP3 remain to be verified, but an increased proton back flow to matrix facilitated by UCP3 may decrease superoxide production thus serve as an early response of antioxidant protection. Our data suggest that this adaptation precedes MnSOD upregulation temporally and functionally, and could be an important molecular mechanism to maintain the integrity of mitochondria at the expense of the efficiency of oxidative phosphorylation under potential oxidative stress. Endurance training attenuated, but did not abolish, UCP3 activation due to the upregulation of MnSOD and ATP synthase. These adaptations optimize the skeletal muscle to reach maximal efficiency of energy metabolism while minimizing hypoxia-induced oxidative stress.

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