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Response of mitochondrial fusion and fission protein gene expression to exercise in rat skeletal muscle

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ABSTRACT

The purpose of this study was to investigate the changes in the gene expression of Mitofusion (Mfn) 1 and 2 and Fission 1 (Fis1) and mitochondrial energy metabolism in response to altered energy demand during prolonged exercise in rat skeletal muscle. Male Sprague–Dawley rats were subjected to an acute bout of treadmill running at various durations and killed immediately or during recovery. Mfn1/2 and Fis1 mRNA and protein contents, reactive oxygen species (ROS) generation, state 3 and state 4 respiration rates, trans-innermembrane potential and ATP synthase activity were measured in isolated muscle mitochondria. We found that (1) Mfn1/2 mRNA contents were progressively decreased during 150 min of exercise, along with decreased Mfn 1 protein levels. Fis1 mRNA and protein contents showed significant increases after 120–150 min of exercise. These changes persisted through the recovery period up to 24 h. (2) Mitochondrial ROS generation and state 4 respiration showed progressive increases up to 120 min, but dropped at 150 min of exercise. (3) State 3 respiration rate and respiratory control index were unchanged initially but decreased at 150 and 120 min of exercise, respectively, whereas ATP synthase activity was elevated at 45 min and returned to resting level thereafter. Our data suggested that the gene expression of mitochondrial fusion and fission proteins in skeletal muscle can respond rapidly to increased metabolic demand during prolonged exercise, which could significantly affect the efficiency of oxidative phosphorylation.

1. Introduction

Mitochondria are highly dynamic organelles whose morphology, distribution, and activity can be regulated by fusion and fission [1]. Mitochondria adopt different shape, localization and motility depending on the cell type and the metabolic demand of the cell. Time-lapse microscopy of living cells reveals that mitochondria take on a wide variety of shapes, ranging from long interconnected tubules to individual small spheres, in mammalian cells. Even in cells with a seemingly stable network of mitochondrial tubules, there are frequent and continual cycles of mitochondrial fusion and fission, opposing processes that exist in equilibrium and serve to maintain the overall architecture of these organelles [2–5].

The central role of mitochondria in cellular energy production was demonstrated by the discovery of the respiratory chain and by the early investigations of oxidative phosphorylation [6]. However, the importance of mitochondrial network integrity was not evaluated in these important functional studies. It has now become clear that mitochondria exist in living cells as a large tubular assembly, extending throughout the cytosol [7], and in close contact with the nucleus, endoplasmic reticulum [8], Golgi network and cytoskeleton [9,10]. While the relationship between mitochondrial morphology and function under various physiological and pathological conditions remains unclear, it is certain that mitochondria participate in a wide range of cellular processes via multiple pathways [11,12].

Several proteins of the key fusion and fission machinery have been identified, and experimental systems have been developed to understand their molecular structure and mechanisms to regulate mitochondrial dynamics. In mammalian cells, mitochondrial fusion process is largely controlled by Mitofusin 1 and 2 (Mfn1/2), the highly conserved GTPases localized in the mitochondrial outer membrane [13]. Downregulaton of Mfn1/2 has been shown to lead to fragmented mitochondria with greatly reduced oxygen consumption and ATP generation, whereas a block in fusion could result in smaller mitochondria with either missing mtDNA or enriched with mutant mtDNA [14–17]. Fis1 and the dynamin-related protein 1 (Drp1) are core components of the mammalian mitochondrial fission machinery. Fis1 is located throughout the outer membrane surface and recruit Drp1 to puncture the fission points. Over-expression of Fis1 leads to

Abbreviations: Mfn1/2, mitofusin1/2; Fis1, fission 1; Drp-1, dynamin-related protein-1; ROS, reactive oxygen species; RCI, respiration control index

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mitochondrial fragmentation [18–20]. Mice lacking either Mfn1 or Mfn2 die in the uterus at approximately embryonic days 10.5–11.5 [5]. Mutation in Mfn2 causes the phenotype of Charcot-Marie-Tooth subtype 2A (CMT2A), a peripheral neuropathy characterized by muscle weakness and sensory loss in the distal limbs [21].

Strenuous physical exercise dramatically alters energy demand to the skeletal muscle and therefore is expected to influence mitochondrial dynamics. However, the effect of exercise on mitochondrial fusion and fission has been studied only sparsely. Cartoni et al. [22] showed that Mfn1/2 mRNA levels were increased in human vastus lateralis muscle 24 h after an acute bout of exercise along with elevated peroxisome proliferator-activated receptor γ co-activator-1α (PGC-1α) and nuclear respiratory factor (NRF)-2 expressions. Garnier et al. [23] demonstrated that in healthy human VO_2peak was dependent on coordinated expression of PGC-1α and its downstream signaling protein levels, and that PGC-1α was positively correlated to Mfn2 and Drp1. However, the interactions between altered muscle metabolic rate and mitochondrial fusion and fission protein expression remain elusive.

In the current study, we employed a prolonged treadmill running protocol with incremental durations to impose differential metabolic stress to rat skeletal muscle and examined Mfn1/2 and Fis1 gene expression. We also assessed respiratory function and ATP synthase activity as indication of mitochondrial oxidative phosphorylation. The data suggest that the dynamic changes in mitochondrial fusion and fission protein expression may be an integral part of mitochondrial adaptation to altered metabolic demand.

2. Materials and methods

2.1. Animals

Male Sprague–Dawley rats (age 8 weeks) were housed in a temperature-controlled (21–22 °C) room with a 12:12-h light–dark cycle. Rats were maintained on a standard rodent chow diet and water ad libitum until the treadmill running experiment began when food was withheld for all experimental groups. All experiments were approved by the Institutional Review Board of the Tianjin University of Sport under the guidelines of the Chinese Academy of Sciences.

2.2. Acute exercise model

Rats were randomly divided to either resting controls (R, $N=10$), or one of the eight exercised groups ($n=10$) with a protocol described previously [24]. All groups started running on a motor-driven treadmill for 15 min at 8.2 m/min, 0° grade (~53% of maximum oxygen consumption [Vo_{2max}]), followed by 15 min at 15 m/min, 5° grade (~64% Vo_{2max}). Thereafter, four groups of rats ran at 19.3 m/min, 10° grade (~76% Vo_{2max}) continuously for 15, 60, 90, and 120 min and killed immediately after exercise, referred to as E-45, E-90, E-120, and E-150, respectively. Four additional groups of rats performed the exercise protocols described above and skilled at 3, 6, 12, or 24 h after running for 150 min (referred to as PE-3h, PE-6h, PE-12h, and PE-24h, respectively). Rats were killed by injection of an overdose of sodium thiopentobarbitol (150 mg/kg body wt). Hindlimb muscles were quickly excised, with one portion used for mitochondrial preparation (see below) and a second portion rapidly frozen between tongs cooled with liquid nitrogen, and stored at −80 °C.

2.3. Mitochondrial isolation

Mitochondria from harvested muscle were prepared using differential centrifugation as described previously [25]. Briefly, muscle samples were washed in *buffer A* (50 mM Tris buffer, pH 7.4, 100 mM KCl, and 5 mM EDTA), minced in *buffer A* supplemented with 1 mM ATP (*buffer B*) (1:5, wt/vol), and incubated with Nagarse (1 U/mg of muscle; Sigma Chemical) for 5 min. Digestion was stopped by addition of *buffer B* (1:30, wt/vol). Muscle samples were then 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) and centrifuged at 5000 × *g* for 10 min. The pellets were resuspended in *buffer B* (1:35, wt/vol) and centrifuged at 800 × *g* for 10 min. The supernatant was retained, whereas the remaining pellets were suspended in *buffer B* (1:35, wt/vol) and centrifuged as above. Both supernatants were then combined and centrifuged at 9000 × *g* for 10 min. The resulting mitochondrial pellets were resuspended in *buffer B* (1:10, wt/vol) and centrifuged again at 9000 × *g* for 10 min. The final mitochondrial pellets were diluted (1:1, wt/vol) in 10 mM Tris, pH 7.4, 1 mM EDTA, 700 mM mannitol, and 220 mM sucrose solution. Mitochondrial protein content was assayed using γ-globulin as a standard according to Bradford [26].

2.4. Mitochondrial respiration

Mitochondrial oxygen consumption was monitored at 25 °C in a thermostatically controlled chamber equipped with a Clarke oxygen electrode (YSI, USA). The 3-ml respiration buffer was prepared as follows: 130 mM KCl, 3.0 mM Hepes, 500 μM EDTA, 2.0 mM KH_2PO_4, 1 mg/ml BSA, 10 mM Tris–HCl pH 7.4 and substrates (2.5 mM L-malate, 5 mM pyruvate). The mitochondrial protein concentration used for respiration measurements was 2 mg. After a stable state 4 respiration rate was established, state 3 respiration rate was obtained by the addition of 200 μM final concentration of ADP. Respiration rates were expressed in nmol O_2/minute/mg protein. The respiratory control index (RCI) was calculated as the ratio of state 3 to state 4 respiratory rates.

2.5. Mitochondrial ROS production

Muscle mitochondrial ROS generation was monitored using the dichlorofluorescin diacetate (H_2-DCFDA) probe. Briefly, the dichlorofluorescin diacetate (H_2-DCFDA) 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 mitochondrial protein 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 H_2-DCFDA (total volume 0.3 ml). The assay mixture was incubated at 37 °C for 15 min to allow the H_2-DCFDA 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 H_2-DCFDA without mitochondria was used to correct the autoxidation rate of H_2-DCFDA. The units were expressed as picomole of DCF formed per minute per milligram of protein.

2.6. 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 [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.

2.7. Mitochondrial ATP synthase activity

ATP synthase activity was determined using a bioluminescence technique [28]. 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 20/20n

Table 1
Real-time PCR primer sequences.

Gene	Forward Primer (5′-3′) Reverse Primer (5′-3′)	Tm	PCR product Size (bp)
mfn1	ccttgtacatcgattcctgggttc	60 °C	143
NM-138976	cctgggctgcattatctggtg		
mfn2	gatgtcaccacggagctgga	59 °C	136
NM-130894	agagacgctcactcactttg		
fis1	gcacgcagtttgaatacgcc	58 °C	113
XM-213746	ctgctcctctttgctacctttgg		
β-actin	cacccgcgagtacaaccttc	60 °C	*207*
NM-031144	cccatacccaccatcacacc		

luminometer (Turku, Finland) and expressed as nmol/min/mg protein.

2.8. RNA extraction

Total RNA was isolated from skeletal muscles by TRIzol reagent according to the manufacturer's instructions. Briefly, ~50 mg of muscle was removed from the freezer and immediately immersed in 1 ml of TRIzol reagent. The muscle was homogenized on ice using a glass homogenizer, and the aqueous and organic phases were separated using 200 μl of chloroform. Total RNA was precipitated using 500 μl of isopropyl alcohol, washed three times with 75% ethanol, redissolved in 15 μl of DEPC-treated H_2O, aliquoted, and stored at −80 °C. The concentration and purity of the RNA was determined using a UV spectrophotometer (Beckman, DU-800, USA) by measuring the absorbance at 260 and 280 nm. Measurements were done in duplicate and had an average coefficient of variation of <10%. The average purity (OD260/OD280) of the samples was ~1.9–2.0. RNA integrity was assessed in a randomly chosen subset of samples using agarose gel electrophoresis and ethidium bromide staining, and the OD ratio of 28S to 18S rRNA was consistently greater than 1 for each sample checked, indicating high-quality RNA.

2.9. Real-time quantitative RT-PCR analysis

Transcript levels of mfn1, mfn2, fis1 and β-actin were measured by real-time RT-PCR. Oligo-dT first-strand cDNA was synthesized from 5 μg total RNA using RevertAid™ M-MuLV Reverse Transcriptase (Fermentas, LTU). Real-time PCR was performed using the SYBR Green technology on an iCycler iQ® multicolor real-time PCR detection system (BIO-RAD, USA). The sequences of the primers and the sizes of the PCR products are shown in Table 1**.** Forward and reverse primers were each designed in a different exon of the target sequence, eliminating the possibility of amplifying genomic DNA. For each set of primers, a basic local alignment search tool (BLAST) search revealed that sequence homology was obtained only for the target gene. PCR amplification was performed in duplicate in a total reaction volume of 20 μl. The reaction mixture consisted of 2 μl diluted template, 10 μl the *SYBR® Premix Ex Taq*™ Kit (TaKaRa, China), and 0.5 μl forward and reverse primers (10 μM). Amplification specificity was controlled by a melting curve analysis and a gel electrophoresis of the PCR product. A melting curve from 65 to 95 °C (0.05 °C/s) at the end of the reaction was used to check the purity and nature of the product. In all cases, a single PCR product was detected. Results were normalized to β-*actin* transcription to compensate for variation in input RNA amounts and efficiency of reverse transcription [29].

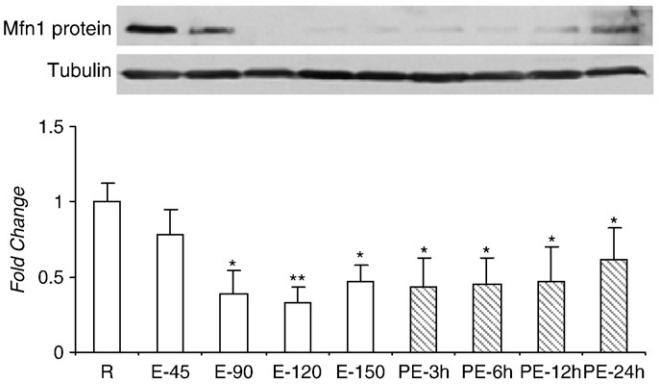

Fig. 2. Western blot analyses of Mfn1 protein in rat skeletal muscle during and post exercise. R, E-45, E-90, E-120, and E-150 stand for rest, an acute bout of exercise for 45, 90, 120, and 150 min, respectively; PE-3h, PE-6h, PE-12h, and PE-24h stand for 3, 6, 12, and 24 h after a single bout of running (150 min), respectively. Values represents the mean ± SEM ($N = 10$). *$p<0.05$, **$p<0.01$ compared with the R group.

2.10. Western blotting analysis

Powdered muscle was homogenized by motor pestle in 0.4 ml of ice cold lysis buffer containing 20 mM Tris (pH 8.0), 137 mM NaCl, 10 mM NaF, 1 mM MgCl, 1 mM Na_3VO_4, 0.2 mM phenylmethylsulfonyl fluoride, 10% glycerol, 1% Triton X-100, 0.1% SDS, and protease inhibitors cocktail. Homogenates were solubilized by end-over-end mixing for 60 min at 4 °C and subjected to centrifugation (15,000 × *g*, 20 min, and 4 °C). Total protein concentration was determined using δ-globulin as standard protein according to Bradford [26]. Whole cell proteins solubilized in Laemmli sample buffer were separated by SDS-PAGE [30] and transferred to PVDF membranes (Millipore). Conventional Western blot analysis was performed using the following antibodies as indicated: β-tubulin antibody (abcam, Cambridge, UK), Mfn1 (Santa Cruz Biotechnology, California), and Fis1 (ALEXIS Biochemicals, Pennsylvania). Western blot analysis was performed using enhanced chemiluminescence (LumiGLO® Chemiluminescent Substrate, KPL, USA).

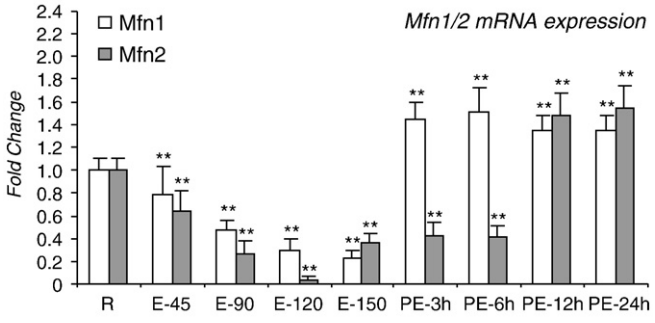

Fig. 1. Real-time PCR analyses of mfn1/2 mRNA expression in rat skeletal muscle during and post exercise. R, E-45, E-90, E-120, and E-150 stand for rest, an acute bout of exercise for 45, 90, 120, and 150 min, respectively; PE-3h, PE-6h, PE-12h, and PE-24h stand for 3, 6, 12, and 24 h after a single bout of running (150 min), respectively. **$p<0.01$ compared with the R group.

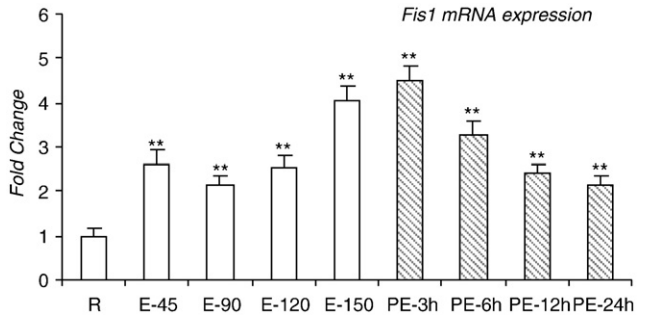

Fig. 3. Real-time PCR analyses of fis1 mRNA expression in rat skeletal muscle during and post exercise. R, E-45, E-90, E-120, and E-150 stand for rest, an acute bout of exercise for 45, 90, 120, and 150 min, respectively; PE-3h, PE-6h, PE-12h, and PE-24h stand for 3, 6, 12, and 24 h after a single bout of running (150 min), respectively. Values represents the mean ± SEM ($N = 10$). **$p<0.01$ compared with the R group.

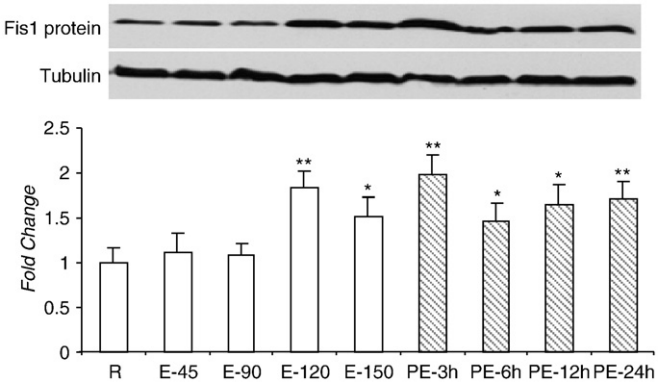

Fig. 4. Western blot analyses of Fis1 protein in rat skeletal muscle during and post exercise. R, E-45, E-90, E-120, and E-150 stand for rest, an acute bout of exercise for 45, 90, 120, and 150 min, respectively; PE-3h, PE-6h, PE-12h, and PE-24h stand for 3, 6, 12, and 24 h after a single bout of running (150 min), respectively. Values represents the mean ± SEM ($N=10$). *$p<0.05$, **$p<0.01$ compared with the R group.

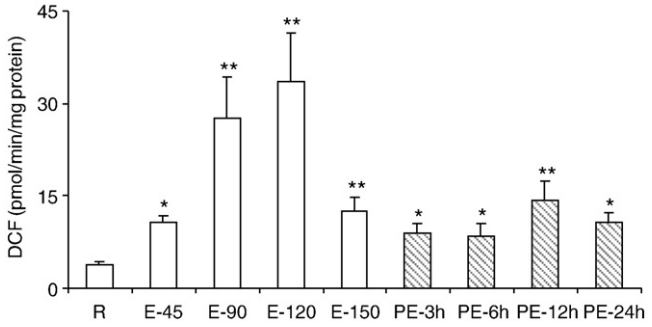

Fig. 5. Rate of DCF formation in rat skeletal muscle mitochondria during and post exercise. R, E-45, E-90, E-120, and E-150 stand for rest, an acute bout of exercise for 45, 90, 120, and 150 min, respectively; PE-3h, PE-6h, PE-12h, and PE-24h stand for 3, 6, 12, and 24 h after a single bout of running (150 min), respectively. Values represents the mean ± SEM ($N=10$). *$p<0.05$, **$p<0.01$ compared with the R group.

2.11. 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 $p<0.05$.

3. Result

3.1. Mfn1/2, Fis1 mRNA and protein

The mRNA contents of both Mfn1 and Mfn2 showed progressive reductions in response to an acute bout of exercise with increased duration (Fig. 1). Mfn1 mRNA levels were decreased by 21%, 42%, 70%, and 77% ($p<0.01$) respectively, in E-45, E-90, E-120 and E-150 compared to R group. Mfn1 mRNA levels were increased by 44%, 52%, 34% and 35% ($p<0.01$), respectively, in PE-3h, PE-6h, PE-12h and PE-24h compared to R group. Mfn2 mRNA levels were decreased by 36%, 73%, 96%, and 64% ($p<0.01$) respectively, in E-45, E-90, E-120 and E-150, compared to R group. It remained to be lowered at PE-3h and PE-6h, but elevated to 148% and 154% ($p<0.01$) of R level at PE-12h and PE-24h, respectively. Consistent with mRNA, Mfn1 protein content was decreased by 61%, 67%, 53%, 57%, 55%, 53% and 38% ($p<0.05$ or $p<0.01$) in E-90, E-120, E-150, PE-3h, PE-6h, PE-12h and PE-24h group, respectively, compared to R group (Fig. 2). As shown in Fig. 3, Fis1 mRNA contents were significantly increased after an acute bout of exercise and reached 264%, 213%, 252%, 406% and 450% of R level ($p<0.01$), respectively, at E-45, E-90, E-120 and E-150. Fis1 mRNA level gradually declined after exercise, but was still ~1–3 fold higher than R group ($p<0.01$). Fis1 protein showed a delayed response compared to mRNA and was increased by 84% ($p<0.01$) and 51% ($p<0.05$) at E-120 and E-150, respectively (Fig. 4); the high Fis1 protein level was maintained throughout the 24 h recovery period post exercise ($p<0.05$ or $p<0.01$).

3.2. Mitochondrial respiratory function

There was a time-dependent change in mitochondrial state 4 respiration rate in response to an acute bout of exercise (Table 2). State 4 respiration rate was 46% and 58% ($p<0.05$) higher at E-90 and E-120 group, respectively, compared to the R group. However, it returned to the resting level at E-150. State 4 respiration rate remained to be significantly elevated above the resting level at PE-3h, PE-12h, and PE-24h groups ($p<0.05$). State 3 respiration rate decreased 31% at E-150 compared to R ($p<0.05$), but was not significantly altered during the post-exercise recovery. Mitochondrial RCI showed a decline at E-120 and E-150, as well as during the whole post-exercise recovery period ($p<0.01$), mainly due to increased state 4 respiration rates.

3.3. Mitochondrial ROS production

As shown in Fig. 5, ROS production was continuously increased ($p<0.05$) from 45 to 120 min of exercise and was 9 fold higher ($p<0.01$) at E-120 compared with R. Interestingly, ROS production plunged dramatically at 150 min but was still threefold higher

Table 2
Mitochondrial respiratory functions in rat skeletal muscle during and post exercise.

Groups	State 3 (nmol O_2/min/mg protein)	State 4 (nmol O_2/min/mg protein)	RCI
R	27.91 ± 2.28	9.61 ± 0.57	2.90 ± 0.13
E-45	29.07 ± 3.13	10.15 ± 0.84	2.61 ± 0.23
E-90	33.77 ± 1.77	14.04 ± 0.62*	2.55 ± 0.17
E-120	28.67 ± 2.23	15.14 ± 1.36*	1.91 ± 0.15**
E-150	19.24 ± 1.42*	8.91 ± 0.67	2.17 ± 0.16**
PE-3h	24.58 ± 2.78	15.73 ± 1.51*	1.69 ± 0.09**
PE-6h	20.75 ± 1.58	12.61 ± 0.96	1.77 ± 0.09**
PE-12h	32.93 ± 1.34	14.79 ± 1.38*	2.25 ± 0.19**
PE-24h	27.67 ± 1.61	13.91 ± 1.06*	1.96 ± 0.18**

R, E-45, E-90, E-120, and E-150 stand for rest, an acute bout of exercise for 45, 90, 120, and 150 min, respectively; PE-3h, PE-6h, PE-12h, and PE-24h stand for 3, 6, 12, and 24 h after a single bout of running (150 min), respectively. RCI, Respiratory control index. Data are means ± SEM ($N=10$). *$p<0.05$, **$p<0.01$ compared with the R group.

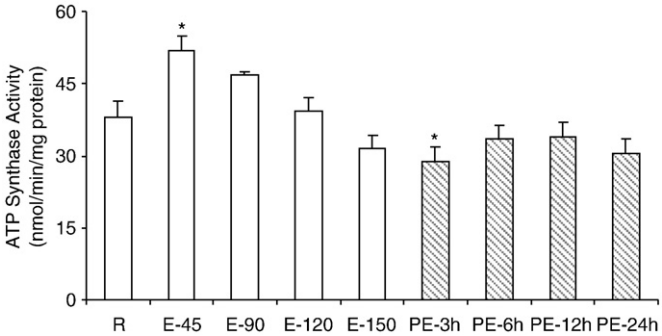

Fig. 6. Mitochondrial ATP synthase activity in rat skeletal muscle during and post exercise. R, E-45, E-90, E-120, and E-150 stand for rest, an acute bout of exercise for 45, 90, 120, and 150 min, respectively; PE-3h, PE-6h, PE-12h, and PE-24h stand for 3, 6, 12, and 24 h after a single bout of running (150 min), respectively. Values represents the mean ± SEM ($N=10$). $p<0.05$ compared with the R group.

($p<0.05$) than the resting level. ROS production remained significantly elevated during the 24-h post-exercise period ($p<0.05$).

3.4. Mitochondrial ATP synthase activity

As shown in Fig. 6, mitochondrial ATP synthase activity was transiently increased ($p<0.05$) at E-45 group, followed by a gradual return to basal levels. ATP synthase activity was significantly lower in the PE-3h group ($p<0.05$) but remained constant thereafter.

4. Discussion

Up till recently, few studies have reported changing status of the gene expression of mitochondrial fusion and fission protein in response to exercise in skeletal muscle [22,23].

The most important finding of the present study is that an acute bout of prolonged exercise suppressed the gene expression of fusion protein Mfn1/2 whereas increased the expression of fission protein Fis1, and that the magnitudes of these alterations depended on exercise duration. These findings suggest that mitochondrial fusion/fission protein expression could be rapidly altered in response to changing energy demand in skeletal muscle. During heavy muscular contraction, ATP requirement is dramatically increased, which is matched by an elevated mitochondrial respiration supported by both substrate (NADH and FADH) supply and O_2 consumption. However, as exercise intensity and duration increase, the coupling of oxygen consumption and ATP production (oxidative phosphorylation) could be negatively influenced by two factors: an increased proton leak that enhances state 4 respiration and superoxide anion formation [31,32], and an upregulation of upcoupling protein 3 (UCP3) [33]. The former process could cause an oxidative stress and damage mitochondrial integrity; the latter process, while alleviating cross-membrane proton gradient thus reducing ROS formation, enhances heat production known to disturb mitochondrial oxidative phosphorylation coupling [34]. Furthermore, both processes increase net consumption of substrate and O_2 but decrease the efficiency of ATP production. Thus, evolutionary pressure forces mitochondria to develop mechanisms to cope with the above problems to ensure cell survival.

Two potential strategies for mitochondria to maintain the efficiency of oxidative phosphorylation and avoid oxidative damage are (1) to increase mitochondrial inner membrane density and mitochondrial number via biosynthesis, which has been clearly demonstrated in endurance trained muscle and will not be discussed here [35,36] ; and (2) to undergo morphological changes via fusion and fission [1,4,5]. It has been postulated that elongation of mitochondria enables a rapid transmission of membrane potential across a greater distance within a cell [37]. Fragmentation of mitochondrial network may facilitate recruitment of mitochondria to cellular compartment in need of ATP, such as in neuronal protrusion [11]. Furthermore, recent studies show that RNAi of dynamin-related protein (DRP)-1, another key fission protein, induces alterations in the mitochondrial network organization with a slower rate of proliferation and that mitochondrial energy production is impaired in DRP1 deficient cells [38]. In view of the above notions, our data showing increased Fis1 expression and a downregulation of Mfn1/2 in response to heavy exercise may indicate a tendency towards more fragmented mitochondrial network, which could be advantageous in terms of ATP production. Indeed, state 3 respiration and ATP synthase activity were maintained relatively constant during the first 120 min, and so was RCI until 90 min of exercise. However, without morphological analysis, we cannot confirm whether altered fusion and fission protein expression were associated with changes in mitochondrial shape, size and division in the present study.

Although over-expression of Mfn inhibits mitochondrial propagation, excessive downregulation of Mfn could also disturb mitochondrial respiratory function and homeostasis. For example, obesity-induced Mfn2 deficiency in rat skeletal muscle could repress nuclear-encoded subunits of electron transport chain [39]. We found that a dramatic decrease in Mfn1/2 mRNA (by 70–90%) and Mfn1 protein (by 50–60%) after 120 min exercise were associated with increased ROS generation and state 4 respiration, widely regarded as indication of reduced inner membrane integrity [40]. Meanwhile, oxidative phosphorylation was also less coupled as shown by decreased RCI. It is note worthy that at 150 min there was a dramatic drop in ROS production and state 4 respiration rate compared to 120 min, when the changes in Mfn1 (Fig. 2) and Fis1 (Fig. 4) protein levels were leveled off despite additional metabolic demand. We previously attributed these observations to elevated muscle UCP3 gene expression which might alleviate cross-inner membrane proton gradient thereby serving as antioxidant function [33]. This adaptation, however, caused a decrease in state 3 respirations and attenuated ATP synthase activity, thus sacrificing the efficiency of oxidative phosphorylation.

Whereas altered fusion/fission protein expression can affect mitochondrial function, altered energy metabolism has been shown to impact on the dynamics of mitochondrial networks structure as well. It is the proton pumping coupled with mitochondrial respiration that constitutes the direct driving force for fusion of mitochondrial outer membrane with opposing membranes of liposome [41–44]. When ADP is added to a respiratory state in which only phosphate acceptor is deficient (i.e. state 4 to state 3 transition), a dramatic change in the manner of inner membrane folding takes place [45,46]. In addition, the electron opacity of the matrix increases as the volume of the matrix decreases, but total mitochondrial volume does not appear to change during this transition [45,46]. This is called the "condensed conformation." Isolated mitochondria have been found to oscillate between the orthodox and condensed conformations during reversible transitions between state 3 and state 4. Since exhaustive exercise represent a state when substrates and high-energy phosphates are depleting in the skeletal muscle, it is not surprising that these metabolic conditions could dramatically affect mitochondrial morphology and hence fusion/fission protein expression.

The exact mechanisms controlling mitochondrial fusion and fission are still elusive. However, peroxisome proliferator-activated receptor γ co-activator-1α (PGC-1α), ROS and nuclear factor (NF) κB have been identified as potential regulators in some recent studies. For example, Cartoni et al. [22] showed that an acute bout of exercise increased Mfn1/2 mRNA and protein levels in human leg muscle along with elevated PGC-1α and nuclear respiratory (NRF)-2 expression. Garnier et al. [23] demonstrated in healthy humans that peak VO_2 was dependent on coordinated expression of PGC-1α, which was lineally correlated to Mfn2 and Drp1 levels in human leg muscle. Soriano et al. [47] showed in L6E9 myocytes that cold- and β_3-agonist-induced Mfn2 expression was severely hampered with the inhibition of PGC-1α, whereas cells transfected with high PGC-1α showed dramatically higher Mfn2 expression. Furthermore, PGC-1α stimulated ERRα binding at −413/−398 motifs on the Mfn2 promoter. In line of these findings we have recently demonstrated that exercise-induced upregulation of PGC-1α and mitochondrial biogenic pathway are redox-sensitive, i.e. inhibition of ROS with allopurinol decreased PGC-1α and PGC-1α-controlled gene expression of mitochondrial biogenesis in rat skeletal muscle [48]. Moreover, inhibition of NFκB with pyrolidine dithiocarbamate (PDTC) was found to prevent a training-induced downregulation of Mfn2 in rat skeletal muscle [49]. Thus, mitochondrial fusion and fission dynamics seem to be integrated into several other important cellular processes that determine the overall welfare of the organelle and the cell during exercise, although the exact role of each of the above factors remains unclear and requires further investigation.

In the current study we examined the Mfn1/2 and Fis1 expression during the post-exercise period. Our data showed that Mfn1 protein

remained downregulated whereas Fis1 protein level was elevated significantly above the resting levels even a day after an acute bout of prolonged exercise. Associated with these changes were increased state 4 respirations and ROS production, as well as decreased RCI. These data suggest that extreme energy demand and metabolic stress could cause mitochondrial dysfunction which is hard to reverse. Potential structural damage to the mitochondrial membrane might have also occurred as indicated by increase state 4 respirations [40]. Several previous studies reported that after an acute bout of exhaustive exercise mitochondrial in skeletal muscle became more fragmented [50], and that increased apoptotic activities were observed in these muscles [51]. The alteration of mitochondrial fusion and fission protein expression may provide a clue to these observations, because an upregulation of fission protein has been shown as the overture to mitochondrial fragmentation and apoptosis [52]. It was noticed that mRNA for Mfn1/2 were fully recovered and showed "super-compensation" 24 h after an acute bout of exercise, a phenomenon never reported before. Although the physiological significance of this finding is still unclear, since it is well known that Mfn2 protect cells from oxidative damage and inhibits apoptosis, it is tempting to speculate that damaged and/or fragmented mitochondria were being repaired, which requires a high level of fusion protein to participate [53].

In summary, there are mutual adaptations between the dynamic changes of mitochondrial fusion–fission and energy metabolism during exercise. However, the exact relationship between the dynamic changes of mitochondrial morphology and energy metabolism, as well as the detailed mechanism, has yet to be further investigated, especially how ROS and PGC-1α are involved in regulation of mitochondrial fusion and fission protein gene expression.

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