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Voluntary wheel exercise alters the levels of miR-494 and miR-696 in the skeletal muscle of C57BL/6 mice

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ABSTRACT

The discovery of miRNAs has brought the focus of physiologists to post-transcriptional regulation of the skeletal muscle. However, the field of how miRNAs are involved in regulating mitochondrial biogenesis and apoptosis of the skeletal muscle following endurance training is still in its infancy. Twelve male C57BL/6 mice were randomly assigned to either control group (Group C) or voluntary wheel running group (Group E). The Group C was housed in cages mounted with fixed wheels, while mice of Group E were allowed to run on wheels freely for 8 weeks. It was found that miR-494 and miR-696 were significantly decreased in the gastrocnemius muscle after 8 week voluntary wheel exercise, accompanied with an increase in the mRNA expression of NRF1, BIM and Bcl-XL, an increase in the protein content of PGC-1α, and a decrease in the protein content BIM. The lack of correlation between miR-494 and TFAM and BIM, as well as between miR-696 and PGC-1α suggests that even though miR-494 and miR-696 are sensitive miRNAs in response to exercise training, other factors or miRNAs might also be important during the regulation of mitochondrial biogenesis and apoptosis.

1. Introduction

MicroRNAs (miRNAs) are a class of small non-coding RNAs that could regulate gene expressions post-transcriptionally by degrading mRNA molecules or inhibiting mRNA translation (Ambros, 2004). Mature miRNAs are 18–24 nucleotides in length, which functions primarily by binding to the complementary region in the 3′-untranslated regions of mRNAs, thereby regulating translation of mRNAs to amino acids (Flowers et al., 2015). Computational predictions of miRNA targets suggest that up to 30% of protein-coding genes might be regulated by miRNAs (Sassen et al., 2008).

Physical activity plays a role in regulating various bodily functions, such as cellular metabolism, cardiovascular function and muscular functions etc. Endurance exercise has been shown to cause an increase in the enzymes of TCA cycle, mitochondrial respiratory chain and β-oxidation pathway in the skeletal muscle. The cellular responses to endurance exercise are quite complex, involving both transcriptional regulation, post-transcriptional regulation and allosteric regulation (Joseph et al., 2006; Coffey and Hawley, 2007). In the recent years, the discovery of miRNAs, which was speculated to fine tune gene expression, has brought the focus of physiologists to post-transcriptional regulation. However, up to now, the field of how miRNAs are involved in regulating the biology of skeletal muscle following endurance exercise training is still in its infancy. Existing studies revealed that the expression of miR-1, miR-133a, miR-133b were significantly decreased in the skeletal muscle following 12 week endurance training, while were increased after a single bout of endurance exercise (Nielsen et al., 2010). Besides, miR-9, miR-23a/b and miR31 were significantly downregulated in response to acute exercise (Russell et al., 2013).

Mitochondria are ubiquitous double membrane-bound organelles in the eukaryotic cells. It is well established that mitochondria are essential to energy production, substrate metabolism and apoptosis etc. The normal functioning of mitochondria depends on the optimal balance between mitochondrial biogenesis, mitochondrial dynamics and mitophagy, which forms mitochondrial quality control machinery. Mitochondrial biogenesis in the mammalians is subject to subtle physiological control, considered to be responsive to exercise intervention as an adaptation to increased oxygen demand. Although rarely reported, two miRNAs have been suggested to play a part in regulating mitochondrial biogenesis of skeletal muscle following exercise training. miR-696 was found downregulated in the skeletal muscle of mice following 4 wk. treadmill training, accompanied with increased protein level of PGC-1α (Aoi et al., 2010). Besides, the expression of miR-494 was significantly decreased after 7 d swimming training, which was accompanied by an increase in TFAM (Yamamoto et al., 2012). However, it is yet to know

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whether and how miR-494 and miR-696 are involved in the regulation of mitochondrial biogenesis under other animal exercise intervention settings, such as voluntary wheels running. Compared with treadmill training or swimming exercise, wheel running is a type of endurance training that best mimics the natural physical activities of mice, less forceful and traumatic yet ensuring adequate training intensity. In addition, miR-494 was reported to be involved in the regulation of apoptosis in non-small-cell lung cancer by directly targeting BIM (Romano et al., 2012). However, it needs to be explored whether miR-494 could regulate apoptosis of the skeletal muscle through BIM following exercise training.

Therefore, the aim of the present study is to investigate the changes of miR-696 and miR-494 in the skeletal muscle of mice following voluntary wheel running, as well as explore their potential roles in regulating mitochondrial biogenesis and apoptosis.

2. Material and methods

2.1. Animals and experimental design

Male C57BL/6 mice, obtained from Shanghai SLAC Experimental Animal center (Shanghai, China), were housed in an air-conditioned room on a 12 h light-dark cycle with food and water ad libitum. The temperature and humidity were maintained at 20 ± 1 °C and 50%, respectively. After one-week acclimatization, the mice were randomly assigned to either control (Group C, $n = 6$) or voluntary wheel running (Group E, $n = 6$) group ensuring that body weight was similar between groups. None of the mice were previously subjected to structured exercise intervention. The experimental protocol strictly followed guidelines put forth by Animal Research Ethics Committee of East China Normal University.

2.2. Exercise intervention

Hamster-sized plastic cage wheel was mounted in cage of 460 × 300 × 200 mm. Digital counters installed on the top of the wheels were used to measure running distance. The mice were housed individually so that the running distance could be recorded each morning for each animal, after which the counter was reset. The mice of Group E were allowed to run on the wheels freely for 8 weeks, while the cages of Group C were mounted with fixed wheels.

2.3. IGTT test

At completion of the exercise intervention period, the mice were subjected to intraperitoneal glucose tolerance test (IGTT). After overnight fasting, venous blood was collected from the tail for measurement of baseline glucose level at $t = 0$ min, and then at $t = 30$, 60, 90 and 120 min after an intraperitoneal injection of glucose (2 g/kg body weight) with a handheld glucometer (One Touch Ultra).

2.4. Tissue sampling

At completion of the IGTT test, the mice in both groups C and E were euthanized by cervical dislocation after overnight fasting. The gastrocnemius muscle (GS) of both sides were removed, snap frozen in liquid nitrogen, and then stored at −80 °C for further analysis.

2.5. Quantitative real-time PCR

Real-time PCR was performed using total RNA samples obtained from muscle tissues. Total RNA was isolated from ~100 mg of frozen gastrocnemius muscle using TRIzol (Invitrogen, Singapore) according to the manufacturer's directions. The mRNA expression of PGC-1α (peroxisome proliferator-activated receptor gamma coactivator 1-α), TFAM (mitochondrial transcription factor A), NRF1 (nuclear respiratory factor 1), BIM (BCL2 like 11), Bcl-XL (BCL2-like 1), Bcl-2 and Bax were quantified with Step One instrument (ABI) and software (Applied Biosystems, USA) using SYBR Green PCR Master Mix (Toyobo, Japan). Double-stranded cDNA was synthesized from about 1 μg of total RNA using a ReverTra Ace qPCR RT Kit (Toyobo, Japan). Forward and reverse primers (Table 1) for the target genes were designed based on sequences available in UCSC Genome Bioinformatics (http://genome.ucsc.edu/) using the Primer3web (http://primer3.ut.ee/), and were confirmed for specificity using Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). GAPDH was used as a control house-keeping gene. All samples were run in duplicate and data were analyzed according to the $2^{-\Delta\Delta Ct}$ method. The purity of the amplified product was checked through analysis of the melting curve carried out at the end of amplification. A value of 1 was arbitrarily assigned to Group C, against which the values of Groups E were reported.

2.6. miRNA expression

The miRNA expression was quantified in real-time using miRNA assays for miR-494 and miR-696 according to the manufacturer's instructions (RiboBio, China). Briefly, reverse transcription reaction was performed with miRNA RT primers and total RNA for 60 min at 42 °C, followed by 10 min at 70 °C. miRNA qPCR was then performed with RT product and miRNA primers for 40 cycles at 95 °C for 2 s, followed by 60 °C for 20 s and then 70 °C for 10 s. U6 snRNA was used as endogenous control for analysis.

2.7. Western blotting

The GS was weighed and homogenized in lysis buffer containing 50 mM Tris HCl, 150 mM NaCl, 1 mM EDTA, 0.2 mM PMSF and 1% NP-40, with pH of 7.4. The homogenate was centrifuged at 8000*g* for 10 min at 4 °C, resolved in SDS buffer, and then boiled for 5 min at 100 °C. The protein concentration of the supernatant was determined with the method of BCA using BCA protein assay kit (Thermo Fisher Scientific, USA). Equal amount of protein was run on 10% SDS-PAGE (120V, Bio-Rad, USA), and protein was transferred to PVDF membrane (1 h, 1.2 mA/cm^2, Criterion blotter, Bio-Rad). After staining and destaining with Ponceau S, the membranes were blocked with 5% nonfat dry milk powder (Shanghai Sangon, China) in Tris-buffered saline containing 0.1% Tween 20 (TBST) for 1 h at room temperature. The blots were incubated overnight with a 1:200 dilution of the primary antibodies (see Table 2) in 5% TBST at 4 °C on a shaker. After washing three times for 10 min each in 5% TBST, the membranes were incubated with a 1:5000 dilution of the secondary antibodies (see Table 2) in 5% TBST for 1 h at room temperature. The membranes were washed three times in TBST for 10 min each again. The membrane was examined

Table 1
Primer sequences for real-time PCR.

Gene	Primer Sequence
PGC-1α (forward)	AAG TGT GGA ACT CTC TGG AAC TG
PGC-1α (reverse)	GGG TTA TCT TGG TTG GCT TTA TG
TFAM (forward)	TGC AGT TTC TTG GTC AGC AT
TFAM (reverse)	TGC ACA ACT GGT AAC CAT CA
NRF1 (forward)	GCA CCT TTG GAG AAT GTG GT
NRF1 (reverse)	CTG CAT GGG GCT GAT CTT AT
BIM (forward)	TGG TGT CCT GAG TGA TTC TTC A
BIM (reverse)	CAA TTG CAC AGA GAC ATT TGG C
Bcl-XL (forward)	CTA GAG CCT TGG ATC CAG GAG
Bcl-XL (reverse)	GTA GCA ATG GTG GCT GAA GAG
Bcl-2 (forward)	TTG TAA TT CAT CTG CCG CCG
Bcl-2 (reverse)	AAT GAA TCG GGA GT TGG GGT
Bax (forward)	CGA ATT GGA GAT GAA CTG GAC AG
Bax (reverse)	CTA GCA AAG TAG AAG AGG GCA AC
GAPDH (forward)	AAC TTT GGC ATT GTG GAA GG
GAPDH (reverse)	ACA CAT TGG GGG TAG GAA CA