Effect of Electrical Stimulation at Different Time on Mitochondrial Function of C2C12 Myotubes

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Abstract

Objective: To study the effect of different time of electrical stimulation on mitochondrial function of C2C12 myotubes, and further explore its molecular mechanism.

Methods: An electrical stimulation was given 7 days after C2C12 myotubes differentiation, of which intensity was 30 ms, 3Hz, and the stimulation time was 60 mins, 120 mins, and 180 mins, respectively. A total of four experimental 4 groups, including control group (Con), 60 mins group (E60), 120 mins (E120) and 180 mins (E180). Microscope was used to observe the muscular myotubes form; Kits were to detect MDA, SOD and ROS; Western blot was used to detect the expression of autophagy proteins and mechanism proteins, including PGC1, p-ULK, SIRT1 and SIRT3; Flow cytometry technology was used to detect muscle mitochondrial membrane potential.

Results: There was no significant difference of C2C12 myotubes form after different electrical stimulation. Compared with the control group, E60 had no significant difference of mitochondrial membrane potential (p>0.05); but MDA, ROS, SIRT3 increased significantly (p<0.05), p-ULK and PGC1 increased significantly (p<0.01), SIRT1 decreased significantly (p<0.05). In E120, MDA, ROS, SIRT3 and PGC1 increased significantly (p<0.01), SOD and mitochondrial membrane potential decreased significantly (p<0.05). In E180, MDA and ROS increased significantly (p<0.01), SOD and mitochondrial membrane potential decreased significantly (p>0.01).

Conclusion: Moderate electrical stimulation (60 and 120 mins) can significantly activate oxidative stress, and further promote SIRT3, PGC1 and p-ULK expression, and further promote the mitochondrial membrane potential, while excessive stimulation (180 mins) has the opposite effects.

Keywords: Electrical stimulation; C2C12 muscle myotubes; Sirt; Mitochondrial function

Introduction

In recent years, many researchers have used different exercise types or intensity of exercise programs to explore the phenomenon and mechanism of oxidative stress. Many studies have confirmed that an acute strenuous exercise leads to an imbalance between oxidation and antioxidant system in body, an enhancement in lipid peroxidation and a-significantly increase in MDA level in skeletal muscle. The mitochondrial membrane potential and free calcium ion content were significantly reduced and MDA content was significantly increased in mitochondrial membrane potential and free calcium ion content were significantly reduced and MDA content was significantly increased in skeletal muscle of rats after two weeks of moderate intensity treadmill exercise. An acute strenuous exercise leads to an imbalance between oxidation and antioxidant system in body, an enhancement in lipid peroxidation and a-significantly increase in MDA level in skeletal muscle. The mitochondrial membrane potential and free calcium ion content were significantly reduced and MDA content was significantly increased in skeletal muscle of rats after two weeks of moderate intensity treadmill exercise. Mitochondria, as the center of energy metabolism, not only maintain various physiological processes in resting state, but also play an extremely important role in movement state. Therefore, it is more meaningful to study the role of mitochondrial function in motor body. Other studies have shown that [3-5] p-ulk and sirt 1/3 (silent information regulatory factor 2 related enzymes 1/3) have an important regulatory effect on the level of oxidative stress. They can reduce reactive oxygen free radicals and improve the living environment. C2C12 (mouse C3H muscle myoblast) cells were used in this study. The differentiated C2C12 myotubes could complete the same contraction as in vivo skeletal muscle, while avoiding the effects of other organs, tissues, cells and hormones in vivo experiments. The effects of the mitochondrial function can be studied in real time with different exercise combination. The research provided a theoretical basis for sports injury and recovery.

Materials and Methods

Research material

C2C12 (mouse C3H muscle myoblast); DMSO, DEPC, L-glutamine (sigma, USA); HEPES (MBCHEM, India); fetal bovine serum, DMEM medium, horse serum (GIBCO, USA); MDA, SOD, ROS kits (Nanjingjiancheng, China); anti-Sirt1, Sirt3, p-ULK (CST, USA); anti-PGC1 (abcam, USA); Tetramethylrhodamine methyl ester, perchlorate (TMRM, Shanghaijieling, China).

Cell culture

C2C12 cells were cultured in growth medium containing 4.5 g/L glucose, 4 mM glutamine, 10% FBS, 100 ug/mL penicillin, and 50 ug/mL streptomycin at 37°C under a 5% CO₂ atmosphere. Cells were then plated on 35 mm culture dishes at a density of 2 × 10⁵ cells/well in 2 mL of growth medium. Three days after plating, the cells had reached 90%-95% confluence (day 0). Differentiation was induced by incubating cells in differentiation medium containing 4.5 g/L glucose, 4 mM Glutamine, 2% HS, 100 ug/mL penicillin, and 50 ug/mL streptomycin. The differentiation medium was changed every 24 h.

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Electrical stimulation

We used custom built electrodes made of 1 mm platinum wire for electrical stimulation of the C2C12 myotubes. Two half-circle electrodes were aligned to the side of the well, in order to obtain a maximal area of stimulation. At first, platinum wire electrodes were sterilized with 70% ethanol and subsequently dried under ultraviolet light. On differentiation day 7, C2C12 myotubes were given one electrical stimulation exposure (15V, 3Hz, 30ms, Grass S-48 stimulator). There were four groups: Con (without stimulation); E60 (with stimulation of 60 minutes); E120 (with stimulation of 120 minutes); E180 (with stimulation of 180 minutes).

Determination of MDA, SOD and ROS

Enzyme assays were performed on cell lysates. MDA, SOD and ROS were measured using commercial assay kits. All metabolite data were normalized to the corresponding cellular protein content. Firstly, MDA (Malondialdehyde) was tested by TBA (Thiobarbituric acid) method. In brief, MDA can be condensed with TBA to form a red product, which has a maximum absorption peak at 532 nm by visible spectrophotometer. Secondly, SOD was tested by NBT method. In brief, under aerobic conditions, azablue tetrazole is reduced to blue methylhydrazone easily, which has a maximum absorption at 560 nm. SOD inhibits the formation of methylhydrazone. So the deeper the blue, the lower will be the enzyme activity. Thirdly, ROS was tested by DCFH-DA method. In brief, DCFH-DA can be changed into DCFH. Briefly, cells were washed with cold phosphate buffer saline (PBS) twice and directly lysed with Laemmli sample buffer, then centrifuged (12000 g, 10 min), and the supernatant was removed. The BCA Protein Assay Kit was then used to measure total protein concentration (µg/cm²). The left samples were then run on 8-15% SDS-PAGE, followed by transfer to a nitrocellulose membrane (Bio-Rad–USA). The membrane was blocked for 2 h at room temperature with 5% BSA and 0.05% Tween-20 in phosphate buffer saline. Next, the membrane was incubated with anti-Sirt1, anti- Sirt3, anti- p-ULK (CST), and anti-PGC1 (abcam) overnight at 4°C. The membrane was washed and incubated with rabbit-peroxidase-conjugated secondary antibody at room temperature for 2 h and the immune-reactive bands were visualized using a chemi-luminescence detection system (Imuno-Star, Bio-Rad). Relative band intensity was quantified using Image J software after normalizing with β-actin band intensity.

Western blotting

Briefly, cells were washed with cold phosphate buffer saline (PBS) twice and directly lysed with Laemmli sample buffer, then centrifuged (12000 g, 10 min), and the supernatant was removed. The BCA Protein Assay Kit was then used to measure total protein concentration (µg/cm²). The left samples were then run on 8-15% SDS-PAGE, followed by transfer to a nitrocellulose membrane (Bio-Rad–USA). The membrane was blocked for 2 h at room temperature with 5% BSA and 0.05% Tween-20 in phosphate buffer saline. Next, the membrane was incubated with anti-Sirt1, anti- Sirt3, anti- p-ULK (CST), and anti-PGC1 (abcam) overnight at 4°C. The membrane was washed and incubated with rabbit-peroxidase-conjugated secondary antibody at room temperature for 2 h and the immune-reactive bands were visualized using a chemi-luminescence detection system (Imuno-Star, Bio-Rad). Relative band intensity was quantified using Image J software after normalizing with β-actin band intensity.

Determination of mitochondrial membrane potential in myotubes by flow cytometry

TMRM shows fluorescent staining aggregating in mitochondria selectively. Once released to the cell plasma, fluorescence quenching. So TMRM method used to analyze and observe the mitochondrial channel pore activity. In belief, Mitochondria was extracted according to the extraction kit description, and then add a fluorescent probe 100 nm TMRM to dye the mitochondria in dark for 30 minutes. 4, 11000 g of centrifugation for 10 minutes. After discarding the supernatant, clean the cock solution twice, 4, centrifuging 11000 g, 10 minutes each time. After discarding the supernatant, the mitochondrial membrane potential was measured by adding the cock solution.

Statistical analysis

SPSS software, version 16.0, was used to analyse all data presented as means and standard deviation (M ± SD). One-way ANOVA and Tukey’s post hoc tests were carried out to determine the differences in several time points and two different conditions. Two-way ANOVA were carried out to determine the differences between time and treatment effects. P<0.05 was considered statistically significant.

Results

Effects of electrical stimulation on myotubes morphology of C2C12

C2C12 myotubes that differentiated for 7 days were thick and had the same contractility as in vivo muscles. As can be seen from the data in Figure 1, there were a large number of elongated myotubes. And there were a large number of myotubes with normal structure in the four images. Therefore, there was no significant difference in the morphology of myotubes between different groups.

Effects of electrical stimulation on MDA, ROS and SOD of C2C12

As can be seen from the data in Table 1, compared with the control group, MDA and ROS increased significantly at 60 min after electrical stimulation (p<0.05); MDA and ROS increased significantly at 120 min and 180 min after electrical stimulation (p<0.01); SOD decreased significantly at 120 min (p<0.05) and 180 min (p<0.01) after electrical stimulation.

Expression of mechanism protein in C2C12 myotubes after electrical stimulation

As can be seen from the data in Figure 2, compared with the control group, the expression of Sirt3, p-ULK and PGC1 increased significantly increased significantly (p<0.01) and Sirt1 decreased significantly (p<0.05) at 60 min of electrical stimulation. The expression of Sirt3 and PGC1 increased significantly at 120 min of electrical stimulation (p<0.01). The expression of all proteins did not change significantly at 180 min of electrical stimulation.

<table>
<thead>
<tr>
<th></th>
<th>Con</th>
<th>E60</th>
<th>E120</th>
<th>E180</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA (nmol/mL)</td>
<td>3.65 ± 0.14</td>
<td>4.37 ± 0.21*</td>
<td>4.95 ± 0.09**</td>
<td>5.21 ± 0.15**</td>
</tr>
<tr>
<td>SOD (U/mL)</td>
<td>648.6 ± 14.7</td>
<td>665.2 ± 16.0</td>
<td>582.6 ± 13.7*</td>
<td>550.1 ± 10.8**</td>
</tr>
<tr>
<td>ROS (U/mL)</td>
<td>21.7 ± 12.4</td>
<td>88.2 ± 16.7*</td>
<td>155.4 ± 11.2**</td>
<td>147.4 ± 10.9**</td>
</tr>
</tbody>
</table>

*p<0.05; **p<0.01

Table 1: Effects of electrical stimulation on MDA, ROS and SOD of C2C12.
Effect of electrical stimulation on mitochondrial membrane potential of C2C12 myotubes

From the data of Figure 3 and Table 2, we can know that compared with the control group, the mitochondrial membrane potential decreased slightly at 60 min after electrical stimulation (p>0.05); And the mitochondrial membrane potential decreased significantly at 120 min after electrical stimulation (p<0.05), and then decreased more significantly at 180 min after electrical stimulation (p<0.01).

Discussion

Reactive oxygen species, the superoxide radicals and its derivatives produced in the electron transport of aerobic respiration, are considered to be the major factors mediating exercise-induced mitochondrial biosynthesis [7]. Loss of balance between the ROS produced by exercise and the body’s antioxidant system, results in a series of adaptive stress physiologic responses known as exercise oxidative stress. The level of reactive oxygen species is closely related to exercise. Exercise activates reactive oxygen species quickly, and the recovery period gradually decreased as the movement time continues to accelerate [8]. Compared with the quiet state, the production and physiological effects of reactive oxygen species are very different during exercise. In resting state, the level of reactive oxygen species is generally very low, and the damage to mitochondrial function is small. During exercise, the level of reactive oxygen species, oxidative stress and cell injury increase. Excessive reactive oxygen species stimulates mitochondrial lipid peroxidation and produces more malondialdehyde. Malondialdehyde crosslinks with mitochondrial membrane lipids to form (schiff) enamine base, which affects mitochondrial electron transport and function [9].

![Figure 3: Results of TMRM of C2C12 myotubes after electrical stimulation.](image)

\*p<0.05; \**p<0.01

**Figure 2: Effect of electrical stimulation on mechanism proteins of C2C12 myotubes.**

**Table 2: Effect of electrical stimulation on TMRM of C2C12 myotubes.**

<table>
<thead>
<tr>
<th></th>
<th>Con</th>
<th>E60</th>
<th>E120</th>
<th>E180</th>
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<tbody>
<tr>
<td>TMRM</td>
<td>11.13 ± 0.13</td>
<td>9.87 ± 0.09</td>
<td>7.32 ± 0.08*</td>
<td>6.57 ± 0.11**</td>
</tr>
</tbody>
</table>

*\*p<0.05; \**p<0.01

However, transient reactive oxygen species caused by exercise also stimulate mitochondrial biosynthesis [7]. Therefore, moderate reactive oxygen species is necessary to maintain mitochondrial function.

Mitochondria are the main source of reactive oxygen species, and also are first attacked by reactive oxygen species. Reactive oxygen species attack mitochondria, leading to mitochondrial dysfunction. Mitochondrial membrane potential is the potential difference between ions in the mitochondrial membrane and ions outside the membrane. It is the basic condition for mitochondrial oxidative phosphorylation and proton transmembrane transport, reflecting mitochondrial function. The decrease of mitochondrial membrane potential is the precursor of cell oxidative damage. In myocardial aging model, we can observe a series of mitochondrial function decrease, such as decrease in mitochondrial membrane potential, increase in proton leakage and increase in reactive oxygen species production [10]. Skeletal muscle is the most important part of energy metabolism. So it is very important to maintain the integrity of structure and function in skeletal muscle mitochondrial. The increase of skeletal muscle degradation and...
apoptosis were related to increased reactive oxygen species, changes in mitochondrial membrane potential and DNA oxidative damage. The mitochondrial structure and function are closely related to exercise ability. Any changes of mitochondria will directly or indirectly affect skeletal muscle function. Exercise is also an important factor affecting the structure and function of skeletal muscle mitochondria. Exercise directly or indirectly interferes with mitochondrial biosynthesis and function through transcription and translation [11]. It found that aerobic exercise is beneficial to maintain the mitochondrial membrane potential of myocardial cells in aging rats in a 12 weeks aerobic exercise study [12].

It is well known that different exercise causes different phenotype changes in cells and tissues. The endurance training is characterized by high oxygen intake, low shrinkage and mitochondrial dependence. Therefore, endurance training improves the oxidative ability and mitochondrial function of skeletal muscle [13]. In this study, it was found that, as the time of electrical stimulation increased, there was no significant change in the shape of the myotubes, but MDA and ROS gradually increased, SOD and mitochondrial membrane potential gradually decreased. The results showed that the electrical stimulation intensity of this experiment stimulated aerobic exercise and successfully resulted in oxidative stress and decreased mitochondrial function. However, the changes of motor oxidative stress and mitochondrial function caused by different electrical stimulation are different, so the mechanism needs to be studied.

The Sirts are NAD+ dependent deacetylase, which regulates multiple cellular processes including metabolic balance. Sirt1 is a redox sensor mainly located in the nucleus. Sirt1 is a major regulator of mitochondrial biosynthesis in skeletal muscle, and regulates the mitochondrial function mainly through deacetylation and PGC1 activation [14-16]. NADH affects downstream substrates such as PGC1 by inhibiting Sirt1 activity, thus regulating mitochondrial biological function [17]. Cell experiments after H2O2 revealed that, cell overoxidation induced phosphorylation of c-JNK, leading to proteinase degradation of Sirt1 [18]. Similarly, compared to aerobic exercise, acute hypoxia induced more intense oxidative stress in vivo, leading to more severe oxidative damage, and decreased Sirt1 and NAD/NADH ratios [19-21]. However, some studies have shown that the deacetylation activity of Sirt1 is not required to maintain mitochondrial function during exercise [22]. Sirt1 has three phosphorylation sites, including Ser27, Ser47 and Thr530. Phosphorylation of Ser27 and Thr530 enhances enzyme activity and nuclear location, while phosphorylation at Ser47 inhibits the deacetylase activity of Sirt1 [23]. In this study, the expression of Sirt1 decreased after 1 hour electric stimulation, but unchanged after 2 and 3 hours. The reason for the result may be that acute hypoxia induced by 1 hour electrical stimulation causes oxidative stress, which leads to degradation of Sirt1. However, there was no change in Sirt1 after 2 and 3 hours electrical stimulation, which also confirmed by previous studies, suggesting that Sirt1 was not need to maintain mitochondrial function.

Sirt3 is a mitochondrial protein that regulates energy balance, oxidative metabolism, oxidative stress and cell damage [24]. Reduced Sirt3 leads to mitochondrial function changes, elevated oxidative stress, activation of JNK, and impaired insulin signals. Although Sirt3 widely targets many substrates as a mitochondrial deacetylase, the exact molecular mechanism about its effect on the oxidative metabolism in skeletal muscle remains unclear [25]. Previous studies have found that PGC1 regulates the activity of Sirt3 as an endogenous stimulant in both skeletal and hepatocytes [26]. Under different pathological and physiological conditions, Sirt3 controls mitochondrial oxidation and ROS production, and activates ROS scavenging enzyme [27-29]. Therefore, it is important to study the affection of Sirt3 on mitochondrial oxidation function and energy balance. In addition, McGee et al. suggested that PGC1 and p-ULK regulated skeletal muscle volume and muscle fiber type [30]. Both Sirt3 and Sirt1 can promote mitochondrial biosynthesis and fatty acid oxidation through PGC1, but in different ways. Sirt3 activates PGC1, and Sirt1 promotes deacetylation of PGC1 [14]. In this study, Sirt3 increased after 1 and 2 hours electrical stimulation. The reason for the result may be that the free radical produced by electrical stimulation mainly comes from mitochondria, which are also the first to be attacked. While expressed Sirt3 mainly in mitochondria and therefore in higher levels after electrical stimulation. Although it has been found that exercise regulate the expression of Sirt3 instead of Sirt1 in the skeletal muscle [31]. But in different environments and organizations, about the work about the collaborative of the two Sirts deserves further study.

This study found that the expression of Sirt3 increased after 1 and 2 hour electrical stimulation, while Sirt1 decreased after 1 hour electrical stimulation, suggesting that the exercise intensity of this experiment does maintain mitochondrial function of skeletal muscle through Sirt3 rather than Sirt1. In addition, compared with control group, P-ULK and PGC1 increased significantly after 1 hour electrical stimulation. PGC1 increased and mitochondrial membrane potential decreased significantly after 2 and 3 hour electrical stimulation. The results showed that moderate exercise could activate oxidative stress, promote the expression of Sirt3, PGC1, p-ULK, and the recovery of mitochondrial membrane potential. While excess movement activates the opposite. In the following study, SI RNA interference will be used to observe the role of Sirt3, PGC1 and p-ULK in the mitochondrial function of skeletal muscle, and to more precisely prove the mechanism of oxidative stress affecting mitochondrial function of skeletal muscle.

Conclusion

Moderate time electrical stimulation (60 and 120 mins) could activate oxidative stress, promote the expression of Sirt3, PGC1, p-ULK, and then promote mitochondrial membrane potential recovery. While excessive time electrical stimulation (180 mins) had the opposite effect. In addition, the changes of mitochondrial membrane potential induced by electrical stimulation were mainly controlled by Sirt3 rather than Sirt1.

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