Effects of Hypoxic Exercise on Skeletal Muscle Mitochondrial Autophagy in Obese Rats

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Abstract
The effects of hypoxic exercise on skeletal muscle mitochondrial autophagy in obese rats were investigated. Sixty-four healthy male rats were randomly divided into four groups: normoxic control group, normoxic exercise group, hypoxic control group and hypoxic exercise group. Mitochondrial membrane potential, mitochondrial ATP synthesis ability and protein expression were compared between the four groups. The mitochondrial membrane potential and ATP synthesis ability of hypoxia group were significantly lower than those of normoxia group (P < 0.05). Mitochondrial membrane potential and ATP synthesis ability in normoxic exercise group were significantly higher than those in normoxic exercise group and hypoxic exercise group (P < 0.05). Compared with normoxic group, COXIV and VDAC-1 in normoxic exercise group were significantly increased (P < 0.05). Compared with normoxic group, COXIV and VDAC-1 in hypoxic group decreased significantly (P < 0.05). Compared with hypoxia group, the expression of Tfam protein and PGC-1 α protein in hypoxic exercise group increased significantly (P < 0.05). Compared with normoxic group, the expression of Tfam protein and PGC-1 α protein in hypoxic exercise group were significantly higher (P < 0.05). Compared with hypoxia group, the expression of Bnip3 and beclin-1 protein increased significantly in hypoxic exercise group (P < 0.05). Chronic hypoxic exposure increases mitochondrial autophagy but inhibits mitochondrial biosynthesis, resulting in a decrease in mitochondrial content. Hypoxic exercise promotes the autophagy of skeletal muscle mitochondria under hypoxic condition, so as to effectively remove damaged mitochondria, promote mitochondrial biosynthesis, and produce healthy mitochondria to maintain mitochondrial quantity threshold and function.

Key words: Hypoxia, Exercise, Obese Rats, Skeletal Muscle, Autophagy of Mitochondria

Efectos del Ejercicio Hipóxico Sobre la Autofagia Mitochondrial del Músculo Esquelético en Ratas Obesas

Resumen
Se investigaron los efectos del ejercicio hipóxico sobre la autofagia mitocondrial del músculo esquelético en ratas obesas. Sesenta y cuatro ratas macho sanas se dividieron al azar en cuatro grupos: grupo de control normóxico, grupo de ejercicio normóxico, grupo de control hipóxico y grupo de ejercicio hipóxico. El potencial
de membrana mitocondrial, la capacidad de síntesis de ATP mitocondrial y la expresión de proteínas se compararon entre los cuatro grupos. El potencial de membrana mitocondrial y la capacidad de síntesis de ATP del grupo de hipoxia fueron significativamente más bajos que los del grupo de normoxia (P <0.05). El potencial de membrana mitocondrial y la capacidad de síntesis de ATP en el grupo de ejercicio normóxico fueron significativamente más altos que los del grupo de ejercicio hipóxico (P <0.05).

En comparación con el grupo normóxico, COXIV y VDAC-1 en el grupo de ejercicio normóxico aumentaron significativamente (P <0.05). En comparación con el grupo normóxico, COXIV y VDAC-1 en el grupo hipóxico disminuyeron significativamente (P <0.05). En comparación con el grupo de hipoxia, COXIV y VDAC-1 aumentaron significativamente en el grupo de ejercicio hipóxico (P <0.05). En comparación con el grupo normóxico, la expresión de la proteína Tfam aumentó significativamente en el grupo de ejercicio normóxico y el grupo de ejercicio hipóxico (P <0.05). En comparación con el grupo normóxico, la expresión de la proteína alfa PGC-1 en el grupo de ejercicio normóxico aumentó significativamente (P <0.05). En comparación con el grupo normóxico, la expresión de la proteína Tfam y la proteína PGC-1 α en el grupo de hipoxia disminuyó significativamente (P <0.05). En comparación con el grupo de hipoxia, la expresión de la proteína Tfam y la proteína PGC-1 α en el grupo de ejercicio hipóxico aumentó significativamente (P <0.05). En comparación con el grupo de normoxia, la expresión de las proteínas Bnip3 y beclin-1 en el grupo de ejercicios de normoxia, el grupo de ejercicios de hipoxia y el grupo de ejercicios de hipoxia fueron significativamente mayores (P <0.05). En comparación con el grupo de hipoxia, la expresión de las proteínas Bnip3 y beclin-1 aumentó significativamente en el grupo de ejercicio hipóxico (P <0.05). La exposición hipóxica crónica aumenta la autofagia mitocondrial pero inhibe la biosíntesis mitocondrial, lo que resulta en una disminución del contenido mitocondrial. El ejercicio hipóxico promueve la autofagia de las mitocondrias del músculo esquelético en condiciones hipóxicas, para eliminar efectivamente las mitocondrias dañadas, promover la biosíntesis mitocondrial y producir mitocondrias saludables para mantener el umbral y la función de la cantidad mitocondrial.

Palabras clave: Hipoxia, ejercicio, ratas obesas, músculo esquelético, autofagia de las mitocondrias

Oxygen is a necessary material to maintain normal activity of the body. Hypoxia is an important pathophysiological feature when the organism is at high altitude, aircraft and other special environment or when the organism has heart failure, chronic obstructive pulmonary disease, anemia and other diseases. Skeletal muscle movement is an important oxygen consumption process, and hypoxia will affect the shape and function of skeletal muscle, resulting in muscle atrophy and decreased working ability. Long-term hypoxia reduces the content of mitochondria and the expression of respiratory chain complex in skeletal muscle, which leads to progressive decrease of muscle quality and endurance. Therefore, the protection of mitochondria quantity and quality is of great significance for improving the hypoxic tolerance of skeletal muscle. Mitochondrial content is mainly determined by mitochondrial biosynthesis and mitochondrial autophagy. The imbalance of the two biological effects leads to the accumulation of damaged mitochondrial volume or the dysplasia of mitochondrial proliferation, which can affect mitochondrial function and cell homeostasis, and is closely related to the occurrence of aging, neurodegenerative diseases and cardiovascular diseases. Mitochondrial autophagy refers to the elimination of damaged or unnecessary mitochondria through a special, selective and purposeful autophagy pathway, which can effectively maintain the physiological activity of cells and timely remove damaged or aging mitochondria. Under normal circumstances, mitochondrial autophagy is stable, but the abnormal state of the body will lead to the enhancement or weakening of mitochondrial autophagy, which is not conducive to the stability of the cellular environment and increase the incidence of metabolic diseases. In addition, mitochondrial autophagy is involved in the quality control of mitochondria, which is important for maintaining the number and function of mitochondria. Hypoxia can induce mitochondrial autophagy through hypoxia inducible factor pathway, suggesting that mitochondrial autophagy is a self-regulatory mechanism of cells adapting to hypoxia stress. In addition, exercise can induce skeletal muscle mitochondrial white phagocytosis and improve muscle cell glucose metabolism. From the point of view of hypoxic exercise, this experiment further explores the effect of hypoxic exercise on mitochondrial autophagy in skeletal muscle.

1. Data and methods

1.1. Experimental animals

64 healthy male rats were selected. Rats were randomly divided into four groups. There were 16 in each group, namely normoxic control group, normoxic exercise group, hypoxic control group and hypoxic exercise group. There was no significant difference in body weight among the four groups. All the rats were obese and fed in ABSL-3 animal room of our hospital. Judgment criteria: 1. The body weight of rats fed with high-fat diet was 20% higher than that of rats fed with ordinary diet; 2. The Lee's index and fat-body ratio of rats fed with
high-fat diet were significantly higher than those fed with ordinary diet; 3. The serum cholesterol and triglyceride of rats fed with high-fat diet were significantly higher than those fed with ordinary diet. During the experiment, rats were fed freely. The room temperature of the animal room was 22±1ºC and the humidity was 55±2%.

1.2. Experimental method

Rats in hypoxic control group and hypoxic exercise group were fed in normobaric hypoxic tent to simulate 11.3% hypoxic state (that is, oxygen concentration higher than 5 000 m above sea level) for 4 weeks. Rats in hypoxic exercise group needed treadmill training in hypoxic tent, and the running slope was 5 degrees and the speed was 15m/min. Continuous training for 4 weeks, training 5 days a week, training 60min every day.

Rats in normoxic exercise group and normoxic control group were fed in normoxic environment at normal pressure. Rats in normoxic exercise group were trained on treadmill in hypoxic tent with the same training method and frequency as those in hypoxic exercise group. All the animals were sacrificed immediately after the last hypoxia. Bilateral quadriceps femoris were separated quickly. Some of the quadriceps femoris were frozen at minus 80ºC for reserve. The rest were prepared immediately.

The skeletal muscle mitochondria were extracted by differential centrifugation. The muscle tissues were chopped and homogenized and added to the extracting medium (1mmol/L EDTA, 0.12mol/L KCL, 5mmol/L MgCl2, 20mmol/L HEPES, pH 7.4), 600×g centrifugation is carried out for 10min, and the supernatant was centrifuged at 17000×g for 10min. The precipitate was suspended in 1ml extraction medium, and the mitochondrial protein was quantified by Coomassie brilliant blue method.

64 rats were grouped.

Atmospheric oxygen → Normoxic movement → hypoxia → Hypoxic exercise → 4 weeks later

Mitochondrial membrane potential, mitochondrial ATP synthesis ability and protein expression were compared between the four groups.

Figure 1. Flowchart

1.3. Observation indicators

(1) Mitochondrial membrane potential of four groups of rats: JC-1 fluorescent probe was used to detect the mitochondrial membrane potential. Different JC-1 fluorescent probes had different polymerization degree and produced different colors in the mitochondrial matrix. The 0.9mlJC-1 staining solution was added to mitochondria, and 10min in water bath at 37ºC centigrade. The red fluorescence and green fluorescence (red fluorescence excitation wavelength 525 nm, emission wavelength 590 nm, green fluorescence excitation wavelength 490 nm, emission wavelength 530 nm) were measured by fluorescence spectrophotometer. The mitochondrial membrane potential was expressed by red and green fluorescence intensity.

(2) Determination of ATP synthesis ability of mitochondria: The synthesis ability of mitochondrial ATP was determined by fluorescein-luciferase luminescence assay. The medium consisted of 0.5 umol/L EDTA, 3.0 umol/L HEPES, 0.25 mol/L sucrose, 0.1 mmol/L malic acid, 1 mmol/L glutamate. Then 0.05 mg mitochondria and 20 umol/L fluorescence enzyme were added in order to record books. The luminescent intensity of the bottom was then added to the 4umol/L ADP to initiate the reaction to record the change of the intensity of luminescence.

(3) Protein expression assay: Western blotting was used to detect the expression of skeletal muscle-related proteins, including peroxisome proliferator-activated receptor gamma-coactivator-1 alpha (PGC-1 alpha) and mitochondrial transcription factor-A (Tfam), Bcl-2/adenovirus E1B19KD interacting protein-3 (Bni), which
regulates mitochondrial autophagy. P3 and beclin-1, cytochrome C oxidase subunit IV (COXIV) and voltage-dependent anion channel 1 (VDAC-1), which reflect the content of mitochondria, were used as internal reference. The protein sample was added into 2× SDS sample buffer, heated at 100°C for 5 min, and then cooled by ice bath. On the vertical electrophoresis apparatus, 10ug protein samples were separated by 12% SDS-PAGE and transferred to PVDF membrane. Resistance I was incubated overnight at 4°C for three times, then incubated at room temperature for one hour with 1:1000 horseradish peroxidase labeled antibody II, and washed thoroughly again. Then, the relative gray value of each strip was scanned and quantified by ECL kit and X-ray film exposure. The gray value of the control group was 100%. The expression of gray value of the other groups accounted for 100% of the gray value of the control group.

1.4. Statistical processing

The experimental data were statistically processed with SPSS22.3. The measurements were expressed as mean (men±SD) and the differences were statistically significant with P < 0.05 by two-way ANOVA.

2. Result

2.1. Effect of hypoxic exercise on mitochondrial membrane potential and ATP synthesis ability of skeletal muscle

The mitochondrial membrane potential and ATP synthesis ability of hypoxia group were significantly lower than those of normoxia group (P < 0.05). Mitochondrial membrane potential and ATP synthesis ability in normoxic exercise group were significantly higher than those in normoxic exercise group and hypoxic exercise group (P < 0.05). As shown in figure 2.

Mitochondrial membrane potential of skeletal muscle | ATP synthesis ability
---|---
\[\text{Atmospheric oxygen} \quad \text{Normoxic movement} \quad \text{hypoxia} \quad \text{Hypoxic exercise}\]
\[\text{Atmospheric oxygen} \quad \text{Normoxic movement} \quad \text{hypoxia} \quad \text{Hypoxic exercise}\]

Figure 2. Effect of hypoxic exercise on mitochondrial membrane potential and ATP synthesis ability of skeletal muscle

2.2. Effect of hypoxic exercise on mitochondrial content of skeletal muscle

Compared with normoxic group, COXIV and VDAC-1 in normoxic exercise group were significantly increased (P < 0.05). Compared with normoxic group, COXIV and VDAC-1 in hypoxic group decreased significantly (P < 0.05). Compared with hypoxia group, COXIV and VDAC-1 increased significantly in hypoxic exercise group (P < 0.05). As shown in Figure 3.

COXIV | VDAC-1
---|---
\[\text{Atmospheric oxygen} \quad \text{Normoxic movement} \quad \text{hypoxia} \quad \text{Hypoxic exercise}\]
\[\text{Atmospheric oxygen} \quad \text{Normoxic movement} \quad \text{hypoxia} \quad \text{Hypoxic exercise}\]

Figure 3. Effect of hypoxic exercise on mitochondrial content in skeletal muscle
2.3. Effect of hypoxic exercise on mitochondrial biosynthesis in skeletal muscle

Compared with normoxic group, the expression of Tfam protein was significantly increased in normoxic exercise group and hypoxic exercise group (P < 0.05). Compared with the normoxic group, the expression of PGC-1 alpha protein in the normoxic exercise group increased significantly (P < 0.05). Compared with normoxic group, the expression of Tfam protein and PGC-1 alpha protein in hypoxia group decreased significantly (P < 0.05). Compared with hypoxia group, the expression of Tfam protein and PGC-1 alpha protein in hypoxic exercise group increased significantly (P < 0.05). As shown in Figure 4.

![Figure 4](image)

**Figure 4.** Effect of hypoxic exercise on mitochondrial biosynthesis in skeletal muscle

2.4. Effect of hypoxic exercise on mitochondrial autophagy in skeletal muscle.

Compared with normoxia group, the expression of Bnip3 and beclin-1 protein in normoxia exercise group, hypoxia exercise group and hypoxia exercise group were significantly higher (P < 0.05). Compared with hypoxia group, the expression of Bnip3 and beclin-1 protein increased significantly in hypoxic exercise group (P < 0.05). As shown in Figure 5.

![Figure 5](image)

**Figure 5.** Effect of hypoxic exercise on autophagy in skeletal muscle mitochondria

3. Discussion

In order to ensure that the quantity and quality of mitochondria are maintained within a certain range, there is a mitochondrial quality control system. The quantity and quality of mitochondria are controlled by mitochondrial ergonomic reconstruction, mitochondrial biosynthesis, mitochondrial fusion, mitochondrial division, mitochondrial DNA repair and mitochondrial autophagy. Aerobic exercise training under hypoxic condition can improve the ATP output of skeletal muscle mitochondria, and effectively inhibit the generation of reactive oxygen species, which belongs to mitochondrial ergonomic reconstruction. Mitochondrial resistance to hypoxia is heterogeneous. When mitochondrial damage is too severe, repair and remodeling functions cannot be activated, which leads to the formation of a large number of reactive oxygen species, thus triggering the mitochondrial apoptosis pathway. Then the mitochondrial network divides, and the mitochondria with low membrane potential cannot fuse again. Then, mitochondrial autophagy is initiated to remove damaged mitochondria, promote mitochondrial biosynthesis and produce healthy mitochondria to maintain mitochondrial quantity.

Mitochondrial respiratory chain marker protein COXIV and mitochondrial outer membrane marker protein VDAC-1 were selected to evaluate the mitochondrial content, which can objectively reflect the changes of
mitochondrial content. Continuous chronic hypoxia will lead to progressive decrease of mitochondrial number density and body density in skeletal muscle of rats, and eventually lead to decrease of mitochondrial content in skeletal muscle. In addition, sustained hypoxia will lead to a more submyocardial distribution of mitochondria, which will reduce the capillary oxygen diffusion distance and compensate for the decrease in mitochondrial content. In this trial, compared with normoxic group, COXIV and VDAC-1 in normoxic exercise group were significantly increased (P < 0.05). Compared with normoxic group, COXIV and VDAC-1 in hypoxic group decreased significantly (P < 0.05). Compared with hypoxia group, COXIV and VDAC-1 increased significantly in hypoxic exercise group (P < 0.05). PGC-1a is the most important regulator of mitochondrial synthesis. Some studies have found that long-term hypoxia in human skeletal muscle leads to the decrease of PGC-1a expression and the progressive decrease of mitochondrial density, indicating that the loss of PGC-1a expression is an important mechanism of inhibiting mitochondrial synthesis under hypoxia. PPARgamma is a binding ligand of PGC-1a, which can effectively reduce the down-regulation of hypoxia on PGC-1a expression, and is another mechanism of chronic hypoxia inhibiting mitochondrial biosynthesis. In this experiment, the expression of Tfam protein in normoxic exercise group and hypoxic exercise group was significantly higher than that in normoxic exercise group (P < 0.05). Compared with the normoxic group, the expression of PGC-1 alpha protein in the normoxic exercise group increased significantly (P < 0.05). Compared with normoxic group, the expression of Tfam protein and PGC-1 alpha protein in hypoxia group decreased significantly (P < 0.05). Compared with hypoxia group, the expression of Tfam protein and PGC-1 alpha protein in hypoxic exercise group increased significantly (P < 0.05). When mitochondrial damage cannot be effectively repaired, mitochondrial autophagy is initiated to clear abnormal mitochondria. Mitochondrial autophagy can lead to a significant increase in Bnip3 and beclin 1 protein expression. Bnip3 protein can anchor the carboxyl terminal transmembrane region on the mitochondrial outer membrane to reduce the inhibition of Bel-2 on beclin-1 and activate mitochondrial autophagy. In this experiment, the expression of Bnip3 and beclin-1 protein in normoxic exercise group, hypoxic exercise group and hypoxic exercise group were significantly higher than those in normoxic exercise group (P < 0.05). Compared with hypoxia group, the expression of Bnip3 and beclin-1 protein increased significantly in hypoxic exercise group (P < 0.05). In addition, the mitochondrial membrane potential and ATP synthesis ability in hypoxia group were significantly lower than those in normoxia group (P < 0.05). Mitochondrial membrane potential and ATP synthesis ability in normoxic exercise group were significantly higher than those in normoxic exercise group and hypoxic exercise group (P < 0.05). Hypoxia can significantly inhibit mitochondrial membrane potential and ATP synthesis ability, indicating that mitochondria are damaged. However, 4-week hypoxia caused dysfunction of mitochondria, which initiated mitochondrial autophagy but was not sufficient to clear the damaged mitochondria. At the same time, hypoxia inhibited mitochondrial biosynthesis and mitochondrial content decreased.

To sum up, chronic hypoxia exposure increases mitochondrial autophagy but inhibits mitochondrial biosynthesis, resulting in a decrease in mitochondrial content. Hypoxic exercise promotes the autophagy of skeletal muscle mitochondria under hypoxic condition, so as to effectively remove damaged mitochondria, promote mitochondrial biosynthesis, and produce healthy mitochondria to maintain mitochondrial quantity threshold and function.

References

