➡ Research Article

Effect of Pre-conditioning of Endurance Training with Different Intensities on Soleus Muscle Atrophy in a Period of Inactivity: The Role of PGC-1α4 Gene

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Abstract

Background: This study aimed to measure PGC-1α4 soleus muscle and to investigate the effect of endurance training pre-conditioning with different intensities on the atrophy response of the soleus muscle to a period of inactivity.

Methods: In this experimental study, 24 male Wistar rats were randomly divided into equal groups of control (C), control inactivity (CI), high-intensity endurance training group (HE) (treadmill speed 30 km/h), and low-intensity endurance training (LE) (treadmill's speed: 10 km/h). After two weeks of familiarization, the endurance training group ran on a treadmill for two weeks (five sessions each week). The animal's lower limbs were then immobilized for seven days. Then the soleus muscle was extracted, and after weighting, the expression of the PGC-1α4 gene was measured using the real-time polymerase chain reaction (real-time PCR) technique. Data were analyzed using SPSS software, version 24.

Results: The expression of the PGC-1α4 gene was significantly higher in the HE group than the CI group. However, compared to the C group, all groups with inactivity intervention showed significantly lower PGC-1α4 gene levels. The ratio of muscle mass to body weight in the C group was significantly higher than the LE and CI groups, and higher in the HE group than the CI group.

Conclusion: Endurance training seems to be able to reduce the destructive effects of inactive atrophy. The higher intensity of these exercises was more effective, which was associated with increased expression of the PGC-1α4 gene.

Keywords: Atrophy, Endurance exercise, Inactivity, PGC-1α4

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Background

Muscle atrophy significantly reduces the ability to perform physical tasks leading to decreased independence and increased mortality (1). Skeletal muscle atrophy occurs in pathological (such as cancer and diabetes) and non-pathological (such as inactivity and muscle disuse) conditions. It has been suggested that the mentioned conditions lead to loss of muscle protein, muscle fibrous atrophy, and functional impairment (2). Atrophy is caused by muscle disuse identified by a decrease in the size, weight, and function of skeletal muscle (3). In muscle disuse, because of the imbalance in synthesis and degradation of the protein, a rapid decrease in muscle size has been observed (4). Anabolic pathways include the mechanical stretching pathways (5), the mammalian target of rapamycin (mTOR) (6), the Wnt/β-catenin pathway, and ribosomal biogenesis (7), the beta-adrenergic receptor pathway (8), plus some unknown and emerging pathways such as nitric oxide (NO) (9), peroxisome proliferator-activated receptor-gamma coactivator-1 alpha (PGC-1α4) (10), and microRNAs (4).

PGC-1α4, one of the isoforms of PGC-1α, promotes muscle growth by activating IGF-1 and inhibiting myostatin (11). Although PGC-1α4 expression decreases in atrophic conditions, its changes in inactivity have not been well established (12). However, the expression of PGC-1α4 in the skeletal muscle of rat causes resistance to cancer and muscle atrophy induced by disuse (13).

Research on the preventive factors in muscle atrophy is scarce (14). Some studies have investigated the effective nutritional and pharmacological approaches in the prevention of atrophy. However, physical activity as a non-pharmacological strategy is effective (15). Physical activity before the period of inactivity or sedentary state can reduce the rate of muscle atrophy (16); however, the factors and mechanisms involved are not clear.

Several studies have shown the role of aerobic exercise pre-conditioning in reducing atrophy after inactivity. Decreasing the expression of PGC-1α gene and protein induced by inactivity, in mice with five weeks of endurance training was lower than not trained mice (17). Also, PGC-1α4 expression was significantly increased in

response to an eight-week exercise program that included resistance training (10). This result was obtained following endurance training as well (18).

Despite the proven positive effects of intensity on muscle adaptation through factors such as metabolic stress, accumulation of metabolites such as lactate, mineral phosphate (Pi), and hydrogen ions (H) that have a significant effect on hormone release, hypoxia, cell swelling, and production of reactive oxygen species which can initiate anabolic pathways for muscle growth and adaptation to energy metabolism (19), the effect of different intensities of endurance training on the role of new factors such as PGC-1α4 in anabolic and catabolic pathways involved in the pretraining of skeletal muscle for confronting atrophy induced by inactivity or not using the limb is not well defined (19).

We aimed to measure PGC-1α4 of muscle tissue and evaluate the effect of pre-conditioning of endurance training with different intensities on the response of the soleus muscle atrophy in a period of inactivity in male rats.

Material and Methods
Techniques and Samples
Twenty-four Wistar rats aged eight weeks (weight: 180 ± 20 g) were used. After 2 weeks of acclimatization in the animal room at a temperature of $22 \pm 3°C$, 40-60% humidity, 24 sleep-wake cycle (12 hours of light and 12 hours of darkness), and free access to water and food, they were randomly divided into four groups of six: the control (C); control of inactivity (CI); low-intensity endurance training (LE); high-intensity endurance training (HE). Induction of immobility was performed in LE and HE groups after two weeks of training (five days a week) and in the CI group performed without training.

Exercise Group Protocol
When groups underwent a familiarization period and the training time progressively reached 30 minutes in all groups, the LE and HE groups trained at 10 and 30 m/min, respectively. To match the stress on the treadmill, group C experienced walking on a treadmill at 5 m/s for 15 minutes (20, 21).

Induction of Immobility by Hanging Lower Limb
After anesthetizing the rat, a wire was passed through the tail, and a ring made from wire then connected it to the screw that had hung from the lid of the container with a clamp. This period lasted for seven days (22).

Isolation and Tissue Preparation
After deep anesthetizing with ketamine (75 mg/kg) and xylazine (10 mg/kg), blood samples were taken from the heart, and the soleus muscles were removed. The muscles were rinsed in saline and weighed to determine

hypertrophy/atrophy and were frozen (-80°C) until further tests.

Evaluation of PGC-1α4 Gene Expression in Soleus Muscle Tissue
RNA Extraction
This step was done by the RNA extraction kit (CinnaGen Inc., Tehran, Iran). For achieving this goal, tissues (50 mg) were lysed by trizol solution and completely homogenized with a tissue homogenizer and then the aqueous phase was separated using 0.25 mL of chloroform. Extracted RNA was washed and dehydrated by 1ml cold ethanol (70%). Then sterile water was added (5.1 µL/mg tissue). The quantity and quality of the obtained RNA were checked by measuring the ratio of the optical density of 260/280 nm using Nanodrop™ spectrophotometer (Nanodrop; Thermo Fisher Scientific, Wilmington, DE, USA).

cDNA Synthesis
cDNA Synthesis kit (Thermo Fisher Scientific, Inc., Waltham, MA, USA) was used for cDNA synthesis. For each sample, three steps of cDNA synthesis were performed. Thus, at first, 8 µL of the extracted RNA was mixed with 0.8 µL of DNase I enzyme, 2 µL of 10x buffers, and DEPC water then brought the mixture up to a total volume of 20 µL. The final product was gently mixed without vortexing and then incubated in the thermocycler with the following program: 5 min at 55°C, 15 minutes at 25 °C, 30 minutes at 42 °C (cDNA synthesis step by RT enzyme), 5 minutes at 95°C (to inactivation of RT enzyme).

After completion of the thermocycler steps, 280 µL of injection water was added and stored at -20°C for use in quantitative polymerase chain reaction (qPCR). Also, for each cDNA sample, a positive control sample with a b2m primer was prepared as an internal control and test for the presence of cDNA. The samples were mixed slowly and without vortex and placed in a real-time PCR set (StepOne, Applied Biosystems, USA) with the following program: 10 minutes at 95°C (initial denaturation), 10 seconds at 95°C (denaturation), 15 seconds at 60°C (primers binding), and 20 seconds at 72°C (extension). The reaction was repeated from the second stage onwards for 40 cycles. Threshold cycles (Cts) related to the reactions were extracted by real-time PCR software and finally, the mean three times Cts was recorded.

Quantification of Target Gene Expression Levels
To calculate gene expression levels, the $2^{-\Delta\Delta CT}$ ("delta-delta Ct") method was used.

Statically Analysis
All results were expressed in different groups as aean ± SD. The data were analyzed using SPSS software, version 21. After checking the normality of the data

with Kolmogorov-Smirnov test, One-way analysis of variance (ANOVA) and Tukey's post hoc tests were used for statistical analysis and comparison between groups. Pearson's correlation test was used to evaluate the relationship between PGC-1α4 gene expression levels and soleus muscle hypertrophy. In all cases, $P<0.05$ was considered statistically significant.

Results

Mean Body Weight

The mean body weight at the beginning and end of the study in all groups is shown in Table 1. There was a significant difference in the final weight between the four groups ($F_{3, 23} = 13.02$, $P<0.01$). The final weight in group C was significantly higher than groups CI ($P=0.000$), LE ($P=0.001$), and HE ($P=0.041$). Also, the final weight of the HE group was significantly higher than the CI group ($P=0.033$).

Mean Soleus Muscle Weight and its Ratio to Body Weight

The mean weight of the soleus muscle and its ratio to body weight, as an important indicator of hypertrophy and muscle atrophy in different groups, is shown in Table 2. There was a statistically significant difference between the four groups in this regard ($F_{3, 23} = 10.1$, $P<0.01$). Tukey's post-hoc test showed that this index was significantly higher in the HE group compared with the CI group. On the other hand, this index was significantly lower than the C group in LE ($P=0.006$) and CI ($P<0.001$) groups. This ratio was lower in the HE group compared with the C group ($P=0.215$).

PGC-1α4 Levels of Soleus Muscle Tissue

There was a significant difference in the PGC-1α4 level

Table 1. The Mean±SD Weight of the Groups (n=6) at the Beginning and End of the Exercise

Groups	Initial Weight (g)	Final Weight (g)
C	183.20±3.20	311.33±7.71*
HE	182.44±2.67	297.50±9.73#
LE	185.17±4.42	289.33±7.71
CI	184.27±2.24	283.16±7.71

* $P<0.05$ as compared to other groups; # $P<0.05$ as compared to CI group.
Control (C); Control of Inactivity (CI); Low-intensity endurance training (LE); High-intensity endurance training (HE).

Table 2. The Mean±SD Weight of the Soleus Muscle in Different Groups (n=6) and its Ratio to Bodyweight

Groups	Weight of the Soleus Muscle (mg)	Sol /BW (mg/g)
C	150.83±4.66	0.48±0.02*
HE	137.33±4.71	0.46±0.01#
LE	127.83±5.52	0.44±0.02
CI	120.50±1.87	0.42±0.02

* $P<0.05$ as compared to CI and LE groups; # $P<0.05$ as compared to CI group.
Control (C); Control of inactivity (CI); Low-intensity endurance training (LE); High-intensity endurance training (HE); soleus muscle's weight to body weight (Sol/BW)

in soleus muscle tissue in different groups ($F_{3,23} = 27.43$, $P<0.01$). The PGC-1α4 level in the groups that had inactivity intervention (HE, LE, and CI) was significantly lower than the C group ($P<0.01$). In the HE group, the PGC-1α4 level was significantly higher than the CI group (without exercise, $P<0.01$). Also, the PGC-1α4 level in the LE group was higher than the CI group, but this difference was not statistically significant (Figure 1).

Relationship Between PGC-1α4 Gene Expression and Soleus Muscle Hypertrophy

The results of Pearson's correlation test did not show a significant relationship between PGC-1α4 gene expression values and soleus muscle hypertrophy in the three groups (Table 3).

Discussion

This study showed the beneficial effects of pre-conditioning with a period of endurance exercise training in reducing the harmful effects of seven days of non-training on the soleus muscle in rats. This period of pre-conditioning greatly reduced the loss of muscle mass in the endurance exercise groups (HE and LE) compared with the control group. Moreover, the expression levels of proteogenesis pathways in endurance exercise groups were higher than in the control group. Accordingly, these findings reinforce the hypothesis that endurance exercise pre-conditioning reduces harmful changes in the soleus muscle.

We found that endurance training reduced atrophy after inactivity. This result was similar to another study showing less reduction in muscle hypertrophy and expression of the PGC-1α gene and protein in the group that had five weeks of endurance exercise before inactivity (17). Other researchers reported an increase in PGC-1α4 gene expression in response to the exercise and resistance training program (18, 23).

Concerning PGC-1α4 levels and hypertrophy index, after the inactivity period, the endurance training groups (HE and LE groups) and the CI group had lower results compared to the control group (without intervention), but the soleus muscle hypertrophy index was significantly increased in the HE group compared with the CI group by 9.5%. This increase was also seen in the LE group (4.7%), but was not statistically significant. This increase can probably be related to the higher intensity of exercise activity in the HE group. On the other hand, similar to the results of the hypertrophy index, the level of PGC-1α4 in

Table 3. Correlation Between PGC-1α4 Gene Expression Level and Soleus Muscle Hypertrophy in Different Groups (n=6)

Correlation of Variables	HE	LE	CI
Gene expression level and muscle hypertrophy	r=0.23 (P=0.65)	r=-0.68 (P=0.13)	r=-0.44 (P=0.37)

Control of Inactivity (CI); Low-intensity endurance training (LE); High-intensity endurance training (HE).

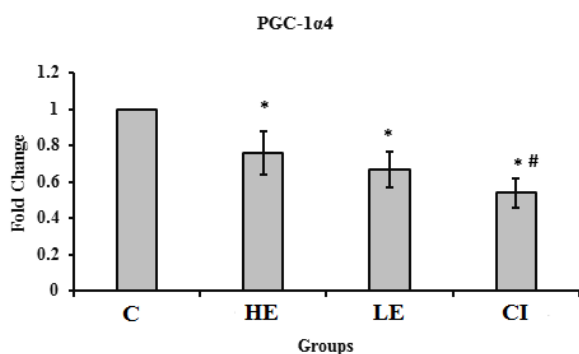**Figure 1.** Mean of the PGC1-α4 level of soleus muscle tissue in different groups
* $P<0.05$ as compared to C group; # $P<0.05$ as compared to HE group
Control (C); Control of Inactivity (CI); Low-intensity endurance training (LE); High-intensity endurance training (HE)

the groups with inactivity intervention was significantly lower than the control group (without exercise and inactivity). However, the PGC-1α4 gene expression level in the soleus muscle of the HE group was significantly higher than the CI group (without exercise intervention). Also, in the period of inactivity, the effects of inactivity on soleus muscle mass have been shown. The intensity of exercise increases the expression of the PGC-1α4 gene, which can play an important role in the process of hypertrophy and the prevention of atrophy. The proteogenesis pathway of PGC-1α4 is likely to be further stimulated by increasing the intensity of endurance training, affecting slow-twitch fibers such as the soleus muscle. Although most research have shown an association between PGC-1α4 and muscle hypertrophy with resistance training, studies have shown that PGC-1α4 also increases with endurance exercise (18, 24) and hypoxia (25). In general, PGC-1α4 promotes muscle growth by activating IGF-1 and inhibiting myostatin and prevents the processes of muscle atrophy because of inactivity or underload and disease. Endurance exercise may suppress muscle atrophy mechanisms by reducing inflammatory cytokines in the long-term in muscles, reducing free oxygen species, and increasing PGC-1α gene expression. In the period of lack of training and weightlessness, the expression of proteins of these genes and the mentioned changes remain at a high level. On the other hand, direct stimulation of IGF-1 activates the growth pathways related to the PI3K-AKT-mTOR cell cascade and prevents atrophy in the long-term, and promotes protein production. However, according to the results of the present study, inactivity, even when the rats trained, was able to significantly reduce the levels of PGC-1α4 and soleus hypertrophy index compared with the control group. Muscle growth and expression of PGC-1α4 gene in the soleus muscle during exercise were much higher than the control group, for this reason, with inactivity; a larger reduction slope was created. It seems that the higher the baseline level of a physiological factor, the more significant the effect of reduction interventions.

It seems that increasing the intensity of endurance training can further stimulate factors such as higher metabolic stress, accumulation of stimulant metabolites such as lactate, mineral phosphate (Pi), and hydrogen ions (H) that affect hormonal release, hypoxia, cell swelling, and the production of reactive oxygen species and further stimulate anabolic pathways for muscle growth and adaptation to energy metabolism (19). One of the most important growth hormone stimuli involved in skeletal muscle hypertrophy is lactate accumulation, which can stimulate NO to release more growth hormone in addition to NO stimulation.

Higher expression of PGC-1α4 gene in the period of inactivity in HE and LE groups compared to the CI group can also be attributed to adaptations related to antioxidant defenses because in the period of inactivity, by reducing the adaptation of muscle fibers, free radicals increase and destroy muscle fibers, especially slow-twitch fibers, leading to muscle atrophy.

The positive correlation between PGC-1α4 gene expression level and soleus muscle mass in the HE group indicates that high-intensity training has been able to prevent muscle mass loss in the inactivity period by higher pre-conditioning changes than the LE group. Because of the lack of measurement of muscle mass at the end of the last training step and before the start of the inactivity period, it is not clear whether endurance training with higher intensity caused more hypertrophy in the soleus muscle or it had prevented the further reduction of muscle mass during the inactivity period. The reason for the lack of a positive relationship between muscle hypertrophy index and expression level of the PGC-1α4 gene in the soleus muscle of the HE group may be related to the sample size of the study (six heads). By measuring the expression level of the two main targets of the PGC-1α4 gene, IGF-1, and myostatin, it may be possible to talk more accurately about the effects of endurance exercise as an important factor in pre-preparation for inactivity or injury.

Conclusion
The results of this study showed that endurance exercise as a non-pharmacological intervention can create beneficial effects and adaptations in the soleus muscle, which can greatly prevent destruction, atrophy, and reduced muscle force in the period of inactivity through pre-conditioning. The higher the intensity of this type of exercise, the greater the pre-conditioning level. One of the most important indicators is the PGC-1α4 gene expression level, but to determine the more accurate and clear paths, more studies are needed in the upstream and downstream paths of this indicator.

Authors' Contribution
All those who have mentioned their names as authors in this article confirm that they have contributed to the preparation of content,

concept, design, analysis, and interpretation of data as well as drafting the manuscript. All authors are responsible for writing this article.

Ethical Approval

Conflict of Interests

None declared.

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