Original Article

Role of Endurance Training in Preventing Pathological Hypertrophy via Large Tumor Suppressor (LATS) Changes

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ABSTRACT

Background: One of the negative effects of cardiac sympathetic hyperactivity is pathologic hypertrophy. Recent studies have indicated that large tumor suppressor (LATS) is one of the molecules which play a critical role in cardiomyocyte apoptosis. Considering the preventive role of exercise training, we evaluated the effects of endurance training on LATS gene expression and its upstream pathway in the present study.

Methods: Eighteen male Wistar rats were randomly divided into 2 groups: endurance and control. Endurance training was performed for 8 weeks, 1 hour per day, and 6 days per week on the treadmill at a 15° inclination. Pathologic hypertrophy was induced with the injection of 3 mg/kg^{-1} of isoproterenol for 7 days; and after 24 hours, the left ventricle was separated and the gene expressions of LATS, MST, and MAP4K were measured. The apoptosis cells of the left ventricle were counted via TUNEL assay. The data were analyzed using the *t*-test and the Mann–Whitney test.

Results: The gene expressions of LATS and MAP4K in the training group decreased significantly ($P \leq 0.001$). In addition, the apoptosis levels of cardiomyocytes in the training group decreased and the left ventricular weight increased significantly. There were no differences in MST gene expression between the groups ($P = 0.061$).

Conclusions: Our results showed that endurance exercise training diminished LATS suppression by reducing the expression of MAP4K, preventing the propagation of apoptosis induced by hypertrophy in the cardiomyocytes of the Wistar rats. ***(Iranian Heart Journal 2019; 20(3): 52-59)***

KEYWORDS: Endurance training, LATS, MST, MAP4K, Induced pathologic hypertrophy

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Cardiac failure, which is induced by pathologic hypertrophy, is a major cause of mortality worldwide. [1] Excessive sympathetic activity causes pathologic hypertrophy and exerts such negative effects on the heart as hypertrophy, apoptosis, and fibrosis. [2] Large tumor suppressor (LATS) is an important signaling molecule known for its role in decreasing cell proliferation and apoptosis and is, thus, responsible for maintaining organs. [3] LATS is a key mediator and the main regulator of the kinase cascade, whose downregulation leads to the higher activity of YAP and tumorigenesis in the rat model. [4] In the heart, LATS is responsible for regulating the responses of cardiomyocytes, including hypertrophy, apoptosis, and autophagy. Moreover, following cardiac injury, the phosphorylation of LATS through macrophage-stimulating protein (MST) activation increases the apoptosis of cardiomyocytes, and its inhibition results in hypertrophy. [5] In fact, the MST gene expression is a probable cause of increased apoptosis in cardiomyocytes. [6] With the inactivation of MST and LATS, Lin et al [5] in 2014 observed a considerable increase in heart size. In another study, the LATS gene knockout in adults led to the proliferation of cardiomyocytes. MST was initially thought to be the factor commencing the Hippo kinase cascade [7] and the initial signal for the activation of LATS in mammals. [8] Currently, however, it is known that kinases regulating LATS activity are not limited to MST, and the MST gene elimination cannot completely prevent LATS activity. [4] Recent studies have revealed that, parallel with MST, the mitogen-activated protein kinase kinase kinase kinase (MAP4K) family plays a role in LATS activation. In cardiomyocytes, MAP4Ks activate LATS, thus suppressing hypertrophy. [9, 10] Therefore, MST and MAP4K both regulate LATS to some extent (Meng, 2015). Signals leading to the activation of these 2 factors include, among others, stress-energy and intracellular density. [7] Some of these signals also affect exercise pathways, and the factor affecting this pathway regulates numerous genes in exercise. [11]

Various studies support the fact that regular training protects the heart [12] and its protective effects against cardiac muscle injury act in a pleiotropic manner. Training decreases cardiac muscle fibrosis and cardiomyocyte apoptosis. [13] The maintenance of the number of cardiomyocytes and increasing the growth of viable cardiomyocytes are 2 important factors in regulating the maintenance of cardiac muscle function in response to stress. [14] Silva et al [2] in 2014 demonstrated that endurance training on the treadmill was able to prevent fibrosis and apoptosis caused by the injection of isoproterenol and induced hypertrophy in rats. Thus, given the efficacy of training in preventing apoptosis, the question is whether endurance training can prevent cardiac fibrosis and apoptosis by suppressing LATS. If so, what is the effect of endurance training on the MST and MAP4K genes as the initiators of this signaling pathway?

METHODS

This experimental study was conducted in the Animal Laboratory, Faculty of Physical Education and Sports Sciences, University of Tehran. After ethical approval (IR.UT.SPORT.REC.1397.014) was obtained, 18 Wistar rats weighing approximately between 180 and 200 g were prepared from Pasteur Institute of Iran, Tehran. The rats were randomly assigned to 2 groups of 9 (endurance training vs control). The number of the rats in the control group was reduced to 8 due to death caused by drug injection in the final week of the study period.

The animals were kept in special cages at a mean temperature of 22 ± 2 °C, a humidity of 50% ± 5, and a 12:12 hour light/dark cycle in accordance with ethical considerations. Standard water and food were freely available to the animals.

Training intervention

After a 2-week adaptation to the new environment and familiarity with the treadmill, the rats underwent the training intervention consisting of 8 weeks of endurance training on the treadmill at a slope of 15° which was a combination of the training protocols proposed by Kemi et al [15] in 2005 and Wisloff et al [16] in 2001. The training protocol was performed based on Table 1.

For the induction of the same stress as the training group, the control group was placed on the treadmill twice a week at the speed of 9 m/min on the slope of 0, which causes no physiological response. [16]

Test for estimating maximum oxygen consumption

To determine maximum oxygen consumption in the rats, after 10 minutes of warm-up at the speed of 40–50% of the maximum oxygen consumption, we added 0.3 km/h to the initial speed. This trend was continued every 2 minutes up to the point where the rat was unable to run. [16]

Pathological hypertrophy induction

At the end of 8 weeks of training, to induce hypertrophy, we injected 3 mg/kg of isoproterenol (Sigma-Aldrich) subcutaneously once a day for 7 days. [17] Twenty-four hours after the final injection, the rats were anesthetized with the subcutaneous injection of 50 mg of ketamine and 10 mg of xylazine (per kg body weight), the heart and the left ventricle were immediately removed by a histologist, who weighed them on a very sensitive scale and stored them at -80 °C for the following measurements.

TUNEL staining

The apoptosis diagnosis technique was performed using the TUNEL Kit (Roche Company, Germany) based on the manufacturer's procedure manual. After TUNEL staining, the samples were viewed with a fluorescent microscope (Zeiss LSM 5). To count the apoptosis cells, we enumerated 5 fields in each group. The apoptotic cells in this tissue were visible as bright green points demonstrating apoptotic cell labeled during the TUNEL staining process. The nuclei of the cells were made red in color. Moreover, the green positive TUNEL cells were combined with the red nuclei and turned orange; thus, they became distinguishable from the healthy red cells and easy to count.

Gene expression

The expression of the genes of interest was examined using real-time PCR. After the primer was designed, RNA was extracted from all the tissues based on the manufacturer's protocol (QIAGEN, Germany) and converted into cDNA using reverse transcriptase. The procedure of cDNA synthesis was performed according to the manufacturer's protocol (Fermentas USA). Then, cDNA was used for reverse transcription and gene expression was determined using PCR. Gapdh was considered to be the reference gene. Forty cycles were considered for each real-time PCR cycle, and the temperature of each cycle was 94 °C for 20 seconds, 58–60 °C for 30 seconds, and 72 °C for 30 seconds. The melting diagram was plotted to examine the accuracy of the PCR reactions.

Statistical Analysis

The mean ± the standard error of the mean (SEM) was used for the descriptive statistics. The Shapiro–Wilk test was used to determine the normal distribution of the data. The independent *t*-test and the non-parametric Mann–Whitney test were applied in order to compare the mean of the training and control groups. The data were analyzed using SPSS software, version 24, at a significant level of $\alpha \leq 0.05$.

Table 1. Endurance training protocol (15° incline)

Warm-up	1 Week	2 Weeks	3 Weeks	4–8 Weeks	Cool-up
10 min 40-50 Vo_2max	30 min 65-75 Vo_2max	40 min 65-75 Vo_2max	50 min 65-75 Vo_2max	60 min 65-75 Vo_2max	10 min 40-50 Vo_2max

RESULTS

According to the results of the Shapiro–Wilk test, the *t*-test was used to compare the differences between the groups for MST1 and MAP4K and the non-parametric Mann–Whitney test was applied for LATS. The primer sequence of the genes and their ID number are shown in Table 2.

Real-time PCR showed that the LATS gene expression was significantly decreased in the training group compared with the control ($P < 0.001$) (Fig. 1). Although the MST1 gene expression increased after endurance training, the independent *t*-test results did not show any significant increase ($P = 0.061$). The results of these changes are presented in Figure 2. As is shown in Figure 3, following endurance training, the MAP4K gene expression was significantly lower than that in the control group ($P < 0.001$). Figure 4 shows that the ratio of the left ventricle to the heart as a result of endurance training was significantly higher than that of the control group ($P < 0.001$). The results of TUNEL staining showed that the number of apoptotic cells in the control group was significantly higher than that of the control group ($P < 0.001$).

Table 2. Primer sequence and associated ID number based on the reference bank

Gene	Forward Primer	Reverse Primer	ID
MAP4K	5′-GAGGCTGTGGGAATGGTTGGA-3′	5′-GTAGACGGAAGGTGAGGGTGG-3′	11184
LATS1	5′-TGTGATTGGTGGAGTGTTGGTG-3′	5′-TGAGGTGGGATGTGAAGAGAAG-3′	308265
MST1	5′-GCAATAGCTCAGTCCTTC-3′	5′-CTCGGTGTATATCTCACTCT-3′	24566
Gapdh	5′-AAGTTCAACGGCACAGTCAAGG-3′	5′-CATACTCAGCACCAGCATCACC-3′	108351137

DISCUSSION

After evaluating the results of gene expression, TUNEL staining, and weighing the left ventricles of the rats, we found that the MAP4K and LATS genes significantly decreased, while there was a nonsignificant increase in the level of MST variations. The left ventricle-to-body weight ratio had a significant increase in the training group, and TUNEL staining indicated a reduction in the level of apoptosis in the training group by comparison with the control group.

Isoproterenol leads to a stable adrenergic stimulation and, thus, cardiac hypertrophy, which is of an abnormal and pathologic type. [1] In contrast, endurance training increases the thickness and volume of the left ventricle and is deemed physiologic hypertrophy in the heart. [11] As the cardiomyocytes of adult mammals have little proliferative ability, this increase in heart size while exercising or during disease mainly occurs through cardiomyocyte hypertrophy. [5] In the present study, the significant increase in the weight of the left ventricle compared with the body weight in the training group and in comparison, with the control group demonstrated the effects of the training intervention in inducing physiologic hypertrophy in cardiomyocytes. TUNEL staining also showed that cardiomyocyte apoptosis resulting from the pathological hypertrophy induced by isoproterenol had occurred in both groups. However, the

significant reduction in cardiomyocyte apoptosis in the training group confirmed the effectiveness of the training protocol in decreasing apoptosis and its preventive role in the incidence of pathological hypertrophy. Similarly, in other studies, 8 weeks of endurance training caused an increase of between 17% and 32% in the size of cardiomyocytes in mice. [11] Silva et al [2] confirmed the anti-apoptosis effects of endurance training in counteracting the effects of intense sympathetic activity.

The differences between physiologic and pathologic hypertrophy at the molecular level is due to the response of cardiomyocytes to different types of stimulations. [11] Gabriel et al [11] in 2016 found that removing the LATS gene in the heart of adult mice increased cardiomyocyte proliferation and decreased the formation of scar tissue. Lin et al [5] in 2014 introduced LATS as the regulator of various responses of cardiomyocytes, including hypertrophy, apoptosis, and autophagy. Many studies have examined pathological conditions. For instance, the negative regulation of LATS increases cardiac hypertrophy in response to an increased pathological overload on cardiomyocytes. [18] Matsui et al [19] in 2008 concluded that LATS was essential and adequate for the negative regulation of the cardiac ventricle mass, and although LATS is necessary for the apoptosis resulting from increased overload on cardiomyocytes, it cannot commence apoptosis on its own at the starting point. LATS affects both the growth and death of cardiomyocytes, and it principally regulates heart size and acts as a negative regulator of cardiac hypertrophy. The question addressed in this study was the role of LATS in physiologic hypertrophy, and whether it still plays an inhibitory role in this type of hypertrophy. [9] The significant results of the present study indicated that endurance training on a 15° slope led to a significant decrease in the LATS gene expression, which may be an important reason for the reduction in the left ventricular cardiomyocyte apoptosis in the training group. Nevertheless, what is the upstream pathway involved in LATS regulation during training? Several mechanisms have been proposed for LATS regulation, and various genes regulate LATS levels and activity. [3] One of the most important genes is MST, which is known as an upstream kinase and is responsible for LATS activation. [10] Considering the findings of other studies, a rise in MST expression is accompanied by an increase in LATS in murine heart and increased cardiomyocyte apoptosis.[6] Although the MST gene expression in the training group of the present study had a nonsignificant increase, even this amount of increase is inconsistent with the existing information. Thus, it is expected that the effect of training on LATS variations in rats be a function of a regulating signal other than MST. Based on the results of other studies, LATS can be regulated in the absence of MST [10] and play a role in cell proliferation and death. [20] In fact, it has been shown that, parallel with MAP4K, MST can directly phosphorylate LATS and thus activate it. [10] Meng et al [7] in 2015 separately removed MST and MAP4K and still observed an increased LATS phosphorylation. It was only after the simultaneous removal of both MST and MAP4K kinase that LATS phosphorylation was suppressed. In the present study, the significant reduction in MAP4K in parallel with the reduction in LATS can demonstrate the effect of training on regulating the LATS gene expression and, thereby, reducing the induced pathological hypertrophy. Moreover, it should be kept in mind that MAP4K and MST are both effective in regulating the LATS gene expression. In this study, since a decrease was seen in MAP4K, the minor and nonsignificant increase in MST in the training group failed to enhance the LATS gene expression. Consequently, apoptosis had a more limited extension in the training group than in the control group.

At any rate, the causes for a decrease in the LATS gene expression through the upstream MAP4K gene due to training require further studies. MAP4K is a pleiotropic kinase that can affect various factors, including mTOR for tissue growth and LATS for growth suppression. [21] How and through what mechanism exercise can reduce MAP4K and LATS in pathological conditions can be examined in future studies.

CONCLUSIONS

The results of the present study showed that endurance training on a 15° slope treadmill suppressed the LATS gene expression by reducing MAP4K and, thus, preventing the development of apoptosis as a result of induced hypertrophy in the cardiomyocytes of the rats. It was also found that this training protocol had no effect on the variations of the MST gene expression in pathological conditions.

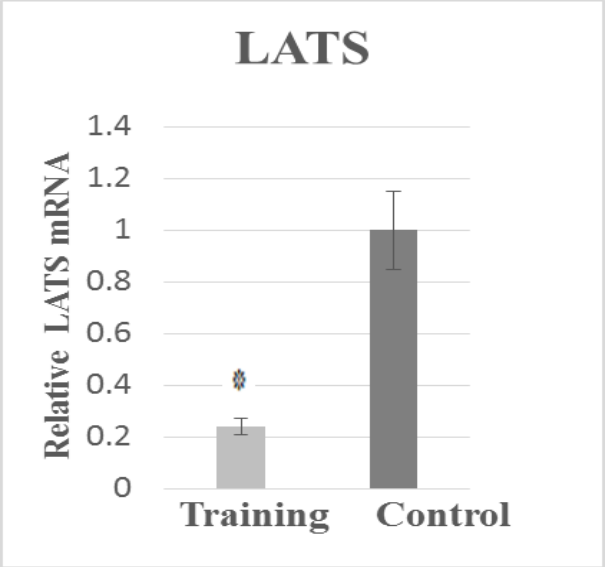

Figure 1. Relative amount of the LATS gene expression
*Significant difference with the control group ($P < 0.001$)

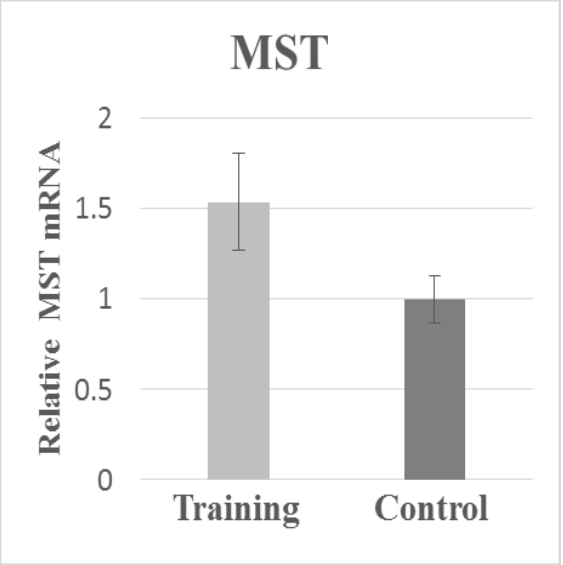

Figure 2. Relative MST gene expressio

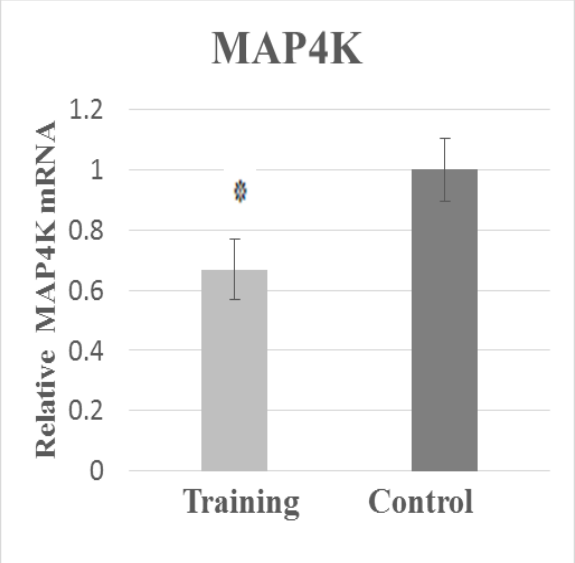

Figure 3. Relative amount of the MAP4K gene expression
*Significant difference with the control group ($P < 0.001$)

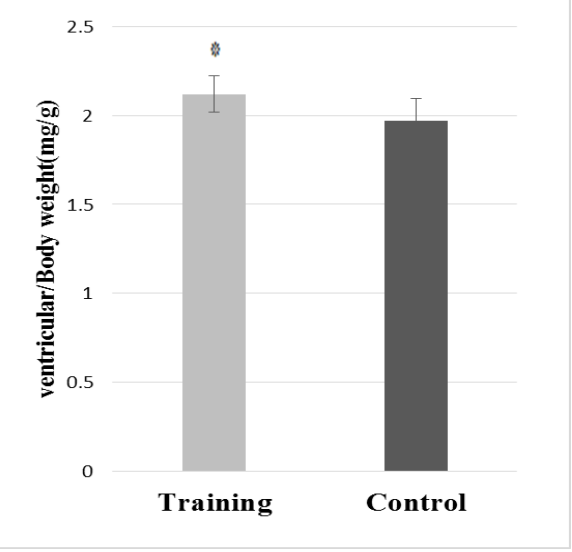

Figure 4. Ratio of the left ventricular weight to body weight
*Significant difference with the control group ($P < 0.001$)

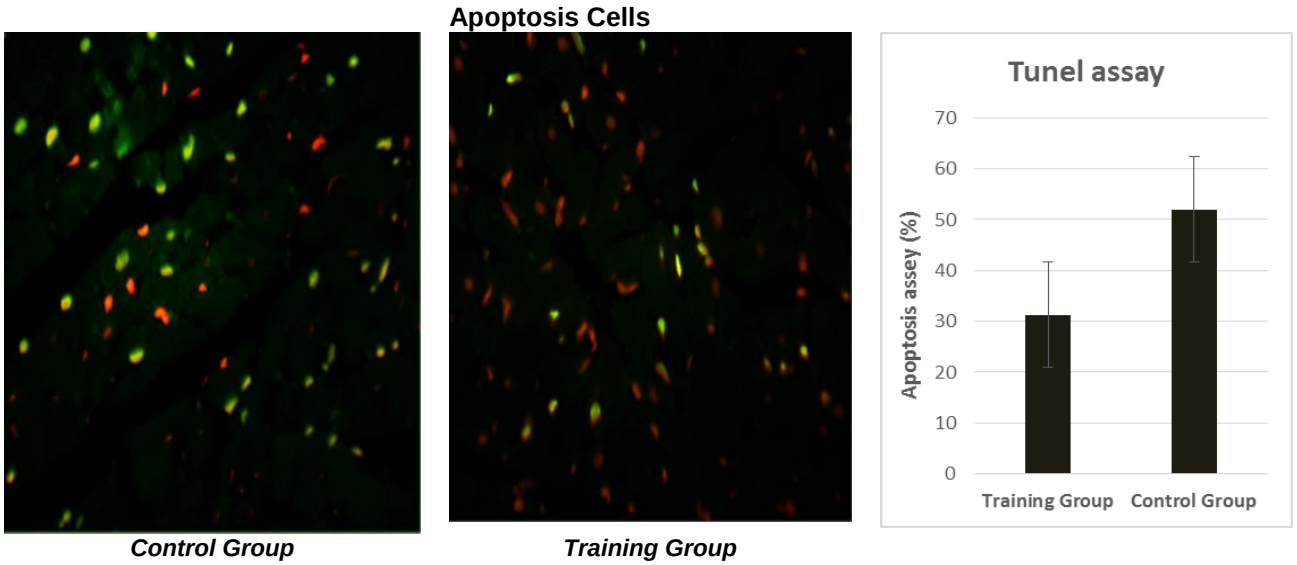

Figure 5. TUNEL staining, indicating the apoptosis rates in the 2 groups
Green spots represent apoptotic cells.

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