

Aerobic Training-induced Upregulation of YAP1 and Prevention of Cardiac Pathological Hypertrophy in Male Rats

Abstract

Background: Pathological hypertrophy is one of the negative consequences of cardiac sympathetic hyperactivity. Recent studies have shown that YAP1 plays a critical role in cardiomyocytes hypertrophy. Considering the preventive role of exercise training in cardiovascular diseases, the present study was conducted to examine the effect of aerobic exercise training on YAP1 gene expression and its upstream components. **Methods:** Eighteen male Wistar rats were randomly divided into aerobic training and control groups. Aerobic training was performed one hour/day, five days per week, for eight weeks, on a treadmill at 65-75% VO_2 max. Pathological hypertrophy was induced by injecting 3 mg/kg⁻¹ of isoproterenol for seven days. The left ventricle was separated, and YAP1, 3-mercaptopyruvate sulfurtransferase (MST), large tumor suppressor (LATS), and mitogen-activated protein 4 kinase (MAP4K) gene expressions were assessed and YAP1 protein levels were also assessed by western blotting. Cell apoptosis was detected by TUNEL assays. The between-group differences were evaluated using the T-test and P value <0.05 was considered statistically significant. **Results:** There were no significant between-group differences in MST gene expression ($P = 0.061$); meanwhile, in the training group, LATS and Map4K expressions were suppressed, followed by a significant increase in YAP1 expression ($P < 0.001$). Compared to the control group, the left ventricular weight increased significantly in the training group while the cardiomyocyte apoptosis decreased. **Conclusions:** The results showed that, by reducing LATS, aerobic training-induced YAP1 upregulation can help prevent the propagation of cardiomyocyte apoptosis due to pathological conditions.

Keywords: Aerobic exercise, cardiac hypertrophy, large tumor suppressor, mammalian sterile 20 like kinase, YES associated protein1

Introduction

Normal cardiac growth in response to exercise is mainly due to the physiological hypertrophy of cardiomyocytes. This situation is not associated with any functional and structural disorders of the heart. In contrast, pathologic hypertrophy is associated with the loss of cardiomyocytes and cardiac injury.^[1,2] Various pathways have been introduced as hypertrophic mediators in the heart.^[1] The Hippo pathway has recently been characterized as a potential key player in the regulation of cardiomyocytes.^[3] This pathway is capable of activating a kinase cascade that eventually activates the transcriptional coactivator YAP, leading to an increased expression of the downstream genes responsible for cell proliferation and growth. 3-Mercaptopyruvate sulfurtransferase (MST) and large tumor

suppressor (LATS) kinases are the upstream components of the Hippo pathway in mammals. LATS is directly activated by MST, which, in turn, inactivates YAP.^[4,5] Some studies have demonstrated that the increased expression of YAP leads to an increase in cardiomyocytes hypertrophy.^[6,7] Moreover, in the case of heart failure, the upregulation of YAP could result in an increased proliferation of cardiomyocytes.^[8-10] Many pathways that are regulated by exercise training have also been shown to contribute to the regulation of the Hippo pathway.^[2,4,11] It is therefore plausible that exercise training has influenced YAP as a component of the Hippo pathway, and YAP may thus act as a mediator in exercise adaptations.^[8] Furthermore, studies have shown that aerobic training is associated with physiological left ventricular hypertrophy.^[4,11] So, the question is: What is the role of YAP and its upstream genes

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in response to aerobic training and also in preventing pathological hypertrophy?

Methods

Sample processing

This experimental study was conducted at the University of Tehran. Eighteen Wistar rats^[12] (8 weeks-old, weighing 180-200 g) were randomly (with excel software) assigned into two groups of nine (training vs. control groups). The number of controls was reduced to eight due to the death of one of the rats as a result of isoproterenol injection in the final week of the study period. The rats were housed three per each cage at a mean temperature of $22 \pm 2^\circ\text{C}$, humidity of $50\% \pm 5$, and a 12:12 h light/dark cycle in accordance with the ethical considerations of clinical research.^[13] Standard water and food were freely available to the rats throughout the study.

Training intervention

After a two-week adaptation to the new environment and familiarity with the treadmill, the training group underwent an eight-week aerobic exercise training intervention on the treadmill with 15° inclination. The intensity of exercise was 65-75% VO_2 max and the time of training was progressive. The duration of each session was 30 min at the beginning of the program and extended to 60 min/session in the last five weeks.^[14,15] For inducing similar stress as in the training group, the control group ran on the treadmill twice a week with a speed of 9 m/min at a slope of 0° , which causes no physiological response.^[15]

Test for estimating maximum oxygen consumption

To determine the maximum oxygen consumption, after 10 min of warm-up at the speed of 40-50% of the maximum oxygen consumption, the speed was increased 0.03 m/min every 2 min to exhaustion, that is, when the rat was no longer able to keep running on the treadmill.^[15]

Pathological hypertrophy induction

At the end of training, to induce pathological hypertrophy, all animals were injected subcutaneously with 3 mg/kg⁻¹ of isoproterenol (Sigma-Aldrich) once a day for seven days,^[16] and 48 hours after the final injection, the rats were anesthetized by injection of 50 mg/kg⁻¹ ketamine and 10 mg/kg⁻¹ xylazine. The hearts were immediately removed. Then, the hearts and dissected left ventricle were weighted and then stored at -80°C for the following analysis.

TUNEL staining

The apoptosis diagnosis technique was performed using a TUNEL kit (Roche Company, Germany) based on the manufacturer's procedure manual. After TUNEL staining, the samples were viewed with a fluorescence microscope (Zeiss LSM 5). For counting the apoptosis cells, five fields were enumerated in each group. The apoptotic cells in this

tissue were visible as bright green points labeled during the TUNEL staining process. The nuclei of the cells were dyed red. The green TUNEL positive cells were combined with the red nuclei and dyed orange so that they could be distinguished from the healthy red cells and easily counted.

Gene expression

After designing the primer, ribonucleic acid (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). The cDNA was then used for reverse transcription and gene expression was determined using polymerase chain reaction (PCR). GAPDH was considered as the reference gene. Forty cycles were considered for each real-time PCR cycle, and the cycles' temperature included 94°C maintained for 20 s, $58-60^\circ\text{C}$ for 30 s, and 72°C for 30 s. The melting diagram was plotted to examine the accuracy of the PCR reactions. Primers were listed in Table 1.

Western blot analysis

Left ventricles were homogenized and centrifuged. The protein content was assessed by the Lowry method and then separated by polyacrylamide gel electrophoresis (Bio-Rad) via 4-20% gradient polyacrylamide gels containing 0.1% sodium dodecyl sulfate for ~ 2 h at 95 V. After electrophoresis, the proteins were transferred to polyvinylidene fluoride (PVDF) membranes (Roth) for 80 min at 80 V (Bio-Rad). Nonspecific sites were blocked overnight at 4°C in tris buffered saline (TBS) containing Tween and 5% nonfat milk (Sigma-Aldrich). Membranes were then incubated for 2 h with primary antibodies directed against the proteins of interest at room temperature. Then, the protein abundance of YAP1 and GAPDH were determined in heart samples. Following incubation with primary antibodies, membranes were washed extensively with PBS-Tween and then incubated with secondary antibodies for 1 h at room temperature. After washing, membranes were developed using 3, 3'-diaminobenzidine (DAB) substrate and images were analyzed using the Image J software.

Statistical analysis

Mean \pm SEM was used as descriptive statistics. The Shapiro-Wilk test was used to determine the normal distribution of the data. The independent *t*-test was used to evaluate between-group differences. Also, 95% confidence intervals (CIs) were reported. Data were analyzed using Statistical Package for the Social Sciences (SPSS) version 24 at a significance level of $\alpha < 0.05$.

Results

The evaluation of the effects of aerobic training on the alteration of YAP1 showed mRNA levels of YAP1 (95% CI:

5.01–11.15; $P = 0.03$) and its protein levels ($P = 0.01$) were significantly upregulated in training group as compared with control [Figure 1]. In addition, the results of TUNEL staining showed that aerobic training led to a significant decrease in apoptosis of the left ventricular cardiomyocytes compared with the control group ($P < 0.001$), [Figures 2 and 3]. Also, Figure 3 shows that the left

ventricular weight to body weight ratio was significantly higher in the training group compared with control as a result of aerobic training. The results of PCR showed β myosin heavy chain (β -MHC) was significantly suppressed in the aerobic group (95% CI: -0.77– -0.17; $P < 0.001$). LATS expression decreased significantly in the training group compared with control (95% CI: -18.63– -45.07; $P < 0.001$). Also, to determine which pathway affected the LATS alteration, MST and mitogen-activated protein 4 kinase (MAP4K) expression levels were measured as shown in Figure 4. Although MST expression increased after aerobic training, the independent t -test did not reveal a significant increase (95% CI: 0.04–1.25; $P = 0.061$). On the other hand, MAP4K expression was significantly lower in training than that in the control group (95% CI: -0.65– -0.02; $P < 0.001$).

Discussion

The analysis of the data revealed that the gene and protein expression of YAP increased significantly in the training group, while the expression levels of LATS, MAP4K, and β -MHC were significantly suppressed. There was no significant difference in MST changes. The results showed a decrease in the apoptosis rate in the training group compared to the controls. Also, the left ventricle weight was significantly higher in the training group than that in the controls. The Hippo pathway function is to deactivate YAP to maintain the cells in the quiescent state. YAP is activated in the case of tissue injury or the suppression of the Hippo pathway, which could then lead to tissue repair.^[17] The present findings showed that, compared to the control group, the expression of YAP was increased in the left ventricular cardiomyocytes in the training group. The increase in YAP expression might indicate the possibility of aerobic training causing stress in cardiomyocytes, similar to the early phase of cardiac injury, which leads to an increased YAP expression.^[6] In this study, increased YAP expression was also associated

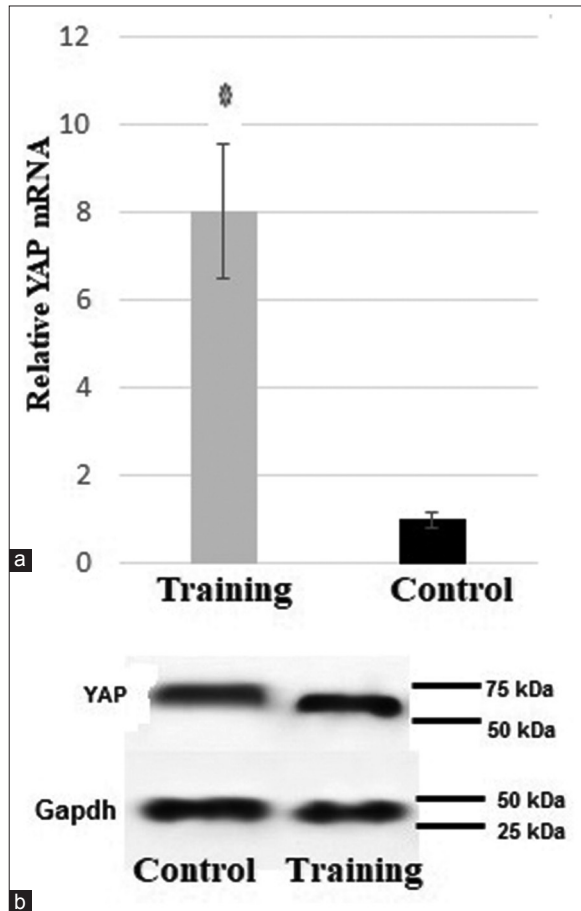


Figure 1: (a) The Relative YAP1 gene expression (b) The protein expression levels of YAP1 assessed by Western blotting and Gapdh was used for controlling the loading ($P < 0.001$)

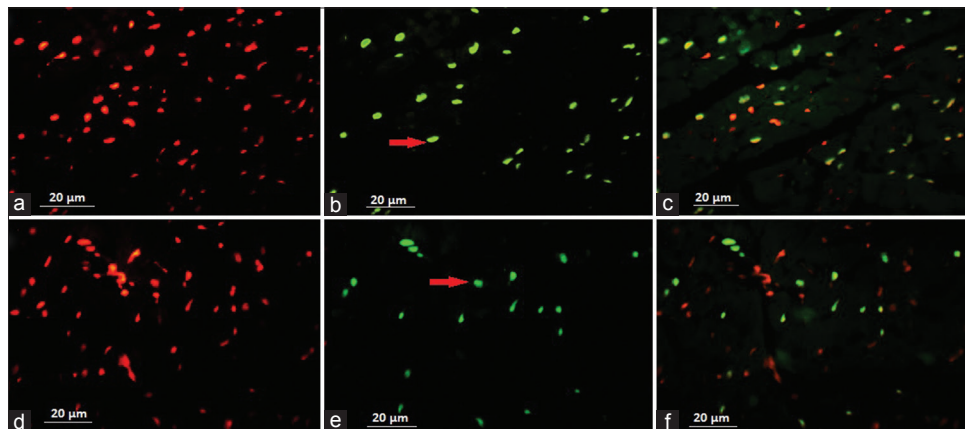


Figure 2: TUNEL Apoptosis Cells staining indicates the apoptosis rates in the two groups; magnification: 400 \times ; Green spots represent the apoptotic cells. (a)nuclei in control group; (b) apoptotic cell in control group; (c) merge in control group. Positive reaction: 53%. (d) nuclei in training group; (e) apoptotic cell in training group; (f) merge in training group. Positive reaction: 33%

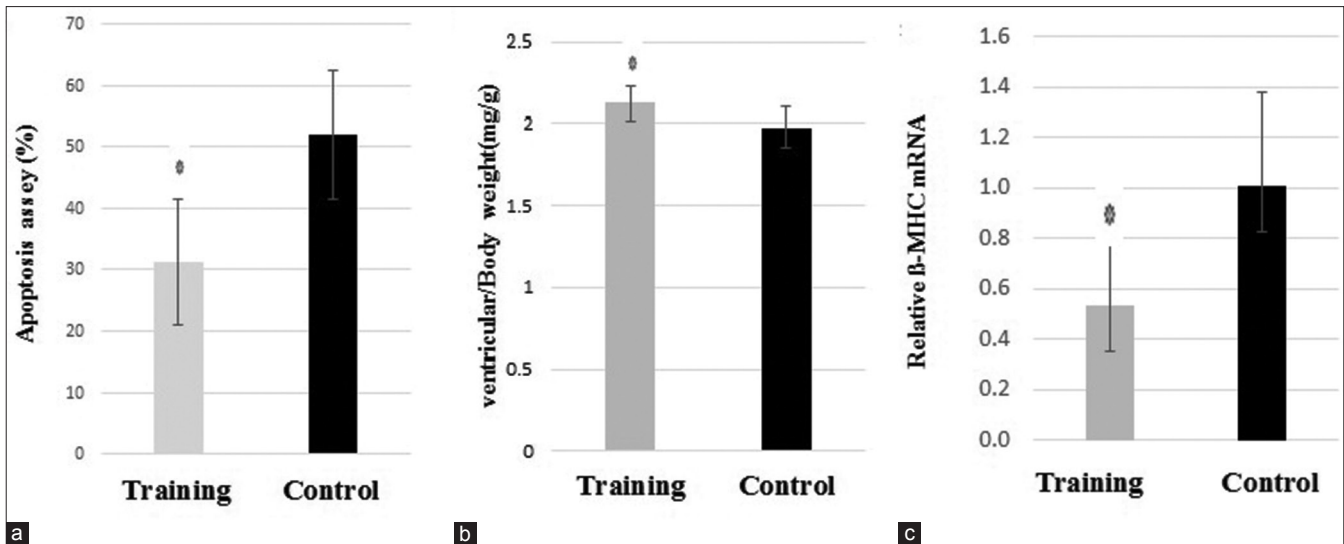


Figure 3: (a) TUNEL Apoptosis Cells Percents indicates the apoptosis rates in the two groups (b) The ratio of left ventricular weight/body weight (mg/g); (c)The Relative β-MHC gene expression. *Significant difference from control group ($P < 0.001$)

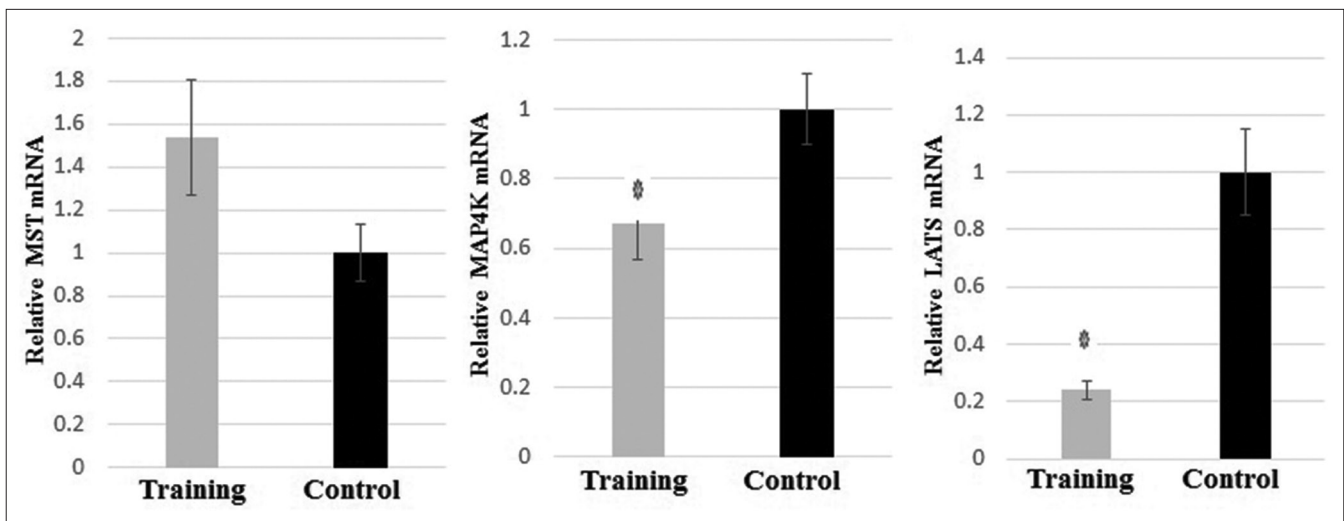


Figure 4: (a) The Relative MAP4K gene expression (b) The relative amount of MST gene expression (c) The relative amount of LATS gene expression. * Significant difference from control group ($P < 0.001$)

with suppressed LATS, which is the most crucial factor in the regulation of YAP in mammals.^[4] LATS gene expression negatively regulates the function of YAP in adults' heart.^[6] LATS is activated by MST, which is known as an upstream kinase involved in the Hippo pathway.^[5] The present findings showed that aerobic training induces a moderate non-significant increase in MST. Given the increase in MST, LATS gene expression was also expected to be elevated or at least remain stable, but on the contrary, its expression reduced significantly. LATS and YAP have been shown to act independently and be regulated in the absence of MST.^[17,18] Meanwhile, in parallel with MST, MAP4K can also directly activate LATS.^[17] Therefore, considering the significant reduction in MAP4k in this study, decreased LATS expression can be mediated by this reduction and lead to reduced pathological cardiac

hypertrophy as well. In the present study, a significantly lower rate of apoptosis was observed in cardiomyocytes of the training group, which is similar to the results reported by Silva *et al.* (2014). This finding indicates that aerobic training has positive effects on the reduction in the number of apoptotic cardiomyocytes in cardiac failure.^[19] Other corroborating research demonstrated that regular exercise can reverse or at least attenuate the impact of pathological remodeling.^[20,21] Increased YAP expression has been shown to protect cardiomyocytes against apoptosis following chronic myocardial infarction.^[22] In a study conducted by Windmueller *et al.* (2015), the increased expression of YAP was found to potentially improve cardiovascular disorders in hypertrophic cardiomyopathy,^[23] and inversely, the lack of YAP expression could result in a significant increase in myocardial fibrosis and apoptosis in response

Table 1: Primer sequence and associated ID number based on the reference bank

Gene	Forward primer	Reverse primer	ID
Yap1	5'-ACTGGAGGAGGATGGAGGGA-3'	5'-AGGGTGTCTGGCTGATGGTGT-3'	363014
LATS1	5'-TGTGATTGGTGGAGTGTGGTG-3'	5'-TGAGGTGGGATGTGAAGAGAAG-3'	308265
MAP4K	5'-GAGGCTGTGGGAATGGTTGGA-3'	5'-GTAGACGGAAGGTGAGGGTGG-3'	11184
MST1	5'-GCAATAGCTCAGTCCTTC-3'	5'-CTCGGTGTATATCTCACTCT-3'	24566
β-MHC	5'-TCCAGCTGAAAGCAGAAAGAG-3'	5'-CCTGGGAGATGAACGCATAAT-3'	29557
Gapdh	5'-AAGTCAACGGCACAGTCAAGG-3'	5'-CATACTCAGCACCAGCATCACC-3'	108351137

to myocardial infarction in mice.^[7] The enhanced levels of YAP1 in the training group could thus have been responsible for the reduced apoptosis rate. Many studies have reported LATS to be an important factor in the induction of apoptosis in cardiomyocytes.^[10] One other reason for the reduction of apoptotic cardiomyocytes in the training group in this study could be the effect of aerobic exercise on reducing LATS gene expression. In this study, the significant reduction in apoptosis was associated with a significant increase in left ventricular weight in the rats in the training group in relation to their body weight and compared to the control group; meanwhile, the reduction in the training group in the mRNA levels of β-MHC as a marker of pathological hypertrophy suggests that training was associated with physiological hypertrophic responses. Considering the role of YAP in the reduction of apoptosis and cardiomyocyte hypertrophy,^[9] it could be assumed that the exercise protocol in this study induced the expression of YAP, and YAP was responsible for the reduction of apoptosis and physiological cardiac hypertrophy. In contrast to the present findings, cardiac YAP activation after myocardial infarction (MI) was not able to reduce cardiomyocyte apoptosis but could increase hypertrophy^[24]; the results of another study also confirmed this finding and showed that decreased cardiomyocytes apoptosis in YAP knockout mice but decline in cardiac hypertrophy^[6] and The increased hypertrophy in the present research may be explained by noting the findings of previous reports, which showed that aerobic training increases the mechanical load and causes physiological cardiomyocyte hypertrophy.^[11] YAP activation has been presumed to regulate the activation of the mammalian target of rapamycin (mTOR) pathway,^[25] which is one of the well-known pathways in physiological cardiac hypertrophy.^[20] The cross-talk between YAP and mTOR may thus play a role in physiological cardiac hypertrophy.

Together, these findings support the assumption that aerobic exercise training can prevent cardiomyocyte apoptosis and enhance physiologic hypertrophy through its impact on YAP.

Conclusions

It could be inferred from the present findings that aerobic exercise training can suppress the Hippo pathway and increase the expression of YAP in the myocardium of rats

and thereby prevent pathological cardiac hypertrophy. These phenomena were associated with a reduction in the apoptosis rate and increased physiological cardiomyocyte hypertrophy.

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Conflicts of interest

There are no conflicts of interest.

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