



The role of Hippo signaling pathway in physiological cardiac hypertrophy

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Abstract

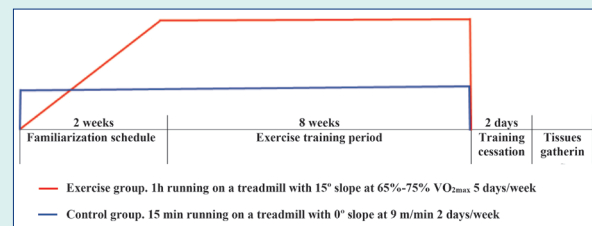
Introduction: The role of Hippo signaling pathway, which was identified by genetic studies as a key regulator for tissue growth and organ size, in promoting physiological cardiac hypertrophy has not been investigated.

Methods: Fourteen male Wistar rats were randomly assigned to the exercise and control groups.

The exercise group ran 1 hour per day, 5 days/week, at about 65%-75% VO_{2max} on the motor-driven treadmill with 15° slope, and the control group ran 15 min/d, 2 days/week at 9 m/min (0° inclination), throughout the eight-week experimental period. Forty-eight hours after the last session, hearts were dissected and left ventricles were weighed and stored for subsequent RT-PCR analysis.

Results: Despite a significant increase in the MAP4k1 expression levels in the exercise group ($P=0.001$), the Mst1 expression was inhibited compared to the control group ($P<0.001$) which was followed by suppression of Lats1 expression ($P=0.001$). Compared with the control group, significant increases were observed in heart weight/body weight ($P=0.024$) and left ventricular weight/body weight ($P=0.034$) ratios in the exercise group. The H&E staining confirmed the cardiac hypertrophy that may be partly due to a significant increase in Yap1 expression level compared with the control group ($P<0.001$), which was confirmed by Western blot analysis.

Conclusion: Increased MAP4K1 expression did not influence Lats1 activation. The exercise training protocol suppressed Mst1 and Lats1 (Hippo pathway) and caused an increase in Yap1 expression level, which led to physiological cardiac hypertrophy in healthy rats. Further studies are suggested to apply this exercise protocol for the prevention and/or rehabilitation of cardiovascular disease and health promotion.



Introduction

The heart is a muscle that pumps blood throughout the circulatory system to deliver adequate oxygen and nutrients to tissues such as skeletal muscles and itself which may lead to improved athletic performance and health promotion.^{1,2} Most of the heart mass is composed of the cardiomyocytes. Despite, mammalian cardiomyocytes are terminally differentiated soon after birth, characteristics of the cardiac tissue lead to an increase in the heart mass through the hypertrophy of individual cardiomyocytes.³ Cardiomyocyte hypertrophy is characterized by enhanced protein synthesis and an increase in cell size, especially the left ventricle. There are two different classes of cardiac hypertrophy. In the pathological hypertrophy

(cardiac dysfunction), which is associated with conditions like hypertension or injuries, inadequate capillaries to supply nutrients and oxygen leads to cardiac hypoxia and remodeling. In contrast, physiological hypertrophy (normal cardiac function) occurs during normal growth of children, as well as in response to exercise training that provokes the physiological cardiac hypertrophy, enhances its function, and it is not considered as a cardiac disease risk factor due to an expanded capillary network to supply adequate blood flow and nourishment,^{2,4} however, those beneficial effects depend on duration and intensity of exercise. Physiological cardiac hypertrophy is regulated by both organ-extrinsic stimuli such as circulating growth factors, hormonal, and nutritional status and also organ-



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intrinsic mechanisms that control the heart size via genetic signaling pathways such as PI3K-Akt and mTOR with their downstream targets, p70S6 and eEF2 which are not fully understood.^{5,6} The earlier studies demonstrated the critical and important role of the Hippo signaling pathway in cardiomyocyte proliferation and hypertrophy, cardiac regeneration, and controlling the heart size. The mammalian genome of the Hippo contains Ste20 family kinases (Mst1 and Mst2), an adaptor protein Salvador, Salv/WW45, two proteins MOBKL1A and MOBKL1B, often collectively referred to as Mob1, and two large tumor suppressor kinases (Lats1 and Lats2),⁷⁻⁹ that phosphorylates and inactivates yes-associated protein (Yap) and transcriptional coactivator with PDZ-binding motif (Taz), the downstream transcriptional regulators of the signaling pathway.¹⁰ In contrast, in the inactive state of Hippo pathway, accumulation of Yap and Taz in the nucleus enhances gene expression for organ growth by interacting with other proteins such as SMAD and OCT4.¹¹ The versatile interactions between Akt, MAP4Ks, and Hippo kinase network including Mst1, Lats1, Yap and Taz have been documented,¹²⁻¹⁴ but none of them included the exercise training protocols. Several studies have been conducted on rodents to evaluate the positive effect of treadmill running on ventricular mass and cardiomyocyte dimensions, and also to investigate the effect of endurance exercise on heart weight and its ratio to body weight,^{15,16} however, recently it has been shown that eight weeks of endurance treadmill running has no impact on heart weight/body weight ratio in mice.¹⁷ To date, the molecular mechanism of adaptive cardiac hypertrophy, especially Hippo pathway, in response to exercise training has not been investigated and the contribution of the Hippo signaling pathway to physiological hypertrophy is unclear. Given that the duration and intensity of exercise have a positive impact on the cardiovascular system and athletic performance, the purpose of the present study was to investigate the effects of an eight-week endurance treadmill running at 15° inclination on cardiac hypertrophy and alteration in Mst1, Lats1, and Yap1 expression levels. In addition, because MAP4Ks had been identified as a direct Lats 1/2-activating kinase,¹⁴ assessment of MAP4K1 expression levels was included in this study.

Materials and Methods

Experimental design

Fourteen male Wistar rats, eight-week-old (180-200 g), were randomly assigned to either exercise (n=7) or control (n=7) groups. Three-four rats were housed per standard cage and maintained in a light-dark cycle of 12 hours, 22 ± 2°C, 50 ± 5% relative humidity, and had access to pelleted rodent diet and water ad libitum. Throughout the study, the same person handled the animals in each group and all experimental protocols were conducted during the dark cycle. The body weight was measured every week.

Exercise training

All rats completed a two-week familiarization schedule, 5 days/week, which consisted of running on the motor-driven treadmill with stainless steel grids at the end of the lines to provide an electrical stimulus (0.5 mA, 1 stimulus of 200-ms duration every second). For the exercise group, the intensity of running gradually increased in speeds and grades to achieve the initial condition of the main exercise training protocol. To determine exercise intensity, before starting the main protocol and at the every other weekend, VO_{2max} (maximum oxygen consumption as an endurance capacity index) test was performed with uphill-running on the treadmill (15° inclination) as previously described.¹⁸ Briefly, each rat warmed up for 10 minutes at 40-50% VO_{2max} , and then treadmill velocity was increased by 0.03 m/s every 2 minutes until the rat was unable to run further. During the eight-week experimental period, all rats in the exercise group ran 1 hour per day, 5 days/week, at about 65%-75% VO_{2max} on the treadmill with 15° slope.¹⁹ Each session included 10 minutes warm-up and cool-down periods at 50%-60% VO_{2max} with 0° slopes. Given that 15 minutes running at 0° slope at 9 m/min does not yield any training response, the treadmill running skill was maintained in control rats for 2 days/week throughout the study (Fig. 1).¹⁸

Cardiac harvesting

Forty-eight hours after the last exercise session, the rats in the two groups were anesthetized with 50 mg/kg ketamine hydrochloride and 10 mg/kg xylazine intraperitoneally. Then the animals were euthanized by draining the blood directly from their hearts and the hearts were excised and flushed free of blood in saline and were weighed after absorbing the excess water by tissue paper. Thereafter, all dissected left ventricles were weighed and stored at -80°C for qRT-PCR, Western blot, and Histological analyses.

Gene expression

Quantitative real-time PCR

Approximately 40-50 mg of each left ventricle was homogenized and according to manufacturer's instruction, TRizol reagent (Qiazol, cat. no. 79306, USA) was used to isolate total RNA. Purity and concentration of RNA were determined spectrophotometrically by NanoDrop 2000 (Thermo Scientific, Rockford, USA), and 1% agarose gel stained with Nancy-520 was used to determine RNA integrity electrophoretically (Sigma-Aldrich, Sao Paulo, SP, Brazil). cDNA was synthesized from 2 mg of total RNA using a cDNA synthesis kit (Fermentas, Glen Burnie, MD, USA). The quantitative real-time polymerase chain reaction (qRT-PCR) was run to assess the messenger RNA (mRNA) levels of the target genes and reference gene (GAPDH) separately. Amplifications were performed with an ABI Prism 7500 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) by using SYBR Green/High ROX qPCR Master Mix (Ampliqon, Denmark) and purity of the amplification products was confirmed

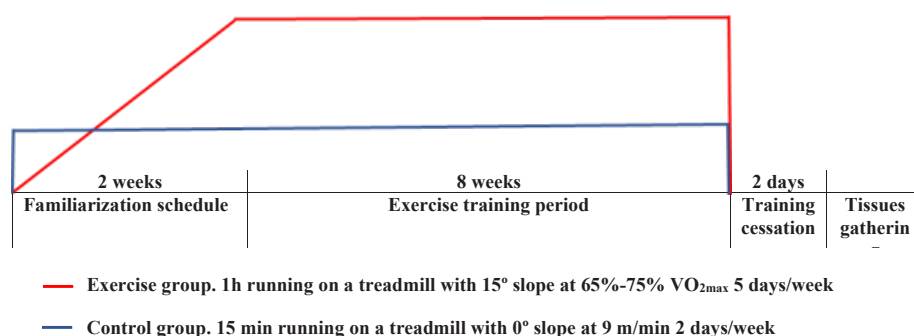


Fig. 1. Illustration of familiarization period and exercise training protocol including duration and intensity.

using melting point dissociation curves. According to the manufacturer method, compared cycle threshold (Ct) values were expressed. GAPDH was considered as housekeeping gene and fold-change was determined using $2^{-\Delta\Delta Ct}$.

The primer sequences used in this study are shown in Table 1.

Western blot analysis

Homogenized left ventricles in RIPA buffer (cytomatingene) with a protease inhibitor cocktail (Sigma) centrifuged at 15000 rpm for 10 minutes at 4°C and supernatants were collected to assess protein content by the Lowry method. Polyacrylamide gel electrophoresis (Bio-Rad) via 4%–20% gradient polyacrylamide gels containing 0.1% sodium dodecyl sulfate for 2 hours at 95 V was applied for electrophoresis and separated proteins were transferred to PVDF membranes (Roth) for 80 minutes at 80 V (Bio-Rad). Nonspecific sites were blocked overnight at 4°C in TBS containing Tween and 5% non-fat milk (Sigma). Membranes were incubated 2 hours with primary antibodies directed against the proteins of interest at room temperature. The protein abundance of Yap1 and Gapdh were determined in left ventricle samples. Following incubation, membranes were washed extensively with PBS-Tween and then incubated with secondary antibodies 1 hour at room temperature. Thereafter, membranes were developed using DAB and related images were captured and analyzed using the ImageJ software.

Histological analysis

All left ventricle tissues were fixed with formalin (10%),

embedded in paraffin, and then, the samples were cut into sections (3 μm thick). To evaluate histological changes, the paraffinized sections were stained using hematoxylin and eosin (H&E). Images of the stained sections were taken (×400 magnification).

Statistical analysis

Data are expressed as mean ± standard error of the mean (SEM). Between-group differences were evaluated using independent Student's *t* test and Mann-Whitney *U* test for normally and non-normally distributed data, respectively, using SPSS version 24. Statistical significance was accepted at $P < 0.05$.

Results

Findings of the present study indicate that compared with the control group, the eight-week endurance treadmill running at 15° slope had a positive effect on the left ventricular hypertrophy of rats in the exercise group which could be due to Mst1, Lats1, and Yap1 genes alteration.

The effect of exercise training on Hippo signaling-related genes

Left ventricular expression levels of Mst1, Lats1, and Yap1 genes were evaluated using qRT-PCR analysis to reveal the molecular phenotype of physiological cardiac hypertrophy related to Hippo signaling. The eight-week endurance training protocol caused a significant reduction in expression levels of Mst1 ($P < 0.001$) in the left ventricles of the exercise group compared with the control group (Fig. 2A). It has been demonstrated that MAP4Ks, as components of the expanded Hippo pathway, in parallel to Mst1/2 regulate the Lats1/2 and Yap/Taz. The results

Table 1. The primer sequences of genes

Gene name	Gene ID	Forward Sequence	Reverse Sequence
Mst1	24566	5'-GCAATAGCTCAGTCCTTC-3'	5'-CTCGGTGTATATCTCACTCT-3'
Lats1	308265	5'-TGTGATTGGTGGAGTGTGGTG-3'	5'-TGAGGTGGGATGTGAAGAGAAG-3'
Yap1	363014	5'-ACTGGAGGAGGATGGAGGGA-3'	5'-AGGGTGTCTGGCTGATGGTGT-3'
MAP4K1	292763	5'-GAGGCTGTGGGAATGGTTGGA-3'	5'-GTAGACGGAAGGTGAGGGTGG-3'
GAPDH	108351137	5'-AAGTCAACGGCACAGTCAAGG-3'	5'-CATACTCAGCACCAGCATCACC-3'

showed that there was a significant increase in MAP4k1 expression level compared with the control group ($P=0.001$) after eight weeks of endurance exercise training (Fig. 2B). Despite the increased MAP4K1 expression, according to the Hippo signaling pathway and due to reduced Mst1 level, the Lats1 expression was significantly suppressed in the exercise group (Fig. 2C) compared with the control group ($P=0.001$). It is noteworthy that the increased MAP4K1 expression level alone was not able to inhibit the Yap1 expression in the exercise group. As shown in Fig. 2D, following the suppression of Lats1, the expression levels of Yap1 increased significantly in the exercise group compared with the control group ($P<0.001$).

Also, Western blot analysis was used for more verification of Yap1 expression. The results confirmed a significant increase in Yap1 ($P<0.001$) compared with the control group (Fig. 3A-B).

The effect of endurance exercise training on body, heart, and left ventricular weights and their ratio

The physiological characteristics of the rats are presented in Table 2. There were no significant between-group differences in body, heart, and left ventricular weights. The morphological cardiac changes including increased heart and left ventricular weight (7.37% and 7.90% respectively) and especially the heart weight/body weight and left ventricular weight/body weight ratios in the exercise group were significantly higher than the control group (9.38%; $P=0.024$ and 9.64%; $P=0.034$ respectively), indicating that the training protocol improved cardiac hypertrophy, which was confirmed by H&E staining. The results revealed that the left ventricular hypertrophy in the exercise group (Fig. 3C) is higher than the control group (Fig. 3D).

Discussion

To the best of our knowledge, this is the first study that examines the effect of exercise training on the Hippo signaling pathway to reveal the cellular signaling responsible for uphill-endurance treadmill running-induced physiological hypertrophy in cardiac tissue. Although no significant changes in HW/BW ratio after eight weeks of endurance treadmill running in mice has been reported,¹⁷ the significant increased HW/BW (9,38%, $P=0.024$) and LVW/BW (9,64%, $P=0.034$) ratios in our exercise group (Table 2) confirmed the physiological hypertrophy effects of endurance exercise on cardiac tissue.²⁰ Protein synthesis and consequently physiological cardiac hypertrophy depend on the activation of systemic growth factors and related signaling pathways such as IGF-1/PI3K/Akt/mTOR.²¹ Also, Mst1/2, Lats1/1, and Yap/Taz, the mammalian core Hippo signaling components, are considered as important regulators of cell proliferation, differentiation, and organ growth. Given that cardiomyocyte hypertrophy is regulated by both Hippo and Akt-mTOR networks, the cross-talk between Hippo and Akt-mTOR signaling components has been studied, but in non-exercise training models. In this regard, it has been shown that Akt phosphorylates the Hippo signaling components to provoke the signaling induction responsible for cardiomyocyte hypertrophy.^{12,22} It has been shown that Akt phosphorylates Mst1, well known as an apoptosis component, to promote cell survival by protecting Mst1 from apoptotic cleavage,¹³ however, evidence gathered from some studies indicate that Mst1/2 are not essential for Lats1/2 activation.¹⁴ On the other hand, inactivated WW45 (Salv) which forms an active complex with Mst kinases, promoted cardiomyocyte proliferation and heart size.²³ The result of the present study is consistent with those reports and showed that

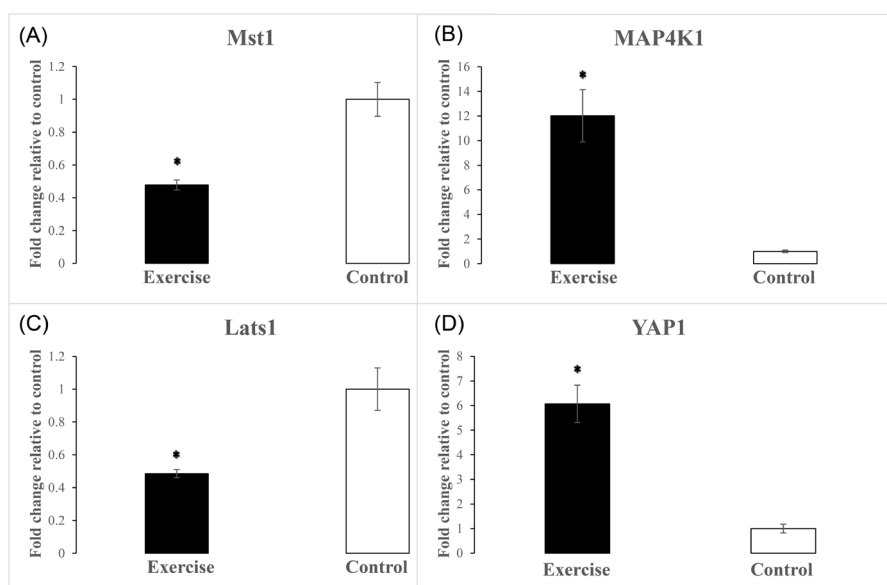


Fig. 2. The effect of uphill-endurance treadmill running on alteration of left ventricular mRNA expression levels of Hippo signaling components. * Indicates a significant difference of Mst1 (A), MAP4K1 (B), Lats1 (C), and Yap1 (D) gene expression levels from the control group ($n=7$ per group). $P\leq 0.001$.

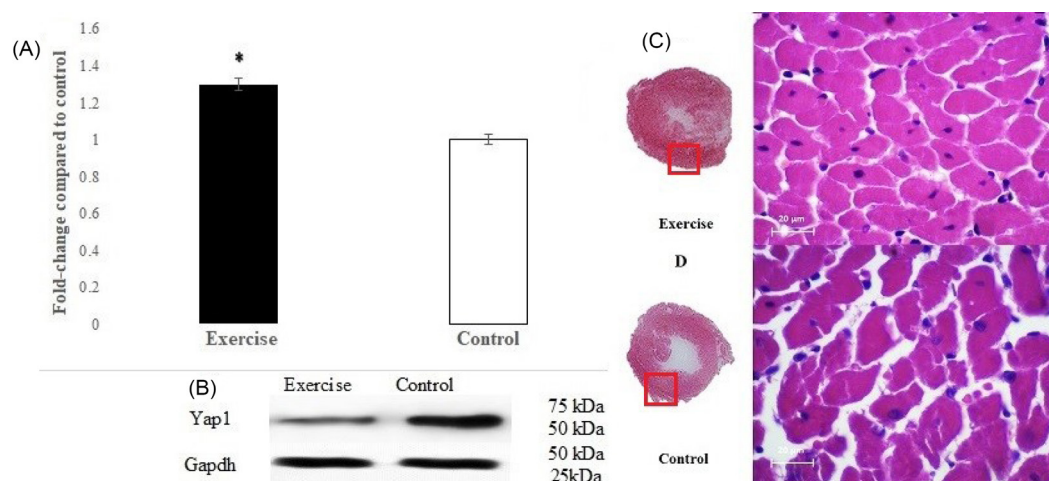


Fig. 3. (A) Significant difference ($P < 0.001$) from the control group in left ventricular Yap1 protein expression. (B) Protein expression levels of Yap1 in exercise and control groups, assessed by Western blotting, whereas Gapdh was used as a control for loading. (C and D) H&E stained left ventricle sections of rats in exercise and control groups. Compared with the control group, uphill-endurance treadmill running for eight weeks caused increased hypertrophy in the left ventricle that may partly be due to the alterations of Hippo signaling components. ($n = 7$ per group)

compared with the control group, Mst1 expression levels reduced in response to upstream regulators (Fig. 2A). Considering the Hippo signaling cascade, Mst is activated in response to upstream regulators and can phosphorylate Lats1/2. Phosphorylated Lats1/2, in turn, inhibits the transcriptional cofactors Yap and Taz by phosphorylating multiple serine residues.²⁴ Apart from this, recently, it has been reported that Mst1/2 are not essential for the Lats1/2 activation, and also MAP4Ks are considered as alternative kinases for phosphorylating Lats1/2 and deletion of both MAP4Ks and Mst1/2 is necessary to suppress phosphorylation of Lats1/2 and Yap/Taz. In addition, the deletion of Lats1/2 abolishes Yap/Taz phosphorylation.¹⁴ Our results showed that following the reduction of Mst1 levels, Lats1 expression levels reduced compared with the control group (Fig. 2C), whereas, MAP4K1 expression level increase (Fig. 2B). Thus, the increased MAP4K1 expression levels concomitant with reduction of Mst1 confirm the necessity of both MAP4Ks and Mst1/2 deletion to suppress phosphorylation of Lats1/2 and consequently Yap/Taz.¹⁴ There is convincing evidence that the Lats1 tumor suppressor can phosphorylate and inactivates the transcription regulator Yap, the major

downstream effector of the mammalian Hippo pathway, in vitro and in vivo. Depending on phosphorylation site, overexpression of Mst/Lats leads to phosphorylation and inactivation of Yap oncogenic function. Phosphorylated Yap is sequestered and localized in the cytoplasm as a transcriptionally inactive form. Conversely, non-phosphorylated Yap exists in the nuclear and regulates gene expression by down-regulating or up-regulating many oncogenes or tumor suppressor genes to promote cardiomyocyte adaptation.²⁵ It has been shown that inactivated lats1/2 and consequently Yap/Taz enhance cardiomyocyte regeneration in adult mice with myocardial infarction.²⁶ Indeed, depending on the condition, Hippo pathway involves in both physiological and pathological hypertrophy. The effect of endurance exercise training on the alterations of Hippo components expression level and consequently cardiomyocyte hypertrophy has not been directly investigated but the role of Yap in muscle hypertrophy has been reviewed and suggested. Indeed, there is an interaction between the Hippo and Akt-mTOR pathways. In fact, overexpression of Yap/Taz, increases the phosphorylation of Akt to prevent cardiomyocyte apoptosis, and inversely, Yap is phosphorylated by Akt,

Table 2. Physiological characteristics of the rats

Variables	Exercise (n=7)	Control (n=7)	P value	% Change
BW (g)	321.57 ± 9.67	326.29 ± 7.55	0.707	- 1.45
HW (mg)	1010.71 ± 38.49	941.29 ± 27.25	0.167	7.37
LVW (mg)	694.43 ± 24.39	643.57 ± 13.18	0.092	7.90
HW/BW%	3.15 ± 0.09 *	2.88 ± 0.04	0.024	9.38
LVW/BW%	2.16 ± 0.07 *	1.97 ± 0.03	0.034	9.64
LVW/HW%	0.688 ± 0.012	0.685 ± 0.014	0.885	0.44

BW, body weight; HW, heart weight; LVW, left ventricular weight in exercise and control groups ($n = 7$ per group). * indicates significant difference from control group. Data are presented as Mean ± SEM.

for example in an exercise training condition, and thereby, its transcriptional role is suppressed. Also, Yap induces cardiomyocyte hypertrophy through the upregulation of miR-206.^{27,28} Recently, It has been shown that endogenous Yap has a critical role in hypertrophic and survival adaptive role to acute overload pressure.²⁹ In this regard, increased HW/BW and LVW/BW ratios in our subjects (Table 2) are consistent with the result of a study that showed inactivated Hippo pathway components in the mouse heart, promoted cardiomyocyte proliferation and heart size²³ and Yap has been identified as a major Hippo effector in cardiomyocyte development.³⁰ Moreover, our findings support the view in which physical exercise, such as endurance running, cause adult's cardiac growth³¹ and more intense uphill-treadmill running in the present study may be considered as a more powerful stimulator. Additionally, Western blots confirmed that Yap1 protein expression in the exercise group significantly increased compared with the control group (Fig. 3A-B). Increased Yap1 expression can lead to protein synthesis in the heart and consequently, cardiac hypertrophy which was occurred in the exercise group and confirmed by H&E staining (Fig. 3C-D).

Conclusion

The results of the present study showed that the increased Yap1 expression level induced by endurance treadmill running at 15° inclination caused cardiomyocyte hypertrophy in healthy rats. In accordance with Hippo signaling cascade, compared with Mst1, MAP4K1 does not affect the activation of Lats1 in response to exercise training. Cardiac hypertrophy and consequently enhanced cardiac contractility contribute to improving cardiac pump capacity that may lead to more blood and oxygen delivery to the heart itself and also other tissues like skeletal muscle³² which may help to promote individual and public health. Also, these findings may help to make a more effective exercise training program for rehabilitation and/or prevention of cardiovascular disease.

Funding sources

The present study was conducted with no external financial support.

Ethical statement

All experiments were reviewed and approved by the research ethics committee of sport sciences research institute of Iran.

Competing interests

There is no conflict of interest to be declared.

Authors' contribution

MGH and AT have the same contribution in designing, conducting, data gathering, statistical analyzing, and writing the manuscript. MGH also reviewed and prepared the final version of the manuscript.

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Research Highlights

What is the current knowledge?

- ✓ Heart is a vital organ in health promotion.
- ✓ Genetic studies have established the role of Hippo pathway in organ size regulation.
- ✓ Its role in physiological cardiac hypertrophy has not been investigated.

What is new here?

- ✓ Exercise training inhibited Mst1 expression levels, followed by suppression of Lats1.
- ✓ The significantly increased expression of MAP4K1 did not influence Lats1 activation.
- ✓ The exercise protocol increased Yap1 expression which was confirmed by Western blotting.
- ✓ Confirmed cardiac hypertrophy by H&E staining may partly be due to increased Yap1.
- ✓ These findings may help to make a more effective exercise training program for rehabilitation and/or prevention of cardiovascular disease.

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