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ORIGINAL ARTICLE

Swimming exercise training-induced left ventricular hypertrophy involves microRNAs and synergistic regulation of the PI3K/AKT/mTOR signaling pathway

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Abstract
Purpose Swimming exercise leads to a nonpathological, physiological left ventricular hypertrophy. However, the potential molecular mechanisms are unknown. We investigated the role of microRNAs (miRNA) regulating the cardiac signal cascades were studied in exercised rats.
Methods Female Wistar rats were assigned into two groups: (1) sedentary control (SC), (2) swimming exercise (SE). The rats in the SE group completed a 1-h swimming exercise, 5 times/week/8-week with 5 % body overload. miRNA, phosphoinositide-3-kinase catalytic alpha polypeptide (PIK3α), phosphatase and tensin homolog (PTEN) and tuberous sclerosis complex 2 (TSC2) gene expression analysis were performed by real-time PCR in heart muscle. Moreover, we assessed cardiac protein expression of ERK1/2, PI3K/AKT/mTOR, PTEN and TSC2.
Results Cardiac phospho^{ser473}-AKT and phospho^{Ser2448}-mTOR were, respectively, increased by 46 and 38 % in the SE group when compared with SC group. miRNAs-21, 144, and 145 were, respectively, up-regulated in the SE group (152 %, 128, and 101 % relative increases), but miRNA-124 was decreased by 38 %. In SE group, PIK3α (targeted by miRNA-124) gene expression increased by 213 %, and Pten (targeted by miRNAs-21 and 144), and TSC2 (targeted by miRNA-145) were, respectively, decreased by 51 and 55 %. In addition, the swimming exercise increased protein levels of PIK3α (36 %) and phospho^{Thr1462}-TSC2 (48 %), while it decreased PTEN (37 %) and TSC2 (22 %), which induced activation of PI3K/AKT/mTOR signaling pathway.
Conclusion These findings are consistent with a model in which exercise may induce left ventricular hypertrophy, at least in part, changing the expression of specific miRNAs targeting the PIK3/AKT/mTOR signaling pathway and its negative regulators.

Keywords Swimming · Heart · Hypertrophy · microRNA · PI3K · ERK

Abbreviations

ANP	Atrial natriuretic polypeptide
LVH	Left ventricular hypertrophy
mTOR	Mammalian target of rapamycin
miRNA	microRNAs
PIK3α	Phosphoinositide-3-kinase catalytic alpha polypeptide
PIP2	Phosphatidylinositol-4,5-bisphosphate
PIP3	Phosphatidylinositol-3,4,5-trisphosphate
PTEN	Phosphatase and tensin homolog
PKC	Protein kinase C
TSC2	Tuberous sclerosis complex 2
α-Actin	Skeletal muscle α-actin
α-MHC	α-Myosin heavy chain
β-MHC	β-Myosin heavy chain

Introduction

Left ventricular hypertrophy (LVH) induced by swimming exercise training is an important physiological mechanism that compensates for chronic increases in hemodynamic

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overload. This phenotype is associated with sarcomeres added in series and in parallel to lengthen the cardiac cell. The increased cross-sectional area contributes to increased ventricular stroke volume and cardiac output, which improves aerobic capacity. In contrast, pathological LVH in cardiovascular diseases is associated with increased fibrosis and lowered aerobic capacity, contributing to maladaptive remodeling in conditions, such as hypertension, heart failure, and myocardial infarction (Dorn 2007; Heineke and Molkentin 2006; McMullen and Jennings 2007). The physiological LVH can capably exert cardioprotection in patients with cardiovascular diseases. Therefore, this research in the mechanism of exercise-induced LVH is useful to human subjects.

Both physiologic and pathologic hypertrophy are induced by endocrine, paracrine, and autocrine regulatory circuits that directly signal myocyte growth through membrane-bound receptors, which in turn activate intermediate signal transduction pathways within the cytoplasm. Signaling pathways, such as MAPK, PI3K/AKT/mammalian target of rapamycin (mTOR), protein kinase C, calcineurin-nuclear factor of activated T cells, and regulated shuttling of class II histone deacetylases are all critical for myocyte growth (Heineke and Molkentin 2006).

The ERK signaling pathway, which belongs to the MAPK family, signals a cascade consisting of a sequence of successively acting kinases that finally results in the phosphorylation and activation of terminal kinases, such as p38, JNK, and ERK (Garrington and Johnson 1999). The ERK signaling pathway is launched in cardiac myocytes by several receptor families containing receptor tyrosin kinases (insulin-like growth factor and fibroblast growth factor receptors), G protein-coupled receptors (angiotensin II, adrenergic and endothelin-1 receptors), and cardiotrophin-1 (gp 130 receptor); it is also triggered by different stress and stretch stimuli (Sugden and Clerk 1998). Following their activation, each ERK phosphorylates a wide array of intracellular targets that includes transcription factors, resulting in the reprogramming of cardiac gene expression.

In addition, some studies have also reported that the PI3K/AKT/mTOR signaling pathway is a major regulator of cell growth, metabolism, survival and angiogenesis (Guertin and Sabatini 2007), and operates in the transformation and progression of LVH (Aoyagi and Matsui 2011). Although the upstream activator of this pathway is not yet completely understood, recent studies have reported that IGF-1 is responsible for the activation of hypertrophic signaling including PI3K/AKT/mTOR (Kim et al. 2008), and FAK can control AKT via interaction with the p85 subunit of PI3K (Franchini 2012). PI3K consists of three classes based on the combination of isoforms of catalytic and regulatory subunits. PI3K, the Class I component of the PI3K enzyme family, converts the plasma membrane lipid phosphatidylinositol-4, 5-bisphosphate (PIP2) to phosphatidylinositol-3,4,5-trisphosphate (PIP3), which initiates the activation of downstream signaling molecules containing pleckstrin-homology (PH) domains, such as phosphatidylinositol-dependent kinase 1 (PDK1), Bruton's tyrosine kinase (Btk), ADP-ribosylating factor 6 (ARF6), and AKT (Cantley 2002). AKT is a well-characterized serine threonine kinase downstream of PI3K and consists of three isoforms: AKT1, AKT2, and AKT3. Two of these—AKT1 and AKT2—are chiefly expressed in the heart (Muslin and DeBosch 2006). As with PI3K, due to the growth promoting effects of PIP3, the amount of this phospholipid is tightly controlled by several lipid phosphatases, including the SH2-containing 5′-inositol phosphatase (SHIP) 2 (Vanhaesebroeck et al. 1997) and phosphatase and tensin homolog (PTEN). PTEN, however, is the only enzyme capable of directly antagonizing PI3K by dephosphorylating the D3 phosphate of PIP3 (Maehama and Dixon 1998, 1999).

mTOR is a large serine-threonine protein kinase belonging to the phosphatidylinositol kinase-related kinase (PIKK) family (Yang and Guan 2007). Receptor tyrosine kinase (RTK) and Akt activation, as well as elevated amino acids, activate mTOR via both translation and phosphorylation-dependent mechanisms, leading to changed metabolism and increased growth mediated by changes in gene transcription and translation (Oudit et al. 2004; Schmelzle and Hall 2000). mTOR phosphorylates and activates p70S6K, which is a short isoform of the ribosomal S6 kinase (S6K1) (Schmelzle and Hall 2000). Ribosomal S6 kinases (S6K1 and S6K2) are critical regulators of cell growth, which they achieve through the control of protein translation. Both mTOR and p70S6K are expressed in the heart and in the vascular smooth muscle cells (Crackower et al. 2002; Shioi et al. 2003). However, there is only limited data about the mechanisms of swimming exercise involved in PIK3/PTEN/AKT/mTOR signaling, microRNA (miRNA; miR) and LVH. The purpose of this research was to interpret these mechanisms of exercise training on physiological LVH.

miRNAs have recently emerged as a large group of short (18–25 nucleotides), non-coding, small RNA molecules that negatively regulate gene expression. Although little is yet known about the specific biological functions of most miRNAs, these small molecules are believed to constitute a large gene regulatory network that can impact the expression of up to 30 % of total cellular proteins. There is increasing data supporting the roles of miRNAs in the regulation of a range of physiological responses, including development (Harfe 2005), cellular apoptosis (Lynam-Lennon et al. 2009), differentiation (Zhang et al. 2009), proliferation (Kaddar et al. 2009), and cancer (Catto et al. 2011; Cao et al. 2012; Qiang

et al. 2011; Uziel et al. 2009). Moreover, recent studies have shown the roles played by miRNAs in different forms of cardiovascular disease and pathological LVH (Van Rooij and Olson 2007; Van Rooij et al. 2008). Studies have further demonstrated that miRNAs may also be important for normal development and in physiological LVH (DA Silva Jr et al. 2012; Fernandes et al. 2011; Soci et al. 2011). The present study hypothesized that swimming exercise alters specific miRNAs that regulate their cardiac signaling genes and tip the balance of PI3K/AKT/mTOR and ERK signaling to contribute to physiological LVH.

Materials and methods

Animal care and exercise protocols

All care policies and procedures in this study conform to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH publication No.85–23, revised 1996) and approved by the Ethics Committee for the Use of Experimental Animals at Yangzhou University. The study used female normotensive Wistar rats (180–200 g, $n = 32$). They were fed a standard diet and exposed to a 12-h light–dark cycle. They were housed in a space that maintained a constant room temperature (21 ± 3 °C) and humidity (50 ± 10 %). The rats were randomly assigned to two groups: (1) sedentary control (SC, $n = 16$), (2) swimming exercise (SE, $n = 16$). For 8 weeks, from Monday to Friday, the rats in the SE group completed a 1-h swimming exercise. Exercise training of the rats was performed by placing rats in a swimming pool (150 cm × 60 cm × 70 cm) filled with warm water to a depth of 60 cm. The pool was divided by plastic barriers into eight lanes. To avoid interaction, each rat was placed into an individual lane. The water temperature was maintained at 31 ± 1 °C. The exercise duration and workload were gradually increased until the rats could swim for 60 min wearing caudal dumbbells weighing 5 % of their body weight. Subsequently, the duration and dumbbells maintained constant. All animals were weighed once a week.

In contrast, the SC group was exposed to the water twice weekly—they were placed in the swimming apparatus at these junctures for 10-min sessions. The O_2 uptake for rats swimming individually is about 50–65 % of the maximum oxygen uptake. This low-intensity, long-period swimming exercise protocol is effective for the promotion of cardiovascular adaptations and for the increase of the muscle oxidative capacity. These protocols have previously been reported (DA Silva Jr et al. 2012; Fernandes et al. 2011; Oliveira et al. 2009; Soci et al. 2011).

Blood pressure and heart rate

24 h after last exercise session blood pressure (BP) and heart rate (HR) were recorded. The hemodynamic parameters of the rats were measured by the tail-cuff method, which used a blood pressure analyzer (BP-98A; Softron, Tokyo, Japan) after the rat had has been placed in a restrainer for a minimum of 5 min in quiet. The recorded data indicated the average of all values of systolic, diastolic, heart rate, and mean arterial pressure over the entire recording time of 30 min.

Samples preparation

After the last bout of exercise, the rats, who had fasted overnight, were decapitated and tissue samples (hearts) were harvested, weighed, kept frozen at −80 °C, and used within 1 month for microRNA, mRNA, and protein assays.

Measurement of cardiac hypertrophy

To measure cardiac mass, the hearts were stopped at diastole by perfusion of 14 mM KCl. After the rats' heart weights (HW) were measured, the left ventricle (LV) was dissected corresponding to the remaining tissue upon the removal of both the atria and the free wall of the right ventricle (RV). The interventricular septum remained as part of the LV. Left cardiac hypertrophy was assessed by the measurement of the ratio of LV weight to HW in milligrams and HW in milligrams to animal body weight (BW) in grams (LV/BW in mg mg^{-1} and HW/BW in mg g^{-1}). The LV was cut in 8 μm with a freezing microtome (AF100AS; Scotsman frimont, Italy) and subsequently stained with hematoxylin and eosin (HE) in order to capture an image of the heart structures. Two random sections from each animal were visualized using light microscopy at 40× magnification. Myocytes with a visible nucleus and intact cellular membranes were chosen for diameter determination. The width of individually isolated cardiomyocytes were displayed on a viewing screen that was manually traced, across the middle of the nuclei, with a digitizing pad and determined by a computer-assisted image analysis system (ScopePhoto 3.0 for Scope Technology). For each heart, 20 visual fields were assayed.

RNA extraction and miRNA microarray

Total RNA was harvested using TRIzol (Invitrogen, USA) and the miRNeasy mini kit (QIAGEN, German) according to manufacturer's instructions. After having taken an RNA quantity measurement using the NanoDrop 1000 Specthophotometer (NanoDrop Technologies, USA) and standard denaturing agarose gel electrophoresis, the samples from three

animals in each group were pooled and labeled using the miRCURY™ Hy3™/Hy5™ Power labeling kit (Exiqon, Vedbaek, Denmark). They were then hybridized on the miRCURY™ LNA Array (v.16.0) (Exiqon, Vedbaek, Denmark). Next, the slides were scanned using the Axon GenePix 4000B microarray scanner (Axon Instruments, Foster City, CA). Scanned images were then imported into GenePix Pro 6.0 software (Axon) for grid alignment and data extraction. Replicated microRNAs were averaged and miRNAs with intensities ≥50 in all samples were chosen for calculating the normalization factor. An electronic link to the miRNA microarray platform is available at http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GPL11434. Expressed data were normalized using median normalization. After normalization, differentially expressed miRNAs were identified through fold change filtering. TargetScan software was used to search the miRNAs' target to the PI3K/AKT/mTOR signaling pathway.

TaqMan miRNA assay

Reverse transcription (RT)

cDNA for miRNA analysis was synthesized from total RNA using gene-specific primers according to the TaqMan MicroRNA Assay protocol (Applied Biosystems, CA, USA). The 15 μl reactions obtained by the TaqMan MicroRNA Reverse Transcription Kit protocol (Applied Biosystems, CA, USA) were incubated in a Thermal Cycler (Applied Biosystems, CA, USA) for 30 min at 16 °C, 30 min at 42 °C, and 5 min at 85 °C. They were then maintained steadily at 4° C.

Real-time polymerase chain reaction (PCR) for miRNA

The real-time PCR quantification was performed by using the TaqMan MicroRNA Assay protocol (Applied Biosystems, CA, USA). The 20 μl PCR reaction solution contained 10 μl TaqMan Universal PCR master mix II (2×), 1.33 μl RT product, 7.67 μl nuclease-free water, and 1 μl of primers and probe mix from the TaqMan MicroRNA Assay protocol for microRNA-21, 124, 144, and 145. The reactions were performed at 95 °C for 10 min, and then in 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Samples were normalized by evaluating the U6 gene. The use of microRNA microarray was not meant for global profiling, but candidates were restricted to those that play a role in the expression of genes in the PI3K/AKT/mTOR signaling pathway.

Quantification of mRNA expression of signal molecules

After reverse transcription, the relative gene expression was measured by real-time PCR using the Applied Biosystems 7500 Real-Time PCR System (ABI, USA). RNAs were isolated from LV using TRIzol (Invitrogen, USA). 500 ng total RNA each was used to synthesize cDNA, using the PrimeScript™ RT Master Mix (TaKaRa, Japan). Primers were designed by using Primer Premier 5.0 and assessed by using Oligo 7.37 software. The nucleic acid sequences were obtained from NCBI, and primer sequences were matched using Blast software. The real-time PCR was performed according to SYBR Premix EX Taq II (Tli RNaseH Plus) kit protocol (TaKaRa, Japan). The expression of β-actin was measured as an internal control for sample variation in the PCR reaction. The oligonucleotides primers are shown in Table 1.

PCR product generation was monitored by measuring the increase in fluorescence caused by the probe presence in the TaqMan MicroRNA Assay at each annealing phase or by the binding of SYBR green to double-stranded DNA. A melt curve observed in the SYBR green analysis was generated at the end of the reaction to demonstrate that only one product was amplified. Each assay was performed in triplicate. The relative quantities of the target gene expression of sedentary rats vs. trained rats were compared after normalization to the values of internal control (ΔCT). Fold change in the mRNA expression was calculated using the differences in ΔCT values between two samples (ΔΔCT) and equation

Table 1 Quantitative real-time PCR primer

Genes	Sequences
ANP	F: 5′-CTT CGG GGG TAG GAT TGA C-3′
	R: 5′-CTT GGG ATC TTT TGC GAT CT-3′
α-Actin	F: 5′-ACC ACA GGC ATT GTT CTG GA-3′
	R: 5′-TAA GGT AGT CAG TGA GGT CC-3′
α-MHC	F: 5′-CGA GTC CCA GGT CAA CAA G-3′
	R: 5′-AGG CTC TTT CTG CTG GAC C-3′
β-MHC	F: 5′-CAT CCC CAA TGA GAC GAA G-3′
	R: 5′-AGG CTC TTT CTG CTG GAC A-3′
PIK3α	F: 5′-CAT GGA TGC TTT GCA GGG TTT-3′
	R: 5′-CCA GAT GTT CTC CAT GAT TCG GA-3′
PTEN	F: 5′-AGA CCA TAA CCC ACC ACA GC-3′
	R: 5′-TAC ACC AGT CCG TCC TTT CC-3′
TSC2	F: 5′-ACT GAC ATT GGA CGG CTG AG-3′
	R: 5′-CAG GTG GGA CTG TGA TAC GG-3′
β-actin	F: 5′-AGA GGG AAA TCG TGC GTG AC-3′
	R: 5′-AGG AAG GAA GGC TGG AAG AGA-3′

Genes: *ANP* atrial natriuretic polypeptide, *α-actin* skeletal muscle α-actin, *α-MHC* α-myosin heavy chain, *β-MHC* β-myosin heavy chain, *PIK3α* phosphoinositide-3-kinase catalytic alpha polypeptide, *PTEN* phosphatase and tensin homolog, *TSC2* tuberous sclerosis complex 2

$2^{-\Delta\Delta CT}$. The results are expressed as a percentage of the sedentary control group.

Western blotting analysis

The protein levels of ERK1/2, phospho$^{Thr202/Tyr204}$-ERK1/2, phosphoinositide-3-kinase catalytic alpha polypeptide (PIK3α), PTEN, AKT1, phospho^{ser473}-AKT, TSC2, phospho^{Thr1462}-TSC2, mTOR, and phospho^{Ser2448}-mTOR in the LV were analyzed by western blotting. The tissues (100 mg) were homogenized in a cell lyses solution that included 100 mM Tris–HCl, 50 mM NaCl, 1 % Triton X-100, and a protease inhibitor cocktail (1:100, Sigma-Aldrich, MO, USA). Solventless tissues were removed by centrifugation at 10,000×*g*, 4 °C, for 10 min. Samples were loaded and subjected to SDS-PAGE in 8 % polyacrylamide gels. After electrophoresis, proteins were electro-transferred to nitrocellulose membrane (Amersham Biosciences, NJ, USA). Equal loading of samples (50 μg) and even transfer efficiency were monitored using a 0.5 % Ponceau S staining of the blot membrane. The blot membrane was then incubated in a blocking buffer (5 % nonfat dry milk, 10 mM Tris–HCl, PH 7.6, 150 mM NaCl, and 0.1 % Tween 20) for 2 h at 27 °C and then incubated overnight at 4 °C with: mouse anti-ERK1/2 and anti-phospho$^{Thr202/Tyr204}$-ERK1/2 monoclonal antibody (1:2,000); rabbit anti-PIK3α monoclonal antibody (1:1,000); rabbit anti-PTEN polyclonal antibody (1:1,000); mouse anti-AKT1 and phospho^{ser473}-AKT monoclonal antibody (1:1,000); rabbit anti-TSC2 and anti-phospho^{Thr1462}-TSC2 polyclonal antibody (1:1,000); and rabbit anti-mTOR and phospho^{Ser2448}-mTOR monoclonal antibody (1:1,000). All of the primary antibodies were purchased from Cell Signaling Technologies (Beverly, MA, USA). Binding of the primary antibody was detected with peroxidase-conjugated secondary antibodies, and enhanced chemiluminescence reagents (Amersham Biosciences, NJ, USA) were used to visualize the autoradiogram, which was later exposed to photographic film. The film was developed and the bands were analyzed using Scion Image software (Scion Corporation based on NIH image). Cardiac β-actin expression levels were used to normalize the results. The results are expressed as a percentage of control.

Statistical analysis

All data were analyzed using SPSS v. 17 and statistical significance was set at $P = 0.05$. Each data were presented as mean ± SD. Differences between two groups were analyzed by using an one-way ANOVA.

Results

Blood pressure and heart rate

The systolic blood pressure, diastolic blood pressure, mean blood pressure, and heart rate data of the SC and SE groups are presented in Table 2. There were no differences in blood pressure between the two groups, but the heart rate in the SE group was significantly lower than the SC group's after 8 weeks of the swimming exercise(298.6 ± 13.2 bpm vs. 340.8 ± 11.5 bpm; $P < 0.05$).

Measurement of cardiac hypertrophy

Before and after the swimming exercise, the body weight of the SC group was nearly the same as that of the SE group. LV/BW and HW/BW ratios were used as indices of hypertrophy. The results referring to BW, LV/BW, and HW/BW are summarized in Table 3. The HW of the SE group increased after the swimming exercise by 38.7 % (4.3 ± 0.45 mg/g; $P < 0.05$); by comparison, the SC group was lower (3.1 ± 0.16 mg/g). Likewise, the LV hypertrophy demonstrated in the SE group was 28.6 % (2.7 ± 0.13 mg/g; $P < 0.01$), again higher than the SC group (2.1 ± 0.09 mg/g). The increase in the diameter of the LV myocyte in the SE group (13.6 ± 1.3 μm), when

Table 2 Hemodynamic Parameters

Groups	SBP (mm Hg)	DBP (mm Hg)	MBP (mm Hg)	HR (bpm)
SC	125.2 ± 3.6	98.1 ± 7.6	114.7 ± 6.8	340.8 ± 11.5
SE	123.1 ± 6.8	95.8 ± 5.3	110.4 ± 6.1	298.6 ± 13.2*

Each data are mean ± SD

SBP systolic blood pressure, *DBP* diastolic blood pressure, *MBP* mean blood pressure, *HR* heart rate

* $P < 0.05$, SE group vs. SC group

Table 3 Cardiac hypertrophy

Groups	BW(g)	HW/BW (mg/g)	LV/BW (mg/g)	Myocyte Diameter (μm)
SC	274.8 ± 17.9	3.1 ± 0.16	2.1 ± 0.09	11.3 ± 1.2
SE	267.2 ± 13.4	4.3 ± 0.45*	2.7 ± 0.13**	13.6 ± 1.3*

Each data are mean ± SD. At least 100 cardiocytes in randomly selected fields of sections were assessed to estimate the mean myocyte diameter in each group

BW body weight, *LV* left ventricular weight, *HW* heart weight

* $P < 0.05$; ** < 0.01, SE group vs. SC group

compared with the SC group (11.3 ± 1.2 μm; $P < 0.05$; see Table 3; Fig. 1) confirmed the increase in LV/BW ratio.

Molecular markers of left ventricular hypertrophy

Both the up-regulation of the atrial natriuretic polypeptide (ANP) and the skeletal muscle α-actin, and the decrease in the ratio of α/β-myosin heavy chain (α/β-MHC) are the characters of pathological cardiac hypertrophy during fetal development. The mRNA levels of these four genes were determined in the LV of the SC and SE groups by RT-PCR. The data recorded in this study demonstrated that the swimming exercise did not alter the gene levels of ANP, skeletal muscle α-actin, or α/β-MHC, when compared with the SC group. The data are shown in Fig. 2.

Signaling pathway assays

To confirm whether the swimming exercise induces the activation of the ERK and/or AKT/mTOR signaling pathway, the protein levels of ERK1/2, phospho$^{Thr202/Tyr204}$-ERK1/2, AKT1, phospho^{ser473}-AKT, mTOR and phospho^{Ser2448}-mTOR were tested by using the western blot in the LV. As shown in Fig. 2, the swimming exercise did not change the protein levels of ERK1/2, phospho$^{Thr202/Tyr204}$-

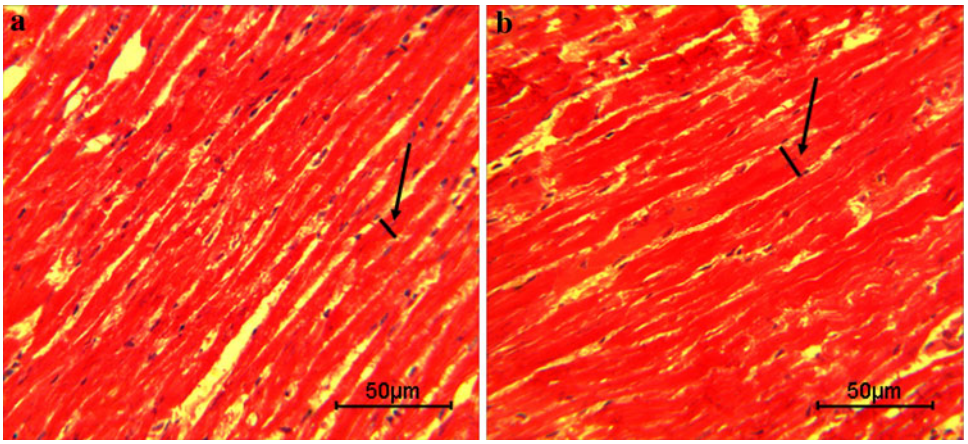

Fig. 1 Effect of swimming exercise on the cardiomyocytes diameter (μm). Representative histological sections of LV myocytes diameter in the sedentary control (**a**), swimming exercise (**b**). The *arrows* indicate the lines showing the width of individually-isolated cardiomyocytes that was manually traced, across the middle of the nuclei, visualized by light microscopy (×40). The diameter of the LV myocyte is increased in the swimming exercise (13.6 ± 1.3 μm), when compared with the sedentary control (11.3 ± 1.2 μm; $P < 0.05$)

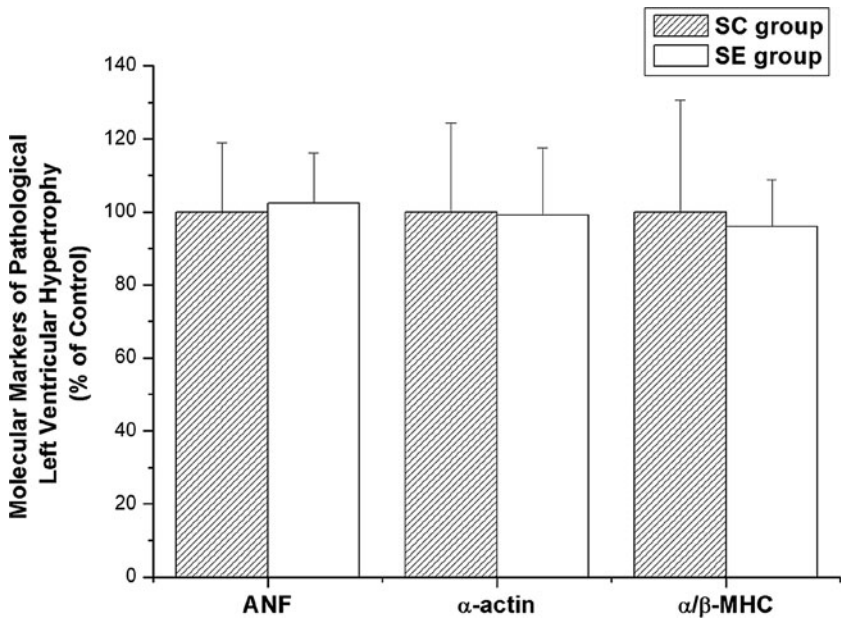

Fig. 2 Effect of swimming exercise on molecular markers of pathological left ventricular hypertrophy. Each data are presented as mean ± SD. Atrial natriuretic factor (ANF), skeletal muscle α-actin (α-actin), the ratio of α and β-myosin heavy chain (α/β-MHC). There are no significant differences between the SC and SE groups

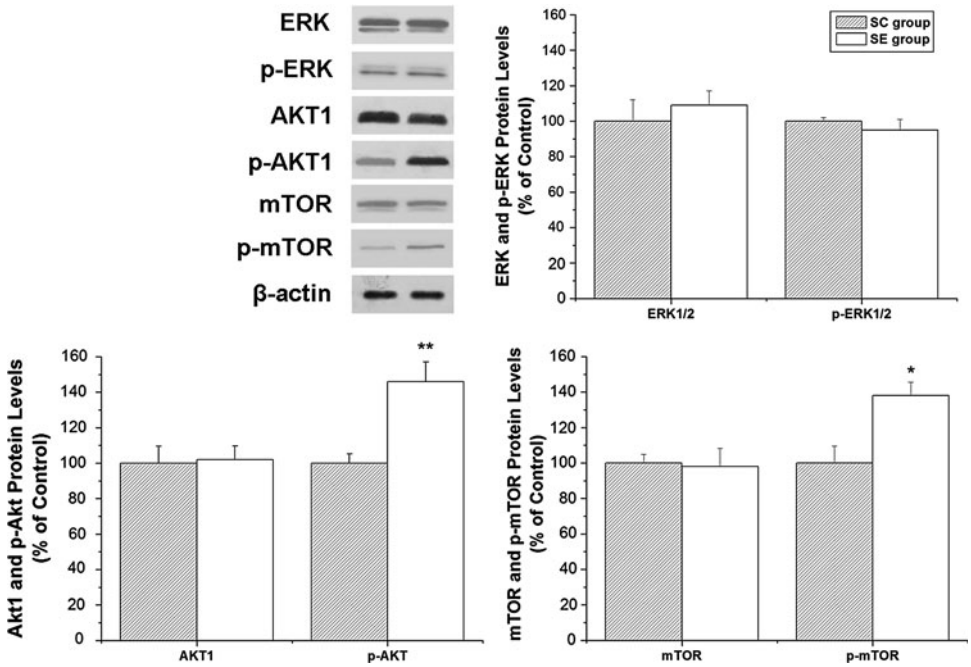

Fig. 3 Effect of swimming exercise on the ERK and PI3K/AKT/mTOR signaling pathways. Each data are presented as mean ± SD. ERK1/2, phospho$^{Thr202/Tyr204}$-ERK1/2, AKT1, phospho^{ser473}-AKT (p-AKT), the mammalian target of rapamycin (mTOR) and phospho^{Ser2448}-mTOR (p-mTOR) protein levels analyzed by the western blot accompanied by their representative blots from sedentary control (SC) and swimming exercise (SE) group. Targeted bands were normalized by β-actin. *SE group vs. SC group, $P < 0.05$; **SE group vs. SC group, $P < 0.01$

ERK1/2, AKT1 or mTOR, but the phospho^{ser473}-AKT and phospho^{Ser2448}-mTOR were respectively increased by 46 % ($P < 0.01$) and 38 % ($P < 0.05$) in the SE group when compared with SC group (see Fig. 3).

microRNA analysis by microarray

The microarray analysis of miRNA was restricted to those miRNAs that underwent a significant change from the baseline (the ratio of SC/SE was ≥2 or ≤0.5 fold). Figure 4a shows the miRNA targeting phosphatase and tensin homolog (PTEN): In the SC group, the relative expression value of miRNA-21 was 381 ± 21 arbitrary units (AU), and the value of miRNA-144 was 2,250 ± 178 AU. In the SE group, the values were 1,150 ± 32 (202 % increase; $P < 0.01$) and 4820 ± 194 (114 % increase; $P < 0.01$) AU, respectively, in comparison with the SC group. In addition, in the SE group, the swimming exercise increased the relative expression value of miRNA-124, targeting PIK3α. The value was decreased by 54 % (1,015 ± 119 AU; $P < 0.01$), when compared with SC (2,205 ± 48 AU). Finally, the swimming exercise increased miRNA-145, targeting tuberous sclerosis complex 2 (TSC2, tuberin). This is a 116 % increase (4,555 ± 128 AU; $P < 0.01$) over the SC group (2,105 ± 161 AU).

microRNA analysis by RT-PCR

To confirm the microRNAs that targeted associated genes in physiological LVH, the miRNAs-21, 124, 144, and 145 were quantified by RT-PCR. miRNAs-21, 144, and 145 were respectively up-regulated in the SE group (152, 128, and 101 % relative increases over the SC group; $P < 0.01$), whereas miRNA-124 was down-regulated in the SE group (38 % decrease compared with the SC group; $P < 0.05$). The miRNA expression in the SE group, when compared with the SC group demonstrated the microarray results. The data are shown in Fig. 4b.

miRNAs targeting molecules involving in PI3K/AKT/mTOR assays

To assess the effect of the swimming exercise on the mRNA expression of the miRNA targeting genes PIK3α, PTEN, and TSC2, mRNA expression levels were determined by using RT-PCR in the heart. mRNA levels of PIK3α showed a significant up-regulation by 213 % in the SE group when compared with the SC group ($P < 0.01$). PTEN and TSC2 gene expressions were respectively decreased by 51 % and 55 % in the SE group when compared with the SC group, as shown in Fig. 2 ($P < 0.05$). The data are shown in Fig. 5a, c, e, respectively).

Similar results were obtained for the proteins, which were assessed by western blotting, associated with PI3K/AKT/mTOR signaling pathway. Figure 5b, g, respectively, shows that the swimming exercise increased the SE group's protein levels of PIK3α (36 % increase; $P < 0.05$) and phospho^{Thr1462}-TSC2 (48 %; $P < 0.01$), while it decreased PTEN (37 %; $P < 0.05$) and TSC2 (22 %; $P < 0.05$) as shown in Fig. 5d, f. The data imply that PIK3α, PTEN, and TSC2 probably correlate with the development of physiological LVH.

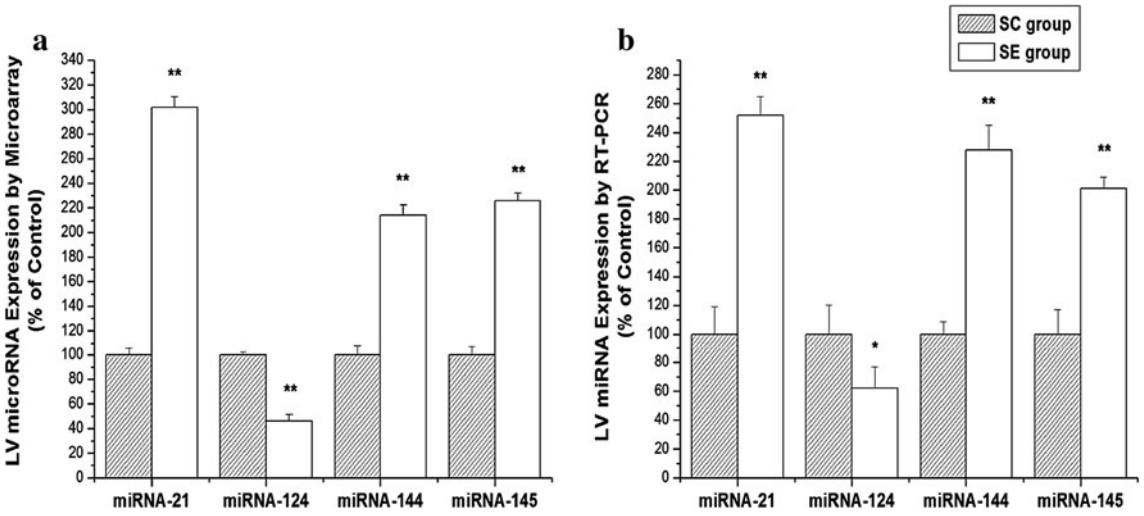

Fig. 4 Effect of swimming exercise on specific microRNAs (miRNAs). miRNAs associated with phosphatase and tensin homolog (PTEN; miRNA-21 and 144), phosphoinositide-3-kinase catalytic alpha polypeptide (PIK3α; miRNA-124) and tuberous sclerosis complex 2 (TSC2; miRNA-145) assessed by microarray (**a**). Determination of miRNAs by RT-PCR (**b**). Targeted miRNAs were normalized by U6 levels. *SE group vs. SC group, $P < 0.05$; **SE group vs. SC group, $P < 0.01$

Discussion

The present study's data demonstrates that the swimming exercise-induced physiological LVH is not correlated with pathological cardiac hypertrophy markers as shown in Fig. 2 and the ERK1/2 signaling pathway but is rather related to the active PI3K/AKT/mTOR signaling pathway. Moreover, exercise changed the expression of specific miRNAs that target PIK3α, PTEN, and TSC2.

The LV/BW ratio, myocyte diameter, and sedentary bradycardia confirmed the exercise-induced adaptations of physiological LVH (Medeiros et al. 2004; Oliveira et al. 2009). In contrast to molecular markers for pathological hypertrophy (Purcell et al. 2007), the physiological LVH reported here was not associated with the activation of fetal genes, such as ANP, skeletal muscle α-actin, or the ratio of α/β-MHC. In this study, no molecular markers of pathological hypertrophy were changed in the SE group.

Some previous studies reported that the ERK1/2 signaling pathway becomes activated in cardiac myocytes in response to virtually every type of stress stimulation examined to date (Bueno and Molkentin 2002). For example, neuroendocrine effectors, G protein-coupled receptor agonists, receptor tyrosine kinase agonists, cytokines, reactive oxygen species, and stretch all induce ERK1/2 activation and, in most cases, a hypertrophic response, suggesting that ERK1/2 directly programs growth itself (Bueno and Molkentin 2002). In another study, although ERK1/2 phosphorylation was eliminated at the baseline after pressure overload stimulation, it did not diminish the hypertrophic response to overload stimulation induced by exercise (Purcell et al. 2007). In addition, the studies have shown that inhibition of MEK-ERK1/2 does not antagonize hypertrophic morphology or cytoskeletal organization in response to agonist treatment in culture (Bueno and Molkentin 2002; Purcell et al. 2007). Although the use of cultured cardiomyocytes has provided key clues, little consensus exists on the causal relationship between physical cardiac hypertrophy and ERK1/2 signaling. In our study, the development of LVH after exercise did not appear to involve the ERK1/2 signaling pathway. In the SE group, the protein expression of ERK1/2 showed no significant difference between the SC and SE groups. Additionally, the protein levels of phospho$^{Thr202/Tyr204}$-ERK1/2 were not altered by the SE group's swimming exercise. Thus, the study proves that ERK1/2 signaling is not active in physical LVH induced by an 8-week swimming exercise.

The activation of the phosphoinositide-3-kinase catalytic alpha polypeptide (PIK3α)/AKT signaling cascade was abundantly reported in previous studies of myocardial hypertrophy (McMullen et al. 2003, 2007). In dominant negative PI3K transgenic (dnPI3K) mice, pathological cardiac hypotrophy induced by ascending aortic constriction in Ntg and dnPI3K mice had no significant difference (McMullen et al. 2003). Interestingly, exercise-induced physiological cardiac hypotrophy was attenuated as compared to Ntg mice (McMullen et al. 2003). PIK3α (p110α)-dependent hypertrophy was protective in animal models of hypertrophic cardiomyopathy and heart failure (McMullen et al. 2003, 2007). Therefore, p110α plays a crucial role in physiological hypertrophy but not in pathological hypertrophy. Consistent with this conclusion, in our study, PIK3α (p110α) was assessed by the western blot, and the

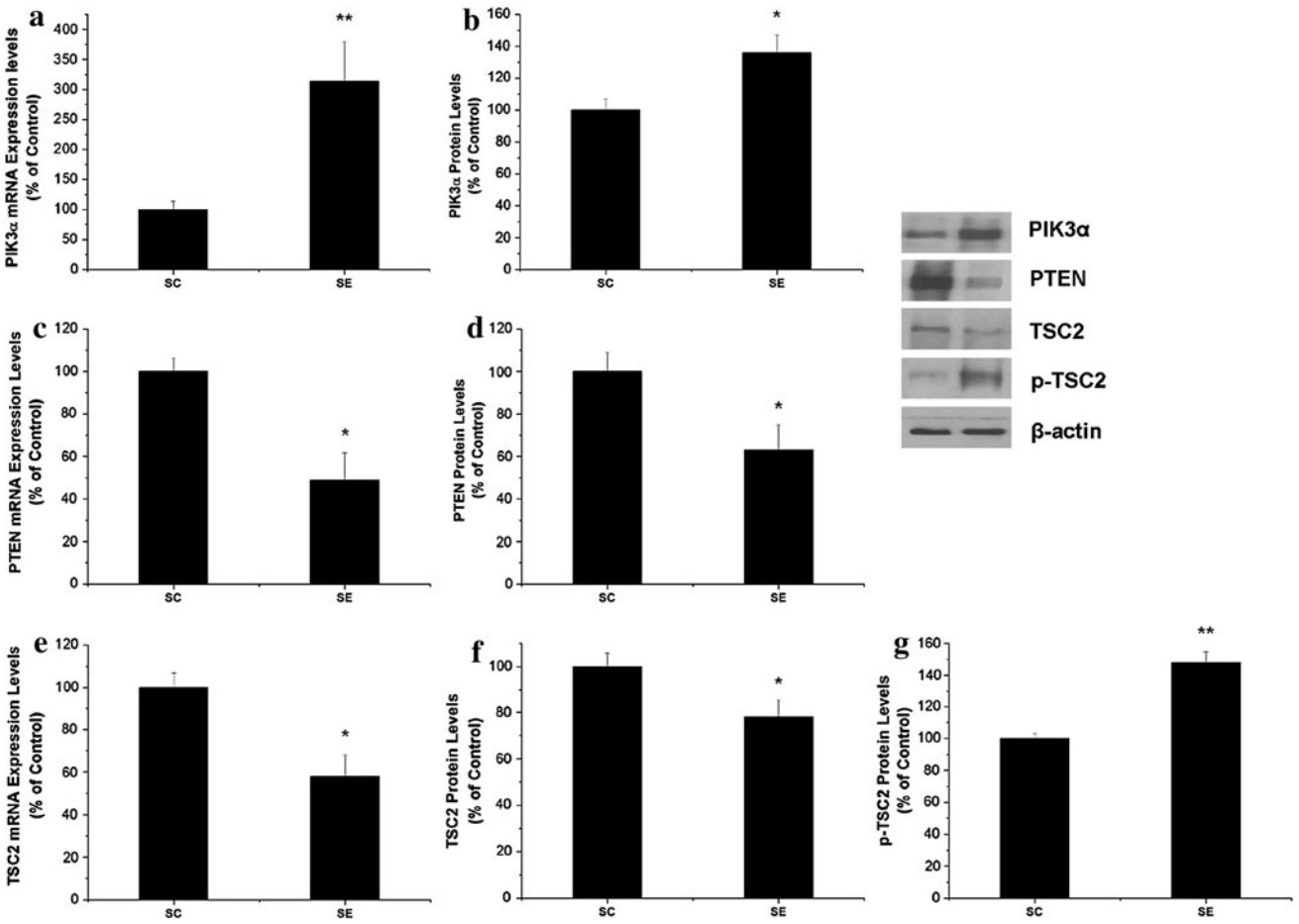

Fig. 5 Effect of swimming exercise on expression levels of microRNA targeting molecules. Each data are reported as mean ± SD. Group: SC indicates sedentary control; SE, swimming exercise. Phosphoinositide-3-kinase catalytic alpha polypeptide (PIK3α, **a**), phosphatase and tensin homolog (PTEN, **c**) and tuberous sclerosis complex 2 (TSC2, **e**) mRNA expression levels were analyzed by RT-PCR. In addition, PIK3α (**b**), PTEN (**d**), TSC2 (**f**) and phospho^{Thr1462}-TSC2 (p-TSC2; **g**) protein levels were assessed by the western blot accompanied by their representative blots from the sedentary control (*SC*) and swimming exercise (*SE*) groups. Targeted bands were normalized by β-actin. *SE group vs. SC group, $P < 0.05$; **SE group vs. SC group, $P < 0.01$

data showed that the PIK3α protein was significantly increased in the LV.

AKT1 has been shown to be required for the development of physiological hypertrophy induced by exercise training (DeBosch et al. 2006) and the regulation of normal cardiac growth (Cho et al. 2001b). On the other hand, AKT2 plays an important role in insulin-regulated glucose homeostasis, as well as in cardiomyocyte survival (Cho et al. 2001a; Etzion et al. 2010; Garofalo et al. 2003). AKT3 expression is higher in the brain than it is in the skeletal muscle and liver (Brodbeck et al. 1999). Based on these opinions (Brodbeck et al. 1999; Cho et al. 2001a, 2001b; DeBosch et al. 2006; Etzion et al. 2010; Garofalo et al. 2003), AKT1 was chosen as candidate, since it may play a critical role in LVH induced by swimming exercise in our study. However, for full activation of AKT, the phosphorylation of Ser473 in a C-terminal regulatory domain is essential (Andjelkovic et al. 1999). Thus, we also analyzed the phospho^{Ser473}-AKT levels in hearts. In Akt-deficient mice, even though AKT knockout mice share similar heart and cardiac functions with wild-type mice, in the former, exercise-induced cardiac hypertrophy was attenuated. Furthermore, other groups (Condorelli et al. 2002; Matsui et al. 2002; Shioi et al. 2002) previously demonstrated that mice with cardiac-specific overexpression of a constitutively active form of AKT showed significant cardiac hypertrophy, apparently due to increased cardiomyocyte size in transgenic mice. Plenty of studies concluded that cardiac AKT caused the deterioration of the cardiac function in models of cardiac hypertrophy or ischemia. However, the alteration of AKT1 expression was consecutive and irreversible in transgenic models in cardiac hypertrophy, and the roles of AKT in exercise-induced LVH have been extremely limited in physiological condition (DeBosch et al. 2006; Soci et al. 2011). To evaluate the roles of AKT1, we tested the protein levels of AKT1

and phospho^{Ser473}-AKT. We concluded that the AKT1 is activated by upstream PIK3α via phosphorylation in the LVH induced by the swimming exercise in the SE group. It should be noted that hormonal changes, in response to overnight fasting before the rats were decapitated, likely exert an effect on phosphorylation at Ser473 (Shao et al. 2000). Nevertheless, some studies have shown that there were no significant differences in phospho^{Ser473}-AKT between control and overnight fasted subjects (Leontieva et al. 2012; Vendelbo et al. 2012; Nakashima et al. 2006).

Activated AKT phosphorylates downstream molecules such as Glucose transporter (GLUT), Glycogen synthase kinase (GSK)-3, and mTOR (Matsui and Rosenzweig 2005). mTOR expression has also been found to be associated with the attenuation of cardiac hypertrophy via inhibition of mTOR with rapamycin in both human and animal models (McMullen et al. 2004; Shioi et al. 2003; Soesanto et al. 2009). However, transgenic mice with cardiac-specific overexpression of either dominant negative or constitutively active mTOR showed cardiac hypertrophy similar to wild-type animals, following physiological and pathological stimuli (Shen et al. 2008). These results suggest that cardiac mTOR seem have little effect on hypertrophy. In contrast, a study in cardiac-specific mTOR-deficient mice showed that cardiac mTOR is necessary for preserving cardiac function under LV pressure overload (Zhang et al. 2010). Furthermore, other research generated cardiac-specific transgenic mice overexpressing wild-type mTOR and observed protection of cardiac function under LV pressure overload (Song et al. 2010). Based on the results of our work, the phospho^{Ser2448}-mTOR was significantly increased by the swimming exercise, alcrccuruthough the mTOR protein showed little change between the SC and SE groups. Ser2448 is the position that was phosphorylated by the upstream molecular in PI3K/AKT/mTOR (Navé et al. 1999), and phospho^{Ser2448}-mTOR could transmit a signal to its downstream signal molecules, such as 4E-BP1, p70S6K (Fang et al. 2001).

The present study demonstrates the effect of a swimming exercise on PIK3α, AKT, mTOR and the phosphorylation formations of AKT and mTOR in rats' hearts. The discovery reveals that the PI3K/AKT/mTOR signaling axis is activated and implies that this signaling plays a critical role in physiological LVH induced by an 8-week swimming exercise.

Next, two components associated with the PI3K/AKT/mTOR signaling pathway named PTEN and TSC2 were studied in our work. PTEN is a major negative regulator of PI3K/AKT (Maehama and Dixon1998; Maehama and Dixon 1999). It plays an important role in many cellular functions, such as cell migration (Leslie et al. 2007), PDGF-induced membrane ruffling (Leslie et al. 2000), IGF-1/insulin sensitivity (Lackey et al. 2007), and oncogenesis (Leslie and Downes 2004). In many instances, the loss of PTEN leads to enhanced tumor formation (Kishimoto et al. 2003). However, the loss of PTEN in a striated muscle does not lead to transformation in muscle (Crackower et al. 2002; Wijesekara et al. 2005). Interestingly, striated muscle specific PTEN null mice have been reported to have an approximate 50 % increase in heart mass (Crackower et al. 2002). A more recent study has shown the role of PTEN in the regulation of cardiomyocyte hypertrophy in rats (Xu et al. 2012). In accordance with these conclusions, our data show that the PTEN protein level was decreased in physiological LVH in the SE group when compared with the SC group. Thus, PTEN may participate in the hypertrophy induced by the swimming exercise in this study. On the other hand, similar to PTEN but an inhibitor of mTOR, TSC2 is another negative regulator of PI3K/AKT/mTOR signaling. TSC2 heterodimerizes with TSC1 (hamartin) and acts as a GTPase-activating protein for the mTOR activator Rheb (Huang and Manning 2008). Phosphorylation of TSC2 by AKT kinase promotes its dissociation from harmartin, resulting in the formation of Rheb-GTP, which activates mTOR (Huang and Manning 2008; Wullschleger et al. 2006; Zoncu et al. 2011). Furthermore, Ther1462 of the TSC2 is known to be directly phosphorylated by AKT, leading to the destabilization of the TSC1/2 complex and activation of mTOR (Inoki et al. 2002). In an angiotensin II induced cardiac hypertrophy, the degradation of TSC2 activated the mTOR complex 1, thereby resulting in increased protein synthesis and enlarged cell size (Wang et al. 2013). It was reported that the depletion of shp2 by specific small interfering RNA induced the hypertrophy of neonatal rat ventricular myocytes due to the increased phosphorylation of TSC2 at Thr1462 (Marin et al. 2008). The present results show that there was a decrease in the TSC2 protein and an increase in phospho^{Thr1462}-TSC2. Thus, our results provide a clue to how swimming exercise induced activation of PI3K/AKT/mTOR signaling.

Another aspect of the present study is the correlation of miRNAs with signaling-related molecules. The implication of specific miRNA-regulating PI3K/AKT/mTOR signaling molecules in cardiac hypertrophy induced by a swimming exercise has rarely been reported (DA Silva Jr et al. 2012; Soci et al. 2011). Every target prediction of miRNAs is based on the 3′untranslated region of mRNA of PI3K/AKT/mTOR components in the web-based bioinformatics TargetScan, Miranda, and PicTar. Confirming these predictions, the literature provides further details about PI3K/AKT/mTOR signaling regulation. The PIK3α has been recently shown to be regulated by miRNA-124 (Lang and Ling 2012). Some studies (Ling et al. 2012; Zhang et al. 2013) have demonstrated that PTEN is regulated by miRNAs -21 and -144, and the positive and negative roles of

miR-21 have been reported in cardiomyocyte hypertrophy in vitro (Cheng et al. 2007; Tatsuguchi et al. 2007).

Although the control of expression of TSC2 involving miRNA-145 has not been elucidated, microRNA.org has predicted that the TSC2 is a target of miRNA-145. However, a recent study reported that the PI3K inhibitor, LY29, suppressed phosphorylation of AKT and C/EBP-β, and the up-regulation of miRNA-145 was found simultaneously (Sachdeva et al. 2012). C/EBP-β is an important functional target of ErbB4-related signaling pathways, particularly AKT1, in exercise-induced cardiomyocyte hypertrophy (Boström et al. 2010). More recent research showed that the miRNA-143/145 cluster involved inhibition of both the PI3K/AKT and the MAPK signaling pathway (Noguchi et al. 2013). These limited results suggest that the miRNA-145 regulatory system involving AKT may contribute to the down-regulation of TSC2 in physiological LVH, via the PI3K/AKT signaling pathway.

This present study reveals the potential molecular mechanisms that are responsible for the results. miRNA targets multiple genes, but targeted genes are controlled by specific miRNAs (Van Rooij and Olson 2007; Van Rooij et al. 2008). Decreased expression of miRNA indicates improvement of the target gene. This appears to be the case with microRNA-124 because the comparison of the SE and SC groups revealed an increase in the PIK3α gene in the SE group, whereas the microRNA-124 was down-regulated. By the same principle, the increased expression of the miRNAs reflects the inhibition expression of target genes. In the SE group, the PTEN gene exhibited a lower level, whereas the miRNAs that target the PTEN, microRNA 21, and 144 were up-regulated, a fact demonstrated through comparison with the SC group. Furthermore, the data also imply that the increase in microRNA-145 is accompanied by the decrease of the TSC2 gene after the swimming exercise. Thus, the 8-week swimming exercise affects the expression of miRNAs and thereby probably regulates their specific target genes.

Conclusion

Exercise is widely recognized as an important lifestyle factor in anti-hypertension and improving cardiac health. This work reveals, at least in part, the biochemical and molecular mechanisms of exercise involved in physiological cardiac hypertrophy. The results of the swimming exercise of trained rats indicate explicitly that LVH is physiological and is associated with the activation of the PI3K/AKT/mTOR signaling pathway. In contrast, the ERK1/2 signaling is unavailable in exercise-induced LVH. In addition, there is a reciprocal differential expression of specific miRNAs and genes, which are the components of PI3K/AKT/mTOR signaling. Together, these effects might provide the additional aerobic capacity required by the hypertrophic heart in exercised animals. The results suggest that a decrease in miRNA-124 could up-regulate the PIK3α gene, while an increase of microRNA-21 and 144 expressions inhibit PTEN levels, and an increase of microRNA-145 probably suppresses TSC2 expression. All of these microRNAs indirectly regulate PI3K/AKT/mTOR signaling by directly regulating their target genes (PIK3α, PTEN, and TSC2). These results increase our understanding of the mechanisms of physiological LVH, and imply that a basis for treatment to prevent the development of pathological LVH might be to regulate specific miRNAs, probably through antisense or small interfering RNA, so as to activate PI3K/AKT/mTOR signaling via PIK3α, PTEN, and TSC2.

Declaration The experiments comply with the current laws of the country in which they were performed.

Conflict of interest The authors declare that they have no conflict of interest.

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