Aerobic Exercise Training–Induced Left Ventricular Hypertrophy Involves Regulatory MicroRNAs, Decreased Angiotensin-Converting Enzyme-Angiotensin II, and Synergistic Regulation of Angiotensin-Converting Enzyme 2-Angiotensin (1-7)


Abstract—Aerobic exercise training leads to a physiological, nonpathological left ventricular hypertrophy; however, the underlying biochemical and molecular mechanisms of physiological left ventricular hypertrophy are unknown. The role of microRNAs regulating the classic and the novel cardiac renin-angiotensin (Ang) system was studied in trained rats assigned to 3 groups: (1) sedentary; (2) swimming trained with protocol 1 (T1, moderate-volume training); and (3) protocol 2 (T2, high-volume training). Cardiac Ang I levels, Ang-converting enzyme (ACE) activity, and protein expression, as well as Ang II levels, were lower in T1 and T2; however, Ang II type 1 receptor mRNA levels (69% in T1 and 99% in T2) and protein expression (240% in T1 and 300% in T2) increased after training. Ang II type 2 receptor mRNA levels (220%) and protein expression (332%) were shown to be increased in T2. In addition, T1 and T2 were shown to increase ACE2 activity and protein expression and Ang (1-7) levels in the heart. Exercise increased microRNA-27a and 27b, targeting ACE and decreasing microRNA-143 targeting ACE2 in the heart. Left ventricular hypertrophy induced by aerobic training involves microRNA regulation and an increase in cardiac Ang II type 1 receptor without the participation of Ang II. Parallel to this, an increase in ACE2, Ang (1-7), and Ang II type 2 receptor in the heart by exercise suggests that this nonclassic cardiac renin-angiotensin system counteracts the classic cardiac renin-angiotensin system. These findings are consistent with a model in which exercise may induce left ventricular hypertrophy, at least in part, altering the expression of specific microRNAs targeting renin-angiotensin system genes. Together these effects might provide the additional aerobic capacity required by the exercised heart. (Hypertension. 2011;58:00-00.) ● Online Data Supplement

Key Words: aerobic exercise training ■ cardiac hypertrophy ■ renin angiotensin system ■ microRNAs ■ angiotensin II receptors ■ ACE2 ■ angiotensin (1-7)

Left ventricular hypertrophy (LVH) induced by aerobic exercise training is an important physiological compensatory mechanism in response to chronic increases in hemodynamic overload. This phenotype is associated with sarcomeres added in series to lengthen the cardiac cell, as well as in parallel. 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, leading to high mortality.1-3 Several studies have reported that the renin-angiotensin (Ang) system (RAS) plays an important role in the progression of LVH.4-6 However, there are only limited data about the mechanisms of exercise training involved in RAS and LVH: The aim of this study was to elucidate these mechanisms of exercise training on physiological LVH.

Pathological LVH occurs with arterial hypertension,7,8 myocardial infarction,9,10 and heart failure.11 These disease states are also associated with increased local cardiac RAS levels, represented by augmented angiotensinogen, angiotensin-converting enzyme (ACE), and angiotensin II (Ang II). Blockade of the classic RAS promotes therapeutic benefits to patients with essential hypertension and cardiovascular disease.8 Cardiac Ang II is implicated in the induction of fibrosis but is not required for LVH.12 LVH is produced without the participation of Ang II in transgenic animal models for RAS.
components and in Ang II type 1 (AT₁) receptor activation by mechanical stress. A novel cardiac RAS includes ACE2, which is an essential regulator of heart function and plays a pivotal role in Ang (1–7) formation. This novel RAS is implicated in vasodilatation and control of fibrosis.

Previous findings suggest that ACE2 maintains the important balance between the Ang II and Ang (1–7), favoring cardiovascular homeostasis. However, the role of exercise training in the cardiac ACE2-Ang (1–7) axis is unknown. We have shown that AT₁ receptor blockade prevents physiological LVH induced by resistance training and by aerobic exercise training. Moreover, exercise training promoted LVH by cardiac RAS stimulation independent of the systemic RAS.

Several genes are regulated by microRNAs (miRNAs). MiRNAs are endogenous, small, and noncoding RNAs, which are targeted to specific genes and function as negative regulators of gene expression by inhibiting translation or promoting degradation of target miRNAs. Recent studies have shown the roles played by miRNAs in different forms of cardiovascular disease and pathological LVH. However, miRNAs may be important for normal development and in physiological cardiac hypertrophy induced by aerobic exercise training. In the present study, it was hypothesized that exercise training alters specific miRNAs that regulate their target cardiac RAS genes and tip the balance of classic RAS genes in favor of the novel RAS genes to contribute to physiological LVH.

Materials and Methods

Animal Care
All of the protocols and surgical procedures used were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the ethics committee of the University of Sao Paulo School of Physical Education and Sport. Female normotensive Wistar rats (190 to 220 g, n = 42) were used.

The animals were housed 3 to 5 per cage at a controlled room temperature (22°C) with a 12-hour dark-light cycle and fed standard laboratory animal chow. The rats were randomly divided into 3 experimental groups, each with 14 rats: (1) sedentary (S; n = 7); (2) swimming trained with protocol 1 (T1; n = 7); and (3) swimming trained with protocol 2 (T2; n = 7). Each group was subdivided into 2 groups, one for hemodynamic, biochemical, and molecular studies and the other for morphological and histological studies. For the detailed Material and Methods section, please see the online Data Supplement, available at http://hyper.ahajournals.org.

Results

Table 1. Hemodynamic Parameters

<table>
<thead>
<tr>
<th>Groups</th>
<th>SBP, mm Hg</th>
<th>DBP, mm Hg</th>
<th>MBP, mm Hg</th>
<th>HR, bpm</th>
</tr>
</thead>
<tbody>
<tr>
<td>S</td>
<td>127.6 ± 3.9</td>
<td>97.6 ± 10.3</td>
<td>113.5 ± 7.3</td>
<td>344.8 ± 12.1</td>
</tr>
<tr>
<td>T1</td>
<td>123.3 ± 8.5</td>
<td>96.4 ± 5.2</td>
<td>110.7 ± 6.7</td>
<td>301.2 ± 15.3*</td>
</tr>
<tr>
<td>T2</td>
<td>123.0 ± 8.4</td>
<td>94.3 ± 8.9</td>
<td>108.3 ± 9.0</td>
<td>309.0 ± 14.0*</td>
</tr>
</tbody>
</table>

Values are mean ± SD. SBP indicates systolic blood pressure; DBP, diastolic blood pressure; MBP, mean blood pressure; HR, heart rate.

*Significant difference vs S, P < 0.05.

Table 2. Cardiac Hypertrophy

<table>
<thead>
<tr>
<th>Groups</th>
<th>BW, g</th>
<th>LV/BW, mg/g</th>
<th>RV/BW, mg/g</th>
<th>Myocyte Diameter, µm</th>
</tr>
</thead>
<tbody>
<tr>
<td>S</td>
<td>245.8 ± 9.4</td>
<td>2.5 ± 0.06</td>
<td>0.59 ± 0.04</td>
<td>11 ± 1.1</td>
</tr>
<tr>
<td>T1</td>
<td>232.2 ± 6.4</td>
<td>2.8 ± 0.14*</td>
<td>0.68 ± 0.06*</td>
<td>13.2 ± 1.3*</td>
</tr>
<tr>
<td>T2</td>
<td>234.7 ± 17.3</td>
<td>3.2 ± 0.12† ‡</td>
<td>0.80 ± 0.08‡ ‡</td>
<td>14.4 ± 1.3*</td>
</tr>
</tbody>
</table>

Values are mean ± SD. BW indicates body weight; LV, left ventricular weight; RV, right ventricular weight.

Significant difference vs S, †P < 0.05; ‡P < 0.01.

Cardiac Hypertrophy

Body weight (BW) before and after swimming training was similar among all of the studied groups. Left ventricle (LV) and right ventricle (RV)/BW ratios were used as indices of hypertrophy. The absolute values referring to BW, LV/BW, and RV/BW in all of the groups of rats are summarized in the Table 2. LVH obtained by T1 and T2 was 13% (2.8 ± 0.14 mg/g; P < 0.05) and 27% (3.2 ± 0.12 mg/g; P < 0.01), respectively, in comparison with the S group (2.5 ± 0.06 mg/g). RV hypertrophy obtained by T1 and T2 was 15% (0.68 ± 0.06 mg/g; P < 0.05) and 35% (0.80 ± 0.08 mg/g; P < 0.01), respectively, in comparison with the S group (0.59 ± 0.04 mg/g). The increase in LV/BW ratio observed with swimming training was further confirmed by the increase in LV myocyte diameter in T1 (13.2 ± 1.3 µm) and T2 (14.4 ± 1.3 µm) groups when compared with the S group (11.0 ± 1.1 µm; P < 0.05; Table 2). Figure S1, available in the online Data Supplement at http://hyper.ahajournals.org, shows representative histological sections of increase in LV myocyte diameter in T1 and T2 in comparison with the S group.

Molecular Markers of Pathological LVH

Pathological cardiac hypertrophy is characterized by the induction of genes normally expressed during fetal development, such as atrial natriuretic factor, skeletal muscle α-actin, and a decrease in the ratio of αβ-myosin heavy chain. The mRNA levels of these 4 genes were assessed in the LV of S and trained groups (T1 and T2) by real-time PCR. The results of this study showed that swimming training did not modify atrial natriuretic factor gene expression. Similarly, T1 training did not change the gene levels of skeletal α-actin and αβ-myosin heavy chain, although T2 exercise significantly reduced the LV levels of skeletal α-actin by 53% (P < 0.05) and increased the LV levels of αβ-myosin heavy chain by 98% (P < 0.05), when compared with the S group.

RAS Biochemical Analysis

To evaluate the role of swimming exercise training in systemic RAS, the serum ACE activity and plasma renin activity were measured. Figure 1A shows that there was an increase in serum ACE activity of 3.8% in T1 (P value not significant [NS]) and 23.5% in T2 (P < 0.05) in comparison...
with the S group. Plasma renin activity also was increased by 20% in T1 (P=NS) and 126% in T2 (P<0.01) when compared with the S group (Figure 1A). In contrast, there was a reduction in local cardiac ACE activity of 11% (P=NS) and 15% (P=NS) in the RV and LV, respectively, in the T1 group. When the T2 group was compared with the S group, there was a decrease of 40% (P<0.05) and 32% (P<0.05) in RV and LV, respectively (Figure 1B). Interestingly, Figure 1B also shows that LV ACE2 activity was increased by 12% in T1 (1708±354 unit of fluorescence/min per milligram; P=NS) and 41% in T2 (2160±218 unit of fluorescence/min per milligram; P<0.01) when compared with the S group (1531±174 unit of fluorescence/min per milligram).

**RAS Molecular Analysis**

In order to test whether swimming exercise training modulates cardiac RAS gene expression, real-time PCR was used to assess ACE, ACE2, AT1, and Ang II type 2 (AT2) receptor gene expression in the heart. mRNA levels of ACE showed a small decrease and ACE2 mRNA a small increase in both trained groups but without significance (data not shown). AT1 receptor gene expression increased in T1 (69%; P<0.05) and T2 (99%; P<0.01) when compared with the S group (Figure 4B). In addition, AT2 receptor gene expression increased by 26% (P=NS) in T1 and 332% in T2 (P<0.001); T1 differed from T2 (P<0.001; Figure 4D).

Similar results were obtained for RAS proteins and peptide levels determined by Western blotting and high-performance liquid chromatography, respectively. Figure 2A shows that swimming exercise training decreased cardiac angiotensinogen levels by 26% (P<0.05) in T1 and 44% in T2 (P<0.05) when compared with the S group. Because angiotensinogen is a substrate for Ang I production, this reduction was accompanied by a decrease of 25.6% (P<0.05) in cardiac Ang I levels in T1 and 44% in T2 (P<0.001) when compared with the S group (Figure 2B). The next step was to measure the levels of ACE, because it is responsible for converting Ang I into Ang II. Accordingly, Figure 2C shows that cardiac ACE levels were decreased by 22% (P=NS) in T1 and 31% in T2 (P<0.05) in comparison with the S group. This reduction was accompanied by a decrease of 23% (P<0.001) in Ang II levels in T1 and 20% in T2 (P<0.001) in comparison with the S group (Figure 2D), indicating an attenuation of the ACE-Ang II axis induced by swimming exercise training.

Swimming exercise training also had an effect on the protein and peptide levels of novel RAS, ACE2, and Ang (1-7) in the heart. As shown in Figure 3A, swimming training increased ACE2 protein expression in both trained groups (68% in T1 and 91% in T2; P<0.05) when compared with the S group. There was increased Ang (1-7) formation (160% in T1 and 120% in T2; P<0.01; Figure 3B) in comparison with the S group. Figure 3C shows an increase in the Ang (1-7)/Ang II ratio in both trained groups (180% in T1, P<0.05, and 160% T2, P<0.001) in comparison with the S group, suggesting an aerobic training-mediated increase in Ang (1-7) formation from Ang II by ACE2.

Protein expression of the AT1 receptor, in concert with the increase in AT1 receptor mRNA levels, was 2.4-fold greater in T1 (P<0.05) and 3.0-fold greater in T2 (P<0.05) when compared with the S group (Figure 4A). In addition, AT2 receptor protein expression was increased 1.6-fold in T1 (P=NS) and 2.2-fold (P<0.05) in T2 (Figure 4C).

**MiRNAs Analysis by Microarray**

Microarray analysis of miRNA was restricted to those miRNAs that underwent a significant change from baseline. Figure 5A shows miRNAs targeting ACE: in the S group the relative expression value of miRNA-27a was 1760±108 arbitrary units (AU). In the T1 group the value was 2225±78 AU, (26% increase in comparison with S; P<0.05), and in T2 group the expression was 3218±30 AU (83% increase in comparison with S; P<0.01). In addition, T1 differed from T2 (P<0.01). Similarly, in the S group the relative expression value of miRNA-27b was 3409±89 AU. In the T1 group the value was 4341±124 AU, (27% increase in comparison with S; P<0.05), and in the T2 group the expression was 4939±59 AU (45% increase in comparison with S; P<0.01). In addition, T1 differed from T2 (P<0.01). Figure 5A also shows miRNA targeting ACE2: in the S group the relative expression value of miRNA-143 was 6556±157 AU; T1=6095±83 AU was not significant when compared with S; however, in the T2 group the expression was 4249±32 AU.
MiRNAs Analysis by Real-Time PCR

To confirm the miRNAs that targeted RAS genes in physiological LVH, the miRNAs 27a, 27b, and 143 were quantified by real-time PCR. MiRNAs 27a (S: 1.0 ± 0.08, T1: 1.52 ± 0.06, and T2: 2.04 ± 0.13 AU) and 27b (S: 1.0 ± 0.08, T1: 1.30 ± 0.05, and T2: 1.59 ± 0.08 AU) were upregulated in T1 and T2 in comparison with S, whereas miRNA-143 (S: 1.0 ± 0.11, T1: 0.82 ± 0.02, T2: 0.58 ± 0.05 AU) was downregulated in T2 in comparison with S. The miRNA expression in T1 and T2 in comparison with the S group confirmed the microarray results.

Discussion

The results of the present study show that swimming exercise training induced physiological LVH, is not correlated with pathological cardiac hypertrophy markers, increased AT1 and AT2 receptor expression, decreased cardiac ACE and Ang II.
levels and increased ACE2 and Ang (1-7) levels, and altered the expression of specific miRNAs that target RAS genes.

The LV/BW ratio, myocyte diameter, and resting brady-cardiac confirmed the exercise-associated adaptations of physiological LVH.12,20 In contrast to markers for pathological hypertrophy,26 the physiological LVH reported here was not associated with activation of fetal genes, such as atrial natriuretic factor, skeletal muscle α-actin, and β-myosin heavy chain. There were no pathological hypertrophy markers in the exercised-trained T1 or T2 groups.

The development of LVH after training did not appear to involve Ang II, which was decreased, supporting recent evidence from several transgenic animal models that increased formation of local Ang II in the heart does not directly develop hypertrophy, except when excess cardiac Ang II enters the circulation and causes an increase in blood pressure.12–14 Xiao et al15 reported that, in mice expressing ACE only in the heart, although they exhibit the same ligand binding affinity, AT2 receptor plays a cardioprotective role in opposing deleterious cardiac remodeling in cardiovascular disease. In contrast to the pathological condition, an increase in AT2 receptor expression in the heart could aid vasodilatation induced by aerobic exercise training. This modulation might

Figure 4. Effect of exercise training on angiotensin (Ang) II type 1 (AT1) and Ang II type 2 (AT2) receptor gene and protein expression in the heart. Data are presented as mean±SD. Cardiac AT1 receptor (A) and AT2 receptor (C) protein expression analyzed by Western blotting accompanied by their representative blots from sedentary (S) and trained groups (T1 and T2). Targeted bands were normalized for cardiac α-tubulin.

Figure 5. Aerobic exercise training also increased AT2 receptor mRNA and protein expression in the LV. Studies suggest that AT1 and AT2 receptors may serve opposing functions in the heart, although they exhibit the same ligand binding affinity.4,12 The role of AT2 receptors in cardiac regulation is not fully understood. The AT2 receptor has been associated with dephosphorylation and inactivation of growth factor–activated mitogen-activated protein kinase pathways for cell growth.30,31 The AT1 receptors have an indirect, membrane-transactivating step to stimulate the epidermal growth factor receptor.36,37 Inhibition of the epidermal growth factor receptor directly prevents Ang II–induced LVH in rats.31 The mechanical activity of exercise could, therefore, activate the epidermal growth factor receptor pathway via mechanical activation of AT1, even in the absence of Ang II.

Aerobic exercise training also increased AT2 receptor genes and protein expression in the LV. Studies suggest that AT1 and AT2 receptors may serve opposing functions in the heart, although they exhibit the same ligand binding affinity.4,12 The role of AT2 receptors in cardiac regulation is not fully understood. The AT2 receptor has been associated with dephosphorylation and inactivation of growth factor–activated mitogen-activated protein kinase and inactivation of extracellular signal–regulated kinase 1/2, providing a protective role in the heart.52 Furthermore, AT2 receptor activates NO and bradykinin, inducing vasodilation.33 Yang et al34 demonstrated that, in transgenic animals, the overexpression of AT2 receptors preserved LV function after myocardial infarction. The results of the present study suggest that the AT2 receptor plays a cardioprotective role in opposing deleterious cardiac remodeling in cardiovascular disease. In contrast to the pathological condition, an increase in AT2 receptor expression in the heart could aid vasodilatation induced by aerobic exercise training. This modulation might
Ang (1-7) can reduce hypertension-induced cardiac remodeling through a direct effect on the heart and raise the possibility that pathologies associated with ACE2 inactivation are partly mediated by a decrease in Ang (1-7) production.\textsuperscript{38,39}

\( \text{AT}_1 \) receptor blockade augmented the plasma Ang (1-7)/Ang II ratio, suggesting increased generation of Ang (1-7) from Ang II.\textsuperscript{20} In addition, Crackower et al.\textsuperscript{19} showed that deletion of ACE2 in mice resulted in elevated cardiac and plasma Ang II, together with impaired cardiac contractility and exhibited LV dilatation. Therefore, ACE2 might protect against pathological LVH by reducing Ang II concentration and increasing Ang (1-7) generation.\textsuperscript{6,20–23,38,39}

Thus, the mechanism of aerobic exercise training to prevent LVH could occur by diminished vascular resistance, leading to increased cardiac flow, attributed to the reduction in ACE and Ang II levels and the vasodilator effects of the ACE2-Ang (1-7) expression, mediating the release of different vasoactive factors, such as NO, prostaglandins, and bradykinin.\textsuperscript{40,41}

Another aspect of this report is the correlation of miRNAs with Ang-related genes. The implication of specific miRNA-regulating RAS genes in cardiac hypertrophy induced by exercise training has not been reported previously. The target predictions of miRNAs are all based on 3\textsuperscript{\prime} untranslated regions of miRNA of RAS components in Web-based bioinformatics TargetScan 4.2 and 5.1, MiRanda, and PicTar. Confirming these predictions, the literature provides more details of Ang gene regulation through quantitative PCR and Ang gene measurements. The ACE gene has been shown recently to be regulated by miRNA-27a and 27b.\textsuperscript{32} It has been demonstrated that the ACE2 gene is regulated by miRNA-143.\textsuperscript{45}

This study reveals potential molecular mechanisms for the results. MiRNAs target multiple genes, but targeted genes are controlled by specific miRNAs.\textsuperscript{28,26} Increased expression of miRNA indicates inhibition of the target gene. This appears to be the case with miRNA-27a and -27b, because ACE decreased by 22\% (T1) and 31\% (T2) in comparison with the control, whereas miRNA-27a increased by 26\% (T1) and 88\% (T2) when compared with the control, and miRNA-27b increased by 27\% (T1) and 44\% (T2). By the same principle, decreased expression of miRNAs reflects increased expression of target genes. Although in the T2 group in which the expression of ACE2 was highest, the miRNAs that target the ACE2 gene, miRNA-143, were at their lowest level of expression when compared with the control or T1. Thus, aerobic exercise training exerts an effect on the expression of miRNAs and thereby might regulate their specific target genes.

**Perspectives**

Exercise is widely recognized as an important lifestyle factor in lowering hypertension and improving cardiac health. This study reveals some of the biochemical and molecular mechanisms of aerobic exercise training involved in physiological, nonpathological cardiac hypertrophy. The results clearly indicate that, in aerobic exercise trained animals, LVH is physiological and associated with decreased ACE and Ang II versus increased ACE2 and Ang (1-7) and increased \( \text{AT}_1 \) and \( \text{AT}_2 \) receptors. In addition, there was a reciprocal differential expression of specific miRNAs and these genes. These findings are consistent with a model in which exercise may influence these changes, at least in part, altering the expres-
sion of specific miRNAs targeting RAS genes. Together, these effects might provide the additional aerobic capacity required by the exercised heart. The results imply that a decrease in miRNA-143 could upregulate cardioprotective genes in the heart, and an increase of miRNA-27 expression inhibits ACE levels. These results suggests that a basis for treatment to prevent of the development of pathological LVH might be to inhibit specific miRNAs, probably with antisense or small interfering RNA, to inhibit ACE and Ang II and increase ACE2 and Ang (1-7).

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Disclosures

None.

References

15. Xiao HD, Fuchs S, Bernstein EA, Li P, Campbell DJ, Bernstein KE. Mice expressing ACE only in the heart show that increased cardiac angiotensin II is not associated with cardiac hypertrophy. Am J Physiol Heart Circ Physiol. 2008;294:659–667.


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Aerobic exercise training induced LVH involves regulatory microRNAs, decreased ACE-ANG II, and synergistic regulation of ACE2-ANG (1-7).

Short title: Effect of aerobic exercise training on cardiac RAS.

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DETAILED METHODS

Exercise Training Protocols

Protocol 1 (T1) low intensity, moderate volume exercise: training consisted of swimming sessions of 60-minute duration, 5 days a week, for 10 weeks, which were carried out between 11:30 AM and 1:30 PM. Protocol 2 (T2) low intensity, high volume exercise: Animals assigned to T2 performed the same swimming training protocol as T1 until the end of the 8th week. On the 9th week rats trained twice a day, swimming sessions of sixty-minute duration with four hours interval between sessions. In that week swimming sessions were carried on from 07:30 to 08:30 AM and from 12:30 AM to 1:30 PM. On the 10th week rats trained three times a day, with sessions of sixty-minute duration with four hours interval between sessions. In that week swimming sessions were carried on from 07:30 to 08:30 AM, from 12:30 AM to 1:30 PM, and from 5:30 to 6:30 PM. The aim of increasing training frequency (T2) was to induce the magnitude of cardiac hypertrophy.

Animals were trained in a swimming apparatus specially designed to allow individual exercise training of rats. The system consisted of two coupled 700 L water glass tanks of different dimensions. The outer tank measures 60 cm in diameter, 1.60 cm in width and 90 cm in height. The inner tank is divided into 14 lanes with a surface area of 20 x 20 cm per lane and a depth of 60 cm. A heating system kept the water temperature between 30-32°C and a water filter with a flow capacity of 420 L.h⁻¹ was used to clean the swimming apparatus (1).

It was showed in our previous study that this swimming-training protocol did not stress the animals as it did not alter plasma catecholamine concentration (2). Exercise duration and workload were increased gradually until rats could swim for 60 minutes wearing caudal dumbbells weighing 5% of their body weight. Thereafter, duration and dumbbells were kept constant. All animals were weighed once a week and the workload adjusted to body weight variations. Sedentary groups were placed in the swimming apparatus for 10 minutes twice a week without workload to control for being in the water. O₂ uptake of rats swimming individually is about 50-65% of maximum oxygen uptake. This low intensity long period training protocol is effective for promotion of cardiovascular adaptations and for increase of muscle oxidative capacity. These protocols have already been used previously in our laboratory (1,2).

Hemodynamic Parameters: Blood Pressure (BP) and Heart Rate (HR)

Twenty-four hours after the last training session BP and HR were recorded. Rats were anesthetized (Ketamine 90 mg.kg⁻¹ and Xylazine 10 mg.kg⁻¹, i.p.) and a cannula (PE-50) was inserted into the carotid artery and emerged through the back of the neck. Twenty-four hours after the cannula was implanted, this cannula was connected to a strain-gauge transducer (P23 Db; Gould-Statham). Arterial blood pressure was recorded on a beat to beat basis (AT/CODAS) at a frequency of 1000Hz for 30 min in quiet, conscious, unrestrained rats. 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

At the end of the experimental period the rats were decapitated and blood and tissue samples (heart) were harvested, weighed, frozen, stored at -80°C and used within 1 month for enzymes assay, miRNA, mRNA and protein preparation. To determine plasma
angiotensin II the first 3 ml of trunk blood (a mixture of venous and arterial blood) was rapidly collected in chilled glass tubes containing a mixture of potassium EDTA (25 mmol), o-phenanthroline (0.44 mmol), pepstatin A (0.12 mmol), and 4-chloromercuribenzoic acid (1 mmol). This mixture of protease inhibitors prevented the in vitro production and degradation of angiotensin peptides (3). To determine the renin activity the blood was collected with EDTA (25 mmol). The blood was centrifuged the plasma was separated and stored at -20°C.

Measurement of Cardiac Hypertrophy

To measure cardiac mass, the hearts were stopped at diastole by perfusion of 14 mM KCl. After the heart was weighed, the left ventricle (LV) was dissected corresponding to the remaining tissue upon removal of both atria and the free wall of the right ventricle (RV). The interventricular septum remained as part of the LV. Cardiac hypertrophy was assessed by the measurement of the ratio of LV and RV weight in milligrams to animal body weight (BW) in grams (LV/BW and RV/BW in mg.g\(^{-1}\)). The LV was fixed in 6% formaldehyde and embedded in paraffin, cut in 5 μm sections, from the level of papillary muscle and subsequently stained with hematoxylin and eosin (HE) for the visualization of cellular structures. Two randomly selected sections from each animal were visualized by light microscopy using an oil immersion objective with a calibrated magnification (x400). Myocytes with 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 (Quantimet 520; Cambridge Instruments). For each animal, approximately 20 visual fields were analyzed.

RAS Biochemical Analysis

Cardiac and Serum angiotensin-converting enzyme (ACE) activity:

ACE activity was determined in heart tissue and serum using Abz-FRK(Dnp)P-OH derivatives as substrates by continuously measuring the fluorescence according to Alves et al. (4). Heart samples were quickly harvested, homogenized in 0.1 M Tris-HCl buffer, pH 7.0, containing 50 mM NaCl and centrifuged at 1000 g for 10 min. The assays were performed at 37°C in 0.1 M Tris-HCl buffer, pH 7.0, containing 50 mM NaCl and 10 μM ZnCl₂. The hydrolysis rate of the intramolecularily quenched fluorogenic substrate Abz-FRK-(Dnp)P-OH (10 uM) incubated with aliquots of heart homogenate and serum for 30 min at 37°C was assessed to obtain ACE enzymatic activity. Fluorescence increments along the time were read at 420nm emission: 320nm excitation. Heart and serum ACE activity were expressed as UF.min\(^{-1}\).mg\(^{-1}\)of protein x 1000. The protein content was determined by the Bradford methods (5) by using bovine serum albumin as the standard (Bio-Rad Protein Assay).

Cardiac angiotensin-converting enzyme 2 (ACE2) activity:

ACE2 activity was determined in heart tissue by the same method described above. However, this method uses a fluorescent peptide Abz-APK(Dnp)-OH in 0.2 M Tris-HCl buffer, 200 mM NaCl, 2 μg BSA, pH 7.5 which is hydrolyzed with high affinity by ACE2 (Kcat/Km = 3.5 x 10^4 M\(^{-1}\).s\(^{-1}\)). ACE2 activity was expressed in UF.min\(^{-1}\).mg\(^{-1}\)of protein.

Plasma renin activity assay:

Plasma renin activity (PRA) was determined by angiotensin radioimmunoassay, using a commercial kit (REN-CT2, CIS Bio International, Gif-sur-Yvette, France). This
assay allows direct measurement of PRA. Results were quantified in a Gama Counter, and the enzyme activity was expressed as ng Ang I/mL/h.

**MiRNAs and RAS Molecular Analysis**

**RNA extraction and MiRNA Microarray**

Frozen LV samples (100 mg) were homogenized in Trizol and RNA was isolated according to the manufacture (Invitrogen Life Technologies, CA, USA). Following extraction, the RNA total concentration was quantified using NanoDrop Spectrophotometer (Nano-Drop Technologies, USA) and checked for integrity by EtBr-agarose gel electrophoresis. MiRNA was isolated using the mirVana™ qRT-PCR- miRNA Isolation Kit (Ambion, TX, USA). RNA from two animals in each group was pooled and used for miRNA expression analysis (LC Science, TX, USA) with the Agilent platform. The arrays consist of 15,000 features including rat probes for 349 miRNAs based on Sanger miRBASE 13.0. The Agilent miRNA platform requires 100ng of total RNA per labelling reaction. The quality of all RNA samples was checked using the miRMAX microarray. Results are expressed as arbitrary units (a.u.). TargetScan program was used to search the miRNAs target to the RAS.

**Real-Time Reverse Transcriptase-Polymerase Chain Reaction:**

The relative gene expression of α-MHC (alfa-Myosin Heavy Chain), β-MHC (beta-Myosin Heavy Chain), ANP (Atrial Natriuretic Peptide), skeletal α-actin, ACE, ACE2, AT1a, and AT2 mRNA in the LV was analyzed by real-time PCR. In addition, miRNAs-27a, 27b and 143 also were quantified by real-time PCR.

**cDNA synthesis**

RNA were primed with 0.5 µg/µl oligo dT (12–18 bp) (Invitrogen Life Technologies, CA, USA) to generate the first strand DNA. Reverse transcription (RT) was performed using SuperScript™ II Reverse Transcriptase (Invitrogen Life Technologies, CA, USA).

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 TaqMan MicroRNA Reverse Transcription Kit protocol (Applied Biosystems, CA, USA) were incubated in Thermal Cycler for 30 min at 16°C, 30 min at 42°C, 5 min at 85°C and then held at 4°C.

**Real-Time PCR:**

Prior to analyzing samples, a standard curve for each amplicon was obtained using serial dilutions of cDNA to determine amplification primer efficiency and the amount of material for each reaction. Primers were designed using Primer 3 software ([http://frodo.wi.mit.edu/cgi-bin/primer3/primer3 www.cgi](http://frodo.wi.mit.edu/cgi-bin/primer3/primer3 www.cgi)). DNA sequence was obtained from GenBank and primers were made in separate exons to distinguish by size PCR products derived from cDNA from those derived from genomic DNA contaminants. The mRNA expression were assessed by oligonucleotides primers as follows: for **α-MHC:** (sense: 5’-CGA GTC CCA GGT CAA CAA G-3’, antisense: 5’-AGG CTC TTT CTG CTG GAC C-3’); for **β-MHC:** (sense: 5’-CAT CCC CAA TGA GAC GAA G-3’, antisense: 5’-AGG CTC TTT CTG CTG GAC A-3’); for **ANP:** (sense: 5’- CTG CCT CCC AAC GAG TTA GGA TAG GAT TGA C-3’, antisense: 5’-CTT GGG ATC TTT TGC GAT CT-3’); for **skeletal α-actin:** (sense: 5’-ACC ACA GGC ATT GTT CTG GA-3’, antisense: 5’-TAA GGT AGT CAG TGA GGT CC-3’); for **ACE:** (sense: 5’ CTG CCT CCC AAC GAG TTA GAA 3’, antisense: 5’-CGG GAC GTG GCC ATT ATA TT 3’); for **ACE2:** (sense: 5’ CAT
TGG AGC AAG TGT TGG ATC TT 3', antisense: 5' GAG CTA ATG CAT GCC ATT CTC A 3'); for AT1a (sense: 5'-CAC AAC CCT CCC AGA AAG TG-3’, antisense: 5'-AGG GCC ATT TTG TTT TTC TG-3’) and for AT2 (sense: 5'- GGC CTG TTT GTC CTC ATT GC -3’, antisense: 5'- CAC GGG TTA TCC TGT TCT TC -3’).

Real-time PCR quantification of the target mRNAs was performed with a SYBR Green PCR Master Mix, (Applied Biosystem, CA, USA) using ABI PRISM 7700 Sequence Detection System (Applied Biosystem, CA, USA). The expression of cyclophilin (sense: 5’-AAT GCT GGA CCA AAC ACA AA -3’, antisense: 5’-CCT TCT TTC ACC TTC CCA AA -3’) was measured as an internal control for sample variation in RT reaction. An aliquot of the RT reaction was used for 50 cycle PCR amplification in the presence of SYBR green fluorescent dye according to a protocol provided by the manufacturer (Applied Biosystems, CA, USA).

In order to accurately detect mature miRNAs and confirm array results, real-time PCR quantification was performed using TaqMan MicroRNA Assay protocol (Applied Biosystems, CA, USA). The 20 μl PCR included 1.33 μl RT product, 10 μl TaqMan Universal PCR master mix II (2x), 7.67 μl nuclease-free water and 1 μl of primers and probe mix of the TaqMan MicroRNA Assay protocol for miRNAs- 27a (INV 408) , 27b (INV 409) and 143 (INV 466). The reactions were incubated in a 96-well optical plate at 95°C for 10 min, followed by 40 cycles of 95°C for 15s and 60° for 1 min. Samples were normalized by evaluating U6 expression.

PCR product generation was monitored by measuring the increase in fluorescence caused by the binding of SYBR green to double-stranded DNA or by the probe presence in TaqMan MicroRNA Assay at each annealing phase. A dissociation curve observed in SYBR green analysis was generated at the end of the reaction to verify that a single product was amplified. Each heart sample was analyzed in triplicate. Relative quantities of target gene expressions of sedentary rats vs. trained rats were compared after normalization to the values of internal control (ΔCT). Fold change in mRNA expression were calculated using the differences in ΔCT values between the two samples (ΔΔCT) and equation 2^ΔΔCT. Results are expressed as % of control.

Western Blotting analysis

The protein expression of angiotensinogen, ACE, ACE2, AT1 and AT2 receptors in the left ventricle was analyzed by western blotting. The frozen ventricles (100 mg) were homogenized in cell lyses buffer containing 100 mM Tris-HCl, 50 mM NaCl, 1% Triton X-100 and protease inhibitor cocktail (1:100, Sigma-Aldrich, MO, USA). Insoluble heart tissues were removed by centrifugation at 10,000 × g, 4° C, 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 with the use of 0.5% Ponceau S staining of the blot membrane. The blot membrane was then incubated in a blocking buffer (5% nonfat dry milk, 10mM Tris-HCl, pH 7.6, 150 mM NaCl, and 0.1% Tween 20) for 2h at room temperature and then incubated overnight at 4°C with mouse anti-AT1 receptor monoclonal antibody and rabbit anti-AT2 receptor polyclonal antibody (1:1000 and 1:800, respectively; Abcam, Cambridge, UK), mouse anti-ACE clone 2E2 monoclonal antibody (1:1000, Chemicon International, CA, USA), goat anti-ACE2 and anti-Ang I/II precursor polyclonal antibody (1:1000 and 1:500, respectively; Santa Cruz Biotechnology Inc., CA, USA). Binding of the primary antibody was detected with
the use of 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 α-tubulin expression levels were used to normalize the results. Results are expressed as arbitrary units (a.u.).

**Angiotensin I, II and (1-7) Quantification by High Performance Liquid Chromatography (HPLC)**

Left ventricle was weighed and homogenized in 100 mM sodium phosphate buffer pH 7.2, 340 mM sucrose and 300 mM NaCl, containing protease inhibitor cocktail (1:100, Sigma-Aldrich, MO, USA). The samples were centrifuged at 10,000 × g, 4° C, 20 min.

The extraction of angiotensins was held in Oasis C18 columns (Waters, MA, USA) previously activated with methanol (5 mL), tetrahydrofuran (5 mL), hexane (5 mL), methanol (5 mL) and water (10 mL). After activation, the samples were applied to the columns, washed with water and eluted in ethanol / acetic acid / water in the proportion 90% / 4% / 6%. The eluted fractions were lyophilized and resuspended in 500 uL of mobile phase A (5% acetonitrile in 0.1% orthophosphoric acid) and filtered with 0.22 mm membrane for analysis by high-performance liquid chromatography (HPLC, Shimadzu System, Japan).

The angiotensin of each sample were separated on a reversed phase column ODS Aquapor 300 (250 x 4.6 mm), 7μ (PerkinElmer’s Browlee Columns) using the gradient from 5–35% of mobile phase B (95% acetonitrile in 0.1% phosphoric acid) under a flow of 1.5 mL / min for 40 min. The angiotensins were identified by comparing them with the retention time of standard angiotensins. Results were expressed as pmol/g of tissue.

**Statistical analysis**

Results are represented as mean ± SD. Statistical analysis was performed using one-way ANOVA. P values <0.05 were accepted as statistically significant. Tukey’s post hoc test was used for individual comparisons between means when a significant change was observed with ANOVA.

**REFERENCES**


**Supplementary Figure Legend**

**Figure S1.** Effect of swimming exercise training on the cardiomyocytes diameter (μm). Representative histological sections of LV myocytes diameter in the sedentary (A), T1- swimming training protocol 1 (B) and T2- swimming training protocol 2 (C). 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 (x400). Significant difference vs. *S, P<0.05.*