Research Article

Exercise Training Restores the Cardiac Microrna-16 Levels Preventing Microvascular Rarefaction in Obese Zucker Rats

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Keywords

Obesity · Exercise training · Heart · Angiogenesis · MicroRNA-16

Abstract

Objective: To evaluate the effects of aerobic exercise training (AET) on cardiac miRNA-16 levels and its target gene *VEGF* related to microvascular rarefaction in obese Zucker rats (OZR).

Methods: OZR (n = 11) and lean (L; n = 10) male rats were assigned into 4 groups: OZR, trained OZR (OZRT), L and trained L (LT). Swimming exercise training lasted 60 min, 1x/day/10 weeks, with 4% body weight workload. Cardiac angiogenesis was assessed by histological analysis (periodic acid-Schiff) by calculating the capillary/fiber ratio. The protein expressions of VEGF, VEGFR2, and CD31 were evaluated by western blot. The expression of miRNA-16 was evaluated by real-time PCR. **Results:** Heart rate decreased in the trained groups compared to sedentary groups. The cardiac capillary/fiber ratio was reduced in OZR compared to L, LT and OZRT groups, indicating that aerobic exercise training (AET) was capable of reversing the microvascular rarefaction in the obese animals. miRNA-16 expression was increased in OZR compared to L, LT and OZRT. In contrast, its target, VEGF protein expression was 24% lower in OZR compared to L group, which has been normalized in OZRT group. VEGFR2 protein expression was increased in trained groups compared to their controls. CD31, a endothelial cells marker, showed increased expression in OZRT compared to OZR, indicating greater vascularization in OZRT group. **Conclusion:** AET induced cardiac angiogenesis in obese animals. This revascularization is associated with a decrease in miRNA-16 expression permissive for increased VEGF protein expression, suggesting a mechanism for potential therapeutic application in vascular diseases.

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Introduction

Obesity is a multifactorial disease in which environmental and genetic factors interact, predisposing cardiovascular diseases [1]. Studies indicate that lifestyle modification based on adequate diet and exercise remains the mainstay of treatment for obesity [2, 3].

Pathological cardiac hypertrophy and dysfunction associated with metabolic disorders have been reported in obesity [4–6]. The obese Zucker rat (OZR), a genetic model of morbid obesity, presents many of the same cardiovascular deficits noted in obese humans including myocardial hypertrophy [5, 7], cardiac microvascular rarefaction [8], and exercise intolerance [5, 9]. Aerobic exercise training (AET) has been known to play a key role in the prevention and treatment of obesity and in the non-pharmacological treatment of cardiovascular remodeling [5, 10–12]; however, underlying mechanisms remain to be further determined.

MicroRNAs (miRNAs) are a new class of gene regulators which can bind most commonly, but not exclusively, to 3'-untranslated regions (3'-UTR) of messenger RNAs (mRNAs) of protein-coding genes and negatively regulate their expression [13, 14]. MiRNAs have been shown to play a key role in cardiovascular structure and function under physiological and pathological conditions [11, 15–20]. Kuehbach et al. [17] showed, for the first time, that reduction of the miRNA-regulating enzyme Dicer impairs angiogenesis in vitro and in vivo; however, only a few specific miRNAs targeting endothelial cell function and angiogenesis have been identified.

miRNA-16 plays a well-known role in the control of angiogenesis and vascular integrity and is involved in the survival, maintenance, and formation of new capillaries [18–20]. Vascular endothelial growth factor (VEGF) and anti-apoptotic protein Bcl-2 have been identified by in silico analyses and subsequently validated as targets to miRNA-16 in endothelial cells, showing that mimetic of this miRNA reduced VEGF and Bcl-2 expression, whereas a specific antagomir enhanced their expression [18–22]. Recently, we showed that AET restored skeletal muscle miRNA-16, -21, and -126 expression in hypertension. This was paralleled by normalization of VEGF, eNOS, and PI3KR2 levels, as well as of the pro-apoptotic (Bad) and anti-apoptotic (Bcl-2, and Bcl-x) mediators, indicating that balance between angiogenic and apoptotic factors may prevent microvascular abnormalities in hypertension [23]. However, the effects of AET on miRNAs in obesity still remain unknown.

Therefore, in the present study, we investigated whether AET regulates the miRNA-16 expression and its target VEGF related to microvascular rarefaction in OZR, contributing to vascular integrity and angiogenesis in AET-induced obese animals.

Material and Methods

Animal Care

20-week-old male Zucker rats from the Medical School, Federal University of São Paulo, were randomly assigned to 4 experimental groups: lean sedentary (L, n = 5), lean trained (LT, n = 5), obese sedentary (OZR, n = 5) and obese trained (OZRT, n = 6). The animals were housed 3–5 per cage at a controlled room temperature (22–24 °C), with a 12-hour dark-light cycle, were fed standard rat chow, and had ad libitum access to water. The rats were identified and weighed weekly. All of the protocols and surgical procedures were in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and were approved by the local ethics committee of the University of São Paulo (No. 1023/07).

Exercise Training Protocol

The swimming training was performed according to the protocol developed by Medeiros et al. [24] in a swimming system with water heated between 30 and 32 °C. The AET protocol consisted of swimming sessions of 60 min duration, 5 days a week, for 10 weeks, with 4% caudal body weight workload. This training

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Fig. 1. Experimental protocol.

was characterized by a low to moderate intensity and long duration, ideal for the increased oxidative capacity of skeletal muscle and cardiovascular adaptations [23–26].

Cardiovascular Measurements

Resting systolic blood pressure (SBP) and heart rate (HR) were measured in conscious rats using a computerized tail-cuff system (IITC Life Science, Woodland Hills, CA, USA). Rats were acclimatized to the apparatus during daily sessions over 4 days, 1 week before starting the experimental period. SBP and HR were determined after AET protocol.

Tissue Harvesting

24 h after the last training session, the rats were killed by decapitation, and tissue samples were harvested. The heart was removed, and the left ventricle (LV) was weighed and normalized by tibia length to determine cardiac hypertrophy. The tissues were frozen at -80°C and used for histologic, miRNA, and protein preparations (fig. 1).

Cardiac Capillary-to-Fiber Ratio Measurements

The LV was fixed in 6% formaldehyde and embedded in paraffin, cut into $5\text{-}\mu\text{m}$ sections from the level of papillary muscle, and subsequently stained with periodic acid-Schiff (PAS) to enable visualization of the capillary structure. Cardiac capillary-to-fiber ratio was quantified by a 10×10 grid optically superimposed on each of five non-overlapping fields at $200\times$ magnification, randomly distributed, using a computer-assisted morphometric system (Quantimet 500; Leica, Cambridge, UK). To calculate the capillary-to-fiber ratio, the total number of capillaries was divided by the total number of fibers counted in the same field. Only vessels with a diameter $<10\text{ }\mu\text{m}$ were counted, which would largely comprise capillaries. All analyses were conducted by a single observer blinded to rat identity.

miRNA Expression by Real-Time PCR

Cardiac miRNA-16 expression was determined using real-time PCR. Frozen LV samples (100 mg) were homogenized in Trizol, and RNA was isolated according to the manufacturer's instructions (Invitrogen Life Technologies, Carlsbad, CA, USA). After extraction, the total RNA concentration was quantified using a NanoDrop Spectrophotometer (Nano-Drop Technologies, Wilmington, DE, USA) and checked for integrity by ethidium bromide-agarose gel electrophoresis.

cDNA for miRNA analysis was synthesized from total RNA using gene-specific primers according to the TaqMan MicroRNA Assay protocol (Applied Biosystems, Foster City, CA, USA). The $15\text{ }\mu\text{l}$ reactions obtained

by the TaqMan MicroRNA Reverse Transcription Kit protocol (Applied Biosystems) were incubated in a thermal cycler for 30 min at 16 °C, for 30 min at 42 °C, and for 5 min at 85 °C.

The 20 µl PCR included 1.33 µl RT product, 10 µl TaqMan Universal PCR master mix II (2×), 7.67 µl nuclease-free water, and 1 µl of primers and probe mix of the TaqMan MicroRNA Assay protocol. The reactions were incubated in a 96-well optical plate at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Samples were normalized by evaluating U6 expression. Each LV sample was analyzed in triplicate. Relative quantities of target gene expressions of sedentary versus trained rats were compared after normalization to the values of reference gene (ΔCT). Fold-changes in miRNA expression were calculated using the differences in ΔCT values between the two samples ($\Delta\Delta CT$) and equation $2^{-\Delta\Delta CT}$. Results were expressed as percentage (%) of control.

Protein Expression by Immunoblotting

The protein levels of VEGF, VEGFR2, and CD31 in the LV were analyzed by western blotting. The frozen LV (100 mg) were homogenized in cell lyses buffer containing 100 mmol/l Tris-HCl, 50 mmol/l NaCl, 1% Triton X-100 and protease and phosphatase inhibitor cocktail (1:100; Sigma-Aldrich, St. Louis, MO, USA). Insoluble LV tissues were removed by centrifugation at $3,000 \times g$ at 4 °C for 10 min. Samples were loaded and subjected to SDS-PAGE in polyacrylamide gels (6–15%) depending on the protein molecular weight. After electrophoresis, proteins were electro-transferred to the nitrocellulose membrane using semi-dry system (BioRad Biosciences, Hercules, CA, USA). Equal loading of samples (30 µg) and even transfer efficiency were monitored using 0.5% Ponceau S staining of the blot membrane. The blot membrane was then incubated in a blocking buffer (5% non-fat dry milk, 10 mmol/l Tris-HCl (pH 7.6), 150 mmol/l NaCl, and 0.1% Tween 20) for 2 h at room temperature and then incubated overnight at 4 °C with rabbit anti-VEGF polyclonal antibody (MW: 43 kDa; Abcam, Cambridge, MA, USA), rabbit anti-Flk-1/VEGFR2 polyclonal antibody (MW: 200 kDa; Santa Cruz Biotechnology, Dallas, TX, USA) and mouse anti-CD31 monoclonal antibody (MW: 83 kDa; Abcam). Mouse anti-GAPDH monoclonal antibody (MW: 36 kDa; Abcam) was also used. Binding of the primary antibody was detected with the use of peroxidase-conjugated secondary antibodies and enhanced chemiluminescence reagents (Amersham Biosciences, Waltham, MA, USA) and detection was performed in a digitalising unit (ChemiDoc; BioRad Biosciences). The bands were analyzed using Image J software (ImageJ Corporation based on NIH image). LV GAPDH expression was used to normalize the results. The results were expressed as percent of control.

Statistical Analysis

Data are expressed as mean $\pm$ SEM. Statistical analysis was performed using two-way ANOVA. A value of $p < 0.05$ was considered statistically significant. The Tukey post hoc test (STATISTICA software; StatSoft, Tulsa, OK, USA) was used for individual comparisons between means when a significant change was observed with ANOVA. The Pearson coefficient of correlation was used to analyze the correlation between parametric data.

Results

Cardiovascular Measurements

Table 1 summarizes SBP and HR results of the groups L, LT, OZR, and OZRT after the AET period. SBP was increased in obese groups of animals compared to lean animals groups without characterizing hypertension. Indeed, HR was decreased significantly after 10 weeks of AET in LT and OZRT groups when compared with sedentary groups (L and OZR).

Cardiovascular Structure

LV capillary/fiber ratio was used to analyze cardiac angiogenesis. LV capillary rarefaction was observed in OZR compared with L. Interestingly, AET counteracts cardiac microvascular rarefaction in OZRT compared to L (fig. 2A).

LV/tibia length ratio was used as an index of cardiac hypertrophy. LV hypertrophy was observed in OZR compared with L. AET was effective to decrease LV hypertrophy toward control levels in OZRT compared to the OZR (fig. 2B).

Fig. 2. Cardiovascular morphology. Left ventricle capillary/ fiber ratio (**A**) and left ventricle hypertrophy (**B**) data were obtained after the aerobic exercise training (AET) period in lean (L), lean trained (LT), obese Zucker rats (OZR) and obese Zucker rats trained (OZRT) groups. Data are reported as means $\pm$ SEM. * $p < 0.05$ vs. L, LT and OZRT; † $p < 0.05$ vs. L and LT.

Table 1. Cardiovascular parameters

	Groups			
	L	LT	OZR	OZRT
SBP, mm Hg	112.0 $\pm$ 2.5	114.0 $\pm$ 3.6	122.0 $\pm$ 2.3*	125.0 $\pm$ 3.5
HR, bpm	439 $\pm$ 16	380 $\pm$ 14†	440 $\pm$ 19	386 $\pm$ 13†

SBP = Systolic blood pressure; HR = heart rate.

* $p < 0.05$ vs. lean groups (L and LT).

† $p < 0.05$ vs. sedentary groups (L and OZR).

Effect of Exercise Training on Cardiac miRNA-16 in Obesity

Our data show that cardiac miRNA-16 expression is increased in OZR compared to L. In contrast, AET restored miRNA-16 expression in OZRT toward control levels. There was no difference in cardiac miRNA-16 between L and LT (fig. 3A).

Bioinformatic approaches using the TargetScan and miRBase software show conserved sites for miRNA-16 among different species, such as human, mouse and rat (fig. 3B). The in silico predicted and validated targets included mRNAs encoding several regulators of endothelial cells functions and growth factors such as VEGF. In fact, VEGFA 3'-UTR has highly complementary to the seed region of the miRNA-16 (fig. 3C).

Interestingly, there is a negative correlation between miRNA-16 expression and angiogenesis ($r = -0.73$, $p < 0.05$), suggesting that the increased miRNA-16 expression was accompanied by microvascular rarefaction. In contrast, AET was effective in preventing these parameters (fig. 3D).

Effect of Exercise Training on Angiogenic Markers in Obesity

The protein levels of VEGF, VEGFR2, and CD31 were assessed in the LV of L, LT, OZR and OZRT by western blot. The results showed that obesity decreased cardiac VEGF protein

Fig. 3. Cardiac miRNA-16 expression evaluated by real-time PCR (**A**), conserved sites for miRNA-16 among different species (**B**), VEGFA 3'UTR highly complementary to the seed region of the miRNA-16 (**C**), and negative correlation between miRNA-16 expression and capillary/ fiber ratio (**D**) were showed in lean (L), lean trained (LT), obese Zucker rats (OZR) and obese Zucker rats trained (OZRT) groups. Data are reported as means $\pm$ SEM. *p < 0.05 vs. L, LT and OZRT.

expression. In contrast, AET was effective to restore the LV levels of VEGF in OZRT when compared to L (fig. 4A). Similar results were found for the cardiac CD31 expression (fig. 4C). In addition, AET increased cardiac VEGFR2 expression in trained groups compared with sedentary groups (fig. 4B). Representative blots of VEGF, VEGFR2, CD31, and GAPDH are shown in figure 4D. This reflects one of the means by which there is a vascular remodeling in the hearts of obese and trained obese animals.

Discussion

The risk of cardiovascular disease is significantly reduced by appropriate lifestyle modifications such as the practice of AET. However, the exact mechanisms by which AET counteracts the development and progression of cardiovascular disease are unclear. Our results showed AET is an important non-pharmacological strategy to prevent cardiac capillary rarefaction induced by obesity. AET counteracts miRNA and target gene expression favoring LV angiogenesis. The major finding emerging from this study is that AET restored the levels of cardiac miRNA-16 and VEGF in obese animals which is associated with vascular remodeling. In fact, miRNA-16 was negatively correlated with cardiac angiogenesis, indicating potential mechanisms involved in obesity-associated disorders. Considering the potential clinical benefits of AET in manipulating angiogenesis, the signaling pathways regulating the capillary growth process have formed a major focus for cardiovascular studies.

We previously demonstrated that OZR were significantly heavier throughout the experimental protocol compared to L. In contrast, AET alleviates exacerbated weight gain accom-

Fig. 4. Angiogenic markers. VEGF (A), VEGFR2 (B) and CD31 (C) protein expression were evaluated by western blot. Representative blots of VEGF, VEGFR2, CD31 and GAPDH (D). Groups: lean (L), lean trained (LT), obese Zucker rats (OZR) and obese Zucker rats trained (OZRT). Data are reported as means $\pm$ SEM. * $p < 0.05$ vs. L, LT and OZRT; # $p < 0.05$ vs. L and OZR.

panied by a reduction of triglycerides, cholesterol, and fat mass toward control levels in OZRT compared to OZR [5]. In the present study, AET did not change blood pressure and induced rest bradycardia, recognized as important AET marker.

As expected, with age OZR developed pathological cardiac hypertrophy and cardiac dysfunction concomitant with increased myocardial fat content, as well as apoptosis [4–7, 27]. Similarly our data confirm the presence of cardiac hypertrophy in OZR and the preventive effect of AET on cardiac maladaptive morphological changes in OZRT. Moreover, we previously demonstrated that OZR developed cardiac dysfunction when compared to L, and AET was effective in correcting this parameter [5]. Cardiac dysfunction is linked to pathological cardiac hypertrophy which is associated with reduced vascular network.

Toblli et al. [8] showed that OZR presented substantial reduction in myocardial capillary density along with a significant decrease in both VEGF in the myocardium and eNOS expression in myocardial capillaries compared to L. This was reversed in those OZR that received treatment with perindopril. Similar results were observed in the present study. Capillary rarefaction was observed in OZR compared to L. Interestingly, AET counteracts cardiac microvascular rarefaction in OZRT. AET stimulates angiogenesis which is regulated by a net balance between positive (angiogenic) and negative (angiostatic) regulators of blood vessel growth, and are followed by functional changes which improve blood flow [10, 23, 26, 28]. However,

the underlying molecular mechanisms involved in microvascular remodeling remain to be further determined.

miRNAs emerged as important regulators of gene expression and are implicated in most, if not all, cellular processes and several diseases [13–20, 23, 25, 29]. miRNAs are able to target many genes with the possibility to modulate multiple pathways related to vascular remodeling and either can promote or inhibit structural changes of the vessel [17–20, 30]. Studies, both in vitro and in vivo, indicate a role for Dicer-dependent miRNAs in vascular signaling and functions related to angiogenesis [17–19]. In fact, endothelial miRNAs are potential therapeutic targets to correct capillary rarefaction and defective angiogenesis in cardiovascular diseases [17–19, 23, 30, 31]. Fish et al. [31] showed the loss of vascular integrity and hemorrhage during embryonic development by knockdown of miRNA-126. miRNA-126 targets sprouty-related protein 1 (SPRED1) and phosphoinositol-3 kinase regulatory subunit 2 (PI3KR2), which negatively regulates VEGF signaling via VEGF receptor (VEGFR) 2, mitogen-activated protein kinase, and phosphatidylinositol 3-kinase (PI3K) pathways involved in angiogenesis [10, 18, 19, 23, 29, 31].

VEGF is a major pro-angiogenic factor whose main functions are related to survival, proliferation, enhanced cell migration, and invasion of endothelial cells, thus promoting angiogenesis [10, 23, 32]. Tang et al. [33] showed that deletion of VEGF in specific tissues led to significant reduction in capillary density with tissue cell apoptosis.

Chamorro et al. [22] demonstrated that miRNA-16 and miRNA-424 inhibited in vitro endothelial function and angiogenesis by modulating the expression of VEGF, VEGFR2 and fibroblast growth factor receptor-1 (FGFR1). Lentiviral overexpression of miRNA-16 reduced the ability of endothelial cells to form blood vessels in vivo. Furthermore, in the same study, HUVEC overexpressing miRNA-16 showed reduced levels of both FGFR1 and VEGFR2. Dejean et al. [21] also demonstrated that miRNA-16 directly interacted with VEGF mRNA at the 3'-untranslated region and that the regulation of VEGF by miRNA-16 occurs at the translational level. Interestingly, miRNA-16 is consistently found to be upregulated in cardiomyocytes during cardiac ischemia and heart failure [16]. miRNA-16 also has been shown to induce apoptosis in leukemic cells by targeting the anti-apoptotic protein Bcl-2 and to block cell cycle progression [21].

Specifically, we observed changes in the expression of miRNA-16 and its target gene in LV of the OZR. This is the first investigation demonstrating that increased cardiac expression of miRNA-16 is paralleled by a reduction of VEGF expression in obese animals. AET re-established these parameters in obese rats. In addition, AET also increased VEGFR2 protein expression in trained animals which was accompanied by correction of CD31 levels, an angiogenic marker, in OZRT. This response was also paralleled by the correction of cardiac capillary rarefaction in OZRT induced by AET, indicating that permanency of the newly formed capillaries may depend on the balance between pro-angiogenic and anti-angiogenic factors. These findings advance our understanding of the mechanisms by which miRNAs modulate vascular homeostasis.

Conclusion

AET has been known to play a key role in the prevention and treatment of obesity-associated disorders and improves patient outcome and quality of life. This study demonstrates that miRNA played a role in the cardiac revascularization of AET-induced obese animals. In OZR, miRNA-16 was increased with a concomitant reduction in its target VEGF, which could be associated with cardiac microvascular rarefaction in obesity. AET not only normalized these levels of miRNAs, but also promoted angiogenesis, including enhancement of angio-

genic factors such as CD31 and VEGFR2. Taken together, these data indicate how AET may restore the balance between injury and repair of vascular processes in obese animals. Thus, the understanding the contribution of these miRNAs to the entire spectrum of vascular remodeling processes is important, especially as these miRNAs may have great potential as therapeutic targets for treatment of various cardiovascular diseases.

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Disclosure Statement

None.

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