Exercise Training Prevents the Microvascular Rarefaction in Hypertension Balancing Angiogenic and Apoptotic Factors

Role of MicroRNAs-16, -21, and -126

Tiago Fernandes, Flávio C. Magalhães, Fernanda R. Roque, M. Ian Phillips, Edilamar M. Oliveira

Abstract—Aerobic exercise training (ET) lowers hypertension and improves patient outcomes in cardiovascular disease. The mechanisms of these effects are largely unknown. We hypothesized that ET modulates microRNAs (miRNAs) involved in vascularization. miRNA-16 regulates the expression of vascular endothelial growth factor and antiapoptotic protein Bcl-2. miRNA-21 targets Bcl-2. miRNA-126 functions by repressing regulators of the vascular endothelial growth factor pathway. We investigated whether miRNA-16, -21 and -126 are modulated in hypertension and by ET. Twelve-week-old male spontaneously hypertensive rats (SHRs; n = 14) and Wistar Kyoto (WKY; n = 14) rats were assigned to 4 groups: SHRs, trained SHRs (SHR-T), Wistar Kyoto rats, and trained Wistar Kyoto rats. ET consisted of 10 weeks of swimming. ET reduced blood pressure and heart rate in SHR-Ts. ET repaired the slow-to-fast fiber type transition in soleus muscle and the capillary rarefaction in SHR-Ts. Soleus miRNA-16 and -21 levels increased in SHRs paralleled with a decrease of 48% and 25% in vascular endothelial growth factor and Bcl-2 protein levels, respectively. Hypertension increased Bad and decreased Bcl-x and endothelial NO synthase levels and lowered p-Badser112/Bad ratio. ET in SHR-Ts reduced miRNA-16 and -21 levels and elevated vascular endothelial growth factor and Bcl-2 levels. ET restored soleus endothelial NO synthase levels plus proapoptotic and antiapoptotic mediators in SHR-Ts, indicating that the balance between angiogenic and apoptotic factors may prevent microvascular abnormalities in hypertension. miRNA-126 levels were reduced in SHRs with an increase of 51% in phosphoinositol-3 kinase regulatory subunit 2 expression but normalized in SHR-Ts. Our data show that ET promoted peripheral revascularization in hypertension, which could be associated with regulation of select miRNAs, suggesting a mechanism for its potential therapeutic application in vascular diseases. (Hypertension. 2012;59[part 2]:513-520.) • Online Data Supplement

Key Words: exercise training • angiogenesis • hypertension • microRNA • VEGF • Bcl-2

Aerobic exercise training (ET) is an established, nonpharmacological tool for prevention and treatment of hypertension, involving a decrease in the incidence of cardiovascular events.1-3 ET improves endothelial function, counteracts microvascular rarefaction, and decreases blood pressure in hypertension.1-3 However, the molecular mechanisms underlying these effects by ET in hypertension are poorly understood. Endothelial microRNAs (miRNAs) are potential therapeutic targets for tackling capillary rarefaction and defective angiogenesis in hypertension.4 We hypothesized that ET modulates specific angiogenesis-related miRNAs in hypertension.

miRNA profiles of endothelial cells have been reported and several highly expressed miRNAs identified with angiogenic factors as putative mRNA targets (proangiogenic miRNAs: -17 to -92 cluster,-126, 130a, -210, -296, -378, and let-7f; antiangiogenic miRNAs: -15b, -16, -20, -21, -92a, -221, -222, and -328), according to prediction algorithms.5-7 However, the specific targets and functions in endothelial cells related to angiogenesis have only been characterized for a few of these miRNAs. Among the miRNAs involved in the survival, maintenance, and formation of new capillaries, miRNA-16, -21, and -126 play a well-known role in the control of angiogenesis and vascular integrity.4-11

In animals with knockdown of miRNA-126, endothelial cell migration was impaired during vessel growth.5,10,11 Molecular level analysis revealed that miRNA-126 repressed sproauty-related protein 1 and phosphoinositol-3 kinase regulatory subunit 2 (PI3KR2, also known as p85-β), which negatively regulates vascular endothelial growth factor (VEGF) signaling via VEGF receptor (VEGFR) 2, mitogen-activated protein kinase, and phosphatidylinositol 3-kinase (PI3K) pathways.5,10,11 Indeed, VEGF and antiapoptotic protein Bcl-2 have been identified by bioinformatic approaches and subsequently validated as targets to miRNA-16 in endothelial cells, showing that mimetics of these miRNAs reduced...
VEGF and Bcl-2 expression, whereas specific antagonists enhanced theirs expression.4–8 Overexpression of miRNA-16 reduced proliferation, migration, and cord formation of endothelial cells in vitro, and lentiviral overexpression of miRNA-16 reduced the ability of endothelial cells to form blood vessels in vivo.8 miRNA-21 is also an antiapoptotic miRNA targeting Bcl-2, suggesting its important roles in regulating cell-intrinsic angiogenic activity.4,7,9,11 However, the expression and regulation of miRNAs in vascular physiology and disease are currently unknown.

To test this, we analyzed the effects of ET on the expression of miRNA-16, -21, and -126 in hypertensive and normotensive rats. Furthermore, the effects of ET on its validated targets for angiogenesis-related factors (VEGF, PI3KR2, and endothelial NO synthase [eNOS]), as well as key mediators of the apoptotic pathway (Bcl-2), were studied.

**Methods**

**Animal Care**

Twelve-week-old normotensive male Wistar Kyoto (WKY) rats and spontaneously hypertensive rats (SHRs) were randomly assigned to 4 experimental groups: WKY (n=7), swimming-trained WKY (WKY-T; n=7), SHR (n=7), and swimming-trained SHR (SHR-T; n=7). The animals were housed 3 to 5 per cage at a controlled room temperature (22°C); with a 12-hour dark-light cycle; were fed standard rat chow; and had ad libitum access to water. All of the protocols and surgical procedures used 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 Sao Paulo (No. 2007/35). ET protocol consisted of swimming sessions of 60 minutes of duration, 5 days a week, for 10 weeks, with 4% casual body weight workload, as described previously.12,13

**Cardiovascular Measurements**

Resting systolic blood pressure (SBP) and heart rate (HR) were measured in conscious rats, with the use of a computerized tail-cuff system (BP2000, Visitech system). Rats were acclimatized to the apparatus during daily sessions over 4 days, 1 week before starting the experimental period. BP and HR were determined before and after the ET protocol.

**Physiological Markers of Aerobic ET**

Aerobic work capacity was determined by exercise tolerance and peak oxygen uptake. In addition, skeletal muscle citrate synthase activity was determined by spectrophotometry. For detailed Methods, please see the online Data Supplement, available at http://hyper.ahajournals.org.

**Muscle Fiber Typing and Cross-Sectional Area**

The myosin ATPase reaction was used to identify the muscle fiber type and the fiber cross-sectional area, as described previously.14 For detailed Methods, please see the online Data Supplement.

**Capillary:Fiber Ratio**

Capillary:fiber ratio of the soleus muscle was evaluated after myofibrillar ATPase histochemical reaction at pH 10.3, as described previously.15 For detailed Methods, please see the online Data Supplement.

**mRNA and miRNA Analysis**

The PI3KR2 mRNA and miRNA-16, -21, and -126 levels in the skeletal muscle were analyzed by real-time PCR. For detailed Methods, please see the online Data Supplement.

| Table. Cardiovascular Measurements |
|-----------------|--------|--------|
| Groups          | SBP, mm Hg | HR, bpm |
| Before training |        |        |
| WKY             | 127±3.0  | 390±12.2 |
| WKY-T           | 124±1.6  | 393±8.2  |
| SHR             | 184±3.9* | 409±7.7  |
| SHR-T           | 184±2.9* | 415±6.5  |
| After training  |        |        |
| WKY             | 132±3.9  | 383±11.8 |
| WKY-T           | 131±3.7  | 322±14.2†|
| SHR             | 207±5.4*§ | 407±11.2 |
| SHR-T           | 162±4.4*‡§ | 338±7.8‡ |

Values are mean±SEM. Systolic blood pressure (SBP) and heart rate (HR) data were obtained before and after the exercise training (ET) period in Wistar Kyoto rat (WKY), trained WKY (WKY-T), spontaneously hypertensive rat (SHR), and trained SHR (SHR-T) groups.

*P<0.001 vs WKY and WKY-T.
†P<0.01 vs WKY after training.
‡P<0.01 vs WKY and SHR after training and all of the groups before training.
§P<0.05 vs SHR and SHR-T before training.

**Protein Expression Analysis**

The protein levels of VEGF, VEGFR1, VEGFR2, eNOS, Bcl-2, Bcl-x, Bad, and p-BadSer112 in the skeletal muscle were analyzed by Western blotting. For detailed Methods, please see the online Data Supplement.

**Statistical Analysis**

Results are represented as mean±SEM. Statistical analysis was performed using 2-way ANOVA (strain × condition). P values <0.05 were accepted as statistically significant. Tukey post hoc test (Statistica software; StatSoft, Tulsa, OK) was used for individual comparisons between means when a significant change was observed with ANOVA. Relations between variables were analyzed by linear regression analysis. Repeated-measure ANOVA was used to compare before and after ET period measurements.

**Results**

**Cardiovascular Measurements**

The Table summarizes SBP and HR results of the groups WKY, WKY-T, SHR, and SHR-T before and after the ET period. At the beginning of the ET protocol, SHRs had higher SBP when compared with WKY rats (184±3.9 versus 127±3.0 mm Hg, P<0.001, respectively), indicating that hypertension was established. There was no difference in SBP between WKY and WKY-T groups after ET, but SBP was significantly decreased in SHR-Ts versus SHRs (207±5.4 versus 162±4.4 mm Hg, P<0.01, respectively). Similar HR values among groups were found before the ET period. However, HR decreased significantly after 10 weeks of ET in WKY-T (322±14.2 bpm) and SHR-T (338±7.8 bpm) groups compared with sedentary groups (WKY: 393±11.8 bpm; SHR: 407±11.2 bpm; P<0.01).

**Physiological Markers of Aerobic ET**

To verify the efficacy of ET, performance to maximal exercise test and metabolic changes were also evaluated. Exercise tolerance and peak oxygen uptake before the ET period were similar among all of the studied groups. In
contrast, after ET, WKY-T and SHR-T groups showed an increased exercise tolerance represented by total distance run (Figure S1A, available in the online Data Supplement at http://hyper.ahajournals.org) and peak oxygen uptake (Figure S1B, available in the online Data Supplement at http://hyper.ahajournals.org) when compared with untrained groups (WKY and SHR). Parallel to this, biochemical analysis represented by citrate synthase activity in the soleus muscle was significantly higher in exercise-trained rats from WKY-T (40%) and SHR-T (67%) groups when compared with control levels (WKY; \(P<0.05\)). A small difference in the magnitude of enzymatic decrease was observed in SHR (12%) when compared with the WKY group (\(P>0.05\); Figure S1C, available in the online Data Supplement at http://hyper.ahajournals.org).

**Skeletal Muscle Capillary:Fiber Ratio, Cross-Sectional Area, and Fiber-Type Distribution**

Morphological measurements after histological processing revealed important changes in skeletal muscle microcirculation induced by ET in normotensive and hypertensive rats. As expected, capillary rarefaction was observed in SHRs when compared with WKY rats. On the other hand, ET was effective in increasing the capillary:fiber ratio in both WKY-T and SHR-T groups (Figure 1A).

The cross-sectional area of fibers I, IIA and IIX in the soleus muscle did not differ among all of the studied groups (Figure 1B). Despite this, SHRs presented a decrease in percentage of type I fibers and an increase in type IIA and IIX fibers in the soleus muscle. In contrast, ET increased the percentage of type I fibers and decreased the type IIA and IIX fibers in the SHR-T group. In addition, the SHR-T group did not differ from the control group (Figure 1C). The staining patterns of the fibers and capillaries in the soleus muscle are shown (Figure 1D). This response was paralleled by normalization in capillary rarefaction and increased skeletal muscle oxidative activity, which suggests that a shift toward type II fiber in SHRs could have been associated with microvascular damage.

**ET Regulates miRNA-16, -21, and -126 in Hypertension: Relationship With Angiogenesis**

The expression of miRNA-16, -21, and -126 in the soleus muscle was analyzed by real-time PCR for the 4 groups. Figure 3A shows increased miRNA-16 levels in the SHR (30%) compared with WKY group. The effect of ET reduced the miRNA-16 levels by 27% in WKY-T compared with WKY and 32% in SHR-T compared with the WKY group (Figure 2A). Similarly, Figure 2B shows increased miRNA-21 levels in SHRs (36%) compared with WKY rats, and ET lowered the miRNA-21 levels in SHR-Ts to control levels. In addition, miRNA-126 levels were reduced by 29% in SHRs, and ET elevated the miRNA-126 levels in SHR-Ts to control levels (Figure 2C). Notably, there was a significant positive relationship between angiogenesis and the levels of miRNA-16, -21, and -126 (\(r=0.70\), \(r=0.78\), and \(r=0.65\), respectively; \(P<0.05\)), revealing that newly formed capillaries may depend on improvement in miRNA levels induced by ET in hypertension.

**ET Increases Angiogenic Factors in Hypertension**

Representative blots of VEGF, VEGFR1, VEGFR2, and eNOS protein levels are shown in all of the groups studied (Figure 3A). When compared with sedentary WKY rats, ET increased peripheral VEGF and VEGFR2 protein levels in WKY-T rats (Figure 3B and 3D, respectively). In contrast, VEGF protein levels were significantly lower in SHRs when compared with control levels, but they were increased in SHR-Ts by ET (Figure 3B), accompanied by increased VEGFR2 protein levels (Figure 3D). However, there were no significant differences in the VEGFR1 protein levels among all of the studied groups (Figure 3C). Interestingly, as shown in Figure 3E, similar VEGF results were obtained for eNOS protein levels, indicating that VEGF and eNOS could act synergistically for appropriate vascular growth. The target of miRNA-126, PI3KR2 gene expression, was increased in SHRs (51%) compared with WKY rats, but ET reduced PI3KR2 levels of SHR-Ts to control level and reduced it by 39% in WKY-T rats when compared with WKY rats (Figure 3F).

**ET Inhibits Apoptotic Signaling in Hypertension**

Representative blots of Bcl-2, Bcl-x, Bad, and p-Bad\(_{ser112}\) protein levels are shown in all of the groups studied (Figure 4A). The antiapoptotic Bcl-2 and Bcl-x protein levels were similar between the WKY and WKY-T groups (Figure 4B and 4C, respectively). In addition, no changes in proapoptotic Bad protein levels and their phosphorylation at serine 112 were observed in WKY-T rats when compared with control levels (Figure 4D and 4E, respectively). In contrast, SHRs demonstrated a pronounced increase in proapoptotic Bad protein levels and diminished p-Bad\(_{ser112}\):Bad ratio when compared with control levels (Figure 4D and 4E, respectively), accompanied by a decrease in antiapoptotic Bcl-2 and Bcl-x protein levels when compared with WKY rats (Figure 4B and 4C, respectively). Interestingly, as shown in Figure 4, ET restored the proapoptotic and antiapoptotic protein levels in SHR-Ts to control levels.

**Discussion**

In the present investigation, we used SHRs and their WKY controls to study the influence of essential hypertension on miRNAs and changes in skeletal muscle microcirculation. We also studied the relative contribution of ET to countering expression of miRNAs and capillary rarefaction favoring the regression of hypertension. Three major findings emerge from this study indicating that ET restored the levels of peripheral miRNA-16, -21, and -126 associated with revascularization in hypertension, showing potential mechanisms involved in ET-induced angiogenic miRNAs on vascular disease; corrected capillary rarefaction and changes in fiber type distribution in hypertension; and promoted the balance between angiogenic and apoptotic factors to prevent microvascular abnormalities in hypertension.

A major new finding of this study is the effect of ET on regulating specific miRNAs involved in vascularization. In fact, endothelial miRNAs are potential therapeutic targets to correct capillary rarefaction and defective angiogenesis in hypertension. \(^4\) We showed that ET is a powerful stimulus
modulating miRNA-16, -21, and -126. These miRNAs regulate their target mRNAs, which are critical to angiogenesis and apoptosis in the microcirculation.4–7

miRNA-126 has an important validated target, PI3KR2, which is a negative regulator of the VEGF signaling pathway PI3K/Akt/eNOS.5,10,11,16 Studies have shown that siRNA-mediated knockdown of PI3KR2 in cells with reduced miRNA-126 levels was able to rescue the defect in VEGF-dependent Akt phosphorylation, indicating the significance of PI3KR2 as a target. In vitro, miRNA-126 regulates many aspects of endothelial cell biology, including cell migration, organization of the cytoskeleton, capillary network stability, and cell survival. In vivo, the knockdown of miRNA-126 in zebrafish resulted in the loss of vascular integrity and hemorrhage during embryonic development.5,10,11,16 For the first time we observed that hypertension was associated with lower miRNA-126 expression paralleled with an increase in PI3KR2 expression. This would cause an increase in its action of impairing the VEGF angiogenic signaling pathways in SHRs. In agreement, the data of the present study demon-
miRNA-16 occurs at the translational level. The authors also showed that, in vivo, increased miRNA-16 resulted in reduced angiogenesis and tumor growth. A recent study showed that miRNA-16 overexpression reduced proliferation, migration, and cord formation of endothelial cells in vitro, and lentiviral overexpression of miRNA-16 reduced the ability of endothelial cells to form blood vessels in vivo.5 In addition, miRNA-16 has been shown to induce apoptosis in leukemic cells by targeting the antipoptotic protein Bcl-2.17 Similarly, studies have demonstrated that shear stress forces regulate the expression of miRNAs in endothelial cells and that miRNA-21 influences endothelial biology by modulating apoptosis (Bcl-2) and eNOS activity.9,18 In a recent study, Nicole et al19 demonstrated that miRNA-21 plays a role in the integration of hemodynamics and VEGF signaling during angiogenesis. For the first time we also observed that hypertension was associated with higher miRNA-16 and -21 peripheral expression paralleled with a decrease in VEGF and Bcl-2 expression. ET also re-established these parameters in hypertension. This response was paralleled by correction of capillary rarefaction in SHR-Ts induced by ET, indicating that permanency of the newly formed capillaries may depend on the balance between proangiogenic and/or prosurvival factors and antiangiogenic and/or a prodeath factor. These studies advance our understanding of the mechanisms by which miRNAs modulate vascular homeostasis.

It is well established that ET reduces blood pressure in hypertensive individuals, without significant pressure changes in normotensive subjects. In agreement, the present data showed that 10 weeks of ET was effective in reducing 21% of the blood pressure in SHRs and confirmed that ET normalizes capillary rarefaction in the skeletal muscle of SHRs. ET also increased the capillary:fiber ratio in skeletal muscle of WKY rats, as has been demonstrated in other studies.2,3

The increase in capillary supply seems to be a crucial adaptive response to increased blood flow and peak oxygen uptake of exercising skeletal muscle.3,20 On the other hand, muscles not well provided with O2 and nutrients in the presence of cardiovascular risk factors and cardiovascular disease have been reported to exhibit a skeletal muscle fiber type shift toward type II fibers.21 A significant alteration in fiber type distribution was observed in the soleus muscle of SHRs. There was a decrease in slow-twitch fibers and a simultaneous augmented percentage of type II fibers, paralleled by a reduced capillary:fiber ratio and a small decrease in citrate synthase activity. The present study provides the first evidence that ET prevented changes in the fiber type composition of the soleus muscle in SHRs.

Several mechanisms have been proposed to explain how ET reduces blood pressure in hypertension.22 Dysfunctional microcirculation in hypertension would result from excessive vasoconstriction, causing occlusion of resistance arterioles and nonperfusion of capillaries. This would induce capillary rarefaction and decrease parallel conductance of the microcirculation in the skeletal muscle.23-25 In addition, endothelial shear stress leads to sustained release of eNOS and the absence of blood flow and, therefore, of NO, which would
lead to peripheral apoptosis and loss of nonperfused vessels in hypertension.23–25

We found that VEGF and eNOS levels in SHR were reduced and ET restored these to WKY levels. eNOS stimulates VEGF,26 which is one of the major regulators of angiogenesis and cell survival.27 Furthermore, deletion or inhibition of VEGF in specific tissues in adult mice has shown significant reduction in capillary density with tissue cell apoptosis.28 VEGF binds to VEGFR2, well-known as the most important receptor mediating angiogenic signaling.29 VEGF/VEGFR2-mediated endothelial survival and angiogenesis signals are predominantly mediated through PI3K and its downstream target of the serine-threonine kinase Akt.30 These mechanisms increase eNOS-mediated neovascularization and cell migration or by increasing antiapoptotic protein Bcl-2 expression.30,31 In addition, VEGF/PI3K/Akt has been shown to promote cell survival via its ability to phosphorylate Bad. The proapoptotic Bad is the primary target of Akt, and Akt phosphorylates Bad and renders it inactive for apoptotic signaling.32

Rarefaction occurs parallel to activation of an apoptotic pathway that has harmful implications for skeletal muscle angiogenesis and cell survival. Soleus muscle apoptotic signaling was augmented in SHR compared with WKY rats.33 Bcl-2/Bcl-x and Bad are important apoptosis regulatory proteins acting to inhibit and promote apoptogenic protein release, respectively.33 The skeletal muscle of hypertensive rats was found to have increased Bad and decreased Bcl-2 and Bcl-x levels, as well as a lower p-Badser112:Bad ratio. Furthermore, the data of the present study showed that lower NO synthesis accompanied by increased inducible NO synthase expression levels could contribute to a moderate shift in redox homeostasis toward a pro-oxidant state. This suggests the existence of an apoptotic pathway, because SHR-mediated oxidative stress promoted endothelial cell apoptosis associated with loss of microvessels.34

Figure 3. Exercise training effects on angiogenic factors in hypertension. Representative blots of vascular endothelial growth factor (VEGF), VEGF receptor (R)1, VEGFR2, endothelial NO synthase (eNOS), and GAPDH from Wistar Kyoto (WKY), swimming-trained WKY (WKY-T), spontaneously hypertensive rat (SHR), and swimming-trained SHR (SHR-T) groups (A). Soleus muscle VEGF, VEGFR1, VEGFR2, and eNOS protein levels were analyzed by Western blotting, respectively (B through E). Targeted bands were normalized to skeletal muscle GAPDH. Differential expression of phosphoinositol-3 kinase regulatory subunit 2 (PI3KR2) was analyzed by real-time PCR (F). Data are reported as mean±SEM. *P<0.05 vs WKY, SHR, and SHR-T; †P<0.05 vs WKY, WKY-T, and SHR-T; ‡P<0.05 vs WKY-T and SHR; §P<0.05 vs WKY and SHR.
Remarkably, ET restored normal expression of skeletal muscle microcirculation miRNA-16, -21, and -126. This was paralleled by normalization of VEGF, eNOS, and PI3KR2 levels, as well as of the proapoptotic (Bad) and antiapoptotic (Bcl-2, Bcl-x, and p-Badser112:Bad ratio) mediators, indicating that balance between angiogenic and apoptotic factors may prevent microvascular abnormalities in SHRs.

Our study reveals some of the molecular mechanisms of ET in physiological revascularization that are important in ET therapy for hypertension. Taken together, these indicate how multiple pathways culminated in maintenance of vascular health. Thus, ET may restore the balance between injury and repair in vascular processes leading to regression of hypertension.

Perspectives
ET reduces hypertension and improves patient outcome and quality of life by largely unknown mechanisms. This study demonstrates the role of miRNAs in ET-induced amelioration of hypertension. We found that miRNA-16 and-21 were increased with a parallel reduction in their targets VEGF and Bcl-2, and miRNA-126 was decreased with a parallel increase in the target PI3KR2 in SHRs, which could be associated with capillary rarefaction in hypertension. ET not only normalized these levels of miRNAs, reduced blood pressure, but also improved vascularization, including enhancement of angiogenic factors and decrease in apoptotic pathways. These findings increase our understanding of the mechanisms of ET-induced angiogenesis and vascular integrity in hypertension and suggest that these miRNAs are potential therapeutic targets for pathological conditions involving capillary rarefaction. The elucidation of these processes regulated by miRNAs and the identification of novel targets of miRNAs in the pathogenesis of hypertension is a highly valuable strategy that may lead to the development of novel treatment approaches for hypertension.

Sources of Funding
The present investigation was supported by grants from Fundação de Amparo à Pesquisa do Estado de São Paulo (No. 2010/50048-1 and
No. 2009/18370-3). M.I.P. was supported by National Institutes of Health grant 1 R01 HL 077602. T.F. was the recipient of a master’s fellowship from Fundação de Amparo à Pesquisa do Estado de São Paulo (No. 07/56771-4) and recipient of a doctor’s fellowship from Conselho Nacional de Desenvolvimento Científico e Tecnológico (No. 159827/2011-6). E.M.O. was awarded scholarships from Conselho Nacional de Desenvolvimento Científico e Tecnológico (No. 307591/2009-3) in Brazil.

Disclosures

None.

References


Exercise Training Prevents the Microvascular Rarefaction in Hypertension Balancing Angiogenic and Apoptotic Factors: Role of MicroRNAs-16, -21, and -126

Tiago Fernandes, Flávio C. Magalhães, Fernanda R. Roque, M. Ian Phillips and Edilamar M. Oliveira

_Hypertension_. 2012;59:513-520; originally published online January 3, 2012; doi: 10.1161/HYPERTENSIONAHA.111.185801

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2012 American Heart Association, Inc. All rights reserved.
Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://hyper.ahajournals.org/content/59/2/513

Data Supplement (unedited) at:
http://hyper.ahajournals.org/content/suppl/2012/01/03/HYPERTENSIONAHA.111.185801.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Hypertension can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Hypertension is online at:
http://hyper.ahajournals.org//subscriptions/
SUPPLEMENTARY DATA

EXERCISE TRAINING PREVENTS THE MICROVASCULAR RAREFACTION IN HYPERTENSION BALANCING ANGIOGENIC AND APOPTOTIC FACTORS: ROLE OF MICRORNAS-16, 21 AND 126.

Short title: Aerobic Training, Hypertension and Angiogenic miRNA

Tiago Fernandes, MD¹, Flávio C. Magalhães, PhD¹, Fernanda R.R. Redondo, PhD¹, M. Ian Phillips PhD², Edilamar M. Oliveira, PhD¹²

¹Laboratory of Biochemistry and Molecular Biology of the Exercise, School of Physical Education and Sport, University of Sao Paulo, Sao Paulo, Brazil.
²Laboratory of Stem Cells, Keck Graduate Institute, Claremont, CA, USA.

Word count of manuscript: 6000; Word Count of Abstract: 250;
Total Number of Figures: 4. Total Number of Tables: 1.

Address for correspondence:
Edilamar Menezes de Oliveira
School of Physical Education and Sport, University of Sao Paulo.
Laboratory of Biochemistry and Molecular Biology of the Exercise.
Av. Professor Mello Moraes, 65- Cidade Universitária- São Paulo- SP 05508-900-Brazil
Phone: (5511) 3091-2118, Fax: (5511) 3813-5921
E-mail: edilamar@usp.br
DETAILED METHODS

**Graded Treadmill Exercise Test**

Exercise capacity, estimated by total distance run, was evaluated with a graded treadmill exercise protocol for rats. After being adapted to treadmill exercises over 1 wk (10 min of exercise session), rats were placed in the exercise streak and allowed to acclimatize for at least 30 min. Exercise began at 6 m/min with no grade and increased by 3 m/min every 3 min thereafter until exhaustion. WKY and SHR performed the graded treadmill exercise test before and after the experimental period.

**Oxygen Uptake Measurements**

Oxygen uptake (VO₂) was measured by means of expired gas analysis during the graded treadmill exercise described above. Gas analysis was performed using an oxygen and carbon dioxide analyzer (Sable Systems SS3, FC-10a O2/CO2 analyzer, NV, USA). VO₂ was calculated using the measured flow through the metabolic chamber, the expired fraction of effluent oxygen and the fraction of oxygen in room air.

**Skeletal Muscle Oxidative Enzyme Activity**

Muscle samples were taken from the soleus at the time of killing and frozen in liquid nitrogen for future processing. The rats were killed by anesthesia with intraperitoneal sodium pentobarbital (50 mg/Kg). Citrate synthase activity, used as index of physical training, was determined spectrophotometrically in mixed soleus muscle. The enzyme activity was measured in whole muscle homogenates of the WKY, WKY-T, SHR and SHR-T groups, and the amount of the complex resulting from acetyl-CoA and oxaloacetate was determined at 412 nm and 25°C at an interval of 10 min. The solubilized protein extracts from the homogenates were quantified in duplicate by the Bradford method using bovine albumin standards. The citrate synthase activity was then normalized for the total protein content and reported in nanomoles per milligram of protein per minute.

**Muscle Fiber Typing and Cross-Sectional Area.**

Twenty-four hours after the last exercise training session or the graded treadmill exercise test, sedentary and trained rats were killed and the soleus muscle was harvested, immediately frozen in melting isopentane, and stored in liquid nitrogen. Frozen muscle was cut into 10-µm cross sections from the proximal to the distal region using a cryostat (Micron HM505E; Zeiss, Walldorf, Germany). Muscle sections were then incubated for myofibrillar ATPase activity after alkali (myosin ATPase, pH 10.3) or acid preincubation (myosin ATPase, pH 4.6). The myosin ATPase reaction was used to identify the muscle fiber type. Type I fibers reacted deeply after acid preincubation at pH 4.6 and lightly after formaldehyde pretreatment and alkali preincubation at pH 10.3. The inverse occurred with type II muscle fibers. Fiber typing and the fiber cross-sectional area were evaluated in muscle at ×200 magnification and further analyzed on a digitizing unit connected to a computer (Image-Pro Plus; Media Cybernetics, Silver Spring, MD). The total number of each fiber type was counted to calculate the numerical fiber type composition (I, IIA and IIX). The cross-sectional area of each fiber type was measured for further calculation of the average fiber cross-sectional area. All analyses were conducted by a single observer (T. Fernandes) blinded to rat identity.
**Capillary-to-Fiber Ratio**

Capillary-to-fiber ratio was quantified by a 10 × 10 grid optically superimposed on each of 5 non-overlapping fields at ×200 magnification, distributed in a random manner using a computer-assisted morphometric system (Quantimet 500; Leica, Cambridge, UK). For calculating 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 µm were counted, which would largely comprise capillaries but might also include terminal arterioles or venules. All analyses were conducted by a single observer (T. Fernandes) blinded to rat identity.

**mRNA and miRNA analysis by real-time PCR**

Frozen soleus samples (100 mg) were homogenized in Trizol and RNA was isolated, according to the manufacturer’s instructions (Invitrogen Life Technologies, CA, USA). After extraction, the total RNA concentration was quantified using NanoDrop Spectrophotometer (Nano-Drop Technologies, USA) and checked for integrity by EtBr-agarose gel electrophoresis.

RNA were primed with 0.5 µg/µl oligo dT (12–18 bp) (Invitrogen Life Technologies, CA, USA) to generate first strand DNA. Reverse transcription (RT) was performed using SuperScriptTM 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 a Thermal Cycler for 30 min at 16°C, 30 min at 42°C, 5 min at 85°C.

Real-time quantification of the PI3KR2 mRNA 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 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). 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). The DNA sequence was obtained from GenBank and primers were made in separate exons to distinguish PCR products derived from cDNA by size, from those derived from genomic DNA contaminants. The mRNA expression was assessed by oligonucleotide primers as follows: PI3KR2 (sense: 5’- TGT CTG TCT TCT AAT CCC TTC CCC TGG TG -3’, antisense: 5’- GTA GTA CCA AAG CAG GCT CCC CCA GG -3’), and cyclophilin (sense: 5’-AAT GCT GGA CCA AAC ACA AA -3’, antisense: 5’-CCT TCT TTC ACC TTC CCA AA AA -3’).

In order to accurately detect miRNAs the real-time PCR quantification method 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 (2×), 7.67 µl nuclease-free water and 1 µl of primers and probe mix of the TaqMan MicroRNA Assay protocol for miRNA-16 (INV 0391), -21 (INV 0397) and -126 (INV 2228). 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°C for 1 min. Samples were normalized by evaluating U6 expression.

Each soleus 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
reference gene (ΔCT). Fold changes in mRNA and miRNA expression were calculated using the differences in ΔCT values between the two samples (ΔΔCT) and equation 2-ΔΔCT. Results are expressed as % of control.

**Protein expression by Western Blot**

The frozen soleus muscle (100 mg) were homogenized in cell lyses buffer containing 100 mM Tris-HCl, 50 mM NaCl, 1% Triton X-100 and protease and phosphatase inhibitor cocktail (1:100; Sigma-Aldrich, MO, USA). Insoluble soleus tissues were removed by centrifugation at 3,000 × g, 4° C, 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 (BioRad Biosciences, NJ, USA). Equal loading of samples (30 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% non-fat 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 rabbit anti-VEGF polyclonal antibody, rabbit anti-VEGFR-1 polyclonal antibody, rabbit anti-VEGFR-2 polyclonal antibody and rabbit anti-eNOS polyclonal antibody (Santa Cruz Biotechnology, CA, USA). Rabbit anti-Bcl-x polyclonal antibody, rabbit anti-Bcl-2 polyclonal antibody, rabbit anti-Bad polyclonal antibody and rabbit anti-p-Bad ser112 polyclonal antibody (Cell Signaling Tech., MA, USA) and mouse anti-GAPDH monoclonal antibody (Abcam, Cambridge, UK). 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). Skeletal muscle GAPDH expression levels were used to normalize the results and it expressed as percent of control.
Supplementary Figure Legend

Figure S1. Physiological markers of aerobic exercise training (ET). Exercise tolerance evaluated by distance run (A), oxygen uptake (B) and citrate synthase activity in soleus muscle (C). Measurements were performed in Wistar Kyoto Rats (WKY), trained WKY (WKY-T), Spontaneously Hypertensive Rats (SHR) and trained SHR (SHR-T) before and after the ET period. Data are reported as means ± SEM. *P < 0.05 vs. WKY and SHR, †P < 0.05 vs. before ET.
FIGURE S1