Effects of exercise training and nebivolol treatment on atherosclerotic plaque development, iNOS expression and antioxidant capacity in apoE<sup>−/−</sup> mice

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**Summary**

**Objectives.**—To assess the effects of exercise training and nebivolol either alone or in combination on the atherosclerotic plaque development, iNOS expression and antioxidant capacity in apolipoprotein E deficient (apoE<sup>−/−</sup>) mice.

**Methods.**—Seventeen-week-old male apoE<sup>−/−</sup> mice were divided into four groups of seven animals each: Control (G1); Nebivolol (G2); Exercise (G3); and Nebivolol plus Exercise (G4). G3 and G4 animals were submitted to a swimming training program (60 min/day, 5 days/week) for six weeks. G2 and G4 animals received a daily dose of nebivolol (2.0 μmol/kg) by gavage for six weeks. Atherosclerotic lesion area and iNOS expression in the aorta were measured. Catalase (CAT) and superoxide dismutase (SOD) activities were determined in the liver and kidney.

**Keywords**

Atherosclerosis; Physical activity; Oxidative stress; Nebivolol; ApoE<sup>−/−</sup> mice
1. Introduction

Atherosclerosis is a chronic inflammatory disease that results in formation of plaques in the wall of large- and medium-sized arteries [1]. Factors such as dyslipidemia, physical inactivity, aging, diabetes, hypertension, are involved in such disease [2]. Under pathological conditions, overproduction of nitric oxide occurs after induction and expression of inducible nitric oxide synthase (iNOS) in response to inflammatory agents [3]. The use of therapeutic strategies that inhibit the activity of this enzyme may be of clinical relevance.

Regarding non-pharmacological strategies, the protective role of regular exercise in atherosclerosis has been demonstrated previously [4–6]. Exercise training can reduce and stabilize atherosclerotic plaques [7], although, there is little information on the expression of iNOS in atherogenesis. In respect to pharmacological treatment, the use of nebulol, a β-blocker with a high selectivity for β1-adrenergic receptors, has been shown to increase nitric oxide bioavailability and attenuate the inflammatory activation of endothelial cells [8]. In mouse models nebulol regulates iNOS expression, alleviates oxidative stress in cerebral ischemia [9] and attenuates atherogenesis [10].

In light of the above mentioned, up to date there is no available data on the combined effects of exercise training and nebulol treatment as possible modulators of the iNOS expression and oxidative capacity in the atherogenic process. Thus, the aim of this study was to assess the effects of exercise training and nebulol treatment either alone or in combination on atherosclerotic plaque development, iNOS expression and oxidative capacity in adult apoE−/− mice.

2. Material and methods

2.1. Animals and study design

Male homozygous apo E-deficient (apoE−/−) mice in the C57BL/6 genetic background were purchased from Jackson Laboratory (Bar Harbor, ME, USA) and kept in the Animal House at the Federal University of Viçosa–MG, Brazil. The animals (age: 17 weeks) were randomly divided into four groups of seven animals each:

- control (G1): no exercise and oral administration of saline at a dose of 2.0 μmol/kg/animal/day by gavage;
- nebulol (G2): no exercise and oral administration of nebulol at a dose of 2.0 μmol/kg/animal/day by gavage;
- exercise + nebulol (G3): aerobic exercise and oral administration of nebulol at a dose of 2.0 μmol/kg/animal/day by gavage;
- exercise (G4): aerobic exercise alone.

The exercise protocol has been previously described [7] and consisted of a six-week protocol of aerobic exercise on a four-wheel ergometer with an incline of 10° and at an average speed of 50 m/min. The training frequency and duration were five times a week for 20 min per session.

The nebulol administration was started 14 days before the initiation of the exercise protocol in the morning, immediately after the animals were housed in the animal house. The nebulol was administered as a single oral dose of 0.5 mg/kg/day in a volume of 100 μL/kg/day in order to obtain a final concentration of 5 μg/μL. 

On the 39th day, the animals were sacrificed by cervical dislocation under light anesthesia with 10% (w/v) ketamine hydrochloride (Ketalar®; Pfizer, São Paulo, Brazil) and 2% (w/v) xylazine (Xylazine®; Searle, São Paulo, Brazil).

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- exercise (G3): exercised groups and oral administration of saline at a dose of 2.0 μmol/kg/animal/day by gavage; and
- nebivolol plus exercise (G4): exercised groups and oral administration of nebivolol at a dose of 2.0 μmol/kg by gavage;

Animals from G2 and G4 received a daily dose of nebivolol (Nebilet® 5 mg, BILAB) according Kus et al. [10] throughout the 6 weeks of experiment. Nebivolol solution was prepared fresh every 2–3 days [11].

The animals were housed in collective cages (seven animals per cage) and maintained under standard conditions (12 h light/12 h dark cycle, 22 ± 1°C room temperature). Standard commercial chow (Nuvilab® — composition: 19.0% protein, 56.0% carbohydrate, 3.5% fat, 4.5% cellulose, 5.0% vitamins and minerals, giving a total of 13.87 kJ/g) and filtered water were provided ad libitum, for six weeks.

All experiments were approved by the Institutional Ethics Committee (protocol n° 101/2011) and were conducted in accordance with the national guidelines for the care and use of animals.

2.2. Exercise training protocol

The exercised groups were submitted to a swimming training program, five days/week (Monday to Friday) during six weeks (adapted from Pellegrin et al. [12]). Briefly, in the first week animals swam for 10 min on day 1 (Monday) and with a daily increase of 5 min the training session ended up with 30 minutes on day five (Friday). Then, on the second week animals started swimming 40 min/day with a daily increase of 10 min in the following two days resulting in a session of 60 min/day which was kept until the end of the sixth week of training. Swimming was always performed in the morning, between 7 and 9 am, in a tank filled with water (70 cm depth) kept at 30 ± 2°C. After swimming sessions animals were carefully dried.

2.3. Sample preparation

Forty-eight hours after the last exercise session and 12 hours fasting, animals were weighed and euthanized. A blood sample was collected, immediately centrifuged at 2938 g at 4°C for 10 min and the serum was stored at −80°C for further determination of the lipid profile. The heart was dissected and weighed. Then, a fragment of the gastrocnemius muscle (red portion) was dissected and stored at −80°C for further measurement of citrate synthase (CS) activity. Likewise, the liver and kidney were harvested and stored at −80°C for further measurements of catalase (CAT) and superoxide dismutase (SOD) activities. The entire aorta until the iliac bifurcation was dissected and carefully removed. Then, it was washed thoroughly by injecting phosphate-buffered saline (PBS) and fixed in 10% buffered formalin (pH 7.0). After 24 hours it was transferred to a solution of 70% ethanol.

2.4. Determination of the serum lipid profile

The serum levels of total cholesterol, high density lipoprotein cholesterol (HDL-C) and triglycerides (TG) were determined by the enzymatic colorimetric method (Cobas® automatic analyzer, USA) using commercial kits (Bioclin®, Brazil).

2.5. Measurement of citrate synthase activity

Citrate synthase activity was assayed spectrophotometrically on muscle homogenates as previously described [13].

2.6. Atherosclerotic plaque size measurement

The aorta was embedded in paraffin and sectioned with a rotary microtome. The aortic root was sectioned transversely into 7-μm thick slices equally separated in 28 μm intervals and stained with Masson’s trichrome (modified by Mallory) for quantitative morphometric analysis [14]. The slides were mounted with Entellan® (Merck, Germany) and observed under light microscopy. From the aortic root region, characterized by the presence of the aortic valve, five images of each animal were captured. The size of the plaque was determined as percent lesion area per total lumen area [5,15]. The measurements were performed using the software Image Pro Plus (version 4.5; Media Cybernetics, Sarasota, Florida, EUA).

2.7. Immunofluorescence

Paraffin sections of the aortic arch (7-μm) were placed on immune slides, deparaffinized and rehydrated. For antigen retrieval, sections were then incubated in EDTA buffer (pH 8.0) for 60 minutes at a temperature of 97.5°C. Tissue sections were permeabilized with PBS 0.1 M containing 1% TWEEN 20 (PBST1%) for 5 min twice. Then sections were blocked with 1% low-fat milk powder for 25 min at room temperature and washed with PBST1% for 5 min twice.

For iNOS detection sections were incubated in a humidified chamber overnight with 1:100 dilution of rabbit polyclonal anti-iNOS antibody (sc-8310, Santa Cruz Biotechnology, Inc) at 4°C. Sections were then washed with PBST1% for 5 min twice and labeled with goat anti-rabbit antibody-conjugated FITC (sc-2012, Santa Cruz Biotechnology, Inc.) diluted 1:100 in a dark humidified chamber remaining under the same conditions. Following staining, sections were washed with PBS for 5 min twice and the slides were mounted with Ultra Cruz Mounting Medium containing DAPI (sc-24941 Santa Cruz Biotechnology, Inc).

As a positive control for iNOS expression, apoE−/− mice were injected (i.p.) with 2.5 mg/kg of lipopolysaccharides (Escherichia coli LPS). The animals were euthanized two days after injection and the aorta was collected and processed as previously described [16].

Images for immunofluorescence were obtained and photographed using a fluorescence microscope (BX-50, Olympus, Melville, NY) equipped with digital camera and appropriate filters.

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2.8. Measurement of catalase activity

To determine CAT activity, a portion of liver or kidney tissue (100 mg) was homogenized in 50 mmol/L – 1 phosphate buffer and the resulting suspension was centrifuged at 3000 g at 4 °C for 10-min. The supernatant was used to measure enzymatic activity. CAT activity was determined by the decay rate of hydrogen peroxide (10 mmol/L⁻¹) read on a spectrophotometer at 240 nm, according to Aebi [17]. The amount of protein in the assays of CAT was estimated using the method of Lowry et al. [18] and the results were expressed as units of CAT per milligram of protein.

2.9. Measurement of superoxide dismutase activity

Superoxide dismutase activity was performed according to Dieterich et al. [19]. The method is based on the ability of this enzyme to catalyze the reaction of superoxide \( O_2^- \) to hydrogen peroxide, by monitoring the inhibition of pyrogallol auto-oxidation at 480 nm. Briefly, a fragment of liver or kidney tissue was homogenized in glycine buffer (50 mmol L\(^{-1}\), pH 10.1) and the enzymatic activity was estimated by the inhibition of auto-oxidation using spectrophotometric measurements. The amount of protein in the assays of SOD was estimated using the method of Lowry et al. [18] and SOD activity was calculated as units per milligram of protein.

2.10. Statistical analyses

The Shapiro-Wilk test for normality was initially performed in all data. Animal weights and heart weights were compared using one-way analysis of variance (ANOVA) followed by Tukey’s post-hoc test when necessary. The activities of SOD, CAT and CS, the serum levels of CT, HDL-C and TG, and the atherosclerotic plaque size were compared using Kruskal-Wallis followed by Dun’s post-hoc test when necessary. \( P < 0.05 \) was considered significant. Data are presented as mean ± SE. Data analyses were performed using the statistical software program SigmaPlot® version 11.0 (Systat® Software, Inc).

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### Table 1 Body and heart weights, citrate synthase activity and serum lipid levels of apoE−/− mice.

<table>
<thead>
<tr>
<th></th>
<th>G1</th>
<th>G2</th>
<th>G3</th>
<th>G4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Final BW (g)</td>
<td>25.41 ± 0.43</td>
<td>26.05 ± 0.33</td>
<td>26.24 ± 0.49</td>
<td>26.10 ± 0.22</td>
</tr>
<tr>
<td>HW (g)</td>
<td>0.28 ± 0.02</td>
<td>0.27 ± 0.01</td>
<td>0.31 ± 0.03</td>
<td>0.32 ± 0.02</td>
</tr>
<tr>
<td>CS (μmol/min/mg protein)</td>
<td>10.1 ± 0.43a</td>
<td>10.07 ± 0.42a</td>
<td>14.32 ± 0.34</td>
<td>13.51 ± 0.66</td>
</tr>
<tr>
<td>TC (mg/dL)</td>
<td>339.83 ± 16.17</td>
<td>290.00 ± 24.21</td>
<td>345.29 ± 13.15</td>
<td>381.00 ± 32.98</td>
</tr>
<tr>
<td>HDL-C (mg/dL)</td>
<td>22.50 ± 6.98</td>
<td>24.85 ± 6.82</td>
<td>23.57 ± 3.16</td>
<td>23.5 ± 2.35</td>
</tr>
<tr>
<td>TG (mg/dL)</td>
<td>83.57 ± 9.10</td>
<td>64.29 ± 8.25</td>
<td>82.29 ± 5.38</td>
<td>77.17 ± 5.54</td>
</tr>
</tbody>
</table>

Data are means ± SE of seven animals in each group. BW: body weight; HW: heart weight; TC: total cholesterol; HDL-C: high-density lipoprotein cholesterol; LDL-C: low-density lipoprotein cholesterol; TG: triglycerides; CS: citrate synthase; G1: control; G2: nebivolol; G3: exercise; G4: nebivolol plus exercise.

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3. Results

3.1. Basic physiological parameters

The basic physiological data from the experimental groups are presented in Table 1. The final body and heart weights were not statistically different between groups. The citrate synthase activity was significantly higher in exercised than in non-exercised animals. Regarding serum lipid profile, total cholesterol, HDL-C and TG levels did not differ statistically between groups.

3.2. Atherosclerotic plaque size

Fig. 1A shows photomicrographs of aortic root cross-sections. The mean data (Fig. 1B) demonstrate that the atherosclerotic plaque was smaller in animals treated with nebivolol (G2) as compared to control animals (G1). Nevertheless, either in mice from exercise or nebivolol plus exercise groups the attenuation of the plaque development did not reach statistical significance.

3.3. iNOS expression

Fig. 2 shows that iNOS was expressed in all treatments, being located mainly in the adventitia layer of the artery. The aorta of animals injected with LPS was used as controls, confirming iNOS expression.

In addition to confirming the presence of iNOS, it was demonstrated that this enzyme was localized in the cytoplasm and co-localized within the cell nuclei (Fig. 3).

3.4. Antioxidant enzyme activities in the liver and kidney

The swimming training was able to modify CAT and SOD activities in the liver. Animals from exercised groups (G3 and G4) exhibited lower values for CAT activity than those from non-exercised groups (G1 and G2) (Fig. 4A). In addition, SOD activity was reduced in G3, as compared to G1 and G2 groups (Fig. 4B).

In the kidney, however, neither exercise nor drug treatment changed \( P > 0.05 \) the activity of CAT \((G1 = 5.02 ± 0.94 \text{ u/mg protein})\) and \((G2 = 4.98 ± 0.41 \text{ u/mg})\).
4. Discussion

In this study we used the apoE−/− mice to investigate the effects of exercise training and nebivolol treatment on atherosclerotic plaque development, iNOS expression and oxidative capacity.

To assess the effectiveness of the swimming training on the mice aerobic capacity the CS activity was evaluated. Our results showed that the swimming training used increased the skeletal muscle oxidative capacity in these animals. Similar findings were previously reported [6,25].

Concerning the lipid profile, total cholesterol, HDL-C and TG levels were not statistically different between groups.

Similar results were reported by others [5,6,11] who examined the isolated effects of either nebivolol [11] or exercise [5,6] on this animal model. It is possible that the duration of treatments employed by us was not sufficient to develop a significant change in the serum lipid profile.

Regarding the atherosclerotic plaque development, we observed a smaller lesion area in the aortic roots from nebivolol treated mice, as compared with their control. This effect was probably due to a direct vascular action of this drug on the endothelium. Similar results were obtained by Kus et al. [10] who evaluated the effect of nebivolol on the same animal model. Nebivolol action appears to be related to increased activity of the endothelial nitric oxide synthase [20], which stimulates nitric oxide production and hence vasodilatation [21]. In contrast, exercise training did not affect the atherosclerotic plaque development. The lack of efficiency of the exercise program in changing luminal stenosis was also observed by Schlotter et al. [22] in LDL−/− mice submitted to treadmill running for 16 weeks. It is possible that our exercise training duration was not enough to affect the lesion area. Despite that, according to Arce-Esquível et al. [23], even if exercise training does not affect plaque size, beneficial adaptations through changes in hemostasis, improved resistance to cardiac ischemia, and/or functional changes in vascular cells can be induced by exercise. Furthermore, studies from our laboratory demonstrated atheroprotective effects of exercise training on both LDL−/− [24] and apoE−/− mice [25].

Immunofluorescence was used to document the presence of iNOS in the aorta root of apoE−/− mice. In fact, immuno-detectable signals were located essentially in the aorta adventitia layer in all groups. iNOS is known as an important proinflammatory factor during the development of atherosclerosis [26] and it is not detectable under normal physiological conditions [27]. It is noteworthy that although the increased synthesis of nitric oxide induced by iNOS has positive effects, exaggerated quantities have cytotoxic effect [28]. The role of iNOS in the progression of atherosclerosis is supported by observations that atherosclerotic lesion areas were smaller in apoE−/−/iNOS−/− mice compared with apoE−/− mice [29,30].

The physiological or pathophysiological role of adventitial inflammation remains partly unknown. The traditional concept of vascular inflammation consider the inflammatory responses initiating at the luminal surface, which penetrate the vessel wall and are a result of the in situ interaction of monocytes/macrophages and endothelial cells. However, growing evidences support a new paradigm, the "outside in" response, in which vascular inflammation is initiated in the adventitia and progresses inward toward the intima [31]. In this sense, adventitial myofibroblasts can differentiate into myofibroblasts that migrate, proliferate and secrete cytokines, and play a critical role in the adventitia response to the lesion [32]. This hypothesis was first suggested by Zhang et al. [33] who noted the production of iNOS in aortic adventitial myofibroblasts of rats treated with lipopolysaccharide. Accordingly, the present study demonstrated that adventitial inflammation may be a component of atherosclerotic vascular proliferation, and adventitial cytotoxic nitric oxide production by iNOS may serve to modulate the atherosclerotic process. Despite that, no effect of either exercise training or nebivolol treatment was observed in the iNOS expression.

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Figure 2  iNOS expression detected by immunofluorescence in the experimental groups. LPS: Mice (control) injected with LPS. iNOS was stained using FITC (green) and nuclei were stained using DAPI (blue). The first image from the left presents the nuclei staining, the second image shows iNOS staining and the third is a superimposed image. The arrows indicate iNOS expression. Bar: 20 μm. G1: control; G2: nebivolol; G3: exercise; G4: nebivolol plus exercise.

Figure 3  iNOS expression detected by immunofluorescence in the cytoplasm and cell nuclei. Nuclei were stained using DAPI (blue, first image from the left) and iNOS was stained using FITC (green, second image). The third image from the left is a superimposed image. The arrows indicate iNOS expression. Bar: 10 μm.
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Oxidative stress is one of the factors linking hypercholesterolemia to the pathogenesis of atherosclerosis, and liver tissue contains enzymes that contribute to the antioxidant defense [34]. We evaluated the effects of exercise training and nebivolol treatment on antioxidant enzymes in the liver. Our results showed that SOD activity was reduced in exercised animals. Moreover, nebivolol seems to have attenuated the decline in SOD activity in response to exercise. Such decrease in SOD activity under these conditions is not clear. This result may be due to the reduced activity of the rats during the weeks of confinement in the cage, since under this condition the production of radical oxygen is smaller thus reducing SOD activity [35].

Concerning CAT activity, it was reduced in animals submitted to exercised (G3) and exercise plus nebivolol (G4). Some studies have demonstrated an increase in CAT activity [24] while others showed either decrease [36] or no change [25]. Such inconsistencies may be due to methodological differences.

The reduced activity of SOD and CAT in the liver of exercised animals may be related to decreased production of free radicals in these groups. It is also possible that high levels of oxidative damage have been generated by an excessive stress caused by swimming exercise [37]. Such response can be evidenced by the significant increase in hepatic malondialdehyde, and significant decrease in the glutathione concentration, and in the activities of CAT, SOD and glutathione-S-transferase, suggesting an oxidative stress induced by exercise. However, further studies are needed to confirm these interrelationships.

Atherosclerosis is associated with vascular functional and structural changes in various organs, including the kidneys. According to Bonomini et al. [38], 20-week old apoE−/− mice exhibit a reduced antioxidant capacity in relation to 6-week old animals. Additionally, renal disease is associated with oxidative stress [39]. Our results showed no significant differences between treatments for the SOD and CAT activities in the kidney. Thus, it suggests that both exercise training and nebivolol treatment used here were not effective in increasing the kidney antioxidant capacity in this animal model.

Finally, this study has some limitations. First, since iNOS was not quantified, the comparisons between groups are difficult. And second, since a marker of oxidative stress (i.e. malondialdehyde) was not measured, it is difficult to confirm whether the treatments used here were stressful to the animals or not.

5. Conclusions

In conclusion, while aerobic exercise training reduces antioxidant activity in the liver, nebivolol treatment attenuates atherosclerotic plaque development in apoE−/− mice, but the expression of iNOS is not affected. The combination of treatments does not add on benefits.

Disclosure of interest

The authors declare that they have no competing interest.

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