Conjecturas

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Effect of chronic treatment with apocynin and resisted exercise on endothelial dysfunction of spontaneously hypertensive rats

Efeitos do tratamento crônico com apocinina e do exercício resistido na disfunção endotelial em ratos espontaneamente hipertensos

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ABSTRACT

Aim: Analyze the effects of apocynin treatment associated with resisted training in blood pressure, pressor response to angiotensin II and hypotensive responses to acetylcholine and sodium nitroprusside in spontaneously hypertensive rats (SHR). Plasmatic oxidative damage and protein expression of enzyme nitric oxide synthase (eNOS) and nicotinamide adenine dinucleotide phosphate oxidase subunit 4 (NOX4) were analyzed in aortas of SHR. Main methods: Male SHR were distributed in groups: sedentary (S); in resisted/weight training in ladder (T); sedentary in chronic treatment with apocynin (30 mg/kg, p.o.) (SA) and in chronic treatment with apocynin and in resisted training (TA). Significance: Apocynin treatment was able to prevent the development of hypertension and reduce oxidative stress. When associated to resisted training, apocynin reduced oxidative stress but it was not able to reduce blood pressure caused by the intensity of the training and increase of heart rate.

Keywords: Resisted exercise; Reactive oxygen species; Hypertension; Inbred SHR; NAD(P)H oxidase.

RESUMO

Objetivo: Analisar os efeitos do tratamento com apocinina associado ao treinamento resistido na pressão arterial, resposta pressora à angiotensina II e respostas hipotensoras à acetilcolina e nitroprussiato de sódio em ratos espontaneamente hipertensos (SHR). O estresse oxidativo plasmático e a expressão proteica da enzima óxido nítrico sintase (eNOS) e nicotinamida adenina dinucleotídeo fosfato oxidase subunidade 4 (NOX4) foram analisados em aortas de SHR. Principais métodos: Os machos SHR foram divididos nos grupos: sedentários (S); no treinamento resistido/com pesos em escada (T); sedentários em tratamento crônico com apocinina (30 mg/kg, p.o.) (SA) e em tratamento crônico com apocinina e em treinamento resistido (TA). Significância: O tratamento com apocinina foi capaz de prevenir o desenvolvimento de hipertensão e reduzir o estresse oxidativo. Quando associada ao treinamento resistido, a apocinina reduziu o estresse oxidativo, mas não foi capaz de reduzir a pressão arterial causada pela intensidade do treinamento e aumento da frequência cardíaca.

Palavras- Chave: Exercício resistido; Espécies reativas de oxigênio; Hipertensão; Ratos endogâmicos SHR, NADPH oxidase.

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INTRODUCTION

According to Nabha et al. (2005), the development of hypertension is preceded by endothelial dysfunction and oxidative stress. The endothelium, which is located in the innermost layer of blood vessels, is responsible for controlling vessel dilatation and contraction, either in response to changes in blood flow or vasoactive agents such as nitric oxide (NO) (Carvalho et al., 2001). NO is a potent endogenous vasodilator synthesized in the endothelium by the enzyme nitric oxide synthase (eNOS), acts as a relaxing factor for vascular smooth muscles and plays a fundamental role in the regulation of vascular tone. In physiological conditions, there is a balance in the release of this relaxing factor. However, in the case of arterial hypertension, the vasodilator effect is reduced and leads to endothelial dysfunction (Carvalho et al., 2001).

Endothelial dysfunction can generate several imbalances in the production or release of contractile and relaxing factors because it causes a decrease in NO production, increase of reactive oxygen species (ROS) and consequently muscle tone, vascular permeability and leukocyte adhesion to the vessel wall with acceleration of intravascular coagulation, increased proliferation of smooth muscle cells and hypertrophy and or hyperplasia of the vascular wall (Carvalho et al., 2001). Thus, oxidative stress is also considered a factor of endothelial imbalance. When cells are exposed to excessive ROS levels, this may decrease NO production or bioavailability. This condition is due to a physiological imbalance between the generation of oxidizing compounds and the action of antioxidant defense systems (Barbosa et al., 2010).

Some antioxidant therapies seem to promote results in the control of blood pressure (BP), among which apocynin can be a promising tool (Saleem et al., 2018). Apocynin is considered an important preventive agent for vascular diseases, because it has anti-inflammatory and antihypertensive effects (Baumer et al., 2007; Pachanová et al., 2009; Perassa, 2013; Feng et al., 2018).

National and International Guidelines and data from the National Health and Nutrition Survey recommend lifestyle modifications and the inclusion of physical exercise as the first-line therapy for hypertension prevention. There is evidence of Class I, Level B, that 150 minutes per week of physical activity can be considered as an alternative method to supplement antihypertensive medication (Cornelissen, Smart, 2013). However, few articles in the literature have discussed the benefits of high-intensity

resisted exercises in hypertension control and prevention. The objective of our study was to analyze the effects of apocynin treatment associated or not with resisted training in modulating blood pressure, pressor response to angiotensin II, and hypotensive response to acetylcholine and sodium nitroprusside in spontaneously hypertensive rats (SHR). It was also analyzed plasmatic oxidative damage, eNOs and nicotinamide adenine dinucleotide phosphate oxidase subunit 4 (NOX4) expression in aortas from SHR. Further research on the action of this model of physical exercise and especially on its interaction with apocynin is needed (Battagin, 2010).

MATERIALS AND METHODS

All the experiments carried out in this study were previously approved by CEEA-FOA/UNESP (protocol nº 00131-2016). The funding of the research in relation to the expenditure of materials was partly due to its own funding and to the resources available in the laboratory. There was no sponsor for the development of the research.

Animals

In this study, 88 male spontaneously hypertensive rats (SHR) with approximately 4 weeks of life and 40g of body weight were used.

They were obtained from a lineage maintained by the laboratory of the Department of Basic Sciences of the Faculty of Dentistry, Araçatuba Campus, UNESP. The animals received standard feed and water ad libitum and were kept under controlled temperature conditions (22-24 °C) with 12h:12 h light:dark cycle. The rats were divided into four experimental groups: sedentary SHR (S) n = 21; SHR in resistance training (T) n = 21; sedentary SHR in chronic treatment with apocynin (SA) n = 19; and SHR in chronic treatment with apocynin and resisted training (TA) n = 27.

Exercise protocol for training

At four weeks of age, the rats of the training groups were motivated to climb a 1.13 x 0.18m ladder, with 2 cm steps, and inclination of 80°, by means of a soft touch on their tail to initiate the movement. This process of familiarization with the adder was

repeated for three nonconsecutive days, the first time without the device on their tail and in the other two times with the device.

After these three days, the rats began weight training to find out the maximum load. The first load was adjusted according to the rats' total body weight (TBW), which was 75% of the TBW for two consecutive days; in the 5th day of training, the load was adjusted. From the 6th day onwards, the load was readjusted to 75% of the TBW and 30g were added per climb, until the rat was unable to climb up the ladder; the last load that prevented the rat to climb completely the ladder was the maximum load. These two weeks were used for familiarization with the ladder and the loads, and for readjustment and investigation of the maximum load to be used.

The resisted training protocol was started with the rats in their 6th week of life. The protocol consisted of climbing the ladder with a fixed loader adapted to the rat tail. The device was attached to the tail in the proximal portion with a piece of adhesive tape. With the device attached to the tail, the rats were placed at the bottom of the ladder and stimulated until reaching the top, in a house with a diameter of 20 x 20 x 20 cm, where they would rest for 120 seconds. The animals repeatedly climbed the ladder four times with 85% of the maximum load. The load was readjusted every two weeks considering a possible increase in muscle strength upon body weight gain. Climbing was performed three times a week for two months; at the end, the rats had completed the 14-week training protocol. The resisted training protocol used was based on training adapted by Hornberger, Farrar, (2004).

Chronic treatment with apocynin

The rats of the SA or TA group were treated from the 4th to the 14th week of life with apocynin at a dose of 30 mg/kg/day. This dose of apocynin was chosen based on studies (Hayashi et al., 2005; Baumer et al., 2007; Pechanová et al., 2009; Oelze et al., 2001) that observed the non-toxic effects of apocynin on animals. The apocynin was diluted in drinking water; the volume of water used was determined based on the volume of water consumed per day by the animals. The rats were weighed at each week of development in order to correct the administered dose. At the end of the 14th week of treatment, the rats were used in the experiments.

Cannula implantation surgery

One day after treatment with apocynin or training, the animals were first anesthetized with Ketamine (45 mg/kg ip) and xylazine (5 mg/kg ip) and right after that, a polyethylene cannula (PE10 connected to PE50) filled with saline was implanted inside the abdominal aorta, through the femoral artery for recording the pressure, and another cannula was implanted in the femoral vein for administration of the complexes or the vehicle. These cannulas were passed under the skin to exit at the nape of the neck of the animal. After surgery, the animals were kept in individual cages and received standard feed and water ad libitum. The animals were used within 24 h after the surgeries.

MAP and HR record

Mean arterial pressure (MAP) and heart rate (HR) were continuously recorded in the awaken rats using a pressure transducer (AD instruments) and an amplifier (AD instruments) connected to the arterial cannula. The effects of the drugs acetylcholine (2 and 10 µg/kg), sodium nitroprusside (10 and 35 µg/kg) and ANGII (20, 50, 100, 150, 200 and 250 ng/kg) on MAP and HR levels were evaluated. The resulting values were obtained using the software 4 Chart AD instruments.

Experimental Protocol

After a time interval of 30 minutes for (blood pressure) BP stabilization, 2 and 10 ug/kg of acetylcholine (Sigma Aldrich) were given through bolus injections in the arterial cannula. Only after five minutes for responses stabilization, the effect of the different doses was analyzed. Then, 1mL of saline was given to wash the cannula 10 minutes after the last administration. Doses 10 and 35 µg/kg of sodium nitroprusside (Sigma Aldrich) were applied. Stabilization and lavage with saline solution were repeated and then doses of 20, 50, 100, 150, 200 and 250 ng/kg of angiotensin II (Sigma Aldrich) were injected.

Western Blotting

Evaluation of protein expression of eNOS and NOX4 in the aortas of the SHR

Thoracic segments of the aorta were removed from the animals, dissected, immediately pulverized and frozen in liquid nitrogen and stored in a freezer at -80 °C. The tissues were grinded in liquid nitrogen and homogenized separately in RIPA buffer (65.2 mM Tris-base, 154 mM NaCl, 1% NP-40, 0.25% sodium deoxycholate and 0.8 mM EDTA, supplemented with a cocktail of protease inhibitors - Protease Inhibitor Mix - GE Healthcare) using a sonicator (Sonics Vibra Cell).

Then, the homogenates were centrifuged (4 °C, 4000 rpm for 15 minutes) for separation of supernatant. The protein of the supernatant was measured by the method of Lowry et al. (1951), using bovine serum albumin solution as a standard. One hundred micrograms (100 µg) of total protein were mixed with Laemmli buffer containing mercaptoethanol and subjected to electrophoresis on 8% polyacrylamide gel (for eNOS antibody) or 10% (for NOX4 antibody) and then transferred to the nitrocellulose membrane.

After transfer, the nitrocellulose membranes were blocked in 6% nonfat milk andTris-saline buffer with Tween 20 (TBS-T) for 1 hour at room temperature. The membranes were then incubated with the following primary antibodies: eNOS (1: 2500, SC-654) and NOX 4 (1: 1500, SC 11407) overnight at 4 °C. After incubation with primary antibody, the membranes were washed for fifteen minutes, 3 times with TBS-T buffer and incubated with anti-rabbit secondary antibody to eNOS (1: 2000, SC) and NOX 4 (1: 1000, SC) for one hour at room temperature. Afterwards, the membranes were incubated with the chemiluminescent peroxidase substrate (ECL Kit - Amersham ECL Prime Western Blotting Detection Reagent) and exposed to a radiographic film for detection of bands. β-actin (1: 8000, SAB A5441) was used for normalization of the results. Band intensity was quantified by optical densitometry in the Scion Image software. The results were compared between groups.

Determination of oxidative stress

The rats were pre-anesthetized in a chamber saturated with halothane (5 mL) and then euthanized by decapitation. Then, blood samples from each animal were separately collected in tubes with heparin (0.2 ml). Substances that reacted to 2-thiobarbituric acid (TBARS) were determined in hemolyzed erythrocytes as an indicator of the peroxidized

lipid level. Briefly, erythrocyte samples of the homogenates were mixed with 1 ml of trichloroacetic acid (10%) for precipitation of proteins. Then they were taken to the centrifuge (1000 rpm/3 min) and 1 ml of thiobarbituric acid (0.67%) was added. The samples were then heated in boiling water for 15 minutes. The amount of TBARS was determined by measuring the absorbance of 535 nm in a Hitachi U-1100 spectophotometer. The results were expressed as nmol/mg of protein using the appropriate molar extinction coefficient ($\varepsilon = 1.56 \times 10^5\ M^{-1}\ cm^{-1}$).

STATISTICAL ANALYSIS

The results were expressed as mean ± standard error of the mean (SEM) of the values obtained and later compared between the groups by the statistical test of variance (one way - ANOVA) and Tukey's post-test, using GraphPad Prism 5.0 (GraphPad Software Corporation, La Jolla, CA, USA). The differences between groups were considered significant when $p < 0.05$ for all analyses.

The percentage of variation in MAP (% Δ MAP) was calculated by the difference in MAP values before and after administration of the drugs.

The optical densitometry values of the bands were obtained using the Scion Image software. The results regarding the NOX4 and eNOS bands were normalized by those of the β-actin bands.

The amount of TBARS was determined by measuring the absorbance of 535 nm in a spectrophotometer (Hitachi U-1-100) and expressed as nmol/mg protein.

RESULTS

Treatment with apocynin and/or physical training does not affect the weight of SHR

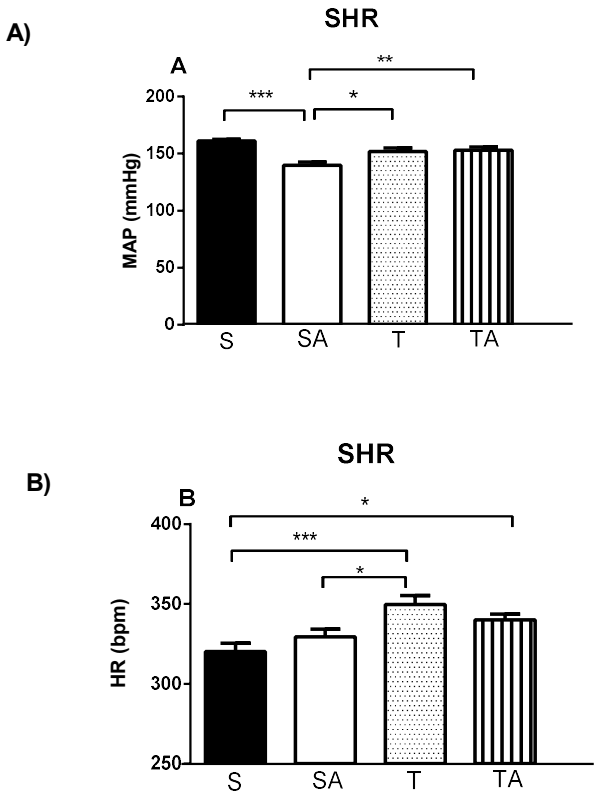
There was no difference in weight ($p = 0.95$) among the animals of the different groups (S: 135.9 ± 20.64 g; SA: 125.2 ± 20.64 g; T: 141.9 ± 21.41 g; TA: 130.5 ± 21.92 g, n = 8/group).

Treatment with apocynin, but not physical training, reduces MAP and increases HR of SHR

The MAP values of sedentary SHR were higher (S: 161.2 ± 1.3 mmHg, n = 16) than those of SHR in SA group (139.9 ± 2.6 mmHg, n = 16). The SHR in the T (151.8 ± 3.3 mmHg, n = 14) and TA (153 ± 2.8 mmHg, n = 22) groups had no reduction of MAP when compared to the S group (Figure 1A). The data showed that chronically apocynin-treated SHR (30 mg/kg) prevented hypertension. Resisted training in SHR did not change the MAP of the animals. When treatment with apocynin was associated with resisted exercise, there was no prevention of the development of hypertension.

HR values of sedentary SHR (320.3 ± 5.2 bpm, n = 14) and SA (329.5 ± 4.7 bpm, n = 14) were lower than in the T (349.8 ± 5.5 bpm, n = 13), TA (340.1 ± 3.5 bpm, n = 15) groups (Figure 1B). Although the resisted exercise did not change MAP, it caused an increased HR in the animals.

Figures 1A e 1B - Mean Arterial Pressure (MAP, in A) and Heart Rate (HR, in B) of Sedentary SHR (S) n= 16, Sedentary and apocynin-treated SHR (SA) n= 16, Trained SHR (T) n= 14 and Trained and apocynin-treated SHR (TA) n= 22. In A

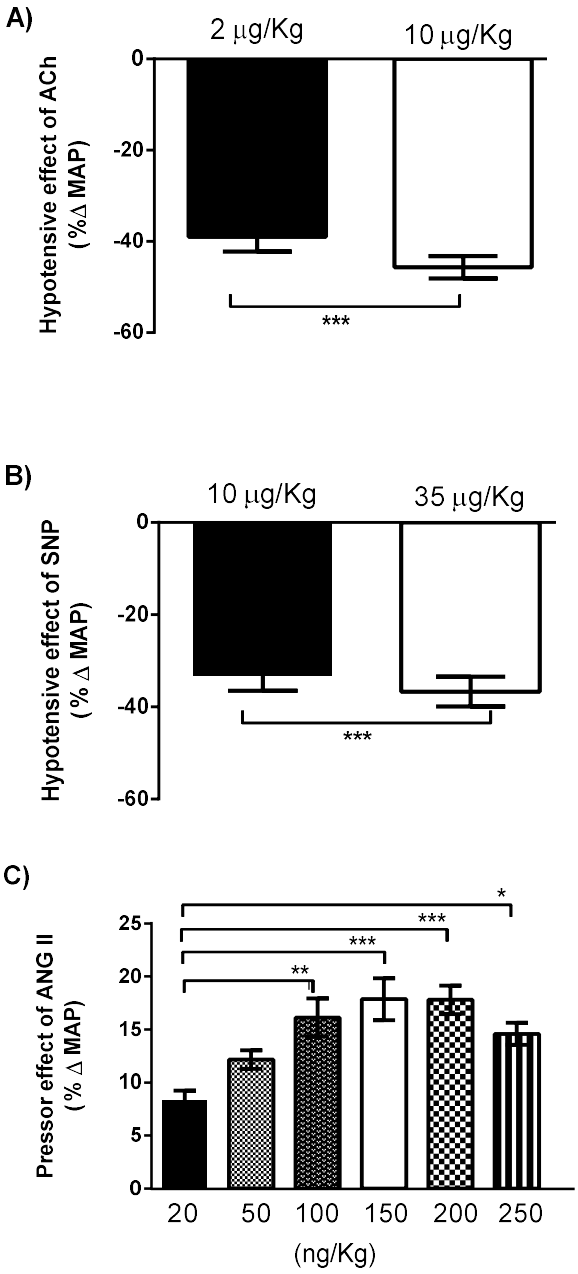


Hypotensive effect of acetylcholine and sodium nitroprusside and pressor effect of ANG II in the Sedentary group (control)

Acetylcholine promoted a dose-dependent hypotensive response at doses of 2 and 10 μg/kg, in %ΔMAP (2 μg/kg: -39.03 ± 3.19 %, n = 12; 10 μg/kg: -45.68 ± 2.46 %, n = 11; Figure 2A). Sodium nitroprusside also promoted dose-dependent hypotensive response at the doses tested in %ΔMAP (10 μg/kg: -33.39 ± 3.15 %, n = 11; 35 μg/kg: -36.71 ± 3.22 %, n = 12; Figure 2B). The administration of ANG II promoted a dose-dependent increase in MAP when administered at doses of 20 ng/kg in %ΔMAP (8.37 ± 0.86 %, n = 10), 50 ng/kg (12.18 ± 0.86 %, n = 10), 100 ng/kg (16.14 ± 1.79 %, n = 10), 150 ng/kg (17.86 ± 1.98%, n = 10), 200 ng/kg (17.81 ± 1.32%, n = 9; Figure 2C). We noted that at the dose 250 ng/Kg in %ΔMAP (14.60 ± 1.05 %, n = 9), the values were lower when compared to the dose of 200 ng/kg in %ΔMAP (17.81 ± 1.32 %, n = 9) (Figure 2C).

Figures 2A, 2B, 2C- Percentage of variation in mean arterial pressure (%Δ MAP) in Sedentary SHR (n= 16) after bolus infusion of Acetylcholine (ACh, in A) at doses of 2 and 10

µg/kg, Sodium Nitroprusside (SNP, in B) at doses of 10 and 35 µg/kg, and Angiotensin II (ANG II, in C) at doses 20, 50, 100, 150, 200, 250 µg/kg. In A and B.



Treatment with apocynin and training change the hypotensive effect of acetylcholine on SHR

In sedentary, sedentary and chronically apocynin-treated, and trained groups, a trend towards a greater effect was observed associated with higher doses, in %ΔMAP (S: 2 µg/kg: -39.03 ± 3.19 %, n=12; 10 µg/kg: -45.68 ± 2.46 %, n = 11; SA: 2 µg/kg: -39.36 ± 2.57 %, n = 8; 10 µg/kg: -46.70 ± 4.04 %, n = 9; T: 2 µg/kg: -40.23 ± 2.10 %, n = 11; 10 µg/kg: - 47.43 ± 2.58 %, n = 9, Figure 3A). In trained and chronically apocynin-treated SHR, the hypotensive effect of the dose 10 µg/kg was greater than the effect of the dose 2 µg/kg (TA: 2 µg/kg: - 41.82 ± 1.90 %, n = 19; 10 µg/kg: - 51.57 ± 2.16 %, n = 19, Figure 3A).

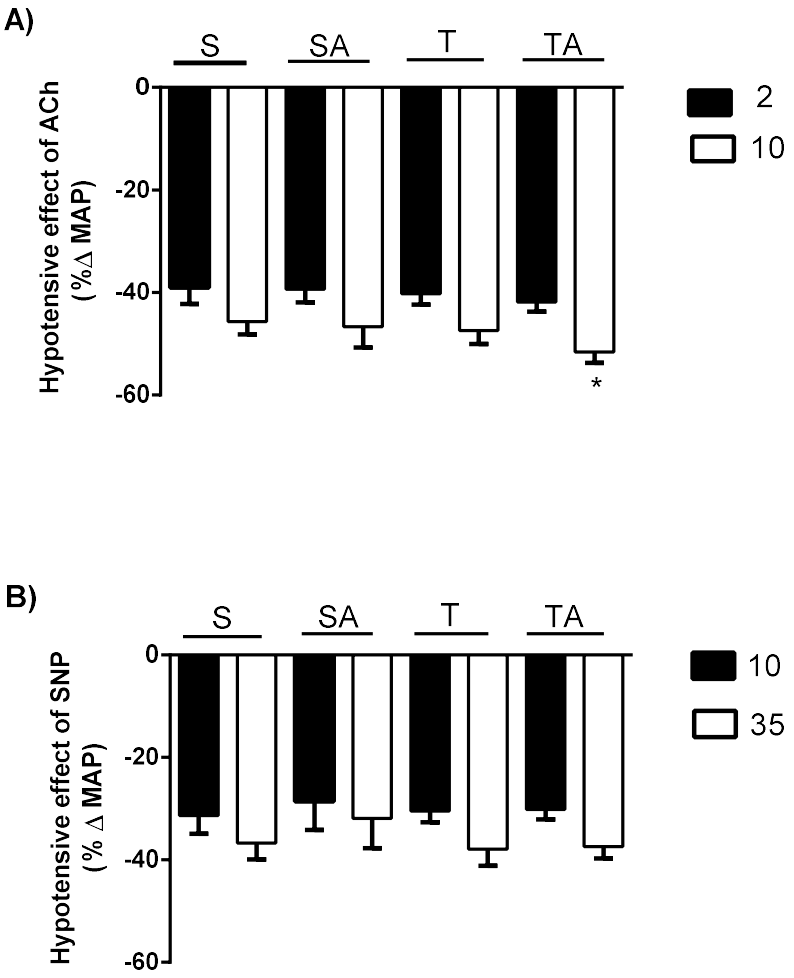
No differences in the hypotensive effects of acetylcholine were observed among groups.

Treatment with apocynin and/or training did not affect the hypotensive effect of sodium nitroprusside in the different groups

Sodium nitroprusside responses at the tested doses of 10 and 35 µg/kg suggest that there is a dose-dependent relationship, although no statistical differences between doses in the different groups were observed, in %ΔPAM (S: 10 µg/kg: - 31.30 ± 3.55 %, n = 12; 35 µg/kg: - 36.71 ± 3.22 %, n = 12; SA: 10 µg/kg: - 28.57 ± 5.56 %, n = 6; 35 µg/kg: - 31.86 ± 5.88 %, n = 6; T: 10 µg/kg: - 30.47 ± 2.20 %, n = 7; 35 µg/kg: - 37.86. ± 3.25 %, n = 8; TA: 10 µg/kg: - 30.14 ± 1.93 %, n = 17; 35 µg/kg: - 37.40 ± 2.34 %, n = 17; Figure 3B). We observed that there was no statistical difference (p = 0.24) in the hypotensive responses of sodium nitroprusside among groups.

Figure 3A e 3B- Hypotensive effect of Acetylcholine (Ach, in A) at doses of 2 and 10 µg/kg and Sodium Nitroprussiate (SNP, in B) at doses of 10 and 35 µg/kg express in (%Δ MAP) in Sedentary SHR (S) (n = 16); Sedentary and Chronically apocynin-treated SHR (SA) (n

= 16); Trained SHR (T) (n = 14); and Trained and Chronically apocynin-treated SHR (TA) (n = 22).



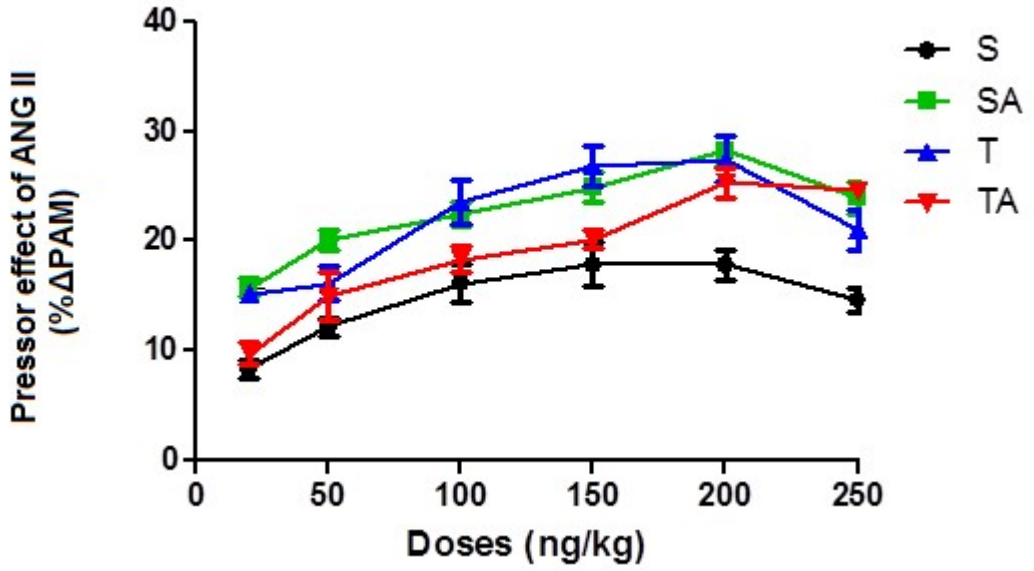
Pressor effect of angiotensin II at doses of 20, 50, 100, 150, 200, 250 µg/kg in different groups

We observed increased pressor responses to angiotensin II at doses of 100, 150 and 200 ng/kg in different groups. When we analyzed the dose of 250 ng/kg, we noticed lower pressure responses in relation to 200 ng/kg (Figure 4).

The sedentary and apocynin-treated (SA) and trained (T) groups presented higher pressor responses at the doses of 20, 50, 100 and 150 ng/kg when compared to the sedentary group (S) and to the trained and apocynin-treated group (TA).

It is also noteworthy that the pressor effect in the sedentary group was lower when compared to the other groups.

Figure 4- Pressor Effect of Angiotensin II (ANG II) express in (%Δ MAP) in Sedentary SHR (S) (n = 16); Sedentary and Chronically apocynin-treated SHR (SA) (n = 16); Trained SHR (T) (n = 14); and Trained and Chronically apocynin-treated SHR (TA) (n = 22) after bolus infusions of ANG II at doses of 20, 50, 100, 150, 200, 250 µg/kg.



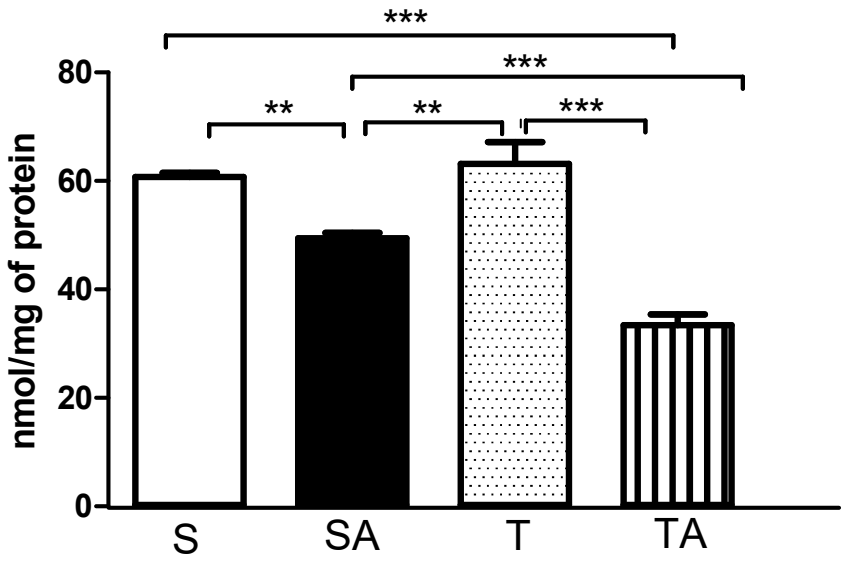
Treatment with apocynin associated or not with training reduces plasma oxidative damage in SHR

Systemic oxidative damage can be evaluated by lipid peroxidation in erythrocytes by the method that determines the concentration of thiobarbituric acid reactive substances (TBARS).

Oxidative damage found in apocynin-treated SHR (49.43 ± 1.00 nmol/mg protein, n = 5) was lower than that observed in sedentary SHR (60.74 ± 0.73 nmol/mg protein, n = 5; Figure 5).

There were no differences between the sedentary and trained groups. However, apocynin treatment associated with training (33.40 ± 2.01 nmol/mg protein, n = 5; Figure 5) was more effective in reducing lipid peroxidation when compared to the other groups.

Figure 5 - Thiobarbituric acid reactive substances (TBARS, in nmol/mg protein) in SHR erythrocytes. The bars represent the mean ± SEM of the results obtained in hemolysates of the different groups: Sedentary SHR (S) (n =5), Sedentary and apocynin-treated SHR (SA) (n = 5), Trained SHR (T) (n =5) and Trained and apocynin-treated SHR (TA) (n =5).

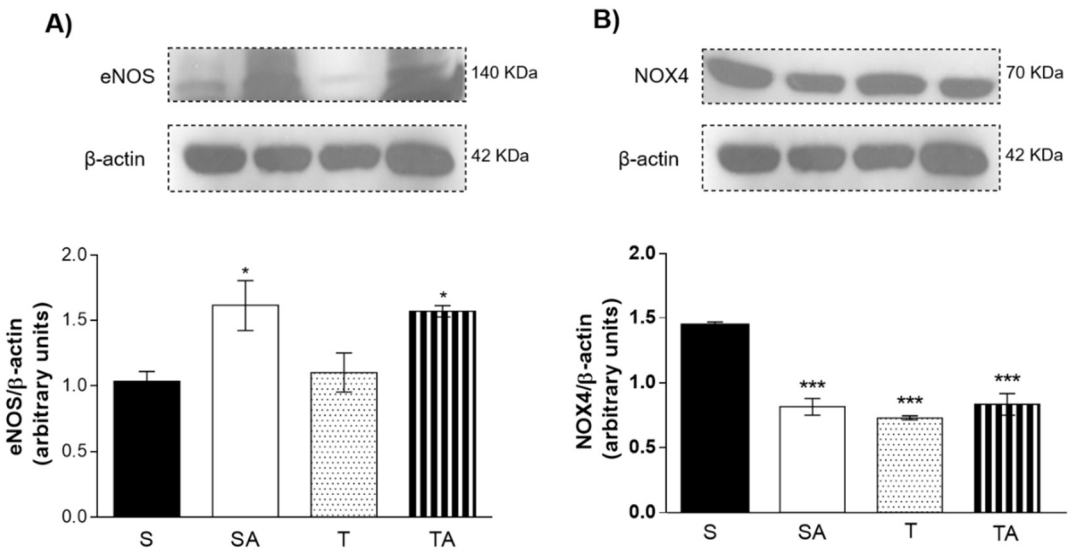


Treatment with apocynin and association between treatment and training increase the protein expression of eNOS, and training alone reduces the expression of NOX4 in aortic homogenates of SHR

There were significant differences in eNOS expression in different groups. We observed an increased eNOS expression in aortas of the groups SA (1.65 ± 0.19 AU, n = 1) and TA (1.56 ± 0.043 AU, n = 1) when compared with the Sedentary group (S: 1.03 ± 0.07 AU, n = 1). There was no significant difference for the trained group (T: 1.10 ± 0.14 AU, n = 1) when compared to sedentary group (Figure 6B).

A reduction in NOx4 protein expression was observed in SA (0.81 ± 0.06 AU, n = 4), T (0.72 ± 0.01 AU, n = 4) and TA (0.83 ± 0.08 AU, n = 5) when compared to S (1.44 ± 0.02 AU, n = 3) group (Figure 6A).

Figure 6- Protein expression of the enzyme endothelial Nitric Oxide Synthase (eNOS, in A) and isoform (NOX4, in B) in aortic homogenates of SHR. The bars represent the mean ± SEM of the results obtained in aortas of the different groups: Sedentary SHR (S) (n =5), Sedentary and apocynin-treated SHR (SA) (n =5), Trained SHR (T) (n =5) and Trained and apocynin-treated SHR (TA) (n = 5).



DISCUSSION

We observed that chronic treatment with apocynin did not affect the weight of the animals. In a previous study, we had demonstrated that apocynin does not alter the weight or the naso-anal length of treated SHR (Perassa et al., 2016) suggesting that apocynin

treatment did not alter rat´s development. In this study, we also demonstrated that resisted training, whether or not associated with apocynin treatment, does not interfere with the development of the animals.

In this study, chronic treatment with apocynin, maintained from the 4th to the 14th week of life (30 mg/kg, orally), attenuated the increase of mean arterial pressure in SHR, as previously demonstrated by Perassa et al. (2016) and Tain et al. (2012), in which apocynin treatment was started at week 4 and maintained for 8 weeks, suggesting that apocynin treatment was able to prevent high BP in SHR. However, resisted training, whether or not associated with apocynin treatment, was not able to reduce BP of SHR.

According to Comess, Fenster (1981), the increase in systolic blood pressure (SBP) and HR during and shortly after muscular work has already been confirmed since 1889. However, despite the apocynin treatment to reduce MAP in SHR (Beswick et al., 2001; Hu et al., 2006; Costa et al., 2009; Unger, Patil, 2009), when we associated it with resistance training, no reduction of MAP was noticed, probably due to the increase in HR induced by training in the animals and its direct relation with the maintenance of increased BP values. According to Polito, Parinatti (2003), acute HR responses seem to be directly associated with the number of repetitions of the exercise. In our study, the rats were submitted to a resisted exercise protocol using 85% of the maximum load, with 4 repetitions, considered by Forjaz et al. (2003) as a high intensity exercise. Probably this load and this number of repetitions resulted in an increased HR and consequently maintenance of the increased MAP values of SHR treated or not with apocynin.

Concerning the promotion of vasodilation and reduction of peripheral vascular resistance, our data on the hypotensive effect of acetylcholine and sodium nitroprusside demonstrated a dose-dependent relationship between the doses tested. We also confirmed the dose-dependent relationship of the pressor effect of angiotensin II I at the doses of 100, 150 to 200 ng/kg in the group of SHR undergoing training and in the other groups. The actions of angiotensin II are explained by their binding to angiotensin II type I and type II receptors, which have similar binding affinity to ANG II, but with opposite effects (Williams et al., 2010). The vasoconstriction mediated by the interaction of angiotensin II II with type I receptor is associated with increased superoxide anion production by NOx activation in the vascular wall (Chung et al., 1998; Touyz, Schiffrin, 1999). The type II receptor is involved in fetal growth and development, cell differentiation, apoptosis, and vasodilation (Chung et al., 1998; Touyz, Schiffrin, 1999; Grishko et al., 2003).

In our study we obtained pressor effects of angiotensin II at doses of 100, 150 to 200 ng/kg, possibly associated with the activation of type I receptors, which are responsible for the vasoconstricting actions of angiotensin II, and are more expressed than the type II receptors (Kemp et al., 2016). When 250 ng/kg was administered, we observed a decreased pressor response in relation to the dose of 200 ng/kg due to possible activation of type II receptors.

In order to analyze the possible hypotensive effects of both acetylcholine and sodium nitroprusside, we compared the responses related to the doses used in the different groups. There was a statistically significant increase in the vasodilatory effect of acetylcholine at 10 ng/kg for group TA. Several studies have demonstrated an increase in the hypotensive response to acetylcholine with apocynin treatment and training (Goto et al., 2003; Costa et al., 2009; Unger, Patil, 2009). These studies, along with our findings, suggest that the association between apocynin treatment and resisted training increases peripheral vasodilation mediated by acetylcholine, which is likely associated with a significant reduced MAP if heart rate is not maintained at high levels as previously noted. We also evaluated the hypotensive effects of sodium nitroprusside. In our study, treatment with apocynin and/or training did not alter the hypotensive effect of sodium nitroprusside doses tested in the different groups. These results were similar to the findings of Unger, Patil (2009) and Perassa et al. (2016) and reinforce the suggestion that apocynin reverses endothelial dysfunction and prevents the development of hypertension in SHR.

Regarding the vasoconstricting responses to angiotensin II, when we compared the different doses in the different groups, the SA and T presented increased pressor responses compared to the others. In contrast, we found that the pressor response to Ang II in the group TA was also higher, but only at the dose of 200 ng/kg; from this dose upwards, we noticed a slight decrease of the pressor effect in all the groups. We note that both the group T and TA result in a greater angiotensin II -mediated pressor response in SHR.

In our study, we observed that at the dose of 250 ng/kg, angiotensin II promoted a hypotensive effect on SHR of the groups S, SA and T, suggesting that this effect is possibly due to the increased interaction of angiotensin II with type II receptors and peripheral vasodilation. When comparing the different groups with the different doses, we noticed that all groups presented greater pressure response than the group S. In the group T, it is suggested that the intensity of training before administration of angiotensin

II further increased the pressor effect of angiotensin II when compared to the control group. When we associated apocynin treatment with training, there was still no decrease in the pressor response to angiotensin II, again showing that training intensity may have contributed to the vasoconstrictor effect. Other experiments should be done to investigate the mechanisms involved in these effects.

It is well established in the literature that one of the causes of endothelial dysfunction is the oxidative stress caused by the imbalance between the production of reactive oxygen species (ROS) and the endogenous antioxidant defenses (Cai, Harrison, 2000). In the present study, as well as in Perrasa et al. (2016), oxidative damage in SA was lower than that observed in S and that when associated with training (TA), there was a more effective reduction of lipid peroxidation when compared to the other groups. We observed that resisted training (T) alone was not effective in reducing lipid peroxidation when compared to S. Dillard et al. (1978) were the first to demonstrate that physical exercise can lead to an increase in lipid peroxidation.

In our study, when training and apocynin treatment were associated, we noticed a greater reduction in lipid peroxidation. Increased oxidative stress that can be caused by the high intensity and strenuous exercise probably promotes a transient increase in the activity of antioxidant enzymes (Atalay et al., 1996). Our results suggested that activation of antioxidant enzymes associated with training and the treatment with apocynin could significantly decrease lipid peroxidation.

The results obtained in our study showed that both SA and T promoted alterations in the protein expression of NOX in aortic homogenates of SHR. We can observe a reduction in protein expression of NOX4 in all the groups when contrasted with the S.

In agreement with the results of Perassa et al. (2016), we observed an increase in eNOS expression in aortic homogenates of SA. In our study we also found significant differences in protein expression in the TA group. Faria et al. (2017) also found no changes in eNOS and inducible nitric oxide synthase (iNOS) expression under resisted exercise. We suggest a possible modulation of apocynin, which may help to reverse endothelial dysfunction.

Thus, to better explain the results obtained in this study, a schematic summary was elaborated to demonstrate the effects of chronic apocynin treatment, resisted exercise and association between apocynin treatment and resistance exercise in the blood pressure of SHR.

CONCLUSIONS

Apocynin reduced MAP by mechanisms involving reduction of plasmatic oxidative damage, reduction of NOX4 expression and increased eNOS expression in SHR aortas. Resisted training, in turn, increased HR, plasmatic oxidative damage, and despite reducing NOX4 expression in aortas, reduced eNOS expression in these tissues, contributing to the increase of MAP in SHR. The association of apocynin treatment with resisted training did not alter the MAP of the rats, but reduced plasmatic damage, reduced NOX4 expression and increased eNOS expression in the aortas of SHR, similar to that observed with chronic treatment with apocynin.

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