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Beneficial effect of enalapril in spontaneously hypertensive rats cardiac remodeling with nitric oxide synthesis blockade

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Abstract

Aims. To study the efficiency of an angiotensin converting enzyme inhibitor on the blood pressure (BP) and the myocardium remodeling when spontaneously hypertensive rats (SHRs) are submitted to nitric oxide synthesis (NOs) blockade (with L-NAME) and simultaneously treated.
Methods. Young adult male SHRs were separated in four groups (n = 5) and treated for 20 days: Control, L-NAME, L-NAME+Enalapril, and Enalapril. The alterations of the BP, heart mass/body mass ratio and stereological parameters for myocytes, connective tissue and intramyocardial vessels were studied among the groups.
Results. The SHRs with NOs blockade showed a great modification of the myocardium with extensive areas of reparative and interstitial fibrosis and accentuated hypertrophy of the cardiac myocytes (cross sectional area 60% higher in animals taking L-NAME than in Control SHRs). Comparing the SHRs with NO deficiency (L-NAME group), the Control SHRs and the Enalapril treated SHRs significant differences were found in the BP and in all stereological parameters. The NO deficiency caused an important BP increment in SHRs that was partially attenuated by Enalapril. This Enalapril effect was more pronounced in Control SHRs. A significant increment of the intramyocardial vessels was observed in NO deficient SHRs and Control SHRs treated with Enalapril demonstrated by the stereology (greater microvascular densities in treated SHRs).
Conclusion. Enalapril administration showed a beneficial effect on vascular remodeling and myocardial hypertrophy in SHRs. In SHRs with NO blockade, however, the beneficial effect of Enalapril occurred only in vascular remodeling.

Keywords: nitric oxide - spontaneously hypertensive rats - enalapril - L-NAME - blod pressure - vascular remodeling

Introduction

Among the target-organ lesions in hypertensive disease, one with great impact is the cardiac hypertrophy, characterized by an increase of the cardiac mass, the size of the myocytes, and the extracellular matrix, with collagen deposition [1]. In hypertrophied hearts usually occurs a vascular remodeling, depending not only on the blood pressure (BP) levels, but also on the myocardial morphologic alterations, predisposing the heart to the occurrence of ischemic events [2]. Several antihypertensive drugs affect the natural history of the hypertensive disease

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causing prevention or regression of the myocardial morphologic alterations associated to the hypertension in clinical or experimental studies [1, 3, 4].

Spontaneously hypertensive rats (SHR), originated from successive crossings of hypertensive animals by Okamoto and Aoki [5], are very popular in experimental studies [6], although many other lineages of hypertensive rats exists [7]. SHR colonies are pre-hypertensive in the first 6 to 8 weeks of life, becoming definitively hypertensive after 12 to 14 weeks [8]. In adult life, the vascular resistance of the SHR is increased by making this animal very used in the hypertension research (stable chronic model similar to the human hypertension) [8, 9, 10].

The Goldblatt II (renovascular model, 2 kidneys, 1 clip) and the DOCA-salt models as well as the nitric oxide synthesis (NOs) blockade (induced by administration of an arginine analogous, L-NAME) are other usual models of experimental hypertension [8, 11]. The NOs blockade model is based on the regulation of the vascular tonus by the endothelium and the consequent arterial constriction and hypertension. As firstly observed by Furchgott and Zawadski [12], the endothelium is not just a physical barrier, but a structure with a pivotal role for the vasodilatation. The former "endothelium derived relaxing factor" (EDRF) is now identified as the NO [13-14]. The NO and the associated enzymes (the NO synthases) were defined not only in the endothelium but also in other organs and tissues, including the central nervous system [15-17].

The decrease of the NO in animals treated with L-NAME induces sustained and dose-dependent hypertension. The mechanism that causes and sustains the hypertension is multifactorial, and not just the vasomotor changes are responsible for the hypertension and for the alterations in the target-organs [18-21].

In hypertensive individuals the reduction of the BP is associated with reduction of the cardiac hypertrophy . However, experimental data suggest that several classes of antihypertensive agents have different effects on the left ventricular mass [22]. The class of antihypertensive known as "angiotensin-converting enzyme (ACE) inhibitors" reduces the BP and inhibits the generation of the hemodinamically active octapeptide angiotensin II (Ang II) from the inactive decapeptide angiotensin I (Ang I) [23]. The use of ACE inhibitors can lead to a partial or complete reversion of the hypertension and associated target-organ lesions in a NO-deficient model of hypertension [24, 25]. The purpose of the present work is to study the effects of an ACE inhibitor on the BP, correlated with the myocardium remodeling, when treated SHRs are simultaneously submitted to the NOs blockade.

Material and methods

Animals

Twenty adult male SHR were obtained from colonies maintained at the State University of Rio de Janeiro. The animals were 120 days old and had the tail BP superior to 140 mmHg at the beginning of the study. All animals were individually housed and maintained in a room with controlled temperature (21 ± 1°C), humidity (60 ± 10%), to 12-h-dark/light cycle (artificial lights, 7:00-19:00 h) and exhaustion cycle (15 min/h).

Rats were given food (Nuvilab®, Brazil) and water *ad libitum*. The animals stayed in cages for acclimatization during the first seven days. During this period, the average daily water intake was determined. This volume was used to dissolve drugs and to ensure the total intake of the planned daily drug dosage. All procedures were carried out in accordance with conventional guidelines for experimentation with animals (NIH Publication No. 85-23, revised 1996). The experimental protocols used in this study were approved by the Ethics Committee for Animal Experimentation at the State University of Rio de Janeiro.

The BP and body mass were weekly verified in all animals. The BP was verified through the non-invasive method of the tail-cuff plethysmography (Letica LE 5100, Panlab®). The rats were randomly divided in four groups of five animals, each and maintained for a period of 20 days:

a) Control (C) group: the animals were manipulated and sacrificed like the animals of the experimental groups; they only received water and food *ad libitum*.
b) L-NAME (L) group: the animals received L-NAME 30 $mg.kg^{-1}.day^{-1}$ (hydrochloride of N_{ω}-nitro-L-arginine-methyl-ester – Sigma Chemical Co.) dissolved in the drinking water.
c) L-NAME+Enalapril (L+E) group: the animals received L-NAME 30 $mg.kg^{-1}.day^{-1}$ plus an angiotensin-converting enzyme inhibitor, Enalapril maleate 15 $mg.kg^{-1}.day^{-1}$ ((S)-N-(1-(Ethoxycarbo-

Table 1. Descriptive statistics (mean ± standard deviation) of the Volume Weighted Nuclear Volume (VWNV), Heart mass/Body mass (HB) ratio and blood pressure (BP).
(*) is significant between consecutive weeks; ($) is significant between the initial BP and the BP in week 2; (#) is significant between the BP in weeks 1 and 3; (&) is significant between initial BP and the BP in week 3.

Groups	VWNV (μm^3)	HB ratio (%)	BP (mm Hg)			
			initial	week 1	week 2	week 3
Control (C)	89±55	0.27±0.01	155±5	154±8	162±9*	157±16
L-NAME (L)	279±198	0.30±0.05	151±4	190±101*	214±13*$	226±15*#&
L-NAME+Enalapril (L+E)	151±73	0.28±0.01	154±4	166±8*	180±19	211±7*#&
Enalapril (E)	64±31	0.24±0.02	155±8	146±8	137±6$	118±6*#&
Probability						
C vs. L	<0.03	NS	NS	0.0002	0.0002	0.0002
C vs. L+E	NS	NS	NS	0.04	0.04	0.0002
C vs. E	NS	0.04	NS	NS	0.007	0.0002
L vs. L+E	NS	NS	NS	0.0007	0.0007	NS
L vs. E	<0.03	0.005	NS	0.0002	0.0002	0.0002
L+E vs. E	NS	NS	NS	0.005	0.0003	0.0002

nyl)-3-phenylpropyl)-Ala-Pro maleate – Sigma Chemical Co.) dissolved in the drinking water.

d) Enalapril (E) group: the animals received Enalapril maleate 15 $mg.kg^{-1}.day^{-1}$ dissolved in the drinking water.

Biometrical and stereological analyses

In the afternoon of the 21st day, BP and body mass were measured and rats were deeply anesthetized (intraperitonial Thiopental 15 $mg.kg^{-1}$) and sacrificed (intracardiac injection of 10% KCl to stop heart in diastole). The heart was excised, dissected by separating the atria from the ventricles and the right ventricle from the left ventricle. In the day of the sacrifice the heart mass/body mass (HB) ratio was determined as $HB = (^{HM}/_{BM})$ (%).

The myocardial random and isotropic fragments were obtained by cutting the heart using the *orientator* method [31]. These fragments were place for 48 h at room temperature in fixative (freshly prepared 4% w/v formaldehyde in 0.1 M phosphate buffer pH 7.2), embedded in Paraplast plus® and sectioned in 3 and 10 μm thickness. Sections were stained by hematoxylin-eosin, Masson's trichrome and picro-sirius red. The analysis used a video-microscopic system composed of a Leica DMRBE microscope (x100 objective, numerical aperture 1.25) and a Kappa video camera. The test system M42 was put upon the screen of the monitor and calibrated with Leitz micrometer 1/100 mm [26].

The volume density (V_V) was determined by point counting to cardiac myocytes, connective tissue and intramyocardial vessels, $V_V=P_P/P_T$ (P_P is the number of points hitting the structure, and P_T is the total number of test points).

The length density of the vessels (L_V) was determined as $Lv=2 \cdot Q_A$ (Q_A is the number of cross-sectioned structures in the testing area).

The surface density (S_V) was determined to cardiac myocytes and intramyocardial vessels as $Sv=2 \cdot I / L_T$ (I is the number of intersections hitting the structure, and L_T is the total length of the test line).

The estimation of the volume-weighted mean nuclear volume (VWNV) was made by the "point-sampled intercepts" in isotropic and uniform random sections [27]. Five fields were analyzed per section, three sections of the animal tissue, and five animals per group (75 fields per group). The test-system consisting of parallel lines associated with test points was superposed on each microscopic field. The direction of the lines on the sample was randomly determined. For each point inside the unbiased counting frame, which hits a nucleus, the

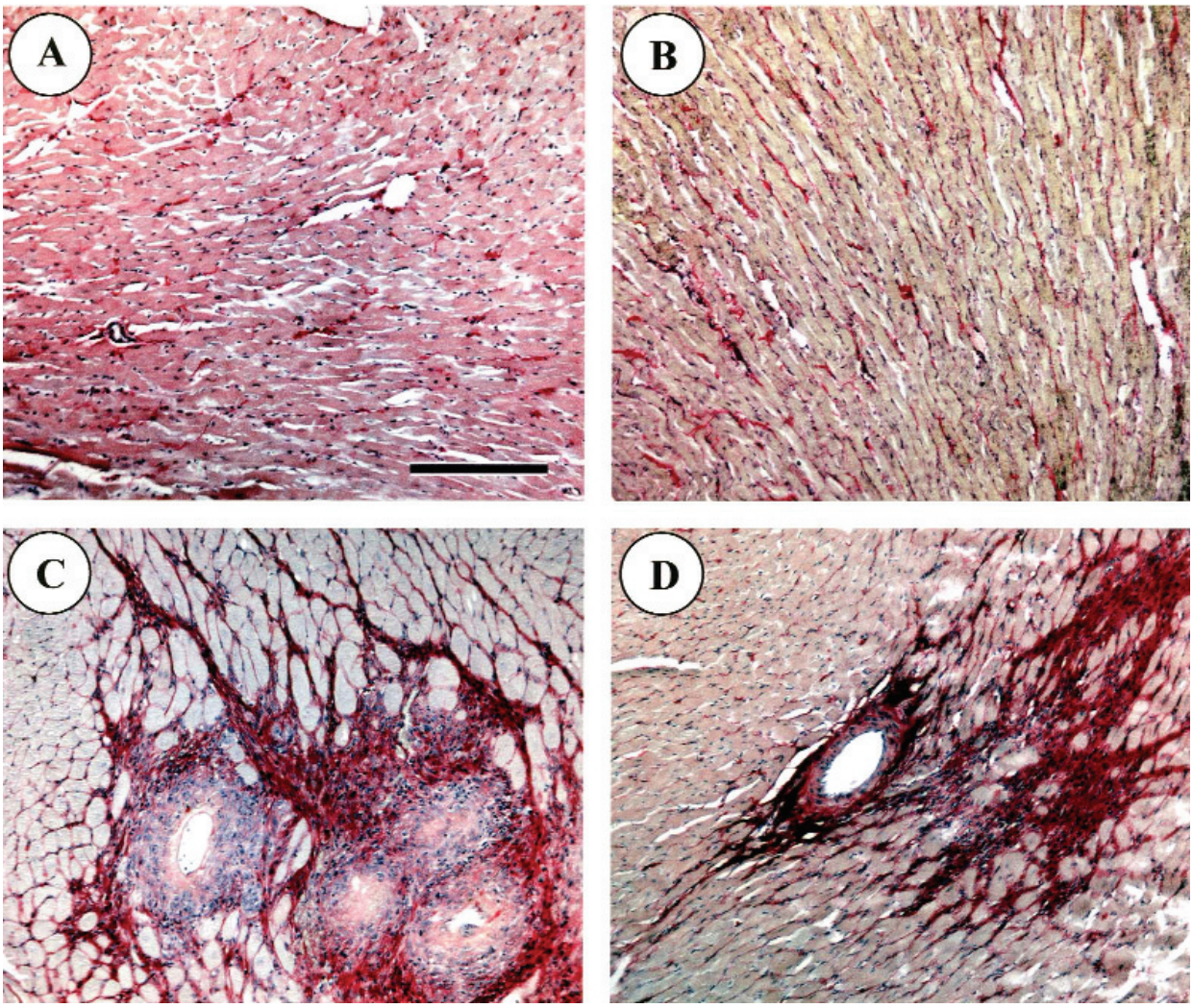

Fig. 1 Photomicrographs of the myocardium in spontaneously hypertensive rats (SHR) treated or not treated with nitric oxide inhibitor (L-NAME) and angiotensin-converting enzyme inhibitor enalapril. All sections were stained with Picro Sirius red and observed with non polarized light (calibration bar = 200μm). **A** and **B** – Good preservation of the myocardium in untreated SHR and SHR treated with enalapril. **C** – L-NAME treated SHR showing hypertrophied myocytes, interstitial and reparative fibrosis (red color) and tunica media hypertrophy of the intramyocardial vessels (some arterial branches are almost occluded). **D** – SHR treated with a combination of L-NAME and enalapril showing an attenuation of the myocyte hypertrophy in comparison with the L-NAME group but a still extensive interstitial fibrosis (red color).

nuclear intercept through the point was measured. The intercept length was measured by a 32-mm long logarithmic l^3 ruler composed of 15 classes, where the width of any class is approximately 17% larger than the preceding class [28]. Each individual intercept was cubed, and the mean of all these values was multiplied by π/3 in every case to give VWNV.

The numerical myocyte nuclear density in the plane, *i.e.*, the number of nuclear profiles per area (Q_A[m]) was also determined using a frame with 6,430 μm². The cross-sectional area of the cardiac myocytes (A[m]) was determined by the ratio between the V_V and the Q_A [26].

Statistical analysis

Differences of the biometrical parameters were analyzed using the ANOVA and the Newman-Keuls test, and of the stereological parameters using the Kruskall-Wallis

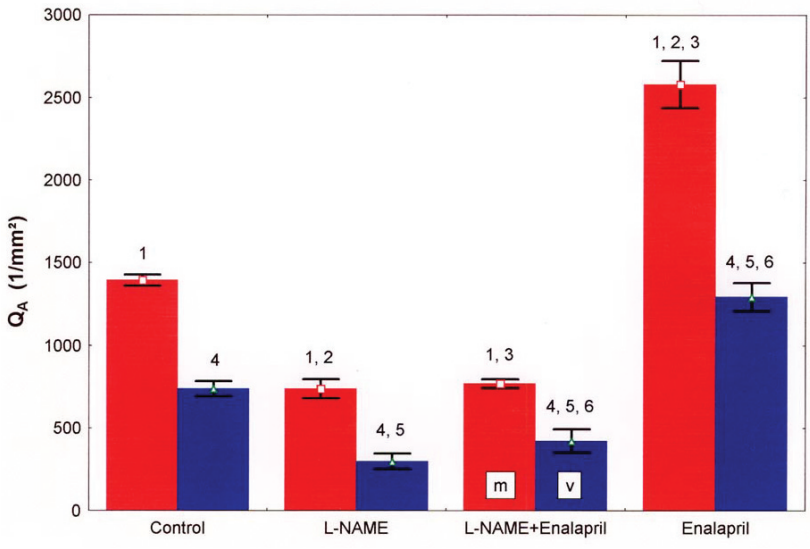

Fig. 2 Numerical density per area (QA) of cardiac myocytes (m) and intramyocardial vessels (v) among the groups. Numbers over the bars show significant difference ($p<0.05$).

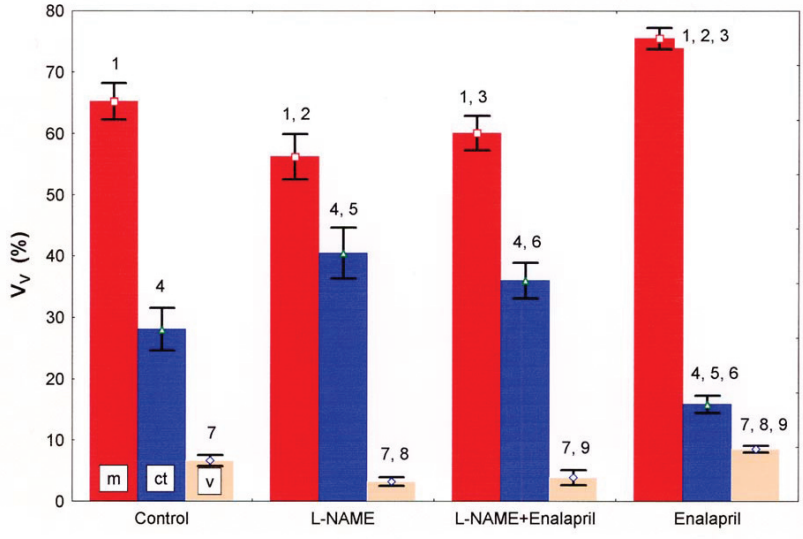

Fig. 3 Volume densities (Vv) of cardiac myocytes (m), connective tissue (ct) and intramyocardial vessels (v) among the groups. Numbers over the bars show significant difference ($p<0.05$).

non-parametric test, followed by the Kolmogorov-Smirnov test (Statistica version 5.5, Statsoft®), performed to determine which differences were significant (the differences between groups were tested with significance level of 0.05) [29].

Results

Table 1 and Fig. 1 to 6 summarize the results. Fig. 1 compares the myocardial structure among the groups.

Blood pressure and cardiac hypertrophy

The HB ratio was significantly smaller in the E group when compared to the L or C groups (-22% and -13%, respectively). The BP in the L group increased every week; during the last week there was an increase of 50% since the initial BP. Final BP of L+E group was 37% higher comparing to initial levels. The BP in the E group decreased 25% concerning the initial BP of this group. There were no differences in BP when L and L+E groups were compared at the end of experiment (Table 1).

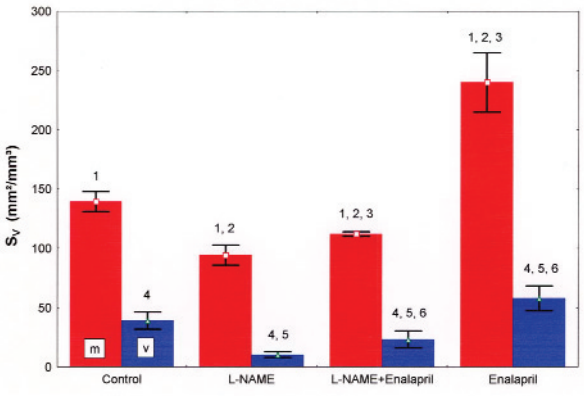

Fig. 4 Surface densities (Sv) of cardiac myocytes (m) and intramyocardial vessels (v) among the groups. Numbers over the bars show significant difference ($p<0.05$).

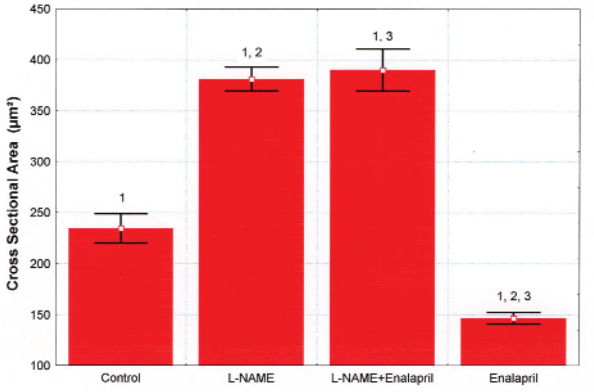

Fig 5 Cardiac myocytes cross sectional area among the groups. Numbers over the bars show significant difference ($p<0.05$).

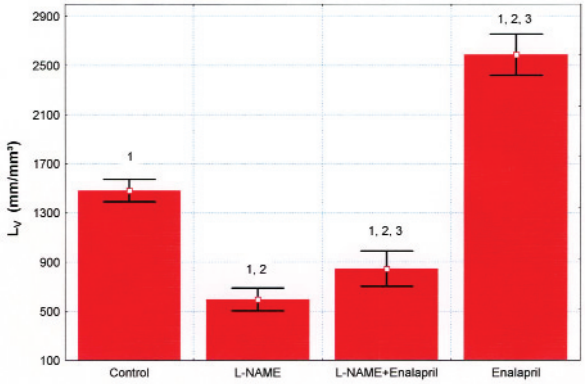

Fig. 6 Length density (Lv) of intramyocardial vessels among the groups. Numbers over the bars show significant difference ($p<0.05$).

Greatest values for intramyocardial vessels and smallest values for connective tissue were found in E. The V_V[v], the S_V[v] and the L_V[v] were greater in the E group, with more than 30, 50 and 70% than the C group, more than 160, 450 and 300% than the L group, and, finally, more than 120, 150 and 200% than the L+E group. Contrarily, the V_V[ct] of the E group was 40% smaller than the C group and more than 50% smaller than the L and L+E groups (Fig. 3, 4 and 6). The NO deficient SHRs treated with Enalapril (L+E group), compared with untreated NO deficient SHRs (L group), showed an increase of Q_A[v] (+40%, Fig. 2), an increase of S_V[v] (+125%, Fig. 4) and an increase of L_V[v] (+40%, Fig. 6).

Stereology

In the Enalapril treated group cardiac myocytes were more abundant and have small size. Comparing with the E group the Q_A[m] was 70% smaller in the L+E and L groups and 40% smaller in the C group (Fig. 2). The V_V[m] was 20% smaller in the L+E and L groups and 10% smaller in the C group (Fig. 3). The S_V[m] was also greater in the E group than in the other groups (Fig. 4), but the A[m] and the VWNV were smaller in the E group, more than 30% smaller than in the C group, and more than 50% smaller than the L and L+E groups (Fig. 5 and Table 1). Differences between the L and L+E groups were not significant except for S_V[m] (Fig. 4).

Discussion

The present study observed the impact of the L-NAME administration in young adult SHRs, as well as the beneficial effect of Enalapril in these animals. Important structural myocardial alterations could be observed and quantified after three weeks of treatment. The SHRs showed cardiac hypertrophy, interstitial fibrosis and rarefaction of intramyocardial vessels. The present work studied five animals per group. A reason for the choice of five is that if something is found to increase (or decrease) in all five cases, then the probability that this is due to chance is $P = (½)^5 < 0.05$, and the experiment

could be conclusive, whereas this would not be the case with four or fewer animals per group [30].

The chronic use of competitive inhibitors of NOs, such as L-NAME, is related to sustained hypertension and myocardial hypertrophy, which is partially or totally reduced by antihypertensive drugs [1, 3, 4, 31-34]. ACE inhibitors are particularly useful in reducing myocardial mass [1, 35]. Although there is an intimate relation between NOs and Ang II [36], the effects of ACE inhibitors is independent of NOs expression in endothelium and NOs activity [32, 37, 38]. The L-NAME administration causes more intense myocardial ischemic alterations than other models of hypertension as Goldblatt II [39], with extensive ischemic necrosis and substitution fibrosis [39, 40].

In SHRs the endothelium-mediated vasodilatation is impaired and the expression of endothelial NOs is reduced in coronary and cerebral arteries [38]. L-arginine reduces left ventricular hypertrophy in SHRs without reducing BP [41] but not in salt-sensitive rats [42]. SHRs have more ACE activity in the aorta and in the coronary arteries than age-matched Wistar-Kyoto rats, although the plasma enzyme activity does not appear to be different. The impact of the treatment of SHR with ACE inhibitors or AT_1 receptor antagonist suggests a pivotal role of Ang II in SHR hypertension [11].

Different studies reported that the NO deficiency in Wistar rats causes cardiac hypertrophy [1, 32, 33, 43, 44], but this matter is still controversial [45-47]. A greater HB ratio was found in untreated SHRs than in matched-age Wistar-Kyoto rats [33], but in SHRs with and without NO deficiency the HB ratio was not significantly different [48]. In the present study, the Enalapril efficiently treated the normal SHRs cardiac hypertrophy, but not the cardiac hypertrophy observed in NO deficient SHRs. Previous studies reported that Wistar-Kyoto rats, the original strain of SHRs, respond in different ways to other antihypertensive drugs, as Nifedipine (a calcium channel blocker) or Hydralazine (a vasodilating antihypertensive agent) not as favorable as the treatment with ACE inhibitors [34, 49, 50].

SHRs with NOs blockade showed an outstanding reduction in the cardiac myocytes population. The decline in myocyte number seems to be due mainly to necrosis, as extensive areas of substitution fibrosis are present, probably by scarring of myocardial infarctions. These morphological features are in accordance with previous studies [1, 3, 43, 44, 47, 51]. The Enalapril treatment did not cause significant differences in the degree of fibrosis from untreated L-NAME SHRs, both presenting large areas of necrosis. This fact was also reported in Wistar-Kyoto rats [52].

Hypertrophied myocardium has a decrease in the capillary to fibers ratio, as the number of capillaries remains the same and connective tissue and A[m] are increased. This leads to a relative ischemia, which is proportional to hypertrophy degree as observed in Wistar-Kyoto rats after 8 weeks of L-NAME administration [53]. In hypertension vascular cell hypertrophy and hyperplasia, as well as deposition around the vessels of extracellular matrix proteins, particularly collagen and elastin, occur. The process of vascular remodeling is mediated by some growth factors, as PDGF (platelet-derived growth factor) and TGF-β (transforming growth factor β), stimulated by shear stress in the vessel wall [54]. These alterations can even more prejudice the vascularization of myocytes. In the present study, the NO deficient SHRs had an impressive reduction of intramyocardial vessels with important perivascular fibrosis.

Nifedipine seems to induce angiogenesis in 24 weeks old SHRs, but not a significant decrease in the myocardial mass [55]. The L-NAME administration inhibits the NO production and NO has a pivotal role in angiogenesis [56]. The myocardial hypertrophy accompanying the NO-deficient hypertension has some typical characteristics of hypertrophy induced by pressure overload, *i.e.*, increased size of cardiac myocytes and relative rarefaction of the capillary bed [53]. Recent stereological study [57] reported greater vascular surface and length densities in young adult SHRs suggesting increased capillarization (but not angiogenesis) in these hypertensive rats.

In the present study, the effect of the Enalapril treatment in NO-deficient SHRs was not significant in attenuating myocyte hypertrophy, but the intramyocardial microvasculature was significantly improved. The literature reported a better microcirculation with ACE inhibitors [4] and increase in cellular activity in endothelium [58]. Instead of better microcirculation, in the present study, L-NAME and L-NAME+Enalapril treated SHRs had extensive areas of healed infarctions (similar results a with previous report) [52]. These data suggest that this

microvascular improvement does not have a great impact in preventing major cardiovascular ischemic events. Many other factors must be involved in the pathogenesis of these infarctions, besides the relative rarefaction of capillaries. One of these can be the antiaggregant effect of NO [59], which is lost in the administration of L-NAME, possibly leading to thrombosis on instable coronary plaques. In this study, rats were analyzed after the period of normal cardiac angiogenesis, which ceased almost completely at 45 days of life [57]. Other studies demonstrated a better vascularization in the SHR treated with ACE inhibitors since 5 weeks of age or from the intrauterine life, but not in adult SHR [60-62].

In conclusion, the Enalapril administration showed a beneficial effect on vascular remodeling and myocardial hypertrophy in SHRs. In SHRs with NO blockade, however, the beneficial effect of Enalapril concerned only vascular remodeling. Further studies are still necessary to fully understand the pathophysiology of the cardiac alterations in experimental hypertension using NO synthesis blockade and ACE inhibitors in the SHR model.

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