Enalapril attenuates cardiorenal damage in nitric-oxide-deficient spontaneously hypertensive rats

Leila M. M. PEREIRA, Daniele G. BEZERRA, Denise L. MACHADO and Carlos A. MANDARIM-DE-LACERDA
Laboratory of Morphometry and Cardiovascular Morphology, Biomedical Centre, Institute of Biology, State University of Rio de Janeiro, Av. 28 de Setembro, 20551-030 Rio de Janeiro, Brazil

ABSTRACT
Stereological structural alterations of the heart and kidney were studied in four groups (n = 5) of spontaneously hypertensive rats (SHRs) treated for 30 days: (i) control, (ii) N\textsuperscript{G}-nitro-L-arginine methyl ester [L-NAME; nitric oxide (NO) synthesis inhibitor] alone, (iii) enalapril alone and (iv) L-NAME plus enalapril. Blood pressure (BP) was elevated significantly in NO-deficient SHRs (rats receiving L-NAME) or significantly lower in enalapril-treated SHRs. Co-administration of L-NAME and enalapril caused a 20% decrease in BP compared with untreated SHRs. NO-deficient SHRs had a decrease in body mass, but this loss of body mass was prevented efficiently in the enalapril-treated group. Enalapril treatment decreased the left ventricular (LV) mass index in SHRs, even in animals with NO synthesis blocked. NO deficiency in SHRs caused a larger decrease in the number of LV cardiomyocyte nuclei, which had a negative correlation with both LV mass index and BP. The volume-weighted glomerular volume (VWGV) separated the SHRs into two groupings: (i) control and NO-deficient SHRs, and (ii) enalapril- and L-NAME plus enalapril-treated SHRs. There was a significant difference between these two groupings, with VWGV being more than 15% smaller in the latter compared with the former grouping. The present findings reinforce the evidence that enalapril efficiently treats genetic hypertension, and demonstrate that this effect is observed even when NO synthesis is inhibited. Enalapril administration also decreases cardiac and renal structural damage caused by genetic hypertension, as well as by the interaction between genetic hypertension and NO deficiency.

INTRODUCTION
Early studies on vascular disease in animal models with hypercholesterolaemia, diabetes and hypertension have established that interfering with the function of nitric oxide (NO) is a major consequence of, and it is potentially related to, the cardiovascular risk factor [1]. Acute and chronic administration of NO synthase inhibitors, such as N\textsuperscript{G}-nitro-L-arginine methyl ester (L-NAME), can increase regional vascular resistance and raise the blood pressure (BP) [2–4] and alter renal function in a dose-dependent manner in rats [5,6]. Given acutely, L-NAME has an antinatriuretic effect that is overridden by the increase in perfusion pressure, and both endothelin and angiotensin II (Ang II) play a role in the systemic and renal haemodynamic alterations associated with impaired NO availability [7,8].

The renin–angiotensin–aldosterone system (RAAS) plays an important role in renal and cardiac functions as part of the system’s overall control of water and

Key words: enalapril, heart, hypertension, kidney, nitric oxide (NO), stereology.
Abbreviations: ACE, angiotensin-converting enzyme; ACEI, ACE inhibitor; Ang II, angiotensin II; BP, blood pressure; L-NAME; N\textsuperscript{G}-nitro-L-arginine methyl ester; LV, left ventricular; [cm\textsuperscript{3}] LV cardiomyocyte nuclei number; NO, nitric oxide; [cm\textsuperscript{3}], numerical density of cardiomyocyte nuclei; RAAS, renin–angiotensin–aldosterone system; SHR, spontaneously hypertensive rat; VWGV, volume-weighted mean glomerular volume.
Correspondence: Dr Carlos Alberto Mandarim-de-Lacerda (e-mail mandarim@uerj.br).
salt homeostasis [9]. Studies focusing on RAAS inhibition led to the discovery of angiotensin-converting-enzyme (ACE) inhibitors (ACEIs), which proved efficacious in the treatment of hypertension, various cardiovascular disorders and renal diseases [10,11]. ACEIs were described as antihypertrophic substances in spontaneously hypertensive rats (SHRs) [12].

In prevention studies, the blockade of RAAS markedly reduced hypertension and the cardiac and renal morphological alterations associated with l-NAME administration in Wistar rats [13–15]. However, administration of l-NAME to genetically hypertensive rats enhances the hypertension-causing factors which greatly increase morbidity [16]. The present study was undertaken to investigate the efficiency of ACEI treatment on cardiac and renal structural alterations in SHRs subjected to NO synthase inhibition. The focus of the study was on BP control and the quantitative alterations of the myocardium and glomeruli.

**MATERIAL AND METHODS**

**Samples and procedures**

Mature male SHRs (n = 20; 25-weeks old) were obtained from colonies maintained at the State University of Rio de Janeiro. All animals were housed individually in a temperature- (21 ± 1 °C) and humidity-controlled (60 ± 10 %) room, subjected to a 12-h dark/light cycle (artificial lights, 19.00–07.00 hours) and air-exhaustion cycle (15 min/h). Animals were acclimatized for 1 week. During this period, the average daily water intake per animal was determined to guarantee the total intake of the planned daily drug dosage.

BP and body mass of the rats were measured weekly. BP was verified in conscious rats through the non-invasive tail-cuff plethysmography method (Letica LE 5100; Panlab, Barcelona, Spain). The animals were kept separately in four groups (n = 5 per group) for 30 days as follows: (i) control group receiving food daily (Labina; Purina, Sao Paulo, Brazil) and fresh water *ad libitum*; (ii) l-NAME group (NO-deficient group) receiving 40 mg·day⁻¹·kg of body weight⁻¹ l-NAME (Sigma, St Louis, MO, U.S.A.) dissolved in the drinking water; (iii) enalapril group receiving 30 mg·day⁻¹·kg of body weight⁻¹ enalapril maleate (Sigma) dissolved in the drinking water; and (iv) l-NAME plus enalapril group receiving l-NAME and enalapril simultaneously at a similar dose and route of administration as indicated for groups receiving l-NAME and enalapril alone.

On the morning of day 31 of experimentation, animals were anaesthetized (sodium thiopental, intraperitoneally) and killed by exsanguination.

All procedures were carried out in accordance with the Conventional Guidelines for Experimentation with Animals (NIH Publication No. 85-23, revised 1996). The experimental protocols used in the present study were approved by the Ethics Committee for Animal Experimentation, State University of Rio de Janeiro.

**Tissue processing**

At the end of the experiment, the heart and kidneys were removed. The ventricles were separated from the atria and were dissected and weighed separately. The interventricular septum was considered as a portion of the left ventricle. The left ventricular (LV) volume was determined according to the submersion method [17] in which the water displacement due to organ volume was recorded by weighing. As the specific gravity (σ) of isotonic saline is 1.0048, the volume is obtained by the equation: volume = weight/σ, or simply volume = weight [18]. The LV mass index was determined as LV mass/body mass and was used to evaluate LV hypertrophy.

Tissue fragments (LV and left kidney) were incubated for 48 h at room temperature in fixative [freshly prepared 4 % (w/v) formaldehyde in 0.1 M sodium phosphate buffer (pH 7.2)] [19], embedded in Paraplast plus® (Sigma), sectioned at 5 µm thickness and stained with Sirius Red (heart) or Masson Trichrome (kidney).

**Determination of structural alterations in the myocardium**

An optical dissector was used to calculate the numerical density of cardiomyocyte nuclei (Nv[cmn]) [20], defined by two parallel sections separated by a 3 µm distance (automatically controlled with the motorized stage of the Leica microscope). Estimated Nv[cmn] was determined by analysing 15 random dissector pairs for each specimen using the equation: $N_{v[cmn]} = Q^-/(t \times A_T)$, where $Q^-$ is the number of nuclei seen in focus only in the dissector’s ‘look-up’ plane when they were partly or totally inside the frame and did not intersect the right and inferior exclusion edges or their extension, $t$ is the thickness and $A_T$ was the dissector’s test area. The ‘look-up’ and the ‘look-down’ planes were determined over a frame area of 3260 µm² [21–23]. The LV cardiomyocyte nuclei number (N[cmn]) was calculated as the product of Nv[cmn] and the LV volume (measured using the Scherle’s method) [17,24].

**Determination of structural alterations in the kidney**

The cortex of the left kidney was analysed according to the vertical section design [25]. The kidneys were divided in two halves and both were placed on the cut surface. One half was rotated randomly and the other was rotated by 90° with respect to the first. Four sections were systematically uniformly sampled at random and were all vertical sections [26]. An estimate of the volume-weighted mean glomerular volume (VWGV) was made using the ‘point-sampled...
intercepts’ method [27]. For this procedure, five fields were analysed per section, with three sections per kidney and five animals per group (75 fields per group). A test system consisting of parallel lines associated with test points was superimposed on each microscopic field. The direction of the lines on the sample was determined randomly. Each point inside the unbiased counting frame, which hits a glomeruli intercept through the point, was measured. The measurement of the intercept length was performed using a 32-mm long logarithmic \( l_3 \) ruler composed of a series of 15 classes, where the width of any class was approx. 17% larger than the preceding class [28]. Each individual intercept was cubed, and the mean of all values was multiplied by \( \pi/3 \) in every case to obtain VWGV.

Statistical analysis
Biometry and VWGV differences between the groups were tested by ANOVA and the Newman–Keuls post-hoc test. Differences in the cardiomyocyte nuclei number between groups, two by two, were tested with the non-parametric Mann–Whitney test. Pearson’s coefficient of correlation and the corresponding linear regression fitted by the least-squares method were determined for \( \text{N}[\text{cmn}] \) against BP and LV mass index. The level of 0.05 was used for statistical significance [29]. All analyses were performed using GraphPad Prism® version 4.0 for Windows® (GraphPad Software, San Diego, CA, U.S.A.).

RESULTS
Effects of enalapril on BP and body mass in SHR
BP was significantly higher in NO-deficient SHRs (rats receiving \( \text{l-NAME} \)) and significantly lower in enalapril-treated SHRs compared with untreated controls. BP in NO-deficient SHRs was more than 40% higher than untreated SHRs, whereas a 25% decrease in BP was found in enalapril-treated SHRs. Co-administration of \( \text{l-NAME} \) and enalapril resulted in a 20% decrease in BP compared with untreated SHRs at the end of the study (Figure 1).

A decrease in body mass was observed in NO-deficient SHRs (more than 10% when compared with untreated animals), but enalapril treatment efficiently prevented this (results not shown).

Effects of enalapril on the myocardium in SHR
Enalapril treatment decreased the LV mass index in SHRs, even in animals in which NO synthesis was inhibited (Figure 2). NO deficiency in SHRs resulted a greater decrease in \( \text{N}[\text{cmn}] \). In SHRs treated with enalapril, \( \text{N}[\text{cmn}] \) was 70% higher than in the control rats, and it was 130% higher than in NO-deficient rats (Figure 3). \( \text{N}[\text{cmn}] \) was negatively correlated with both BP (Figure 4) and LV mass index (results not shown).

Effects of enalapril on the kidney in SHR
Analysis of VWGV allowed the SHRs to be separated into two major groupings. The first grouping consisted of the control and the NO-deficient SHRs, and the second grouping was composed of the enalapril- and the \( \text{l-NAME} \) plus enalapril-treated SHRs. No difference in VWGV was found within the groupings (i.e. control
Figure 3  N(cm²) in the different groups

P < 0.05 when compared with the control group (a), the L-NAME group (b) or enalapril group (c), as determined by Mann–Whitney test.

Figure 4  Correlation and linear regression of N(cm²) as the dependent variable against BP as the independent variable

The individual values for each of the different group are marked (+). Solid line is the regression equation: N(cm²) = 528 × 10⁴ + 257; Pearson's coefficient of correlation r = −0.76 (P < 0.001). The regression equation with LV mass index as the independent variable is: N(cm²) = 805 × 10³ + 265; r = −0.53 (P < 0.02). Broken lines indicate 95% confidence intervals.

compared with NO-deficient SHRs); however, there was a significant difference between the two groupings, with VWGV being more than 15% lower in the second grouping (enalapril and L-NAME plus enalapril SHRs) compared with the first grouping (Figure 5).

DISCUSSION

In the present study, cardiac hypertrophy (measured by the increase in LV mass index) was not affected significantly by the inhibition of NO synthesis (L-NAME administration). The administration of a dose of enalapril causing hypotension was efficient in decreasing BP and LV hypertrophy and attenuating/preventing the loss of

LV cardiomyocytes and glomerular hypertrophy in both control and NO-deficient SHRs. This is an important finding, given that LV hypertrophy occurrence in SHRs seems to be genetically determined, because it is observed before BP elevation [30], and suggests that enalapril may act on this morbid condition of genetic hypertension. In contrast with SHRs, LV hypertrophy and a decrease in cardiomyocyte number caused by NO synthesis inhibition were efficiently treated with the use of enalapril (ACEI) or verapamil (Ca²⁺-channel blocker) in formerly normotensive Wistar rats [14].

NO is an important molecular messenger that plays a pivotal role in vascular relaxation, neuronal transmission and immune modulation, and is essential for cardiovascular homeostasis in Wistar rats [6,14,31,32]. In SHRs, BP normally increases with age and, in the present study, SHRs showed an additional BP increment when L-NAME was administered (usual BP in adult male SHRs from our colony is 170 ± 9.6 mmHg; mean ± S.D.). Data in the literature provide enough evidence that ACEIs are able to treat LV hypertrophy in pressure and volume overload of hearts in SHRs [8,15,33,34]. The beneficial effect of ACEIs in the treatment of hypertension is well known, resulting in BP control and a decrease in mortality of approx. 20%, along with a similar decrease in the ACE activity [35,36].

The ventricular hypertrophic process results from a continuous interaction between vasoconstriction and growth-supporting factors on one hand and vasodilatation and antiproliferative factors on the other [37]. Various classes of antihypertensive agents have different effects on LV mass, but the mechanisms by which antihypertensive agents attenuate LV hypertrophy are still controversial and seem to be multifactorial (whether the regression of cardiovascular alterations is only due to a decrease in BP or a consequence of growth factor inhibition remains controversial) [38,39]. There are two possible mechanisms by which ACEIs exert their beneficial effects on cardiovascular remodelling.
First, ACEIs may act specifically on ACE to inhibit the formation of Ang II. Secondly, because ACE is identical with kininase II, its elevation may have inhibited the breakdown of bradykinin, such that the resultant increase in NO release might have contributed to the beneficial effects of ACEIs [40]. Table 1 compares the different studies using ACEIs in recent years to treat LV hypertrophy and its consequences. 

Administration of 1-NAME in formerly normotensive Wistar rats causes myocardial ischaemia, followed by injury and cell death mainly through necrosis, but also through apoptosis [41–43], and enalapril is efficient in attenuating this loss of cardiomyocytes [8] and also glomerular hypertrophy [15] in NO-deficient Wistar rats. SHRs have a normal decrease in cardiomyocytes with age [44]. In the present study, NO-deficient SHRs had a decrease in N[cmn] compared with the control SHRs after 4 weeks of treatment. Administration of enalapril to control or NO-deficient SHRs during a similar period of 4 weeks appeared to attenuate this loss of LV cardiomyocytes.

The question of apoptosis occurrence due to hypertension and heart failure is still controversial. When young rats are compared with aged rats, myocardial changes suggest the loss of cardiomyocytes with simultaneous hypertrophy of remnant cardiomyocytes in aged rats [45]. Inhibition of NO synthesis increased apoptotic cardiomyocyte death and local ACE mRNA expression in ischaemia/reperfusion-injured myocardium, suggesting that NO, ACE and apoptotic cardiomyocyte death are related to each other during myocardial injury [46]. Renovascular hypertension induces a rapidly developing LV hypertrophy characterized by marked cardiac remodelling and substantial loss of cardiomyocytes [47]. Increased numbers of apoptotic cells are present in failing SHR hearts, suggesting that apoptosis might be a mechanism involved in the decrease in myocyte mass that accompanies the transition from stable compensation to heart failure in this model. Administration of captopril (an ACEI), which ameliorates heart failure in this model, was associated with a decrease in apoptosis that accompanies heart failure [48]. Cardiomyocyte apoptosis continues at a high level after myocardial infarction and contributes to adverse cardiac remodelling. Omapatrilat-treated rats (a drug which causes simultaneous inhibition of both ACE and neutral endopeptidase) have less cardiomyocyte apoptosis and less adverse cardiac remodelling compared with rats treated with a selective ACEI, neutral endopeptidase or placebo [49]. However, other experimental studies failed to detect significant differences in the number of terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL)-positive cells in Sprague–Dawley rats and also in failing and non-failing SHRs [50].

### Table 1: Different ACEI treatments in SHRs and their results in the literature

<table>
<thead>
<tr>
<th>Authors</th>
<th>ACEI</th>
<th>Dose (mg·day⁻¹·kg⁻¹)</th>
<th>Duration</th>
<th>Conclusions draw by the authors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Childs et al. [56]</td>
<td>Enalapril</td>
<td>25</td>
<td>2 to 9 weeks</td>
<td>LV mass index was regressed</td>
</tr>
<tr>
<td>Kett et al. [57]</td>
<td>Enalapril</td>
<td>25 to 30</td>
<td>4 to 10 weeks</td>
<td>Treatment did not prevent vessel wall hypertrophy of both the interlobular and arcuate arteries</td>
</tr>
<tr>
<td>Brilla et al. [58]</td>
<td>Lisinopril</td>
<td>20</td>
<td>32 weeks</td>
<td>LV hypertrophy completely reverted</td>
</tr>
<tr>
<td>Mitchell et al. [59]</td>
<td>Zofenopril</td>
<td>0.07 % g/rat chow</td>
<td>6 months</td>
<td>LV mass index was regressed</td>
</tr>
<tr>
<td>One et al. [60]</td>
<td>Quinapril</td>
<td>3</td>
<td>3 weeks</td>
<td>Reversed l-NAME; exacerbated severe nephrosclerosis</td>
</tr>
<tr>
<td>Ennis et al. [33]</td>
<td>Enalapril</td>
<td>20</td>
<td>5 weeks</td>
<td>Completely regressed cardiac hypertrophy</td>
</tr>
<tr>
<td>Francischetti et al. [61]</td>
<td></td>
<td>15 to 30</td>
<td>3 weeks</td>
<td>High dose of enalapril decreased relative LV mass</td>
</tr>
<tr>
<td>Rizzoni et al. [37]</td>
<td>Enalapril</td>
<td>1 to 25</td>
<td>8 weeks</td>
<td>Decreased LV mass index and LV collagen concentration</td>
</tr>
<tr>
<td>Susic et al. [62]</td>
<td>Enalapril</td>
<td>30</td>
<td>12 weeks</td>
<td>Enhanced vascularization in the adolescent SHRs with a decrease in LV hypertrophy. Decreased LV hypertrophy, but did not enhance vascularization in the adult SHRs</td>
</tr>
<tr>
<td>Black et al. [34]</td>
<td>Perindopril</td>
<td>0.1 and 1</td>
<td>7 to 14 and 16 to 24 weeks</td>
<td></td>
</tr>
<tr>
<td>Saleh and Jurjus [63]</td>
<td>Enalapril</td>
<td>150 mg/l</td>
<td>21 weeks</td>
<td>Prevented LV, aortic arch and kidney hypertrophies and the thickening of the aortic media layer seen in untreated SHRs</td>
</tr>
<tr>
<td>Gasparo et al. [64]</td>
<td>Enalapril and valsartan</td>
<td>1 and 5</td>
<td>4 weeks</td>
<td>Improved endothelial function and tissue injury in SHRs receiving l-NAME</td>
</tr>
<tr>
<td>Zorzi et al. [44]</td>
<td>Enalapril</td>
<td>15</td>
<td>3 weeks</td>
<td>Beneficial effect on vascular remodelling and myocardial hypertrophy. In SHRs with NO blockade, the beneficial effect of enalapril occurred only in vascular remodelling</td>
</tr>
<tr>
<td>Present study</td>
<td>Enalapril</td>
<td>30</td>
<td>4 weeks</td>
<td>Reduction of LV and glomerular hypertrophies</td>
</tr>
</tbody>
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Experimental and clinical studies have demonstrated that antihypertensive agents have a protective effect in various types of kidney diseases. The major mechanism by which antihypertensive drugs exert renoprotective effects is due to a decrease in BP, and ACEIs appear to be the most effective drugs offering renoprotection [51]. When considering renal function and morphology, the long-term effect of L-NAME in primarily normotensive Wistar rats or SHRs resulted in intrarenal vascular, tubular and glomerular injury with a consequent decrease in renal function [7,13,52]. The short-term pressor and renal vascular responses to Ang II are apparently mediated by the Ang II type 1 (AT1)-receptor in both SHRs and Wistar–Kyoto rats, and the renal vascular response to Ang II is enhanced in SHRs compared with Wistar–Kyoto rats when endogenous production of Ang II is minimized by captopril pretreatment [53]. ACEIs delay the progressive loss of function due to hypertension by normalizing the glomerular haemodynamics and attenuating the glomerular and interstitial fibrosis [11,54,55]. In the present study, we observed glomerular hypertrophy in control and NO-deficient SHRs, which was attenuated by enalapril treatment.

In conclusion, the results of the present study reinforce the findings that the ACEI enalapril efficiently treats genetic hypertension, and demonstrate that this effect occurs even when NO synthesis is inhibited. Enalapril administration also attenuated cardiac and renal structural damage caused by genetic hypertension and by the interaction between genetic hypertension and NO deficiency as well.

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