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Renal cortex remodeling in nitric oxide deficient rats treated with enalapril

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Abstract

The kidney NO synthase is one of the most important renal controlling systems. This paper aims the quantification of renal cortical components involved in blood pressure regulation under NOs blockade. Spontaneous hypertensive rats (SHRs) are submitted to chronic blockade of NOs by L-nitro-arginine-methyl-ester (L-NAME) and an ACE inhibitor (enalapril) in comparison with the normotensive Wistar rats. Twenty SHRs and 5 Wistar rats were divided in 5 groups and observed for 21 days for blood pressure (BP) and serum creatinine: control Wistar (5) (C-W), control SHR (5) (C-SHR), L-SHR (5) - received L-NAME 30 mg/kg/day, L+E-SHR (5) - received L-NAME and Enalapril maleate 15 mg/kg/day, E-SHR (5) - received Enalapril maleate. A quantitative morphometric study (glomerular density, Q_A[gl], interstitium volume density, Vv[i], tubular surface and length densities, Sv[t] and Lv[t]) were performed at the end. The BP reached 226±15 mmHg in L-SHR group. The BP difference between the L-SHR and the C-SHR groups was significant from the first week while the E-SHR group became significant from the second week. At the end of the experiment the BP of the E-SHR group was similar to the BP in the C-W group. The Q_A[gl] was similar among C-SHR, L-SHR and L+E-SHR groups and no difference was found between E-SHR and C-W groups. In the L-SHRs serum creatinine was greatly increased, and microscopy showed thickening of arteriolar tunica media with an increase of the wall-to-lumen ratio, perivascular fibrosis, inflammatory infiltrated, tubular atrophy and interstitial fibrosis with focal segmental glomerulosclerosis. The use of enalapril was not completely efficient in reducing BP and morphological injury when the hypertension of SHRs was increased with the NOs blockade suggesting that NO deficiency-induced hypertension is not entirely mediated by the RAAS.

Keywords: Hypertension - kidney - nitric oxide - rennin-angiotensin system - ACE inhibitor - stereology

Introduction

The nitric oxide (NO) is a potent endothelial vasodilator with a short half-life produced in many essential places, such as: central nervous system, cardiovascular and gastrointestinal system, immune system and urinary system [1, 2]. In the kidney, NO is produced by endothelial, mesangial and macula densa cells and is involved in the regulation of the glomerular and medullar microcirculation [3]. This helps to maintain the relatively low vascular resistance that is characteristic for the kidney [4]. The NO blockade causes systemic hypertension [5, 6].

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The NO produced within the kidney controls the glomerular filtration rate, the total renal and medullar blood flow, the pressure natriuresis, the epithelial sodium transport and the production of various vasoactive factors including renin, indicating that NO is one of the most important systems controlling renal function [7, 8]. Renal morphologic alterations with NO synthase inhibition include global glomerulosclerosis, collapse of capillary loops, adhesion to parietal layer of Bowman's capsule, focal segmental glomerulosclerosis, tubular atrophy, focal tubular necrosis areas and extensive interstitial fibrosis [9, 10].

The spontaneously hypertensive rats (SHR) are a genetic model of hypertension. The renal vasculature of the SHR, like resistance vessels of other beds in experimental and genetic forms of hypertension, is characterized by increases in the wall-to-lumen ratio due to vascular hypertrophy and remodeling [11].

Quantification of glomerular structure is important in order to aid the identification of changes in renal structures. The number, size and distribution of glomeruli, cells and other components contain important information about the function and organization of the kidney [12] and it can be obtained by modern stereological methods that are accurate (unbiased) and precise (reproducible) [13, 14].

The present study aims to analyze the renal cortical remodeling and renal function when SHRs are or not simultaneously submitted to chronic blockade of NO and treated with an angiotensin-converting enzyme inhibitor (ACEi) in comparison with the normotensive Wistar strain rats.

Material and methods

Sample and procedures

Twenty SHRs and five Wistar strain rats, all males, obtained from colonies maintained in the State University of Rio de Janeiro, were used in this study. At the beginning of the study animals were 120 days old, SHR weighting 251±49 g (mean±SD) and a systolic blood pressure (BP) of 152±6 mmHg and Wistar rats weighting 260±72 g and BP of 127±5 mmHg.

Rats were individually housed in temperature-controlled (21±1ºC) and humidity-controlled (60±10%) room submitted to a 12h-dark/light cycle (artificial lights, 7:00-19:00h) and to air exhaustion cycle (15min/h). Rats were given food (Nuvilab®, Rio de Janeiro, Brazil) *ad libitum*. 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.

After the time of acclimatization (one week) the average daily water intake *per animal* of experimental groups was determined and this volume was used to dissolve drugs and guarantee the total intake of the planned daily drug dosage. The BP and the body mass were verified weekly in conscious rats. The BP was verified through the non-invasive method of the tail-cuff plethysmography (Leica LE 5100, Panlab®).

The animals were divided in 5 groups of 5 animals each and maintained for 21 days alive. The groups were: Control Wistar group (C-W) - rats received water and food *ad libitum*, Control SHR group (C-SHR) - rats received water and food *ad libitum*. L-NAME SHR group (L-SHR) - rats received daily L-NAME (Hydrochloride of L-Nω-nitro-arginine-methyl-ester, Sigma Chemical Co., St. Louis, Lot 10K1583) 30 mg/kg/day dissolved in drinking water, L-NAME+Enalapril SHR group (L+E-SHR) - rats received daily L-NAME 30 mg/kg/day and Enalapril maleate ((S)-N-(1-Ethoxycarbonyl)-3-phenylpropyl)-Ala-Pro maleate, Sigma Chemical Co., St. Louis, Lot 38H0500) 15 mg/kg/day dissolved in the drinking water, Enalapril SHR group (E-SHR) - rats received daily Enalapril maleate 15 mg/kg/day dissolved in drinking water.

Tissue processing and renal function analysis

On the 22nd day of the experiment, rats were deeply anesthetized (Thiopental 15mg/kg intraperitoneally); blood sample was taken by right atrium puncture and then sacrificed by exsanguination. Both kidneys were removed and weighted according to Scherle's method (immersed in physiological saline solution) [15]. The left kidneys were divided in two halves and were cut in vertical sections to become the renal tissue isotropic to

Table 1 Stereology of the renal cortical structure (mean ± SD). Groups: C-W is control Wistar group; C-SHR is control SHR group; LN-SHR is L-NAME SHR group; LN+E-SHR is L-NAME plus Enalapril SHR group; E-SHR is Enalapril SHR group. Abbreviations: Vv[i] is interstitial volume density, Sv[t] is tubular surface density and Lv[t] is tubular length density. Mann-Whitney test: in signaled cases, when compared, $p<0.05$; if: [*a*] when compared with C-W, [*b*] with C-SHR, [*c*] with L-SHR and [*d*] with E-SHR.

Groups	Vv[i] (%)	Sv[t] (mm^2/mm^3)	Lv[t] (mm/mm^3)
C-W	20.7 ± 1.3	48.0 ± 2.6	614.9 ± 18.1
C-SHR	24.5 ± 0.9 [a]	46.3 ± 2.4	586.2 ± 79.2
L-SHR	26.7 ± 2.1 [a]	36.3 ± 3.5 [a][b]	502.5 ± 70.4 [a]
L+E-SHR	25.2 ± 1.7	37.7 ± 1.6 [a] [b]	458.4 ± 58.6 [a] [b]
E-SHR	22.5 ± 2.2	53.5 ± 3.7 [a] [b] [c] [d]	659.0 ± 92.6 [c] [d]

estimate the absolute stereological parameters, as described later. Renal fragments were placed for 24h at room temperature in fixative, freshly prepared 4% w/v formaldehyde in 0.1M phosphate buffer pH 7.2, embedded in Paraplast plus®, and sections were systematically uniformly random sampled. Sections had 3 μm thickness and were stained with Picro Sirius red. Serum creatinine was determined by alkaline picrate method (Labset kit) using the Mega Bayer automatic analyzer.

Stereology and statistical analysis

The stereological parameters were determined by observing 15 microscopic fields *per* animal (75 fields per group) using a video-microscope (Leica model DMRBE microscope, Kappa CF 15/5 video-camera and a Sony triniton monitor).

The glomerular density per area (Q_A[gl]), that is the number of glomeruli in a test-area, was determined by counting of glomeruli using a systematic uniform random scheme. An orthogonal grid with 0.28 mm^2 was used and glomeruli were counted only considering well preserved structures and not crossing the forbidden line [16].

Other stereological parameters were analyzed by a test-system with cycloids that was put upon the screen of the monitor and calibrated (Leitz micrometer 1mm/100). The minor axes of the cycloids were arranged in parallel with the defined vertical axis. The number of intersections between the surface traces and the cycloid arcs (I_L) was counted to estimate the cortical tubules surface density ($S_V:=2.I_L$) (:= is used because it is an estimation) [12]. The reference volume was estimated by point counting using the test points that hit the renal cortex (P_T). The number of points hitting tubules and interstitium (P_P) was counted to estimate the volume density of these structures ($V_V:=P_P/P_T$). The number of the tubules was counted in a two-dimensional test frame area (Q_A[tub]) of $1.2x10^4$ mm^2 allowing estimate of the tubule length density ($L_V:=2.Q_A$[tub]).

Differences comparing the groups were tested with ANOVA and Student-Newman-Keuls to BP and serum creatinine data. To analyze the stereological parameters we used the non-parametric tests of Kruskal-Wallis followed by the Mann-Whitney post test with significant level of 0.05 [17].

Results

Results are summarized in Tables 1 and Figs. 1-4. The BP reached 226±15 mmHg (mean±SD) in L-SHR group. The BP difference between the L-SHR and the C-SHR groups was significant since the first week of L-NAME administration. The difference between the C-SHR and the E-SHR groups became significant since the second week of enalapril administration ($p = 0.02$). At the end of the experiment the BP of the E-SHR group was similar to the BP in the C-W group (Fig. 1).

The stereology of the renal cortex showed the small amount of the interstitium in C-W group,

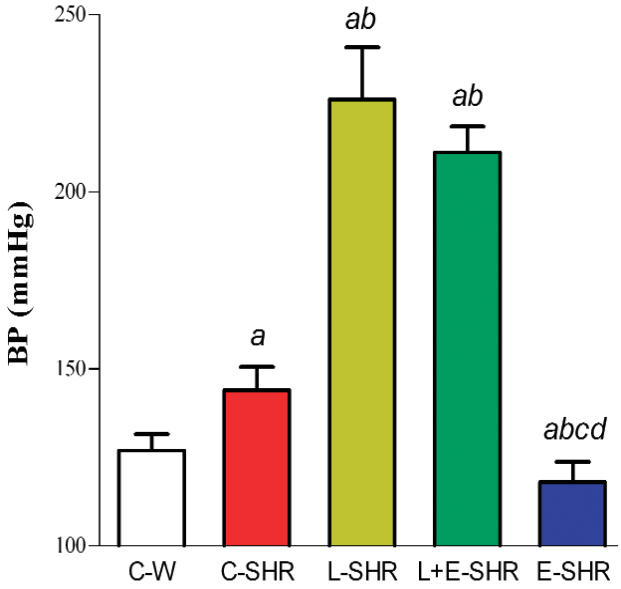

Fig. 1 Blood pressure evaluation. The values are means and SD at the end of the study (120th day). C-W is control Wistar group; C-SHR is control SHR group, LN-SHR is L-NAME SHR group; LN+E-SHR is L-NAME plus Enalapril SHR group; E-SHR is Enalapril SHR group. ANOVA and Student-Newman-Keuls test: in signaled cases, when compared, $p<0.05$; if: [*a*] when compared with C-W, [*b*] with C-SHR, [*c*] with L-SHR and [*d*] with E-SHR.

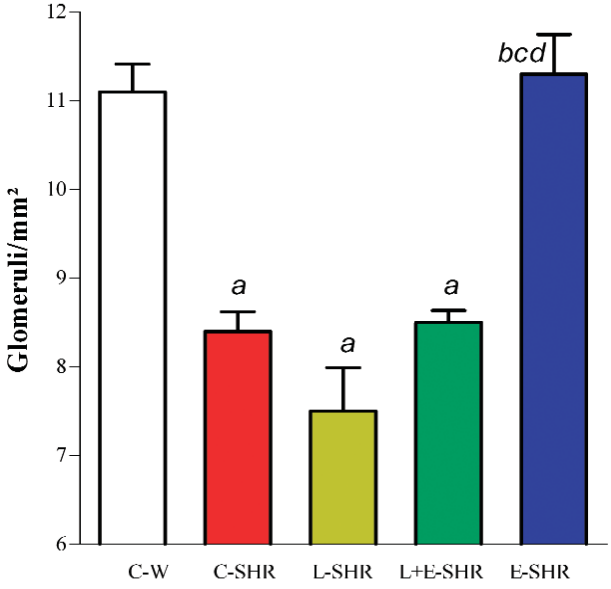

Fig. 2 Density per area of glomeruli evaluation. The values are means and SD. C-W is control Wistar group; C-SHR is control SHR group, LN-SHR is L-NAME SHR group; LN+E-SHR is L-NAME plus Enalapril SHR group; E-SHR is Enalapril SHR group. Kruskal-Wallis and Mann-Whitney test: in signaled cases, when compared, $p<0.05$; if: [*a*] when compared with C-W, [*b*] with C-SHR, [*c*] with L-SHR and [*d*] with E-SHR.

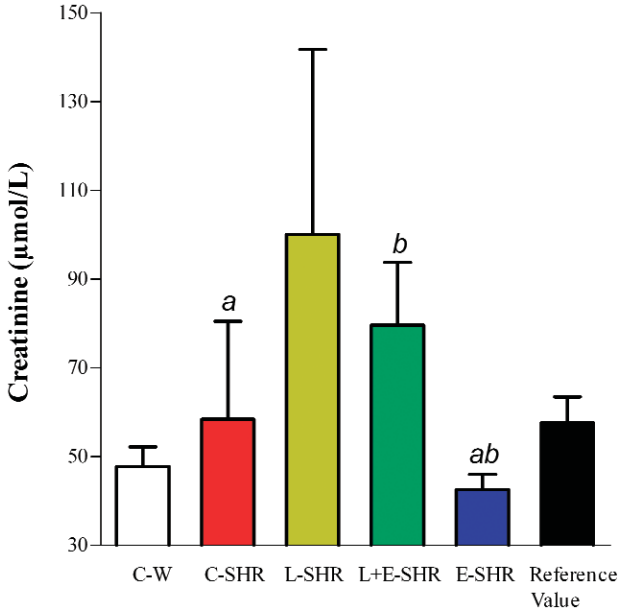

Fig. 3 Serum creatinine evaluation. The values are means and SD. C-W is control Wistar group; C-SHR is control SHR group, LN-SHR is L-NAME SHR group; LN+E-SHR is L-NAME plus Enalapril SHR group; E-SHR is Enalapril SHR group. ANOVA and Student-Newman-Keuls test: in signaled cases, when compared, $p<0.05$; if: [*a*] when compared with C-W, [*b*] with C-SHR, [*c*] with L-SHR and [*d*] with E-SHR.

smaller than in the C-SHR and the L-SHR groups but not different than the enalapril treated SHRs. The cortical tubuli showed great differences among the groups. The Sv[t] and the Lv[t] decreased with the administration of L-NAME and increased with the administration of enalapril. The E-SHR group showed Sv[t] and Lv[t] greater than C-SHR and C-W groups (Table 1).

A reduction in glomerular density occurred in C-SHRs significantly different from the C-W group ($p=0.008$). The Q_A[gl] reduction was around 30% in the L-SHR group compared with the C-W group. The C-SHR, L-SHR and L+E-SHR showed similar Q_A[gl]. However, the Q_A[gl] was greater in E-SHR group than in the C-SHR, L-SHR and L+E-SHR groups ($p=0.008$). The Q_A[gl] was maintained and no difference was found between E-SHR and C-W groups (Fig. 2).

The serum creatinine was greatly increased in SHRs submitted to NOs blockade. The enalapril treatment did not efficiently decrease the serum creatinine in these rats but it did in SHRs without NOs blockade (Fig. 3).

The microscopic appearance of the renal cortex was similar in the C-W and the E-SHR groups. In the L-SHR group, all glomeruli had important alterations, characterized by global or segmental glomerular sclerosis. The Bowman's capsule exhibited thickening. Glomerular obsolescence as well as tubular atrophy and extensive interstitial fibrosis were common findings. The tubular structures showed ischemic areas and vacuolization. We observed thickening of medium layer, some of these occluding the vascular lumen with "onion-skin" aspect. The L+E-SHR group showed less extensive glomerular lesions as well as less tubular injury (Fig. 4).

Discussion

The present study of the renal cortical structure intensified the natural hypertension of SHRs with the hypertension caused by the administration of a NOs blocker and treated the animals with ACE inhibitor. The SHRs submitted to chronic NOs blockade developed severe hypertension with consequent renal cortex damage. The administration of orally active NOs blocker caused stable hypertension and glomerulosclerosis in Wistar rats [18, 19] and SHRs [9, 20]. The treatment with enalapril reduced the BP in SHRs close to the normotensive Wistar rats agreeing with Francischetti and co-workers [21].

Experimental evidence suggested that a delicate balance exists between the vasoconstrictor Angiotensin II (Ang II) and NO and these two molecules appear to have antagonistic effects not only on vascular tone, but also in such diverse areas as vascular remodeling and renal function [22]. The use of the ACE inhibitor enalapril in this work was not completely efficient in reducing BP when the hypertension of SHRs was increased with the NOs blockade suggesting that NO deficiency-induced hypertension is not entirely mediated by the RAAS. Normally, the NO counteracts to vasoconstrictor effect of AngII in normotensive rats, but the protective mechanism is impaired in SHRs [23].

Ang II stimulates NO release from human proximal tubular cells [24]. However, the contribution of NO to the regulation of Ang II-induced renal vasoconstriction seems to be different between hypertensive and normotensive rats [23]. It is possible that local interaction mediated by autocrine and paracrine systems could modulate vasoconstriction and vasodilatation as well as architectural remodeling of the vascular bed and the imbalance between NO and Ang II [25].

The kidney is a target organ of hypertension. The renal disease secondary to hypertension develops progressively up to chronic renal failure with loss of glomeruli and several morphological and quantitative alterations [26, 27]. Renal lesions caused by NOs blockade are glomerulosclerosis, interstitial fibrosis and microvascular lesions [28]. In the present study these lesions were marked in SHRs submitted to NOs blockade and the enalapril treatment attenuated the lesions mainly in tubules and vessels. In SHRs without NOs blockade the enalapril administration was efficient in the preservation of the cortex structure agreeing with Ono and co-workers [29] and Pereira and Mandarim-de-Lacerda [10].

The kidney in mature SHRs normally shows reduction in the glomerular density [30]. We observed efficiency of the enalapril treatment to preserve the number of glomeruli in SHRs reaching similar values to the normotensive Wistar rats. Glomerular injury was substantially more intense in SHRs submitted to NOs blockade and even enalapril treatment was not protective to preserve the number of glomeruli in these SHRs.

In general, quantitative parameters showed a worsening in renal cortical structure in SHRs submitted to NOs blockade than SHRs treated only with enalapril. Stereology showed interstitial cortical fibrosis in SHRs submitted to NOs blockade that was correlated with the renal function loss evaluated by increase of the serum creatinine. The reference values to Wistar rats serum creatinine is median = 60 μmol/L (upper and lower 2.5% are 70 and 52 μmol/L, respectively) [31].

Finally, we could hypothesize that the ACEi are not efficient to sustain the renal function and preserve the cortical fibrosis in NO deficient SHRs probably because ACEi also was not efficient to reduce the BP in these rats [32, 33]. Further investigation is required to clarify the mechanisms underlying the progression of the renal injury and renal function when the genetic hypertension is associated with NO release inhibition.

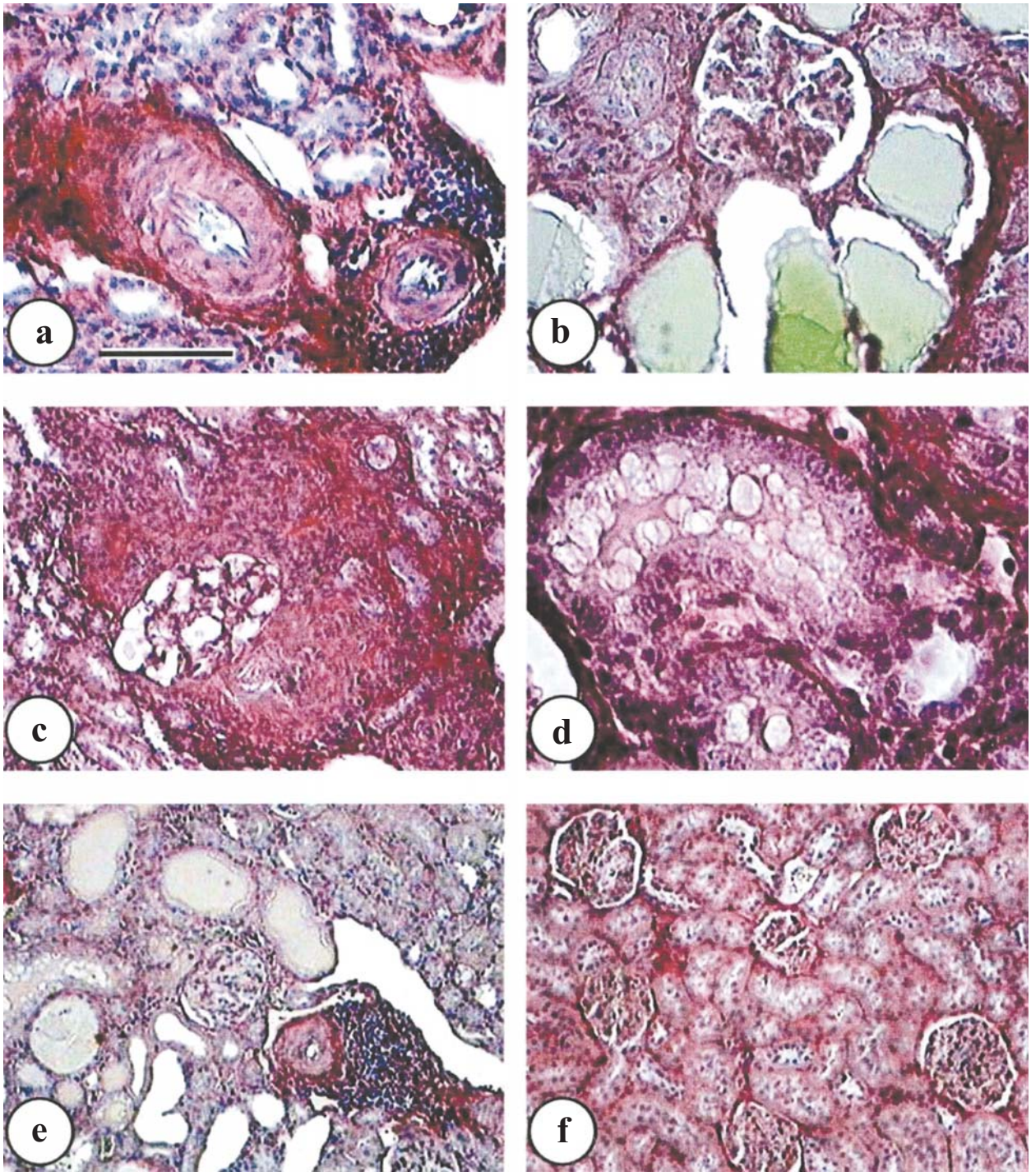

Fig. 4 Photomicrographies of the renal cortex showing renal lesions induced by nitric oxide synthesis (NOs) blockade in spontaneously hypertensive rats (SHRs) (a-d), SHRs with NOs blockade treated with angiotensin converting enzyme (ACE) inhibitor (e) and SHRs without NOs blockade treated with ACE inhibitor (f). Staining: picro sirius red. (a) Thickening of arteriolar tunica media with and important lumen narrowing (increase of the wall-to-lumen ratio). Extensive perivascular fibrosis and inflammatory infiltrated (bar=80μm). (b) Tubular atrophy and dilatation of the tubular lumen with hyaline casts associated to interstitial fibrosis and focal segmental glomerulosclerosis (bar=80μm). (c) Advanced glomerular lesions with several tuft-capsule adhesions areas associated to extensive interstitial fibrosis (bar=160μm). (d) Proximal tubular segment showing extensive vacuolization (bar=40μm). (e) Severe vascular injury with increased wall-to-lumen ratio, perivascular fibrosis, inflammatory infiltrated and glomerulotubular sclerosis (bar=160μm). (f) Preserved renal cortical structures (bar=160μm).

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