Title:

RENAL MITOCHONDRIAL DYSFUNCTION IN SPONTANEOUSLY HYPERTENSIVE RATS IS ATTENUATED BY LOSARTAN BUT NOT BY AMLODIPINE

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Abstract

Mitochondrial dysfunction is associated with cardiovascular damage; however, data on a possible association with kidney damage are scarce. Here we aimed at investigating whether a) kidney impairment is related to mitochondrial dysfunction, and b) angiotensin II blockade, as compared to Ca\(^{2+}\)-channel blockade, can reverse potential mitochondrial changes in hypertension. Eight-week-old male spontaneously hypertensive rats (SHR) received water containing losartan (40 mg/kg/day, SHR+Los), Amlodipine (3 mg/kg/day, SHR+Amlo), or no-additions (SHR) for 6-months. Wistar-Kyoto rats (WKY) were normotensive controls. Glomerular and tubulointerstitial damage, systolic blood pressure, and proteinuria were higher, and creatinine clearance was lower in SHR versus SHR+Los and WKY. In SHR+Amlo, blood pressure was similar to WKY, kidney function was similar to SHR, and renal lesions were lower than in SHR, but higher than in SHR+Los. In kidney mitochondria from SHR and SHR+Amlo, membrane potential, nitric oxide synthase, Mn-superoxide dismutase and cytochrome oxidase activities, and uncoupling protein-2 content were lower than in SHR+Los and WKY. In SHR and SHR+Amlo, mitochondrial hydrogen peroxide production was higher than in SHR+Los and WKY. Renal glutathione content was higher in SHR and SHR+Amlo relative to SHR+Los and WKY, but in the latter groups glutathione was relatively more reduced. Tubulointerstitial α-smooth muscle actin labeling was inversely related to Mn-superoxide dismutase activity and UCP2 content. These findings suggest that oxidant stress is associated with renal mitochondrial dysfunction in SHR. The mitochondrial-antioxidant actions of losartan may be an additional or alternative way to explain some of the beneficial effects of AT1-receptor antagonists.

Keywords: kidney disease, nitric oxide, mitochondria, oxidative stress, hypertension.
Introduction

Arterial hypertension is one of the main causes of end-stage renal failure, and is also an important risk factor for the progression of glomerular and tubulointerstitial diseases to chronic renal failure (31).

The pathogenesis of renal damage from hypertension is incompletely understood. A possible explanation is an increased production of oxidants in the vasculature (2, 30) and the kidney (56), a well-documented finding in experimental and clinical hypertension. However, the pathways that lead from oxidant-induced damage to cellular function decay are poorly identified.

Mitochondria are energy producing organelles that also conduct other key cellular tasks. They are involved in the regulation of tissue oxygen gradients (54), the modulation of apoptosis (9), and hydrogen peroxide signaling (6). Hence, mitochondrial damage may lead to the impairment of various aspects of tissue functioning.

Hypertension is associated with the deterioration of mitochondrial energy production in the brain (18), liver (12), and heart (1). The involvement of mitochondrial dysfunction in the pathogenesis of cardiovascular damage associated with several forms of physiological stress, including hypertension, was recently reviewed (41). In the myocardium of spontaneously hypertensive rats (SHR), evidences that point to the occurrence of mitochondrial dysfunction include the decreases of cytochrome oxidase activity (13), ATP production (1), and inorganic phosphate translocator activity (47). In the kidney, hypertension was shown to have no effect on both mitochondrial lipid oxidation markers and manganese superoxide dismutase (Mn-SOD) content (36), whereas renal MAO activity was lower in hypertensive than in normotensive rats (49). However, reports addressing a wider spectrum of kidney mitochondrial function indicators in hypertension are lacking.

Angiotensin II can induce oxidant stress by stimulating the generation of both NO (38) and NAD(P)H oxidase-derived superoxide (44), thereby enhancing peroxynitrite formation. Angiotensin
II receptor blockers and ACE inhibitors are known to protect from renal injury in hypertension, and at least part of this protection seems to be independent of blood pressure reduction (37).

In previous work, we showed that ACE inhibitors and AT1 receptor blockers can improve antioxidant status and attenuate oxidant stress (14-16). Recently, we reported that long-term use of enalapril or losartan improves mitochondrial function and structure in aged rats (17).

The aim of the present study was to assess the impact of hypertension on kidney mitochondrial function, and the effects of angiotensin II receptor blockade on potential mitochondrial changes in the spontaneously hypertensive rat (SHR). Lowering of blood pressure with a calcium channel blocker, i.e. an antihypertensive agent acting through a mechanism distinct from angiotensin II receptor blockade, was used for comparison.

Materials and Methods

Animals and treatments

All the experiments were approved by the Hospital Alemán Ethics Committee, and the Teaching and Research Committee. They were conducted according to the NIH Guide for the Care and Use of Laboratory Animals. Inbred, eight week-old male SHR (Laboratory of Experimental Medicine, Hospital Alemán, Buenos Aires, Argentina, Charles River Laboratories, Wilmington, MA) were randomly divided into 3 groups of 8 animals each. During the following 6 months the rats were administered drinking water containing either losartan (40 mg/kg/day, SHR+Los), or amlodipine (3 mg/kg/day, SHR+Amlo), or water with no additions (SHR). Eight month-old Wistar-Kyoto rats (WKY) that had received drinking water with no additions since birth were used as normotensive controls. Rats had free access to standard rat chow (Cargill, Buenos Aires), and were housed in individual cages maintained at 21 ± 2°C and a 12 h light/darkness cycle (7am-7pm). Systolic blood pressure, evaluated by tail plethysmography (Narco Bio-Systems, Austin, TX), proteinuria, and
Creatinine clearance (Randox Laboratories Ltd, Crumlin, N. Ireland) were determined at the beginning of the study, and once a month thereafter.

Body weight was determined at the beginning and the end of the study. Rats were anesthetized with pentobarbital (40 mg/kg, IP), blood was drawn from the thoracic aorta, and a systemic perfusion was carried out with NaCl 0.9% (w/v) immediately before excision of the kidneys. One kidney was used for mitochondrial isolation. The other was fixed in phosphate-buffered 10% formaldehyde (pH 7.2), and embedded in paraffin. Three-micron sections were cut and stained with hematoxylin-eosin, and Masson's trichrome. Unless otherwise stated, all reagents were from Sigma Chemical Co. (St. Louis, MO).

**Immunolabeling**

Renal α-SM-Actin (α-SMA) was used as an early marker of the fibrotic process. α-SMA was detected with anti-mouse α-SMA, and immunolabeling was revealed with a modified avidin-biotin-peroxidase complex technique (Vectastain ABC kit, Universal Elite, Vector Laboratories, CA).

**Histological evaluation**

Histological sections were analyzed using a Nikon E400 light microscope (Nikon Instrument Group, Melville, NY). Image-Pro Plus 4.5.1.29 software (Media Cybernetics, LP, Silver Spring, MD) was used to evaluate the sizes of a) glomerular areas, and b) areas of positive α-SMA immunolabeling in the glomerulus and periglomerular tubulointerstitium. Ten consecutive cortical glomerular areas per animal were examined. Tissues were subjected to blinded analysis, and the following score was used to grade renal lesions: 0 = absent; 1 = mild; 2 = moderate; 3 = severe; 4 = very severe.
Isolation of mitochondria

Kidney mitochondria were isolated as previously described (17). For nitric oxide synthase (NOS) activity determination, mitochondria were further purified in a self-forming Percoll gradient (17). The distribution of marker enzymes for contamination with other subcellular fractions was assessed in all the groups studied, and no statistical differences were found among them. Contaminations were in the low range as exemplified by values found in the WKY group: 0.13% glucose-6-phosphatase, 0.10% 5’-nucleotidase, 2.90% acid phosphatase, and 0.30% catalase, respectively. Electron microscopy examination of the purified mitochondrial fraction confirmed the absence of contaminating organelles or organelle fragments. Protein content was assayed according to Bradford using BSA as standard, as previously described (17).

Mitochondrial membrane potential

Mitochondrial membrane potential ($\Delta\Phi$) was evaluated after loading isolated kidney mitochondria with rhodamine 123, and calculating the ratio of fluorescence at 520 nm and 497 nm excitation wavelengths, and 529 nm emission wavelength (46). Mitochondria were incubated in a medium containing 150 mM sucrose, 5 mM MgCl$_2$, 5 mM potassium phosphate, 20 mM K-HEPES, pH 7.4, in the presence of 0.2 mg mitochondrial protein, 0.24 µM Rh 123, 10 mM glutamate and 5 mM malate, at 28°C. Mitochondrial accumulation of Rh123 is dependent on membrane potential. The method takes advantage of the red shift in Rh123 absorption and emission fluorescence spectra that occurs when the dye accumulates in mitochondria. The maximum difference in the excitation spectra between coupled and uncoupled mitochondria occurs at 497 and 520 nm. The above mentioned wavelength shift results in a much greater change in the magnitude of the 520/497 excitation fluorescence ratio than the change in the intensity measured at each wavelength. To calibrate the fluorescence ratio for estimation of $\Delta\Phi$, a calibration curve was constructed by plotting 520/490 excitation fluorescence ratios (obtained in the presence of mitochondria and DNP [0, 1, 2,
4, 8, and 16 µM] to attain discrete levels of $\Delta \Phi$, and the corresponding $\Delta \Phi$ values (obtained by using the Nernst equation: $\Delta \Phi = 59 \log ([R_{123}]_{in}/[R_{123}]_{out})$; where $[R_{123}]_{in}$ and $[R_{123}]_{out}$ were calculated as described in (46).

*Hydrogen peroxide production*

Mitochondrial $\text{H}_2\text{O}_2$ production was determined by fluorometry at 350 nm and 460 nm excitation and emission wavelengths, respectively, as described in (27) with modifications. The reaction medium contained 0.23 M mannitol, 0.07 M sucrose, 20 mM Tris-HCl (pH 7.4), 0.8 µM horseradish peroxidase, 1 µM scopoletin, 0.3 µM SOD, 30 µM sodium azide, 6 mM malate, 6 mM glutamate, 3 µM antimycin, and 0.050 mg mitochondrial protein/ml. $\text{H}_2\text{O}_2$ (0.05-0.35 µM) was used as standard. Antimycin was used to maximize hydrogen peroxide detection, since baseline/normal ROS production was either barely detectable or undetectable in the WKY and SHR+Los groups, but not in the SHR and SHR+Amlo groups.

*Mitochondrial enzyme activities*

Mitochondrial NOS activity was determined by conversion of $^{14}$C-arginine to $^{14}$C-citrulline, in a solution containing 50 mM potassium phosphate, pH 5.8, 1 µM flavin adenine dinucleotide, 1 µM flavin adenine mononucleotide, 10 µM tetrahydrobiopterine, 0.1 µM calmodulin, 300 µM CaCl$_2$, 100 µM NADPH, 60 mM valine, 50 µM arginine, 0.025 µCi $^{14}$C-arginine, and 0.15 mg mitochondrial protein. Assay mixtures were incubated at 37°C for 5 min. The reaction was stopped by adding 3 volumes of 2 mM EDTA, 20 mM HEPES buffer, pH 5.5, followed by 6 volumes of a Dowex exchange resin (BioRad, Hercules, CA). Aliquots of the supernatant were used for scintillation counting (29). Mn superoxide dismutase (Mn-SOD) activity was determined in the presence of 2 mM NaCN after the inhibition of cytochrome c reduction by superoxide anion at 550
nm, as previously described (17). One unit of SOD was defined as the amount of enzyme necessary to cause a 50% inhibition of the reduction of cytochrome c (20 µM) by superoxide anion generated by the xanthine (50 µM)-xanthine oxidase (5 nM) system. NADH/cytochrome c oxidoreductase activity was used to examine electron transfer through Complexes I–III, and was determined by following the reduction of cytochrome c (25 µM) at 550 nm, in a medium containing 100 mM potassium phosphate, pH 7.2, 0.2 mM NADH, 0.5 mM KCN and 0.1 mg mitochondrial protein, as described in (25) with modifications. Rotenone (1 µM) was used to assess background activity. Cytochrome oxidase activity was measured by following the oxidation of reduced cytochrome c (50 µM) at 550 nm, in a solution containing 50 mM potassium phosphate, pH 7.0, and 0.1 mg mitochondrial protein (11). Citrate synthase activity was determined in mitochondria that had been subjected to 3 freeze-thaw cycles, as described by Robinson et al (43) with modifications, and was used to normalize all other mitochondrial parameters. Briefly, citrate synthase activity was determined at room T° by following the reduction of 5,5′-dithiobis-(2-nitrobenzoic) acid (0.1 mM) at 412 nm, in a reaction medium containing 100 mM Tris-HCl, pH 8.1, 0.4 mM acetyl-coenzyme A, and 75 mg of mitochondrial protein. After 1 min, sodium oxaloacetate (OAA) (final concentration = 0.5 mM) was added, and the absorbance was recorded for another minute. To calculate enzyme activity, the change in absorbance recorded before addition of OAA (corresponding to acetyl-CoA hydrolase activity) was subtracted from that recorded after addition of OAA, and ε = 13.6 mM⁻¹ cm⁻¹ for reduced DTNB was used.

Western blot analysis of uncoupling protein 2 (UCP-2)

Mitochondrial proteins were separated on SDS-12.5 % polyacrylamide gels, and transferred to PVDF membranes by liquid electroblotting (MiniProtean 3, BioRad), in a solution containing 25 mM Tris, 190 mM glycine, 20 % methanol, pH 8.3, at 110 V for 90 min. After transfer, membranes were blocked by incubating in phosphate buffered saline (PBS)-5 % dry low-fat milk for 1 h at
room temperature. UCP-2 antibody (Santa Cruz Biotechnology Inc, Santa Cruz, CA) was diluted in 2 % dry low-fat milk, and incubated overnight at 4ºC. Membranes were washed with PBS-0.1 % Tween 20, and incubated with peroxidase-conjugated donkey anti-goat secondary antibody for 90 min at room temperature. After washing with PBS-0.1 % Tween 20, bound peroxidase-conjugated antibody was revealed with diaminobenzidine tablets. Western blots were photographed using a digital camera, and analyzed with Scion Image software (Scion Corporation, Frederick, MD). The amount of protein loaded into gels was normalized by staining membranes with Ponceau Red.

Reduced (GSH) and oxidized (GSSG) glutathione, and protein-bound sulfhydryl group determination.

To prevent conversion of GSH to GSSG, tissue samples were homogenized with 4 vol of 0.33 M HClO4 immediately after collecting the kidneys, and the resulting suspensions were centrifuged at 5,000 g for 10 min. The pellets, containing acid-precipitated proteins, were used to determine protein-bound sulfhydryl groups with Ellman’s reagent (48). After neutralization with 1.75 M K3PO4, the supernatants were used for a) GSSG+GSH determination using the 5,5´-dithiobis (2-nitrobenzoic acid) spectrophotometric assay; and b) GSSG determination by following NADPH oxidation at 340 nm (17). Results are expressed as nanomol of GSH equivalents per gram of wet tissue, and nanomol of GSSG per gram of wet tissue, respectively.

Statistical methods

Values in text, figures, and tables are means ± SE. Statistical analyses were performed by one-way analysis of variance (ANOVA) and F test (Statview SE+Graphics v 1.03 (Abacus Concepts, Berkeley, CA) to establish the significance of differences among groups. Multiple regression analysis tests and a matrix of correlation coefficients (GraphPad Prism, GraphPad Software, Inc., San Diego, CA, USA) were performed to assess the contributions of mitochondrial function
parameters to tubulointerstitial lesion scores, and the association between variables, respectively. P values < 0.05 were considered significant.

Results

Animal outcome

Data in the WKY group are used as a reference to the location of basal values in healthy animals. Hematocrit, serum glucose, cholesterol, triglycerides, and sodium and potassium levels showed no differences among the groups. In WKY, values were as follows, Hematocrit: 49±0.2 %; Serum glucose: 5.8±0.5 mM, Serum cholesterol: 0.8±0.1 mM, Serum triglycerides: 0.4±0.1 mM; Serum sodium: 144.8±3.1 mEq/L; Serum potassium: 4.9±0.1 mEq/L.

Body weight, proteinuria and creatinine clearance values at baseline, and end-of-study body weight showed no differences among the study groups (Table 1).

Baseline blood pressure in the SHR, SHR+Los, and SHR+Amlo groups was significantly higher than in the WKY group. Figure 1 shows the monthly evolution of systolic blood pressure. In the SHR group, systolic blood pressure increased progressively during the first three months of the study and remained stably high thereafter. After one month of treatment, and up to the end of the study, systolic blood pressure in the SHR+Los and SHR+Amlo groups was significantly lower than in the SHR group. At the end of the study, SHR group systolic blood pressure was 43%, 46% and 59% higher than in the SHR+Los, SHR+Amlo and WKY groups, respectively. In the SHR+Los and SHR+Amlo groups, blood pressure decreased progressively during the first three months of treatment; however, it remained significantly higher than in WKY. During the last three months of treatment, both losartan and amlodipine lowered blood pressure to values that were similar to those observed in WKY. In the SHR group, end-of-study proteinuria was 7 and 13 times higher than in the SHR+Los and WKY groups, respectively; whereas in the SHR+Amlo group proteinuria was
similar to that found in the SHR group. End-of-study creatinine clearance was significantly lower in the SHR and SHR+Amlo groups relative to the SHR+Los and WKY groups.

**Histological and immunohistochemical parameters**

Results of the histological evaluation are shown in Table 2. Glomerular and tubulointerstitial lesion scores were significantly higher in the SHR group relative to the WKY group. In the SHR+Los and SHR+Amlo groups lesion scores were significantly lower than in the SHR group, and in the SHR+Los group lesion scores were similar to those found in the WKY group. In the SHR+Los group, glomerular area, and \( \alpha \)-SM-Actin immunolabeling in glomeruli (Figure 2) and cortical tubulointerstitium were significantly lower than in the SHR and SHR+Amlo groups, and similar to those observed in WKY.

**Effect of losartan treatment on kidney mitochondrial function**

Figure 3 shows the effects of losartan and amlodipine treatments on mitochondrial function parameters. Mitochondrial protein recovery was as follows: SHR, 8.38±0.81; WKY, 6.97±1.21; Los, 6.70±0.92; Amlo, 8.19±1.58 mg protein/g wet tissue (no statistical differences between groups). Mitochondrial citrate synthase activity showed no differences among the groups studied (WKY = 232.0 ± 2.99; SHR = 228.8 ± 3.86; SHR+Los = 230.7 ± 2.11; SHR+Amlo = 228.7 ± 3.41 \( \mu \)mol CoA/min.mg protein). To account for potential differences in mitochondrial yield among the groups, mitochondrial function parameters were normalized to citrate synthase activity. Mitochondrial membrane potential was significantly lower in the SHR group relative to the SHR+Los and WKY groups (-48% and -44%, respectively). In the SHR+Amlo group, mitochondrial membrane potential was similar to that observed in the SHR group. NOS activity in mitochondria was significantly lower in the SHR and SHR+Amlo groups than in the SHR+Los (-71% and -49%, respectively) and WKY groups (-77% and -59%, respectively). Mitochondrial \( \text{H}_2\text{O}_2 \)
production was significantly higher in the SHR and SHR+Amlo groups relative to the SHR+Los (86% and 82%, respectively) and WKY (76 % and 72 %, respectively) groups. Mn-SOD activity was lower in the SHR and SHR+Amlo groups relative to the SHR+Los (-86% y -89%, respectively) and WKY (-85 % and -88 %, respectively) groups. In the SHR+Los group, UCP-2 protein level was 3 times and 79 % higher than in the SHR and SHR+Amlo groups, respectively. UCP-2 protein content was lower in the SHR (-82 %), SHR+Los (-29 %), and SHR+Amlo (-61 %) groups relative to the WKY group. Mitochondrial H₂O₂ production was inversely related to UCP-2 protein content (r = -0.813, p = 0.0027). The effects of losartan and amlodipine treatments on the activities of two enzymes of the mitochondrial electron transport chain are shown in Figure 4. In the SHR+Amlo group, NADH/cytochrome c oxidoreductase activity was significantly lower than in SHR, WKY, and SHR+Los groups. Cytochrome oxidase activity was significantly lower in the SHR and SHR+Amlo groups than in the WKY and SHR+Los groups.

Effect of losartan and amlodipine treatments on glutathione and protein-bound sulfhydryl group status in the kidney

Data on thiol status in the kidney are shown in Figure 5. Reduced glutathione (GSH) content in the kidney was moderately higher in the SHR group relative to the SHR+Los and WKY groups. In the SHR+Amlo group, GSH was significantly lower than in the SHR, SHR+Los and WKY groups (-76 %, -62 % and -70 %, respectively). The level of renal glutathione disulfide (GSSG), the oxidation product of GSH, was 3 to 4 times higher in the SHR group relative to the SHR+Los, SHR+Amlo, and WKY groups. The GSH/GSSG ratio, an index of glutathione redox status, was significantly lower (-70 %) in the SHR and SHR+Amlo groups relative to both the SHR+Los and WKY groups. Kidney protein-bound sulfhydryl group content was significantly lower in the SHR+Amlo group than in the SHR, SHR+Los and WKY groups.
Correlations between histochemical and mitochondrial function data

Multiple regression analysis showed that tubulointerstitial lesion scores were inversely related to Mn-SOD activity ($p = 0.0004$), and UCP-2 content ($p = 0.0447$), with an $R^2 = 0.804$ ($p<0.0001$); whereas the contributions of NOS activity and mitochondrial membrane potential were not significant.

Discussion

The present results show that in SHR, hypertension occurs in concurrence with a decline of kidney mitochondrial function. Also, losartan and amlodipine treatments were equally effective in reducing blood pressure, but only losartan prevented mitochondrial dysfunction and attenuated structural and functional changes in the kidney. Amlodipine, a calcium channel blocker with vascular selectivity, is an effective antihypertensive agent that displays in vivo antioxidant activity (20); however, evidences for nephroprotection by this agent are divergent (22). Amlodipine was used to assess whether the potential actions of losartan on kidney mitochondrial function were related to the blood pressure lowering effect of this drug, or specifically to angiotensin II receptor blockade and attenuation of angiotensin II-stimulated oxidant production. In the present study, comparison of the effect of losartan and amlodipine suggests that at least part of the mitochondrial protection afforded by losartan occurred beyond blood pressure lowering. In agreement with these results, previous studies from our laboratory showed that, in normotensive aging mice, inhibition of the renin-angiotensin-system (RAS) protects cardiac and hepatic mitochondria in the absence of blood pressure changes (26). Also, in rats with experimental diabetes, AII receptor blockade prevents mitochondrial decay without significant reduction of blood pressure (unpublished results).

As a consequence of AT1 receptor activation, an excessive production of superoxide anion in vascular cells and the kidney occurs in SHR, as well as in other animal models of hypertension (30), and in humans (2). RAS blockade limits renal injury in adult SHR, providing evidence for a crucial
role of the RAS in this model (60). In line with other reports, the present study shows that losartan forestalled the deterioration of kidney function and structure in SHR, as indicated by the observed prevention of both creatinine clearance decline and elevation of proteinuria in SHR animals. Also, glomerular size and α-SM-Actin immunolabeling, an early marker of kidney fibrosis, were significantly reduced in the kidney by losartan, but not amlodipine, treatment.

Markers of oxidant stress are increased in SHR as compared to WKY (34). Accordingly, we show here that SHR exhibit signs of oxidant stress in the kidney, as indicated by a higher oxidation of the glutathione pool, when compared to WKY. Also, losartan, but not amlodipine, reduced oxidant stress, as revealed by a) the maintenance of the glutathione pool in a relatively more reduced status, b) the preservation of Mn-SOD activity, and c) the attenuation of UCP-2 content reduction in SHR+Los rats, relative to SHR. In a variety of conditions, oxidant stress is associated with increased mitochondrial lipid oxidation (3), inhibition of electron transport (40), mtDNA damage (57), and inactivation of mitochondrial enzymes (50, 59). In line with these findings, we observed an impairment of kidney mitochondrial membrane potential, and a reduction of cytochrome oxidase activity in SHR relative to WKY, which were blunted by losartan but not by amlodipine treatment. In SHR and SHR+Amlo, the observed lower cytochrome oxidase activity (complex IV) is not at variance with a higher rate of H$_2$O$_2$ production since the main sources of mitochondrial superoxide are within complexes I and III. Also, the concurrence of low NADH cytochrome c oxidoreductase activity and high rate of H$_2$O$_2$ production, found in SHR+Amlo, can be explained taking into account that in addition to complex I and III, other mitochondrial superoxide sources are the ubiquinone pool, electron transfer flavoprotein and electron transfer flavoprotein quinone oxidoreductase.

In order to evaluate mitochondrial function we selected a “non-classical” group of indicators, such as: membrane potential, UCP-2 content, Mn-SOD and mitochondrial NOS activities. In addition, electron transfer through complex I-III, and cytochrome oxidase activity were measured. We chose
to determine mitochondrial membrane potential after considering that most of the energy of the proton gradient (generated in the course of mitochondrial electron transport) is necessarily expressed as membrane potential. This is because to maintain a proper intramitochondrial pH, the pH gradient component of the proton motive force cannot exceed one pH unit. In addition, mitochondrial membrane potential drives vital mitochondrial functions such as ATP synthesis, Ca2+ pumping from the cytosol to the matrix, import of mitochondrial proteins, and active metabolite transport (42). UCP-2 content was determined in view of accumulating evidences that strongly suggest a role for this protein in both the attenuation of excessive mitochondrial superoxide production (and therefore in protection against disease) and the modulation of cellular signaling (4). Mn-SOD was chosen because by converting mitochondrial superoxide into hydrogen peroxide (a proposed mitochondrially-derived cellular messenger), it diverts superoxide from reacting with NO and inhibits the formation of mitochondrial peroxynitrite, an oxidant that is known to inactivate Mn-SOD (39). The determination of mitochondrial NOS activity was decided upon based on data that point to NO as a physiological regulator that acts directly on the mitochondrial respiratory chain, and as a modulator of mitochondrial redox signaling (10).

The apparent discrepancy between an increased rate of mitochondrial hydrogen peroxide production (Fig 3C) and a decreased activity of Mn-SOD (Fig 3D), in SHR and SHR+Amlo, may be explained considering that hydrogen peroxide measured under the assay conditions used in this study (ie. in the presence of externally added SOD) is the sum of hydrogen peroxide derived from Mn-SOD-related conversion of superoxide in the matrix plus hydrogen peroxide derived from conversion of superoxide (released from mitochondria into the cytosol) (24) by added SOD in the reaction medium. In consequence, the present data suggest that, in SHR and SHR+Amlo, a decrease in Mn-SOD activity resulted in higher matrix levels of superoxide, which upon release to the cytosol was converted into hydrogen peroxide in the assay medium.
Growing evidence supports the concept that a major function of uncoupling proteins is to increase the proton conductance across the inner mitochondrial membrane, resulting in decreased membrane potential. Since mitochondrial superoxide production is maximal at higher membrane potentials, uncoupling proteins would reduce superoxide production (4). This is in accordance with the observed inverse correlation between UCP-2 protein content and H$_2$O$_2$ production in kidney mitochondria. In this line, UCP-2 was proposed to act as an oxidant stress compensating mechanism, and a protective role for this protein in cellular pathophysiological processes that involve ROS has been suggested (33). It has been indicated that altered expression of UCP-2 is related to the pathophysiology of hypertension in SHR-SP (19). In addition, several agents that upregulate NOS also increase UCP expression, possibly to prevent excessive O$_2^{•−}$ production (8).

Consequently, the observed modulation of NOS activity in mitochondria and UCP-2 protein level suggests that the protective action that losartan exerts on mitochondrial function and structure may rely on a mechanism that involves both, NO, and UCP-2. These results are in agreement with our previous findings in aging rats (17).

Since, as mentioned above, UCP-2 is known to increase proton leak resulting in lower mitochondrial membrane potential, there is an apparent discrepancy between the present UCP-2 and membrane potential data. In fact, those groups displaying lower UCP-2 contents also show lower membrane potential values (Fig 3 A and E). This can be explained taking into consideration that UCP-2 content, and not activity, was determined in this study. UCP-2-related proton leak is activated, as needed, by overproduction of matrix superoxide, and by lipid oxidation products (4). This is consistent with the present findings showing lower Mn-SOD activity in the SHR and SHR+Amlo groups (Fig 3C), suggesting an elevation of matrix superoxide levels, which after activating UCP-2 proton leak, may have led to the observed lower mitochondrial membrane potentials in those groups (Fig 3 A). Conversely, in the SHR+Los and WKY groups, UCP-2 content was higher than in the SHR and SHR+Amlo groups, but this protein may have been less active in
SHR+Los and WKY due to the lower superoxide levels that resulted from a higher activity of Mn-SOD, and a lower rate of superoxide production (detected as hydrogen peroxide, Fig 3 C). From the present data, it is apparent that activation of low UCP-2 levels in SHR and SHR+Amlo was sufficient to reduce mitochondrial membrane potential but not to diminish superoxide production, suggesting that superoxide production was enhanced at other mitochondrial sites, in addition to the electron transport chain. Also, considering that Mn-SOD plays a major role in inhibiting intramitochondrial peroxynitrite formation (39), and that peroxynitrite can increase proton leak (7), the observed lower mitochondrial membrane potential in SHR and SHR+Amlo (Fig 3D) may be the result of decreased Mn-SOD activity, which by enhancing superoxide levels may have increased peroxynitrite formation and proton leak.

We also show that losartan, but not amlodipine, treatment prevented the decrease in NOS activity observed in mitochondria from SHR rats. A lot of controversy exists over the existence of mitochondrial NOS (5). Recently, evidence was provided to support the anchorage of endothelial NOS (eNOS) to the outer mitochondrial membrane (21). Also, moderate levels of hydrogen peroxide can activate eNOS, whereas higher levels block enzyme activation (52). This observation is in line with our data showing increased hydrogen peroxide production in SHR and SHR+Amlo, in association with lower NOS activity in mitochondria. A decreased activity of NOS in mitochondria is expected to lead to a decreased steady-state level of NO. NO of mitochondrial origin was proposed to modulate mitochondrial metabolism and ROS production (45). The inhibition of respiration by NO binding to mitochondrial cytochrome oxidase was suggested to trigger a variety of actions, which include: a) the regulation of mitochondrial superoxide production, which can influence hydrogen peroxide generation and the consequent downstream cell signaling; b) the modulation of tissue oxygen gradients; and c) the impediment of mitochondrial cytochrome c release, conducting to the inhibition of apoptosis (6). Hence, in SHR, loss of control
over mitochondrial NO production can have a severe impact over cell function, leading to detrimental tissue responses.

Whenever mitochondrial derangement prompts electron transport inhibition, electrons are forwarded into an increased generation of reactive oxygen species (ROS) (55). This is in agreement with the observed increase of H₂O₂ production in mitochondria from the SHR group. Increased mitochondrial ROS generation was suggested to underlie tissue injury associated with hyperglycemia (35); thus, it may also participate in the deterioration of kidney structure and function in SHR. (4). It has been suggested that increased ROS content may impact on mitochondrial membrane fluidity and composition, which may in turn affect the capacity of mitochondria to generate sufficient membrane potential to adequately respond to cell energy demands (23). In losartan-treated animals, the prevention of excess mitochondrial hydrogen peroxide production, observed in this study, may have contributed to the maintenance of mitochondrial membrane potential within the values displayed by non-hypertensive rats. Altered antioxidant content, and increased ROS production may impair mitochondria-derived redox signaling, potentially modifying cellular regulatory pathways.

In addition to enhancing superoxide production through the activation of NAD(P)H oxidase, Ang II was shown to stimulate mitochondrial reactive oxygen species production leading to the reduction of mitochondrial membrane potential (28). Based on these evidences, we propose that the contrasting effects displayed by losartan and amlodipine treatments on mitochondrial function may be a consequence of losartan-mediated blockade of AngII actions. In this context, Ang II is associated with downregulation of PPAR-alpha (53), a transcription factor that stimulates the expression of nuclear genes involved in mitochondrial fatty acid oxidation, and of UCP-2 (32) and Mn-SOD (51) genes. Consequently, losartan, but not amlodipine, by upregulating PPAR-alpha may have enhanced not only UCP-2 and Mn-SOD contents, but also the generation of electron donors for the respiratory chain and ATP production.
Considering that the mitochondrial preparations used in this study were obtained from whole kidney homogenates, the observed mitochondrial function changes cannot be ascribed to a particular cell type. However, rat kidneys consist of approximately 20% cortical, and 80% medullar tissue, with total medullar interstitium volume amounting to about 13%. In addition, of all the cell types that compose the kidney, medullar tubular cells have the highest number of mitochondria. In this context, it seems reasonable to assume that the data gathered in this study provide an approximation to tubular cell mitochondria responses to losartan and amlodipine treatments.

In this study we focused on mitochondria as generators of superoxide, while the effects of RAS inhibition on other angiotensin II-related superoxide sources were not analyzed. Angiotensin II stimulates superoxide production mainly by enhancing NAD(P)H oxidase activity and eNOS uncoupling (30); therefore, lowering of superoxide generation from these sources may also be involved in the protective effects displayed by losartan treatment.

Finally, data showing that in the tubulointerstitium, lesion scores are inversely related to Mn-SOD activity and UCP-2 content, suggests that, in addition to cytoplasmic oxidant stress resulting from membrane-bound oxidase activation described elsewhere, the mitochondrial dysfunction that accompanies hypertension may underlie the deterioration of kidney structure.

In summary, the current findings are consistent with a scenario where AngII-related oxidant stress is associated with mitochondrial dysfunction in the kidney. Accordingly, AT1 blockade displays antioxidant effects, and preserves kidney mitochondria favoring the maintenance of adequate tissue function. Consequently, the renal protective effects of AT1 receptor blockers in essential hypertension may be related to the improvement of mitochondrial function, and this may be an additional or alternative way to explain some of the beneficial effects of this type of drug recently reported in clinical studies.

More specific approaches are needed to address if mitochondrial dysfunction is the result of high AngII and hypertension, and causes renal damage. Recently, a causal relationship was found
between a mitochondrial mutation and hypertension suggesting that mitochondrial dysfunction can precede the emergence of hypertension (58).
Acknowledgments

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References


TABLE and FIGURE LEGENDS

Table 1: Body weight, proteinuria and creatinine clearance.

Data are expressed as Mean ± SEM (n = 8 animals for each group). Body weight: non significant differences between groups were found. Basal proteinuria and creatinine clearance: non significant differences between groups were found. End-of-study proteinuria and creatinine clearance: ‡p<0.01 SHR and SHR+Amlo vs. SHR+Los and WKY.

Table 2: Histological and immunohistochemical parameters.

In the SHR and SHR+Amlo groups glomerular area was significantly higher than in the WKY and SHR+Los groups. Tubulointerstitial α-SM-actin staining showed non significant differences among groups, and glomerular α-SM-actin staining was lower in SHR+Los group compared with the SHR and SHR+Amlo groups. Data are expressed as Mean ± SEM. (n = 8 animals for each group) (a)p<0.001 vs. WKY; b)p<0.001 vs.SHR; c)p<0.01 vs. SHR+Amlo; d)p<0.001 vs. SHR+Amlo; e)p<0.05 vs. SHR; f)p<0.05 vs. SHR and SHR+Amlo).

Figure 1: Monthly evolution of systolic blood pressure

Values are Mean ± SEM, n = 8 animals for each group, at each time point. (*p<0.01 versus all other groups, †p<0.05 versus WKY, #p<0.01 versus WKY).

Figure 2: Histological glomerular photomicrographs, α-SM Actin, 400x.

Representative photomicrographs show differences regarding glomerular size and α-SM Actin labeling in groups: A. SHR; B. SHR+Los; C. SHR+Amlo; D. WKY

Figure 3: Renal mitochondrial function parameters.

Data were normalized to citrate synthase (CS) activity (nmol CoA/min.mg protein)
A. Mitochondrial membrane potential in groups SHR and SHR+Amlo was lower than in groups SHR+Los and WKY. B. NOS activity in mitochondria was lower in groups SHR and SHR+Amlo than in groups SHR+Los and WKY. C. Mitochondrial H$_2$O$_2$ production in groups SHR and SHR+Amlo was higher than in groups SHR+Los and WKY. D. Manganese-SOD activity was lower in groups SHR and SHR+Amlo than in groups SHR+Los and WKY. E. UCP-2 protein content was lower in groups SHR and SHR+Amlo than in groups SHR+Los and WKY. In the SHR+Los group, UCP-2 protein content was lower than in the WKY group. Data are expressed as Mean ± SEM (n = 8 animals for each group) (*p<0.05 vs. SHR+Los, WKY; †p<0.05 vs. WKY).

**Figure 4:** NADH/cytochrome c oxidoreductase and cytochrome oxidase activities in kidney mitochondria. Data were normalized to citrate synthase (CS) activity (nmol CoA/min.mg protein). A. NADH cytochrome c oxidoreductase activity in group SHR+Amlo was significantly lower than in groups SHR, SHR+Los and WKY. B. Cytochrome oxidase activity was lower in groups SHR and SHR+Amlo than in groups SHR+Los and WKY. Data are expressed as Mean ± SEM (n = 8 animals for each group) (#p<0.05 vs. SHR, SHR+Los and WKY; *p<0.05 vs. SHR+Los and WKY).

**Figure 5:** Glutathione and protein-bound sulphydryl group status in the kidney. A. GSH content in group SHR+Amlo was significantly lower than in groups WKY, SHR and SHR+Los. B. GSSG content in group SHR was higher than in groups WKY, SHR+Los and SHR+Amlo. C. GSH/GSSG ratio in groups SHR was lower and SHR+Amlo in relation to groups WKY and SHR+Los. D. Protein-bound sulphydryl group content in group SHR+Amlo was lower than in groups WKY, SHR and SHR+Los. Data are expressed as Mean ± SEM (n = 8 animals for each group) (*p<0.05 vs. SHR, SHR+Los, WKY; #p<0.05 vs. SHR+Los, SHR+Amlo, WKY; †p<0.005 vs. SHR+Los, WKY).
Table 1.

<table>
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<th>Characteristic</th>
<th>SHR</th>
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<th>SHR+Amlo</th>
<th>WKY</th>
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<tr>
<td>Baseline</td>
<td>278.2 ± 17.7</td>
<td>275.8 ± 15.7</td>
<td>279.9 ± 16.8</td>
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<td>End-of-study</td>
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<td><strong>Proteinuria (mg/day)</strong></td>
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<td>Baseline</td>
<td>1.8 ± 0.3</td>
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<td>5.9 ± 0.4</td>
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<td><strong>Creatinine Clearance (ml/min)</strong></td>
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<td>1.77 ± 0.07</td>
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Table 2.

<table>
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<th>SHR+Los</th>
<th>SHR+Amlo</th>
<th>WKY</th>
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<td>Glomerular lesion score</td>
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<td>1.00±0.15&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>Tubulointerstitial lesion score</td>
<td>1.92±0.08&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.40±0.10&lt;sup&gt;b,d&lt;/sup&gt;</td>
<td>1.30±0.20&lt;sup&gt;a,e&lt;/sup&gt;</td>
<td>0.13±0.09</td>
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<td>Glomerular area (µ²)</td>
<td>30751±1583</td>
<td>19945±1135&lt;sup&gt;f&lt;/sup&gt;</td>
<td>26870±1552</td>
<td>17946±2191&lt;sup&gt;f&lt;/sup&gt;</td>
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<td>Tubulointerstitial α-SM Actin (µ²)</td>
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<td>1226±149</td>
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<td>Glomerular α–SM-Actin (µ²)</td>
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<td>89±24&lt;sup&gt;f&lt;/sup&gt;</td>
<td>257±52</td>
<td>122±25</td>
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</tbody>
</table>
Figure 1.

Systolic Blood Pressure

- SHR
- SHR + Losartan
- SHR + Amlodipine
- WKY

mm Hg vs Months
Figure 2.
Figure 3.

A) Membrane Potential/CS

B) NOS/CS

C) H₂O₂ Production/CS

D) MnSOD/CS

E) UCP-2/CS
Figure 4.

A) NADH cytochrome c oxidoreductase/CS

B) Cytochrome Oxidase/CS
Figure 5.

A) GSH

B) GSSG

C) GSH/GSSG

D) Protein bound –SH