Renal mitochondrial dysfunction in spontaneously hypertensive rats is attenuated by losartan but not by amlodipine

Elena M. V. de Cavanagh,4 Jorge E. Toblli,2 León Ferder,3 Bárbara Piotrkowski,1 Inés Stella,4 and Felipe Inserra4

1Physical-Chemistry Department, School of Pharmacy and Biochemistry, University of Buenos Aires; 2Laboratory of Experimental Medicine, Hospital Aleman, Buenos Aires, Argentina; 3Department of Physiology, Ponce School of Medicine, Ponce, Puerto Rico; and 4Laboratory of Experimental Nephrology, Institute of Cardiovascular Research, School of Medicine, University of Buenos Aires, Buenos Aires, Argentina

Submitted 24 August 2005; accepted in final form 5 January 2006

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 H2O2 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 monoamine oxidase 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.

ANG II can induce oxidant stress by stimulating the generation of both nitric oxide (NO) (38) and NAD(P)H oxidase-derived superoxide (44), thereby enhancing peroxynitrite formation. ANG II receptor blockers (ARB) and ANG I-converting enzyme 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 ANG I-converting enzyme 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 ANG II receptor blockade on potential mitochondrial changes in the SHR. Lowering of blood pressure with a calcium channel blocker, i.e., an antihypertensive agent acting through a mechanism distinct from ANG II receptor blockade, was used for comparison.

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
MATERIALS AND METHODS

Animals and treatments. All of the experiments were approved by the Hospital Aléman Ethics Committee and the Teaching and Research Committee. They were conducted according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Inbred, 8-wk-old male SHR (Laboratory of Experimental Medicine, Hospital Aléman, Buenos Aires, Argentina; Charles River Laboratories, Wilmington, MA) were randomly divided into three groups of eight animals each. During the following 6 mo, the rats were administered drinking water containing either losartan (40 mg·kg⁻¹·day⁻¹, SHR+Los), or amlodipine (3 mg·kg⁻¹·day⁻¹, SHR+Aml), or water with no additions (SHR). Eight-month-old Wistar-Kyoto (WKY) rats that had received drinking water with no additions since birth were used as normotensive controls. Rats had been maintained on drinking water containing either losartan (40 mg·kg⁻¹·day⁻¹, SHR+Los), or amlodipine (3 mg·kg⁻¹·day⁻¹, SHR+Aml), or water with no additions (SHR). Eight-month-old Wistar-Kyoto (WKY) rats 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, Argentina) and were housed in individual cages maintained at 21 ± 2°C and a 12:12-h light-darkness cycle (7 AM to 7 PM). Systolic blood pressure, evaluated by tail plethysmography (Narco Bio-Systems, Austin, TX), proteinuria, and creatinine clearance (Randox Laboratories, Crumlin, UK) 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 0.9% NaCl (wt/vol) 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-micrometer sections were cut and stained with hematoxylin and eosin and Masson’s trichrome. Unless otherwise stated, all reagents were from Sigma Chemical (St. Louis, MO).

Immunolabeling. Renal α-smooth muscle 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, Burlingame, 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 1) glomerular areas and 2) 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 NO 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 of 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. Electron microscopy 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 (ΔΨ) was evaluated after loading isolated kidney mitochondria with rhodamine 123 (Rh123) 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 MgCl₂, 5 mM potassium phosphate, 20 mM potassium-HEPES, pH 7.4, in the presence of 0.2 mg mitochondrial protein, 0.24 μM Rh123, 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-nm-to-497-nm excitation fluorescence ratio than the change in the intensity measured at each wavelength. To calibrate the fluorescence ratio for estimation of ΔΨ, a calibration curve was constructed by plotting 520-nm-to-497-nm excitation fluorescence ratios obtained in the presence of mitochondria and dinitrophenol (0, 1, 2, 4, 8, and 16 μM) to attain discrete levels of ΔΨ, and the corresponding ΔΨ values obtained by using the Nernst equation: ΔΨ = 59 log ([R123]in/[R123]out), where R123 concentration inside mitochondria ([R123]in) and R123 concentration outside mitochondria ([R123]out) were calculated as described in Ref. 46.

H₂O₂ production. Mitochondrial H₂O₂ production was determined by fluorometry at 350-nm and 460-nm excitation and emission wavelengths, respectively, as described in Ref. 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. H₂O₂ (0.05–0.35 μM) was used as standard. Antimycin was used to maximize H₂O₂ detection, since baseline/normal reactive oxygen species (ROS) production was either barely detectable or undetectable in the WKY and SHR+Los groups, but not in the SHR and SHR+Aml groups.

Mitochondrial enzyme activities. Mitochondrial NOS activity was determined by conversion of [1⁴C]arginine to [1⁴C]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 CaCl₂, 100 μM NADPH, 60 mM valine, 50 μM arginine, 0.025 μM [l⁴C]arginine, and 0.15 mg mitochondrial protein. Assay mixtures were incubated at 37°C for 5 min. The reaction was stopped by adding three volumes of 2 mM EDTA, 20 mM HEPES buffer, pH 5.5, followed by six volumes of a Dowex exchange resin (BioRad, Hercules, CA). Aliquots of the supernatant were used for scintillation counting (29). 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 μM) 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 Ref. 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 three 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 temperature by following the reduction of 5,5′-dithiobis-(2-nitrobenzoic) acid (DTNB) (0.1 mM) at 412 nm, in a reaction medium containing 100 mM Tris·HCl, pH 8.1, 0.4 mM sodium acetylcoenzyme 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
the addition of OAA (corresponding to acetyl-CoA hydrolase activity) was subtracted from that recorded after the addition of OAA, and emission = 13.6 mM/cm for reduced DTNB was used.

Western blot analysis of uncoupling protein 2. Mitochondrial proteins were separated on SDS-12.5% polyacrylamide gels and transferred to polyvinylidene difluoride membranes by liquid electrophotography (MiniProtein 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. Uncoupling protein 2 (UCP-2) antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was diluted in 2% dry low-fat milk and transferred to polyvinylidene difluoride membranes by liquid electrophotography. 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, Frederick, MD). The amount of protein loaded into gels was normalized by staining membranes with Ponceau Red.

Reduced and oxidized glutathione and protein-bound sulfhydryl group determination. To prevent conversion of reduced glutathione (GSH) to oxidized glutathione (GSSG), tissue samples were homogenized with 4 volumes 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 K₂PO₄, the supernatants were used for 1) GSSG+GSH determination using the DTNB spectrophotometric assay; and 2) GSSG determination by following NADPH oxidation at 340 nm (17). Results are expressed as nanomoles of GSH equivalents per gram of wet tissue and nanomoles of GSSG per gram of wet tissue, respectively.

Statistical methods. Values in text, Figs. 1–5, and Tables 1–2 are means ± SE. Statistical analyses were performed by one-way ANOVA and F-test (Statview SE+Graphics version 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, San Diego, CA) 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; and 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 3 mo of the study and remained stably high thereafter. After 1 mo 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 3 mo of treatment; however, it remained significantly higher than in WKY. During the last 3 mo 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 SHR + Amlo 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

---

**Table 1. Body weight, proteinuria, and creatinine clearance**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>SHR</th>
<th>SHR + Los</th>
<th>SHR + Amlo</th>
<th>WKY</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, g</td>
<td>278.2 ± 17.7</td>
<td>275.8 ± 15.7</td>
<td>279.9 ± 16.8</td>
<td>282.0 ± 18.4</td>
</tr>
<tr>
<td>End of study</td>
<td>334.6 ± 17.3</td>
<td>319.4 ± 16.9</td>
<td>340.1 ± 19.4</td>
<td>334.2 ± 20.3</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Proteinuria, mg/day</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
</tr>
<tr>
<td>End of study</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Creatinine clearance, ml/min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
</tr>
<tr>
<td>End of study</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 8 animals for each group. SHR, spontaneously hypertensive rats; WKY, Wistar-Kyoto; Los, losartan; Amlo, amlodipine. Body weight: nonsignificant differences between groups were found. Basal proteinuria and creatinine clearance: nonsignificant differences between groups were found. End-of-study proteinuria and creatinine clearance: *P < 0.01: SHR and SHR + Amlo vs. SHR + Los and WKY.
Figure 3 shows the effects of losartan and amlodipine treatments on mitochondrial function. Figure 3 shows the effects of losartan and amlodipine treatments on kidney mitochondrial function. Figure 3 shows the effects of losartan and amlodipine treatments on kidney mitochondrial function. Figure 3 shows the effects of losartan and amlodipine treatments on kidney mitochondrial function. Figure 3 shows the effects of losartan and amlodipine treatments on kidney mitochondrial function. Figure 3 shows the effects of losartan and amlodipine treatments on kidney mitochondrial function. SHRs and WKYs were normalized to citrate synthase activity. The yield among the groups, mitochondrial function parameters were normalized to citrate synthase activity. The yield among the groups, mitochondrial function parameters were normalized to citrate synthase activity. The yield among the groups, mitochondrial function parameters were normalized to citrate synthase activity. The yield among the groups, mitochondrial function parameters were normalized to citrate synthase activity. The yield among the groups, mitochondrial function parameters were normalized to citrate synthase activity. Mitochondrial citrate synthase activity showed no differences among the groups studied (WKY = 228.8 ± 3.86; SHR + Los = 230.7 ± 2.11; SHR + Amlo = 228.7 ± 3.41 μmol CoA·min⁻¹·mg protein⁻¹). To account for potential differences in mitochondrial yield among the groups, mitochondrial function parameters were normalized to citrate synthase activity. The ∆Φ was significantly lower in the SHR group relative to the SHR + Los and WKY groups (−48 and −44%, respectively). In the SHR + Amlo group, ∆Φ 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 H₂O₂ 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 and 82%, respectively) and WKY (85 and 88%, respectively) groups. In the SHR + Los group, UCP-2 protein level was three 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 Fig. 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 Fig. 5. 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, SHRs and WKYs were normalized to citrate synthase activity. The yield among the groups, mitochondrial function parameters were normalized to citrate synthase activity. The yield among the groups, mitochondrial function parameters were normalized to citrate synthase activity. The yield among the groups, mitochondrial function parameters were normalized to citrate synthase activity. The yield among the groups, mitochondrial function parameters were normalized to citrate synthase activity. The yield among the groups, mitochondrial function parameters were normalized to citrate synthase activity. Mitochondrial citrate synthase activity showed no differences among the groups studied (WKY = 228.8 ± 3.86; SHR + Los = 230.7 ± 2.11; SHR + Amlo = 228.7 ± 3.41 μmol CoA·min⁻¹·mg protein⁻¹). To account for potential differences in mitochondrial yield among the groups, mitochondrial function parameters were normalized to citrate synthase activity. The ∆Φ was significantly lower in the SHR group relative to the SHR + Los and WKY groups (−48 and −44%, respectively). In the SHR + Amlo group, ∆Φ 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 H₂O₂ 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 and −89%, respectively) and WKY (−85 and −88%, respectively) groups. In the SHR + Los group, UCP-2 protein level was three 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 Fig. 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 Fig. 5. 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+/H11001 Los, and WKY groups (76, 62, and 70%, respectively). The level of renal GSSG, the oxidation product of GSH, was three to four times higher in the SHR group relative to the SHR+/H11001 Los, SHR+/H11001 Amlo, and WKY groups. The GSH-to-GSSG ratio, an index of glutathione redox status, was significantly lower (70%) in the SHR and SHR+/H11001 Amlo groups relative to both the SHR+/H11001 Los and WKY groups. Kidney protein-bound sulfhydryl group content was significantly lower in the SHR+/H11001 Amlo group than in the SHR, SHR+/H11001 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 $\Delta \Phi$ 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 ANG II receptor blockade and attenuation of ANG 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, ANG II 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-SMA 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 compared with 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, compared with WKY. Also, losartan, but not amlodipine, reduced oxidant stress, as revealed by 1) the maintenance of the glutathione pool in a relatively more reduced status, 2) the preservation of Mn-SOD activity, and 3) 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 ΔΦ 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 H2O2 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 H2O2 production, found in SHR+Amlo, can be explained, taking into account that, in addition to complexes I and III, other mitochondrial superoxide sources are the ubiquinone pool, electron transfer flavoprotein, and electron transfer flavoprotein quinone oxidoreductase.

To evaluate mitochondrial function, we selected a “nonclassical” group of indicators, such as membrane potential, UCP-2 content, Mn-SOD, and mitochondrial NOS activities. In addition, electron transfer through complexes I–III and cytochrome oxidase activity were measured. We chose to determine ΔΦ 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 protons motive force cannot exceed 1 pH unit. In addition, ΔΦ 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 H2O2 (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 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 H2O2 production (Fig. 3C) and a decreased activity of Mn-SOD (Fig. 3D), in SHR and SHR+Amlo, may be explained considering that H2O2 measured under the assay conditions used in this study (i.e., in the presence of externally added SOD) is the sum of H2O2 derived from Mn-SOD-related conversion of superoxide in the matrix plus H2O2 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
Amlo, a decrease in Mn-SOD activity resulted in higher matrix levels of superoxide, which, upon release to the cytosol, was converted into H$_2$O$_2$ 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 stroke-prone SHR (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 laboratory’s previous findings in aging rats (17).

Since, as mentioned above, UCP-2 is known to increase proton leak, resulting in lower $\Delta\Phi$, 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 by 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 $\Delta\Phi$ in those groups (Fig. 3A). 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 H$_2$O$_2$, Fig. 3C). From the present data, it is apparent that activation of low UCP-2 levels in SHR and SHR + Amlo was sufficient to reduce $\Delta\Phi$ 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 $\Delta\Phi$ 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 mitochon-
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 H$_2$O$_2$ can activate eNOS, whereas higher levels block enzyme activation (52). This observation is in line with our data showing increased H$_2$O$_2$ 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 1) the regulation of mitochondrial superoxide production, which can influence H$_2$O$_2$ generation and the consequent downstream cell signaling; 2) the modulation of tissue oxygen gradients; and 3) 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 ROS (55). This is in agreement with the observed increase of H$_2$O$_2$ 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 H$_2$O$_2$ production, observed in this study, may have contributed to the maintenance of $\Delta \Phi$ within the values displayed by nonhypertensive 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 ROS production, leading to the reduction of $\Delta \Phi$ (28). Based on this evidence, we propose that the contrasting effects displayed by losartan and amloidipine treatments on mitochondrial function may be a consequence of losartan-mediated blockade of ANG II actions. In this context, ANG II is associated with downregulation of peroxisome proliferator-activated receptor-α (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 amloidipine, by upregulating peroxisome proliferator-activated receptor-α 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 ~20% cortical and 80% medullar tissue, with total medullar interstitium volume amounting to ~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 amloidipine treatments.

In this study, we focused on mitochondria as generators of superoxide, while the effects of RAS inhibition on other ANG II-related superoxide sources were not analyzed. ANG 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 suggest 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 ANG II-related oxidant stress is associated with mitochondrial dysfunction in the kidney. Accordingly, AT$_1$ blockade displays antioxidant effects and preserves kidney mitochondria favoring the maintenance of adequate tissue function. Consequently, the renal protective effects of AT$_1$ 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 whether mitochondrial dysfunction is the result of high ANG II 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**

The authors thank Andrea Biscochea and Marcelo Ferder for technical assistance.

**GRANTS**

This work was partially supported by grants from the University of Buenos Aires B042, ANPCYT (PICT-01–08951), and Consejo Nacional de Investigaciones Científicas y Técnicas (0738/98).

**REFERENCES**


11. Capaldi RA, Marusich MF, and Taanman JW. *Chen L, Tian X, and Song L. Doroshchuk AD, Postnov A, Afanas’eva GV, Budnikov E, and Post-


17. Doroshchuk AD, Postnov A, Afanas’eva GV, Budnikov E, and Post-

18. Ohtsuki T, Matsumoto M, Suzuki K, Taniguichi N, and Kamada T. Mitochondrial lipid peroxidation and superoxide dismutase in rat hyper-


22. Ohtsuki T, Matsumoto M, Suzuki K, Taniguichi N, and Kamada T. Mitochondrial lipid peroxidation and superoxide dismutase in rat hyper-


26. Ohtsuki T, Matsumoto M, Suzuki K, Taniguichi N, and Kamada T. Mitochondrial lipid peroxidation and superoxide dismutase in rat hyper-


30. Ohtsuki T, Matsumoto M, Suzuki K, Taniguichi N, and Kamada T. Mitochondrial lipid peroxidation and superoxide dismutase in rat hyper-


