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Long-Term Blood Pressure Control Prevents Oxidative Renal Injury

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ABSTRACT

Arterial hypertension is a leading contributor to the progression of chronic renal disease. Short-term studies had addressed the role of oxidative stress in hypertensive nephropathy. We have now studied oxidative stress and caspase activation in a long-term model of hypertensive renal injury. Nontreated spontaneously hypertensive rats with uninephrectomy displayed severe arterial hypertension over a 36-week follow-up. Uncontrolled high blood pressure in the context of modest renal mass reduction resulted in significant histological renal injury. Blood pressure control by the angiotensin-converting enzyme (ACE) inhibitor, quinapril, or the AT_1 receptor antagonist, losartan, decreased the degree of renal injury. Hypertensive renal injury was associated with evidence of activation of the apoptotic pathway (increased activation of caspase-3) and local renal (increased staining for 4-hydroxy-2-nonenal) and systemic [increased serum levels of 8-iso-prostaglandin $F_{2\alpha}$ (8-iso-$PGF_{2\alpha}$)] lipid oxidation when compared with normotensive control rats. In addition, severe hypertension decreased the renal antioxidant defenses, as exemplified by decreased expression of Cu/Zn superoxide dismutase. Treatment with quinapril or losartan decreased caspase-3 activation, 4-hydroxy-2-nonenal staining, and 8-iso-$PGF_{2\alpha}$ levels and increased Cu/Zn superoxide dismutase expression. These results suggest that hypertension-associated oxidative stress and its consequences may be decreased by either ACE inhibition or AT_1 receptor antagonist, emphasizing the role of angiotensin II in hypertensive renal damage. *Antioxid. Redox Signal.* 7, 1285–1293.

INTRODUCTION

ARTERIAL HYPERTENSION is a leading cause of end-stage renal disease and contributes to the progression of other forms of chronic renal disease (22, 29). Hypertensive nephrosclerosis is characterized by glomerular and arterioarteriolar sclerosis, inflammatory cell infiltration, interstitial fibrosis, and tubular atrophy (14). Loss of renal parenchymal cells is prominent (17). However, the pathophysiological processes that result in progressive loss of renal cells are poorly understood. Oxidative stress in blood vessels and the kidney can accompany hypertension in many models, including the spontaneously hypertensive rats (SHR) (for reviews, see 26, 33). Oxidative stress contributes to chronic inflammation (3) and to apoptotic cell death (2, 11, 21). Apoptosis is an active form of cell death that plays an important role in maintaining cell number homeostasis in both health and disease (for reviews, see 16, 20). Unregulated excessive apoptosis promotes renal cell loss in progressive chronic nephropathies, including hypertensive nephrosclerosis (20). However, data on the response of renal cell apoptosis to adequate blood pressure control are scarce, and the molecular mechanisms are poorly

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understood. Intracellular proteases, such as caspases, play a prominent role in apoptosis and promote oxygen radical production from mitochondria (20, 21).

The present study was designed to investigate the role of oxidative stress in the long-term progressive renal damage associated with hypertension and to address possible molecular mechanisms. SHR, an experimental model considered to be similar in many aspects to hypertension in humans, were studied. To further assimilate the model to the human situation, renal mass was reduced. This more closely mimics the effect of high blood pressure in patients with coexistent renal disease. Furthermore, rats were followed long-term, as high blood pressure is a chronic condition that, in humans, damages the kidney over many years. In addition, we have studied the renal response to tight blood pressure control with angiotensin-converting enzyme (ACE) inhibitors and angiotensin II receptor antagonists. These drugs are widely used in hypertensive patients with renal disease.

MATERIALS AND METHODS

Drugs

The ACE inhibitor quinapril (as powdered hydrochloride salt), kindly provided by Pfizer (Barcelona, Spain), and the angiotensin II type 1 receptor (AT_1) antagonist losartan potassium (Merck Sharp & Dohme, Madrid, Spain) were administered in the drinking water.

Experimental model

Studies were performed in male SHR and normotensive Wistar–Kyoto (WKY) rats (Criffa, Barcelona, Spain). To accelerate renal damage, 12-week-old SHR underwent unilateral nephrectomy (UNX) as previously described (10) and were then randomized to the following groups: UNX-SHR, nontreated animals with spontaneous development of the disease ($n = 7$); quinapril + UNX-SHR, animals that received 16 mg/kg/day quinapril ($n = 8$); losartan + UNX-SHR, animals that received 30 mg/kg/day losartan ($n = 8$). WKY rats of the same age were studied as normotensive control ($n = 7$). Animals were followed for 36 weeks, and allowed free access to water and food in a controlled light, temperature, and humidity environment.

Systolic blood pressure (SBP) was measured weekly in conscious animals by a tail-cuff sphygmomanometer (NARCO Biosystems, Austin, TX, U.S.A.). Three independent SBP measurements per animal were averaged and recorded at each session.

At 48 weeks of age, all rats were anesthetized with pentobarbital sodium (5 mg/100 g of body weight; B. Braun Medical SA, Barcelona, Spain) and sacrificed. The kidneys were perfused with cold sodium saline and quickly removed. Kidney samples were snap-frozen in liquid nitrogen and kept at −80° C, or fixed in 4% paraformaldehyde (24 h) and paraffin-embedded.

Renal histopathological studies

Paraffin-embedded renal sections (4 μm thick) were stained with Masson's trichrome (Bio-Optica, Milan, Italy). A kidney injury score was calculated using the following semiquantitative index: 0, no changes; 1, focal changes that involve 25% of the sample; 2, changes affecting >25 to 50%; 3, changes involving >50 to 75%; 4, lesions affecting >75%. The injury score was calculated by the sum of this semiquantitative assessment of glomerular damage (mesangial cell proliferation and matrix expansion), tubulointerstitial injury (tubular dilation and/or atrophy), interstitial fibrosis, and inflammatory cell infiltrate. Two independent pathologists scored the kidney injury in a blinded fashion.

Human kidney specimens

Kidney samples were obtained by percutaneous renal biopsy from patients undergoing diagnostic evaluation at the Division of Nephrology, Universidad Austral, Valdivia, Chile. The renal biopsies from seven patients with essential hypertension and with the classic pattern of benign nephrosclerosis were studied.

Control human kidney specimens ($n = 3$) were taken from normal portions of renal tissue from patients who underwent surgery because of localized renal tumors.

For light microscopy, kidney tissues were fixed in 4% buffered formalin, dehydrated, and embedded in paraffin by conventional techniques.

Immunohistochemistry

Immunostaining of active caspase-3, Cu/Zn superoxide dismutase (SOD), and 4-hydroxy-2-nonenal (4-HNE) was performed in paraffin-embedded 4-μm-thick renal sections (8). Sections were dewaxed and rehydrated. After quenching of endogenous peroxidase activity, sections were incubated with a polyclonal anti-cleaved caspase-3 (Asp^{175}) antibody (Cell Signaling Technology, Inc., Beverly, MA, U.S.A.) 1:50 in phosphate-buffered saline (PBS), a polyclonal anti-Cu/Zn SOD antibody (Stressgen Biotechnologies, Victoria, BC, Canada) 1:1,500 in PBS, or a mouse monoclonal anti-4-HNE antibody (Oxis Health Products, Portland, OR, U.S.A.) diluted 1:75 in PBS overnight at 4°C in a humid atmosphere. Thereafter, sections were processed with a corresponding secondary biotinylated antibody (for caspase-3 and Cu/Zn SOD; Amersham, Bioscience, U.K.) or anti-IgG peroxidase antibody (for 4-HNE; Amersham, Bioscience, U.K.) at 1:200, and with avidin-biotin complex containing horseradish peroxidase (ABComplex; DAKO, Glostrup, Denmark). Immunostaining was detected with 3,3′-diaminobenzidine (Sigma, Madrid, Spain), and sections were counterstained with Mayer's hematoxylin (Sigma). The specificity of the antibodies was verified by controls lacking the primary antibody, producing no background. Two independent observers evaluated the inmunostaining results in a blinded fashion.

Immunohistochemistry quantification

The surface labeled by anti-Cu/Zn SOD or anti-4-HNE antibodies was evaluated by quantitative image analysis using a KZ 300 imaging system 3.0 (Zeiss, München-Hallbergmoos, Germany). In brief, the percentage of the stained area was calculated as the ratio of suitable binary thresholded image and the total field area. For each sample, the mean

staining area was obtained by analysis of the entire sample. For caspase-3, the number of positive cells was counted.

Serum levels of 8-iso-prostaglandin $F_{2\alpha}$ (8-iso-$PGF_{2\alpha}$)

8-iso-$PGF_{2\alpha}$ is a marker of oxidative stress. Serum levels of 8-iso-$PGF_{2\alpha}$ were measured using the commercially available StressXpress™ 8-iso-$PGF_{2\alpha}$ enzyme-linked immunosorbent assay kit (Stressgen Bioreagents, Victoria, BC, Canada) according to the manufacturer's protocol. The assay uses a rabbit polyclonal antibody specific for 8-iso-$PGF_{2\alpha}$ to bind competitively to either 8-iso-$PGF_{2\alpha}$ in the sample or 8-iso-$PGF_{2\alpha}$ covalently attached to alkaline phosphatase. The alkaline phosphatase activity resulted in color development of the substrate. Color intensity was proportional to the amount of 8-iso-$PGF_2\alpha$–alkaline phosphatase conjugate bound and inversely proportional to the amount of 8-iso-$PGF_{2\alpha}$ in the samples. The concentrations in the samples are quantified by interpolating absorbance readings from a standard curve generated with the calibrated 8-iso-$PGF_{2\alpha}$ standard provided and expressed as picograms per milliliter.

Antioxidant gene expression

Cu/Zn SOD gene expression was analyzed by real-time reverse transcription–polymerase chain reaction (RT-PCR). Total RNA was isolated from homogenized kidney with TRIZOL (Invitrogen, San Diego, CA). Real time RT-PCR was performed on a TaqMan ABI 7700 Sequence Detection System using heat-activated *Taq* DNA polymerase (Amplitaq Gold). After an initial hold of 2 min at 50°C and 10 min at 95°C, the samples were cycled 40 times at 95°C for 15 s and 60°C for 60 s. For all quantitative cDNA analysis, the ΔCt technique was applied (6). Glyceraldehyde-3-phosphate dehydrogenase and 18S rRNA served as housekeeping genes and were amplified in parallel with the gene of interest. The expression of the target gene was normalized to both different housekeeping transcripts.

We used the assay on demand for Cu/Zn SOD: Rn 00566938. All reagents, probes, and primers were obtained from Applied Biosystems (Foster City, CA, U.S.A.). All measurements were performed in duplicate. Controls consisting of double-distilled water were negative in all runs.

Statistical analysis

Results are expressed as means ± SEM. Significance was established by the GraphPAD Instat (GraphPAD Software, San Diego, CA, U.S.A.) using *t* test, Mann–Whitney test (nonparametric *t* test), ANOVA, or Kruskal–Wallis test when appropriate. Differences were considered significant if the *p* value was <0.05.

RESULTS

SBP

Figure 1 shows the SBP measurements. Nontreated UNX-SHR displayed severe arterial hypertension with an average SBP of 212.8 ± 1.9 mm Hg, significantly higher ($p < 0.001$) than that of all other groups. The two treatment regimens used achieved tight SBP control (122 ± 1.1 and 135.1 ± 0.7 mm Hg for quinapril and losartan, respectively), although the SBP in the losartan group was significantly higher than in the quinapril group ($p < 0.05$).

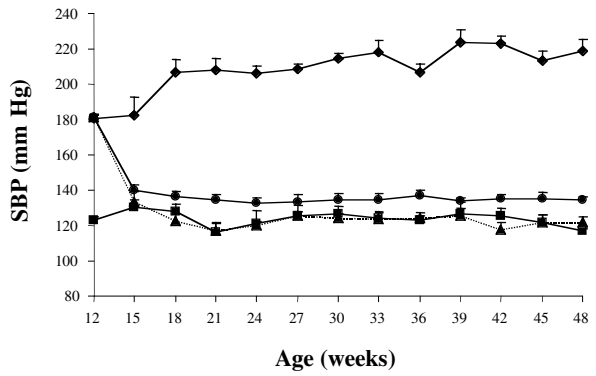

FIG. 1. Evolution of SBP. Measurements were done in UNX-SHR (◆), losartan (30 mg/kg/day)-treated UNX-SHR (●), quinapril (16 mg/kg/day)-treated UNX-SHR (—▲—), and normotensive rats (WKY) (■). Losartan and quinapril were administered from week 12 to week 48. Results are expressed as means ± SEM (n = 7–8 animals per group). Average SBP was significantly higher ($p < 0.001$) in UNX-SHR than all other groups. SBP in the losartan group was significantly higher than in the quinapril group ($p < 0.05$).

Kidney damage

Uncontrolled high blood pressure in the context of renal mass reduction resulted in significant histological renal injury. Renal injury was characterized by mesangial expansion, tubular atrophy, and interstitial fibrosis and inflammation (Fig. 2). Treatment with quinapril or losartan decreased the degree of renal injury (Fig. 2).

Activation of apoptotic mechanisms

Caspase-3 is the central executioner caspase in the course of apoptosis (20). Caspase-3 is activated by cleavage by other caspases. Hypertensive renal injury was associated with evidence of activation of the apoptotic pathway, such as the presence of activated cleaved caspase-3 (Fig. 3). Treatment with either quinapril or losartan decreased, but did not normalize, caspase-3 activation.

Oxidative stress

Oxidative mechanisms play a key role in apoptosis. In hypertensive rats, evidence of oxidative stress was found both in the kidney and systemically. 4-HNE is an aldehyde product of polyunsaturated fatty acid oxidation, which is a marker of oxidative injury of lipids. In the kidney of hypertensive rats, increased staining for 4-HNE was noted in both glomeruli and tubules (Fig. 4). In addition, increased serum levels of 8-iso-$PGF_{2\alpha}$, another marker of lipid oxidation, were observed (Fig.

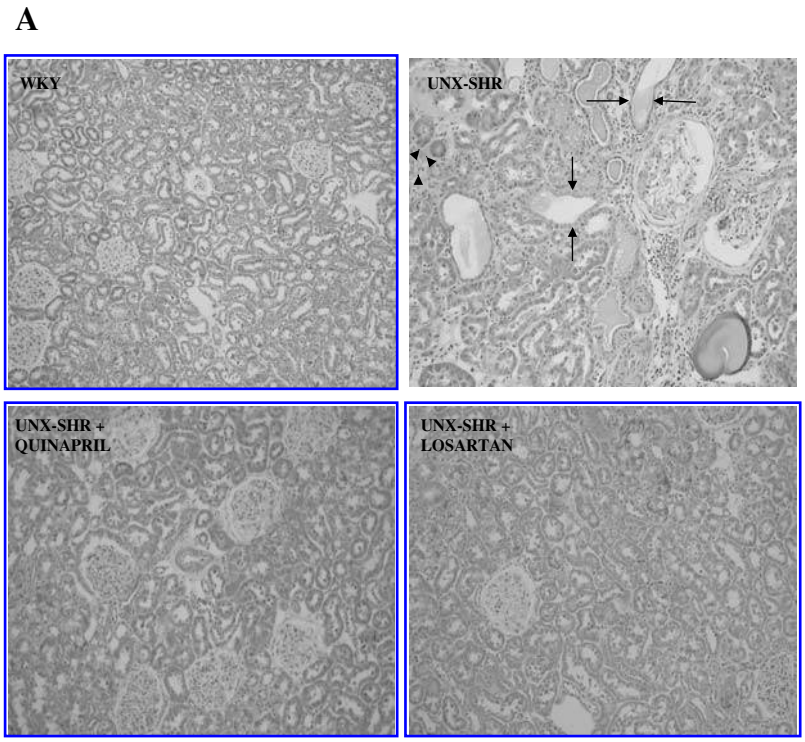

FIG. 2. Hypertensive renal injury is prevented by quinapril or losartan. (**A**) Representative images of the renal pathology of the different treatment groups at week 48. Note signs of tubular atrophy, including dilated tubules (arrows) and interstitial fibrosis (arrowheads), in untreated UNX-SHR, compared with UNX-SHR treated with quinapril or losartan and normotensive (WKY) rats (Masson's trichrome staining; magnification, ×10). (**B**) Data represent means ± SEM of the renal injury score (n = 7–8 animals per group). *p < 0.001 compared with other groups.

5) when compared with normotensive control rats. Treatment with either quinapril or losartan reduced both indicators of oxidative injury (Figs. 4 and 5).

Oxidative stress results from the imbalance between reactive oxygen species (ROS) generation and destruction. A variety of antioxidant enzymes protect from oxidative stress. Cu/Zn SOD is one of such enzymes (7). The expression of Cu/Zn SOD protein was decreased in untreated hypertensive rats (Fig. 6). Treatment with either quinapril or losartan restored Cu/Zn SOD expression (Fig. 6). Although there was trend for lower Cu/Zn SOD mRNA expression in untreated hypertensive rats, it did not reach statistical significance (Fig. 6).

Human renal biopsies

In renal sections of hypertensive patients, Cu/Zn SOD was detected mainly in tubular epithelial cells, and was almost undetectable in glomeruli and renal arteries. The staining score was significantly lower in hypertension than in control kidneys (Fig. 7A). On the other hand, 4-HNE staining was almost undetectable in normal renal sections, but significantly increased in hypertensive patients (Fig. 7B).

DISCUSSION

Our results show that long-lasting hypertension with modest renal mass reduction leads to severe renal lesions. In addition, tight control of blood pressure with drugs targeting angiotensin II prevents long-term renal injury. We had previously demonstrated that tight control of blood pressure with an ACE inhibitor, AT_1 antagonist, or triple therapy was associated with decreased proteinuria, renal lesions, and renal

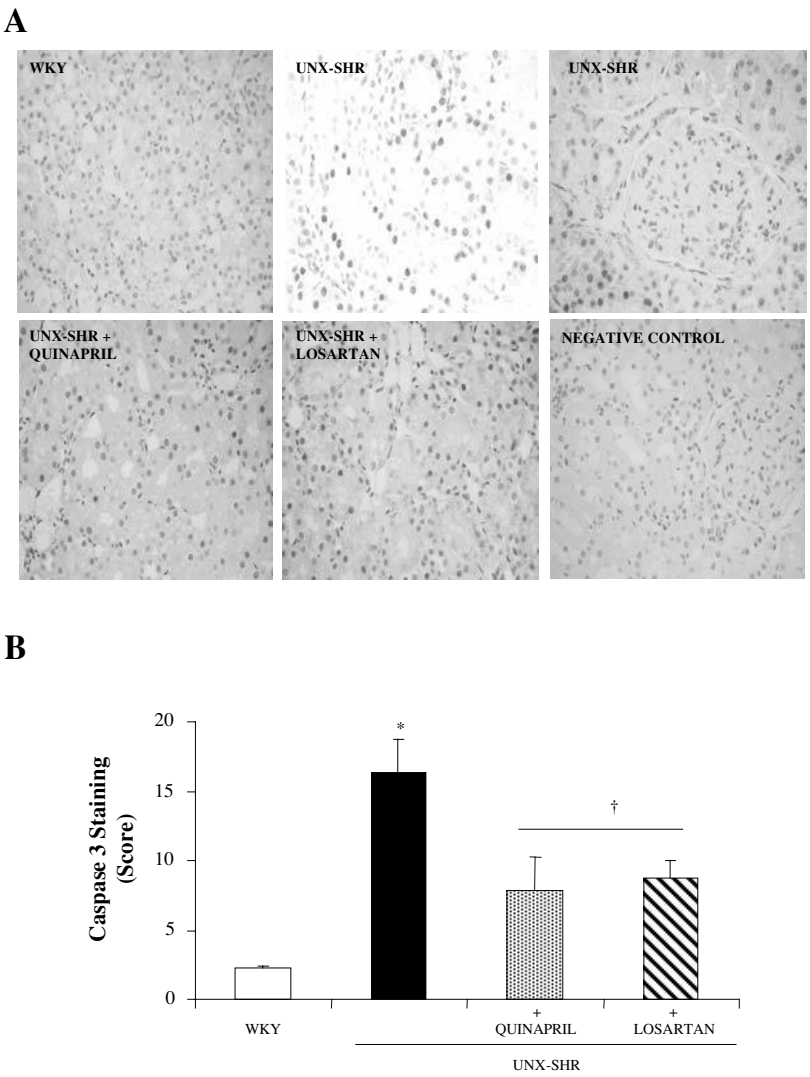

FIG. 3. Hypertensive renal injury is associated with caspase-3 activation. (**A**) Localization of activated cleaved caspase-3 in kidney sections. Note that renal tubules are the main site of caspase-3 activation in UNX-SHR (magnification, ×20). (**B**) Semiquantification of cleaved caspase-3 immunostaining in renal cells. Treatment with either quinapril or losartan decreased, but did not normalize, caspase-3 activation. Data represent means ± SEM (n = 7–8 animals per group). *$p < 0.05$ compared with all other groups; †$p < 0.05$ compared with normotensive rats.

cell apoptosis rate in a subacute (5 weeks) model resembling malignant hypertension (25). In the present study, we extend these observations to a model of a more common clinical condition: long-standing hypertension in the context of chronic kidney disease. The present study differs from previous publications in the long-term nature of the follow-up. It extends previous observations of the beneficial effect of short-term treatment of hypertension with ACE inhibitors or angiotensin II antagonists on oxidative stress and renal cell apoptosis (9, 25, 32), and improves our understanding of the nephroprotective effect of blood pressure control with these compounds. Short-term results of therapy may not be relevant to the human condition, which persists for decades.

Hypertension is accompanied by oxidative stress of large blood vessels and certain organs, including the kidney (15, 26). Oxidative stress has multiple potential consequences: it stimulates growth-signaling pathways, induces expression of proinflammatory genes, alters contraction–excitation coupling, and impairs endothelial function (28). In our model of hypertension-induced renal injury, which resembles chronic hypertension in the setting of modest renal mass reduction and older age in humans, we have found oxidative stress in the kidney, characterized by elevated renal 4-HNE staining and serum levels of 8-iso-$PGF_{2\alpha}$, both markers of oxidative injury of lipids. Moreover, decreased renal Cu/Zn SOD gene and protein levels were found, reflecting a diminution of the antioxidant defenses. Similar changes were found in renal biopsies from hypertensive patients. These data indicate that oxidative stress is a common feature of kidney damage associated with hypertension, and our experimental model is a good tool to investigate long-term consequences of this disease.

At present, the cause of the oxidative stress in arterial hypertension is unclear. Both increased production and decreased clearance of ROS may have a role. Among possible mediators of the oxidative stress, angiotensin II has been singled out (26). Angiotensin II directly promotes oxidative stress in cultured cells and in the kidneys and other organs and systems *in vivo* (12, 23, 27, 34). Current evidence suggests that angiotensin II increases intracellular ROS concentration through both AT_1 and AT_2-receptor activation (26). *In vivo,* angiotensin II increases urinary 8-iso-$PGF_{2\alpha}$, decreases certain isoforms of renal SOD, and promotes changes in the proliferation of regulatory genes in the kidney (5, 34). In-

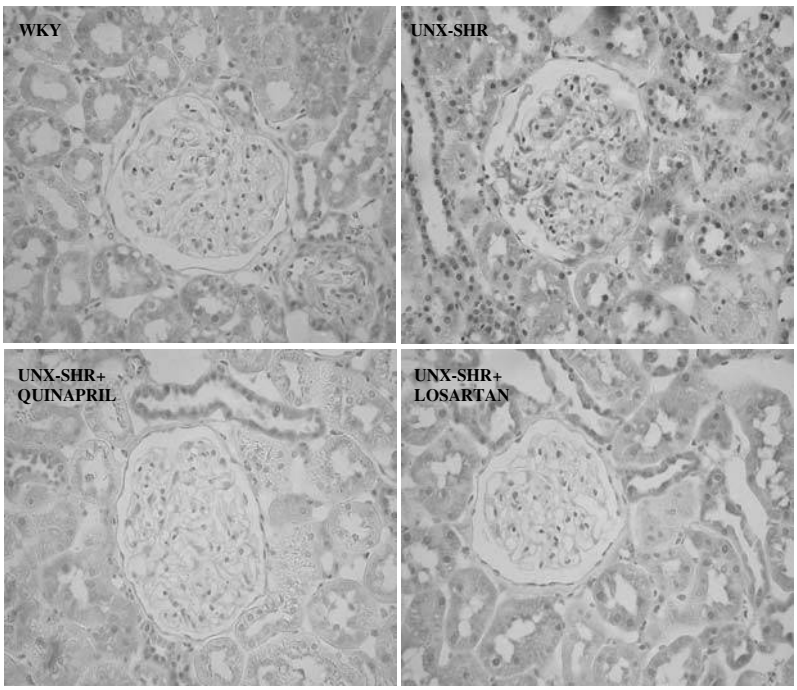

FIG. 4. Hypertensive renal injury is associated with local lipid peroxidation. (**A**) Localization of 4-HNE in kidney sections. Note glomerular staining and increased tubular staining in untreated UNX-SHR compared with UNX-SHR treated with quinapril or losartan and control normotensive rats (magnification, ×20). (**B**) Semiquantification of 4-HNE immunostaining in renal cells. Data represent means ± SEM (n = 7–8 animals per group). *p < 0.0001 compared with all other groups.

deed, ACE inhibitors decreased oxidative end-organ injury in a subacute model of rat hypertension (27). The beneficial effect of ACE inhibitors or angiotensin II antagonists in our system may be directly related to interference with angiotensin II. However, the design of the study does not allow differentiating between general consequences of tight blood pressure control or specific consequences of interference with angiotensin II by the use of these agents.

Regarding disposal of oxygen radicals, a number of enzymes reduce superoxide (O_2^-), which is formed as an intermediate. One such family of enzymes is the SODs, which catalyze the reaction of O_2^- with an electron and two protons to form hydrogen peroxide (H_2O_2) (7). Excess O_2^- production leads to decreased nitric oxide (NO) bioavailability and may promote hypertension (1). Indeed it has been found that the administration of a SOD mimetic to SHR rats normalizes mean arterial pressure (24). Studies in Cu/Zn SOD knockout mice have suggested that lack of this enzyme leads to increased O_2^- levels, loss of NO, and decreased endothelium-mediated vasodilatation (7). In other models with enhanced oxidant stress, namely, subtotal nephrectomy and lead-in-

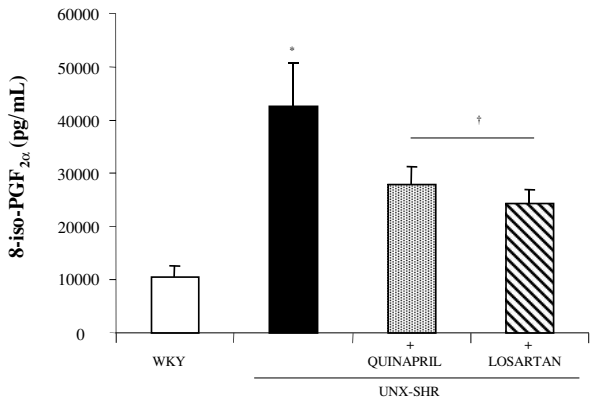

FIG. 5. Hypertensive renal injury is associated with systemic lipid peroxidation. Serum 8-iso-$PGF_{2\alpha}$ concentration was increased in untreated UNX-SHR compared with UNX-SHR treated with quinapril or losartan and control normotensive rats. Data are shown as means of each group ± SEM (n = 7–8 animals per group). *p < 0.05 compared with all other groups; †p < 0.05 with respect to WKY.

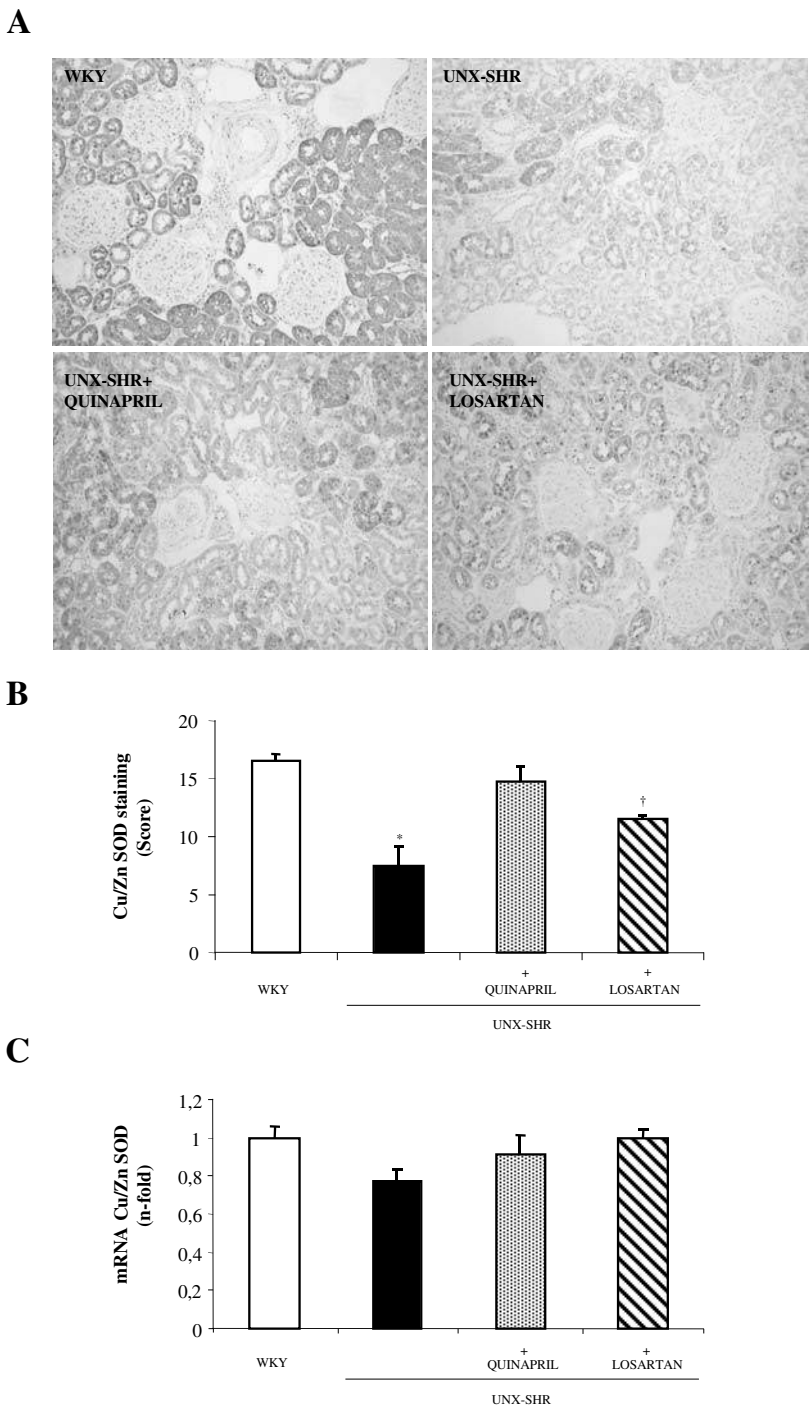

FIG. 6. Hypertensive renal injury is associated with decreased antioxidant defenses. (**A**) Localization of Cu/Zn SOD in kidney sections. Note localization in tubules and decreased expression in untreated UNX-SHR compared with UNX-SHR treated with quinapril or losartan and control normotensive rats (magnification, ×10). (**B**) Semiquantification of Cu/Zn SOD immunostaining in renal cells. *$p < 0.05$ compared with all other groups; $^{\dagger}p < 0.05$ with respect to WKY. (**C**) mRNA expression of Cu/Zn SOD, in arbitrary densitometry units. Data represent means ± SEM (n = 7–8 animals per group).

duced hypertension, renal expression of Cu/Zn SOD has been found to be decreased or increased (30, 31). However it was unchanged in the kidneys of young SHR (1). By contrast, we found that decreased Cu/Zn SOD in SHR with modest reduction in renal mass followed long-term. These differential findings emphasize one of the goals of our study: to address the effects of chronic hypertension and older age, as most patients with modest renal mass reduction are old and hypertensive.

In the experimental model of hypertension-induced renal damage and in kidney samples from hypertensive patients, we have observed elevated oxidative stress associated with caspase-3 activation, a marker of apoptosis. Oxidative stress directly promotes apoptosis (2, 11, 21). Angiotensin II may also promote apoptosis (4, 12, 35). In cultured mesangial cells, angiotensin II caused apoptosis by oxidative stress mechanisms (12). In addition, oxidative stress promotes inflammation (3). Inflammatory cells infiltrating the kidneys are known to express lethal cytokines, such as tumor necrosis factor and Fas ligand (13, 18, 19).

In summary, oxidative stress persists long-term in hypertensive animals with renal mass reduction. Oxidative stress is as-

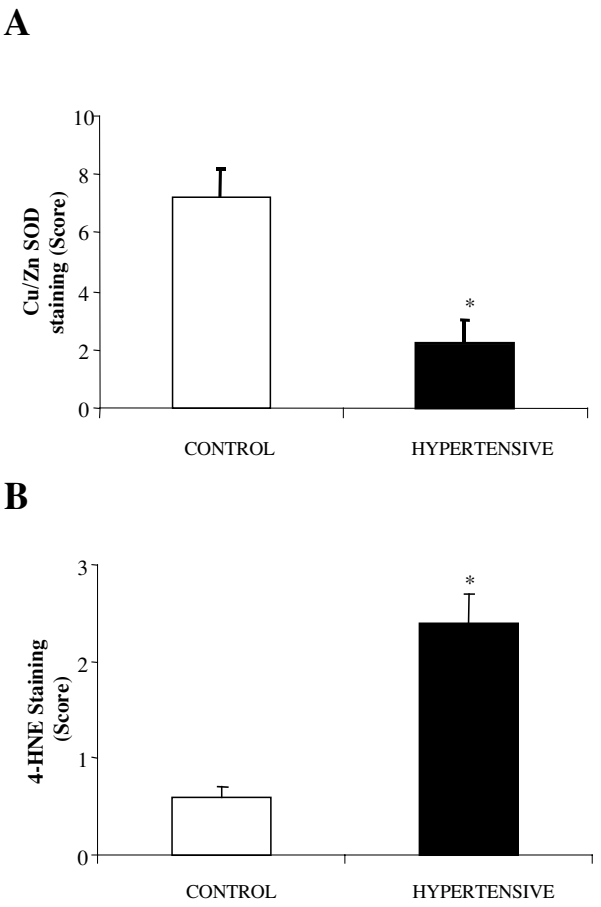

FIG. 7. Hypertensive human renal injury is associated with lipid peroxidation and decreased antioxidant defenses. Semiquantification of Cu/Zn SOD (**A**) and 4-HNE immunostaining (**B**) in human kidney samples is shown. *$p < 0.05$ versus control human kidneys. Data represent means ± SEM ($n = 7$ hypertensive human samples and $n = 3$ control human kidneys).

sociated with tissue injury and activation of apoptotic pathways. Decreased renal Cu/Zn SOD may contribute to oxidative stress. Long-term treatment with the ACE inhibitor quinapril or the angiotensin II antagonist losartan not only lowers blood pressure, but also prevents oxidative stress and tissue injury.

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ABBREVIATIONS

ACE, angiotensin-converting enzyme; AT_1, angiotensin II type 1 receptor; AT_2, angiotensin II type 2 receptor; 4-HNE, 4-hydroxy-2-nonenal; 8-iso-$PGF_{2\alpha}$, 8-iso-prostaglandin $F_{2\alpha}$; NO, nitric oxide; O_2^-, superoxide; PBS, phosphate-buffered saline; ROS, reactive oxygen species; RT-PCR, reverse transcription–polymerase chain reaction; SBP, systolic blood pressure; SHR, spontaneously hypertensive rats; SOD, superoxide dismutase; UNX, uninephrectomy; WKY, Wistar–Kyoto.

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