Renal expression of COX-2, ANG II, and AT$_1$ receptor in remnant kidney: strong renoprotection by therapy with losartan and a nonsteroidal anti-inflammatory

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Gonçalves, Anderson Ricardo Roman, Clarice Kazue Fujihara, Ana Lúcia Mattar, Denise Maria Avancini Costa Malheiros, Irene de Lourdes Noronha, Gilberto de Nucci, and Roberto Zatz. Renal expression of COX-2, ANG II, and AT$_1$ receptor in remnant kidney: strong renoprotection by therapy with losartan and a nonsteroidal anti-inflammatory. Am J Physiol Renal Physiol 286: F945–F954, 2004. First published December 16, 2003; 10.1152/ajprenal.00238.2003.—Chronic renal injury can be mediated by angiotensin II (ANG II) and prostanoids through hemodynamic and inflammatory mechanisms and attenuated by individual suppression of these mediators. In rats with 5⁄6 renal ablation (Nx), we investigated 1) the intrarenal distribution of COX-2, ANG II, and the AT$_1$ receptor (AT$_1$R); 2) the renoprotective and antiinflammatory effects of an association between the AT$_1$R blocker, losartan (Los), and the gastric sparing anti-inflammatory nitrofurflurbiprofen (NOF). Adult male Munich-Wistar rats underwent Nx or sham operation (S), remaining untreated for 30 days, after which renal structure was examined in 12 Nx rats (Nxpre). The remaining rats were followed during an additional 90 days, distributed among 4 treatment groups: NxV (vehicle), NxLos (Los), NxNOF (NOF), and NxLosNOF (Los/NOF). Nxpre rats exhibited marked aluminuria, hypertension, glomerulosclerosis, interstitial expansion, and macrophage infiltration, accompanied by abnormal glomerular, vascular, and interstitial COX-2 expression. ANG II appeared in interstitial cells, in contrast to S, in which ANG II was virtually confined to afferent arterioles. Intrarenal AT$_1$R expression shifted from mostly tubular in S to predominantly interstitial in Nxpre. All these changes were aggravated at 120 days and attenuated by Los and NOF monotherapies. Los/NOF treatment arrested renal structural injury and ANG II expression and reversed hypertension, albuminuria, and renal inflammation. In conclusion, abnormal expression of COX-2, ANG II, and AT$_1$R may be key to development of renal injury in Nx. Concomitant COX-2 inhibition and AT$_1$R blockade arrested renal injury and may represent a useful strategy in the treatment of chronic nephropathies.
In the present study, we evaluated in Nx rats the renal distribution of both the AT_1R and the COX-2 isofrom, as well as the variation of their renal expression with time. In addition, we estimated the amount of ANG II present in glomerular arterioles and in the cortical interstitium. To evaluate the role of these mediators in progression, Nx rats were treated with nitrofuribiprofen (NOF), a nonsteroidal anti-inflammatory drug (NSAID) with low gastrointestinal toxicity, or losartan potassium (Los), an AT_1R blocker. In an attempt to obtain more effective renal protection, a group of Nx rats receiving a combined NOF/Los treatment was studied as well.

**Methods**

Seventy-seven adult male Munich-Wistar rats, obtained from a local breeding colony, with initial weights of 240 to 260 g, were used in this study. Under anesthesia with pentobarbital sodium (50 mg/kg ip), nephrectomy was performed by removal of the right kidney and ligation of the appropriate left renal artery branches, thus ensuring the infarction of at least two-thirds of the left kidney. Twelve sham-operated (S) rats underwent anesthesia, ventral laparotomy, and manipulation of the renal pedicles, without any removal of renal mass. After recovering from anesthesia, the animals were returned to their original cages, given free access to tap water and standard chow (0.5% Na, 22% protein), and maintained at 23 ± 1°C under a 12:12-h light-dark cycle. All experimental procedures were in strict accordance with our institutional guidelines and with international standards for manipulation and care of laboratory animals, being previously approved by the local Research Ethics Committee.

**Experimental groups.** Thirty days after Nx, the tail-cuff pressure (TCP) was measured by an indirect method (11). The animals were then placed in metabolic cages for determination of daily urinary albumin excretion rate (U_abV). Animals that at this time had failed to develop hypertension (defined as TCP ≥ 140 mmHg) or albuminuria (U_abV ≥ 50 mg/day) were excluded from the study. The kidneys of 12 Nx rats were then perfused-fixed with Duboscq-Brazil solution (0.45% picric acid in a mixture of ethanol, formaldehyde, and acetic acid) after a brief saline washout and prepared for light microscopic and immunohistochemical analysis as described below. This group, designated Nx_sec, was used to evaluate the extent of renal injury at 30 days after Nx and served to assess the therapeutic efficacy of treatments started thereafter. The remaining Nx rats were then followed for an additional 3 mo (up to 4 mo after nephrectomy) after having been distributed among four experimental groups: Nx (n = 12), Nx rats receiving inert vehicle; Nx_NOF (n = 14), Nx rats receiving NOF (Nicox, Sophia Antipolis), 7.5 mg/kg dissolved in a mixture of DMSO (Nicox, Sophia Antipolis) and acetic acid; and Los (Merck Sharp & Dohme, Rahway, NJ) dissolved in the drinking water at 20 mg/dl, corresponding to a daily ingestion of ~50 mg/kg; and Nx_NOF/Los (n = 13), rats given simultaneous NOF and Los treatments. The concentration of Los in the drinking water was adjusted to compensate for variations in daily water intake to ensure that both Nx_NOF and Nx_NOF/Los groups received a constant dosage of 50 mg·kg⁻¹·day⁻¹. A group of S rats (n = 12) was also followed. To mimic the situation usually found in clinical practice, all drug treatments were started only 30 days after renal ablation, when the process of progressive renal injury associated with this model is already in motion. The distribution of rats into the four Nx groups was performed in such a way as to ensure that both mean TCP and albumin excretion rates were similar among groups. The mean initial blood pressure values (in mmHg) and albuminuria values (in mg/day) are shown in Table 1. None of these differences was statistically significant.

**Experimental protocol.** To evaluate the efficiency of NOF as a COX blocker, the daily urinary excretion of thromboxane B_2 (TXB_2), a derivative of thromboxane A_2 (TXA_2), was measured by radioimmunoassay (NEN, Boston, MA) 60 days after renal ablation (30 days of treatment). Because, after renal mass reduction, most of the urinary prostanooids is believed to originate from renal synthesis (26), we also estimated the thromboxane production per nephron by factoring urinary TXB_2 by the total number of nephrons, assumed to be 6 × 10^4 in S and 10^4 in Nx.

All groups were followed until 120 days after surgery (90 days of treatment), with monthly determination of TCP and U_abV (11). At the end of the study, rats were anesthetized with pentobarbital sodium (50 mg/kg ip), and a blood sample was collected from the abdominal aorta to assess serum creatinine (S_creat). The renal tissue was then perfusion-fixed with Duboscq-Brazil solution after saline washout and prepared for morphological analysis as described below.

**Histological analysis.** Sections 4-μm thick were stained by periodic acid-Schiff reaction or Masson trichrome. All morphometric evaluations were performed in a blinded fashion by a single observer. The extent of glomerular sclerosis (GS) was evaluated by attributing a score to each glomerulus according to the apparent extent of the tuft area affected by the sclerotic injury, as described previously (11). At least 100 glomeruli were evaluated for each rat. A GS index (GSI) was calculated for each rat as the weight average of all individual glomerular scores thus obtained (11). To evaluate the extent of renal interstitial expansion, the fraction of renal cortex occupied by interstitial tissue staining positively for extracellular matrix components was estimated by a point-counting technique in 25 consecutive microscopic fields, at a final magnification of ×100 under a 176-point grid (11).

**Immunohistochemical analysis.** All immunohistochemical studies were performed in 4-μm-thick, paraffin-embedded renal sections. Sections were mounted on glass slides coated with 2% gelatin, deparaffinized, and rehydrated using standard techniques. Sections were then exposed to microwave irradiation in citrate buffer to

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**Table 1. Renal and systemic functional parameters before treatment (30 days after renal mass reduction) and at the end of the study (120 days after renal mass reduction)**

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>BW (120 days), g</th>
<th>LKW (120 days), g</th>
<th>TCP, mmHg</th>
<th>U_abV, mg/day</th>
<th>S_creat (120 days), mg/dl</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>30 days</td>
<td>120 days</td>
<td>30 days</td>
</tr>
<tr>
<td>S</td>
<td>12</td>
<td>359±6</td>
<td>1.6±0.1</td>
<td>119±3</td>
<td>112±3</td>
<td>2.0±0.2</td>
</tr>
<tr>
<td>Nx_pre</td>
<td>12</td>
<td>253±3^a</td>
<td>1.2±0.1^a</td>
<td>172±3</td>
<td>ND</td>
<td>104.3±7.1^a</td>
</tr>
<tr>
<td>Nx</td>
<td>12</td>
<td>273±9</td>
<td>1.2±0.1</td>
<td>184±5</td>
<td>216±6^ab</td>
<td>92.6±7.5^a</td>
</tr>
<tr>
<td>Nx_NOF</td>
<td>14</td>
<td>279±12^a</td>
<td>1.2±0.1</td>
<td>178±4^a</td>
<td>174±9^a</td>
<td>96.2±12.9</td>
</tr>
<tr>
<td>Nx_NOF/Los</td>
<td>14</td>
<td>270±12^a</td>
<td>1.2±0.1^a</td>
<td>189±5^b</td>
<td>175±6^a</td>
<td>97.6±9.7^a</td>
</tr>
<tr>
<td>Nx_NOF</td>
<td>13</td>
<td>291±6^b</td>
<td>1.3±0.1^b</td>
<td>177±7</td>
<td>144±7</td>
<td>97.6±9.7^a</td>
</tr>
</tbody>
</table>

Values are means ± SE, n = No. of rats; BW, body weight; LKW, left kidney weight; TCP, tail-cuff pressure; U_abV, urinary albumin excretion; S_creat, serum creatinine concentration; ND, not determined; S, sham-operated; Nx, 5/6 nephrectomy; Nx_sec, 30 days after Nx; Nx_NOF, Nx_NOF/Los, and Nx_NOF/Los/Nof. Nx rats treated with vehicle, losartan, nitrofuribiprofen (NOF), and combination therapy, respectively. ^P < 0.05 vs. S. ^P < 0.05 vs. Nx_sec. *P < 0.05 vs. Nx_NOF. **P < 0.05 vs. Nx_NOF/Los.
enhance antigen retrieval and preincubated with 5% normal rabbit (for ED-1) or horse (for COX-2, AT1, and ANG II) serum in Tris-buffered saline to prevent nonspecific protein binding. Incubation with the primary antibody was always carried out overnight at 4°C in a humidified chamber. Negative control experiments for all antigens were performed by omitting incubation with the primary antibody.

For macrophage detection, a monoclonal mouse anti-rat ED-1 antibody (Serotec, Oxford, UK) was used. After being washed, sections were incubated with rabbit anti-mouse Ig (Dako, Glostrup, Denmark), then with an alkaline phosphatase anti-alkaline phosphatase (Dako) complex. Finally, sections were developed with a fast-red dye solution, counterstained with Mayer’s hemalum, and covered with Kaiser’s glycercin-gelatin (Merck, Darmstadt, Germany).

COX-2-, ANG II-, and AT1R-positive cells were detected by an indirect streptavidin-biotin alkaline phosphatase technique. The primary (monoclonal mouse anti-rat) antibody for COX-2 was purchased from Transduction Laboratories (Lexington, KY). For ANG II detection, a monoclonal rabbit anti-human ANG II (Peninsula Lab, San Carlos, CA) was used, whereas AT1 R was detected with a monoclonal rabbit anti-rat AT1R antibody (RD1, Flanders, NJ). Sections were preincubated with avidin and biotin solutions to block nonspecific binding of these compounds (Blocking Kit, Vector Labs, Burlingame, CA). After being washed, the sections were incubated at room temperature with rat-adsorbed biotinylated anti-mouse or anti-rabbit IgG (Vector Labs) for 45 min, then with a streptavidin-biotin-alkaline phosphatase complex (Dako) for an additional 30 min. Sections were finally incubated with a freshly prepared substrate, consisting of naphthol AS-MX-phosphate and developed as described above.

The extent of renal infiltration by macrophages, ANG II-positive cells, and COX-2-positive cells was evaluated in a blinded manner at ×250 magnification and expressed as cells per square millimeter. For each section, 25 microscopic fields, each corresponding to an area of 0.06 mm², were examined. Because interstitial AT1R in Nx rats was often so densely expressed as to preclude the individualization of positively stained cells, AT1R expression had to be estimated by a point-counting technique similar to the one employed to determine % INT. This technique allowed us to assess the distribution of AT1R among several compartments of the renal cortex (glomeruli, vessels, tubules, and interstitium). The glomerular expression of COX-2 was evaluated by counting positively stained cells in a total of 100 glomeruli/rat, and the results were expressed as cells/100 glomeruli. The fraction of stained macula densa regions was also estimated. The expression of COX-2 in arteries/arterioles was evaluated by counting the number of stained cells in a total of 50 cortical vessels and given as cells/50 vascular profiles.

Statistical analysis. One-way ANOVA with paired comparisons according to the Newman-Keuls formulation was used in this study (9). The Spearman correlation coefficient was used to evaluate the existence of significant linear correlation between parameters obtained in individual rats. Because GSI and albumin excretion rates behaved as continuous variables with nonnormal distribution, an approximately Gaussian distribution was obtained in all groups by performing log transformation of these data before statistical analysis. For similar reasons, parameters expressed as proportions underwent arcsine transformation before analysis (9). P values < 0.05 were considered significant.

RESULTS

Renal and systemic parameters obtained at 30 (before treatment) and 120 days after Nx are given in Table 1. Nx groups exhibited limited growth compared with S. In all Nx groups except NxLos/NOF, body weights were not statistically different from those observed before treatment. Average food intake was similar among groups. The left kidney weight was similar among Nx groups 120 days after Nx. Figure 1 shows, in a graphic manner, TCP as a function of time. TCP rose markedly in the NxV group, reaching 216 ± 6 mmHg 120 days after surgery (vs. 112 ± 3 in S, P < 0.05, and 172 ± 3 mmHg in Nxpre, P < 0.05). After 30 days of treatment (60 days after Nx), TCP was unchanged by NOF monotherapy but fell by ~40 mmHg in both NxLos and NxLos/NOF. Los monotherapy lost part of this initial antihypertensive effect with time, TCP returning to pretreatment levels 120 days after renal ablation. Because NOF monotherapy had no antihypertensive effect, TCP reached similar values in NxLos and NxLos/NOF rats 120 days after Nx. Although these values were significantly lower than in NxV, TCP did not differ statistically from the respective Nxpre values, remaining markedly elevated compared with S. In the NxLos/NOF group, the initial antihypertensive effect was maintained throughout the study (Fig. 1). As a consequence, final TCP values in this group were only moderately elevated compared with S (144 ± 7 mmHg, P < 0.05 vs. S and NxV) and significantly lower than those verified before treatment (P < 0.05 vs. Nxpre).

As expected, U alb/V (Table 1) was markedly increased at 30 days of surgery (Nxpre group), reaching 104.3 ± 7.1 mg/day (P < 0.05 vs. S). Albuminuria was aggravated at 120 days after Nx (178.5 ± 43.1 mg/day, P < 0.05 vs. S and P > 0.05 vs. Nxpre). Monotherapy with LOS numerically decreased U alb/V relative to untreated Nx (112.9 ± 14.7 mg/day, P > 0.05 vs. NxV) and prevented further increases in albuminuria, which remained at levels similar to those observed in the Nxpre group. Monotherapy with NOF significantly reduced albuminuria relative to pretreatment values (72.6 ± 11.2 mg/day, P < 0.05 vs. NxV, NxLos, and Nxpre). Combined Los+NOF treatment exerted a much more efficient antiproteinuric action than any of the monotherapies, reversing albuminuria to 26.2 ± 3.5 mg/day (P < 0.05 vs. NxV, NxLos, and Nxpre).

As expected, Scr levels were markedly increased by renal mass reduction. None of the monotherapies promoted a significant decline in Scr relative to either NxV or Nxpre. In the Los+NOF group, Scr was significantly reduced compared with either NxV or Nxpre (1.0 ± 0.1 vs. 1.2 ± 0.1 in Nxpre, P < 0.05 and 1.4 ± 0.2 mg/day in NxV, P < 0.05).

As shown in Table 2, urinary excretion of TxB2, measured at 30 days of treatment, was slightly increased in NxV compared with S. In MX mice, urinary excretion of TxB2 was decreased, suggesting a decreased production of this metabolite.
with S (20.6 ± 1.7 vs. 17.8 ± 1.9 ng/day in S, P > 0.05). Calculated TxB2 excretion per nephron was increased sevenfold in Nx rats 60 days after surgery (2.1 ± 0.2 vs. 0.3 ± 0.1 pg-nephron⁻¹·day⁻¹ in S, P < 0.05). As expected, total urinary TxB2 excretion was markedly depressed compared with S and NxV in NxNOF and NxLOS/NOF rats (7.0 ± 1.0 and 8.6 ± 0.8 ng/day, respectively, P < 0.05 vs. S and NxV). TxB2 excretion in the NxNOF and NxLOS/NOF groups appeared even more depressed when factored by the estimated number of nephrons, reaching values close to those verified in S (0.7 ± 0.1 and 0.9 ± 0.1 ng-nephron⁻¹·day⁻¹, respectively, P < 0.05 vs. NxV and P > 0.05 vs. S). Monotherapy with Los resulted in a slight but statistically significant reduction in the calculated TxB2 excretion per nephron relative to Nx (1.7 ± 0.2 ng-nephron⁻¹·day⁻¹, P < 0.05 vs. NxV).

Glomerular segmental sclerotic lesions were evident 30 days after surgery (Nxpre group), the GSI reaching values almost 20-fold higher than in S (Table 3). Ninety days later (120 days after surgery), considerable progression of glomerular injury had occurred. In NxV rats, the GSI attained values almost 10-fold as high as in Nxpre and 200-fold higher than in S. Treatment with any of the monotherapies was associated with a less pronounced increment of the GSI, although the respective differences relative to the NxV group were not statistically significant. Combined Los/NOF treatment arrested the progression of glomerular injury, keeping the GSI at levels close to those verified in the Nxpre group (43 ± 11 vs. 19 ± 4 in Nxpre, P > 0.05). There was a significant correlation between GSI and TCP (r = 0.74, P < 0.01). Interstitial expansion was also a prominent component of renal injury after Nx (Table 3), %INT reaching values more than threefold higher than in Nxpre 120 days after Nx. Unlike NOF and Los monotherapies, combined Los/NOF treatment significantly attenuated the progression of interstitial expansion (4.8 ± 0.5 vs. 7.8 ± 0.7 in NxV, P < 0.05).

**Immunohistochemistry.** Thirty days afterNx (Nxpre group), macrophage infiltration in the renal tissue (Table 3), assessed by the density of ED-1-positive cells, more than quadrupled compared with S values (131 ± 12 vs. 29 ± 3 cells/mm² in S, P < 0.05). This was aggravated 120 days after surgery, macrophage density reaching values of 178 ± 23 cells/mm² in the NxV group. None of the monotherapies had any significant influence on macrophage infiltration. However, the combined Los/NOF treatment reduced the renal macrophage density to levels significantly lower than in the Nxpre group (81 ± 6 cells/mm² vs. Nxpre, P < 0.05). A significant linear correlation (r = 0.67, P < 0.001) was observed between the macrophage density and the GSI.

Table 2. Urinary thromboxane B2 excretion 60 days after renal ablation (30 days of treatment)

<table>
<thead>
<tr>
<th>Group</th>
<th>Total TxB2 Excretion, ng/day</th>
<th>Estimated per Nephron TxB2 Excretion, pg-nephron⁻¹·day⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>S</td>
<td>17.8±1.9</td>
<td>0.3±0.1</td>
</tr>
<tr>
<td>Nx</td>
<td>20.6±1.7</td>
<td>2.1±0.2*</td>
</tr>
<tr>
<td>NxLOS</td>
<td>16.5±2.4</td>
<td>1.7±0.2*</td>
</tr>
<tr>
<td>NxNOF</td>
<td>7.0±1.0††</td>
<td>0.7±0.1††</td>
</tr>
<tr>
<td>NxLOS/NOF</td>
<td>8.6±0.8*†††</td>
<td>0.9±0.1†††</td>
</tr>
</tbody>
</table>

Values are means ± SE. TxB2, thromboxane B2. *P < 0.05 vs. S. †P < 0.05 vs. Nx. ‡P < 0.05 vs. NxLOS.

Figure 2A shows a typical staining pattern for ANG II in an afferent arteriole obtained from an S rat (the efferent arteriole, not stained, is shown as well). In Fig. 2B, ANG II-positive cells unrelated to vascular tissue are shown in the renal cortical interstitium of an Nx rat 120 days after ablation. Most of these ANG II-positive cells appeared in association with inflamed areas. Figure 3 shows the intensity of both modalities of ANG II expression in graphic form. Afferent arteriolar ANG II expression was deeply depressed in untreated Nx (0.04 ± 0.02 positively stained arterioli/mm² in Nxpre and 0.03 ± 0.02 in NxV vs. 1.55 ± 0.16 in S, P < 0.05) and was not influenced by any of the treatments. By contrast, the density of ANG II-positive cells in the interstitium was already markedly increased at 30 days after ablation (nxpre group) compared with S. These values were tripled 120 days afterNx (nxv group). The increasing interstitial ANG II expression was completely arrested and kept at pretreatment levels by the combined Los/NOF treatment. None of the monotherapies had any significant effect on interstitial ANG II expression.

AT/R expression patterns were shown in Figs. 4 and 5. In the S group, AT1R expression was almost entirely confined to the tubular compartment and only sparsely expressed at glomeruli, vessels, and interstitium. Renal mass reduction increased only numerically the total renal expression of AT1R but promoted a drastic change in its intrarenal distribution. In the Nxpre group (30 days afterNx), total renal AT1R expression was clearly shifted to the interstitial area. This pattern became even more marked 120 days after ablation (nxv group), when AT1R expression at the tubules declined to very low levels (Fig. 5). None of the treatments promoted any significant change in the intensity or distribution of AT1R expression in Nx rats.

As described previously (9, 49), COX-2 was constitutively expressed in cells of the macula densa region of S rats (Fig. 6A). Only rare COX-2-positive cells were found in glomeruli, vessels, or interstitium of intact kidneys. In accordance with previous observations of this laboratory (9), renal mass reduction numerically increased the frequency of macula densa staining positively for COX-2 (Table 4). Monotherapy with NOF or Los further augmented the expression of COX-2 at the macula densa, although only with the latter was this change statistically significant (Table 4). Combined Los/NOF treatment nearly doubled COX-2 expression at the macula densa (Table 4).

As previously reported by this laboratory (9), there was a dramatic elevation in the density of COX-2-positive cells at glomeruli (Fig. 6B), vessels (Fig. 6C), and interstitium (Fig.

Table 3. Parameters of renal injury and inflammation 120 days after renal ablation (90 days of treatment)

<table>
<thead>
<tr>
<th>Group</th>
<th>GSI</th>
<th>%INT</th>
<th>Md (cells/mm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S</td>
<td>1 ± 1</td>
<td>0.6±1</td>
<td>29±3</td>
</tr>
<tr>
<td>Nxp</td>
<td>19±4*</td>
<td>2.5±0.3</td>
<td>131±12</td>
</tr>
<tr>
<td>Nvx</td>
<td>189±38ab</td>
<td>7.8±0.7</td>
<td>178±23*</td>
</tr>
<tr>
<td>Nxl</td>
<td>134±31ab</td>
<td>6.8±1.0</td>
<td>155±19*</td>
</tr>
<tr>
<td>Nxs</td>
<td>130±37ab</td>
<td>7.9±1.2</td>
<td>140±13</td>
</tr>
<tr>
<td>NxsNOF</td>
<td>43±11abcd</td>
<td>4.8±0.5</td>
<td>81±6*</td>
</tr>
</tbody>
</table>

Values are means ± SE. GSI, glomerular sclerosis index; %INT, fractional cortical interstitial area; Md, extent of macrophage infiltration. *P < 0.05 vs. S. †P < 0.05 vs. Nxp; ‡P < 0.05 vs. Nvx; §P < 0.05 vs. Nxsnc; ††P < 0.05 vs. NxsNOF.
6D) 30 days after renal mass reduction, which was exacerbated after 120 days (Table 4). Monotherapies had no effect on the frequency of glomerular, vascular, or interstitial COX-2-positive cells, whereas combined Los/NOF treatment reduced vascular and glomerular COX-2 expression to values indistinguishable from those seen in S (Table 4). There was a strong positive correlation ($r = 0.83$, $P < 0.001$) between the glomerular density of COX-2-positive cells and the GSI.

### DISCUSSION

As expected, % renal ablation promoted growth retardation, systemic arterial hypertension, impaired renal function, and severe albuminuria. These functional changes were accompanied by severe glomerulosclerosis, as well as expansion and cellular infiltration, it appears to be linked to regulation of sodium balance. According to this view, vascular ANG II would be depressed by the extracellular fluid volume expansion known to occur in the Nx model (17). By contrast, "interstitial" ANG II seems to be insensitive to volume expansion and, given its association with interstitial expansion and cellular infiltration, it appears to be linked to renal inflammation. The origin of this interstitial ANG II was not addressed in the present study. ANG II may have been produced locally, because tubular epithelial cells, macrophages, and myofibroblasts all possess the appropriate biochemical machinery (12, 29, 39). Alternatively, ANG II may have originated outside the renal interstitium and undergone internalization by local cells after binding to AT1R (44, 57). Local ANG II may have had a proinflammatory and profibrotic effect at the renal interstitium. ANG II stimulates inflammatory cells such as lymphocytes (25) and activates nuclear factor-κB in monocytes (36). In addition, ANG II stimulates the release of MCP-1 by vascular smooth muscle cells (36). In renal tissue, ANG II stimulates the proliferation of mesangial cells, glomerular endothelial cells (53), and myofibroblasts (55), as well as the secretion of chemokines and growth factors such as RANTES (52), PDGF (8), and MCP-1 (36). In addition, ANG II strongly stimulates collagen synthesis and renal fibrosis by activating TGF-β (54) and the MAPK/ERK pathway (41). Blockade of interstitial ANG II is a possible explanation for the...
well-known beneficial effect of RAS suppressors in progressive nephropathies, also observed in the NxLos and NxLos/NOF groups.

In agreement with previous observations (15), renal expression of AT1R in S rats was typically expressed in tubules (T), vessels (V) and, to a much lesser extent, glomeruli (G). B: in Nxpre rats (30 days after ablation), AT1R expression was massively shifted to the interstitium. C: in NxV rats (120 days after ablation), interstitial AT1R expression was even more intense, whereas tubular and vascular AT1R expression was much weaker than in the Nxpre group.

The potential role of prostanoids in the pathogenesis of progressive nephropathies has long been acknowledged. The stimulation of podocytes by complement fractions can increase the local synthesis of prostanoids (40). Similarly, nonimmune mechanisms such as mesangial stretching can augment the expression of COX and enhance the production of its derivatives (1). Accordingly, studies of the Nx model showed increased urinary excretion of prostanoids per nephron (26).

Increased production of prostanoids can enhance inflammation and, therefore, accelerate renal injury. Prostanoids derived from COX-2 are thought to modulate proliferation and activation of T lymphocytes (20). Dendritic cells, described in the remnant kidney (33), constitutively express COX-2 and utilize prostanoids as an autocrine stimulus for cytokine secretion and for their own proliferation (51). In addition to its well-known vasoconstrictor effect (24), TxA2 stimulates the expression of adhesion molecules and of MCP-1 in endothelial cells (21), promotes the proliferation of mesangial cells (4), and enhances platelet aggregation and extracellular matrix production (5, 24).

Previous studies have shown that the renal cortical expression of COX-2 increases after 5/6 renal ablation, whereas the expression of COX-1 remains unchanged (18, 49). We showed recently that a large fraction of the excess COX-2 expressed in remnant kidneys localizes in glomeruli, vessels, and the interstitium, especially in areas of injury and inflammation (9). The present study confirms these observations, reinforcing the concept that COX-2 can exert a dual role in this model: at the MD, COX-2 appears to exert a physiological effect, possibly related to sodium homeostasis. At “anomalous” locations such as...
glomeruli and vessels, COX-2 and its products would mediate inflammation and structural injury. The consistent presence of COX-2 in damaged areas, and the strong correlation between the intensity of its expression and parameters of renal injury, strengthens the notion that COX-2-derived prostanoids play an important pathogenic role in this model. The mechanisms by which COX-2 may have been induced in these areas are obscure. COX-2 may have been activated by ANG II anomalously produced in the interstitium (56), by stretching of mesangial cells resulting from glomerular hypertension (1) and/or by the action of other mediators such as TNF-α and interleukin-1β (7, 31). Once synthesized, prostanoids can further activate COX-2, thereby contributing to amplify and perpetuate the inflammatory process (42). The protective effect of chronic treatment with either COX-2-specific inhibitors (9, 48) or NOF (10) lends further support to the notion that prostanoids play a fundamental role in the pathogenesis of progressive renal injury in the Nx model.

Consistent with previous observations, Los monotherapy lowered blood pressure by 40 mmHg 1 mo after treatment was started, although TCP returned to pretreatment levels at the end of the study. In addition, Los limited UaV, GSI, interstitial damage, macrophage infiltration, and ANG II-positive cell infiltration (11). However, protection conferred by losartan monotherapy was only partial, because progression of renal inflammation and of renal structural injury was not arrested in the NxLos group. There are at least three possible reasons for the limited efficacy of Los treatment in this study. First, rats failing to attain blood pressures higher than 140 mmHg or albuminuria in excess of 50 mg/dl 30 days after nephrectomy were excluded from the study to ensure that the attending nephropathy had a progressive nature. Second, unlike in most previous studies of this model, treatments were started only 30 days after nephrectomy, when renal injury was already established. Third, rats were followed up to 4 mo after renal mass reduction, whereas in most other studies of this model observations were ended at 2 mo or less. In the face of the unusual severity of renal injury, the relative resistance to Los treatment was not unexpected. At any instance, these findings agree with previous experimental observations, obtained in this laboratory and elsewhere (11, 19), as well as in large clinical trials (3), all of which indicate that, once set in motion, progressive nephropathies can be attenuated, but not detained, by isolated treatment with AT1R blockers or ACE inhibitors. As a whole, these observations suggest that events antecedent to the initiation of treatment may be of crucial importance in the pathogenesis of renal injury associated with this model. Additionally, the relative inefficiency of Los monotherapy in the present study may reflect the presence of ANG II-independent inflammatory events, as well as the rerudescence of arterial hypertension after the first few weeks of treatment. Finally, it is conceivable that the “conventional” dose of Los employed in the present study, although high enough to exert full vascular effect, was insufficient to effectively block the enormous

### Table 4. Renal COX-2 expression and its distribution

<table>
<thead>
<tr>
<th>Group</th>
<th>Glomeruli, + cells/100 glom.</th>
<th>MD, + MD/100 glom.</th>
<th>Vascular, + cells/50 vasc. profiles</th>
<th>Interstitial, + cells/mm²</th>
</tr>
</thead>
<tbody>
<tr>
<td>S</td>
<td>2.0 ± 0.7</td>
<td>18 ± 1.5</td>
<td>1.4 ± 0.8</td>
<td>0.9 ± 0.2</td>
</tr>
<tr>
<td>NxPre</td>
<td>30.4 ± 4.1</td>
<td>24 ± 1.6</td>
<td>27.9 ± 10.2</td>
<td>10.4 ± 2.7</td>
</tr>
<tr>
<td>NxV</td>
<td>106.2 ± 27.7</td>
<td>21.5 ± 4</td>
<td>30.3 ± 7.6</td>
<td>10.8 ± 2.0</td>
</tr>
<tr>
<td>NxLos</td>
<td>60.5 ± 22.0</td>
<td>31.8 ± 5</td>
<td>18.0 ± 8.7</td>
<td>9.9 ± 4.0</td>
</tr>
<tr>
<td>NxLos/NOF</td>
<td>97.6 ± 30.7</td>
<td>26.3 ± 4</td>
<td>29.6 ± 11.8</td>
<td>8.8 ± 2.4</td>
</tr>
<tr>
<td>NxLos/NOF</td>
<td>6.1 ± 1.5</td>
<td>44.4 ± 1.4</td>
<td>2.6 ± 1.6</td>
<td>5.9 ± 1.1</td>
</tr>
</tbody>
</table>

Values are means ± SE. MD, macula densae; + cells, cells staining positively for COX-2; + MD, COX-2-positive maculae densae. *P < 0.05 vs. S; **P < 0.05 vs. NxPre; ***P < 0.05 vs. NxV; ****P < 0.05 vs. NxLos; †P < 0.05 vs. NxLos/NOF.
amount of AT1R already present at the renal tissue when treatment was started.

NOF is a nonsteroidal anti-inflammatory compound with low gastrointestinal toxicity, presumably due to its NO-releasing properties (10, 47). It appears unlikely that NO released by NOF has a direct therapeutic effect because the NOF molecule is rapidly degraded in the intestinal lumen, releasing flurbiprofen (10). However, we cannot exclude the possibility that nitroso proteins, which have a much longer half-life than NO itself, propagate a possible protective effect of NO released into the intestinal lumen (37). Flurbiprofen is a potent inhibitor of both COX isozymes (45). In the present study, this property was demonstrated by the marked reduction in urinary TxB2 excretion obtained in the NxNOF and NxLos/NOF groups. Monotherapy with NOF reduced proteinuria and attenuated the progression of glomerular injury and the intensity of local macrophage infiltration as effectively as monotherapy with Los, even though NOF exerted no effect on blood pressure. However, as in the case of Los treatment, progression of renal injury was not arrested. Moreover, no protective effect was observed regarding interstitial expansion or glomerular and vascular COX-2 expression. In addition, the effects of NOF were much more modest than those obtained when treatment was begun immediately after surgery (10), once again underlining the pathogenic importance of early events in this model.

Treatment of Nx rats with the Los/NOF combination promoted a significant regression of hypertension, albuminuria, and inflammatory signs such as macrophage infiltration and tissue COX-2 expression, whereas the parameters of structural tissue injury remained stable, or were strongly attenuated, compared with pretreatment levels. The protection achieved with combined treatment was much greater than that obtained with either drug alone. On the basis of the present study, one cannot exclude the hypothesis that the success of combined treatment was due to a particularly effective hemodynamic action, although previous observations from this laboratory (10) indicated that NOF had no significant effect on glomerular hemodynamics. Because treatment with NOF alone had no effect on blood pressure, it seems unlikely that the hemodynamic effect of LOS was directly intensified by its association with NOF. Therefore, the efficacy of combined treatment was likely due to the simultaneous blockade of the hemodynamic and proinflammatory actions of ANG II and COX derivatives and by abrogation of the complex interplay between hypertension and inflammation (34). The present findings support previous observations of the Nx model, which similarly indicated the superiority of the combination of a RAS suppressor with an anti-inflammatory agent (11, 13, 33). It is noteworthy that combined treatment afforded partial regression of the nephropathy associated with Nx even though it was started 1 mo after surgery, when renal injury was already established. This observation suggests that both continued stimulation of AT1 receptors and production of prostanoids continue to play an important pathogenic role even during the late phases of the process, necessitating vigorous and persistent treatment to prevent further renal deterioration.

Despite the promising response of Nx rats to combined treatment, it must be noted that NSAIDs are potentially nephrotoxic drugs, especially in the setting of chronic renal insufficiency. NOF promoted no glomerular filtration rate decline in these and in previous studies (10). However, there have been numerous anecdotal reports of acute renal failure, hyperkalemia, hypertension, and edema attributed to NSAIDs (14, 16), although few studies have directly assessed the objective risk associated with the clinical use of these compounds (38, 46). It is possible that simultaneous administration of a RAS suppressor, by limiting or suppressing the vascular effect of ANG II, reduces the risk of excessive renal vasoconstriction in the presence of a COX inhibitor (32). Nevertheless, any future systematic use of COX inhibitors in chronic nephropathies, alone or in combination, will necessitate the development of careful clinical studies to assess the safety of these compounds in this particular group of patients.

In summary, combined treatment with Los/NOF partially reversed the nephropathy and renal inflammation associated with the Nx model, showing much more effective protection than with either drug alone. Clinical studies are needed to establish whether this scheme may eventually become a new weapon in the limited arsenal currently available to attenuate or prevent human progressive nephropathies.

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