An extremely high dose of losartan affords superior renoprotection in the remnant model

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Background. Rats subjected to 5/6 renal ablation (NX) exhibit large renal amounts of angiotensin II (Ang II) and of its main receptor, AT-1R. At previously used doses, AT-1R blockers (ARB) offer only partial renal protection. A possible explanation for this limited effect is that these doses are insufficient to block most of the abnormally expressed AT-1R. We investigated whether extremely high doses of the ARB, losartan (L), offer better protection than conventional doses in the NX model.

Methods. Thirty days after NX, tail-cuff pressure (TCP), albuminuria (U albV, mg/day), glomerulosclerosis index (GSI), fractional interstitial area (%INT), and macrophage infiltration (MØ) were evaluated in a separate group (NXpre). The remaining rats were then subdivided among four groups: NX + V, receiving vehicle; NX + L50, treated with L, at the "conventional" dose of 50 mg/kg/day; NX + L500, receiving L, 500 mg/kg/day; and NX + HH, receiving hydrochlorothiazide and hydralazine to lower blood pressure to a similar extent as in group L500.

Results. After a month of treatment, blood pressure and renal vascular resistance were lowest in group L500. Glomerular pressure was lowered by a similar extent by L50 and L500, while GFR was similar among groups. U albV, TCP, and renal injury were only partially reduced by L50 120 days after renal ablation. By contrast, L500 arrested renal inflammation and glomerular/interstitial injury at pretreatment levels, and promoted regression of hypertension and U albV, causing no apparent untoward effects.

Conclusion. The renal protection afforded by ARB in NX is dose dependent. Maximal protection may require doses several fold higher than those currently employed.

Chronic nephropathies, which lead to glomerulosclerosis, renal fibrosis, and end-stage renal failure, can be initiated by immune and nonimmune mechanisms. Whatever their origin, the propagation and perpetuation of chronic nephropathies require the participation of inflammatory events such as macrophage infiltration [1], fibroblast proliferation [2], and excessive production of extracellular matrix [3]. These processes require, in turn, the occurrence of a complex chain of cellular events that include the proliferation of lymphocytes and macrophages [4], the synthesis of cytokines and chemokines [5], the release of growth factors [6], and the expression of adhesion molecules [7].

Angiotensin II (Ang II) is deeply involved in the pathogenesis of chronic nephropathies. The generalized vasoconstriction and enhanced proximal sodium absorption promoted by Ang II, primarily aimed at defending blood pressure and the extracellular volume, can elevate systemic and intraglomerular pressure, leading to mechanical aggression to the walls of glomeruli and arterioles. Recent evidence has shown that, besides this “hemodynamic” effect, Ang II participates directly in the inflammatory response that follows initial mechanic or immunologic insult. In renal and nonrenal tissue, Ang II stimulates the expression of a host of inflammatory mediators such as growth factors [8, 9], cytokines and chemokines [10, 11], and adhesion molecules [12], as well as the proliferation of lymphocytes [13]. In addition, inflammatory cells can produce Ang II [14], thus generating a positive feedback mechanism that could contribute to the propagation and perpetuation of chronic inflammation and to the development of irreversible injury to the renal parenchyma. The production of “inflammatory” Ang II must be influenced by local rather than systemic hemodynamic factors, since the available models of chronic renal disease, usually associated with sodium retention, would tend to depress rather than stimulate the renin-angiotensin system.

Evidence that Ang II originated in the renal tissue participates in local inflammatory events and in the development of progressive nephropathies has steadily accumulated in recent years. In the 5/6 renal ablation model (NX), angiotensinogen mRNA can be shown in the glomeruli [15], whereas Ang II itself appears in tubules, mesangial cells, and interstitial cells in the kidneys.
weeks following nephrectomy [16, 17]. The intensity of interstitial Ang II expression associates closely with the severity of renal injury [17, 18]. Similar findings were obtained in the chronic nitric oxide inhibition model [19, 20]. More recent studies showed that, in both models, AT-1 receptors are massively expressed in inflamed interstitial areas [17, 19], consistent with the hypothesis that Ang II exerts a pathogenic role in this process.

Angiotensin-converting enzyme inhibitors (ACEI) and AT-1 receptor blockers (ARB) have been intensely utilized in the treatment of progressive nephropathies [21–23]. Although the advent of these compounds represented a major therapeutic advance, they fail to completely arrest the progression of advanced disease, only postponing the need for renal replacement therapy [21, 22]. In experimental nephropathies, this limitation becomes evident in studies in which therapy with these compounds is started late in the course of the disease, allowing substantial previous progression of renal injury [17, 24, 25].

Doses of ACEI and ARB currently employed in clinical practice and even in experimental protocols are based essentially on the observation of the maximal effects of these drugs on blood pressure. These hemodynamic effects depend basically on the availability of AT-1 receptors on vascular smooth muscle cells. However, we have recently shown that, in the 5/6 renal ablation model, AT-1 receptors are intensely expressed in inflamed areas of the renal parenchyma [17]. This finding raised the possibility that “conventional” doses of these drugs may be insufficient to completely neutralize the anomalous activation of the renin-angiotensin system, thus helping to explain their failure to achieve complete renal protection. Since these drugs are very well tolerated, it is conceivable that, at doses currently considered as “extremely high,” they may confer much more efficient renal protection than “conventional” doses. In accordance with this notion, the protective effect of the ARB, irbesartan, was shown to be dose dependent in a large trial of diabetic nephropathy [26].

In the present study, we investigated whether the use of an “extremely high” dose (one order of magnitude higher than “conventional” doses) of the ARB, losartan potassium (L), would be well tolerated and exert a superior renoprotective effect in the NX model. Treatments were initiated 30 days after renal ablation, thus mimicking what usually occurs in the management of human progressive nephropathies. We also verified whether a possible salutary effect of high-dose L treatment would be associated with amelioration of systemic hypertension, of glomerular hypertension, or of the associated inflammatory process, or would instead involve a combined effect of these mechanisms.

**METHODS**

One hundred thirty-seven adult male Munich-Wistar rats, weighing initially between 230 and 260 g were utilized in this study. Rats were obtained from a local facility at the University of São Paulo, and maintained at 23 ± 1°C, with relative air humidity at 60 ± 5% and under 12-hour light/dark cycle. Five-sixth nephrectomy (NX) was performed after ventral laparotomy under anesthesia with sodium pentobarbital, 50 mg/kg IP, by removal of the right kidney and ligation of 2 branches of the left renal artery, resulting in the infarction of two thirds of the left kidney. Sham-operated rats (S) underwent anesthesia and manipulation of the renal pedicles, without removal of renal mass. After recovering from anesthesia, the animals were returned to their original cages, which were kept warmed during the following 24 hours. All animals were given free access to tap water and standard chow (0.5 Na, 22% protein). All experimental procedures were approved by the local Research Ethics Committee (CAPPesq, process no. 368/02), and developed in strict conformity with our institutional guidelines and with international standards for manipulation and care of laboratory animals.

**Experimental groups**

Thirty days after renal ablation, tail-cuff pressure (TCP) and the daily urinary albumin excretion (UαlβV) were determined in all rats. Rats failing to increase TCP above 160 mm Hg or UαlβV above 30 mg/day were not included in the study. Twenty-two of the rats that fulfilled these criteria were utilized as pretreatment control subjects, and were followed no further (group NXpre). The remaining 96 NX animals were subdivided into 4 additional experimental groups: NX+V (N = 26), receiving pure tap water; NX+L50 (N = 25), receiving the ARB losartan potassium (L), 200 mg/L in the drinking water, corresponding to an average daily intake of 50 mg/kg; NX+L500 (N = 22), receiving L, 2000 mg/L in the drinking water, corresponding to an average daily intake of 500 mg/kg/day. This was the maximal L dose not resulting in growth stunting; NX+HH (N = 23), receiving hydralazine, 100 mg/L in the drinking water, corresponding to an average daily intake of 24 mg/kg/day, and hydrochlorothiazide, 25 mg/L in the drinking water, corresponding to an average daily intake of 6 mg/kg/day. This last group was included to evaluate the degree of renal protection that would be afforded by decreasing TCP to a similar extent as in group NX+L500, without suppressing the renin-angiotensin system. Nineteen sham-operated rats (group S) received pure tap water only and served as control subjects. To reproduce the situation usually encountered in clinical practice, all drug treatments were started only 30 days after renal ablation, when chronic
renal injury associated with this model is known to be under progression [17]. The distribution of NX rats into the experimental groups described above (except for NXpre) was performed in such a fashion as to ensure that initial values for both mean TCP and UalbV were similar among groups.

Functional studies

Thirty days after treatments were started (60 days after renal ablation), 8 rats from each of the groups described above were anesthetized with inactin, 100 mg/kg, IP, and prepared for whole kidney and glomerular hemodynamic studies, in which mean arterial pressure (MAP), glomerular filtration rate (GFR), renal plasma flow (RPF), renal vascular resistance (RVR), and glomerular hydraulic pressure (Poc) were determined. The details of the experimental procedures and analytic techniques utilized in these studies are given elsewhere [4].

At the end of each experiment, the kidneys were perfused with saline solution at the MAP and fixed in situ, still at the MAP, with Dubosq-Brasil solution. The renal tissue was then prepared for morphologic evaluation.

Long-term studies

Rats from groups S (N = 11), NX+V (N = 18), NX+L50 (N = 17), NX+L500 (N = 14), and NX+HH (N = 15) were followed during 120 days after ablation (90 days of treatment), with monthly determination of TCP and UalbV. Since initial and final values for TCP and UalbV were obtained in the same animals, direct-paired comparisons could be performed. At the end of this period, all rats were anesthetized with sodium pentobarbital, 50 mg/kg, IP, and blood samples were drawn from the abdominal aorta for determination of plasma sodium and potassium concentration. The kidneys were then perfusion-fixed as described above. The same procedure had been adopted for the 22 rats from group NXpre (pretreatment reference), studied 30 days after renal ablation.

Histologic techniques and histomorphometry

After fixation, the kidneys were weighed and 2 mid-cortical sections were postfixed in buffered 4% formaldehyde. The renal tissue was then embedded in paraffin using standard sequential techniques, and prepared for assessment of glomerular, interstitial, and vascular injury, as well as for immunohistochemical analysis, as described previously [4].

Morphometric evaluations were always performed in a blinded manner by a single observer in 4-μm thick sections. For each rat, the severity of glomerulosclerosis (GS) was estimated, in sections stained with periodic acid–Schiff (PAS), by attributing to each glomerulus a score that reflected the fraction of the tuft area taken by the sclerosing lesion, as described previously [4]. A GS index (GSI) was calculated for each rat as the weighted average of the individual glomerular scores obtained in this manner multiplied by 100. At least 120 glomeruli were examined for each rat. The extent of interstitial expansion was assessed by estimating, using a point-counting technique [27], the percentage of the renal cortical area occupied by interstitial tissue (%INT). This procedure was carried in Masson-stained sections by examining 25 consecutive microscopic fields, at a final magnification of 100×, under a 176-point grid.

Immunohistochemistry

Immunohistochemical procedures were always carried on 4-μm thick, paraffin-embedded renal sections, mounted on glass slides coated with 2% gelatin. Sections were initially deparaffinized and rehydrated using standard techniques, then exposed to microwave irradiation in citrate buffer to enhance antigen retrieval, and preincubated with 5% normal rabbit (for ED-1) or horse (for AT-1 and Ang II) serum in Tris-buffered saline (TBS) to prevent nonspecific 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 antirat ED-1 antibody (Serotec, Oxford, UK) was used. After washing, sections were incubated with rabbit antirabbit immunoglobulins (Dako, Glostrup, Denmark), then with an alkaline phosphatase antialkaline phosphatase (APAAP; Dako) complex. Finally, sections were developed with a fast-red dye solution, counterstained with Mayer’s hemalaun, and covered with Kaiser’s glycercin-gelatin (Merck, Darmstadt, Germany).

Ang II- and AT1R-positive cells were detected by an indirect streptavidin-biotin alkaline phosphatase technique. For Ang II detection, a monoclonal rabbit antihuman Ang II (Peninsula Lab., San Carlos, CA, USA) was used, whereas AT1R was detected with a monoclonal rabbit antirat AT1R antibody (RDI, Flanders, NJ, USA). Sections were preincubated with avidin and biotin solutions to block nonspecific binding of these compounds (Blocking Kit, Vector Labs, Burlingame, CA, USA). After washing, the sections were incubated at room temperature with rat-adsorbed biotinylated antirabbit IgG (Vector Labs) for 45 minutes, then with a streptavidin-biotin-alkaline phosphatase complex (Dako) for an additional 30 minutes. 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 and Ang II-positive cells was evaluated in a blinded manner at ×250 magnification and expressed as cells/mm². For each section, 25 microscopic fields, each corresponding to an area of 0.06 mm², were examined. Since interstitial 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).

Statistical analysis

Differences among different groups were analyzed using one-way analysis of variance (ANOVA) with pairwise post-test comparisons by the Neuman-Keuls method [28]. Since GSI and albumin excretion rates exhibited a strong non-Gaussian distribution, log transformation of these data was performed prior to statistical analysis. For similar reasons, parameters expressed as proportions underwent arcsine transformation before analysis [28]. Differences between TCP and U_alb/V measurements obtained in the same rats at 30 and 120 days after ablation were analyzed using the Student paired t test. P values less than 0.05 were considered significant.

RESULTS

Functional studies

Functional parameters obtained at 30 days of treatment (60 days after ablation), as well as those verified in group NX_pre (pretreatment reference values obtained at 30 days after ablation), are given in Table 1. Growth was stunted in all groups subjected to renal ablation. However, there was no difference in body weight among the NX groups, except for group NX_pre, which was followed during only 30 days. Kidney weight (KW) was also lower in this group. In the remaining NX groups, KW was similar to that in group S, indicating the occurrence of marked renal hypertrophy, since renal mass had been initially reduced by 5/6. MAP reached 132 ± 5 mm Hg 30 days after ablation (group NX_pre), a value significantly higher than that in group S (114 ± 2 mm Hg, P < 0.05). L50 treatment (group NX+L50) attenuated hypertension (138 ± 4 mm Hg, P < 0.05 vs. NX+V and S). Both L500 and HH treatments brought MAP to values not statistically different from those obtained in S or NX_pre (124 ± 3 mm Hg and 112 ± 5, respectively, P < 0.05 vs. NX+V, and P > 0.05 vs. S, NX_pre, and NX+L50). There were no significant differences among the NX groups regarding GFR, which in each of them was approximately half as high as in S (P < 0.05). A similar pattern was observed for RPF, except for group NX+L500, in which FPR was significantly higher than in the remaining NX groups. RVR increased by more than 250% in group NX_pre (37 ± 6 mm Hg/mL/min vs. 14 ± 1 in S, P < 0.05). Renal vasoconstriction was aggravated 60 days after ablation (RVR rose to 48 ± 4 mm Hg/mL/min in group NX+V, P < 0.05 vs. S and P > 0.05 vs. NX_pre), and attenuated in groups L50 and HH (34 ± 3 and 33 ± 7 mm Hg/mL/min, respectively, P < 0.05 vs. S, and P > 0.05 vs. NX+V). L500 treatment brought RVR to levels significantly lower than in group NX+V, and not statistically different from S or NX_pre (24 ± 2 mm Hg/mL/min, P < 0.05 vs. NX+V, P > 0.05 vs. S and NX_pre).

Long-term studies

Four out of 18 rats (22%) were lost in group NX+V. Only 1 out of 17 and 1 out of 15 rats (6 and 7%, respectively) died in groups NX+L50 and NX+HH, respectively, whereas mortality was zero in group NX+L500. As in the functional studies, renal ablation always promoted growth stunting, whereas no significant differences were

Table 1. Hemodynamic studies after 30 days of treatment (60 days after ablation)

<table>
<thead>
<tr>
<th></th>
<th>BW (g)</th>
<th>LKW</th>
<th>MAP (mm Hg)</th>
<th>GFR (mL/min)</th>
<th>RPF (mm Hg/mL/min)</th>
<th>RVR (mm Hg/mL/min)</th>
<th>PG (mm Hg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S (N = 8)</td>
<td>333 ± 9</td>
<td>1.5 ± 0.03</td>
<td>114 ± 2</td>
<td>1.4 ± 0.1</td>
<td>4.6 ± 0.4</td>
<td>14 ± 1</td>
<td>53 ± 1</td>
</tr>
<tr>
<td>NX_pre (N = 8)</td>
<td>263 ± 5a</td>
<td>1.1 ± 0.6a</td>
<td>132 ± 5a</td>
<td>0.7 ± 0.1a</td>
<td>2.2 ± 0.2a</td>
<td>37 ± 6a</td>
<td>66 ± 3a</td>
</tr>
<tr>
<td>NX+V (N = 8)</td>
<td>286 ± 9ab</td>
<td>1.4 ± 0.1b</td>
<td>158 ± 4ab</td>
<td>0.6 ± 0.1a</td>
<td>2.0 ± 0.1a</td>
<td>48 ± 4ab</td>
<td>78 ± 4ab</td>
</tr>
<tr>
<td>NX+L50 (N = 8)</td>
<td>291 ± 6ab</td>
<td>1.5 ± 0.1b</td>
<td>138 ± 4ac</td>
<td>0.7 ± 0.1a</td>
<td>2.5 ± 0.2a</td>
<td>34 ± 3ac</td>
<td>64 ± 3ac</td>
</tr>
<tr>
<td>NX+L500 (N = 8)</td>
<td>288 ± 6ab</td>
<td>1.5 ± 0.1b</td>
<td>124 ± 3c,d</td>
<td>0.7 ± 0.1a</td>
<td>3.2 ± 0.3e,b,c</td>
<td>24 ± 2a,b,c,d</td>
<td>59 ± 1b,c</td>
</tr>
<tr>
<td>NX+HH (N = 8)</td>
<td>292 ± 6ab</td>
<td>1.6 ± 0.1b</td>
<td>112 ± 5b,c,d</td>
<td>0.6 ± 0.1a</td>
<td>2.5 ± 0.3a</td>
<td>33 ± 7a</td>
<td>70 ± 3a,c,e</td>
</tr>
</tbody>
</table>

Abbreviations: BW, body weight; LKW, left kidney weight; MAP, mean arterial pressure; GFR, glomerular filtration rate; RPF, renal plasma flow; RVR, renal vascular resistance; PG, glomerular hydraulic pressure. Results expressed as mean ± SE. aP < 0.05 vs. S; bP < 0.05 vs. NX_pre; cP < 0.05 vs. NX+V; dP < 0.05 vs. NX+L50; eP < 0.05 vs. NX+L500.
observed among the NX groups. Similar findings were obtained regarding left kidney weight (LKW), except for group NX+L500, in which LKW, unlike in the functional studies, was significantly lower than in group NX+V (data not shown). The behavior of TCP and U_{alb}V at 30 and 120 days after ablation is represented in Figure 1A and B, respectively. Initial (30-day) TCP values were uniformly elevated among all NX groups compared to S, and similar, as expected, to those found in group NX_{pre} (201 ± 9 mm Hg, P < 0.05 vs. S). At 120 days after ablation, hypertension was aggravated in group NX+V (231 ± 4 mm Hg, P < 0.05 vs. the respective 30-day value). L50 treatment prevented further elevation of blood pressure, which nevertheless remained at severely hypertensive levels (203 ± 8 mm Hg, P < 0.05 vs. S and NX+V). Treatment with L50 lowered TCP below pretreatment levels (177 ± 7 mm Hg, P < 0.05 vs. S, NX+V, NX+L50, and pretreatment levels). HH therapy was even more effective, bringing final TCP to levels similar to those seen in S (157 ± 6 mm Hg, P < 0.05 vs. NX+V, NX+L50, and pretreatment levels, P > 0.05 vs. S). Initial (30-day) albuminuria was uniformly increased among groups, and about 16-fold higher than in S. Albuminuria was aggravated 120 days after ablation in untreated NX rats (129 ± 13 mg/24h in group NX+V, P < 0.05 vs. S). Treatment with L50 promoted a numerical decrease in U_{alb}V (100 ± 17 mg/24h in group NX+L50, P < 0.05 vs. S and P > 0.05 vs. NX+V). With L500 treatment, albuminuria regressed relative to pretreatment values (46 ± 7 mg/24h, P < 0.05 vs. S, pretreatment values, NX+V, and NX+L50). HH promoted no amelioration of albuminuria (120 ± 13 mg/day, P < 0.05 vs. S, pretreatment values, and NX+L500, P > 0.05 vs. NX+V).

The GSI in the several groups is represented in Figure 2. In group NX_{pre} (pretreatment reference), GSI was 15 ± 5, significantly higher than in group S (0.13 ± 0.09), showing that glomerular injury already existed 30 days after ablation. These lesions were exacerbated 120 days after ablation (139 ± 43 in group NX+V, P < 0.05 vs. groups S and NX_{pre}). L50 treatment reduced GSI numerically relative to NX+V, although this difference was not statistically significant (68 ± 23, P < 0.05 vs. S and NX_{pre}, P > 0.05 vs. NX+V). By contrast, GSI remained at levels not statistically different from pretreatment values in group NX+L500 (22 ± 6, P < 0.05 vs. S, NX+V, and NX+L50, P > 0.05 vs. NX_{pre}). HH treatment had a similar effect on GSI as was obtained with the L50 regimen (67 ± 13, P < 0.05 vs. S, NX_{pre}, and NX+L500, P > 0.05 vs. NX+V). A quantitative analysis of renal interstitial expansion, expressed by the fraction of the cortical area occupied by interstitial tissue (%INT), can be seen in Figure 3. In group NX_{pre}, %INT was 10-fold as high as in group S (2.2 ± 0.5% vs. 0.2 ± 0.1 in S, P > 0.05). The interstitial area was markedly expanded 120 days after ablation, reaching 8.5 ± 1.2% in group NX+V (P < 0.05 vs. S and NX_{pre}). L50 and HH treatments promoted slight numerical, not statistically significant, decreases in %INT relative to untreated NX (6.2 ± 0.8% and 5.8 ± 0.7%, respectively, P < 0.05 vs. S and NX_{pre}, P > 0.05 vs. NX+V). High-dose L treatment L500 reduced %INT to 4.1 ± 0.6 in group NX+L500, a value significantly different from that in group NX+V.

The extent of renal interstitial macrophage infiltration is represented in Figure 4. In group NX_{pre}, the interstitial macrophage density more than tripled compared to that in S (71 ± 10 vs. 17 ± 2 cells/mm², P < 0.05). Macrophage infiltration was exacerbated in group NX+V 120 days after ablation, reaching 133 ± 17 cells/mm² (P < 0.05 vs. S and NX_{pre}). Neither L50 nor HH ameliorated the intensity of macrophage infiltration (107 ± 20 and 121±14 cells/mm² in groups NX+L50 and NX+HH, respectively,
Fig. 2. Glomerulosclerosis index (GSI) 120 days after renal ablation in groups S (sham), NX_pre (30 days after renal ablation, used as pretreatment reference), NX+V (no treatment), NX+L50 (losartan, 50 mg/kg/day), NX+L500 (losartan, 500 mg/kg/day), and NX+HH (hidralazine, 24 mg/kg/day, and hydrochlorothiazide, 6 mg/kg/day). Results expressed as mean ± SE. *P < 0.05 vs. S; †P < 0.05 vs. NX_pre; ‡P < 0.05 vs. NX+V; §P < 0.05 vs. NX+L50; ¶P < 0.05 vs. NX+L500.

Fig. 3. Fraction of cortical area occupied by interstitium (%INT) in groups S (sham), NX_pre (30 days after renal ablation, used as pretreatment reference), NX+V (no treatment), NX+L50 (losartan, 50 mg/kg/day), NX+L500 (losartan, 500 mg/kg/day), and NX+HH (hidralazine, 24 mg/kg/day, and hydrochlorothiazide, 6 mg/kg/day). Results expressed as mean ± SE. *P < 0.05 vs. S; †P < 0.05 vs. NX_pre; ‡P < 0.05 vs. NX+V.

Fig. 4. Intensity of interstitial macrophage infiltration (in cells/mm²) in groups S (sham), NX_pre (30 days after renal ablation, used as pretreatment reference), NX+V (no treatment), NX+L50 (losartan, 50 mg/kg/day), NX+L500 (losartan, 500 mg/kg/day), and NX+HH (hidralazine, 24 mg/kg/day, and hydrochlorothiazide, 6 mg/kg/day). Results expressed as mean ± SE. *P < 0.05 vs. S; †P < 0.05 vs. NX_pre; ‡P < 0.05 vs. NX+V; §P < 0.05 vs. NX+L50; ¶P < 0.05 vs. NX+L500.

Fig. 5. Microphotography showing cells staining positively for angiotensin II (darker areas), detected by immunohistochemistry in an afferent arteriole (4-μm thick section, ×200).

P < 0.05 vs. S and NX_pre, and P > 0.05 vs. NX+V). By contrast, L500 treatment kept macrophage infiltration at levels not statistically different from those observed in group NX_pre (68 ± 8 cells/mm², P < 0.05 vs. S, NX+V, and NX+HH). Figure 5 shows the immunohistochemical expression of Ang II in an afferent arteriole, such as typically observed in sham-operated control subjects. A quantitative analysis of this expression in Ang II-positive profiles/mm² is given in Figure 6, showing that, in the NX groups, this value was invariably lower than in group S. Figure 7 shows a typical inflamed renal interstitial area infiltrated by Ang II-positive cells, whereas Figure 8 shows the extent of this infiltration among the several groups. In group NX_pre, the density of Ang II-positive cells was 5.2 ± 0.9 cells/mm², over 2-fold higher than in S (P < 0.05). At 120 days, this value reached 11.2 ± 2.3 cells/mm² in group NX+V (P < 0.05 vs. S and NX_pre). Neither L50 nor HH treatment had a significant effect on this parameter, while L500 therapy kept it at levels indistinguishable from pretreatment values (5.1 ± 0.8 cells/mm², P < 0.05 vs. NX+V, P > 0.05 vs. S and NX_pre).

A typical pattern of the AT1R expression in the S group is shown in Figure 9A, in which AT1R appears mostly in tubules, whereas glomeruli, vessels, and the renal interstitium show only weak AT1R expression. As shown previously in this laboratory [17], renal mass reduction changed dramatically the AT1R intrarenal distribution...
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Fig. 6. Quantitative analysis of the Ang II content (in cells/mm²) of afferent arterioles in groups S (sham), NXpre (30 days after renal ablation, used as pretreatment reference), NX+V (no treatment), NX+L50 (losartan, 50 mg/kg/day), NX+L500 (losartan, 500 mg/kg/day), and NX+HH (hidralazine, 24 mg/kg/day, and hydrochlorothiazide, 6 mg/kg/day). Results expressed as mean ± SE. aP < 0.05 vs. S.

Fig. 7. Microphotography showing cells staining positively for angiotensin II (darker areas), detected by immunohistochemistry, in an interstitial inflamed area in a rat from group NX+V (4-µm thick section, ×400).

pattern at 120 days (Fig. 9B), when most of the AT1R expression was located in the interstitial compartment, especially at inflamed areas. AT1R distribution was not affected by L50 or HH treatments, whereas L500 restored the pretreatment pattern (Fig. 9C). To better evaluate the intrarenal AT1R distribution, the ratio between its interstitial and tubular expressions was calculated in all groups (Fig. 10). In S, this ratio was very small (0.09 ± 0.02), indicating an almost complete predominance of the tubular expression of AT1R. Interstitial shifting was already evident 30 days after ablation (0.5 ± 0.1 in group NXpre, P < 0.05 vs. S), and reached a maximum 120 days after ablation (9.1 ± 2.9 in group NX+V, P < 0.05 vs. S and NXpre). Treatment with L50 or HH promoted a numerical, not statistically significant decrease in this parameter (3.6 ± 0.8 and 4.9 ± 0.9, respectively, P < 0.05 vs. S and NXpre, P > 0.05 vs. NX+V). L500 treatment reduced the interstitial/tubular ratio to 1.3 ± 0.2 (P < 0.05 vs. all the other groups).

Group NX+V exhibited a modest but significant hyperkalemia at day 120 compared to S (4.4 ± 0.2 mmol/L vs. 3.4 ± 0.1 in S, P < 0.05). Treatment with either L50 or L500 promoted no further elevation of plasma K⁺ levels (4.4 ± 0.2 and 4.5 ± 0.1, respectively, P < 0.05 vs. S and P > 0.05 vs. NX). Not unexpectedly, plasma K⁺ concentration was brought to normal levels by the HH treatment (3.5 ± 0.1, P < 0.05 vs. S and P < 0.05 vs. NX). No variation was observed among groups regarding plasma sodium levels (data not shown).

DISCUSSION

As expected, 5/6 renal mass removal led to progressive hypertension, albuminuria, glomerulosclerosis, and interstitial expansion, all of which may have been underestimated given the 22% mortality in the untreated group, since the animals that succumbed were likely those with worst renal injury. As in previous studies [4, 29, 30], renal injury was clearly associated with severe and progressive systemic and glomerular hypertension, which likely played a major pathogenic role. However, abundant experimental evidence [4, 15, 31, 32] now indicates that the development of progressive renal injury requires that hemodynamic stress be coupled with inflammatory events, such as the severe and progressive macrophage infiltration seen in NX rats. In vitro evidence suggests
that inflammation can actually be caused directly by mechanical stimulation [31–34].

As described previously in association with the NX model [17] and the chronic NO inhibition model [19, 35], Ang II could be detected by immunohistochemistry at 2 main intrarenal locations: at the final portion of the afferent arterioles (AA), and the renal interstitium. In normal S rats, Ang II-positive cells were easily seen at AA, but appeared infrequently at the interstitial area. This pattern was inverted 30 days after ablation, at which time the arteriolar expression of Ang II had fallen precipitously, while a large number of Ang II-positive cells had appeared in areas of interstitial inflammation. This abnormality was exacerbated 120 days after ablation, in parallel with the aggravation of renal structural injury. These findings suggest that, at these 2 locations, Ang II exerts quite different functions. At the AA, Ang II seems to participate, as it would be expected, in the process of renal sodium conservation, varying inversely with extracellular volume, being thus depressed under conditions of severe renal mass reduction. By contrast, the interstitial expression of Ang II, rather than reflect the extracellular volume status, appeared intimately associated with the process of interstitial inflammation characteristic of this model. The exact cell origin of this “anomalous” Ang II is unclear, since both tubular and several types of inflammatory cells can produce Ang II, or at least possess the necessary biochemical machinery [16, 19, 36, 37]. Additionally, Ang II may have been produced by tubular cells and undergone internalization after binding to the AT1 receptor, which is known to be expressed by interstitial inflammatory cells [38, 39]. It is worth noting that mechanical stress, such as caused by glomerular hypertension, can enhance the production of Ang II [40], and the expression of the AT1 receptor [41].

Whatever its origin, Ang II is likely to have been involved in the process of chronic inflammation and fibrosis that took place at the renal interstitium. Ang II is known to participate in the activation of lymphocytes and monocytes [13, 42], to stimulate the activation of the nuclear-kappa B factor [11] and the expression of several inflammatory mediators and growth factors [11, 43–46], the proliferation of both inflammatory and parenchymal cells [47, 48], the activation of intracellular inflammatory pathways [49], and the synthesis of extracellular matrix [50, 51].

The renal distribution pattern of the AT1 receptor was already altered at 30 days of ablation, before treatments were begun, with the interstitial/tubular ratio increasing several fold relative to the value observed in S. This shift of the AT1 receptor expression toward the interstitium was dramatically increased at day 120, confirming recent observations of this laboratory using the NX model [17]. The AT1 receptor was shown to be expressed by cells involved in the inflammatory response, such as lymphocytes, macrophages, and myofibroblasts [13, 36, 48], and may therefore have contributed decisively to the intense interstitial inflammation observed in this study. The simultaneous presence of large amounts of Ang II and of its main receptor at the limited space of the renal interstitium is likely to have resulted in intense paracrine and even autocrine stimulation of inflammatory cells, establishing a positive feedback that culminated in the severe destruction of renal parenchyma observed at day 120.

Monotherapy with L at the “conventional” dose of 50 mg/kg/day promoted only modest reduction of hypertension and albuminuria relative to untreated NX rats. Accordingly, the parameters of glomerular and interstitial injury 120 days after renal ablation in group NX+L50 were only slightly lower than, and not statistically different from, those observed in untreated NX rats. These results are consistent with previous observations of experimental models of chronic kidney disease and clinical trials, which indicate that, when initiated late in the course of chronic kidney disease, treatment with ACE inhibitors or AT1 blockers can slow, but not detain, the progression toward advanced renal insufficiency [21–25]. The reasons why only incomplete protection is afforded by late treatment with suppressors of the renin-angiotensin system are currently unclear. However, the present study...
Fig. 10. Quantitative analysis of the ratio between the interstitial and tubular expressions of the AT1 receptor in groups S (sham), NX pre (30 days after renal ablation, used as pretreatment reference), NX + V (no treatment), NX + L50 (losartan, 50 mg/kg/day), NX + L500 (losartan, 500 mg/kg/day), and NX + HH (hidralazine, 24 mg/kg/day, and hydrochlorothiazide, 6 mg/kg/day). Results expressed as mean ± SE. a \( P < 0.05 \) vs. S; b \( P < 0.05 \) vs. NX pre; c \( P < 0.05 \) vs. NX + V; d \( P < 0.05 \) vs. NX + L50; e \( P < 0.05 \) vs. NX + L500.

The superior protection conferred by the high-dose L treatment might be ascribed to the much more intense and long-lasting antihypertensive effect of this regimen compared with that obtained in the L50 group. However, blood pressure was lowered to the same extent by the HH treatment, yet albuminuria and the parameters of renal injury in this group were similar to those found in rats treated with L50, in which hypertension was less well controlled. Thus, the additional protection brought by the high dose L treatment must have been independent of blood pressure and related, in all likelihood, to some intrarenal effect. Indeed, the L500 treatment reduced the RVR to levels half as high as in untreated NX rats. This marked vasodilatation was likely associated with a higher sodium excretion capability, which helps to explain the more efficacious antihypertensive effect of the L500 treatment [56]. In addition, \( P_{OC} \) was 5 mm Hg lower in this group compared with L50. However, it is uncertain whether this difference, which was not statistically significant, can account for the remarkable additional effects of L500 on renal structure.

The additional protection afforded by L500, not satisfactorily explained by its effect on either systemic or glomerular hemodynamics, may be attributed, at least in part, to an anti-inflammatory action. Treatment with L500 markedly limited the renal infiltration by macrophages, which remained close to, or even below, the levels observed before treatments were started. The amount of interstitial cells staining positively for Ang II was equally limited to pretreatment values. In addition, and unlike L50 or HH treatments, L500 shifted the intrarenal distribution of AT-1 receptor toward the pattern observed before treatments, limiting its expression at the interstitium

keeping the respective parameters at pretreatment levels even at 120 days after renal ablation, whereas TCP and \( U_{alb}V \) regressed to values significantly lower than those observed before treatment. In addition, mortality was reduced to zero in this group, compared to 22% in untreated NX rats and 6% in the L50 group. The degree of protection obtained in these NX rats with L500 was higher than with late treatment with enalapril [54], and similar to that achieved with an association of L50 with either mycophenolate mofetil [25] or a nonsteroidal anti-inflammatory [17]. Thus, these results indicate that the renoprotective effect of AT1 blockade is strongly dose dependent, and that achievement of maximal beneficial effects may require doses one order of magnitude higher than those currently employed. This dose dependence of losartan is likely to be observed in humans as well, since clinical studies showed that the human response can be predicted with reasonable accuracy from animal experiments [55], although studies of the kinetics of very high ARB doses in humans have not been performed.

Although L50 treatment did attenuate systemic hypertension, blood pressure was never returned to normal, remaining at the very high levels already observed before treatment. Since hypertension has been pointed out by some investigators to be a major pathogenic factor in the progression of chronic kidney disease [52], the persistently heightened blood pressures in the L50 group might help to explain the narrow difference between the renal injury observed in these rats and in untreated NX rats. However, it must be noted that blood pressure was nearly normalized in the group that received HH treatment, in which renal injury parameters were similar to those of untreated NX rats, suggesting that additional factors had a more decisive pathogenic role. In consistency with previous observations [25, 53], treatment with L50 attenuated glomerular hypertension in NX rats. However, glomerular pressure remained 11 mm Hg above the levels observed in S, which might help to explain the continued progression of glomerular injury in this group. On the other hand, L50 treatment promoted no significant alteration in the intensity of the renal interstitial macrophage infiltration, the renal expression of Ang II-positive cells, or the intrarenal distribution of the AT1 receptor in NX rats. Thus, L50 monotherapy had only partial effect on either the hemodynamic or the inflammatory events associated with the development of progressive nephropathy in the NX model, which helps to explain its limited protective action.

Treatment with L at a dose 10-fold higher than for group L50, and 50-fold higher than those usually employed in experimental studies, arrested the progression of both glomerulosclerosis and interstitial expansion, offers some possible explanations for this limitation.
and enhancing it at the tubules. Together, these observations are consistent with our working hypothesis that, given the intense interstitial expression of the AT-1 receptor, a much larger dose of L (or of any ARB) is necessary to counteract the proinflammatory effects of Ang II. An additional possibility is that the “anomalous” AT-1 receptors possess higher affinity for Ang II than at the vessels, thus requiring higher concentrations of L to be blocked, although at present this possibility must remain entirely speculative, given the absence of appropriate evidence. It is conceivable that AT-1 binding triggers intracellular inflammatory events, such as generation of reactive oxygen species and activation of the NF-kappa-B and MAP pathways [11, 49, 51, 57, 58], recruiting more inflammatory cells, that will in turn produce Ang II and/or express the AT-1 receptor, thus establishing a positive feedback, and causing an exponential growth of the inflammatory infiltrate. The administration of a high dose of L may have interrupted this vicious cycle, thus limiting inflammation and preserving the renal structure more effectively than the “conventional” dose of L.

Despite its remarkable effect on interstitial Ang II, treatment with L500 did not normalize the “physiologic” expression of Ang II at the afferent arterioles. The reason for this apparently unexpected finding is unclear. However, it must be noted that normalization of blood pressure was not achieved with L500 treatment, indicating that some degree of extracellular volume expansion persisted in this group, and that additional measures may be required to adequately enhance sodium excretion by the remnant renal tissue.

It is important to note that L could be administered at an extremely high dose without any perceptible toxic effect. Rats receiving this treatment grew at the same rate as untreated rats or rats receiving the lower L dose. No hypotension was seen in the group L500, in either awake or anesthetized rats, while GFR was identical to that obtained in groups NX+V and NX+L50. Plasma potassium concentration was not higher in group L500 than in the other NX groups, as it might be expected. This finding suggests that, in the NX model, potassium excretion is relatively independent of AT-1 stimulation by Ang II. Circulating aldosterone concentration is actually increased in this model [59], even though the renin levels are depressed [60]. Elevated potassium levels can directly stimulate aldosterone synthesis by the adrenals [61], and the insertion of potassium channels at the luminal membrane of principal cells at the collecting duct [62], leading to enhanced potassium excretion and limiting hyperkalemia. Although the present observations cannot be applied to clinical practice before careful toxicology tests are carried, recent evidence suggests that ARB doses in large excess of those currently recommended may be well tolerated even by patients suffering from chronic kidney disease [63].

CONCLUSION

Late treatment of NX rats with a very high dose of losartan afforded more effective renal protection compared to treatment with “conventional” doses, arresting the parameters of renal injury and inflammation at the levels observed prior to treatment. The superiority of this regimen cannot be satisfactorily explained by its action on systemic or intrarenal hemodynamics, and may be largely due to an anti-inflammatory effect. The present findings indicate that the renal protection conferred by ARB is dose dependent, and that the doses necessary for maximal protection may be several fold higher than those currently adopted. Although hyperkalemia and other toxic effects were not observed in the present study, careful tests are necessary to establish whether high doses of ARB can be equally safe and effective in the clinical management of chronic nephropathies.

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