Losartan-hydrochlorothiazide association promotes lasting blood pressure normalization and completely arrests long-term renal injury in the 5/6 ablation model

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Fujihara CK, Malheiros DM, Zatz R. Losartan-hydrochlorothiazide association promotes lasting blood pressure normalization and completely arrests long-term renal injury in the 5/6 ablation model. Am J Physiol Renal Physiol 292: F1810–F1818, 2007. First published March 6, 2007; doi:10.1152/ajprenal.00521.2006.—The possible long-term renoprotective effects of treatment with thiazides, either as monotherapy or associated with renin-angiotensin suppressors, have not been assessed. We investigated the effect of hydrochlorothiazide (H), alone or combined with losartan (L), in the 5/6 renal ablation model (Nx). Adult male Munich-Wistar rats underwent Nx, remaining untreated for 1 mo. At this time, functional and morphological studies were performed in 21 rats (group Nxpre). The remaining rats were untreated for 1 mo. At this time, functional and morphological studies had not been assessed. We investigated the effect of hydrochlorothiazide (H), alone or combined with losartan (L), in the 5/6 renal ablation model (Nx). Adult male Munich-Wistar rats underwent Nx, remaining untreated for 1 mo. At this time, functional and morphological studies were performed in 21 rats (group Nxpre). The remaining rats were distributed among groups: Nx, no treatment; Nx+L, receiving L, 50 mg·kg⁻¹·day⁻¹ in the drinking water; Nx+H, receiving H, 6 mg·kg⁻¹·day⁻¹ in drinking water; and Nx+L+H, receiving both L and H as described. At 30 days of treatment, systemic and glomerular pressures were markedly elevated in group Nx. Both H and L attenuated hypertension, whereas combined L+H treatment completely normalized both pressures. Eight months after Nx, mortality approached 70% in untreated rats, whereas severe albuminuria, hypertension, glomerulosclerosis, and interstitial expansion were observed. H and L attenuated, but did not prevent, mortality, hypertension, and renal injury. Combined L+H treatment completely prevented mortality, normalized albuminuria and blood pressure, and arrested renal injury at levels found 1 mo after ablation, despite the unusually long period of observation. Combined L+H treatment may represent an effective therapeutic alternative to prevent progression of chronic nephropathies.

chronic kidney disease; thiazide diuretics; 5/6 nephrectomy; chronic renal insufficiency

SUPPRESSION OF THE RENIN-ANGIOTENSIN-ALDOSTERONE SYSTEM (RAS) by converting enzyme inhibitors (ACEI) or AT-1 receptor blockers (ARB) represents a major asset in the struggle to prevent or retard the progression of chronic kidney disease (CKD). Nevertheless, a substantial fraction of treated patients still progress to end-stage renal disease. The reasons why this renoprotective effect is only partial are unclear and may include incompleteness of ANG II suppression (9, 10), continuing stimulation of downstream intracellular events by factors independent of the RAS (35), and failure to completely normalize renal and/or systemic hemodynamics (9). The latter may result from overexpression of tubular sodium transporters (20), which may favor the perpetuation of extracellular volume expansion. We showed previously that, when administered at extremely high doses to rats subjected to 5/6 renal ablation (Nx), the ARB, losartan (L), provided more efficient renoprotection after 4 mo than treatment with “conventional” doses (9). However, even at these high doses protection was still incomplete, since albuminuria and tail-cuff pressure (TCP) never returned to control values. In addition, follow-up beyond 4 mo after ablation showed that severe albuminuria and renal structural damage did develop in these rats, although mortality was drastically reduced (Fujihara CK and Zatz R, unpublished observations). Meyer and co-workers (25) showed that late treatment with a relatively low dose of enalapril reversed glomerular hypertension and attenuated glomerular injury in the Nx model. However, treatment lasted for only 2 mo in that study.

Recent clinical evidence suggested that thiazides may have more powerful antihypertensive and renoprotective action than might be expected from their diuretic potency alone. In the ALLHAT study, chlorthalidone lowered systolic blood pressure and prevented cardiovascular disease more effectively than lisinopril or amldopine (2). In CKD patients, Dussol and co-workers (5) obtained comparable antihypertensive and better natriuretic effect with chlorthalidone than with furosemide. In addition, the antihypertensive effect and cardiovascular risk reduction provided by ACEI or ARB are strongly reinforced by concomitant administration of thiazides (21, 33, 36, 40). However, systematic clinical or experimental studies of the long-term renoprotective effect of these associations have not been performed.

In the present study, we sought to determine whether association of hydrochlorothiazide (H) with L would provide more complete renoprotection than monotherapy with either compound to rats followed up to 8 mo after Nx, as well as the mechanisms underlying their possible interaction.

METHODS

One hundred forty two adult male Munich-Wistar rats, weighing initially 230–260 g, were included in this study. Rats were obtained from a local facility and maintained at 23 ± 1°C, with air humidity at 60 ± 5%, under a 12:12-h light-dark cycle. All animals had free access to tap water and standard chow (0.5 Na, 22% protein). All experimental procedures were approved by the local Research Ethics Committee (CAPPseq, process no. 770/05) and developed in strict conformity with our institutional guidelines and with international standards for manipulation and care of laboratory animals. Nx was performed in one step under anesthesia with pentobarbital sodium, 50 mg/kg ip, by removal of the right kidney and infarction of two-thirds of the left kidney by closure of two or three branches of the left renal

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artery. Sham-operated rats (S) underwent anesthesia and manipulation of the renal pedicles without renal mass reduction. After recovering from anesthesia, the animals were returned to their original cages, which were warmed during the following 24 h.

**Experimental Groups**

Thirty days after Nx, TCP and albuminuria (U_{alb}V) were measured in all rats. TCP was determined by an automated method (Visitech Systems, Apex, NC) (19), under light restraining and after light warming. All rats were preconditioned (at least twice) to the procedure. TCP values were always taken as the average of three consecutive measurements obtained after stabilization of the readings. Urine albumin was measured by an immunodiffusion technique.

Twenty-seven Nx rats with TCP under 160 mmHg or U_{alb}V less than 40 mg/day were excluded from the study. Twenty-one Nx rats fulfilling these criteria constituted a separate group that was utilized as a pretreatment reference (group Nx_pre) in functional and morphological experiments and were followed no further. Ten S rats (group S_pre) were utilized as normal controls for group Nx_pre. The remaining Nx animals were subdivided into four experimental groups: Nx (n = 24), receiving no treatment; Nx + L (n = 22), receiving L, 200 mg/l (50 mg/kg day^{-1}) in the drinking water; Nx + H (n = 22), receiving H, 25 mg/l (6 mg/kg day^{-1}) in the drinking water; Nx + L + H (n = 22), receiving both H and L as described above. Nx rats were distributed in such a way that no statistically significant differences among groups existed regarding pretreatment values for either TCP or U_{alb}V. Likewise, no statistical difference among groups was observed regarding body weight. Twenty-one S rats (group S) served as normal controls for these four groups. To allow renal injury to develop, thus mimicking the usual clinical presentation of CKD, all drug regimens were started only 30 days after Nx.

**Functional Studies**

Seven rats from each group underwent renal hemodynamic studies 30 days after treatments were started (60 days after Nx). Seven rats from group Nx_pre (studied 30 days after Nx) were subjected to the same protocol. Rats were anesthetized with inactin, 100 mg/kg ip, and blood samples were drawn from the abdominal aorta for determination of serum creatinine and plasma aldosterone concentrations. The extent of renal infiltration by macrophages or ANG II-positive cells was assessed by immunohistochemistry, using a monoclonal rabbit anti-human ANG II antibody (Peninsula Laboratories, San Carlos, CA). Sections were preincubated with avidin and biotin solutions to block nonspecific binding (Blocking Kit, Vector Laboratories, Burlingame, CA), incubated at room temperature with rat-adsorbed biotinylated anti-mouse or anti-rabbit IgG (Vector Laboratories) for 45 min, then with a SBAP complex (Dako) for an additional 30 min, naphthol AS-MX-Phosphate, and finally developed as described above. The extent of renal infiltration by macrophages or ANG II-positive cells (in cells/mm²) was evaluated in a blinded manner at 250 magnification. For each section, 25 microscopic fields (corresponding to a total area of 1.5 mm²) were examined.

**Analytic Techniques**

U_{alb}V was determined by radial immunodiffusion. Serum creatinine concentration (S_{crea}) was measured by a colorimetric method. Plasma aldosterone concentration was measured by radioimmunoassay using a commercially available kit (Diagnostic Systems Laboratories, Webster, TX). Sodium and potassium concentrations were assessed by flame photometry (Instrumentation Laboratory, Lexington, MA).

**Statistical Analysis**

Differences among the different groups were analyzed using one-way ANOVA with pairwise posttest comparisons by the Newman-Keuls method (37). Student’s t-test was employed in the comparison between groups Nx_pre and S_pre. Since GSI, the albumin excretion rate and the intensity of interstitial infiltration by ANG II exhibited a strong non-Gaussian distribution, log transformation of these parameters was performed before statistical analysis. For similar reasons, parameters expressed as proportions underwent arcsine transformation before analysis (37). P < 0.05 was considered significant.

**RESULTS**

**One-Month Studies**

Thirty days after renal ablation, before any drug treatment was started (group Nx_pre), body growth was stunted in rats subjected to 5/6 renal ablation compared with sham-operated

**Immunohistochemistry**

All immunohistochemical analyses were carried on 4-μm-thick, paraffin-embedded sections, mounted on glass slides coated with 2% gelatin. Sections were deparaffinized and rehydrated and then heated in citrate buffer to enhance antigen retrieval, and preincubated with 5% normal rabbit (for ED-1) or horse (for ANG II) serum in Tris-buffered saline (TBS), to prevent nonspecific binding. Incubation with the primary antibody was carried out overnight at 4°C and omitted in the negative control experiments. 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 immunoglobulins (Dako, Glostrup, Denmark) and then with an alkaline phosphatase anti-alkaline phosphatase (APAAP; Dako) complex. Sections were then developed with a fast-red dye solution, counterstained with Mayer’s hemalaun, and covered with Kaiser’s glycercin-gelatin (Merck, Darmstadt, Germany).

Cells staining positively for ANG II were detected by an indirect streptavidin-biotin alkaline phosphatase (SBAP) technique, using a monoclonal rabbit anti-human ANG II antibody (Peninsula Laboratories, San Carlos, CA). Sections were preincubated with avidin and biotin solutions to block nonspecific binding (Blocking Kit, Vector Laboratories, Burlingame, CA), incubated at room temperature with rat-adsorbed biotinylated anti-mouse or anti-rabbit IgG (Vector Laboratories) for 45 min, then with a SBAP complex (Dako) for an additional 30 min, naphthol AS-MX-Phosphate, and finally developed as described above.
Table 1. Functional and morphologic parameters obtained in Spre and in rats with Nxpre killed 1 mo after surgery (having received no drug treatment)

<table>
<thead>
<tr>
<th></th>
<th>TCP, mmHg</th>
<th>MAP, mmHg</th>
<th>U AlbV, mg/day</th>
<th>GFR, ml/min</th>
<th>Screat, mg/dl</th>
<th>Pgc, mmHg</th>
<th>Gsi</th>
<th>%Int</th>
<th>Mf, cells/mm²</th>
<th>Ang II+, cells/mm²</th>
</tr>
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<tbody>
<tr>
<td>S</td>
<td>139 ± 4</td>
<td>115 ± 2</td>
<td>3 ± 1</td>
<td>1.3 ± 0.1</td>
<td>0.6 ± 0.1</td>
<td>54 ± 1</td>
<td>0.3 ± 0.2</td>
<td>0.1 ± 0.1</td>
<td>23 ± 2</td>
<td>1.2 ± 0.2</td>
</tr>
<tr>
<td>Nxpre</td>
<td>204 ± 7*</td>
<td>130 ± 6*</td>
<td>72 ± 9*</td>
<td>0.7 ± 0.1*</td>
<td>1.1 ± 0.1*</td>
<td>62 ± 1*</td>
<td>21.0 ± 6.6*</td>
<td>3.2 ± 0.4*</td>
<td>119 ± 12*</td>
<td>3.8 ± 0.6*</td>
</tr>
</tbody>
</table>

Values are means ± SE. TCP, tail cuff-pressure; MAP, mean arterial pressure; U AlbV, urinary albumin excretion rate; GFR, glomerular filtration rate; Screat, serum creatinine concentration; Pgc, glomerular hydraulic pressure; Gsi, glomerulosclerosis index; %Int, fraction of cortical area occupied by interstitium; Mf, intensity of interstitial infiltration by macrophages; Ang II+, intensity of interstitial infiltration by cells staining positively for Ang II; Spre, sham-operated rats; Nxpre, 5/6 renal mass reduction. *P < 0.05 vs. Spre.

(Spre) rats (268 ± 6 vs. 298 ± 6 g, P < 0.05). Hemodynamic and morphological parameters obtained at this time are given in Table 1. Compared with Spre, TCP and U AlbV were markedly elevated in group Nxpre. As expected, GFR was reduced and Screat was increased, whereas Pgc was elevated, in group Nxpre. The histomorphometric parameters, Gsi, and %Int, as well as counting of interstitial cells staining positively for ED-1 and ANG II, indicate that renal injury and inflammation were already present in Nx rats at this time.

Renal Hemodynamic Studies (2 Mo After Renal Ablation)

Functional and hemodynamic parameters measured 2 mo after renal ablation (1 mo after drug treatments were started) are given in Table 2. Body growth was limited in all Nx groups compared with S. However, there was no statistically significant difference in body weight (BW) among Nx groups. Left kidney weight (LKw) was only slightly diminished in the Nx groups relative to S, indicating marked renal hypertrophy, given the magnitude of the original renal ablation. MAP was severely elevated compared with S in group Nx. Both L and H monotherapies (groups Nx+L and Nx+H, respectively) significantly attenuated hypertension, which remained at levels similar to those observed in group Nxpre. Combined L+H treatment brought MAP to levels indistinguishable from those obtained in S and significantly lower than in each Nx group. There were no significant differences among Nx groups regarding GFR or RPF, which were uniformly reduced by ~60% compared with S (P < 0.05), showing that renal mass removal resulted in similar functional impairment among groups. RVR increased by more than threefold in group Nx (P < 0.05). Treatment with either L or H reduced RVR numerically compared with Nx. Combined L+H treatment brought RVR to levels not statistically different from those observed in S. Glomerular hypertension reached severe levels in group Nx. Both L and H treatments significantly attenuated glomerular hypertension compared with Nx. In rats receiving L+H, Pgc was brought to levels indistinguishable from those measured in S.

Long-Term Studies (8 Mo After Renal Ablation)

Mortality and estimation of renal function. During the 8-mo follow-up, 12 of 17 rats (71%) were lost in group Nx, whereas 2 of 15 (13%) and 5 of 15 rats (33%) died in groups Nx+L and Nx+H, respectively. By contrast, mortality was zero in group Nx+L+H (Table 3). Eight months after Nx, body growth was restricted in all Nx groups. Screat was fourfold higher in Nx than in S, and twice as high as observed 1 mo after renal ablation (group Nxpre). L and H monotherapies attenuated the rise in Screat to a similar extent. In rats receiving combined L+H treatment, Screat was lower than in either monotherapy group and similar to that observed before treatment (group Nxpre).

Plasma potassium and aldosterone. Severe hyperkalemia was observed in group Nx at the end of the study. All treatments limited hyperkalemia, without normalizing plasma K⁺ concentration. Plasma aldosterone concentration was significantly increased at 8 mo in group Nx and brought by all treatments to values indistinguishable from those seen in sham-operated controls.

TCP and albuminuria. The long-term course of TCP and U AlbV is represented in Fig. 1, A and B, respectively. As described under METHODS, initial TCP values (measured 1 mo after Nx) were uniformly elevated among Nx groups compared with S. TCP was further elevated in group Nx, reaching extremely high levels 8 mo after renal ablation. Both L and H treatments attenuated the elevation of TCP, which nevertheless attained at 8-mo levels as elevated as those observed in untreated Nx rats 30 days after renal ablation. Combined L+H therapy lowered TCP to levels not significantly different from those found in S, an effect that was already apparent 30 days after treatment was started, and persisted until the end of the study.

As in the case of TCP, U AlbV was markedly increased compared with S 30 days after renal ablation, before treatments were started (Fig. 1B). Albuminuria increased steadily thereafter in untreated Nx rats, reaching values 100-fold higher than

Table 2. Functional parameters obtained after 1 mo of treatment (2 mo after surgery)

<table>
<thead>
<tr>
<th></th>
<th>BW, g</th>
<th>LKw, g</th>
<th>TCP, mmHg</th>
<th>MAP, mmHg</th>
<th>GFR, ml/min</th>
<th>RPF, ml/min</th>
<th>RVR, mmHg·ml⁻¹·min⁻¹</th>
<th>Pgc, mmHg</th>
</tr>
</thead>
<tbody>
<tr>
<td>S</td>
<td>347 ±10</td>
<td>1.6 ±0.1</td>
<td>136 ±4</td>
<td>111 ±3</td>
<td>1.4 ±0.1</td>
<td>4.5 ±0.6</td>
<td>14 ±1</td>
<td>53 ±1</td>
</tr>
<tr>
<td>Nx</td>
<td>279 ±8*</td>
<td>1.4 ±0.1*</td>
<td>204 ±1*</td>
<td>151 ±4*</td>
<td>0.5 ±0.1*</td>
<td>1.7 ±0.2*</td>
<td>60 ±1*</td>
<td>82 ±4*</td>
</tr>
<tr>
<td>Nx+L</td>
<td>284 ±5*</td>
<td>1.4 ±0.1*</td>
<td>181 ±14*</td>
<td>135 ±5*</td>
<td>0.6 ±0.1*</td>
<td>1.9 ±0.3*</td>
<td>51 ±10*</td>
<td>63 ±3*†</td>
</tr>
<tr>
<td>Nx+H</td>
<td>288 ±5*</td>
<td>1.4 ±0.1*</td>
<td>197 ±5*</td>
<td>140 ±7*</td>
<td>0.6 ±0.1*</td>
<td>1.8 ±0.2*</td>
<td>48 ±8*</td>
<td>65 ±2*†</td>
</tr>
<tr>
<td>Nx+L+H</td>
<td>292 ±8*</td>
<td>1.3 ±0.1*</td>
<td>137 ±4</td>
<td>§§</td>
<td>99 ±5†‡</td>
<td>0.6 ±0.1*</td>
<td>2.2 ±0.2*</td>
<td>28 ±2‡</td>
</tr>
</tbody>
</table>

Values are means ± SE. BW, body weight; LKw, left kidney weight; RPF, renal plasma flow; RVR, renal vascular resistance; S, sham-operated rats; Nx, 5/6 renal ablation model; L, losartan; H, hydrochlorothiazide. *P < 0.05 vs. S. †P < 0.05 vs. Nx. §P < 0.05 vs. Nx+L. $P < 0.05 vs. Nx+H.
in S 8 mo after ablation. Both L and H monotherapies promoted an initial regression of albuminuria, which nevertheless soon resumed progression, returning to pretreatment levels by the end of the study. In rats receiving combined L+H treatment, albuminuria rapidly regressed to levels not significantly different from S (40-fold lower than pretreatment values) and remained at these low levels, with no upward trend, until the end of the study.

Histomorphometric Studies

Figure 2A shows an example of a normal glomerulus such as found in S rats. An example of moderately severe segmental GS typically found in Nx rats 30 days after ablation is shown in Fig. 2B. Much more severe GS, with occlusion of most capillary loops, such as typically found in untreated Nx rats at 8 mo, is shown in Fig. 2C, along with severe expansion and inflammation of the renal interstitium. Partial attenuation of GS and interstitial inflammation at 8 mo by L and H monotherapies is shown in Fig. 2, D and E, respectively, in which examples of moderate to severe GS can be seen. Sclerotic glomerular lesions were rare and modest in rats treated with combined L and H treatment, as exemplified in Fig. 2F.

The GSI observed 8 mo after renal ablation is represented in Fig. 3A. The severity of glomerular damage in untreated Nx rats is illustrated by the high GSI observed in this group, compared with the S levels and to values obtained in group Nxpre. L treatment promoted only numerical GSI reduction relative to Nx. Similar findings were obtained with H monotherapy. By contrast, GSI in group Nx+L+H remained at levels only slightly higher than in S and comparable to those observed in group Nxpre.

Renal interstitial expansion, evaluated by the fraction of the cortical area occupied by interstitial tissue (%INT), is represented in Fig. 3B. Marked interstitial expansion was observed in group Nx 8 mo after renal ablation compared with S and Nxpre. Both L and H monotherapies promoted statistically significant decreases in %INT relative to Nx. Combined L+H treatment attenuated the increase in %INT, which nevertheless remained elevated compared with S. However, %INT values were comparable to those measured in group Nxpre.

Imunohistochemical Studies

The intensity of renal interstitial macrophage infiltration, represented in Fig. 4A, more than quadrupled in group Nx compared with S. Treatment with either L or H limited the extent of macrophage infiltration only slightly (P > 0.05). By contrast, L+H treatment kept macrophage infiltration at levels significantly lower than in groups Nx, Nx+L, and Nx+H, and comparable to those found in Nxpre, although still higher than in S.

As shown previously in this laboratory (10), ANG II-positive cells were detected in inflamed renal interstitial areas. The estimated intensity of this infiltration among the several groups is shown in Fig. 4B. Eight months after renal ablation, the density of ANG II-positive cells was nearly sixfold higher in group Nx than in S. Neither L nor H monotherapies had any significant effect on this parameter, while combined L+H therapy decreased it at levels significantly lower than in untreated Nx, and comparable to those found in group Nxpre, although still higher than in S.

DISCUSSION

As expected from previous studies (9), untreated Nx rats developed progressive and remarkably severe renal disease,
with obsolescence of most glomeruli, as well as intense interstitial inflammation, undoubtedly reflecting the unusually long follow-up period (8 vs. 2 or 4 mo in most previous studies) adopted in this study. The corresponding renal functional consequences were equally severe: Sc_{\text{crea}}t was quadrupled, and serum K^+ levels approached 6 mmol/l. It must be stressed that, given the high mortality observed in this group, the rats that survived until the end of the study were likely those with the least aggressive renal injury. Therefore, the severity of hypertension, albuminuria, and renal injury in untreated Nx rats was most likely underestimated.

Recent evidence suggests that the pathogenesis of CKD involves a complex interaction between hemodynamic and inflammatory mechanisms (7, 18, 30). Systemic hypertension most likely played a role in the pathogenesis of this process. Likewise, glomerular hypertension has been consistently identified as a pathogenic factor in several experimental models (3, 4, 22, 39). Renal inflammation, with infiltration of macrophages, fibroblasts, and myofibroblasts, along with deposition of extracellular matrix components, has also been pointed out as a crucial factor in the pathogenesis of CKD (7, 18, 32). Abundant in vitro evidence suggests that the cellular events underlying this inflammatory response can be triggered by the mechanical stress imparted to the renal microcirculation by the heightened intracapillary pressure (1, 12, 29).

Previous evidence (9, 10, 32) also indicated that in the Nx model 1) ANG II can be produced locally in the renal tissue; 2) this local production bears little relation to any need for sodium conservation, rather, it follows closely the intensity of renal inflammation, particularly at the interstitium; and 3) local ANG II generation likely contributes significantly to progressive renal injury, especially in the Nx model, in which intense interstitial expression of the AT-1 receptor was observed. This concept has been strengthened by abundant evidence that ANG II can activate several intracellular signaling systems that enhance the production of inflammatory mediators and extracellular matrix components (17, 26, 34). In the present study, as shown earlier, the density of ANG II-positive cells at the renal interstitium was abnormally elevated as early as 30 days after renal ablation and had increased further by the end of the

Fig. 2. A: normal glomerulus from a sham rat. B: moderate glomerulosclerosis 1 mo after renal ablation (group Nx_{pre}, pretreatment reference). C: severe glomerulosclerosis, with obliteration of most capillary loops and extensive interstitial inflammation, 8 mo after renal ablation. D and E: glomerulosclerosis of medium to severe intensity observed in rats treated with losartan (D) and hydrochlorothiazide (E) monotherapies, respectively, 8 mo after renal ablation. F: near normal glomerulus in a rat receiving combined losartan and hydrochlorothiazide treatment, 8 mo after renal ablation. PAS ×200.
study. Given the evidence that the 5/6 renal ablation is associated with volume expansion (4), this finding suggests that this local production of ANG II is related to the pathogenesis of renal injury rather than to the maintenance of sodium balance.

In recent years, it has become increasingly clear that, even though its synthesis is largely governed by ANG II, aldosterone plays an independent role in the pathogenesis of CKD (13, 27). As in the case of ANG II, aldosterone stimulates the synthesis of components of the extracellular matrix, an effect unrelated to sodium conservation (11). Consistent with previous findings (11, 23), the circulating levels of aldosterone were strikingly elevated in Nx rats at 8 mo, a seemingly paradoxical effect considering the evidence that renal mass reduction is associated with a trend for sodium retention (4). The reasons for this apparent incongruence are unclear. Abnormal aldosterone production, which appears early in the course of the renal ablation and of other experimental models (11, 13), may reflect the need to maintain potassium balance (14). In addition, aldosterone can be synthesized independently of the need for sodium or potassium regulation, as indicated by the finding that adipocytes can stimulate mineralocorticoid secretion from adrenocortical cells (6). Moreover, recent evidence suggested that aldosterone can be produced outside the adrenals (38), although the meaning of this finding is presently unclear.

As in earlier studies (9, 10), both TCP and UaI/V fell below pretreatment (but not to control) levels in the initial weeks of L treatment. Although this trend was reversed after a few weeks, the severity of albuminuria and hypertension occurring at 8 mo was significantly attenuated compared with untreated rats. Accordingly, the mortality, the renal insufficiency, and the renal structural injury observed at 8 mo were all mitigated, although not prevented, by L treatment. Previous evidence obtained in this laboratory and elsewhere suggests that the renal and systemic protection offered by ARB and ACEI involves both hemodynamic and anti-inflammatory mechanisms (9, 10, 28). In the present study, systemic and glomerular hypertension were both ameliorated by L, as shown previously (8), reflecting the well-known effects of L on sodium retention, peripheral resistance, and the renal microcirculation. However, neither renal nor systemic hemodynamics was normalized. Moreover, the intensity of renal infiltration by macrophage and ANG II-positive cells was not significantly reduced, helping to explain why L monotherapy provided only incomplete renoprotection.

H and other thiazides have been used for over five decades, usually as an adjuvant, in the treatment of hypertension and

Fig. 4. Intensity (in cells/mm²) of interstitial infiltration by macrophages (A) and ANG II-positive cells (B) 8 mo after renal ablation in groups S, Nx, Nx+L (50 mg·kg⁻¹·day⁻¹), Nx+H (6 mg·kg⁻¹·day⁻¹), and Nx+L+H (receiving both drugs as described). Results expressed as means ± SE. *P < 0.05 vs. S. bP < 0.05 vs. Nx. cP < 0.05 vs. Nx+L. dP < 0.05 vs. Nx+H. The dashed line represents the mean values obtained 30 days after renal ablation in group Nxpre.
CKD, although more recent evidence suggested that the antihypertensive effect of thiazide monotherapy may be as effective as that of other agents (2). H monotherapy has not been used in the Nx model, although an association of H with hydralazine and reserpine was effective as an antihypertensive (but not renoprotective) regimen (4). In the present study, H provided attenuation of mortality, renal insufficiency, and renal structural injury that was comparable to that obtained with L. This effect involved amelioration of systemic/glomerular hypertension, undoubtedly a result of the diuretic action of H, although a more direct effect is possible (41). However, as with L, inflammation was only marginally attenuated. Of particular interest is the finding that plasma aldosterone levels fell in H-treated rats, consistent with the hypothesis that aldosterone secretion was influenced by stimuli related to the pathogenesis of renal injury in this model, rather than to the maintenance of sodium homeostasis.

Diuretics have long been known to enhance the antihypertensive and renoprotective effects of RAS suppressors. Accordingly, the association between thiazides and either ARBs or ACEI has been used to lower blood pressure and limit proteinuria (21, 24, 36, 40). However, the long-term effects of this association on CKD progression had not been systematically examined. Even considering that L and H act through complementary mechanisms, it is somewhat surprising that their association prevented mortality, albuminuria, hypertension, renal injury, and renal insufficiency in such a complete and sustained manner. Interstitial infiltration by macrophages and ANG II-positive cells was limited to values similar to those found 30 days after renal ablation, whereas aldosteronemia was even numerically lower than in S. To our knowledge, this is the first time that rats subjected to Nx, a very aggressive CKD model, are maintained free of complications for such an extended period.

The exact factors that led to the remarkable renoprotective effect obtained with the L+H regimen are obscure and cannot be ascertained on the basis of the present findings. However, it is noteworthy that systemic blood pressure fell precipitously 1 mo after treatment was started, remaining at levels indistinguishable from those observed in sham-operated rats until the end of the study period, 7 mo later. Normalization of blood pressure for such a long period had not been obtained previously in this model, which is characterized by severe and relatively refractory hypertension, especially if treatment is not started immediately after renal ablation, as was the case in the present study. Likewise, glomerular hypertension, a pathogenic factor directly linked to glomerular stretching and to the ensuing cascade of events, was entirely reversed by combined treatment. These observations are consistent with the notion that the remarkable renoprotection obtained with the L+H therapy results from the nearly perfect control of the hemodynamic abnormalities associated with the Nx model. It must be noted, however, that in a previous study of the Nx model (25), late enalapril monotherapy for 3 mo promoted near normalization of BP and P\textsubscript{\text{cr}} yet renal injury, although strongly attenuated, was still substantial. It remains to be determined whether better renoprotection in the Nx model would have been achieved if the ACEI treatment were maintained for a longer period, if a higher ACEI dose were employed, or if a combined ACEI/thiazide therapy were adopted.

The striking renoprotective obtained with the L+H association indicates the occurrence of a strong synergistic interaction between the respective effects of L and H. The exact nature of this cooperation is unclear. It should be noted that, despite its hemodynamic and anti-inflammatory actions, L has a relatively limited natriuretic effect, perhaps because several sodium transporter molecules are overexpressed in remnant nephrons (20). Thus the additional salt-wasting power provided by H may have strengthened the hemodynamic effect of L and allowed its anti-inflammatory action to express fully once the mechanical stretch was removed. This hemodynamic interaction may have been enhanced by a possible vascular effect of H (41). An important additional possibility is that the kaliuretic effect of H greatly alleviated the need for augmented aldosterone production, a view consistent with the low aldosteronemia observed in rats that received L+H treatment, although a similar result was obtained by L and H monotherapies. This effect would add to the anti-inflammatory and antifibrotic effects of L, thus contributing to the protection afforded by combined therapy.

Recent evidence clearly indicated that the relationship between hypertension and renal injury is much more intricate than formerly thought. This may be one of the main reasons why complete normalization of blood pressure in CKD, and particularly in the 5/6 renal ablation model, had not been achieved previously. Whereas the deleterious effects of systemic and glomerular hypertension on renal structure are well established, inflammation of the renal parenchyma, even at subtle levels, may itself be one of the factors causing hypertension. This notion is strongly supported by the finding that both hypertension and renal inflammation can be prevented in the spontaneously hypertensive rat by treatment with mycophenolate mofetil during early life (31). In the present study, the association between L and H may have promoted a “virtuous cycle”: the anti-inflammatory effect of L may have added to the direct anti-hypertensive effect of H (and to that of L itself), causing systemic and glomerular blood pressure to fall and further reducing renal inflammation.

Further experimentation is needed to establish whether similar renoprotection would be obtained if ARB therapy were combined with loop diuretics. However, recent clinical studies have shown that H may exert better antihypertensive action (2) and more effective natriuresis (5) than furosemide. The reasons why the beneficial effect of H might equal or even surpass that of furosemide despite its lower natriuretic power are unclear and may relate to the slower H kinetics compared with furosemide, as well as to a possible direct vascular effect (41).

The potential clinical applications of the present findings are evident, considering the vast experience accumulated with L and H, as well as the low toxicity of these drugs. In clinical practice, thiazides are seldom prescribed once GFR has fallen under 30 ml/min, since thiazides are believed to become ineffective, even at high doses, at advanced stages of CKD (with the possible exception of metalozone). Since solid evidence in this regard is lacking, and considering the results of the present study, it may be necessary to revise this concept. Accordingly, long-term controlled studies of combined L+H therapy in advanced CKD may be warranted. Nevertheless, caution will be needed regarding the well-known untoward effects of thiazides, especially those related to uric acid reten-
tion, absent in rats but not in humans, in which the urate-degrading enzyme, uricase, is lacking (16).

In summary, H or L monotherapies attenuated the severe structural and functional consequences of Nx but failed to prevent the long-term progression of CKD. Concomitant L and H treatment completely arrested progressive renal injury even after an unusually long period of observation, indicating a synergistic interaction. Systemic and glomerular blood pressure normalization may explain the striking renoprotection provided by L+H treatment. Further experimental and clinical investigation is needed to determine whether additional mechanisms are involved, and whether the present findings can be applied in the treatment of CKD.

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REFERENCES


