An association of losartan-hydrochlorothiazide, but not losartan-furosemide, completely arrests progressive injury in the remnant kidney

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Arias SC, Souza RA, Malheiro DM, Fanelli C, Fujihara CK, Zatz R. An association of losartan-hydrochlorothiazide, but not losartan-furosemide, completely arrests progressive injury in the remnant kidney. Am J Physiol Renal Physiol 310: F135–F143, 2016. First published November 4, 2015; doi:10.1152/ajprenal.00388.2015.—We have previously shown that an association of losartan and hydrochlorothiazide, initiated 1 mo after 5/6 nephrectomy (Nx), reversed hypertension and albuminuria and promoted lasting renoprotection. In this new study, we investigated whether equal or even better protection could be obtained by combining losartan and furosemide. Nx was performed in 58 Munich-Wistar rats. One month later, tail-cuff pressure and albuminuria were markedly elevated. At this time, Nx rats were distributed among the following four groups: untreated Nx rats, Nx rats that received losartan, Nx rats that received losartan + hydrochlorothiazide, and Nx rats that received losartan + furosemide. Seven months later, Nx rats exhibited high mortality, severe hypertension, albuminuria, glomerulosclerosis, and interstitial fibrosis. Losartan treatment limited mortality and attenuated the renal and hemodynamic abnormalities associated with Nx. As previously shown, the losartan + hydrochlorothiazide association normalized tail-cuff pressure and albumin, prevented renal injury, and reduced mortality to zero. The losartan + furosemide treatment failed to reduce tail-cuff pressure or albumin to normal and prevented renal injury less efficiently than the losartan and hydrochlorothiazide regimen. The reasons for the differing efficacies of the losartan + furosemide and losartan + hydrochlorothiazide schemes are unclear and may include beneficial natriuretic actions of thiazides, such as vasorelaxation and antiproliferative activity. These results refute the established concept that thiazides and thiazide-like diuretics are ineffective at advanced chronic kidney disease stages. Rather, they suggest that, in view of their renoprotective action, these compounds may even be preferable to loop diuretics in the management of hypertension in advanced chronic kidney disease.

5/6 nephrectomy; furosemide; thiazides; chronic kidney disease

Thiazides and thiazide-like diuretics (TTLD) are considered frontline drugs in the management of primary arterial hypertension (24, 35), especially in association with inhibitors of the renin-angiotensin system (RAS) (42). However, this prominent position is lost in the setting of advanced chronic kidney disease (CKD), in which case the conventional recommendation is that loop diuretics are used as antihypertensives instead, particularly when the glomerular filtration rate (GFR) falls below 30 ml·min⁻¹·1.73 m⁻²·yr⁻¹ (28a, 44). This concept, which is founded on scant evidence (37, 40), has been challenged in recent years by both clinical (3, 11, 14, 15, 27) and experimental (6, 17) observations.

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Combined therapy with diuretics and angiotensin-converting enzyme inhibitors or ANG II receptor blockers has been widely used for the treatment of hypertension, particularly when associated with CKD. In addition, recent clinical observations suggest that the association between angiotensin-converting enzyme inhibitors inhibitors or ANG II receptor blockers and TTLD may not only promote better control of blood pressure but also slow the progression of CKD, even at advanced stages of CKD (12, 19, 22). Evidence that loop diuretics may exert a similar effect is lacking.

We have previously shown that an association between the angiotensin receptor blocker losartan and the thiazide diuretic hydrochlorothiazide exerts a striking renoprotective effect in rats with 5/6 nephrectomy (Nx), a rather aggressive model of CKD, keeping blood pressure levels and renal injury at control levels for 8 mo after renal ablation (17). Reversal of hypertension and arrest of Nx-induced renal injury by losartan + hydrochlorothiazide were observed even when treatment was started at rather advanced stages (6). These findings constituted additional evidence that TTLDs can exert antihypertensive and renoprotective effects even when GFR falls to very low levels.

Although some salutary effects of TTLD may be independent of their action on Na⁺ transport (7, 8, 36, 43, 50), the synergism between losartan and hydrochlorothiazide in the Nx model might be solely attributed to salt depletion owing to the natriuretic properties of hydrochlorothiazide. If this hypothesis is correct, the renoprotection obtained with the losartan + hydrochlorothiazide association in the Nx model should be readily duplicated, and even intensified, when losartan is associated with more powerful natriuretic agents, such as loop diuretics.

In the present study, we investigated whether, in the Nx model, combined treatment with losartan and a loop diuretic, furosemide, would preserve renal structure with equal or better efficiency than the losartan + hydrochlorothiazide association, in consistency with the notion that TTLDs maximize the renoprotection exerted by angiotensin-converting enzyme inhibitors and ANG II receptor blockers by virtue of their natriuretic action.

METHODS

Seventy-four adult male Munich-Wistar rats, weighing between 220 and 260 g, were used in this study. All rats were obtained from a local facility at the Faculty of Medicine, University of São Paulo. All experimental procedures were specifically approved by the local Research Ethics Committee (Comissão de Ética para Análise de Projetos de Pesquisa do Hospital das Clínicas da Faculdade de Medicina da Universidade de São Paulo, under process no. 0955/06) and developed in strict conformity with our institutional guidelines and with international standards for the manipulation and care of laboratory animals. All rats were monitored daily for body weight and

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general condition. Five-sixths renal ablation (Nx) was performed in a single-step procedure after ventral laparotomy under anesthesia with ketamine (50 mg/kg) and xylazine (10 mg/kg im). The right kidney was removed, and two or three branches of the left renal artery were ligated, resulting in the infarction of two-thirds of the left kidney. Sham-operated rats underwent anesthesia and manipulation of the renal pedicles without any removal of renal mass. After surgery, all animals received enrofloxacin and, after full recovery, were given free access to tap water, fed regular rodent chow containing 0.5 Na+ and 22% protein (Nuvital Labs, Curitiba, Brazil), and kept at 23 ± 1°C and 60 ± 5% relative air humidity under an artificial 12:12-h light-dark cycle.

Thirty days after renal ablation, the basic pretreatment conditions were assessed. For this purpose, tail-cuff pressure (TCP) was determined with an optoelectronic automated device (Visitech Systems, Apex, NC) after rats had been preconditioned to the procedure (6). Urinary albumin excretion (UalbV) was assessed by radial immuno-diffusion using conventional techniques. Rats that failed to increase TCP above 170 mmHg or UalbV above 40 mg/day were excluded from the study. The remaining 58 Nx rats were divided into the following 4 experimental groups: untreated Nx rats (Nx group; n = 11), Nx rats that received losartan (50 mg/kg) plus hydrochlorothiazide (6 mg/kg) diluted in the drinking water (NxL group; n = 16), Nx rats that received losartan (50 mg/kg) plus furosemide (6 mg/kg) diluted in the drinking water (NxLF group; n = 17), and Nx rats that received losartan (50 mg/kg) plus furosemide (20 mg/kg) (NxLFH group; n = 14). Nx rats were distributed in such a way that the initial body weight, TCP, and UalbV were similar among experimental groups. All treatments were maintained for 210 days with monthly determination of TCP and UalbV. A group of sham-operated rats (sham group) that received no treatment (n = 16) was followed concomitantly. At the end of the study, rats were anesthetized, blood was collected from the abdominal aorta, and renal tissue was prepared for histomorphometric and immunohistochemical analyses as previously described (6).

Histomorphometric analysis. Morphometric evaluations were always performed in a blinded manner by a single observer. The extent of glomerular injury was estimated by determining the percentage of glomeruli with sclerotic lesions in sections stained by the periodic acid-Schiff reaction. This reaction was also used to evaluate glomerular volume (Vg). The percentage of renal cortical area occupied by interstitial tissue, used as a measure of the degree of interstitial expansion (in %), was estimated in 25 consecutive microscopic fields of Masson-stained sections by a point-counting technique (25) at a final magnification of ×100 under a 144-point grid.

Immunohistochemical analysis. Immunohistochemistry was performed on 4-μm-thick sections mounted on glass slides precoated with 2% silane. Sections were deparaffinized and rehydrated by conventional techniques, heated in citrate buffer for antigen retrieval, and then incubated overnight with the primary antibody at 4°C. For negative control experiments, incubation with the primary antibody and then incubation with an appropriate secondary antibody and then with an alkaline phosphatase anti-alkaline phosphatase complex (Dako). Sections were developed with a fast red dye solution (Sigma-Aldrich). For ANG II and α-SMA detection, a streptavidin-biotin complex for alkaline phosphatase (Dako) was used. Nonspecific binding was prevented with normal horse serum diluted at 1:20 in nonfat milk at 2% in TBS. Primary antibodies were diluted at 1:800 (for α-SMA) and 1:400 (for ANG II). Sections were developed in the same manner as for ED-1 detection. For the visualization of PCNA and collagen-positive stain, sections were pretreated with 30% H2O2 in methanol and preincubated with normal horse serum as described above. Primary antibodies were diluted at 1:100 and 1:200, respectively, in nonfat milk at 2% in TBS. The EnVision Labelled Polymer for peroxidase (Dako) was used before development with diaminobenzidine substrate (Dako).

Double immunostaining was used to visualize the proliferation activity of different segments of the nephron. The identification of proximal cells was performed by detection of AQP1, identification of the thick ascending loop of Henle (TAL) was obtained by detection of the Na+–K+–Cl- cotransporter, and the identification of the distal convoluted tubule (DCT) was performed by detection of the Na+–Cl- cotransporter. For these experiments, sections were pretreated with 30% H2O2 in methanol and preincubated with avidin and biotin blocking solutions (Vector, Burlingame, CA). Nonspecific staining was prevented with normal goat serum diluted at 5% in BSA at 1% in TBS. Sections were incubated overnight with the specific primary antibody diluted at 1% in BSA at 1% in TBS. Appropriate biotinylated secondary antibody was applied, and streptavidin-AP solution (Dako) was used followed by development with fast red dye solution (Sigma-Aldrich). Sections were then incubated once again with avidin and biotin blocking solutions followed by the prevention of nonspecific staining with a mixture of normal horse and rabbit serum diluted at 2% and 5%, respectively, in 2% nonfat milk in TBS. Sections were then incubated overnight with the primary antibody against PCNA at 0.01% in a solution containing 1% BSA and 2% nonfat milk diluted in TBS. The appropriate biotinylated secondary antibodies were applied, and the LSAB-HRP kit (Dako) was used for PCNA detection. Sections were developed with diaminobenzidine substrate (Dako). All sections were counterstained with Mayer’s hematoxylin, dehydrated, and covered with Permount Mounting Media (Thermo Fisher Scientific).

The renal density of macrophages, proliferating cells, and double stains were evaluated in a blinded manner at ×200 magnification. For each section, 50 microscopic fields (corresponding to a total area of 1.6 mm²) were examined. The percentage of renal cortical interstitial area occupied by α-SMA was estimated by the same point-counting technique used to evaluate the degree of interstitial expansion, excluding positively stained blood vessels.

Statistical analysis. Differences among groups were assessed using one-way ANOVA with pairwise posttest comparisons by the Newman-Keuls method (46). P values of <0.05 were considered significant. Results are presented as means ± SE. Calculations were performed using Prism 4.0 (GraphPad Software).

RESULTS

A Kaplan-Meier plot representing survival rates over the duration of the study is shown in Fig. 1. Eight months after renal ablation, no deaths occurred in the sham group. The mortality rate was 62.5% in the Nx group (P < 0.05 vs. the sham group). Losartan treatment reduced the mortality rate to 6.3% (P < 0.05 vs. the Nx group). No deaths occurred in the NxLH group (P > 0.05 vs. the sham and NxL groups and P < 0.05 vs. the NxL group). Mortality was 17.6% in the NxLF group (P < 0.05 vs. the Nx group and P > 0.05 vs. the NxLFH group).

The time course of hypertension and albuminuria is shown in Fig. 2. TCP was markedly elevated in the Nx group along the whole study (Fig. 2A). All treatments promoted an initial
and Nx rats treated with losartan and furosemide (NxLF group). a

body weight, and VG determined 240 days after renal ablation

increased/11011 hydrochlorothiazide treatment, whereas the losartan mono-

normalization of albuminuria was achieved with losartan

time, indicating the development of incipient aging nephropa-

of TCP. However, final TCP levels in rats that received

losartan monotherapy were as high as in the Nx group. In

contrast, rats treated with the losartan + hydrochlorothiazide

association remained normotensive until the end of the study.

In the NxLF group, TCP reached an intermediary value. A similar picture was observed for albuminuria (Fig. 2B): the

sham group exhibited a sixfold increase in albuminuria over tim

compared with values observed before renal ablation. A steady

normalization of albuminuria was achieved with losartan + hydrochlorothiazide treatment, whereas the losartan mono-

therapy and losartan + furosemide regimen provided only temporary limitation of albumin excretion.

Fluid intake was always similar among treated Nx rats and increased −10% from the beginning of treatment to the end of the study. Body weight, serum creatinine, K+/H11011, kidney weight/body weight, and VG determined 240 days after renal ablation are shown in Table 1. Growth stunting was evident in all Nx groups compared with the sham group. No differences in plasma K+ were seen among Nx groups. The kidney weight-
to-body weight ratio in the Nx group was even higher than in the sham group despite the 5/6 renal mass reduction, indicating the presence of marked renal hypertrophy. Losartan treatment had no significant effect on renal hypertrophy, whereas kidney weight/body weight was diminished in the NxLF group (P < 0.05 vs. the Nx group) but not in the NxLH group (P < 0.05 vs. the sham, NxL, and NxLF groups). VG was markedly increased in the Nx group (P < 0.05 vs. the sham group). Losartan and losartan + hydrochlorothiazide treatments had no significant effect on glomerular enlargement (P > 0.05 vs. the sham group and P > 0.05 vs. the Nx group). In the NxLF group, VG was numerically larger than in the Nx group (P < 0.05 vs. the sham group and P > 0.05 vs. the Nx group) and significantly higher than in the NxLH group.

Figure 3 shows the main glomerular and interstitial changes observed 240 days after renal ablation. Most glomeruli exhibited sclerotic lesions in the Nx group (Fig. 3A). Losartan treatment significantly diminished glomerular scle-

rosis to half the value seen in the Nx group. The glomerular sclerosis attenuation was much stronger with combined losartan + hydrochlorothiazide treatment (P < 0.05 vs. the sham, Nx, and NxL groups). Treatment with losartan + furosemide also diminished the percentage of glomerular sclerosis, albeit to a lesser extent than with losartan + hydrochlorothiazide treatment (P < 0.05 vs. the sham, Nx, NxL, and NxLH groups). The findings for the glomerular collagen type I cortical area approximately paralleled those for glomerular sclerosis, being maximal in the Nx group (P < 0.05 vs. the sham group) and unchanged by losartan treatment (P > 0.05 vs. the sham and Nx groups). The degree of interstitial expansion and interstitial collagen type I accumulation followed approximately those obtained for glomerular sclerosis. The degree of interstitial expansion (in %) was markedly increased in the Nx group (P < 0.05 vs. the sham group) and was limited by losartan treatment (P < 0.05 vs. the sham and Nx groups). Further attenuation was obtained with either the losartan + hydrochlorothiazide and losartan + furosemide treatment (P < 0.05 vs. the sham, Nx, and NxL groups). Similar results were obtained for interstitial collagen type I accumulation. However, in this case, the
losartan + furosemide scheme failed to duplicate the strong amelioration obtained with the losartan + hydrochlorothiazide treatment.

Classical inflammation markers are shown in Fig. 4, A–C. The number of macrophages at the interstitial area was markedly elevated in the Nx group (P < 0.05 vs. the sham group). Losartan treatment attenuated macrophage infiltration (P < 0.05 vs. the sham and Nx groups). Losartan + hydrochlorothiazide treatment brought the macrophage number to much lower levels, which nevertheless were still abnormally high (P < 0.05 vs. the sham, Nx, and NxL groups). Losartan treatment had no significant effect on the number or distribution of PCNA-positive cells (63 ± 1 cells/mm²), whereas a smaller proportion was associated with the tubular compartment (11 ± 2 cells/mm²). In untreated Nx rats, the number of PCNA-positive cells was markedly increased in both the interstitial (53 ± 8 cells/mm², P < 0.05 vs. the sham group) and tubular (53 ± 8 cells/mm², P < 0.05 vs. the sham group) compartments. Losartan treatment had no significant effect on the number or distribution of PCNA-positive cells (63 ± 11 cells/mm² in the interstitial compartment and 53 ± 9 cells/mm² in the tubular compartment, P < 0.05 vs. the sham group and P > 0.05 vs. the Nx group). In contrast, the profile observed in the NxL group was very similar to that seen in the sham group (18 ± 2 cells/mm² and 15 ± 2 cells/mm², respectively, P < 0.05 vs. the Nx and NxL groups). The losartan + furosemide

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**Table 1.** Body weight, serum creatinine, K⁺, kidney weight/body weight, and glomerular volume determined 240 days after renal ablation

<table>
<thead>
<tr>
<th>Number of Rats/Group</th>
<th>Body Weight, g</th>
<th>Serum Creatinine, mg/dl</th>
<th>K⁺, mmol/l</th>
<th>Kidney Weight/Body Weight</th>
<th>Glomerular Volume, ×10⁴ μm²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham group</td>
<td>16</td>
<td>413 ± 5</td>
<td>0.2 ± 0.1</td>
<td>4.5 ± 0.2</td>
<td>0.49 ± 0.01</td>
</tr>
<tr>
<td>Nx group</td>
<td>11</td>
<td>300 ± 14ᵇ</td>
<td>2.2 ± 0.2ᵃ</td>
<td>5.6 ± 0.2ᵃ</td>
<td>0.53 ± 0.01ᵃ</td>
</tr>
<tr>
<td>NxL group</td>
<td>16</td>
<td>341 ± 9ᵇ</td>
<td>1.2 ± 0.1ᵇ</td>
<td>5.3 ± 0.2ᵃ</td>
<td>0.49 ± 0.02ᵇ</td>
</tr>
<tr>
<td>NxLH group</td>
<td>17</td>
<td>319 ± 5ᵃ</td>
<td>1.0 ± 0.1ᵇ</td>
<td>5.2 ± 0.1ᵃ</td>
<td>0.45 ± 0.01ᵇ</td>
</tr>
<tr>
<td>NxLF group</td>
<td>14</td>
<td>334 ± 7ᵇ</td>
<td>1.1 ± 0.1ᵇ</td>
<td>5.4 ± 0.2ᵃ</td>
<td>0.56 ± 0.01ᵇ,c,d</td>
</tr>
</tbody>
</table>

Results expressed as means ± SE. The following groups of rats were used: sham-operated (sham) rats (S group), untreated 5/6 nephrectomized (Nx) rats (Nx group), Nx rats treated with losartan (NxL group), Nx rats treated with losartan and hydrochlorothiazide (NxLH group), and Nx rats treated with losartan and furosemide (NxLF group). *P < 0.05 vs. the sham group; †P < 0.05 vs. the Nx group; ‡P < 0.05 vs. the NxL group; ‡‡P < 0.05 vs. the NxLH group.

![Image](http://ajprenal.physiology.org/)

Fig. 3. Glomerulosclerosis (A), glomerular area occupied by collagen type I (B), cortical interstitial area (C), and cortical interstitial area occupied by collagen type I (D). Results are expressed as means ± SE. *P < 0.05 vs. the sham group; †P < 0.05 vs. the Nx group; ‡P < 0.05 vs. the NxL group; ‡‡P < 0.05 vs. the NxLH group.
regimen promoted a numerical decrease in the number of interstitial and tubular PCNA-positive cells, but none of these differences reached statistical significance (46 ± 6 and 39 ± 5 cells/mm², respectively, \( P < 0.05 \) vs. the sham and NxLH groups and \( P > 0.05 \) vs. the Nx and NxL groups). Only few glomerular cells stained positively for PCNA.

The proportions of PCNA-positive cells located in the proximal tubule (PT), TAL, and DCT, as assessed by double staining with specific antigens, are shown in Fig. 6, A–C. In untreated Nx rats, 29 ± 3% of the PT profiles were positive for PCNA (\( P < 0.05 \) vs. the sham group). Losartan treatment diminished, but did not normalize, this proportion (20 ± 2%, \( P < 0.05 \) vs. the sham group), in contrast with the losartan + hydrochlorothiazide association, which significantly reduced PT cell proliferation (11 ± 2%, \( P < 0.05 \) vs. the sham, Nx, and NxL groups). The pattern observed in the NxLH group was similar to that seen in the NxL group (19 ± 4%, \( P < 0.05 \) vs. the sham and NxLH groups and \( P > 0.05 \) vs. the NxL groups).

The fraction of PCNA-positive TAL cells was sharply increased in Nx rats (19 ± 3%, vs. 5 ± 1% in sham rats, \( P < 0.05 \)). Despite numerical decreases, none of the treatments reduced proliferation in this segment.

The proportion of cells staining positively for PCNA in the DCT was significantly increased in the Nx group (4 ± 1% vs. 1 ± 1% in the sham group, \( P < 0.05 \)). A similar fraction was observed in the losartan-treated group (3 ± 1%, \( P < 0.05 \) vs. the sham group and \( P > 0.05 \) vs. the Nx group). The losartan + hydrochlorothiazide regimen lowered this proportion to levels similar to those found in the sham group (1 ± 1%, \( P > 0.05 \) vs. the sham group and \( P < 0.05 \) vs. the Nx and NxL groups). The losartan + furosemide scheme failed to provide any significant reduction in TAL proliferation (4 ± 1%, \( P < 0.05 \) vs. the sham and NxLH groups and \( P > 0.05 \) vs. the Nx and NxL groups).

**DISCUSSION**

Although the Nx model was described nearly 40 yr ago, only a few studies (6, 9, 17) have extended the period of observation beyond 4 mo, making it possible to estimate in more detail the natural course of the disease, its evolution to renal insufficiency, its mortality, and the long-term effect of therapeutic strategies. In the present study, rats were followed for 8 mo after renal ablation. At this time, marked renal and glomerular hypertrophy, glomerulosclerosis, and tubulointerstitial injury were noted in untreated rats, along with intense glomerular and interstitial inflammation and fibrosis, marked interstitial cell proliferation, and severe renal functional decline. It should be noted that, since mortality was high in this group, these observations were possible only in survivors, indicating that
the actual amount of renal damage in untreated Nx rats was most certainly underestimated.

In the present study, as in previous studies (6, 17), therapies were started when renal injury was already established and in progression rather than immediately after renal ablation. Monotherapy with losartan attenuated the progression of albuminuria, temporarily reversed hypertension, and limited renal inflammation, glomerulosclerosis, and interstitial expansion, which nevertheless progressed to advanced renal disease. This incomplete protection mimicked the well-known limitations of RAS inhibitors in the clinical management of CKD.

Combined losartan + hydrochlorothiazide therapy exerted a much more complete protective effect than losartan monotherapy, promoting sustained reversal of hypertension and albuminuria and preventing or strongly attenuating the development of inflammation, renal injury, and glomerulosclerosis. These results confirm previous findings of this laboratory (6, 17) and elsewhere (49) and are in line with several clinical observations of a renoprotective effect of the association of TTLD and RAS inhibitors (1, 3, 19).

The protective effect afforded by the losartan + hydrochlorothiazide association was partially lost when hydrochlorothiazide was replaced by furosemide: although hypertension was strongly attenuated, blood pressure remained elevated compared with control, whereas albuminuria resumed its progression and caused a direct relaxant effect on vascular smooth muscle cells (7, 8, 36, 50). Diazoxide, a thiazide-like compound devoid of natriuretic properties, has long been known as a vasodilator and was, for some time, used as an antihypertensive drug (39). In line with these observations, the renoprotection conferred by the losartan + hydrochlorothiazide association appears to be independent of blood pressure reduction, as...
recently indicated by clinical (19, 28) and experimental (6) observations. These findings suggest that some intrarenal effect of the losartan + hydrochlorothiazide regimen is key to its renoprotective effect. Indeed, the glomerular intracapillary hydraulic pressure (P Giuliani) was shown to be normalized by the losartan + hydrochlorothiazide association 1 mo after renal ablation, an effect not observed with losartan monotherapy (17).

Increased cell proliferation is a hallmark of CKD, assuming particularly intense levels in the Nx model, in which a surge of tubular and interstitial cell division can already be observed a few days after renal ablation (16, 18). In the present study, cell proliferative activity in the tubular compartment was markedly elevated at the end of the study. Hyperplasia was observed at three different nephron segments, namely, the PT, TAL, and DCT, indicating that most, if not all, nephron segments were affected. PCNA overexpression was also observed at inflamed interstitial areas, corroborating previous observations that inflammatory cells proliferate locally in this model (18, 38). Despite its partial protective action, losartan monotherapy had little effect on the extent and intrarenal distribution of cell proliferative activity. In contrast, the losartan + hydrochlorothiazide association largely prevented the marked tubular and interstitial cell proliferation observed in Nx rats, keeping PCNA expression close to control at the end of the study, particularly at the DCT. These observations are in line with previous findings of an in vitro antiproliferative action of indapamide (43) and in vascular smooth muscle cells (20), although these effects have not been specifically reported for hydrochlorothiazide. Additional beneficial effects may have derived from other nonnatriuretic effects of TTLD, such as inhibition of platelet aggregation and vascular permeability (47) and augmentation of circulating klotho (28).

The better protection conferred by the losartan + hydrochlorothiazide association may also partly reflect the differing effects of losartan + hydrochlorothiazide and losartan + furosemide on V_G, whereas losartan + hydrochlorothiazide slightly diminished V_G compared with the Nx groups, losartan + furosemide promoted an equally modest glomerular enlargement, in such a way that a significant difference established between the two groups. These results are in line with the previous finding that furosemide treatment increases V_G in normal rats (32). Glomerular hypertrophy has been pointed out as one of the initiating factors leading to the development of glomerulosclerosis (23, 29), along with an elevation of P Giuliani (5, 17, 18, 31), presumably because these two abnormalities synergistically increase mechanical stretching of the glomerular wall (13), thus leading to glomerular cell proliferation and to the production of inflammatory mediators (21). The action of furosemide on P Giuliani in the Nx model has not been examined, but it is noteworthy that P Giuliani has been previously shown to be elevated in normal rats treated with furosemide for 6–8 wk (33), raising the possibility that persistence of both tuft enlargement and intracapillary hypertension limited any renoprotective effect that the natriuretic action of furosemide might have provided. The mechanisms underlying these effects are unclear. Conceivably, interruption of the tubuloglomerular feedback signal, a well-known effect of loop diuretics not shared by TTLD (34, 41, 48), may have played a role, although this hypothesis can only be speculative in the absence of specific measurements.

Taken together, these observations suggest that the strong renoprotective effect imparted by the addition of hydrochlorothiazide to losartan, not entirely reproduced by the losartan + furosemide association, may result from a combination of hemodynamic and cellular effects that superimpose on the natriuretic actions of hydrochlorothiazide. Since furosemide apparently lacks these additional properties, the beneficial effects of its association with losartan may be restricted to those stemming from its natriuretic properties.

The present experimental observations have two important clinical implications. First, the established concept that TTLDs are ineffective at advanced CKD stages is once again challenged, in keeping with previous clinical and experimental observations (1, 6, 17, 19, 49). Second, contrary to current recommendations, TTLD may be preferable to loop diuretics as an add on to RAS inhibitors, providing more effective antihypertensive action and renoprotection in advanced CKD by virtue of its as yet ill-defined nonnatriuretic properties.

In summary, in partial agreement with our working hypothesis, the association of furosemide and losartan did attenuate the progression of CKD in the 5/6 renal ablation model, but not as effectively as with the losartan + hydrochlorothiazide combined treatment. Hydrochlorothiazide may exert renoprotective actions that are independent from, and/or additive to, its natriuretic effect. In the conservative therapy of advanced CKD, TTLD may be preferable to loop diuretics as an add on to RAS inhibitors, not only for their antihypertensive action but also for their synergistic interaction with those agents to promote renoprotection. Large randomized control studies are needed to confirm the available evidence and establish the basis for a possible change in practice.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

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