Regression of glomerulosclerosis in subtotally nephrectomized rats: effects of monotherapy with losartan, spironolactone, and their combination

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Piecha G, Koleganova N, Gross M-L, Geldyyev A, Adamczak M, Ritz E. Regression of glomerulosclerosis in subtotally nephrectomized rats: effects of monotherapy with losartan, spironolactone, and their combination. Am J Physiol Renal Physiol 295:F137–F144, 2008. First published April 23, 2008; doi:10.1152/ajprenal.00065.2008.—Angiotensin II accelerates and renin-angiotensin system blockade halts progression; blockade with high doses even reverses established glomerulosclerosis. Aldosterone also accelerates progression of glomerulosclerosis, partially independently of angiotensin II. The purpose of this study was to assess the relative ability of an angiotensin receptor type 1 (AT1) blocker, a mineralocorticoid receptor blocker, and their combination to reverse glomerulosclerosis. Sprague-Dawley rats were subjected to subtotal renal ablation (SNX) or sham operation. Eight weeks after surgery, they were either euthanized or allocated to treatment with vehicle, losartan, spironolactone, their combination, or unspecified antihypertensive treatment (dihydralazine) for 4 wk. Renal morphology was evaluated by stereology in tissues obtained using pressure-controlled perfusion fixation. Systolic blood pressure was significantly higher in SNX compared with sham-operated animals and decreased in all treatment groups. Compared with wk 8 after SNX, the glomerulosclerosis index (GSI) had increased further by wk 12 in the vehicle- and dihydralazine-treated groups but was significantly lowered in the SNX+losartan as well as in the SNX+losartan+spironolactone groups and had not progressed further in the SNX+spironolactone group. The study confirms the partial regression of established glomerulosclerosis in subtotally nephrectomized rats after high-dose AT1 receptor blockade. Nonhyperkalemic doses of spironolactone prevented the increase but failed to decrease the GSI below the 8-wk level and preserved podocyte numbers. Combining the AT1 blocker with mineralocorticoid receptor blockade failed to further increase the regression of glomerulosclerosis.

aldosterone escape; renin-angiotensin system

GLOMERULOSCLEROSIS AND INTERSTITIAL fibrosis tend to progress over time even if the primary insult to the kidney is no longer operative (11). Blocking the renin-angiotensin system (RAS) with angiotensin converting enzyme (ACE) inhibitors or angiotensin type 1 receptor (AT1) blockers slows progression in various experimental models and in patients with chronic kidney disease (11). In humans the possibility to reverse established glomerular lesions was clearly demonstrated in diabetic nephropathy after isolated pancreatic transplantation (7). In the renal ablation model in the rat, reversal of glomerulosclerosis was seen after administration of high-dose ACE inhibitors or AT1 blockers (2, 8, 16). In this model, aldosterone accelerated progression of glomerulosclerosis independently of ANG II (10, 13). This finding is clinically relevant because frequently, during prolonged treatment with ACE-inhibitors or AT1 blockers, “aldosterone breakthrough” occurs, i.e., a delayed increase in aldosterone concentration after an initial fall following pharmacological blockade of the RAS (24).

It was the purpose of this study to compare the ability of the angiotensin receptor type 1 blocker losartan, the mineralocorticoid receptor blocker spironolactone, and their combination to reverse established glomerulosclerosis.

MATERIALS AND METHODS

Animals. All animal procedures were approved by the local ethics committee for animal experiments. Male Sprague-Dawley rats weighing 344 ± 81 g were obtained from Charles River (Sulzfeld, Germany) at the age of 8 wk. The animals were housed at a constant room temperature (21 ± 1°C) and humidity (75 ± 5%) and were exposed to a 12:12-h light-dark cycle. The animals received a standard rodent diet ad libitum (ssniff RM-H; Ssniff, Germany) containing 19% protein, 41% carbohydrates, 3.3% fat, 0.24% sodium, and 0.92% potassium. After a 1-wk adaptation period, the animals were randomly allotted to undergo a two-step subtotal nephrectomy (SNX) or sham operation (sham-op), respectively, as described before (1). After the operation, all the animals were followed for 8 wk without additional intervention. After that period, one group of animals was killed and the remaining animals were randomized to receive no treatment or treatment with losartan (250 mg·kg body wt⁻¹·day⁻¹ in the drinking water, MSD Chiropharm, Haar, Germany); spironolactone (15 mg·kg body wt⁻¹·day⁻¹ in the drinking water, Aldactone, Hoffmann-LaRoche, Grenzlach-Wyhlen, Germany); a combination of losartan plus spironolactone (in the same doses); or the nonspecific antihypertensive dihydralazine (20 mg·kg body wt⁻¹·day⁻¹ in the drinking water, Nepresol, Teofarma, Pavia, Italy) for the subsequent 4 wk. The dose of spironolactone was selected after a pilot study in which 80% of animals had died on 80 mg/kg spironolactone (and 30% on 30 mg/kg) in combination with high-dose losartan. Daily water consumption was monitored, and the concentrations of losartan, spironolactone, and dihydralazine were adjusted to maintain a stable daily dose. The following groups were studied:

Sham-op killed at week 8 post-op (n = 15)
Sham-op killed at week 12 post-op (n = 16)
Untreated SNX killed at week 8 post-op (n = 16)
Untreated SNX killed at week 12 post-op (n = 15)
SNX treated with losartan from week 8 to week 12, killed at week 12 post-op (n = 17)
SNX treated with spironolactone from week 8 to week 12, killed at week 12 post-op (n = 19)

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SNX treated with losartan plus spironolactone from week 8 to week 12, killed at week 12 post-op (n = 19)

SNX treated with dihydralazine from week 8 to week 12, killed at week 12 post-op (n = 14)

Body weight and systolic blood pressure (by tail plethysmography) were measured at regular intervals. At weeks 4, 8, and 12, animals were kept in metabolic cages for 24-h urine collection.

Albumin excretion. Urinary albumin excretion was measured using ELISA techniques and rat-specific anti-albumin antibodies as described previously (2).

Perfusion fixation and tissue sampling. The experiment was terminated at 8 or 12 wk after the surgery under general anesthesia (100 mg/kg ketamine hydrochloride, Ketamin, 10%; Essex, Tierarznei, Germany) and 3.0 mg/kg xylazine (Xylazin, 2%; Ceva, Tiergesundheit, Germany). The abdominal aorta was catheterized, blood samples were taken, and retrograde perfusion fixation was performed using 3% glutaraldehyde solution at body temperature for morphological investigations or ice-cold NaCl for immunohistochemistry and Western blotting, respectively (21). The perfusion pressure was kept constant at 120 mmHg. The kidneys were weighed and dissected in a plane perpendicular to the interpolar axis, yielding slices of 1-μm width. Tissue samples were embedded in paraffin; 3-μm sections were prepared and stained with periodic acid-Schiff. Five small pieces of one kidney were selected by area-weighted sampling and embedded in epon-araldite from which semithin sections (0.5 μm) were prepared and stained with methylene blue and basic fuchsins. For immunohistochemical investigations, tissues were fixed in 4% formalin and stained with hematoxylin. Negative controls were performed by omitting the primary antibody. All antibodies were tested to cross-react with rat samples.

The sections were examined under light microscopy at ×400 magnification using a semiquantitative scoring system (0-4): 0 = no expression, 1 = mild expression, 2 = moderate expression, 3 = strong expression, and 4 = very strong expression. The number of podocytes per glomerulus staining positively for desmin was determined.

Western blotting. Samples of kidney cortex were homogenized, and the protein concentration was determined according to Bradford method (Bio-Rad). One hundred microliters of protein was electrophoresed on SDS-polyacrylamide gels. Subsequently, proteins were electrophoresed onto polyvinylidene difluoride membranes (Immobilon-P, pores 0.45 μm, Millipore). The membranes were blocked with 5% nonfat dry milk in Tris-buffered saline with 0.5% Tween 20 (TBS-T) and incubated for 2 h at room temperature with the primary antibody against α-smooth muscle actin (Sigma), TGF-β1 (Santa Cruz Biotechnology), a horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology), and VEGF receptor 2 (Abcam). Exposed films were scanned with an imager (Herolab), and

Table 1. Animal data

<table>
<thead>
<tr>
<th>Group</th>
<th>Baseline Body Weight, g</th>
<th>Final Body Weight, g</th>
<th>Left Kidney Weight (Intact or Remnant), g</th>
<th>Serum Creatinine, mg/dl</th>
<th>Serum Urea, mg/dl</th>
<th>Serum Potassium, mmol/l</th>
<th>Serum Aldosterone, ng/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham-op 8 wk</td>
<td>343±79</td>
<td>510±94</td>
<td>2.17±0.40</td>
<td>0.46±0.02</td>
<td>43.4±4.5</td>
<td>6.6±0.5</td>
<td>196±4.2</td>
</tr>
<tr>
<td>Sham-op 12 wk</td>
<td>355±79</td>
<td>536±65</td>
<td>2.16±0.55</td>
<td>0.46±0.06</td>
<td>53.4±7.0</td>
<td>7.1±0.5</td>
<td>234±4.4</td>
</tr>
<tr>
<td>SNX 8 wk</td>
<td>335±85</td>
<td>447±57[b]</td>
<td>2.72±0.53[b]</td>
<td>0.85±0.09[b]</td>
<td>93.5±13.6[b]</td>
<td>6.2±0.5[b]</td>
<td>172±46</td>
</tr>
<tr>
<td>SNX 12 wk</td>
<td>319±87</td>
<td>472±50[b]</td>
<td>2.59±0.63[b]</td>
<td>0.83±0.11[b]</td>
<td>106.4±19.8[b]</td>
<td>6.9±0.5[b]</td>
<td>366±118[b,c]</td>
</tr>
<tr>
<td>SNX+losartan 8 wk</td>
<td>331±85</td>
<td>449±80[b]</td>
<td>2.32±0.48</td>
<td>0.85±0.18[b]</td>
<td>118.4±24.4[b]</td>
<td>7.5±0.7[b]</td>
<td>257±130[d]</td>
</tr>
<tr>
<td>SNX+spironolactone 8 wk</td>
<td>324±91</td>
<td>467±69[b]</td>
<td>3.17±0.68[b,d,e]</td>
<td>0.80±0.08[b]</td>
<td>106.0±12.3[b]</td>
<td>7.2±0.6[b]</td>
<td>343±153[d,b,c]</td>
</tr>
<tr>
<td>SNX+losartan+spironolactone 8 wk</td>
<td>345±78</td>
<td>470±88[b]</td>
<td>2.50±0.62[b]</td>
<td>0.85±0.10[b]</td>
<td>119.7±22.7[b]</td>
<td>6.9±0.9[b]</td>
<td>180±47[d,b,c]</td>
</tr>
<tr>
<td>SNX+dihydralazine 12 wk</td>
<td>310±55</td>
<td>449±31[b]</td>
<td>3.10±0.49[a,d,e,g]</td>
<td>0.85±0.17[b]</td>
<td>91.2±23.2[b]</td>
<td>7.2±0.6[b]</td>
<td>301±102[a-c,g]</td>
</tr>
</tbody>
</table>

ANOVA NS

Values are means ± SD. Sham-op, sham operation; SNX, subtotal nephrectomy; NS, not significant. Significant differences vs. *Sham-op week 8; *Sham-op week 12; **untreated SNX week 8; ***untreated SNX week 12; **SNX+losartan; **SNX+spironolactone; **SNX+losartan+spironolactone.
the data were analyzed using computer software (EASY Win 32, Herolab).

Statistical analysis. Data are given as means ± SD. For Western blotting, the vehicle-treated sham-op group served as the reference, and the mean value of individual measurements was set as 100%. The value for each animal was expressed as manifold of reference. After the testing for normal distribution, the Kruskal-Wallis test or one-way ANOVA was chosen, followed by Duncan’s multiple-range test, for differences among groups. The results were considered significant when the probability of error ($P$) was <0.05.

RESULTS

Animal data. As shown in Table 1, body weight did not differ between the SNX groups at baseline and at the end of the study. The weight of the left kidney remnant was significantly higher in untreated SNX, SNX + losartan, and SNX + losartan + spironolactone rats compared with the intact left kidney weight in sham-op animals. Plasma urea and creatinine concentrations were significantly higher in all SNX groups compared with sham-op, and no difference was noted between the treatments.

The serum potassium concentration of SNX animals was not altered by any of the treatments (Table 1). Serum aldosterone concentration was significantly higher in untreated SNX rats at week 12 compared with sham-op and untreated SNX rats at week 8 (Table 1). At week 12, it was significantly lower in SNX + losartan and SNX + losartan + spironolactone compared with untreated SNX rats.

Systolic blood pressure. Systolic blood pressure was significantly higher in all SNX groups compared with sham-op from weeks 4 to 8. Delayed treatment with losartan, spironolactone, losartan + spironolactone, or dihydralazine significantly decreased blood pressure in SNX animals (Fig. 1). No significant differences in blood pressure were noted between the treatments.

Urinary albumin excretion rate. The urinary albumin excretion rate was significantly higher in SNX animals compared with sham-op at week 8 and increased further in untreated SNX, SNX rats treated with spironolactone, and SNX rats treated with dihydralazine at week 12 (Fig. 2). In SNX animals treated with losartan alone or in combination with spironolactone, albumin excretion decreased between weeks 8 and 12 and was not significantly different from sham-op rats at week 12.

GSI, TII, and VI. GSI, TII, and VI were significantly higher in all SNX groups compared with sham-op rats. GSI was significantly higher in untreated SNX and SNX + dihydralazine rats at week 12 compared with SNX rats at week 8 (Figs. 3 and 4). Treatment with losartan alone or in combination with spironolactone resulted in significantly lower GSI compared with SNX rats at week 8. No difference in GSI was observed between SNX + spironolactone rats at week 12 and SNX rats at week 8 (Figs. 3 and 4).

TII was significantly higher in untreated SNX rats at week 12 compared with SNX rats at week 8 (Fig. 3). At week 12, TII was significantly lower in SNX rats treated with losartan, spironolactone, and losartan + spironolactone, respectively, compared with untreated SNX rats and was not significantly different from SNX rats at week 8. At week 12, TII of SNX rats treated with dihydralazine was significantly lower than that of untreated SNX, but still significantly higher than that of SNX rats at week 8.
VI was significantly higher in untreated SNX rats at week 12 compared with SNX rats at week 8 and was significantly lower in SNX rats treated with losartan, spironolactone, losartan+spironolactone, and dihydralazine, respectively, compared with untreated SNX rats at week 12 (Fig. 3).

Number and volume of glomeruli and analysis of glomerular cells, and capillaries. The total number of glomeruli per kidney was reduced from an average of 31,401 ± 5,899 to 11,828 ± 2,443 after SNX rats with little variance between the SNX groups, documenting that reduction of nephron number had been precise with little interindividual variation (Fig. 5).

The mean glomerular volume was significantly higher in all SNX groups compared with sham-op animals. It was also significantly higher in untreated SNX rats at week 12 compared with SNX rats at week 8 (Fig. 5). The glomerular volume was significantly lower in SNX animals treated with losartan alone or in combination with spironolactone compared with untreated SNX rats at week 12 and was even lower than in SNX rats at week 8. In SNX+spironolactone rats, it was significantly lower than in untreated SNX rats at week 12 but not different from SNX rats at week 8. In SNX rats treated with dihydralazine, glomerular volume was significantly higher than in SNX rats at week 8 and not different from untreated SNX rats at week 12.

The mean number of podocytes per glomerulus (Table 2) was significantly lower in SNX rats at week 8 and was increased further in untreated animals at week 12. The podocyte volume in SNX rats treated with losartan alone or in combination with spironolactone was not different from that observed in SNX rats at week 8 and was significantly lower than in untreated SNX rats at week 12. In contrast, the number of podocytes in SNX rats treated with dihydralazine was lower than in SNX at week 8 and did not differ from untreated SNX rats at week 12.

The mean podocyte volume was significantly higher in SNX rats at week 8 and was increased further in untreated animals at week 12. The podocyte volume in SNX rats treated with losartan alone or in combination with spironolactone was not different from that observed in SNX rats at week 8 and was significantly lower than in untreated SNX rats at week 12. In SNX rats treated with spironolactone alone, at week 12 podocyte volume was higher than at week 8, but lower than in untreated SNX rats at week 12.
Untreated SNX rats at week 12. Treatment with dihydralazine had no significant effect on mean podocyte volume.

The mean number of cells within the mesangium (Table 2) was significantly higher in SNX rats at week 8 compared with sham-op rats and significantly higher in untreated SNX rats at week 12 compared with SNX rats at week 8. The number of cells within the mesangium was significantly lower in SNX rats treated with losartan and losartan+spironolactone, respectively, and not significantly different in SNX rats treated with spironolactone and dihydralazine, respectively.

At week 12, the mean volume of the cells in the mesangium was comparable in all study groups.

The mean number and mean volume of endothelial cells (Table 2) was not significantly different among the groups.

The length density of glomerular capillaries was significantly lower in all treated SNX groups compared with untreated SNX rats at week 12 compared with week 8. At week 12, accumulation of collagen IV was significantly lower in all treated SNX groups compared with untreated SNX rats. It was significantly lower in SNX+losartan and SNX+losartan+spironolactone rats compared with SNX+spironolactone and SNX+dihydralazine rats, respectively.

Staining for NF-κB in glomeruli was significantly more pronounced in untreated SNX rats at week 12 compared with sham-op and untreated SNX rats at week 8. Such an increase was prevented in SNX rats treated with losartan or losartan+spironolactone (Table 3). Staining for NF-κB in the tubulointerstitium was significantly more intense in SNX+dihydralazine rats compared with other groups (Table 3).

The protein abundance of TGF-β1 in glomeruli and tubulointerstitium was significantly higher in untreated SNX compared with sham-op rats (Table 4). This was prevented...
in SNX + losartan, SNX + losartan + spironolactone, and less effectively in SNX + spironolactone rats.

There was no difference in staining for PDGF-AB between untreated SNX and sham-op rats (Table 4).

The staining for VEGF in glomeruli and tubulointerstitium was significantly less marked in untreated SNX compared with sham-op rats. Treatment with losartan, spironolactone, and losartan + spironolactone resulted in more intense staining for VEGF (Table 4).

The expression of VEGF receptor 2 (flk-1) in glomeruli and tubulointerstitium was significantly higher in untreated SNX (Fig. 6). This was prevented by treatment with losartan, spironolactone, but not in SNX rats treated with dihydralazine (Fig. 6).

**DISCUSSION**

Our study documents that the regression of glomerulosclerosis achieved with losartan was not further augmented by adding nonhyperkalemic doses of spironolactone. While spironolactone alone stopped progression of glomerulosclerosis, addition of spironolactone to losartan failed to cause further reversal of established glomerular changes. The beneficial effects of AT₁ and aldosterone blockade could not be reproduced by blood pressure lowering alone, using dihydralazine. We acknowledge, however, that the systemic blood pressure does not reflect the local intraglomerular pressure which contributes to glomerular damage.

Administration of aldosterone negates the renoprotective effect of angiotensin-converting enzyme (ACE) inhibition in both the stroke-prone spontaneously hypertensive rats (22) and in the renal ablation model (10). Aldosterone blockade was shown to slow the progression of podocyte damage and glomerulosclerosis in Dahl salt-hypertensive rats (19). A delayed increase of aldosterone concentration is seen in the serum of many patients receiving ACE inhibitors or AT₁ blockers (“aldosterone breakthrough”) (24). Our results showed that adding a mineralocorticoid receptor blocker to high-dose AT₁ blockade did not improve regression of glomerulosclerosis but remarkably improved expression of α-SMA, a marker of epithelial-to-mesenchymal transition of tubular cells. High doses...
of the AT_1 blocker effectively controlled serum aldosterone concentration in this short-term experimental setting. The absence of an aldosterone breakthrough in this model may explain the failure of mineralocorticoid receptor blockade to improve the readouts.

Unlike a high-dose ACE inhibitor (2), losartan failed to reduce TII in this study. Whether the renoprotective effects of these two groups are indeed different requires further head-to-head comparison.

In the renal ablation model, regression of glomerulosclerosis was found after the administration of high doses of spironolactone (200 mg·kg^{-1}·day^{-1}) in monotherapy (3). The high dose in this study is the most likely explanation for the difference in results between the present and the above-quoted study. Because of the importance of hyperkalemia as a clinically relevant side effect, we selected deliberately, on the basis of pilot experiments, a dose that, in combination with losartan, failed to cause hyperkalemia but was still high compared with the therapeutic range (9, 26).

Enlargement of glomeruli accompanies development of glomerulosclerosis (29). In line with those findings, glomerular volume 12 wk after SNX was significantly below the starting point volume at 8 wk in animals treated with losartan and the combination of losartan plus spironolactone, indicative of glomerular remodeling. In agreement with the concept that the podocytes, which play a pivotal role in the development of glomerular scarring, are postmitotic cells (18), the number of podocytes did not increase in parallel with the glomerular volume but rather decreased while podocyte volume increased. Further loss of podocytes, however, was completely stopped after the start of treatment with losartan, spironolactone, or their combination, in contrast to blood pressure lowering by dihydralazine. This finding is in line with the concept that increased ANG II signaling via the AT_1 receptor in podocytes induces podocyte loss, glomerulosclerosis, and albuminuria (12). Podocytes have also been shown to be directly damaged by aldosterone (25). In a past study, mineralocorticoid receptor blockade protected against podocyte injury when started at the time of the insult (19). Our results document preservation of podocyte number and lower number of desmin-positive podocytes even when the treatment is started late after kidney injury.

The reversal of glomerulosclerosis after inhibition of the RAS involves restructuring of the glomerular capillary network (1). In vitro evidence showed that VEGF signaling between injured podocytes and glomerular endothelial cells was reduced and could be restored by AT_1 blockade (14). In agreement with this observation, we detected less VEGF in injured glomeruli, and this was restored to normal in animals treated with the AT_1 receptor blocker. Furthermore, spironolactone had a similar effect on VEGF expression, suggesting that aldosterone contributed to suppression of VEGF expression in glomeruli.

Albuminuria was significantly reduced by losartan either in monotherapy or in combination with spironolactone, but not by spironolactone alone, despite its protective effect on podocyte number and to a lesser extent on podocyte volume. This finding is in contrast to clinical observations (5, 23). In an experimental nephrotic syndrome, however, mineralocorticoid receptor blockade reduced proteinuria when started immediately after the insult but failed to reduce proteinuria when treatment was started later (20).

Epithelial-to-mesenchymal transition, which was not directly assessed in the present study, plays a pivotal role in the progression of interstitial fibrosis and is induced by aldosterone (30). One of its consequences is the de novo expression of α-SMA in tubular epithelial cells (15). Spironolactone caused regression of α-SMA in parallel with the prevention of further progression of the TII and deposition of collagen IV.

The profibrotic effects of the activation of the RAS are to a large extent mediated by the profibrogenic cytokine TGF-β (28). Interfering with TGF-β signaling prevents renal damage (4). Our study documents by immunohistochemistry and Western blotting that spironolactone suppressed TGF-β expression but did not amplify its suppression by losartan.

NF-κB-linked regulatory pathways has been demonstrated to mediate ANG II-dependent renal damage (17, 27). In agreement with that observation, regression of glomerulosclerosis was accompanied by downregulation of NF-κB.

In conclusion, in this short-term renal ablation model with no evidence of aldosterone breakthrough, nonhyperkalemic doses of spironolactone, unlike similar lowering of blood pressure with dihydralazine, stopped the progression of renal damage, specifically glomerular scarring and podocyte loss but failed to amplify the protective effect of the AT_1 blocker losartan.

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GRANTS

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REFERENCES


