Nephroprotection of an ET \textsubscript{A}-Receptor Blocker (LU 135252) in Salt-Loaded Uninephrectomized Stroke-Prone Spontaneously Hypertensive Rats

Stephan R. Orth, Jan P. Esslinger, Kerstin Amann, Ute Schwarz, Manfred Raschack, Eberhard Ritz

Abstract—The present study was designed to assess whether the orally active and highly specific endothelin A (ET\textsubscript{A}) receptor antagonist LU 135252 affects progressive renal dysfunction in a hypertensive rat model of renal damage, ie, the uninephrectomized (UNX) stroke-prone spontaneously hypertensive rat (SHRsp). The animals were examined on a normal salt (0.25%) diet and, to sensitize the kidney to hypertensive injury, also on a high salt (3%) diet. Stereological methods were used to quantify indices of glomerulosclerosis, vascular damage, and tubulointerstitial damage. Treatment with LU 135252 (100 mg/kg body wt) did not affect systolic blood pressure (BP) in animals on a normal salt diet during the whole period of the experiment (18 weeks) or in salt-loaded animals until week 10; subsequently, BP was slightly but significantly lower in salt-loaded UNX-SHRsp given LU 135252. Between weeks 6 and 12, 40% of the untreated UNX-SHRsp on a high salt diet, but none on a standard salt diet, died; such mortality was completely prevented by LU 135252. Indices of renal damage were more abnormal in salt-loaded UNX-SHRsp compared with UNX-SHRsp on a normal salt diet. Development of glomerulosclerosis and tubulointerstitial and vascular damage in UNX-SHRsp on high salt was completely prevented by LU 135252. The respective indices were no longer significantly different from those of salt-loaded sham-operated SHRsp controls. In the less severely damaged kidneys of UNX-SHRsp on normal salt, treatment with LU 135252 tended to ameliorate the indices, but the difference was not statistically significant. The results document a role of the ET system, specifically of ET\textsubscript{A} receptors, in the development of progressive renal injury in salt-loaded UNX-SHRsp. LU 135252 completely prevented death and renal damage resulting from salt loading. (Hypertension. 1998;31:995-1001.)

Key Words: endothelin ■ kidney ■ renal failure ■ receptors, endothelin ■ rats, inbred SHRsp

While the role of ETs in the normal kidney has not been well defined, it is certain that ETs play an important role in the diseased kidney, eg, progression of chronic renal failure.1

In a rat model of progressive renal failure,2 renal proproET-1 gene expression and production of ET-1 was increased and correlated with progression of renal insufficiency.3

In rats with renal ablation, ET\textsubscript{A} as well as mixed ET\textsubscript{A/B} receptor antagonists reduced glomerulosclerosis and proteinuria.4,5 Nephroprotective effects of ET receptor antagonists have also been reported in other models of renal damage, ie, lupus nephritis,6 mesangioproliferative nephritis,7 and diabetes.8 Inhibition of progression of renal failure may emerge as an important indication for ET receptor antagonists.9

Available reports differ in that ET receptor antagonists affected systemic BP in some10-13 but not all studies.5,14-18 Furthermore, quantitative analysis of renal lesions was difficult because of disruption of renal architecture and inhomogeneity of compensatory hypertrophy of the residual nephrons after renal ablation.

In the present investigation, we used the new highly specific and orally active nonpeptide ET\textsubscript{A} receptor antagonist LU 135252\textsuperscript{19} to assess its effect on progression of renal injury in the model of UNX-SHRsp. Uninephrectomy of SHRsp was performed to sensitize renal tissue to injury. The effect of LU 135252 on progressive renal damage was compared in UNX-SHRsp fed a normal or high salt diet, respectively.

Methods

Animals and Experimental Design

Male SHRsp (90 to 100 g; Iffa Credo, L’Arbresle, France) were housed in single cages with constant room temperature (21°C) and humidity (75%) under a controlled light-dark cycle. The rats were fed either a normal salt diet containing 0.25% NaCl or a high salt diet containing 3% NaCl (ssni Spezialdiäten GmbH, Soest, Germany). The protein content of the food was 19%. After a 7-day adaptation period, the animals were randomly allotted to three groups receiving either a normal salt diet (experiment 1) or a high salt diet (experiment 2). Experiment 1 (normal salt) included (1) sham-operated, control; (2) uninephrectomized, no treatment (UNX); and (3) uninephrectomized plus LU 135252 (UNX+LU 135252). Experiment 2 (high salt) included (1) sham-operated, salt-loaded control; (2) uninephrectomized, salt-loaded, no treatment (UNX); and (3) uninephrectomized, salt-loaded plus LU 135252 (UNX+LU 135252).

In both experiments, treatment of group 3 with the specific ET\textsubscript{A} receptor antagonist LU 135252 (K\textsubscript{i} values: ET\textsubscript{A} receptor, 1.4 nmol/L; ET\textsubscript{B} receptor, 184 nmol/L) in the food was started 3 days before...
uninephrectomy (right kidney). The concentration in the food was calculated to deliver a daily dose of 100 mg/kg body wt. Daily food consumption was measured to control the amount of LU 135252 ingested. Water was given ad libitum. Anesthesia for uninephrectomy was performed by intramuscular injection of ketamine (100 mg/kg body wt) and xylazine (5 mg/kg body wt). Systolic BP was measured weekly by tail plethysmography.

In experiment 1, blood samples as well as 24-hour urine samples were taken 7 and 15 weeks after uninephrectomy. Eighteen weeks after uninephrectomy, blood samples were obtained, and the experiment was terminated by retrograde perfusion fixation through the abdominal aorta, as described elsewhere.

In experiment 2, blood samples and 24-hour urine samples were obtained 8 weeks after uninephrectomy. Because of excessive mortality of untreated animals, blood samples were obtained 12 weeks after uninephrectomy, and the experiment was terminated prematurely at week 12 by retrograde perfusion fixation through the abdominal aorta.

Morphological Investigations of the Kidney

The kidneys were weighed and dissected in a plane perpendicular to the interpolar axis, yielding slices 1 mm in width. Tissue slices were embedded in paraffin, and 4-μm sections were stained with hematoxylin and eosin/PAS and analyzed using morphometric and stereologic techniques as previously described in detail.

Blood and Urine Measurements

Routine blood and urine parameters were measured with a Hitachi 9–17E. Immunoactive ET-1 concentrations were determined in duplicate by enzyme immunoassay (Bionetica GmbH), according to manufacturer guidelines. Results were expressed as the mean of two determinations. The sensitivity of the assay is 0.1 to 15.6 nmol/L, and cross-reactivities of the antibodies used are <1% with big ET-1-(1–38) and big ET-1-(22–38), <5% with ET-3, and 100% with ET-2. The intraassay and interassay variabilities of the kit are 3.3% and 3.5%, respectively.

Statistics

Data are given as mean ± SD. Statistical analysis was performed using one-way ANOVA, followed by the Bonferroni-Dunn multiple range test. A probability of error of P < .05 was accepted as statistically significant.

Results

Animal Data

Experiment 1 (Normal Salt Diet)

Survival

No deaths were seen in any of the animals.

Systolic Blood Pressure (Table 1)

In UNX-SHRsp, systolic BP gradually increased throughout the experiment. LU 135252 had no significant effect on systolic BP.

Body Weight

Body weight was not different among the three groups (data not shown).

Urinary ET-1 Excretion and Plasma ET-1 Levels

After 7 and 15 weeks there was a tendency for urinary ET-1 excretion in the UNX animals to be higher compared with the sham-operated group, but the difference was not significant. Urinary ET-1 excretion did not differ significantly between UNX and UNX + LU 135252. There were also no significant differences among the three groups concerning plasma ET-1 levels (data not shown).

Blood and Urine Chemistry (Table 2)

Pcr was significantly higher in UNX animals compared with sham-operated animals. In weeks 15 and 18, Pcr tended to be lower in treated animals compared with untreated UNX (not statistically significant). In week 15, Ccr was significantly higher in LU 135252–treated animals compared with untreated UNX (not statistically significant). In week 15, Ccr was significantly higher in LU 135252–treated animals compared with untreated UNX. No significant differences were noted in weeks 7, 15, and 18 concerning plasma Na, K, Ca, Cl, HPO4, protein, cholesterol, and triglycerides. The same was true for hematologic parameters assessed in week 18, ie, white blood cells, platelets, hemoglobin, and mean corpuscular volume; there were also no significant differences of urinary Na, K and Cl excretion rates (data not shown).

Experiment 2 (High Salt Diet)

Survival

The most dramatic effect of LU 135252 was the prevention of early mortality in UNX-SHRsp. No animal died in the sham-operated and the UNX + LU 135252 groups. In contrast, 6 of the 15 untreated UNX animals died before the experiment was terminated 12 weeks after uninephrectomy (1 animal died at week 6, 2 animals at week 10, 2 at week 11, and 1 further animal at week 12). Postmortem showed brain hemorrhage and gastric or duodenal hemorrhage, respectively. The animals were anorexic and showed signs of cachexia.

| TABLE 1. Systolic BP (mm Hg) During Experiment 1 (Normal Salt) |
|--------------------------|----------------|----------------|----------------|----------------|----------------|
| Group                     | Week 1       | Week 6        | Week 10        | Week 14        | Week 17        |
| Sham-operated (n = 15)    | 177 ± 22     | 268 ± 23      | 280 ± 18       | 287 ± 10       | 282 ± 10       |
| UNX (n = 15)              | 171 ± 16     | 275 ± 18      | 284 ± 11       | 282 ± 16       | 281 ± 13       |
| UNX + LU 135252 (n = 15)  | 178 ± 15     | 264 ± 25      | 284 ± 12       | 287 ± 10       | 282 ± 10       |

UNX indicates uninephrectomized SHRsp fed a normal salt diet. A dose of 100 mg/kg body wt of LU 135252 was administered. Values are mean ± SD.
Systolic Blood Pressure (Table 3)
A continuous increase in systolic BP was seen in sham-operated and UNX rats. LU 135252 had no significant effect on systolic BP during the first 10 weeks of the experiment; subsequently, systolic BP was modestly but significantly lower in treated animals.

Body Weight
Body weight of treated UNX rats was comparable to that of sham-operated rats, whereas untreated UNX rats failed to gain weight beyond the 10th week (data not shown).

Urinary ET-1 Excretion (Table 4)
After 8 weeks, ET-1 excretion was significantly increased in untreated UNX but unchanged in LU 135252–treated UNX compared with sham-operated animals.

Structural Changes of the Kidney

Table 2. Blood and Urine Chemistry in Experiment 1 (Normal Salt)

<table>
<thead>
<tr>
<th>Group</th>
<th>Week 7</th>
<th>Week 15</th>
<th>Week 18</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P_D, μmol/L</td>
<td>P_RR, mmol/L</td>
<td>C_Cr×10^-3, mL/s</td>
</tr>
<tr>
<td>Sham-operated</td>
<td>38±3.2§</td>
<td>5.4±0.9§</td>
<td>16.7±6.2</td>
</tr>
<tr>
<td>UNX</td>
<td>40±4.3</td>
<td>6.3±1.2</td>
<td>14.0±2.5</td>
</tr>
<tr>
<td>UNX+LU 135252</td>
<td>41±3.4</td>
<td>6.5±0.5</td>
<td>15.0±2.5</td>
</tr>
</tbody>
</table>

UNX indicates uninephrectomized SHRsp fed a normal salt diet. Values are mean±SD; group size, n=15.
*P<.05 vs UNX; †P<.01 vs UNX; ‡P<.05 vs UNX+LU 135252; §P<.01 vs UNX+LU 135252.

Structural Changes of the Kidney

Experiment 1 (Normal Salt Diet)
Glomerular, Tubulointerstitial, and Vascular Damage
Kidneys of 1 animal of the sham-operated and 1 animal of the UNX+LU 135252 group were poorly perfused and not examined. The glomerulosclerosis index was significantly increased in untreated and treated UNX compared with sham-operated rats (Fig 1A). Tubulointerstitial and vascular damage indices tended to be higher in the untreated UNX group compared with the sham-operated group (not statistically significant) (Fig 1B and 1C). Treatment with LU 135252 tended to prevent tubulointerstitial and vascular damage (not statistically significant) (Fig 1B and 1C). Further histological analysis was not performed in view of the modest changes.

Renal Weight
The weight of the perfusion-fixed left kidney was significantly higher (P<.01 versus sham-operated) in untreated UNX (2.08±0.24 g) and LU 135252–treated animals (1.98±0.28 g) compared with sham-operated controls (1.29±0.22 g); n=15 per group.

Experiment 2 (High Salt Diet)
Glomerular, Tubulointerstitial, and Vascular Damage
Kidneys of 2 sham-operated animals and 1 treated UNX animal were poorly perfused and not examined. The glomerulosclerosis index, tubulointerstitial damage index, and index of vascular damage were significantly increased in untreated UNX. Treatment with LU 135252 completely prevented these structural alterations (Fig 1D through 1F). Fig 2A and 2B show a representative glomerulus. Fig 2C and 2D show the tubulointerstitium and vessels of an untreated UNX and a UNX treated with LU135252, respectively.

Renal Weight
The weight of the perfusion-fixed left kidney was significantly higher (P<.01 versus sham-operated) in untreated

Table 3. Systolic BP (mm Hg) in Experiment 2 (High Salt)

<table>
<thead>
<tr>
<th>Group</th>
<th>Week 1</th>
<th>Week 6</th>
<th>Week 10</th>
<th>Week 11</th>
<th>Week 12</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham-operated (n=12)</td>
<td>171±13</td>
<td>256±19</td>
<td>273±24</td>
<td>270±10*</td>
<td>273±13*</td>
</tr>
<tr>
<td>UNX (n=15)†</td>
<td>170±11</td>
<td>268±22</td>
<td>280±11</td>
<td>288±8</td>
<td>288±10</td>
</tr>
<tr>
<td>UNX+LU 135252 (n=10)</td>
<td>173±18</td>
<td>261±29</td>
<td>277±25</td>
<td>275±8*</td>
<td>270±14*</td>
</tr>
</tbody>
</table>

UNX indicates salt-loaded uninephrectomized SHRsp. Values are mean±SD.
*P<.01 vs UNX; †of these 15 rats, 6 died during the experiment (see “Results”).
UNX (2.07±0.18 g, n=8) and LU 135252–treated UNX (2.12±0.11 g, n=10) compared with sham-operated controls (1.26±0.16 g, n=11).

Renal Cortex and Medulla
Total kidney volume was significantly increased (sham-operated, 1.21±0.15 cm$^3$; UNX, 1.99±0.17 cm$^3$ [P<.01 versus sham-operated]; UNX+LU 135252, 2.04±0.11 cm$^3$ [P<.01 versus sham-operated]) and this was due to a parallel significant increase of cortical and medullary volumes in UNX animals (data not shown). There were no significant differences between untreated and treated rats.

Glomerular Number and Morphology
The total number of glomeruli of the left kidneys in the three groups of SHRsp did not differ significantly: sham-operated, 33 999±2176; UNX, 35 412±8505; and UNX+LU 135252, 39 806±5588. Total glomerular volume was significantly increased in UNX treated with LU 135252, whereas total glomerular volume of untreated UNX was not significantly different from sham-operated controls (Fig 3A). In parallel, mean glomerular volume was also significantly increased in treated UNX rats (Fig 3B). The number of glomeruli per unit of cortical volume (1/mm$^3$) was significantly lower in both UNX groups (Fig 3C).

Discussion
The main finding of this study is that the orally active ET$_{A}$-specific receptor antagonist LU 135252 has a remarkable, probably BP-independent, nephroprotective effect in salt-loaded UNX-SHRsp. This conclusion is based on the observation that in the salt-loaded UNX-SHRsp, the ET$_{A}$ antagonist LU 135252 prevented the increase in P$_{cr}$ and this was due to a parallel significant increase of cortical and medullary volumes in UNX animals (data not shown). There were no significant differences between untreated and treated rats.

![TABLE 4. Blood and Urine Chemistry in Week 8 of Experiment 2 (High Salt)](https://example.com/table4)

<table>
<thead>
<tr>
<th>Group</th>
<th>P$_{cr}$, µmol/L</th>
<th>C$_{cr}$×10$^{-3}$, mL/s</th>
<th>P$_{urea}$, mmol/L</th>
<th>Urinary ET Excretion, fmoL/24 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham-operated</td>
<td>36±4.42†</td>
<td>16.3±3.0†</td>
<td>5.1±0.68†</td>
<td>65±33†</td>
</tr>
<tr>
<td>UNX</td>
<td>44±3.77</td>
<td>11.2±1.7</td>
<td>8.1±1.69</td>
<td>177±83</td>
</tr>
<tr>
<td>UNX+LU 135252</td>
<td>38±3.08†</td>
<td>16.7±2.8†</td>
<td>6.3±0.36†</td>
<td>61±8*</td>
</tr>
</tbody>
</table>

UNX indicates salt-loaded uninephrectomized SHRsp. Values are mean±SD; group size n=10.

In contrast to animals on a high salt diet, there was only a tendency to reduce glomerulosclerosis in UNX-SHRsp on a normal salt diet, but only marginal glomerular, tubulointerstitial, and vascular damage was observed in untreated animals to begin with. In this study, BP was not further increased in SHRsp on a high salt diet. This does not necessarily indicate salt resistance because (1) BP was only measured in surviving animals and we cannot exclude the fact that animals in the high salt group may have died from malignant hypertension, and (2) intermittent increases in BP may not have been reliably detected by tail plethysmography.

The effect of LU 135252 is probably independent of BP: the drug did not influence systolic BP either in the normal salt experiment or during the first 10 weeks of the high salt experiment. Systolic BP was somewhat lower in treated animals beyond the 10th week of the high salt experiment, but this finding does not necessarily indicate an antihypertensive action; it may merely reflect progression of renal damage in untreated salt-loaded rats, leading to a further increase in systolic BP. Thus, although renal ET-1 production is increased in SHR, ETs apparently play no major role in BP elevation, in contrast to other models of hypertensive renal damage, eg, deoxycorticosterone-salt hypertension, in which specific ET$_{A}$ receptor blockade, as well as nonspecific ET$_{A/B}$ receptor blockade, normalizes BP. However, the ET system may play a role in the genesis of progressive renal damage even in the absence of systemic hypertension, as illustrated by the fact that normotensive rodents transgenic for ET-1 and ET-2 develop progressive renal damage.

Urinary ET-1 excretion was significantly increased in the group with the highest degree of renal damage, ie, the salt-loaded UNX-SHRsp. Increased urinary ET-1 excretion has also been reported in other renal-damage models and

![TABLE 5. Blood Chemistry and Hematology in Week 12 of Experiment 2 (High Salt)](https://example.com/table5)

<table>
<thead>
<tr>
<th>Group</th>
<th>P$_{cr}$, µmol/L</th>
<th>P$_{urea}$, mmol/L</th>
<th>Plasma Cholesterol, mmol/L</th>
<th>Plasma Triglycerides, mmol/L</th>
<th>Hemoglobin, g/L</th>
<th>Platelets, ×10$^{12}$/L</th>
<th>WBC, ×10$^{9}$/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham-operated</td>
<td>38±4.67†</td>
<td>5.7±0.52†</td>
<td>1.5±0.24†</td>
<td>0.84±0.24‡</td>
<td>93±6</td>
<td>513±107*</td>
<td>3.4±1.0*</td>
</tr>
<tr>
<td>UNX</td>
<td>52±6.81</td>
<td>8.2±1.41</td>
<td>3.0±0.26</td>
<td>1.1±0.21‡</td>
<td>92±5</td>
<td>376±121</td>
<td>4.6±1.2</td>
</tr>
<tr>
<td>UNX+LU 135252</td>
<td>41±2.78†</td>
<td>6.6±0.82†</td>
<td>1.6±0.11†</td>
<td>0.51±0.12‡</td>
<td>98±4*</td>
<td>584±55‡</td>
<td>4.1±0.9†</td>
</tr>
</tbody>
</table>

UNX indicates salt-loaded uninephrectomized SHRsp; WBC, white blood cells. Values are mean±SD; each group, n=10 except hemoglobin, platelets, and WBC (salt-loaded sham-operated SHRsp, n=12; salt-loaded uninephrectomized untreated UNX, n=8; and salt-loaded uninephrectomized UNX treated with LU 135252, n=9).

*P<.05 vs UNX; †P<.01 vs UNX; ‡P<.01 vs UNX+LU 135252.
correlates with the degree of renal damage. This is true even in humans with kidney disease. In our study, plasma ET-1 levels were not elevated in untreated UNX-SHRsp on a normal or high salt diet, but this finding must be interpreted with caution because circulating ET-1 levels do not necessarily reflect local ET production, which is known to be increased in renal-damage models despite there being no elevation in circulating ET-1. In one study on SHRsp with untouchked kidneys, salt loading had no effect on the renal expression but increased cardiac expression of the preproET-1 mRNA. In SHR, uninephrectomy increases ET-1 gene expression and the amount of the corresponding peptide in mesangial cells, podocytes, and proximal tubules and vessels. ET_A and ET_B receptors are overexpressed in the glomeruli of SHR, and ET_A receptors are overexpressed in vascular smooth muscle cells of intrarenal arteries also. Furthermore, in SHR the receptor affinity for ET-1 is increased.

The inhibitory effect of ET_A receptor blockade on the development of glomerulosclerosis and of interstitial or vascular lesions appears plausible in view of the known renal actions of ET-1, ie, mitogenic effects on mesangial cells and increased expression of fibronectin and collagen. Furthermore, ET-1 stimulates the production of cytokines such as platelet-derived growth factor. In the rat kidney, ET_A receptors have been demonstrated in mesangial cells and ET_B receptors in endothelial and epithelial cells. In hypertensive rats, the specific ET_A receptor antagonist BQ123 reduces glomerular filtration rate by increasing arteriolar resistance. Consequently, ET_A receptor blockade may affect both renal hemodynamics and growth processes. LU 135252 also prevented tubulointerstitial damage in the salt-loaded UNX-SHRsp. The tubulointerstitial space is a major site of ET-1 synthesis; ET_B receptors are the major receptor subtype expressed by rat tubular cells, whereas expression of ET_A receptors is controversial. All three ET peptides constrict rat vasa recta at very low concentrations via ETA and ET_B receptors. Preliminary studies indicate that ET-1 can stimulate collagen I gene expression in human renal interstitial fibroblasts. ET-1 is also chemotactic for monocytes, which play a key role in interstitial renal fibrosis. Increased tubular ET-1 synthesis may induce fibroblast proliferation, interstitial matrix deposition, and infiltration of inflammatory cells, features typical of progressive tubulointerstitial fibrosis.

In view of the observation that LU 135252 completely prevented vascular damage in the salt-loaded UNX-SHRsp, it...
is of interest that ET-1 affects rat vascular smooth muscle cells in vitro via the ETₐ receptor.²⁹

It is unknown whether the beneficial effects of selective ETₐ receptor blockade in the salt-loaded UNX-SHRsp can be extrapolated to other renal damage models and to other species, including humans, because of the known species differences in the renal ET system.⁹ Results of this study should be interpreted cautiously, since it is possible that therapy with LU 135252 may not have completely prevented but only retarded progression of renal damage. Nevertheless, the results are sufficiently encouraging to warrant further studies.

Acknowledgments

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