Calcimimetic R-568 or calcitriol: equally beneficial on progression of renal damage in subtotally nephrectomized rats

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SECONDARY HYPERPARATHYROIDISM (sHPT) is a common feature of chronic kidney disease. Parathyroid hormone (PTH) concentrations increase progressively with diminishing glomerular filtration rate, but it is unclear whether PTH per se modifies progression. Active metabolites of vitamin D are widely used to control sHPT. Moreover, beneficial effects of active vitamin D on progression of chronic kidney disease have been documented (13, 19).

A therapeutic alternative is the use of calcimimetics (R-568 and, in humans, cinacalcet HCl), allosteric activators of the calcium-sensing receptor (CaSR), which reduce PTH secretion and interfere with parathyroid hyperplasia (4, 20, 22). In addition, however, Ogata et al. (17) showed that short-term treatment with R-568 reduces albuminuria and attenuates glomerular and tubulointerstitial lesions in subtotally nephrectomized (SNX) rats.

The present study was designed to compare the effect of the two interventions on albuminuria and morphological lesions of the kidney in the SNX rat. The readouts were morphology and ultrastructure of podocytes, glomerulosclerosis index (GSI), and tubulointerstitial damage index, as well as expression profile of TGF-β1, endothelin-1 (ET-1), and VEGF using immunohistochemistry and mRNA in situ hybridization. In addition, expression of CaSR and vitamin D receptor (VDR) was monitored.

MATERIALS AND METHODS

Animals and Experimental Design

Twelve-week-old male Sprague-Dawley rats (331 ± 85 g body wt; Charles River) were housed under constant room temperature (22 ± 1°C) and relative humidity (75 ± 5%), exposed to a 12:12-h light-dark cycle, given free access to water, and fed a standard rodent diet without vitamin D (19.0% protein, 4.0% fat, 0.9% calcium, and 0.7% phosphorus; Ssniff). The animals were handled in accordance with the German law for protection of animals, and all animal procedures were approved by the local ethics committee for animal experiments (Regierungspraesidium, Karlsruhe, Germany).

After a 7-day adaptation period, rats were randomly subjected to SNX (n = 54) or sham operation (n = 51). As described elsewhere (3), SNX was carried out in two steps: first, under isoflurane anesthesia (Isoflurane, Baxter), the right kidney was removed, and 7 days later, weight-controlled surgical removal of cortical tissue of the hypertrophied left kidney corresponding to 60% of the weight of the right kidney was performed. In sham-operated animals, the kidneys were decapsulated. After the second operation, the animals were randomly assigned to the following study groups: 1) sham-operated and treated with vehicle alone (sham-op + vehicle, n = 16), sham-operated and treated with R-568 (sham-op + R-568, n = 15), 2) sham-operated and treated with calcitriol (sham-op + calcitriol, n = 18), 3) SNX and treated with vehicle alone (SNX + vehicle, n = 17), 4) SNX and treated with R-568 (SNX + R-568, n = 18), and 5) SNX and treated with calcitriol (SNX + calcitriol, n = 17).
Calcimimetic agent R-568 (provided by Amgen, Thousand Oaks, CA) was diluted in 10% 2-hydroxypropyl-β-cyclodextrin (Sigma-Aldrich) and administered subcutaneously (17 mg·kg body wt⁻¹·day⁻¹). The active vitamin D metabolite calcitriol (Calbiochem) was administered subcutaneously (3 ng·kg body wt⁻¹·day⁻¹). The control animals received subcutaneous injections of vehicle alone. The treatment was continued for 12 wk. Body weight was measured every week, and doses were adjusted. Systolic blood pressure (SBP) was measured by tail plethysmography 24 h after the administration of medication at week 12. At weeks 4 and 12, the animals were kept in metabolic cages for 24-h urine collection.

In an additional experiment, R-568-treated SNX rats were paired with vehicle-treated SNX rats. Urine was collected for 24 h at 4 wk after SNX. Blood was collected 2 h after R-568 administration at week 4.

**Urinary Albumin Measurements and Blood Analysis**

Urinary albumin excretion was measured with a rat-specific sandwich ELISA system using the microplate technique and a rabbit anti-rat albumin peroxidase-conjugated antibody, as described in detail elsewhere (19). The blood parameters were determined in samples taken from the abdominal aorta by standard laboratory methods before the animals were killed. Serum 1,25(OH)₂D₃ concentration was measured by RIA. Serum PTH was measured by the two-antibody method using a rat immunoreactive PTH ELISA kit (Immunopectics, San Clemente, CA).

**Tissue Preparation**

At 12 wk after surgery, the abdominal aorta was catheterized under ketamine (100 mg/kg)-xylazine (3 mg/kg) anesthesia, blood samples were taken, and the experiment was terminated by retrograde aortic perfusion. All blood samples were collected 24 h after the last injection of calcitriol, R-568, or vehicle. For morphometric and stereological investigations, eight to nine randomly chosen animals per group were perfused with 3% glutaraldehyde. Perfusion pressure was kept constant at 120 mmHg. The kidneys were weighed and dissected in a plane perpendicular to the interpolar axis, yielding 1-mm-wide slices. Ten small pieces of one kidney were selected by area-weighted sampling for embedding in Epon-Araldite. Tissue slices were also embedded in paraffin; 4-μm sections were prepared and stained with periodic acid-Schiff (PAS). From five randomly chosen resin blocks, semithin (0.5-μm) sections were prepared and stained with methylene blue-basic fuchsin. The kidneys were subsequently investigated by means of morphometry and stereology (3).

The remaining animals were perfused with ice-cold 0.9% NaCl, and the kidneys were removed. One-half of the harvested kidney was fixed by immersion in 4% phosphate-buffered formaldehyde and embedded in paraffin; the other half was snap frozen in liquid nitrogen and stored at −86°C until molecular biology measurements were performed.

**Morphological and Stereological Evaluation**

All investigations were performed in a blinded manner; i.e., the observer was unaware of the study protocol.

**Glomerular, tubular, interstitial, and vascular damage indexes.** GSI was assessed on PAS-stained paraffin sections according to the semiquantitative scoring system (score 0–4) proposed by El Nahas et al. (6). With use of light microscopy and ×400 magnification, the glomerular score of each animal was derived as the arithmetic mean of 100 glomeruli. The tubular and interstitial damage scores were evaluated separately on the same PAS-stained sections, with evaluation of every field of view at ×400 magnification. Tubular damage was scored separately as follows: for tubular dilatation, 0 = none, 1 = dilated tubuli; for tubular atrophy, 0 = none, 1 = signs of atrophy, 2 = apoptosis and desquamation of cells; for intracellular vacuoles, 0 = none, 1 = small vacuoles, 2 = medium vacuoles, 3 = large vacuoles.

**Table 1. Animal data, blood pressure at study end, serum biochemistry, and albumin excretion rate**

<table>
<thead>
<tr>
<th>Group</th>
<th>Final Body Wt, g</th>
<th>Left Kidney Wt, g</th>
<th>SBP, mmHg</th>
<th>Serum Calcium, mmol/l</th>
<th>Serum Phosphorus, mmol/l</th>
<th>Serum PTH, pg/ml</th>
<th>Urinary Albumin Excretion mg/24 h</th>
<th>Release, g/100 glomeruli</th>
<th>GSI</th>
<th>Tubular dilatation</th>
<th>Tubular atrophy</th>
<th>Interstitial damage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham-op</td>
<td>37 ± 2.3</td>
<td>1.2 ± 0.25</td>
<td>135 ± 11</td>
<td>0.55 ± 0.09</td>
<td>1.00 ± 0.06</td>
<td>24.1 ± 3.2</td>
<td>2.92 ± 0.3</td>
<td>0.0 ± 0.00</td>
<td>0.00</td>
<td>0.0 ± 0.00</td>
<td>0.0 ± 0.00</td>
<td>0.0 ± 0.00</td>
</tr>
<tr>
<td>R-568</td>
<td>41 ± 2.7</td>
<td>1.9 ± 0.2</td>
<td>135 ± 12</td>
<td>0.50 ± 0.08</td>
<td>1.00 ± 0.06</td>
<td>24.1 ± 3.2</td>
<td>2.92 ± 0.3</td>
<td>0.0 ± 0.00</td>
<td>0.00</td>
<td>0.0 ± 0.00</td>
<td>0.0 ± 0.00</td>
<td>0.0 ± 0.00</td>
</tr>
<tr>
<td>SNX</td>
<td>48 ± 2.5</td>
<td>2.3 ± 0.3</td>
<td>140 ± 15</td>
<td>0.61 ± 0.06</td>
<td>1.00 ± 0.06</td>
<td>24.1 ± 3.2</td>
<td>2.92 ± 0.3</td>
<td>0.0 ± 0.00</td>
<td>0.00</td>
<td>0.0 ± 0.00</td>
<td>0.0 ± 0.00</td>
<td>0.0 ± 0.00</td>
</tr>
</tbody>
</table>

Values are means ± SD. SBP, systolic blood pressure; PTH, parathyroid hormone; sham-operated, SNX subtotally nephrectomized; NS, not significant; *P*< 0.05 vs. sham-op or vehicle; +P*< 0.05 vs. sham-op + calcitriol; −P*< 0.05 vs. SNX + vehicle.
infiltration.

formula as follows:

\( \text{transsects per area of the capillary tuft} \) (3). The total length of

\( \text{area density (V)} \) of glomerular capillaries (i.e., capillary length in a tuft volume, \( \text{tuft volume} \)) was then determined as follows:

\[ \text{AT}, \text{AT} = \frac{A_{\text{CT}}}{V_{\text{cortex}}} \]

where \( A_{\text{CT}} \) is the total area of the glomerular tuft (5). The mean number of cells per glomerulus and the mean

\( \text{glomerular capillaries per one kidney (} L_{\text{glomerulus}} \text{)} \) was then derived from

\( L_V \) and the total glomerular volume (\( V_{\text{glomerulus}} \)): \( V_{\text{glomerulus}} = V_{\text{glomerulus}} \times V_{\text{cortex}} \) (3). The mean number of cells per glomerulus and the mean

volume of different glomerular cells (podocytes, cells within the mesangium, and endothelial cells) were assessed stereologically in \( \geq 30 \) randomly selected glomeruli per animal on the basis of cell density per volume and volume density of the cell type, as previously described (1).

Analysis of foot process morphology. Podocyte foot processes were visualized by electron microscopy (Zeiss). Forty images per animal were analyzed (\( \times 10,000 \) magnification). Thickness of the glomerular basement membrane (GBM), dimensions of foot processes, and length of GBM covered by foot processes were measured (21) using Vario Vision Pro 3.2 software (Soft Imaging System).

Immunohistochemistry

Sections (4 \( \mu \text{m} \)) thick of formalin-fixed, paraffin-embedded tissue were mounted on silane-coated slides, deparaffinized with xylene, and rehydrated through descending concentrations of ethanol. After antigen retrieval by heating the slides in the target-unmasking fluid (Pan Path, The Netherlands) at 80°C for 20 min, immunohistochemical staining was performed using the streptavidin-biotin method. The following primary antibodies were applied for 60 min at room temperature: monoclonal mouse anti-desmin (1:50 dilution; Dako), rabbit polyclonal anti-fibronectin (1:500 dilution; Sigma), rabbit polyclonal anti-collagen type IV (1:40 dilution; Biotrend), rabbit polyclonal anti-endothelial nitric oxide synthase (eNOS, 1:200 dilution; Affinity BioReagents), sheep polyclonal anti-nitrotyrosine (1:400 dilution; Oxis Research), mouse monoclonal anti-ET-1 (1:20 dilution; Affinity BioReagents), rabbit polyclonal anti-VEGF (1:25 dilution; idLabs), and rabbit polyclonal anti-CaSR (1:200 dilution; Affinity BioReagents). Appropriate biotinylated secondary antibodies (anti-rabbit (BioGenex), anti-mouse (DCS Innovation Diagnostik-Systeme), and anti-sheep (Jackson ImmunoResearch)) were applied for 20 min, then the slides were exposed to streptavidin-

Table 2. Morphological indexes of kidney damage

<table>
<thead>
<tr>
<th>Group</th>
<th>GSI</th>
<th>VDI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham-op + vehicle</td>
<td>0.13±0.04</td>
<td>0.03±0.01</td>
</tr>
<tr>
<td>Sham-op + R-568</td>
<td>0.10±0.03</td>
<td>0.03±0.02</td>
</tr>
<tr>
<td>Sham-op + calcitriol</td>
<td>0.18±0.09</td>
<td>0.08±0.17</td>
</tr>
<tr>
<td>SNX + vehicle</td>
<td>1.66±0.08 ( \times 10^{10} )</td>
<td>0.26±0.14 ( \times 10^{10} )</td>
</tr>
<tr>
<td>SNX + R-568</td>
<td>1.04±0.22 ( \times 10^{10} )</td>
<td>0.10±0.09 ( \times 10^{10} )</td>
</tr>
<tr>
<td>SNX + calcitriol</td>
<td>0.97±0.49 ( \times 10^{10} )</td>
<td>0.14±0.07 ( \times 10^{10} )</td>
</tr>
<tr>
<td>SNX + calcitriol</td>
<td>0.97±0.49 ( \times 10^{10} )</td>
<td>0.14±0.07 ( \times 10^{10} )</td>
</tr>
<tr>
<td>P (by ANOVA)</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

Values are means \( \pm SD \). GSI, glomerulosclerosis index; VDI, vascular damage index. See Table 1 footnote for significance.

Table 3. Glomerular geometry, capillary density, and glomerular cells

<table>
<thead>
<tr>
<th>Group</th>
<th>Mean ( \times 10^3 ) ( \mu \text{m}^3 )</th>
<th>Mean ( \times 10^3 ) ( \mu \text{m}^3 )</th>
<th>Mean ( \times 10^3 ) ( \mu \text{m}^3 )</th>
<th>Mean ( \times 10^3 ) ( \mu \text{m}^3 )</th>
<th>Mean ( \times 10^3 ) ( \mu \text{m}^3 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group</td>
<td>Mean ( \times 10^3 ) ( \mu \text{m}^3 )</td>
<td>Mean ( \times 10^3 ) ( \mu \text{m}^3 )</td>
<td>Mean ( \times 10^3 ) ( \mu \text{m}^3 )</td>
<td>Mean ( \times 10^3 ) ( \mu \text{m}^3 )</td>
<td>Mean ( \times 10^3 ) ( \mu \text{m}^3 )</td>
</tr>
<tr>
<td>Sham-op + vehicle</td>
<td>2.70±0.91</td>
<td>11.0±1.8</td>
<td>6.01±0.52</td>
<td>199±10</td>
<td>397±67</td>
</tr>
<tr>
<td>Sham-op + R-568</td>
<td>2.74±0.48</td>
<td>6.7±3.9</td>
<td>5.81±0.50</td>
<td>191±26</td>
<td>375±59</td>
</tr>
<tr>
<td>Sham-op + calcitriol</td>
<td>3.03±0.55</td>
<td>10.5±2.8</td>
<td>5.70±0.74</td>
<td>199±23</td>
<td>397±43</td>
</tr>
<tr>
<td>SNX + vehicle</td>
<td>6.05±1.46</td>
<td>22.2±6.5</td>
<td>3.92±0.41</td>
<td>141±12</td>
<td>831±127</td>
</tr>
<tr>
<td>SNX + R-568</td>
<td>3.94±2.59</td>
<td>22.6±4.9</td>
<td>4.33±0.63</td>
<td>191±25</td>
<td>424±100</td>
</tr>
<tr>
<td>SNX + calcitriol</td>
<td>4.45±0.86</td>
<td>13.7±3.4</td>
<td>5.23±0.30</td>
<td>189±19</td>
<td>474±45</td>
</tr>
<tr>
<td>P (by ANOVA)</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>0.0002</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

Values are means \( \pm SD \). See Table 1 footnote for significance.

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conjugated alkaline phosphatase (BioGenex). Between the steps, the slides were subjected to two 5-min rinses with Tris-buffered saline. For antigen visualization, the Fast Red Substrate System (Dako) was used. Color development was stopped by addition of water, and, finally, sections were counterstained with hematoxylin. Negative controls were performed without the primary antibody.

The intensity of immunohistochemical staining was examined using light microscopy at ×400 magnification. Semiquantitative scoring (0 = no staining, 1 = weak staining, 2 = mild staining, 3 = strong staining, 4 = very strong staining) was performed as described elsewhere (10).

**Real-Time PCR**

Total RNA was isolated from whole kidneys using the SV Total RNA Isolation System (Promega) according to the manufacturer’s instructions.

### Table 4. Foot process and GBM morphology

<table>
<thead>
<tr>
<th>Group</th>
<th>GBM Covered by FP, %</th>
<th>No. of FP, µm⁻¹</th>
<th>Mean FP Width, nm</th>
<th>GBM Thickness, nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham-op + vehicle</td>
<td>72.4 ± 3.8</td>
<td>2.69 ± 0.30</td>
<td>272 ± 35</td>
<td>167 ± 23</td>
</tr>
<tr>
<td>Sham-op + R-568</td>
<td>75.2 ± 3.0</td>
<td>2.76 ± 0.48</td>
<td>289 ± 68</td>
<td>145 ± 6</td>
</tr>
<tr>
<td>Sham-op + calcitriol</td>
<td>76.4 ± 3.3</td>
<td>2.53 ± 0.35</td>
<td>323 ± 65</td>
<td>170 ± 10</td>
</tr>
<tr>
<td>SNX + vehicle</td>
<td>94.7 ± 2.6a,b,c</td>
<td>1.13 ± 0.44b,c</td>
<td>958 ± 364b,c</td>
<td>244 ± 6b,c</td>
</tr>
<tr>
<td>SNX + R-568</td>
<td>79.1 ± 3.3a,b,c,d</td>
<td>2.28 ± 0.20b,c,d</td>
<td>340 ± 36d</td>
<td>164 ± 10d</td>
</tr>
<tr>
<td>SNX + calcitriol</td>
<td>81.5 ± 3.0a,b,c,d</td>
<td>2.07 ± 0.30b,c,d</td>
<td>395 ± 66d</td>
<td>161 ± 5d</td>
</tr>
<tr>
<td>P (by ANOVA)</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>0.005</td>
</tr>
</tbody>
</table>

Values are means ± SD. GBM, glomerular basement membrane; FP, foot process. See Table 1 footnote for significance.

Fig. 1. Electron microscopic images of podocyte foot processes in vehicle-treated and sham-operated (A), R-568-treated and sham-operated (B), calcitriol-treated and sham-operated (C), vehicle-treated and subtotally nephrectomized (SNX, D), R-568-treated and SNX (E), and calcitriol-treated and SNX (F) animals. Magnification ×10,000.
instructions. RNA concentration was determined photometrically. Reverse transcription was performed with the first-strand cDNA synthesis kit (avian myeloblastosis virus, Roche Diagnostics, Switzerland) using 1 μg of RNA and random primers (3.2 μg final concentration). All PCR were performed on a LightCycler (Roche Diagnostics) using the LightCycler-Faststart DNA Master SYBR Green I kit (Roche Diagnostics). The samples were quantified by GAPDH expression. Primer sequences for TGF-β1 were 5'-CCACATCCCATGAGCATGACC-3' (forward) and 5'-TCATGTTGGACAACTGCTCC-3' (reverse). Specificity of the PCR was confirmed by melting curve analysis. Every sample was quantified using a gene-specific standard curve, and the mean value of three different PCR runs was taken for statistical evaluation.

**In Situ Hybridization**

CaSR mRNA expression was investigated by nonradioactive in situ hybridization. For synthesis of the RNA probe from the sequence NM_016996, the primers 3'-CCGATGACTTCTGGTCCAAT-5' (sense) and 3'-TCTTCAAAGATGCAGAAG-5' (antisense), resulting in a 339-bp PCR fragment, were prepared. The product was amplified with PCR, cleaned, and transfected into JM 109 cells (Promega) using the pGEM-T Easy vector system (Promega). The DNA sequence was cut with restriction enzymes Bca I (for sense sequence) and Nco I (for antisense sequence; Fermentas) and linearized, and the single-chain RNA probes were synthesized with T7 (sense) or Sp6 (antisense) RNA polymerase (Roche Diagnostics) and, finally, labeled with digoxigenin. In the situ hybridization was performed as described previously in detail (19) with probe concentration of 1 ng/μl.

**Statistical Analyses**

Values are means ± SD. After testing for normal distribution, we chose the Kruskal-Wallis test or ANOVA, followed by Duncan’s multiple-range test. Intraindividual changes of SBP and albumin excretion rate were analyzed using Wilcoxon’s matched-pairs test. The results were considered significant when P < 0.05.

**RESULTS**

**Animal Data, SBP, and Urinary Albumin Excretion Rate**

The weight of the left kidney remnants was equal in all SNX groups and significantly higher than the weight of the intact left kidneys of sham-operated animals. Food consumption and body weight were less in the sham-op + R-568 and SNX + R-568 groups. Serum creatinine was significantly higher in all SNX than sham-operated animals. No difference was observed between treatment groups (Table 1).

At the end of the study, SBP was higher in all SNX than sham-operated animals. R-568 or calcitriol had no significant effect on SBP by tail plethymography in sham-operated or SNX animals (Table 1).

Albumin excretion rate increased in all SNX animals at 4 wk (this was not significant, however, in the SNX + R-568 group) and further at 12 wk (in all groups). Albuminuria at 4 and 12 wk was significantly lower in the SNX + R-568 and SNX + calcitriol groups than in the SNX + vehicle group (Table 1). There was no significant difference between the SNX + R-568 and SNX + calcitriol group.

In addition to the experiment with ad libitum-fed rats (see above), a pair-feeding experiment was performed to exclude an artifact from malnutrition. Under the pair-feeding condition, albumin excretion was still significantly (P = 0.014) lower in the SNX + R-568 than in the SNX + vehicle group (2.1 ± 0.9 vs. 4.5 μg/24 h, n = 10).

**Calcium and Phosphorous Metabolism**

Serum calcium concentration did not change significantly in any group, but values tended to be higher in the calcitriol-treated animals. In the pair-feeding protocol, serum ionized calcium 2 h after calcitriol administration was significantly (P < 0.001) lower in the SNX + R-568 than in the SNX + vehicle group (1.11 ± 0.05 vs. 1.32 ± 0.04 mmol/l). Serum phosphorus concentration was significantly higher in the SNX + vehicle and SNX + calcitriol groups but significantly lower in the SNX + R-568 group. As a result, the calcium-phosphorus product was increased in the SNX + vehicle and SNX + calcitriol groups (7.48 ± 1.42 and 7.23 ± 0.97 mmol²/l², respectively) compared with the SNX + R-568 and sham-operated groups (6.58 ± 0.93 and 5.99 ± 0.87 mmol²/l², respectively, P = 0.005).

Serum 1,25(OH)₂D₃ concentration in vehicle-treated SNX rats was not different from that in sham-operated rats (30.8 ± 4.9 and 36.0 ± 12.9 ng/l, respectively). It was significantly (P < 0.001) higher in the SNX + calcitriol (54.6 ± 14.9 ng/l) than in the SNX + vehicle group and tended to be lower in the SNX + R-568 (18.2 ± 3.5 ng/l) than in the SNX + vehicle group.

Serum PTH was significantly higher in vehicle-treated SNX than in sham-operated animals and significantly lower in the SNX + R-568 and SNX + calcitriol groups than in the SNX + vehicle group (Table 1).

**Special Notations**

- **CaSR VDR**
- **Calcium and Phosphorous Metabolism**
- **In Situ Hybridization**
- **Statistical Analyses**

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**Table 5. Immunohistochemical and RNA in situ hybridization of CaSR and VDR in the kidney**

<table>
<thead>
<tr>
<th>Group</th>
<th>Glomeruli</th>
<th>Tubuli</th>
<th>Glomeruli</th>
<th>Tubuli</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham-op + vehicle</td>
<td>0.02 ± 0.04</td>
<td>0.12 ± 0.11</td>
<td>1.09 ± 0.16</td>
<td>0.44 ± 0.24</td>
</tr>
<tr>
<td>Sham-op + R-568</td>
<td>0.09 ± 0.05</td>
<td>1.03 ± 0.39</td>
<td>1.72 ± 0.30</td>
<td>0.44 ± 0.11</td>
</tr>
<tr>
<td>Sham-op + calcitriol</td>
<td>0.04 ± 0.06</td>
<td>0.18 ± 0.12</td>
<td>1.00 ± 0.14</td>
<td>0.62 ± 0.11</td>
</tr>
<tr>
<td>SNX + vehicle</td>
<td>0.01 ± 0.03</td>
<td>0.35 ± 0.20</td>
<td>0.82 ± 0.25</td>
<td>0.10 ± 0.09</td>
</tr>
<tr>
<td>SNX + R-568</td>
<td>0.20 ± 0.13</td>
<td>2.04 ± 0.53</td>
<td>1.79 ± 0.16</td>
<td>0.74 ± 0.11</td>
</tr>
<tr>
<td>SNX + calcitriol</td>
<td>0.08 ± 0.09</td>
<td>0.20 ± 0.20</td>
<td>0.93 ± 0.13</td>
<td>0.38 ± 0.14</td>
</tr>
<tr>
<td>P (by ANOVA)</td>
<td>&lt; 0.001</td>
<td>&lt; 0.001</td>
<td>&lt; 0.001</td>
<td>NS</td>
</tr>
</tbody>
</table>

Values are means ± SD. IHC, immunohistochemical; ISH, in situ hybridization; CaSR, calcium-sensing receptor; VDR, vitamin D receptor. See Table 1 footnote for significance.

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Urinary phosphorus excretion was significantly ($P < 0.001$) higher in the SNX + vehicle and SNX + calcitriol groups ($489 \pm 213$ and $473 \pm 206 \mu mol/24$ h, respectively) than in the SNX + R-568 group ($299 \pm 194 \mu mol/24$ h) and all sham-operated animals. Phosphorus excretion in the sham-op + R-568 group ($200 \pm 86 \mu mol/24$ h) also tended to be lower than in the sham-op + vehicle and sham-op + calcitriol groups ($290 \pm 70$ and $293 \pm 68 \mu mol/24$ h, respectively).

**Morphological Indexes of Renal Damage**

GSI and vascular damage index were significantly higher in the SNX + vehicle group than in sham-operated animals. GSI was significantly lower in the SNX + R-568 and SNX + calcitriol groups than in the SNX + vehicle group. The vascular damage index was significantly lower in the SNX + R-568 group, but not in the SNX + calcitriol group (Table 2). Tubular and interstitial damage scores were significantly higher in kidneys from the SNX + vehicle group than sham-operated animals. Tubular damage in the SNX + vehicle group comprised tubular dilatation, atrophy, desquamation of epithelial cells, and hyaline deposition in the tubular lumen. Calcitriol and R-568 significantly ameliorated tubular dilatation, atrophy, and desquamation of epithelial cells. In the SNX + R-568 and sham-op + R-568 groups, vacuolization of tubular epithelial cells was noted. Interstitial fibrosis and mononuclear cell infiltrates were less pronounced to a similar extent in the SNX + R-568 and SNX + calcitriol groups than in the SNX + vehicle group.

**Glomerular Geometry, Capillarization, and Glomerular Cells**

The extent of renal resection was well standardized, and the total number of glomeruli per kidney was significantly and equally reduced in all SNX animals compared with sham-operated animals (data not shown).

Mean glomerular volume and the relative volume of the mesangial matrix was greater in SNX than in sham-operated animals.
Table 6. Immunohistochemical staining for desmin, VEGF, collagen type IV, fibronectin, and TGF-β1 in glomeruli and tubulointerstitial tissues and TGF-β1 by RT-PCR in whole kidney samples

<table>
<thead>
<tr>
<th>Group</th>
<th>Desmin Positive Cells/Glomerulus</th>
<th>VEGF</th>
<th>Collagen IV</th>
<th>Fibronectin</th>
<th>TGF-β1</th>
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<tr>
<td></td>
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</tr>
<tr>
<td>Sham-op R-568</td>
<td>0.26±0.18</td>
<td>0.47±0.19</td>
<td>0.19±0.07</td>
<td>0.07±0.07</td>
<td>0.03±0.07</td>
</tr>
<tr>
<td>SNX + calcitriol</td>
<td>0.25±0.12</td>
<td>0.42±0.09</td>
<td>0.18±0.12</td>
<td>0.07±0.12</td>
<td>0.05±0.06</td>
</tr>
<tr>
<td>SNX</td>
<td>0.32±0.12</td>
<td>0.52±0.12</td>
<td>0.24±0.09</td>
<td>0.15±0.09</td>
<td>0.07±0.10</td>
</tr>
<tr>
<td>R-568</td>
<td>0.25±0.12</td>
<td>0.42±0.09</td>
<td>0.18±0.12</td>
<td>0.07±0.12</td>
<td>0.05±0.06</td>
</tr>
<tr>
<td>SNX + calcitriol</td>
<td>0.32±0.12</td>
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<td>0.15±0.09</td>
<td>0.07±0.10</td>
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<td>0.15±0.09</td>
<td>0.07±0.10</td>
</tr>
</tbody>
</table>

Values are means ± SD. TI, tubulointerstitial. TGF-β1, transforming growth factor-β1. See Table 1 footnote for significance.

Expression of CaSR and VDR

In tubular cells, protein expression of CaSR assessed by immunohistochemistry was significantly higher in sham-operated animals and the SNX + R-568 group than in any other group. Staining for CaSR was much weaker in glomeruli than in tubular epithelium. CaSR protein expression was significantly more pronounced in tubuli of the SNX + R-568 group than in any other group. Although staining tended also to be more pronounced in the sham-op + R-568 group than in the other sham-operated animals, the difference did not reach statistical significance.

The mRNA transcript for CaSR was present in tubular and glomerular cells of all animals (Table 5, Fig. 2). CaSR mRNA expression was slightly, but significantly, lower in glomeruli of the SNX + vehicle group than in sham-operated animals. CaSR expression was significantly higher in the sham-op + R-568 and SNX + R-568 groups than in vehicle- and calcitriol-treated animals.

VDR protein expression was lower in glomerular cells of the SNX + vehicle group than in sham-operated animals (Table 5) but was higher in the SNX + calcitriol group and even higher in the SNX + R-568 than in the SNX + vehicle group. VDR expression in tubular epithelial cells was not affected by SNX or any of the treatments.

Expression of Desmin and VEGF

The number of podocytes staining for desmin, a marker of injury, was markedly higher in the SNX + vehicle group than in sham-operated animals (Table 6). The number of desmin-positive cells per glomerulus was significantly lower in the SNX + R-568 and SNX + calcitriol groups than in the SNX + vehicle group.

Glomerular and tubulointerstitial VEGF expression was significantly higher in the SNX + vehicle group than in sham-operated animals and less elevated in the SNX + R-568 and SNX + calcitriol groups (Table 6).

Markers of Fibrosis

Immunostaining for collagen IV and fibronectin was used as an index of fibrosis. Collagen IV deposition was significantly greater in the SNX + vehicle group than in sham-operated animals but was lower in the SNX + R-568 and SNX + calcitriol groups than in the SNX + vehicle group (Table 3).

Capillary length density (Table 3), as well as total capillary length per kidney (data not shown), was significantly reduced after SNX. The length density of capillaries was almost normalized in the SNX + R-568 and SNX + calcitriol groups.

Significantly more mesangial and endothelial cells, as well as fewer podocytes, were noted in the SNX + vehicle group than in sham-operated animals (Table 4). In the SNX + R-568 and SNX + calcitriol groups, there were significantly fewer mesangial cells, but there was no difference in the number of endothelial cells (Table 4).

Mean podocyte volume was significantly higher in the SNX + vehicle group than in sham-operated animals. Electron microscopy revealed massive fusion of foot processes and thickening of GBM (Table 4, Fig. 1). These changes were not seen in the SNX + R-568 and SNX + calcitriol groups (Table 4, Fig. 1).
animals and less in the SNX + R-568 and SNX + calcitriol groups. Glomerular staining for fibronectin was higher in the SNX + vehicle group but less pronounced in the SNX + R-568 and SNX + calcitriol groups (Table 6).

Corresponding to the collagen type IV and fibronectin deposition, the expression of the profibrotic cytokine TGF-β1 was significantly more marked in glomeruli of the SNX + vehicle group than in sham-operated animals but less in the SNX + R-568 and SNX + calcitriol groups (Table 6). Expression of TGF-β1 in the tubulointerstitium was higher in the SNX + vehicle group than in sham-operated animals and the SNX + calcitriol group. Treatment with R-568 was associated with higher protein expression of TGF-β1 in sham-operated and SNX animals than in respective vehicle-treated groups.

The TGF-β1 mRNA transcript by RT-PCR in whole kidney tissue was higher in all SNX than sham-operated animals. Treatment had no apparent effect (Table 6).

Markers of Oxidative Stress and Endothelial Dysfunction

Staining for nitrotyrosine, a marker of oxidative stress-induced nitrosylation of proteins, was markedly higher in the glomeruli and tubulointerstitium of all SNX than sham-operated animals. Tubular staining for nitrotyrosine was lower in the SNX + R-568 and SNX + calcitriol groups than in the SNX + vehicle group (Table 7).

Expression of eNOS in glomeruli was lower in the SNX + vehicle group but not significantly different in the SNX + R-568 and SNX + calcitriol groups (Table 7) than in sham-operated animals. No difference in eNOS expression in the tubulointerstitium was observed.

Glomerular ET-1 expression was higher the SNX + vehicle group than in sham-operated animals but lower in the SNX + R-568 and SNX + calcitriol groups than in the SNX + vehicle group.

DISCUSSION

The present study was designed to compare the effects of treatment with the calcimimetic R-568 and calcitriol on albuminuria, kidney morphology, and progression of relevant molecules in the SNX rat. The calcimimetic R-568 and calcitriol were equally effective in reducing indexes of kidney damage. R-568 and calcitriol prevented podocyte loss and ultrastructural and caused better preservation of glomerular capillaries, as well as less glomerulosclerosis and tubulointerstitial damage.

A delayed and less intense rise of albumin excretion was achieved with R-568 and calcitriol, consistent with previous findings in experimental studies (17, 19) and clinical observations (2).

These effects were observed without lowering SBP (by tail plethymography), although hypotensive effects of calcimimetics had been described earlier with intra-arterial measurements (16).

The originally adopted protocol did not eliminate another confounding factor, i.e., differences in food consumption. The necessity to study six groups precluded a pair-feeding protocol, and, possibly as a result of the known nausea effect of calcimimetics, food intake in the R-568-treated animals (both SNX and sham-op) was lower, as also reflected by lower urinary phosphorus excretion rates. The antialbuminuric effect of R-568 was maintained even under pair-feeding conditions, indicating that dietary protein or caloric restriction was not the main mechanism of nephroprotection in this model.

The serum calcium concentration was not different between the groups when measured 24 h after drug administration. The decrease of serum calcium after R-568 administration is known to be transient (8). Under pair-feeding conditions, serum ionized calcium measured 2 h after R-568 administration was significantly lowered. At the dose used, calcitriol did not cause hypercalcemia, in agreement with previous experiments (13, 19). The serum phosphorous concentration and the calcium-phosphorus product were higher in the SNX + vehicle and SNX + calcitriol groups, respectively, than in the SNX + R-568 group. The effects observed in the SNX + R-568 group could not be explained by increased calcitriol, because its concentration tended to be lower in this group than in the SNX + vehicle group.

Reduction of nephron number leads to podocyte injury, which contributes to the development of glomerulosclerosis (15, 18). Our study is the first to show that treatment with the calcimimetic R-568 prevents podocyte loss and hypertrophy. Furthermore, R-568 prevented the changes of podocyte foot processes typical for the renal ablation model. This beneficial effect was also seen with calcitriol, confirming our previous observations (13).

Maintenance of podocyte foot process ultrastructure is an active process necessary for the filtration properties of these cells (15). Foot process fusion was associated with increased proteinuria in vehicle-treated SNX animals, in contrast to the preservation of podocyte foot process ultrastructure in the SNX + R568 and SNX + calcitriol groups. In parallel, expression of desmin, a marker of podocyte stress (7, 13), was

Table 7. Immunohistochemical staining for nitrotyrosine, eNOS, and ET-1 in glomeruli and tubulointerstitium

<table>
<thead>
<tr>
<th>Group</th>
<th>Glomeruli</th>
<th>TI</th>
<th>Glomeruli</th>
<th>TI</th>
<th>Glomeruli</th>
<th>TI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham-op + vehicle</td>
<td>0.09±0.13</td>
<td>0.18±0.15</td>
<td>1.01±0.43</td>
<td>1.63±0.27</td>
<td>0.18±0.15</td>
<td>0.29±0.20</td>
</tr>
<tr>
<td>Sham-op + R-568</td>
<td>0.05±0.07</td>
<td>0.24±0.17</td>
<td>0.69±0.16</td>
<td>1.66±0.10</td>
<td>0.13±0.10</td>
<td>0.42±0.31</td>
</tr>
<tr>
<td>Sham-op + calcitriol</td>
<td>0.05±0.07</td>
<td>0.10±0.08</td>
<td>1.12±0.50</td>
<td>1.53±0.25</td>
<td>0.17±0.15</td>
<td>0.19±0.20</td>
</tr>
<tr>
<td>SNX + vehicle</td>
<td>1.17±0.18</td>
<td>1.87±0.35</td>
<td>0.28±0.18</td>
<td>1.50±0.27</td>
<td>1.22±0.34</td>
<td>1.41±0.54</td>
</tr>
<tr>
<td>SNX + R-568</td>
<td>1.07±0.26</td>
<td>1.26±0.14</td>
<td>1.24±0.57</td>
<td>1.72±0.40</td>
<td>0.45±0.29</td>
<td>1.47±0.23</td>
</tr>
<tr>
<td>SNX + calcitriol</td>
<td>0.75±0.33</td>
<td>1.02±0.40</td>
<td>0.82±0.30</td>
<td>1.30±0.33</td>
<td>0.48±0.10</td>
<td>0.68±0.21</td>
</tr>
<tr>
<td>P (by ANOVA)</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>NS</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Values are means ± SD. eNOS, endothelial nitric oxide synthase; ET-1, endothelin-1. See Table 1 footnote for significance.
seen in the SNX + vehicle group, but not in the SNX + R-568 and SNX + calcitriol groups.

This study also provides molecular information pointing to the mechanisms underlying the renoprotective effects of R-568 and calcitriol. As potential mechanisms, we considered reduction of profibrotic cytokines, reduction of oxidative stress, lowering of PTH, and reversal of endothelial cell dysfunction.

In the glomeruli of SNX animals, we observed a parallel increase of staining for TGF-β/H9252, decrease in collagen IV deposition and GBM thickness. In cultured podocytes, collagen IV synthesis is increased when VEGF signaling is stimulated (5). The finding of more glomerular collagen IV deposition and GBM thickening in uremic animals is interpreted as a response to injury (11) that is obviously ameliorated by R-568 or calcitriol.

The role of oxidative stress is less clear. In the tubulointerstitial space, both interventions reduced staining for nitrotyrosine as a marker of oxidative stress, whereas in the glomeruli, a modest reduction was seen with calcitriol only.

Since both interventions reduced PTH concentration, this must also be discussed as one potential mechanism underlying the renoprotective properties of R-568 and calcitriol, particularly since PTH receptors are expressed by podocytes in vitro (12). Furthermore, in this model, Ogata et al. (17) showed that the effects of the calcimimetic agent and parathyroidectomy are comparable.

Finally, endothelial dysfunction is a well-known consequence of glomerular damage (23). Relevant in this context is that, in glomeruli (but not in the tubulointerstitium) of SNX animals, eNOS expression was diminished and ET-1 expression was increased, as was VEGF expression, in podocytes. These abnormalities were improved by R-568 and calcitriol.

Since glomeruli are known to express CaSR and VDR, an obvious issue was whether expression of these receptors was altered by R-568 and calcitriol, respectively.

In SNX animals, reduction of CaSR was observed in glomeruli and tubulointerstitium by immunohistochemistry and in situ hybridization, respectively. R-568 increased glomerular expression of CaSR by ion situ hybridization in sham-operated and SNX animals. The same was seen in the tubulointerstitium by immunohistochemistry. The changes in glomerular CaSR expression correspond to changes observed by others in the parathyroid glands (9, 14).

In SNX animals, glomerular (but not tubulointerstitial) expression of VDR was diminished. Interestingly, calcitriol and R-568 upregulated glomerular expression of VDR. Upregulation of VDR by the calcimimetic R-568 is of interest, suggesting that treatment with calcimimetics + vitamin D analogs may have additive beneficial effects.

It is unclear whether the findings obtained with R-568 can be extrapolated to cinacalcet, the agent available for use in humans, but this possibility appears somewhat remote.

In conclusion, in an established renal damage model, a calcimimetic and the active vitamin D calcitriol were similarly effective in lowering PTH and ameliorating albuminuria, as well as morphological abnormalities of the kidneys. Whether the beneficial effect of both PTH-lowering agents is mediated exclusively via reduction of PTH cannot be decided on the basis of this study, but differential effects of the two agents on receptor expression suggest some intrinsic effects independent of PTH.

ACKNOWLEDGMENTS

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REFERENCES


