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

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Running title: Nephroprotective action of calcimimetic and calcitriol

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Abstract

Patients with renal insufficiency develop secondary hyperparathyroidism (sHPT). Monotherapy with either active vitamin D or calcimimetics ameliorates sHPT. We compared kidney damage in subtotally nephrectomized (SNX) rats treated with active vitamin D (calcitriol) or the calcimimetic R-568. Male Sprague-Dawley rats were subtotally nephrectomized (SNX), or sham-operated (sham-op) and subsequently randomized into the following treatment groups: SNX+R-568, SNX+calcitriol, SNX+vehicle, sham-op+R-568, sham-op+calcitriol, and sham-op+vehicle. Albuminuria and blood pressure were monitored and kidneys were examined using morphometry, immunohistochemistry, quantitative RT-PCR, and in-situ hybridization. PTH concentrations were lowered to the same extent by the two interventions, although phosphorus and Ca x P levels were reduced only by R-568 treatment. SNX rats developed marked albuminuria which was significantly reduced in fed ad libitum and pair-fed animals treated with R-568 and animals treated with calcitriol. Mean glomerular volume (6.05±1.46 vs. 2.70±0.91 mm³) and podocyte volume (831±127 vs. 397±67 µm³), the degree of foot processes fusion (mean width of foot processes 958±364 vs. 272±35 nm) and GBM thickness (244±6 vs. 267±23 nm), as well as desmin staining were significantly higher in vehicle treated SNX compared with sham-op animals. These changes were ameliorated both with R-568 and calcitriol. In SNX as well as in sham-op animals, expression of the calcium sensing receptor (protein and mRNA) was upregulated by the treatment with the calcimimetic, but not with calcitriol. Calcitriol and R-568 were similarly effective in ameliorating kidney damage.

Key words: secondary hyperparathyroidism, chronic renal failure, nephroprotection, calcimimetics, calcitriol
Introduction

Secondary hyperparathyroidism (sHPT) is a common feature of chronic kidney disease. PTH concentrations increase progressively with diminishing glomerular filtration (GFR), but it is currently 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 or, in humans, Cinacalcet HCl), allosteric activators of the calcium sensing receptor which reduce PTH secretion and interfere with parathyroid hyperplasia (4, 20, 22). In addition, however, Ogata et al. showed that short-term treatment with R-568 reduces albuminuria and attenuates glomerular and tubulointerstitial lesions in subtotally nephrectomised rats (17).

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

Animals and experimental design

Twelve-week-old male Sprague-Dawley (SD) rats weighing 331±85 g were used (Charles River, Germany). All animals were housed under constant room temperature (22±1°C), and relative humidity (75±5%), and exposed to 12 hours light on – 12 hours light off cycle, had free access to water and were fed a standard rodent diet without vitamin D (19.0% protein, 4.0% fat, 0.9% calcium, 0.7% phosphorus) (Sniff, Germany). The animals were handled in accordance with the German law for protection of animals.

After a 7-days adaptation period, rats were randomly allotted to subtotal nephrectomy (SNX) (n=54) or sham operation (n=51). As described before (3) the rats underwent two-step subtotal nephrectomy. Under isoflurane anesthesia (Isoflurane, Baxter, Germany) 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 into following study groups:

1. sham-operated, treated with vehicle alone (sham-op+vehicle, n=16)
2. sham-operated, treated with R-568 (sham-op+R568, n= 15)
3. sham-operated, treated with calcitriol (sham-op+calcitriol, n= 18)
4. SNX, treated with vehicle alone (SNX+vehicle, n= 17)
5. SNX, treated with R-568 (SNX+R568, n= 18)
6. SNX, treated with calcitriol (SNX+calcitriol, n= 17)

The calcimimetic agent R-568 (Amgen, Thousand Oaks, USA) was diluted in 10% 2-hydroxypropyl-β-cyclodextrin, (Sigma-Aldrich, Germany) and administered every 24 hours (17 mg/kg body weight) by subcutaneous injection. The active vitamin D metabolite calcitriol (Calbiochem,
Germany) was administered subcutaneously (135 ng/kg b.w./day). The control animals received s.c. injections of vehicle alone. The treatment was continued for 12 weeks. Body weight was measured every week and the dosing was adjusted. Systolic blood pressure (SBP) was measured by tail plethysmography 24 hours after the administration of medication at week 12. At week 4 and 12 the animals were kept in metabolic cages for 24-h urine collection.

In an additional experiment SNX rats treated with R-568 were pair-fed with vehicle treated SNX. Urine was collected for 24 hours at week 4 post-op. 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 abdominal aorta before sacrifice by standard laboratory methods. Serum 1,25(OH)2D3 concentration was measured by RIA method. Serum PTH level was measured by the two-antibody method using a rat iPTH ELISA kit (Immutopics, Inc., San Clemente, USA).

**Tissue Preparation**

Twelve weeks after surgery the abdominal aorta was catheterized under ketamine/xylazine anesthesia (100 mg/kg, and 3 mg/kg respectively), blood samples were taken, and the experiment was terminated by the retrograde aortic perfusion. All blood samples were collected 24 hours after the last injection of calcitriol, R-568, or vehicle. For morphometric and stereological investigations, randomly chosen 8-9 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 slices of 1-mm width. 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). Five of the resin blocks were randomly chosen, from which semithin sections (0.5 µm) 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 indices:* The glomerulosclerosis index was assessed on PAS-stained paraffin sections according to the semiquantitative scoring system (scores: 0-4) proposed by El Nahas et al. (6). Using light microscopy and a magnification of x400 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, evaluating every field of view at x400 magnification. Tubular damage was scored separately as follows:

- **tubular dilatation:** none (0), dilated tubuli (1);
- **tubular atrophy:** no alteration (0), signs of atrophy (1), apoptosis and desquamation of cells (2);
- **intracellular vacuoles:** none (0), mild, less than 10 cells per field of view involved (1), severe, (2);
- **hyalin** none (0), present (1);
**interstitial fibrosis**: none (0), mild (1), severe (2);

**mononuclear cell infiltration**: none (0), mild (1), severe (2).

Tubular and interstitial scores were expressed as the arithmetic mean of all fields. The vascular damage score was assessed at x100 magnification and the following scheme was adopted: grade 0, no wall thickening; grade 1, mild wall thickening; grade 2, moderate wall thickening; grade 3, severe wall thickening; grade 4, fibrinoid necrosis of the vascular wall.

**Glomerular geometry**: Area density \( A_A \) as an index of volume density \( V_V \) of renal cortex and medulla as well as the number of glomeruli per area \( N_A \) were measured using a Zeiss eyepiece (Integrationsplatte II; Zeiss Co., Oberkochen, Germany) and the point counting method \( (P_P = A_A = V_V) \) at a magnification of x40. All glomeruli on one PAS-stained section were counted to calculate glomerular volume density, area density, and tuft volume. Such a section represents several equidistant slices of the kidney and yields a representative sample of all kidney areas. The measured area of the kidney section was then corrected for tissue shrinkage \( (1.082) \). Total cortex volume \( V_{cortex} \) was calculated from kidney mass and specific weight of the kidney \( (1.04 \, g/cm^3) \). Glomerular geometry was analyzed as follows: Volume density \( V_V \) of glomeruli and tubulointerstitium as well as the area density of the glomerular tuft \( A_{AT} \) were measured by point counting at a magnification of x100 on the same PAS-stained section. The total area of the glomerular tuft \( A_T \) was then determined as \( A_T = A_{AT} \times A_{cortex} \). The number of glomeruli per volume and per kidney as well as mean glomerular volume were calculated as described before \( (1) \).

**Analysis of Glomerular Capillarization and Cellularity**: On five semithin sections per animal, glomerular capillarization and cellularity were analyzed using the point counting method and a 100 point eyepiece (Integrationsplatte II; Zeiss Co.) at a magnification of x1000 (oil immersion) as described previously \( (3) \). Briefly, the length density \( L_V \) of glomerular capillaries (capillary length in a tuft volume in \( mm/mm^3 \)) was determined according to the standard stereological formula, \( L_V = 2Q_A \) (with
Q_A being the number of capillary transects per area of the capillary tuft) (3). The total length of glomerular capillaries per one kidney (L_total) was then derived from L_V and the total glomerular volume (V_{glom}) with V_{glom} = V_{Vglom} \times V_{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 by stereological technique in at least 30 randomly selected glomeruli per animal based on cell density per volume (N_cV) and volume density of the cell type (V_cV) as previously described (1).

**Analysis of foot processes morphology:** Podocyte foot processes were visualized by electron microscopy (Zeiss, Germany). Forty images per animal were analyzed (magnification x10 000). Thickness of 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, Germany)

**Immunohistochemistry**

Four-µm thick sections of formalin-fixed, paraffin-embedded tissue were mounted on silane-coated slides, deparaffinated with xylene and rehydrated through descending concentrations of ethanol. After antigen retrieval (heating the slides in the Target Unmasking Fluid (Pan Path, The Netherlands) at 80°C for 20 minutes immunohistochemical staining was performed using the streptavidin-biotin method. The following primary antibodies were applied for 60 minutes at room temperature: monoclonal mouse anti-desmin (1:50 dilution; DAKO, Germany), rabbit polyclonal anti-fibronectin (1:500, Sigma, Germany), rabbit polyclonal anti-collagen type IV (1:40, Biotrend, Germany), rabbit polyclonal anti-transforming growth factor beta 1 (TGF-β_1) (1:50, Santa Cruz Biotechnologies, USA), rabbit polyclonal anti-endothelial nitric oxide synthase (eNOS) (1:200; Affinity BioReagents, USA), sheep polyclonal anti-nitrotyrosine (1:400, Oxis Research, USA), mouse monoclonal anti-endothelin-1 (1:20; Affinity BioReagents, USA), rabbit polyclonal anti-vascular endothelial growth factor (VEGF) (1:25; idLabs,
Canada), rabbit polyclonal anti-calcium sensing receptor (CaSR) (1:200; Affinity BioReagents, USA). Appropriate biotinylated secondary antibodies (anti-rabbit: BioGenex, USA; anti-mouse: DCS Innovative Diagnostik-Systeme, Germany; anti-sheep: Jackson ImmunoResearch, USA) were applied for 20 minutes and were followed by the streptavidin-conjugated alkaline phosphatase (BioGenex, USA). The slides were rinsed for 2 x 5 minutes with Tris-buffered saline between the steps. For antigen visualization Fast Red substrate (DAKO, Germany) was used. Color development was stopped by adding water, and finally sections were counterstained with hematoxylin. Negative controls were performed by omitting the primary antibody.

The intensity of immunohistochemical staining was examined using light microscopy at a magnification of x400. Semiquantitative scoring (scores 0-4; 0: no staining, 1: weak, 2: mild, 3: strong, 4: very strong staining) was performed as described before (10).

Real time PCR

Total RNA was isolated from whole kidneys using SV Total RNA Isolation System (Promega, Germany) according to the manufacturer’s instructions. RNA concentration was determined photometrically. Reverse transcription was performed with the 1st Strand cDNA Synthesis Kit (AMV) (Roche Diagnostics, Switzerland) using 1 µg RNA and random primers (final concentration: 3.2 µg). All PCR reactions were performed on a LightCycler (Roche Diagnostics, Switzerland) using the LightCycler-Faststart DNA Master SYBR Green I Kit (Roche Diagnostics, Switzerland). The samples were quantified by normalization to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression. Primer sequences for TGF-β1 were: forward 5’-CACCATCCATGACATGAACC-3’, reverse 5’-TCATGTTGGACAACTGCTCC-3’. Specificity of the PCR reaction was confirmed with 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.

To investigate the calcium sensing receptor (CaSR) mRNA expression, nonradioactive in-situ hybridization was performed. For synthesis of the RNA probe from the sequence NM_016996 following primers were prepared: sense: 3' CGGATGACTTCTGGTCCAAT-5'; antisense: 3'-TCTTCACCAAGATGCACGAG-5', resulting in a PCR fragment of 339 bp. The product was amplified with PCR, cleaned and transfected into JM 109 cells (Promega, Germany) using the pGEM T easy Vector system (Promega, Germany). The DNA sequence was cut with restriction enzymes Bcu I (for sense sequence) and Nco I (for antisense sequence) (Fermentas, Germany), linearized, and the single-chain RNA probes were synthesized with T7 (sense), or Sp6 (antisense) RNA polymerases (Roche Diagnostics, Switzerland), and finally labeled with digoxigenine. The in-situ hybridization was performed as described previously in detail (19) with probe concentration 1 ng/μl.

Statistical Analyses

All data are presented as mean±SD. After testing for normal distribution, the Kruskal-Wallis test or ANOVA was chosen, followed by a Duncan multiple range test. Intraindividual changes of systolic BP and albumin excretion rate were analyzed using the Wilcoxon matched pairs test. The results were considered significant when P < 0.05.
Results

Animal Data, Systolic Blood Pressure, 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 R-568 treated sham-op and SNX animals. Serum creatinine was significantly higher in all SNX groups compared with sham operated ones. No difference was observed between treatment groups (table 1).

At the end of the study, systolic blood pressure was higher in all SNX groups compared with sham operated animals. Treatment with R-568 or calcitriol had no significant effect on SBP by tail plethymography in either sham-op or SNX animals (table 1).

The albumin excretion rate increased in all SNX groups at 4 weeks (this was not significant, however, in SNX + R-568) and further at 12 weeks (in all groups). Albuminuria at week 4 and at week 12 was significantly lower in SNX + R-568 and SNX + calcitriol compared to SNX + vehicle (table 1). There was no significant difference between SNX + R-568 and SNX + calcitriol.

In addition to the above experiment with ad libitum fed rats, a pair-feeding experiment was performed to exclude an artifact from malnutrition. Under conditions of pair-feeding albumin excretion in R-568 treated SNX (2.1±0.9 µg/24h; n=10) was still significantly (p=0.014) lower compared to vehicle treated SNX (4.5 µg/24h; n=10).

Calcium and Phosphorous Metabolism

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

Serum 1,25(OH)₂D₃ concentration in vehicle treated SNX (30.8±4.9 ng/l) was not different from sham-op (36.0±12.9). Compared to SNX + vehicle it was significantly (p<0.001) higher in SNX + calcitriol (54.6±14.9) and tended to be lower compared to SNX + vehicle in SNX + R-568 (18.2±3.5).

Serum PTH was significantly higher in vehicle-treated SNX compared with sham-op animals, and significantly lower in SNX + R-568 and SNX + calcitriol animals compared with vehicle-treated SNX animals (table 1).

Urinary phosphorus excretion was significantly (p<0.001) higher in SNX + vehicle (489±213) and SNX + calcitriol (473±206) compared to SNX + R-568 (299±194 µmol/24h) and all sham-op animals. Phosphorus excretion in sham-op + R-568 (200±86) also tended to be lower than in sham-op + vehicle (290±70) and sham-op + calcitriol (293±68).

Morphologic indices of renal damage

The glomerulosclerosis (GSI) and the vascular damage (VDI) indices were significantly higher in vehicle treated SNX compared to sham-op animals. The GSI was significantly lower in SNX animals treated with R-568 and with calcitriol, respectively, than in vehicle treated SNX animals. The VDI was significantly lower in SNX + R-568, but not in SNX animals treated with calcitriol (table 2). Both tubular and interstitial damage scores were significantly higher in kidneys from vehicle treated SNX compared with sham-op animals. Tubular damage in vehicle treated SNX animals comprised tubular dilatation, atrophy, desquamation of epithelial cells, and hyalin in the tubular lumen. Both calcitriol and R-568 treatment significantly ameliorated tubular dilatation, atrophy, and desquamation of epithelial
cells. In the SNX and sham-op groups treated with the R-568, vacuolization of tubular epithelial cells was noted. Interstitial fibrosis and mononuclear cell infiltrates were less pronounced to a similar extent in SNX animals treated with R-568 and with calcitriol, respectively, compared with vehicle treated SNX animals.

**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 groups compared with sham-op (data not shown).

Mean glomerular volume and the relative volume of mesangial matrix was higher in SNX compared with sham-op animals, but was lower in SNX treated with R-568 and calcitriol, respectively, compared with vehicle treated SNX (table 3).

The capillary length density (table 3) as well as the total capillary length per kidney (data not shown) was significantly reduced after SNX. The length density of capillaries was almost normalized in both treated SNX groups.

A significantly higher number of mesangial and endothelial cells as well as a lower number of podocytes were noted in vehicle treated SNX compared with sham-op animals (table 4). In SNX animals treated with R-568 and calcitriol, respectively, the number of mesangial cells was significantly lower, but there was no difference in the number of endothelial cells (table 4).

Compared with sham-op animals, the mean podocyte volume was significantly higher in vehicle treated SNX animals. Electron-microscopy revealed massive fusion of foot processes and thickening of glomerular basement membranes (table 4, figure 1). These changes were not seen in SNX animals treated with R-568 and calcitriol, respectively (table 4, figure 1).
Expression of Ca sensing receptor (CaSR) and vitamin D receptor (VDR)

In tubular cells of sham-op and SNX animals treated with R-568, the protein expression of CaSR assessed by immunohistochemistry was significantly higher than in any other group. The staining for CaSR was much weaker in glomeruli than in tubular epithelium. The CaSR protein expression was significantly more pronounced in tubuli of SNX+R-568 than of any other group. Although the staining in sham-op+R-568 animals tended also to be more pronounced than in the other sham-op groups, 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 vehicle-treated SNX animals compared to sham-op. CaSR expression was significantly higher in sham-op+R-568 animals as well as in SNX+R-568 compared with both vehicle-treated and calcitriol-treated groups.

VDR protein expression was lower in glomerular cells of SNX+vehicle compared to sham-op animals (table 5), but was higher in SNX+calcitriol and even more so in SNX+R-568 animals compared with vehicle treated SNX animals. The expression of VDR 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 vehicle treated SNX compared with sham-op animals (table 6). The number of desmin-positive cells per glomerulus was significantly lower in SNX + R-568 and SNX + calcitriol compared with SNX + vehicle animals.

Glomerular and tubulointerstitial VEGF expression was significantly higher in SNX + vehicle than in sham-op animals and less elevated in SNX + R-568 and SNX + calcitriol animals (table 6).
Markers of fibrosis

Immunostaining for collagen IV and fibronectin was used as an index of fibrosis. Collagen IV deposition was significantly higher in SNX + vehicle compared to sham-op animals and was less in SNX + R-568 and SNX + calcitriol animals. Glomerular staining for fibronectin was higher in SNX + vehicle, but less pronounced in SNX + R-568 and SNX + calcitriol, (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 SNX + vehicle compared with sham-op animals, but less in SNX + R-568 and SNX + calcitriol animals (table 6). The expression of TGF-β1 in the tubulointerstitium was higher in SNX + vehicle compared with sham-op and SNX + calcitriol animals. Treatment with R-568 was associated with higher protein expression of TGF-β1 both in sham-op and SNX animals as compared with respective vehicle-treated groups.

The TGF-β1 mRNA transcript by RT-PCR in whole kidney tissue was higher in all SNX groups compared with sham-op animals. Treatment had no apparent effect (table 6).

Markers of oxidative stress and endothelial dysfunction

The staining for nitrotyrosine, a marker of oxidative stress-induced nitrosylation of proteins, was markedly higher in the glomeruli and tubulointerstitium of all SNX compared with sham-op groups. Tubular staining for nitrotyrosine was lower in SNX + R-568 and SNX + calcitriol animals compared with SNX + vehicle animals (table 7).

Expression of the endothelial nitric oxide synthase (eNOS) in glomeruli was less in SNX + vehicle animals but not significantly different in SNX + R-568 and SNX + calcitriol animals (table 7) compared to sham-op animals. No difference in eNOS expression in tubulointerstitium was observed.
Glomerular *endothelin-1 (ET-1)* expression was higher SNX + vehicle than in sham-op animals, but was lower in SNX + R-568 and SNX + calcitriol than in SNX + vehicle animals.
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 relevant molecules in the subtotally nephrectomised rat. The study documented that treatment with either the calcimimetic R-568 or calcitriol is equally effective in reducing indices of kidney damage.

Both R-568 and calcitriol prevented podocyte loss and ultrastructure 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 both R-568 and calcitriol respectively, consistent with previous findings in experimental studies (17, 19), and clinical observations (2).

These effects were observed without lowering the systolic blood pressure by tail plethymography, although hypotensive effects of calcimimetics had been described earlier with intraarterial measurements (16).

The originally adopted protocol did not eliminate another confounding factor, however, i.e. differences in food consumption. The necessity to study 6 groups precluded a pair-feeding protocol and, possibly as a result of the known nauseating 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 conditions of pair-feeding 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 24h post-dosing. The decrease of serum calcium after the administration of R-568 is known to be transient (8). Under conditions of pair-feeding serum ionized calcium measured 2h post-dose was significantly lowered by R-568. At the dose used, calcitriol did not cause hypercalcemia in agreement with previous experiments (13, 19). The serum phosphorous concentration and the Ca x P product were higher in SNX
animals treated with vehicle and calcitriol respectively than in SNX animals treated with R-568. The effects observed in SNX + R-568 group could not be explained by increased calcitriol because its concentration tended to be lower in this group compared to vehicle treated SNX.

Reduction of nephron number leads to podocyte injury and this 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 treatment 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 SNX+R568 and SNX+calcitriol. In parallel, expression of desmin, a marker of podocyte stress (7, 13), was seen in vehicle treated SNX, but not in SNX+R568 and SNX+calcitriol animals.

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 pro-fibrotic 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-β1, VEGF and collagen IV. Downregulation of TGF-β1 and VEGF in uremic animals treated with R-568 and calcitriol respectively was associated with decreased collagen IV deposition and lower thickness of the GBM. In cultured podocytes collagen IV synthesis is increased when VEGF signaling is stimulated (5). The finding of more glomerular collagen IV deposition and thickening of GBM in uremic animals is interpreted as a response to injury (11) which is obviously ameliorated by R-568 or calcitriol.
The role of oxidative stress is less clear. In the tubulointerstitial space both interventions reduced increased staining for nitrotyrosine as a marker of oxidative stress, while in the glomeruli modest reduction was seen with calcitriol only.

Since both interventions reduced the 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. had shown that the effects of the calcimimetic agent and of parathyroidectomy are comparable (17).

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 the expression of eNOS was diminished and that of endothelin-1 increased as was the expression of VEGF by podocytes. These abnormalities were improved by R-568 and calcitriol.

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

In SNX animals, the CaSR was reduced in glomeruli and in the tubulointerstitium by immunohistochemistry and in-situ hybridisation respectively. R-568 increased glomerular expression of the CaSR by ion situ hybridisation both in sham-op and in SNX animals. The same was seen in the tubulointerstitium by immunohistochemistry. The changes in glomerular CaSR expression correspond to what had been observed by others in the parathyroid glands (9, 14).

In SNX animals, the glomerular (but not the tubulointerstitial) expression of VDR was diminished. Interestingly both calcitriol and R-568 upregulated the glomerular expresison of VDR in the glomerulus. The upregulation of VDR by the calcimimetic R-568 is of interest suggesting that co-treatment with both calcimimetics and 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 in humans, but this possibility appears somewhat remote.
In conclusion, in an established renal damage model, both a calcimimetic and the active vitamin D calcitriol were similarly effective in lowering PTH and in ameliorating albuminuria as well as morphological abnormalities of the kidneys. Whether the beneficial effect of both PTH lowering agents is mediated exclusively via lowering 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.
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References:


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

<table>
<thead>
<tr>
<th>Group</th>
<th>Final body weight [g]</th>
<th>Left kidney (remnant) weight [g]</th>
<th>Systolic blood pressure [mmHg]</th>
<th>Serum creatinine [mg/dl]</th>
<th>Serum calcium [mmol/l]</th>
<th>Serum phosphorus [mmol/l]</th>
<th>Serum PTH [pg/ml]</th>
<th>Urinary albumin excretion at week 4 [mg/24h]</th>
<th>Urinary albumin excretion at week 12 [mg/24h]</th>
<th>ANOVA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham-op + vehicle</td>
<td>506±37</td>
<td>2.3±0.4</td>
<td>125±23</td>
<td>0.55±0.09</td>
<td>2.64±0.18</td>
<td>2.28±0.31</td>
<td>392±218</td>
<td>0.4±0.3</td>
<td>0.4±0.2</td>
<td>P&lt;0.001</td>
</tr>
<tr>
<td>Sham-op + R-568</td>
<td>444±37&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.9±0.2</td>
<td>135±17</td>
<td>0.50±0.06</td>
<td>2.56±0.21</td>
<td>2.41±0.22</td>
<td>288±155</td>
<td>0.3±0.2</td>
<td>0.6±0.4</td>
<td>P=0.001</td>
</tr>
<tr>
<td>Sham-op + calcitriol</td>
<td>498±32&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.3±0.3</td>
<td>125±12</td>
<td>0.61±0.08</td>
<td>2.78±0.15</td>
<td>2.21±0.28</td>
<td>366±266</td>
<td>0.8±0.4</td>
<td>0.5±0.2</td>
<td>NS</td>
</tr>
<tr>
<td>SNX + vehicle</td>
<td>462±41&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.7±0.4&lt;sup&gt;abc&lt;/sup&gt;</td>
<td>168±20&lt;sup&gt;abc&lt;/sup&gt;</td>
<td>1.05±0.36&lt;sup&gt;abc&lt;/sup&gt;</td>
<td>2.66±0.25</td>
<td>2.78±0.56&lt;sup&gt;abc&lt;/sup&gt;</td>
<td>1739±1023&lt;sup&gt;abc&lt;/sup&gt;</td>
<td>15.3±7.7&lt;sup&gt;abc&lt;/sup&gt;</td>
<td>39.0±24.8&lt;sup&gt;abc&lt;/sup&gt;</td>
<td>p&lt;0.05</td>
</tr>
<tr>
<td>SNX + R-568</td>
<td>416±55&lt;sup&gt;abcd&lt;/sup&gt;</td>
<td>2.6±0.5&lt;sup&gt;abc&lt;/sup&gt;</td>
<td>169±12&lt;sup&gt;abc&lt;/sup&gt;</td>
<td>1.16±0.32&lt;sup&gt;abc&lt;/sup&gt;</td>
<td>2.68±0.22</td>
<td>2.46±0.29&lt;sup&gt;d&lt;/sup&gt;</td>
<td>267±163&lt;sup&gt;d&lt;/sup&gt;</td>
<td>2.8±2.7&lt;sup&gt;d&lt;/sup&gt;</td>
<td>17.9±12.7&lt;sup&gt;abcd&lt;/sup&gt;</td>
<td>p&lt;0.05</td>
</tr>
<tr>
<td>SNX + calcitriol</td>
<td>486±40&lt;sup&gt;de&lt;/sup&gt;</td>
<td>2.3±0.3&lt;sup&gt;abc&lt;/sup&gt;</td>
<td>158±21&lt;sup&gt;abc&lt;/sup&gt;</td>
<td>1.09±0.30&lt;sup&gt;abc&lt;/sup&gt;</td>
<td>2.73±0.13</td>
<td>2.66±0.37&lt;sup&gt;ac&lt;/sup&gt;</td>
<td>332±87&lt;sup&gt;d&lt;/sup&gt;</td>
<td>4.5±2.3&lt;sup&gt;abcd&lt;/sup&gt;</td>
<td>16.6±10.7&lt;sup&gt;abcd&lt;/sup&gt;</td>
<td>p&lt;0.05</td>
</tr>
</tbody>
</table>

ANOVA P=0.001 NS P<0.001 P<0.001 NS P<0.001 P<0.001 P<0.001 P<0.001 P<0.001

NS – not significant
<sup>a</sup>p<0.05 vs. Sham-op+vehicle
<sup>b</sup>p<0.05 vs. Sham-op+R-568
<sup>c</sup>p<0.05 vs. Sham-op+calcitriol
<sup>d</sup>p<0.05 vs. SNX+vehicle
<sup>e</sup>p<0.05 vs. SNX+R-568
### Table 2. Morphologic indices of kidney damage

<table>
<thead>
<tr>
<th>Group</th>
<th>Glomerulo-sclerosis index (GSI)</th>
<th>Vascular damage index (VDI)</th>
<th>Tubular damage</th>
<th>Interstitial damage</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Dilatation</td>
<td>Atrophy</td>
</tr>
<tr>
<td>Sham-op +vehicle</td>
<td>0.13±0.04</td>
<td>0.03±0.01</td>
<td>0.00</td>
<td>0.00</td>
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<tr>
<td>Sham-op +R-568</td>
<td>0.10±0.03</td>
<td>0.03±0.02</td>
<td>0.03±0.02</td>
<td>0.00</td>
</tr>
<tr>
<td>Sham-op +calcitriol</td>
<td>0.18±0.09</td>
<td>0.08±0.17</td>
<td>0.03±0.01</td>
<td>0.03±0.01</td>
</tr>
<tr>
<td>SNX +vehicle</td>
<td>1.66±0.08abc</td>
<td>0.26±0.14abc</td>
<td>0.88±0.14abc</td>
<td>0.51±0.21abc</td>
</tr>
<tr>
<td>SNX +R-568</td>
<td>1.04±0.23abcd</td>
<td>0.10±0.00d</td>
<td>0.48±0.29abcd</td>
<td>0.25±0.17abcd</td>
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<tr>
<td>SNX +calcitriol</td>
<td>0.97±0.44abcd</td>
<td>0.14±0.07ab</td>
<td>0.26±0.13abcd</td>
<td>0.21±0.16abcd</td>
</tr>
</tbody>
</table>

ANOVA P<0.001 P<0.001 P<0.001 P<0.001 P<0.001 P<0.001 P<0.001 P<0.001

NS – not significant

a p<0.05 vs. Sham-op+vehicle
b p<0.05 vs. Sham-op+R-568
c p<0.05 vs. Sham-op+calcitriol
d p<0.05 vs. SNX+vehicle
e p<0.05 vs. SNX+R-568
<table>
<thead>
<tr>
<th>Group</th>
<th>Mean glomerular volume [10^6 µm³]</th>
<th>Volume of mesangial matrix [%]</th>
<th>Capillary length density [cm/mm³]</th>
<th>Podocytes</th>
<th>Endothelial cells</th>
<th>Cells within the mesangium</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No/glom.</td>
<td>Mean volume [µm³]</td>
<td>No/glom.</td>
<td>Mean volume [µm³]</td>
<td>No/glom.</td>
<td>Mean volume [µm³]</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>
<td>457±62</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>
<td>430±88</td>
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<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>
<td>516±146</td>
</tr>
<tr>
<td>SNX + vehicle</td>
<td>6.05±1.46abc</td>
<td>22.2±6.5abc</td>
<td>3.92±0.41abc</td>
<td>141±12abc</td>
<td>831±127abc</td>
<td>733±243abc</td>
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<tr>
<td>SNX + R-568</td>
<td>3.94±0.69abcd</td>
<td>12.6±4.4bd</td>
<td>5.33±0.63ad</td>
<td>191±25df</td>
<td>424±100df</td>
<td>614±151b</td>
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<tr>
<td>SNX + calcitriol</td>
<td>4.45±0.86abcd</td>
<td>13.7±3.1bd</td>
<td>5.23±0.30ad</td>
<td>189±19df</td>
<td>474±45d</td>
<td>690±105abc</td>
</tr>
</tbody>
</table>

ANOVA P<0.0001 P<0.0001 P<0.0001 P=0.0002 P<0.0001 P=0.001 NS P<0.0001 NS

NS – not significant

a p<0.05 vs. Sham-op+vehicle
b p<0.05 vs. Sham-op+R-568
c p<0.05 vs. Sham-op+calcitriol
d p<0.05 vs. SNX+vehicle
e p<0.05 vs. SNX+R-568
Table 4. Foot processes (FP) and glomerular basement membrane (GBM) morphology

<table>
<thead>
<tr>
<th>Group</th>
<th>GBM covered by FP [%]</th>
<th>No of FP [1/µm]</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±2.6abc</td>
<td>1.13±0.44abc</td>
<td>958±364abc</td>
<td>244±6abc</td>
</tr>
<tr>
<td>SNX + R-568</td>
<td>79.1±3.3abcd</td>
<td>2.28±0.20abcd</td>
<td>340±36d</td>
<td>164±10d</td>
</tr>
<tr>
<td>SNX + calcitriol</td>
<td>81.5±3.0abcd</td>
<td>2.07±0.30abcd</td>
<td>395±66d</td>
<td>161±5d</td>
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</table>

ANOVA P<0.001 P<0.001 P<0.001 P=0.005

NS – not significant

a p<0.05 vs. Sham-op+vehicle
b p<0.05 vs. Sham-op+R-568
c p<0.05 vs. Sham-op+calcitriol
d p<0.05 vs. SNX+vehicle
e p<0.05 vs. SNX+R-568
**Table 5.** Immunohistochemical (IHC) and RNA *in-situ* hybridization (ISH) of CaSR and VDR in the kidney

<table>
<thead>
<tr>
<th>Group</th>
<th>CaSR - IHC</th>
<th>CaSR – ISH</th>
<th>VDR - IHC</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>
</tr>
<tr>
<td>Sham-op+R-568</td>
<td>0.09±0.05</td>
<td>1.03±0.39&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.72±0.30&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Sham-op+calcitriol</td>
<td>0.04±0.06</td>
<td>0.18±0.12&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.00±0.14&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>SNX+vehicle</td>
<td>0.01±0.03</td>
<td>0.35±0.20&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.82±0.25&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>SNX+R-568</td>
<td>0.20±0.13&lt;sup&gt;abcd&lt;/sup&gt;</td>
<td>2.04±0.53&lt;sup&gt;acd&lt;/sup&gt;</td>
<td>1.79±0.16&lt;sup&gt;ac&lt;/sup&gt;d</td>
</tr>
<tr>
<td>SNX+calcitriol</td>
<td>0.08±0.09&lt;sup&gt;e&lt;/sup&gt;</td>
<td>0.20±0.20&lt;sup&gt;be&lt;/sup&gt;</td>
<td>0.93±0.13&lt;sup&gt;be&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

ANOVA P<0.001 P<0.001 P<0.001 P<0.001 NS

NS – not significant
<sup>a</sup>p<0.05 vs. Sham-op+vehicle
<sup>b</sup>p<0.05 vs. Sham-op+R-568
<sup>c</sup>p<0.05 vs. Sham-op+calcitriol
<sup>d</sup>p<0.05 vs. SNX+vehicle
<sup>e</sup>p<0.05 vs. SNX+R-568
### Table 6. Immunohistochemical staining for desmin, vascular endothelial growth factor A (VEGF), collagen type IV, fibronectin and transforming growth factor beta 1 (TGF-β1) in glomeruli and tubulointerstitium (TI), and TGF-β1 by RT-PCR in whole kidney samples

<table>
<thead>
<tr>
<th></th>
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<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham-op +vehicle</td>
<td>0.26±0.18</td>
<td>0.19±0.07</td>
<td>0.47±0.19</td>
<td>0.07±0.07</td>
<td>0.05±0.10</td>
<td>0.03±0.06</td>
<td>0.09±0.07</td>
<td>0.24±0.10</td>
<td>0.37±0.16</td>
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</tr>
<tr>
<td>Sham-op +R-568</td>
<td>0.25±0.12</td>
<td>0.16±0.12</td>
<td>0.64±0.26</td>
<td>0.07±0.12</td>
<td>0.05±0.05</td>
<td>0.05±0.05</td>
<td>0.07±0.05</td>
<td>0.82±0.19</td>
<td>0.29±0.04</td>
<td></td>
</tr>
<tr>
<td>Sham-op +calcitriol</td>
<td>0.31±0.12</td>
<td>0.15±0.10</td>
<td>0.39±0.19</td>
<td>0.27±0.12</td>
<td>0.20±0.39</td>
<td>0.00±0.00</td>
<td>0.10±0.05</td>
<td>0.25±0.12</td>
<td>0.46±0.11</td>
<td></td>
</tr>
<tr>
<td>SNX +vehicle</td>
<td>6.59±2.09abc</td>
<td>0.82±0.37abc</td>
<td>1.22±0.25abc</td>
<td>1.84±0.43abc</td>
<td>1.73±0.52abc</td>
<td>1.09±0.23abc</td>
<td>0.98±0.18abc</td>
<td>1.39±0.43abc</td>
<td>0.82±0.25abc</td>
<td></td>
</tr>
<tr>
<td>SNX +R-568</td>
<td>2.70±0.34abcd</td>
<td>0.43±0.10abcd</td>
<td>0.90±0.38abcd</td>
<td>0.64±0.50abcd</td>
<td>0.75±0.48abcd</td>
<td>0.29±0.21abcd</td>
<td>0.63±0.25abcd</td>
<td>1.90±0.41abcd</td>
<td>0.67±0.32ab</td>
<td></td>
</tr>
<tr>
<td>SNX +calcitriol</td>
<td>2.53±0.29abcd</td>
<td>0.35±0.22d</td>
<td>0.73±0.30d</td>
<td>0.69±0.14abcd</td>
<td>0.62±0.29abcd</td>
<td>0.81±0.28abcd</td>
<td>0.52±0.16abcd</td>
<td>1.01±0.45abcd</td>
<td>0.83±0.18abc</td>
<td></td>
</tr>
</tbody>
</table>

ANOVA P<0.001 P<0.001 P<0.001 P<0.001 P<0.001 P<0.001 P<0.001 P<0.001 P=0.0003

NS – not significant

ap<0.05 vs. Sham-op+vehicle
bp<0.05 vs. Sham-op+R-568
cp<0.05 vs. Sham-op+calcitriol
dp<0.05 vs. SNX+vehicle
ep<0.05 vs. SNX+R-568
Table 7. Immunohistochemical staining for nitrotyrosine, endothelial nitric oxide synthase (eNOS), and endothelin 1 (ET-1) in glomeruli and tubulointerstitium (TI)

<table>
<thead>
<tr>
<th>Group</th>
<th>Nitrotyrosine [score]</th>
<th>eNOS  [score]</th>
<th>ET-1  [score]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Glomeruli</td>
<td>TI</td>
<td>Glomeruli</td>
</tr>
<tr>
<td>Sham-op+vehicle</td>
<td>0.09±0.13</td>
<td>0.18±0.15</td>
<td>1.01±0.43</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>
</tr>
<tr>
<td>Sham-op+calcitriol</td>
<td>0.05±0.07</td>
<td>0.10±0.08</td>
<td>1.12±0.52</td>
</tr>
<tr>
<td>SNX+vehicle</td>
<td>1.17±0.18abc</td>
<td>1.87±0.35abc</td>
<td>0.28±0.18abc</td>
</tr>
<tr>
<td>SNX+R-568</td>
<td>1.07±0.26abc</td>
<td>1.26±0.14abcd</td>
<td>1.24±0.57d</td>
</tr>
<tr>
<td>SNX+calcitriol</td>
<td>0.75±0.33abcde</td>
<td>1.02±0.40abcd</td>
<td>0.82±0.30d</td>
</tr>
</tbody>
</table>

ANOVA                  | P<0.001               | P<0.001       | P<0.001       | NS            | P<0.001       | P<0.001       |

NS – not significant
a p<0.05 vs. Sham-op+vehicle
b p<0.05 vs. Sham-op+R-568
c p<0.05 vs. Sham-op+calcitriol
d p<0.05 vs. SNX+vehicle
e p<0.05 vs. SNX+R-568
Figure 1. Electron microscopy images of podocytes’ foot processes. Magnification ×10 000.

A: Sham-op + vehicle; B: Sham-op + R-568; C: Sham-op + calcitriol;
D: SNX + vehicle; E: SNX + R-568; F: SNX + calcitriol.

Figure 2. RNA in situ hybridization for Calcium sensing receptor. Magnification ×400.

A: Sham-op + vehicle; B: Sham-op + R-568; C: Sham-op + calcitriol;
D: SNX + vehicle; E: SNX + R-568; F: SNX + calcitriol.
Figure 1. Electron microscopy images of podocytes’ foot processes. Magnification ×10000. A: Sham + vehicle; B: Sham + R-568; C: Sham + calcitriol; D: SNX + vehicle; E: SNX + R-568; F: SNX + calcitriol.
Figure 2. RNA in situ hybridization for Calcium sensing receptor. Magnification x400. A: Sham + vehicle; B: Sham + R-568; C: Sham + calcitriol; D: SNX + vehicle; E: SNX + R-568; F: SNX + calcitriol.