The calcimimetic R-568 prevents podocyte loss in uninephrectomized ApoE -/- mice.

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Abstract

Calcimimetics are indicated for secondary hyperparathyroidism in chronic kidney disease and some data suggest their protective role for progression of renal damage. We aimed at evaluating whether a calcimimetic can slow the progression of kidney damage in uninephrectomized ApoE -/- mice. To this end we compared its effect with that of calcitriol.

12-weeks old male ApoE -/- mice were randomized to undergo sham operation or unilateral nephrectomy (UNX) and subsequently received the calcimimetic R-568 (4 µg/kg/day), calcitriol (0.03 µg/kg/day) or vehicle intraperitoneally. Glomerular number and volume, damage indices (glomerular, vascular and interstitial), and glomerular cells (podocytes, mesangial, endothelial) number and volume were assessed in perfused kidneys after 12-weeks treatment period.

Lower numbers of podocytes per glomerulus were observed in UNX+vehicle compared to sham-op and this was prevented in UNX+R-568 but not in UNX+calcitriol. In parallel, albuminuria was higher in untreated UNX compared to sham-op and the increase was prevented in UNX+R-568. Interstitial fibrosis was more prevalent in vehicle treated UNX compared to sham-op group and this was prevented in UNX treated with R-568 and less effectively with calcitriol. In all UNX groups the weight of residual kidney was significantly higher compared to all sham-op. No difference was observed in serum ionized calcium and systolic blood pressure between the groups.

The calcimimetic R-568 prevented interstitial fibrosis and podocyte loss after uninephrectomy in ApoE -/- mice. Minor renal dysfunction, lack of secondary hyperparathyroidism and hypertension in this model support the hypothesis of direct effects of this compound on glomerular cells.
Introduction

Chronic kidney disease (CKD) progresses over time even if the primary insult is no longer operative (6). Secondary hyperparathyroidism (sHPT) is a common feature of chronic kidney disease. Elevated parathyroid hormone (PTH) concentrations have been demonstrated to contribute to heart fibrosis in uremia (1). PTH is also thought to play a role in progression of kidney damage (24). Both active vitamin D metabolites and calcimimetics are indicated for the treatment of sHPT. Experimental data suggest that lowering PTH with either active vitamin D or calcimimetics may slow progression of advanced kidney damage (20).

In a previous study in a model of more advanced kidney damage we showed that the calcimimetic R-568 and calcitriol prevented podocyte loss and ultrastructure, as well as ameliorated glomerulosclerosis and tubulointerstitial damage (20). This was accompanied by a delayed and less intense rise of albumin excretion. While podocytes express both vitamin D (VDR) (25) and calcium sensing (CaSR) (20) receptors it is unclear whether the nephroprotective effects are mediated directly by calcimimetics or vitamin D respectively or indirectly by PTH lowering.

In vitro data indicate a direct, pro-survival effects of calcimimetics on podocytes and protection from direct toxicity (18). Some data suggest similar podocyte protection by active vitamin D (10).

For this study we chose a model of less severe kidney dysfunction, without rise of blood pressure and presumably without secondary hyperparathyroidism (3). The goal of this study was to evaluate whether a calcimimetic or active vitamin D prevent podocyte damage in vivo in the absence of elevated PTH levels.
**Materials and Methods**

All animals were handled according to written approval from the local authority for animal experiments (Regierungspraesidium Karlsruhe). The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). 12-weeks old male ApoE -/- mice were randomized to undergo sham operation or unilateral nephrectomy (UNX) and received a standard rodent diet containing 19.3% protein, 39.1% carbohydrates, and 3.3% fat. 1.00% calcium, 0.70% phosphate, and 0.68% potassium (Ssniff, Soest, Germany) and tap water ad libitum. The mice after UNX were randomized to receive the calcimimetic R-568 (4 µg/kg/day), calcitriol (0.03 µg/kg/day) or vehicle administered daily intraperitoneally. The dose of R-568, lower than used in rats (21), was determined in a pilot experiment as well tolerated by the mice. The sham-operated mice received the vehicle. The treatment was continued for 12 weeks; body weight was measured weekly.

Blood pressure was measured by tail-cuff plethysmography in conscious mice at week 12 post UNX as described previously (23).

24-hour urine samples were collected in metabolic cages at week 12 of treatment. Blood samples were collected from the abdominal aorta at the time of sacrifice. Serum and urine parameters (sodium, potassium, calcium, phosphate, creatinine, total and HDL cholesterol, triglycerides) and blood cells were analyzed using standard laboratory methods. Parathormone (PTH) was measured in plasma by a mouse-specific ELISA kit according to manufacturer’s recommendations (Immutopics Int., San Clemente, CA, USA).

Urinary albumin was measured by a mouse-specific ELISA kit according to manufacturer’s recommendations (Bethyl Laboratories, Montgomery, TX, USA).
After the 12-week treatment period tissue samples were collected by pressure-controlled perfusion. The abdominal aorta was catheterized under anesthesia (100 mg/kg ketamine hydrochloride and 3.0 mg/kg xylazine). Blood samples were taken; the vasculature was flushed with 0.9% NaCl solution and collected for molecular investigations or fixed with glutaraldehyde and collected for morphologic investigations.

Morphological and Stereological Evaluation

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

Glomerular sclerosis index (GSI) was assessed on PAS-stained paraffin sections according to the semiquantitative scoring system (score 0 – 4) proposed by El Nahas et al. (4). 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.

The indices of tubulointerstitial (TII) and vascular damage (VI) were also assessed at a magnification of ×100 using similar scoring systems (22). The number of glomeruli per kidney and mean glomerular volume were determined using the point counting method as previously described (22). Capillaries and the number and volume of glomerular cells were analyzed in semithin sections at ×1000 magnification using the point-counting method as previously described (22).

Total collagen content was measured in sections stained with 0.1% Sirius red F3BA saturated in picric acid (a percent of Sirius-red stained collagen area to total kidney area)
using a semiautomatic image analysis software (Optimas 6.0, Optimas Corp., Seattle, WA, USA).

Podocyte foot processes were visualized by electron microscopy (Zeiss). Thickness of the glomerular basement membrane (GBM), dimensions of foot processes, and length of GBM covered by foot processes were measured on 40 images per animal at ×10 000 magnification using Vario Vision Pro 3.2 software (Soft Imaging System) as previously described (20).

Western blotting

Samples of kidney tissue from eight to nine animals per group were prepared by homogenization, and the protein concentration was assessed according to Bradford (Protein Assay Kit; Bio-Rad Laboratories, Munich, Germany). Equal amounts of protein (20 µg) were electrophoresed in SDS-PAGE gel and subsequently transferred to Immobilon-P membranes (Millipore Corp., Bedford, MA, USA). The membranes were blocked with 5% non-fat dried milk and then incubated with primary antibody against angiotensin converting enzyme (ACE), ACE2, angiotensin II type 1 and type 2 receptors (Abcam, Cambridge, UK), renin and renin receptor (Santa Cruz Biotechnology, Heidelberg, Germany), vitamin D receptor (VDR; Santa Cruz Biotechnology, Heidelberg, Germany), calcium sensing receptor (CaSR; Affinity BioReagents, Golden, CO, USA). Horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnologies) were used, followed by the ECL kit (GE Healthcare, Buckinghamshire, UK) according to manufacturer’s instruction. Specific bands were quantified by densitometric analyses (ImageJ, NIH, Bethesda, MD, USA).
Immunohistochemistry

Immunohistochemical analysis was performed on paraffin sections using antibodies against VDR (Santa Cruz Biotechnology), CaSR (Affinity BioReagents), desmin (Dako, Copenhagen, Denmark), synaptopodin, ZO-1, Neph-1, p-Bad (Santa Cruz Biotechnology), angiotensin II type 1 and type 2 receptors, and ACE (Abcam, Cambridge, UK) and the streptavidin-biotin technique with alkaline phosphatase as the labeling enzyme. All antibodies had been tested for specificity in mice, and the optimal concentration for staining was evaluated by testing different dilutions in a pilot study. Negative controls were performed by omitting the primary antibody. The staining was analyzed by an investigator blinded with respect to the animal groups. For desmin, p-Bad, angiotensin II type 1 and type 2 receptors, and ACE the average number of stained cells per glomerulus was calculated in all visible glomeruli. For synaptopodin, ZO-1, and Neph-1 stained area was measured in individual glomeruli using Vario Vision Pro 3.2 software (Soft Imaging System) and an average was calculated for each case.

Quantitative stereology of the heart

All investigations were performed by an observer unaware of the animal group. The length density (Lv) of capillaries, i.e. the length of capillaries per unit tissue volume, was measured in eight systematically subsampled areas per section and intercapillary distance was calculated according to a formula by Henquell and Honig as previously described (23).

Statistical analysis

Data are given as mean ± SD or median and range as appropriate. For western blots, the vehicle-treated sham-op group served as reference and the mean value of individual
measurements was set as 100%. One-way analysis of variance (ANOVA) was used followed by Duncan’s multiple range test for differences between groups.
Results

Animal data

There was no difference in final body weight between the groups (tab. 1). The left kidney weight (absolute and relative to body weight) was higher in all three UNX groups without any effect of the treatment. At the end of the study systolic blood pressure was not different between the groups. Albuminuria at the end of the study was significantly higher in UNX compared with sham-op mice. Compared with untreated UNX albumin excretion was significantly lower in UNX treated with R-568 but not in those treated with calcitriol (table 1).

Serum ionized calcium and phosphate concentrations were not different between the groups. After UNX no elevation of serum PTH concentration was observed and the serum PTH concentration was unaffected by any treatment.

Kidney morphology

The glomerulosclerosis and vascular indices were significantly higher in all UNX compared with sham-op without any effect of the treatments (table 3).

The tubulointerstitial index and interstitial fibrosis were significantly higher in all UNX compared to sham-op (table 3). Treatment with R-568 in UNX mice ameliorated interstitial fibrosis and tubulointerstitial damage while treatment with calcitriol was less effective.

Glomerular geometry, capillarization, and glomerular cells

The number of glomeruli was not different between the groups (9962±2749, 8777±1053, 8666±1237, and 8438±1827 for sham-op, UNX+V, UNX+R-568, and
UNX+calcitriol respectively). The average glomerular volume was significantly higher in all UNX groups compared with sham-op (0.95±0.13 x 10^6 µm^3; p<0.001). Compared to UNX+V (1.42±0.32) the average glomerular volume was lower in UNX+R-568 (1.19±0.24) but not in UNX+calcitriol (1.27±0.23).

The glomerular capillary length density was significantly lower in all UNX compared with sham-op and it was higher in UNX+R-568 compared with UNX+V (table 4).

The average number of podocytes per glomerulus was significantly lower in all UNX compared with sham-op and it was higher in UNX+R-568 compared with UNX+V (table 4). The average volume of podocytes was significantly higher in UNX+calcitriol compared with sham-op and UNX+R-568.

The average number of cells in mesangium was significantly higher in UNX+V compared with sham-op and it was significantly lower in UNX+R-568 and UNX+calcitriol compared with UNX+V (table 4). The average volume of cells in mesangium was not different between the groups.

The average number and volume of glomerular endothelial cells was not different between the groups (table 4).

The foot podocyte process effacement was more marked in UNX+V and UNX+calcitriol compared with sham-op and was prevented in UNX treated with R-568 (table 5, fig. 1).
The extranuclear staining for CaSR and VDR both in the tubulointerstitial compartment and in glomeruli was not significantly different between the groups. There was no difference in CaSR and VDR expression by Western blotting as well (fig. 2).

Components of the renin-angiotensin system

The expression of the angiotensin converting enzyme (ACE) was significantly higher in UNX compared with sham-op mice (fig. 3). Compared with untreated UNX it was significantly lower in UNX treated with R-568 but not in UNX treated with calcitriol. By immunohistochemistry ACE was detected in the cytoplasm of glomerular cells. The number of cells in glomeruli stained for ACE was significantly higher in UNX compared with sham-op mice (fig. 4). Compared with untreated UNX it was significantly lower in UNX treated with R-568 but not in UNX treated with calcitriol.

The expression of the angiotensin converting enzyme 2 (ACE2) was significantly higher in all UNX compared with sham-op mice without any significant effect of the treatments (fig. 3).

The expression of the angiotensin II type 1 receptor (AT1R) was significantly higher in UNX compared with sham-op mice (fig. 3). Compared with untreated UNX it was significantly lower in UNX treated with R-568 but not in UNX treated with calcitriol. In glomeruli AT1R stained extranuclear. In UNX mice there was significantly more cells stained for AT1R compared to sham-op (fig. 4). The number of cells positive for AT1R was significantly lower in UNX treated with R-568 compared to untreated UNX. There was no difference in the number of cells positive for AT1R between untreated UNX and UNX treated with calcitriol.
The expression of the angiotensin II type 2 receptor (AT2R) was significantly higher in UNX compared with sham-op mice (fig. 3). Compared with untreated UNX it was significantly lower in UNX treated both with R-568 and calcitriol, but in both these groups it was still higher compared with sham-op mice. In glomeruli AT2R stained the extranuclear and there was no difference in the number of cells stained for AT2R between sham-op and UNX mice (fig. 4).

The expression of renin was significantly higher in UNX compared with sham-op mice (fig. 3). Both R-568 and calcitriol prevented the increase in renin expression in UNX mice.

The expression of the renin receptor was not different between sham-op and untreated UNX mice, but it was significantly higher in UNX treated with calcitriol (fig. 3).

**Podocyte proteins**

Significantly more podocytes per glomerulus were stained positive for desmin in the cytoplasm in UNX mice treated with vehicle compared to sham-op (tab. 6). This increase was ameliorated in UNX mice treated with either R-568 or calcitriol, but the number of desmin-stained cells was still higher compared to sham-op (fig. 4).

Staining for synaptopodin, ZO-1, and Neph-1 in the membrane was significantly less in glomeruli of vehicle-treated UNX mice compared to sham-op (tab. 6). Neither R-568 nor calcitriol influenced staining for synaptopodin. The area stained for ZO-1 and Neph-1 was significantly greater in UNX treated with R-568 or calcitriol compared to those treated with vehicle (fig. 4).
Apoptosis marker

Significantly more cells per glomerulus stained positively (perinuclear) for phosphorylated Bad (p-Bad) in untreated UNX mice compare with sham-op and this was prevented in UNX treated with R-568.

Heart capillary density

The capillary length density was significantly (ANOVA p<0.001) lower, and the mean intercapillary distance was significantly higher, in untreated UNX mice (2210±222 mm/mm$^3$ and 22.9±1.1 µm, respectively) than those in sham-op animals (3080±74 and 19.4±0.2, respectively). The capillary length density was significantly higher, and the mean intercapillary distance was significantly lower, in UNX mice treated with R-568 (2530±121 and 21.4±0.5 respectively) compared with untreated UNX. There was no difference in these parameter between untreated UNX mice and those treated with calcitriol (2262±124 and 22.6±0.6, respectively).
Discussion

In this study in a model of subtle kidney damage we have shown that a calcimimetic R-568 prevents podocyte loss and preserve podocyte ultrastructure in vivo independently of PTH concentration and blood pressure. This observation is important for optimal therapy of chronic kidney disease as currently available treatments are not sufficient to provide complete nephroprotection.

The calcium sensing receptor (CaSR) (20) and the vitamin D receptor (VDR) (25) are present in the podocyte. Therefore both calcimimetics and VDR activators can potentially exert direct effects on these cells. In vitro studies have confirmed that calcimimetics protect podocytes from damage (19). In past in vivo experiments it has not been clear whether the protective effects in the kidney are due to direct actions of these agents or due to lowering of PTH as similar results have been observed with different drugs reducing PTH, as well as with surgical parathyreoidectomy (16). In this study podocyte loss and effacement were observed without significant rise in serum PTH concentration and blood pressure and protective effects were observed without affecting PTH. An increased number of cells in glomeruli of UNX mice stained for phosphorylated Bad – a protein involved in apoptosis (27), which was prevented in UNX mice treated with R-568. This suggests that R-568 ameliorated podocyte apoptosis in line with previous in vitro observations (18).

Angiotensin II is well known to perpetuate kidney and specifically podocyte damage (7). VDR activators have been shown to ameliorate kidney damage by inhibiting the renin-angiotensin system (29) and VDR activation directly suppresses renin gene transcription (28). Calcimimetics also lower renin release (11). In line with those observations we have shown that both calcitriol and R-568 lowered increased renin expression in the remnant kidney. The calcimimetic, however, also decreased the expression of ACE and AT1 receptor specifically
in the glomeruli, which were unaffected by calcitriol. The suppression of several components of the renin-angiotensin system by R-568 may explain the protective effect of the calcimimetic. This was opposed by a specific renin inhibition by calcitriol and failure to prevent podocyte damage in this study. Angiotensin-converting enzyme 2 (ACE2) degrades angiotensin II to angiotensin-(1-7), is expressed in podocytes, and may prevent podocyte damage (13). In this model, however, it seems not to play a role in podocyte protection as its expression was not significantly affected by both treatments.

In this model podocyte loss has been observed without an increase in systemic blood pressure. In agreement with previous studies in ApoE -/- mice blood pressure did not increase after UNX (23). Similarly, the protection of podocyte damage by the calcimimetic was shown without lowering blood pressure. Although calcimimetics were shown to decrease blood pressure (14, 15), this effect had been observed in animals with increased blood pressure and more extensive kidney damage. The indirect method of blood pressure measurement is a limitation of this study.

The serum ionized calcium concentration was not different between the groups when measured 24h post-dosing as the decrease of serum calcium after the administration of R-568 is known to be transient (5). At the dose used, calcitriol did not cause hypercalcemia in agreement with previous experiments (2).

Reduction of nephron number leads to podocyte injury and this contributes to the development of glomerulosclerosis (12).

In line with the decreased podocyte number in UNX mice we observed increased urinary albumin excretion. The reduction of albuminuria by R-568 treatment in UNX mice confirms that the observed preservation of podocyte number and ultrastructure in this group
provides functional benefits. Again, UNX mice treated with calcitriol failed to maintain
podocyte number or to significantly reduce albuminuria. This is in contrast to previous
observations that VDR activation leads to lower albuminuria (9, 20) but those observation
were made in models of more advanced kidney damage with greater increase in albuminuria.
Furthermore, amelioration of albuminuria in those studies was observed together with a
decrease in PTH concentrations. Lowering PTH by parathyreoidectomy is sufficient to reduce
albuminuria in the renal ablation model (17). Our results indicate that the reduction of
albuminuria by a calcimimetic is, at least in part, independent of the effects on PTH.

It was previously shown by Yoshida et al. (26) that at an early stage of
glomerulosclerosis a positive correlation is found between glomerulosclerosis index and
glomerular volume. Podocytes are thought to play a pivotal role in the development of
glomerular scarring (12). We observed only a slight increase and no effect of treatments on
glomerulusclerosis in UNX mice. There were however differences in glomerular volume – in
parallel with the lower podocyte number, glomerular volume was higher in UNX mice treated
with vehicle or calcitriol and glomerular volume was lower in UNX treated with R-568
accompanying the preservation of podocyte number.

In contrast to observations in subtotally nephrectomized rats (20) we observed no
difference in expression of VDR and CaSR between UNX and sham-op mice, most probably
due to the lower severity of kidney damage in the current model.

The quantitative assessment of protein expression in whole cortex lysates repressents
both glomerular and tubular localization and it is a limitation of this study. Semiquantitative
data from immunohistochemical staining provides more insight into glomerular expression of
investigated proteins.
ApoE -/- mice develop accelerated atherosclerosis already after uninephrectomy (23). R-568 ameliorated capillary rarefaction in the heart, indirectly suggesting that it might ameliorate atherosclerotic lesions, which have been demonstrated previously in rats (8).

In conclusion, in a model of subtle reduction of kidney function the calcimimetic R-568 prevented podocyte loss, glomerular enlargement as well as albuminuria. These effects were observed without changes in parathormone concentration and systolic blood pressure suggesting a direct effect on podocytes. Whether such effects can be extrapolated to humans remains unknown. It offers, however, a potential new treatment option for delaying progressive kidney failure.
Table 1. Animal data

<table>
<thead>
<tr>
<th>Group</th>
<th>Body weight [g]</th>
<th>Left kidney weight [mg]</th>
<th>Left kidney/body weight [mg/g]</th>
<th>Systolic blood pressure [mmHg]</th>
<th>Albuminuria [mg/g BW/24h]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham-op</td>
<td>30.1±2.9</td>
<td>245±42</td>
<td>8.4±1.2</td>
<td>108±14</td>
<td>0.31±0.13</td>
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<tr>
<td>UNX</td>
<td>29.3±3.2</td>
<td>312±43*</td>
<td>10.6±0.8*</td>
<td>101±20</td>
<td>0.57±0.33*</td>
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<tr>
<td>UNX+R-568</td>
<td>28.3±3.4</td>
<td>301±43*</td>
<td>10.7±1.2*</td>
<td>106±18</td>
<td>0.35±0.15†</td>
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<td>UNX+Calcitriol</td>
<td>28.4±2.4</td>
<td>289±47*</td>
<td>10.2±1.5*</td>
<td>108±21</td>
<td>0.43±0.31</td>
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ANOVA p=NS p<0.005 p<0.001 p=NS P<0.05

NS-not significant, *-p<0.05 vs. sham-op, †-p<0.05 vs. UNX, ‡-p<0.05 vs. UNX+R-568
### Table 2. Serum parameters

<table>
<thead>
<tr>
<th></th>
<th>serum Ca(^{2+}) [mmol/l]</th>
<th>Serum PTH [pg/ml]</th>
<th>Serum creatinine [mg/dl]</th>
<th>Serum total cholesterol [mg/dl]</th>
<th>Serum triglycerides [mg/dl]</th>
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</thead>
<tbody>
<tr>
<td>Sham-op</td>
<td>1.09±0.08</td>
<td>9.15±10.51</td>
<td>0.22±0.22</td>
<td>489±78</td>
<td>135±61</td>
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<td>UNX</td>
<td>1.13±0.09</td>
<td>5.32±5.28</td>
<td>0.35±0.29</td>
<td>619±74*</td>
<td>149±56</td>
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<td>UNX+R-568</td>
<td>1.12±0.05</td>
<td>4.36±2.01</td>
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<td>156±45</td>
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<td>UNX+Calcitriol</td>
<td>1.07±0.05</td>
<td>5.21±7.13</td>
<td>0.31±0.34</td>
<td>603±73*</td>
<td>173±59</td>
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</table>

ANOVA p=NS p=NS p=NS p<0.05 p=NS

NS-not significant, *-p<0.05 vs. sham-op, †-p<0.05 vs. UNX, ‡-p<0.05 vs. UNX+R-568
Table 3. Kidney damage indices.

<table>
<thead>
<tr>
<th>Group</th>
<th>Glomerulosclerosis index</th>
<th>Vascular damage index</th>
<th>Tubulointerstitial damage index</th>
<th>Area of interstitial fibrosis [%]</th>
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</thead>
<tbody>
<tr>
<td>Sham-op</td>
<td>0.41±0.03</td>
<td>0.12±0.01</td>
<td>0.01±0.02</td>
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<td>UNX</td>
<td>1.08±0.17*</td>
<td>0.38±0.04*</td>
<td>0.64±0.28*</td>
<td>10.9±4.9*</td>
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<td>UNX+R-568</td>
<td>1.06±0.21*</td>
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<td>UNX+Calcitriol</td>
<td>0.99±0.12*</td>
<td>0.35±0.04*</td>
<td>0.46±0.22*</td>
<td>7.8±3.7*</td>
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ANOVA P<0.001 P<0.001 P<0.001 P<0.001

NS-not significant, *p<0.05 vs. sham-op, †p<0.05 vs. UNX, ‡p<0.05 vs. UNX+R-568
<table>
<thead>
<tr>
<th>Group</th>
<th>Glomerular capillary length density [m/mm³]</th>
<th>Podocytes</th>
<th>Average number per glomerulus</th>
<th>Average volume [µm³]</th>
<th>Endothelial cells</th>
<th>Average number per glomerulus</th>
<th>Average volume [µm³]</th>
<th>Cells within mesangium</th>
<th>Average number per glomerulus</th>
<th>Average volume [µm³]</th>
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<tbody>
<tr>
<td>Sham-op</td>
<td>10.3±1.0</td>
<td>129±17</td>
<td>447±54</td>
<td>132±24</td>
<td>186±34</td>
<td>106±12</td>
<td>425±70</td>
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<tr>
<td>UNX</td>
<td>7.8±0.8*</td>
<td>92±8*</td>
<td>507±55</td>
<td>142±32</td>
<td>164±25</td>
<td>161±33*</td>
<td>528±94</td>
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<tr>
<td>UNX+R-568</td>
<td>9.3±1.1*†</td>
<td>110±18*†</td>
<td>471±56</td>
<td>141±11</td>
<td>176±20</td>
<td>115±11†</td>
<td>462±54</td>
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<tr>
<td>UNX+Calcitriol</td>
<td>8.7±0.7*</td>
<td>97±13*‡</td>
<td>569±91*‡</td>
<td>140±14</td>
<td>173±29</td>
<td>115±12†</td>
<td>463±61</td>
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</tbody>
</table>

ANOVA P<0.001 P<0.001 P<0.05 P=NS P=NS P<0.001 P=NS

NS-not significant, *-p<0.05 vs. sham-op, †-p<0.05 vs. UNX, ‡-p<0.05 vs. UNX+R-568
Table 5. Foot process (FP) and glomerular basement membrane (GBM) morphology

<table>
<thead>
<tr>
<th>Group</th>
<th>GBM covered by FP [%]</th>
<th>Number of FP [1/mm]</th>
<th>Mean FP width [nm]</th>
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<tbody>
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<td>90±2</td>
<td>2187±136</td>
<td>415±31</td>
<td>123±7</td>
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<tr>
<td>UNX</td>
<td>93±1*</td>
<td>1849±141*</td>
<td>504±44*</td>
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<td>UNX+R-568</td>
<td>91±2</td>
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<td>92±1</td>
<td>1882±75*†</td>
<td>488±25*†</td>
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ANOVA p<0.05  p<0.001  p<0.001  p=NS

NS-not significant, *-p<0.05 vs. sham-op, †-p<0.05 vs. UNX, ‡-p<0.05 vs. UNX+R-568
Table 6. Immunohistochemical staining for podocyte proteins

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<tr>
<th></th>
<th>Desmin [positive cells/glom]</th>
<th>Synaptopodin [% area]</th>
<th>ZO-1 [% area]</th>
<th>Nephi [% area]</th>
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<tr>
<td>Sham-op</td>
<td>0.44±0.19</td>
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<tr>
<td>UNX</td>
<td>1.13±0.37*</td>
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<tr>
<td>UNX+R-568</td>
<td>0.72±0.22*†</td>
<td>5±3*</td>
<td>18±11†</td>
<td>23±13†</td>
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<tr>
<td>UNX+Calcitriol</td>
<td>0.71±0.23*†</td>
<td>8±8*</td>
<td>16±11†</td>
<td>22±9†</td>
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ANOVA p<0.001 p<0.05 p<0.005 p<0.01

NS-not significant, *-p<0.05 vs. sham-op, †-p<0.05 vs. UNX, ‡-p<0.05 vs. UNX+R-568
**Figure legends**

Fig. 1. Representative pictures of podocyte foot processes in electron microscopy.

Fig. 2. Expression of the vitamin D receptor (VDR) and calcium sensing receptor (CaSR) in the kidney by Western blotting. NS-not significant.

Fig. 3. Expression of the components of the renin-angiotensin system in the kidney by Western blotting. NS-not significant, *-p<0.05 vs. sham-op, †-p<0.05 vs. UNX, ‡-p<0.05 vs. UNX+R-568.

Fig. 4a. Representative pictures of immune staining for angiotensin converting enzyme (ACE), angiotensin II type 1 receptor (AT1R), angiotensin II type 2 receptor (AT2R), and phosphorylated Bad (p-Bad).

Fig. 4b. Representative pictures of immune staining for desmin, ZO-1, Neph-1, and calcium sensing receptor (CaSR).
Acknowledgements:

The study was supported by Amgen.

The skillful technical assistance of Z. Antoni is gratefully acknowledged.
References


ANOVA p=NS

ANOVA p=NS
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