Combination Therapy with an Angiotensin-Converting Enzyme Inhibitor and a Vitamin D Analog Suppresses the Progression of Renal Insufficiency in Uremic Rats

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Monotherapy with angiotensin-converting enzyme inhibitors has been shown to be beneficial in suppressing the progression of experimentally induced kidney diseases. Whether such therapy provides additional benefits when combined with vitamin D or an analog of vitamin D has not been established. Rats were made uremic by 5/6 nephrectomy and treated as follows: Uremic + vehicle (UC), uremic + enalapril (30 mg/L in drinking water; E), uremic + paricalcitol (19-nor; 0.8 μg/kg, three times a week), and uremic + enalapril + paricalcitol (E + 19-nor). A group of normal rats served as control (NC). BP was significantly elevated in the UC and 19-nor groups compared with the NC group but was indistinguishable from normal in the E and E + 19-nor groups. The decrease in creatinine clearance and the increase in the excretion of urinary protein that were observed in the UC group were ameliorated by the use of E alone or by E + 19-nor (P < 0.05 versus UC). The glomerulosclerotic index was significantly decreased in both the 19-nor (P < 0.01) and E + 19-nor groups (P < 0.01) compared with the UC group. Tubulointerstitial volume was significantly decreased in both the E (P < 0.05) and E + 19-nor groups (P < 0.01) compared with the UC group. Both macrophage infiltration (ED-1–positive cells) and production of the chemokine monocyte chemoattractant protein-1 were significantly blunted in E + 19-nor compared with E group. TGF-β1 mRNA and protein expression were increased in the UC group (mRNA: 23.7-fold; protein: 29.1-fold versus NC). These increases were significantly blunted in the 19-nor group (mRNA: 7.1-fold; protein: 8.0-fold versus NC) and virtually normalized in the E + 19-nor group (protein: 0.8-fold versus NC). Phosphorylation of Smad2 was also elevated in the UC group (7.6-fold versus NC) but less so in the 19-nor–treated rats (5.5-fold versus NC). When rats were treated with E + 19-nor, the phosphorylation of Smad2 was normal (1.1-fold versus NC). Thus, 19-nor can suppress the progression of renal insufficiency via mediation of the TGF-β signaling pathway, and this effect is amplified when BP is controlled via renin-angiotensin system blockade.


The progression of chronic kidney disease (CKD) leads to end-stage kidney disease and consequent renal replacement therapy. The renin-angiotensin-aldosterone system (RAAS) regulates extracellular volume homeostasis, which contributes to BP stability. Overactivity of this system is involved in the pathophysiology of kidney disease. Experimental studies in animals suggest that glomerular hypertension, profibrotic factors, and proteinuria contribute to the kidney damage that is associated with an activated RAAS (1). The initial stage in the development of kidney disease is due to a pathologic process that produces nephron injury. The loss of these functioning units results in systemic and glomerular hypertension, with the damage caused by glomerular hypertension being key in the pathophysiology of kidney disease. The hyperfiltration state that is caused by glomerular hypertension induces the local stimulation of the RAAS. This local stimulation causes glomerular injury by further raising glomerular capillary pressure through angiotensin II (AngII)-driven efferent arteriolar vasoconstriction (2). AngII also stimulates cell proliferation and tissue remodeling by enhancing the synthesis of profibrotic cytokines and growth factors, among these, TGF-β1 (3,4). Because the RAAS has an important role in renal pathophysiology, agents that inhibit this system, such as angiotensin-converting enzyme inhibitor (ACEI) or AngII receptor 1 blocker, have been shown to have beneficial effects in patients with CKD (5–14).

Calcitriol, 1,25-dihydroxyvitamin D₃, and its analogs have been shown to have therapeutic potential in attenuating experimentally induced kidney disease (15–18). Treatment with calcitriol suppresses the progression of glomerulosclerosis and albuminuria in subtotally nephrectomized rats (18). Calcitriol (17) and one of its analogs, 22-oxa-calcitriol (16), have been shown to reduce both mesangial cell proliferation and the degree of glomerulosclerosis in the rat model of anti-Thy-1.1 glomerulonephritis. Calcitriol also alleviates chronic allograft nephropathy in rats by altering TGF-β1 and matrix-regarding molecules (19). Although most of these studies focused on the
effect of calcitriol or one of its analogs on glomerular damage, the suppressive effect of calcitriol on renal interstitial myofibroblasts indicates that calcitriol has a potential role in suppressing the development of renal interstitial fibrosis (20). Recently, Tan et al. (21) demonstrated that the vitamin D analog paricalcitol attenuates renal interstitial fibrosis in a mouse model of obstructive nephropathy. Agarwal et al. (22) reported that paricalcitol also has an antiproteinuric effect on patients with CKD.

Activation of TGF-β1 has a crucial role in matrix expansion and in the development of fibrosis in experimentally induced kidney disease (23–26). Binding of TGF-β1 to its cell-surface receptors results in the phosphorylation of receptor-activated TGF-β signaling proteins known as Smads. Individually, Smad2 or Smad3 can interact with the co-Smad Smad4 to form binary complexes that translocate to the nucleus and regulate targeted gene expression. There is a direct link between the vitamin D receptor and Smads, indicating an interaction between calcitriol and the TGF-β signaling pathway (27,28). Because clinical studies suggest that not only RAAS blockers but also calcitriol or its analogs have beneficial effects on the progression of kidney disease (22,29), we tested the effect of combination therapy with the ACEI enalapril and the vitamin D analog paricalcitol on the progression of renal insufficiency in the 5/6 nephrectomy uremic rat model.

### Materials and Methods

#### Experimental Protocol
All studies were approved by the Washington University Animal Studies Committee in accordance with federal regulations. Renal insufficiency was induced by 5/6 nephrectomy in a group of female Sprague-Dawley rats that weighed 225 to 250 g. The 5/6 nephrectomy involves the ligation of several branches of the left renal artery and excision of the right kidney. All animals were fed a diet that contained 0.9% phosphorus and 0.6% calcium (Dyets, Bethlehem, PA). At the onset of renal insufficiency, uremic rats were given the ACEI enalapril in their drinking water (30 mg/L) and treated three times a week for 4

### Table 1. Blood chemistries in normal and uremic rats

<table>
<thead>
<tr>
<th>Parameter</th>
<th>n</th>
<th>Cr (mg/dl)</th>
<th>i-Ca (mg/dl)</th>
<th>Ca (mg/dl)</th>
<th>P (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NC</td>
<td>6</td>
<td>0.6 ± 0.0</td>
<td>5.31 ± 0.07</td>
<td>9.4 ± 0.1</td>
<td>3.9 ± 0.2</td>
</tr>
<tr>
<td>UC</td>
<td>8</td>
<td>2.3 ± 0.3b</td>
<td>4.65 ± 0.23</td>
<td>9.7 ± 0.4</td>
<td>9.8 ± 1.8b</td>
</tr>
<tr>
<td>E</td>
<td>13</td>
<td>1.1 ± 0.1c</td>
<td>5.54 ± 0.09c</td>
<td>10.0 ± 0.3</td>
<td>5.5 ± 0.5d,e</td>
</tr>
<tr>
<td>19-nor</td>
<td>6</td>
<td>1.7 ± 0.2b</td>
<td>5.34 ± 0.19</td>
<td>10.4 ± 0.3</td>
<td>9.7 ± 1.3f</td>
</tr>
<tr>
<td>E+19-nor</td>
<td>14</td>
<td>1.1 ± 0.1c</td>
<td>5.56 ± 0.10c</td>
<td>10.4 ± 0.1f</td>
<td>5.9 ± 0.3d</td>
</tr>
<tr>
<td>ANOVA</td>
<td></td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

Data are means ± SEM. Cr, creatinine; i-Ca, ionized calcium; NC, normal controls; 19-nor, paricalcitol; P, serum phosphorus; UC, uremic controls.

bP < 0.01 versus NC group.

P < 0.01 versus UC group.

P < 0.05 versus UC group.

P < 0.05 versus 19-nor group (Scheffe test).

P < 0.05 versus NC group.
mo with 100 µl of vehicle (propylene glycol) or 0.8 µg/kg 19-nor as follows: Uremic + vehicle (UC), uremic + enalapril (E), uremic + paricalcitol (19-nor), and uremic + E + 19-nor (E + 19-nor). A group of normal rats that were treated with vehicle served as control (NC).

After 5/6 nephrectomy, BP was measured monthly using the Non-Invasive Blood Pressure System XB1000 (Kent Scientific Corp., Torrington, CT). During the last 3 d of the 4-mo treatment period, the rats were placed in metabolic cages and 24-h urine samples were collected. The first 24-h period was discarded, and the average 24-h urinary excretion of protein, calcium (Ca), and phosphorus (P) and the creatinine clearance (Ccr) for the last 2 d of treatment were measured. After 4 mo, rats were killed by exsanguination via the dorsal aorta, and blood

Table 2. Ccr, urinary protein, P, and Ca*

<table>
<thead>
<tr>
<th>Parameter</th>
<th>n</th>
<th>Ccr (ml/min)</th>
<th>Up (mg/d)</th>
<th>Ca (mg/d)</th>
<th>P (mg/d)</th>
</tr>
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<tbody>
<tr>
<td>NC</td>
<td>6</td>
<td>1.31 ± 0.05</td>
<td>30 ± 9 b</td>
<td>2.5 ± 0.4</td>
<td>71.7 ± 9.5</td>
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<td>UC</td>
<td>8</td>
<td>0.32 ± 0.05 b</td>
<td>254 ± 27 b</td>
<td>2.1 ± 0.4</td>
<td>49.7 ± 5.3</td>
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<tr>
<td>E</td>
<td>13</td>
<td>0.53 ± 0.05 b,c</td>
<td>115 ± 16 d</td>
<td>1.8 ± 0.2</td>
<td>65.1 ± 3.9</td>
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<tr>
<td>19-nor</td>
<td>6</td>
<td>0.40 ± 0.06 b</td>
<td>180 ± 24 b</td>
<td>2.9 ± 0.8</td>
<td>49.9 ± 7.0</td>
</tr>
<tr>
<td>E + 19-nor</td>
<td>14</td>
<td>0.55 ± 0.06 b,d</td>
<td>96 ± 11 d,e</td>
<td>2.5 ± 0.4</td>
<td>58.1 ± 4.6</td>
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<td>&lt;0.01</td>
<td>0.42</td>
<td>0.07</td>
</tr>
</tbody>
</table>

aData are means ± SEM. Ccr, creatinine clearance; Up, urinary protein.

bP < 0.01 versus NC group.

cP < 0.05 versus UC group.

dP < 0.01 versus UC group.

eP < 0.05 versus 19-nor group (Scheffe test).

Figure 3. Representative microphotograph of Masson trichrome–stained sections from NC (A) and 5/6 nephrectomized rats that were treated with vehicle (B), E (C), 19-nor (D), or a E + 19-nor (E) for 4 mo. Arrow shows FSGS. Magnification, ×200.
was retained for analytical determinations. The remnant kidney was removed and divided into two sections perpendicular to the longitudinal axis. One piece was rinsed in 1% PBS and fixed in 10% formalin for histologic examination. The renal cortex was dissected from the remaining piece, frozen immediately in liquid nitrogen, and stored at −80°C until analysis.

**Analytical Determinations**

Urine samples were acidified and analyzed for 24-h excretion of creatinine (Cr), total protein, Ca, and P. Serum and urinary levels of P and Cr, as well as urinary protein, were measured by autoanalyzer (COBAS-MIRA Plus, Branchburg, NJ). Total serum and urinary Ca were measured by atomic absorption spectrophotometry (1100B; Perkin-Elmer, Norwalk, CT). Ionized Ca was measured using a Nova 8 electrolyte analyzer (Nova Biomedical, Waltham, MA). Ccr was calculated as previously reported (30). Intact parathyroid hormone (PTH) was measured by an immunoradiometric assay specific for intact rat PTH (Immutopics, San Clemente, CA).

**Histologic Examination**

Kidney tissue was embedded in paraffin, sectioned, and stained with Masson trichrome reagent. One hundred glomeruli were randomly selected for determination of glomerulosclerosis. Glomeruli that exhibited adhesion of the capillary tuft to the Bowman’s capsule, capillary obliteration, mesangial expansion, or fibrotic crescents were defined as glomerulosclerotic. The extent of glomerular damage was expressed as the percentage of glomeruli that exhibited sclerosis. For evaluation of the extent of renal interstitial expansion, the fraction of renal cortex that was occupied by interstitium that stained positively for extracellular matrix components by Masson Trichrome was quantitatively evaluated by a point-counting technique in 10 randomly selected microscopic fields, at a final magnification of ×200 under a 100-point grid (31). Blind analysis was done on all sections by the same observer.

**Real-Time PCR**

Total RNA from kidney was extracted using TRIZol in accordance with the manufacturer’s instructions (Invitrogen, Carlsbad, CA). Five micrograms of total RNA was reverse-transcribed to first-strand cDNA using Superscript II Reverse transcriptase (Invitrogen). Synthesized cDNA was amplified by a standard PCR protocol using SYBR Green Jump Start TaqReady Mix (Sigma, St. Louis, MO) and rat-specific primers for TGF-β1 and monocyte chemotactic protein-1 (MCP-1). Parallel amplifications with primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were performed. The primer set for TGF-β1, MCP-1, and GAPDH primers were purchased from Qiagen (Germany). The primer set for TGF-β1 was purchased from Sigma (St. Louis, MO) and rat-specific primers for TGF-β1 and monocyte chemotactic protein-1 (MCP-1).

**Western Blot Analysis**

Protein expression of TGF-β1, Smad2, phosphorylated Smad2, Smad7, and β-actin were determined by Western blot analysis. Briefly, renal cortex tissue was homogenized in 2 ml of Cell Lysis Buffer (Cell Signaling Technology, Beverly, MA). Samples were centrifuged at 3000 × g for 15 min, and the supernatants were assayed. After being mixed with SDS-PAGE sample buffer and boiled for 5 min, samples (8 μg per lane) were electrophoresed on 4 to 12% SDS polyacrylamide gels and transferred to nitrocellulose membranes for 2 h at 30 V. Membranes were blocked for 30 min with Tris-buffered saline that contained 5% BSA (5% BSA/TBS) and incubated with diluted primary antibody overnight at room temperature in 5% BSA/TBS that contained 0.05% Tween 20. The source and the concentration of each antibody were as follows: Rabbit anti–TGF-β1 (Santa Cruz Biotechnology, Santa Cruz, CA; 1:200), anti-Smad2 and anti–phosphorylated Smad2 (Cell Signaling Technology; 1:500), goat anti-Smad7 (Santa Cruz Biotechnology; 1:500), and rabbit anti–β-actin antibody (Santa Cruz Biotechnology; 1:500). The membranes were washed, and diluted secondary horseradish peroxidase (HRP)-conjugated antibodies were added (anti-rabbit IgG-HRP 1:1000; anti-goat IgG-HRP 1:1000; Santa Cruz Biotechnology). The membranes were again washed and developed using the enhanced

![Figure 4. Quantification of interstitial volume (A) and glomerulosclerosis (B) in rat kidneys from NC and 5/6 nephrectomized rats that were treated with vehicle (UC), E, 19-nor, or a combination of E + 19-nor for 4 mo. Data are means ± SEM (n = 6 to 14). P = 0.0003 for interstitial volume and P < 0.0001 for glomerulosclerosis by ANOVA; *P < 0.01 versus NC; **P < 0.01 and ***P < 0.05 versus UC; &P < 0.01 and ##P < 0.05 versus 19-nor by post hoc Scheffe test.](#)
chemiluminescence system (LumiGLO Reagent; Cell Signaling Technology). Changes in TGF-β1 and Smad7 expression were normalized by correction for the densitometric intensity of β-actin for each sample.

**Immunohistochemistry**

Immunohistochemical staining for ED-1 was performed by using a mouse anti-rat ED-1 mAb (MCA341R; Serotec, Oxford, UK) and a commercial staining kit (Histostain-Plus, mouse; Zymed Laboratories, South San Francisco, CA). Negative control was obtained by substituting mouse preimmune IgG for the primary antibody. The sections were deparaffinized, rehydrated, and microwaved in 0.01 mol/L citrate buffer (pH 6.0) for 10 min to retrieve the antigens. The sections were then treated with 0.6% hydrogen peroxide in methanol for 10 min at room temperature to block endogenous peroxidase and subsequently blocked with 10% preimmune goat serum for 30 min at room temperature. The primary ED-1 antibody (1:100 dilution) or preimmune IgG was added followed by incubation at room temperature for 2 h. Biotinylated secondary antibody was applied, followed by a streptavidin-HRP conjugate. The immune complexes were visualized with 3-amino-9-ethylcarbazole substrate-chromagen. Finally, all sections were counterstained with hematoxylin. The positive cell numbers that were stained with ED-1 were counted manually in the 20 renal cortical areas including the glomeruli at a magnification of ×400. The positive cells were expressed as the number per field.

**Statistical Analyses**

All results were expressed as means ± SEM. One-way ANOVA was used to assess the statistical differences between groups. A post hoc Scheffe test was used to assess the statistical significance in the differences between all possible two-group comparisons. $P < 0.05$ was considered significant.

**Results**

**BP**

Figure 1 depicts the BP values from all groups of rats. Uremic rats developed significant hypertension (systolic BP [SBP] $176 \pm 8$ mmHg). 19-Nor alone did not control the BP (SBP $171 \pm 15$ mmHg). Uremic rats that were treated with E had normal BP (SBP $104 \pm 3$ mmHg), and rats that were treated with E + 19-nor also showed BP levels that were indistinguishable from the NC (SBP $114 \pm 4$ mmHg).

**Serum Chemistries**

Serum chemistries are shown in Table 1. Treatment with 19-nor alone did not affect serum Cr, Ca, and P compared with UC rats. Treatment with E resulted in lower serum Cr levels. This renoprotective effect resulted in lower P and PTH levels in

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*Figure 5. Representative microphotograph of ED-1–stained sections from NC (A) and 5/6 nephrectomized rats that were treated with vehicle (B), E (C), 19-nor (D), or E + 19-nor (E) for 4 mo. Magnification, ×400.*
both groups that were treated with E. Markedly elevated PTH levels (Figure 2) were seen in UC rats (1899 ± 522 pg/ml) compared with the other uremic groups (E 213 ± 74 pg/ml, 19-nor 146 ± 78 pg/ml, E + 19-nor 145 ± 65 pg/ml).

**Urinary Findings**

UC rats had a significantly lower Ccr (0.32 ± 0.05 ml/min; \( P < 0.01 \)) and increased urinary protein excretion (254 ± 27 mg/d; \( P < 0.01 \)) compared with NC rats (Ccr 1.31 ± 0.05 ml/min; urinary protein excretion 30 ± 9 mg/d; Table 2). Treatment with 19-nor inhibited the decrease in Ccr by 25% (0.40 ± 0.06 ml/min) and the increase in urinary protein excretion by 29% (180 ± 24 mg/d) compared with UC rats. The E + 19-nor proved more efficacious (Ccr 0.55 ± 0.06 ml/min \( P < 0.01 \); urinary protein excretion 96 ± 11 mg/d \( P < 0.01 \)). None of the therapeutic interventions had a significant effect on the urinary excretion of Ca or P.

**Renal Histology**

Morphologic evaluations and representative images are shown in Figures 3 and 4. Varying degrees of glomerular sclerosis, from global to segmental sclerosis of the glomerular tuft, were observed in the UC group and affected 61% of glomeruli (P < 0.01 versus NC). 19-Nor alone produced a significant decrease in the percentage of glomeruli that exhibited sclerotic changes (33%) compared with UC rats (P < 0.01). The E + 19-nor further decreased this parameter (12%). An increase in interstitial volume (23%) was also observed in UC rats (P < 0.01 versus NC). E significantly limited the increase in interstitial volume (15%; P < 0.05 versus UC) and, when combined with 19-nor, produced a further improvement (10%; P < 0.01 versus UC).

**Effect on Inflammatory Cell Infiltration**

For determination of the effect on renal inflammation, the infiltration of ED-1–positive macrophages (Figure 5), quantification of the macrophage infiltration (Figure 6), and MCP-1 mRNA expression (Figure 7) in renal tissue were analyzed. The infiltration of ED-1–positive macrophages in UC rats were significantly increased compared with NC rats (P < 0.01). This increase was significantly abrogated in uremic rats that were treated with E (P < 0.05 versus UC). When combined with 19-nor, however, the infiltration of ED-1–positive cells was further reduced (P < 0.05 versus E; Figure 6). MCP-1 mRNA expression in UC rats was also significantly increased compared with NC rats (P < 0.01). Treatment with E alone had no suppressive effect, whereas treatment with E + 19-nor significantly suppressed MCP-1 mRNA expression compared with E alone (Figure 7).

**TGF-β1 mRNA and Protein Expression**

TGF-β1 mRNA expression in the kidneys of UC rats was significantly increased (23.7-fold) relative to NC rats (P < 0.01; Figure 8). This increase was significantly blunted in uremic rats that were treated with 19-nor (P < 0.05; Figure 8). A similar trend was observed in TGF-β1 protein expression (Figure 9). In addition, with E + 19-nor, TGF-β1 protein level was essentially normalized (0.8-fold versus NC; Figure 9).

**Effect on the TGF-β Signaling Pathway**

To examine the effect of the combined therapy of E + 19-nor on the intracellular TGF-β signaling pathway, we investigated the expression of the following components of the TGF-β signaling pathway: Total and phosphorylated Smad2 (Figure 10) and Smad7 (Figure 11). Phosphorylation of Smad2, however, was markedly increased (7.6-fold; P < 0.01) in UC rats compared with NC rats (Figure 10). Phosphorylation of Smad2 was reduced by 28% by treatment with 19-nor alone (P < 0.05), and E + 19-nor resulted in normal phosphorylation (1.1-fold versus NC). The same effect was observed in Smad7 (Figure 11). UC rats had an 11.1-fold increase in Smad7 expression relative to
the NC rats. 19-Nor treatment alone resulted in decreased Smad7 expression (62%; \( P < 0.05 \)). Even more efficacious was the combination of E + 19-nor, which resulted in lower Smad7 expression (0.94-fold versus NC).

**Discussion**

Many clinical studies have shown the beneficial effects of ACEI on delaying the progression of kidney disease (5–8). This has led to the widespread use of ACEI in patients with kidney disease. However, the mechanisms by which the ACEI exert their beneficial effects are not yet fully understood. Despite the benefits gained by the use of ACEI or AngII receptor 1 blocker, renal function continues to decline in the majority of patients, eventually leading to end-stage kidney disease. However, a role for abnormal activation of vitamin D receptor (VDR) in the presentation of diseases such as diabetes, cardiovascular and muscular dysfunction, infectious diseases, autoimmune diseases, and cancer cannot be ignored (32–34). Evidence suggests that calcitriol and the VDR have a crucial role in the regulation of cell proliferation and differentiation and immunomodulation as well as mineral metabolism. This study demonstrates that treatment with the vitamin D analog 19-nor, which has shown therapeutic potential in arresting CKD (22), results in an additional renoprotective effect in 5/6 nephrectomized rats when coupled with ACEI therapy.

The most important factors in the progression of kidney disease are matrix expansion, interstitial fibrosis, tubular atrophy, and glomerulosclerosis. Because overexpression of TGF-β1 is involved in each of these, the TGF-β signaling pathway is considered to be an important modulator of the progression of renal insufficiency. Neutralizing TGF-β1 activity by the use of anti-TGF-β antibodies has been shown to ameliorate...

![Figure 8](image_url)

**Figure 8.** Effect of 4 mo of combination therapy of E + 19-nor on TGF-β1 mRNA expression level in uremic rats. TGF-β1 mRNA expression levels were analyzed by the real-time PCR technique and normalized for the amount of GAPDH mRNA for the NC, UC, E, 19-nor, and E + 19-nor groups. Data are means ± SEM (n = 6 each). \( P = 0.0423 \) by ANOVA; *\( P < 0.01 \) versus NC; **\( P < 0.05 \) versus UC by post hoc Scheffe test.

![Figure 9](image_url)

**Figure 9.** Effect of 4 mo of combination therapy of E + 19-nor on TGF-β1 protein level in uremic rats. (A) TGF-β1 at 12.5 kD of protein and β-actin as a control were analyzed by Western blotting for the NC, UC, E, 19-nor, and E + 19-nor groups. (B) Densitometric quantification of the corresponding bands was performed using an image analyzer. The data are presented after normalization to β-actin expression and depicted as times of respective controls. Data are means ± SEM (n = 5 each). \( P = 0.0050 \) by ANOVA; *\( P < 0.01 \) versus NC; \( \# \)\( P < 0.01 \) versus UC by post hoc Scheffe test.

![Figure 10](image_url)

**Figure 10.** Effect of 4 mo of combination therapy of E + 19-nor on the phosphorylation of Smad2 in uremic rats. (A) The expression of total and phosphorylated Smad2 at 58 kD was analyzed by Western blotting for the NC, UC, E, 19-nor, and E + 19-nor groups. (B) Densitometric quantification of the corresponding bands was performed using an image analyzer. The data are presented after normalization to total Smad2 expression and expressed as means ± SEM (n = 5 each). \( P < 0.0001 \) by ANOVA; *\( P < 0.01 \) versus NC; \( \# \)\( P < 0.01 \) and **\( P < 0.05 \) versus UC; \( \bullet \)\( P < 0.01 \) versus 19-nor by post hoc Scheffe test.
on Smad7 protein level in uremic rats. (A) Smad7 at 51 kD and 
Scheffe test.
in glomerular TGF-
erosis, and albuminuria, which are associated with the reduction
22-oxa-calcitol attenuates mesangial expansion, glomeruloscle-
Makibayashi
ation to
quantification of the corresponding bands was performed us-
its analogs have also been shown to possess a renoprotective
effect in ameliorating glomerular sclerotic injury and fibrogen-
esis of interstitial tissue (15–21). Schwarz et al. (18) reported that
calcitriol treatment reduces glomerulosclerosis and albuminur-
ia in subtotally nephrectomized rats and that this is accom-
panied by a lower expression of TGF-β1 in renal tubules. Makibayashi et al. (16) reported that calcitriol or its analog
22-oxa-calcitol attenuates mesangial expansion, glomeruloscle-osis, and albuminuria, which are associated with the reduction
in glomerular TGF-β1 expression in rats with anti–Thy-1.1 glo-
erulonephritis. Hullett et al. (19) reported that a high dosage of
calcitriol preserves renal function and induces expression of
Smad7, an inhibitor of TGF-β signaling, in a rat model of
chronic allograft nephropathy. These reports suggest that
calcitriol attenuates the progression of experimentally induced
kidney diseases by downregulating the TGF-β signaling path-
way. Calcitriol treatment, however, can lead to hypercalcemia and
hyperphosphatemia, increasing the risk for the progression of
kidney disease. In this study, we used an analog of calcitriol,
19-nor, which has lower calcemic and phosphatemic activities
than the parent compound (39–42). We show that treatment
with 19-nor alone produces a modest but significant renoprotective
effect in 5/6 nephrectomized rats, including inhibition
of the TGF-β signaling pathway through a reduction of TGF-β1
mRNA levels, and that the effect occurs without affecting se-
rum Ca or P. Because no consensus vitamin D responsive
element T but multiple Sp1 binding sites exist in the rat TGF-β1
promoter, it is likely that 19-nor reduction of TGF-β1 mRNA
levels involves the inhibition of TGF-β gene transcription
through binding of a Sp1/VDR complex to Sp1 sites, as dem-
onstrated for calcitriol-induced downregulation of p45/Skp2
(43).

Overexpression of TGF-β1 is a characteristic feature in many
human kidney diseases as well as in the 5/6 nephrectomy rat
model (44). Although ACEI attenuate the overexpression of
TGF-β1, the effect of ACEI therapy in combination with vita-
min D analogs has not been reported. When 19-nor was com-
bined with E, total suppression of TGF-β1 and its signaling
pathway was observed. Vitamin D has been shown to have an
anti-inflammatory effect in experimental kidney disease (17).
Macrophages are present in the experimentally induced kidney
diseases, where they are associated with fibrosis (45). An
important chemokine for macrophages in kidney disease is
MCP-1, and MCP-1 is known to be expressed in the 5/6 ne-
phrectomized rat model (46–48). In one of these studies (46), E
therapy significantly decreased MCP-1 expression. In that
instance, the E concentration in the drinking water was at least
three-fold higher than the concentration that was used in our
study. This may account for the lack of effect on MCP-1 expres-
sion of E monotherapy in our study but an effect in the previ-
ous (46) study. In this study, combination therapy with E +
19-nor caused an additional reduction in macrophage infiltr-
ation and chemokine production. These results suggested that
controlling BP by blocking the RAAS is vital for renoprotection
in the 5/6 nephrectomized rats and that when BP is normally
controlled, 19-nor exerts an additional beneficial effect in part
because of its anti-inflammatory effects on the progression of
renal insufficiency by mediating TGF-β signaling.

One interesting observation in this study is that Smad7,
which has an inhibitory effect on the TGF-β signaling pathway,
was not induced in the E or E + 19-nor rats. Smad7 has been
shown to be downregulated in some kidney disease models
(49,50), and its overexpression ameliorates renal fibrosis in rat
models of ureteral obstruction (51,52) or in a remnant kidney
model (53). Conversely, upregulation of Smad7 in podocytes
has been observed in some human kidney diseases (54). More-
over, Smad7 transcription is activated by binding of Smad3 or
Smad4, which are activators of the TGF-β signaling pathway,
to the Smad7 promoter. This indicates that Smad7 expression
is regulated by the TGF-β signaling pathway (55). These studies
support our data that Smad7 expression was not induced in the
E, E + 19-nor, or NC groups when the activation of the TGF-β
signaling pathway was suppressed and when BP was de-
creased. It is possible that Smad7 is induced during renal
disease as a counterregulatory mechanism to the elevated
TGF-β signaling system. When Smad7 is overexpressed as in
the ultrasound-microbubble–mediated gene transfer studies
(53), Smad7 expression leads to a demonstrative decrease in
renal fibrosis. When E alone or E + 19-nor are used in the
 treatment, the suppression of other pathophysiologic factors

Figure 11. Effect of 4 mo of combination therapy of E + 19-nor
on Smad7 protein level in uremic rats. (A) Smad7 at 51 kD and
β-actin as a control were analyzed by Western blotting for the
NC, UC, E, 19-nor, and E + 19-nor groups. (B) Densitometric
quantification of the corresponding bands was performed using
an image analyzer. The data are presented after normaliza-
tion to β-actin expression and depicted as times of respective
controls. Data are means ± SEM (n = 5 each), P = 0.0098 by
ANOVA; *P < 0.01 and **P < 0.05 versus NC; #P < 0.01 and
##P < 0.05 versus UC; # #P < 0.05 versus 19-nor by post hoc
Scheffe test.
makes it unnecessary for Smad7 expression as a counterregulatory factor.

Conclusion
We demonstrated that 19-nor can suppress the progression of renal insufficiency via mediation of the TGF-β signaling pathway, and this effect is amplified when BP is controlled via renin-angiotensin system blockade. The systemic activation of the VDR is important for regulating not only Ca homeostasis but also hormone secretion, the immune system, cell proliferation, and cell differentiation. Combination therapy with ACEI and 19-nor may represent a novel and beneficial therapeutic strategy for arresting the progression of CKD.

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Disclosures
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