Effect of combining an ACE inhibitor and a VDR activator on glomerulosclerosis, proteinuria, and renal oxidative stress in uremic rats

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Am J Physiol Renal Physiol 302: F141–F149, 2012. First published September 28, 2011; doi:10.1152/ajprenal.00293.2011.—Angiotensin-converting enzyme (ACE) inhibitors ameliorate the progression of renal disease. In combination with vitamin D receptor activators, they provide additional benefits. In the present study, uremic (U) rats were treated as follows: U+vehicle (UC), U+enalapril (UE; 25 mg/l in drinking water), U+paricalcitol (UP; 0.8 μg/kg ip. 3 X wk), or U+enalapril+paricalcitol (UEP). Despite hypertension in UP rats, proteinuria decreased by 32% vs. UC rats. Enalapril alone, or in combination with paricalcitol, further decreased proteinuria (~70%). Glomerulosclerosis and interstitial infiltration increased in UC rats. Paricalcitol and enalapril inhibited this. The increase in cardiac atrial natriuretic peptide (ANP) seen in UC rats was significantly decreased by paricalcitol. Enalapril produced a more dramatic reduction in ANP. Renal oxidative stress plays a critical role in inflammation and progression of sclerosis. The marked increase in p22phox, a subunit of NADPH oxidase, and decrease in endothelial nitric oxide synthase were inhibited in all treated groups. Cotreatment with both compounds inhibited the uremia-induced increase in proinflammatory inducible nitric oxide synthase (iNOS) and glutathione peroxidase activity better than either compound alone. Glutathione reductase was also increased in UC and UP rats vs. UC. Kidney 4-hydroxynonenal was significantly increased in the UC group compared with the normal group. Combined treatment with both compounds significantly blunted this increase, P < 0.05, while either compound alone had no effect. Additionally, the expression of Mn-SOD was increased and CuZn-SOD decreased by uremia. This was ameliorated in all treatment groups. Cotreatment with enalapril and paricalcitol had an additive effect in increasing CuZn-SOD expression. In conclusion, like enalapril, paricalcitol alone can improve proteinuria, glomerulosclerosis, and interstitial infiltration and reduce renal oxidative stress. The effects of paricalcitol may be amplified when an ACE inhibitor is added since cotreatment with both compounds seems to have an additive effect on ameliorating uremia-induced changes in iNOS and CuZn-SOD expression, peroxidase activity, and renal histomorphometry.

vitamin D; ACEI; ANP; iNOS; eNOS

THE RENIN-ANGIOTENSIN-ALDOSTERONE system (RAAS) regulates the homeostasis of extracellular fluid volume, sodium and water balance, and cardiovascular activity (5). In the kidney, continual overactivation of the RAAS results in glomerular hypertension, fibrosis, and proteinuria that leads to progressive renal damage (22, 41). Since the RAAS has an essential role in both renal as well as cardiovascular pathophysiology, agents that inhibit this system such as angiotensin-converting enzyme inhibitors (ACEIs) and angiotensin II receptor blockers (ARBs), have been shown to have beneficial effects in patients with both chronic renal and cardiovascular diseases (17, 24, 31, 37, 43). Angiotensin II also activates NADPH oxidase, inducing oxidative stress in both the renal and cardiovascular systems by inducing free radical generation (1, 57), specifically, reactive oxygen species (ROS). ROS can oxidize proteins and DNA that promote lipid peroxidation at the vascular membrane (18), leading to an inflammatory cascade protagonist by inflammatory cytokines, including, TNF-α, through the initial activation of NF-kB (58).

Fortunately, living organisms have developed a number of antioxidant defenses to protect against damage from oxidative stress. Among others, the antioxidant capacity is determined by the activity/interaction of molecules such as superoxide dismutase (SOD), glutathione peroxidase, reduced glutathione, and vitamins A, C, and E (14, 48). These antioxidants work together in various cell compartments scavenging ROS. The first line of cellular antioxidant defense is SODs. Two of these are copper/zinc SOD (CuZnSOD), which is found in the cytosol, and manganese SOD (MnSOD), found in mitochondria (13). The glutathione redox cycle is also important in protecting against oxidative stress. Glutathione reductase (GR) catalyzes the reduction of oxidized glutathione (GSSG) to reduced glutathione (GSH), a major antioxidant (52). It has been shown that GSH treatment in rats can attenuate renal interstitial fibrosis and prevent injury from oxidative stress after ureteral obstruction (36). The other important enzyme in the glutathione redox cycle is glutathione peroxidase (GPx). GPxs catalyzes the reduction of peroxides including those produced during the production of GSH. In addition, the capacity of the kidney to respond to oxidative injury is also associated with the supply of NADPH, which is necessary for the production of GSH. NADPH oxidase reacts with NADPH to produce the free radical superoxide. NADPH oxidase is a major source of ROS, and its activation is a positive marker for oxidative stress.

Clinical studies also showed that vitamin D analogs decrease morbidity and mortality in patients with chronic kidney disease (10, 23, 51). Active vitamin D compounds have therapeutic potential in attenuating experimentally induced kidney diseases (29, 47, 50). In subtotally nephrectomized rats, calcitriol...
suppresses the progression of glomerulosclerosis and albuminuria (47). The vitamin D analog 22-oxacalcitriol ameliorates glomerular injury in a model of rat glomerulonephritis (29), and paricalcitol attenuates renal interstitial fibrosis in a model of obstructive nephropathy (50). A recent study from our laboratory demonstrated that the combination therapy of paricalcitol and the ACEI enalapril had an added benefit in ameliorating the progression of renal insufficiency by suppressing inflammatory pathways in the uremic rat model (35). The molecular mechanisms behind the actions of paricalcitol, alone or in combination with an ACEI, on oxidative stress and inflammation in the kidney of uremic rats are not fully understood. Therefore, the aim of this study was to investigate the effect of the ACE inhibitor enalapril and the specific vitamin D receptor activator (VDRA) paricalcitol, alone or in combination, on oxidant/antioxidant balance in the kidney using the 5/6 nephrectomized uremic rat model.

**METHODS**

**Animal studies.** 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 weighing from 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. After 7 days of uremia, blood was drawn and the animals were divided into the following groups: 1) UC, uremic control treated with vehicle (100 μl of propylene glycol ip); 2) UE, uremic + enalapril (25 mg/l in drinking water); 3) UP, uremic + paricalcitol (0.8 μg/kg in 100 μl propylene glycol ip, 3 × wk); and 4) UEP, uremic + enalapril + paricalcitol. A group of normal rats also served as control (N). The rats were treated for 3 mo. All animals were fed a high-phosphorus rodent diet containing 0.9% phosphorus and 0.6% calcium. Blood pressures (BP) were obtained before surgery and then monthly using the Non-Invasive BP System XBP1000 (Kent Scientific, Torrington, CT). The last few days of the study, the rats were placed in metabolic cages and urine was collected for 2- to 24-h periods for analysis of urinary creatinine (Cr) and protein excretion. Approximately 20 h after the last treatment, the rats were euthanized by exsanguination via the dorsal aorta and blood was taken for analysis of serum Cr and parathyroid hormone (PTH). Portions of kidney were taken for analysis. Some portions were fixed in formalin for histochmical analysis of glomerular sclerosis and interstitial infiltration. Others were snap frozen in liquid nitrogen and stored at −80°C until later analysis. The left ventricle was separated from the right ventricle and the right and left atria, weighed, and divided. Portions were frozen in RNA-later and stored at −80°C until later analysis.

**Analytic determinations.** Cr and protein were determined by an autoanalyzer (COBAS MIRA plus, Branchburg, NJ). Serum PTH was determined using a Rat PTH ELISA kit from Immunotopics (San Clemente, CA). Cr clearance was calculated as previously reported (8). Urinary protein is expressed as milligrams excreted per 24 h.

**Histochemistry and assessment of glomerulosclerosis and interstitial infiltration.** Kidney tissue was fixed in formalin, 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 Bowman’s capsule, capillary obliteration, mesangial expansion, or segmental tuft sclerosis were defined as glomerulocrotic. The extent of glomerular damage was expressed as the percentage of glomeruli that exhibited sclerosis. The degree of glomerulosclerosis was graded from 1 to 4 points according to the percentage of effected glomeruli (1 = <10%, 2 = 10–20%, 3 = 25–50%, and 4 = >50%). 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 with Masson’s trichrome was quantitatively evaluated by a point-counting technique in 10 randomly selected microscopic fields, under magnification of ×200. Blind analysis was done on all sections by one observer. Involvement of interstitial infiltration was also graded (1–4 points) according to the following percentages (1 = <10%, 2 = 10–20%, 3 = 25–50%, and 4 = >50% of kidney section area).

**Determination of cardiac atrial natriuretic peptide.** The left ventricle was isolated from the euthanized rats and preserved in 500 μl RNA later (Applied Biosystems, Foster City, CA) and stored at −80°C. Total RNA was isolated from ~25 μg of tissue with the RNeasy kit (Qiagen, Valencia, CA), using the added DNase digestion step. Total RNA was quantified and then reverse transcribed into cDNA using SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, CA). Quantitative PCR was carried out using Taqman primer sets (Applied Biosystems) for the following gene transcripts: Nppa Rn00561661_m1 and GAPDH Rn9999916_s1, which was used as a control. Each primer set was received as a 20× mix and was diluted to 1× with Taqman Universal PCR Master Mix (Applied Biosystems), autoclaved water, and cDNA in a 20-μl reaction volume. Quantitative PCR was then carried out on the 7900HT Fast Real-Time PCR System using the following temperature profile: 50°C for 2 min, followed by 95°C for 10 min, then 40 cycles of 95°C for 15 s and 60°C for 1 min. Cycle threshold (Ct) values were normalized to GAPDH and calculated to obtain fold-change values as previously described (28).

**Determination of kidney oxidative stress.** Tissues were rinsed in PBS (pH 7.0), homogenized as 5% in 50 mM phosphate buffer (pH 7.0 and containing 0.1 mM EDTA), and centrifuged at 10,000 g for 15 min at 4°C. The supernatants were then collected and stored at −80°C until assayed. The plate reader used for all analyses was an Automated Microplate reader (Bio-Rad, Hercules, CA). Protein concentrations in tissue homogenates and extracts were estimated according to the method of Read and Northcole (42), using the Coomassie protein assay dye and bovine serum albumin as the standard. GSH was determined as previously described (3), using a commercially available kit from Cayman Chemical (Ann Arbor, MI). The absorbance was measured 405 nm. Glutathione reductase and peroxidase activity were also determined using commercially available kits (703202 and 703102, respectively) also from Cayman Chemical. A total of 20.0 μl of samples and controls were assayed in triplicate and analyzed by reading the absorbance for a period of 7 min at 340 nm. For Western blot analysis, extracted proteins from tissues (40 μg) were resolved on a 12.5% SDS-PAGE running gel and 5% stacking gel. Proteins were then electrotransferred onto nitrocellulose membranes at 120 V for 1 h. After blocking in 5% nonfat powdered milk for 1 h, the membranes were washed and then treated with one of the following antibodies: inducible NO synthase (iNOS), endothelial NO synthase (eNOS), p22phox, Mn-SOD, CuZn-SOD, or β-actin (1:1,000 and 1:5,000), overnight at 4°C (Santa Cruz Biotechnology, Santa Cruz, CA) and Abcam (Cambridge, MA). After washing, the blot was incubated with horseradish peroxidase (HRP)-conjugated secondary antibody IgG (1:5,000 and 1:10,000) for 1 h at room temperature (Santa Cruz Biotechnology). The washed blot was then treated with SuperSignal West Pico Chemiluminescent Substrate for positive antibody reaction. Membranes were exposed to X-ray film (Kodak) for the visualization and scanning of protein bands using Alpha Imager software. 4-Hydroxyenonenal (4-HNE) in kidney tissue was determined using a kit (Cell Biolabs, San Diego, CA). Briefly, BSA standard or kidney tissue extracts (10 μg/ml protein) are adsorbed onto a 96-well plate for 2 h at 37°C. The HNE protein adducts present in the sample or standard are probed with an anti-HNE-His antibody, followed by an HRP-conjugated secondary antibody. The content of HNE protein adducts in an unknown sample is determined by comparison with a standard curve.
Statistical analyses. All data are expressed as means ± SE. One-way ANOVA with the Tukey posttest was used for comparison between uremic groups. Student’s nonpaired t-test was used to compare the normal control group with individual uremic groups.

RESULTS

BP. Systolic BP was well controlled in enalapril-treated rats but steadily increased to levels >160 mmHg in both UC and UP rats (Fig. 1), showing that paricalcitol alone did not control BP.

Serum and urinary findings. Table 1 depicts the body weights, serum Cr, Cr clearances, and PTH levels in all groups of rats. The body weights of uremic animals tended to be slightly less than those of their normal counterparts. As expected, serum Cr was elevated and Cr clearance decreased in all uremic groups compared with normal rats. Not surprisingly, treatment with enalapril (UE and UEP) ameliorated the deterioration in renal function as shown by both lower serum Cr levels and higher Cr clearances compared with uremic control rats (Cr: P < 0.001 and Cr clearance: P < 0.001 for both groups). As expected, serum PTH markedly increased after 3 mo of uremia (N: 83 ± 19 vs. UC: 3,496 ± 862 pg/ml, P < 0.01). Treatment with enalapril or paricalcitol significantly blunted the increase in PTH (UE: 371 ± 100 pg/ml, P < 0.001; UP: 629 ± 208 pg/ml, P < 0.01). More importantly, the combined treatment of enalapril and paricalcitol (UEP) further inhibited the increase in PTH (170 ± 60 pg/ml). This level was not significantly different from that of normal rats. Urinary protein excretion increased markedly in untreated uremic rats (N: 34 ± 3 vs. UC: 234 ± 35 mg/24 h, P < 0.001) (Fig. 2).

Enalapril treatment significantly blunted this increase (UE: 63 ± 6, P < 0.001; UEP: 67 ± 28 mg/24 h, P < 0.001). Paricalcitol treatment alone also produced a decrease in proteinuria (168 ± 17 mg/24 h), despite significant hypertension.

Renal histology. Figure 3 shows histochemical staining of representative kidney sections from each group, and Fig. 4 depicts the quantification for glomerulosclerosis (Fig. 4A) and mononuclear cell interstitial infiltration (Fig. 4B) derived from the analysis of the kidney sections. Masson trichrome-stained kidney sections show that in the kidneys of UC rats (Fig. 3B), tubules are dilated and lined by reactive epithelial cells and lumens contain cellular debris. There is severe interstitial fibrosis and interstitial inflammation and multifocal calcium deposits (Fig. 3B, *). Glomeruli show focal segmental scarring (arrow). Paricalcitol treatment (UP) ameliorates these findings; sections show only moderate interstitial fibrosis, tubular dilatation, and sparse chronic interstitial inflammation (Fig. 3D). Similar results were seen with enalapril treatment (UE; Fig. 3C). Cotreatment with both drugs resulted in further improvement. Minimal interstitial damage and no glomerular or tubular injury were observed in this group (Fig. 3E). Glomerulosclerosis was markedly increased in untreated uremic rats, involving at least 10–20% of glomeruli, P < 0.001 vs. all other groups (Fig. 4A). Treatment with either enalapril or paricalcitol prevented the increase in glomerulosclerosis observed in UC rats. The interstitial infiltration of mononuclear cells was also dramatically increased in UC rats, with involvement reaching 25–50% (Fig. 4B). Treatment with either enalapril or parical-

Table 1. Serum and urinary findings in normal and uremic rats treated with enalapril and/or paricalcitol

<table>
<thead>
<tr>
<th>N (n = 8)</th>
<th>UC (n = 13)</th>
<th>UE (n = 12)</th>
<th>UP (n = 10)</th>
<th>UEP (n = 12)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body wt, g</td>
<td>305.4 ± 5.3</td>
<td>283.6 ± 3.8</td>
<td>281.9 ± 4.2</td>
<td>291.9 ± 6.4</td>
</tr>
<tr>
<td>Serum creatinine, mg/dl</td>
<td>0.58 ± 0.03</td>
<td>1.65 ± 0.14</td>
<td>0.93 ± 0.02</td>
<td>1.50 ± 0.11</td>
</tr>
<tr>
<td>Creatinine clearance, ml/min</td>
<td>0.96 ± 0.07</td>
<td>0.30 ± 0.03</td>
<td>0.56 ± 0.03</td>
<td>0.35 ± 0.02</td>
</tr>
<tr>
<td>Serum PTH, pg/ml</td>
<td>83 ± 19</td>
<td>3,496 ± 862</td>
<td>371 ± 100</td>
<td>629 ± 208</td>
</tr>
</tbody>
</table>

Values are means ± SE. n, Number of rats. N, normal (untreated) group; UC, uremic control rats treated with vehicle; UE, uremic rats treated with enalapril; UP, uremic rats treated with paricalcitol; UEP, uremic rats treated with both enalapril and paricalcitol; PTH, parathyroid hormone. *P < 0.001 vs. UC. **P < 0.01 vs. UC. †P < 0.001 vs. N. ‡P < 0.01 vs. N. §P < 0.05 vs. N. ‡P < 0.01 vs. UE. †P < 0.05 vs. UE. §P < 0.05 vs. UE.
citol blunted this increase, and the combined treatment with both compounds produced a further inhibition.

**Effect on cardiac atrial natriuretic peptide.** Atrial natriuretic peptide (ANP) mRNA levels in cardiac tissue were analyzed by real-time (RT)-PCR (Fig. 5). The expression of ANP mRNA in the hearts of UC rats increased 133-fold compared with that of normal rats [designated as 1 unit (U)]. Treatment with paricalcitol alone inhibited this increase in ANP mRNA (97.4-fold, \( P < 0.01 \)), despite the lack of effect on systemic BP. Treatment with enalapril alone dramatically blunted the response (4.2-fold, \( P < 0.01 \)) seen in uremic rats, while cotreatment with both compounds had an even more marked effect and normalized ANP mRNA levels (1.4-fold, \( P < 0.01 \)).

**Effect on renal NADPH oxidase, MnSOD, and CuZnSOD.** Figure 6 depicts Western blot analysis of kidney protein levels of the NADPH oxidase subunit p22phox, MnSOD, and CuZnSOD. The top panel shows representative Western blots, and the bottom panel shows the quantification of several analyses. p22phox protein expression was increased in UC animals compared with their normal counterparts (UC: 1.80 ± 0.19 vs. N: 1.00 U, \( P < 0.001 \)). Treatment with enalapril or paricalcitol prevented this increase (UE: 0.90 ± 0.10 U, \( P < 0.01 \) and UP: 1.21 ± 0.09 U, \( P < 0.02 \)). Cotreatment with both compounds

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**Figure 3.** Representative microphotographs of Masson trichrome-stained sections from N (A), UC (B), UE (C), UP (D), and UEP rats (E) after 3 mo of treatment. Magnification ×200. The asterisk identifies multifocal calcium deposits, and the arrow indicates glomeruli showing focal segmental scarring. (Please see text for further description of findings.)

**Figure 4.** Quantification of glomerulosclerosis (A) and interstitial infiltration (B) in uremic rats after 3 mo of treatment with enalapril and paricalcitol, alone, or in combination; \( n = 5 \). Quantification was done using a point system from 1 to 4 with points representing the following percentages of involved kidney section area: 1 = <10%, 2 = 10–20%, 3 = 25–50%, and 4 = >50%. Values are means ± SE. *\( P < 0.001 \) vs. N. \( \beta P < 0.01 \) vs. N. \( \gamma P < 0.05 \) vs. N. \#\( P < 0.001 \) vs. UC. \( \alpha P < 0.05 \) vs. UC.

**Figure 5.** Effect of 3 mo of treatment with enalapril and paricalcitol, alone or in combination, on cardiac atrial natriuretic peptide (ANP) mRNA levels. ANP mRNA levels were analyzed by RT-PCR and normalized to GAPDH mRNA levels. RT-PCR was performed on samples of left ventricle. Values are means ± SE; \( n = 6/group \). *\( P < 0.001 \) vs. N. \#\( P < 0.01 \) vs. UC.
had no further effect. Compared with normal rats, mitochondrial MnSOD protein expression was also significantly increased (UC: 1.65 ± 0.22 vs. N: 1.00 U, P < 0.02) in UC animals. Treatment with enalapril or paricalcitol significantly blunted the increase in MnSOD (UE: 1.10 ± 0.07 U, P < 0.01 and UP: 0.90 ± 0.10 U, P < 0.02, respectively). Cotreatment had no further effect. On the other hand, cytosolic CuZnSOD protein expression was significantly decreased (UC: 0.42 ± 0.04 U vs. N: 1.00 U, P < 0.001) by uremia. Enalapril or paricalcitol inhibited this decrease (UE: 0.75 ± 0.06 U, P < 0.01 and UP: 0.63 ± 0.05 U, P < 0.01, respectively). More importantly, cotreatment with both enalapril and paricalcitol further ameliorated the decrease in CuZnSOD protein expression (0.87 ± 0.04 AU, P < 0.05) compared with either compound alone.

Effect on renal iNOS and eNOS. Western blot analysis of iNOS and constitutively expressed eNOS protein levels in kidney is shown in Fig. 7, with the top panel showing representative Western blots and the bottom panel showing the quantification of several analyses. iNOS was markedly induced in uremia (UC: 3.70 ± 0.59 U vs. N: 1 U, P < 0.001). While treatment with either paricalcitol or enalapril blunted this increase, treatment with both compounds was significantly more effective than either compound alone (P < 0.05) and totally prevented iNOS induction (UE: 1.90 ± 0.39, P < 0.001, UP: 2.00 ± 0.39, P < 0.001 and UEP: 0.90 ± 0.28 U, P < 0.001 vs. UC). On the other hand, the expression of eNOS protein was markedly suppressed in uremic rats (UC: 0.44 ± 0.07 U vs. N: 1 U, P < 0.001). Treatment with either enalapril or paricalcitol totally inhibited the eNOS suppression seen in UC rats, and no further effect was seen with the cotherapy of both compounds (UE: 1.90 ± 0.20, P < 0.001, UP: 0.91 ± 0.20, P < 0.001 and UEP: 1.11 ± 0.29 U, P < 0.001 vs. UC).

Effect on glutathione redox cycle. As shown in Fig. 8, glutathione peroxidase activity was markedly increased in UC animals compared with the N group (UC: 535 ± 22 vs. NC: 415 ± 29 nmol·min⁻¹·ml⁻¹, P < 0.002). Treatment with either enalapril or paricalcitol alone prevented this increase, while cotreatment with both compounds was even more effective (UC: 395 ± 51, P < 0.001, UP: 413 ± 50, P < 0.001 and UEP: 349 ± 42 nmol·min⁻¹·ml⁻¹, P < 0.05). In addition, glutathione reductase activity was significantly increased in rats receiving either enalapril or paricalcitol compared with UC rats (UC: 85 ± 9 vs. UC: 122 ± 7, P < 0.05 and UP: 142 ± 21 nmol·min⁻¹·ml⁻¹, P < 0.05).

Effect on kidney 4-HNE. Kidney 4-HNE was significantly increased in the UC group compared with normal (UC: 6.84 ± 0.59 AU vs. N: 2.53 ± 0.24 AU, P < 0.001). Both enalapril and paricalcitol blunted this increase, while cotreatment was significantly more effective (P < 0.05).
0.45 vs. N: 4.99 ± 0.26 μg/ml, P < 0.01). While treatment with enalapril or paricalcitol alone had no significant effect on 4-HNE (UE: 5.96 ± 0.56 μg/ml vs. UP: 6.96 ± 0.98 μg/ml), combined treatment with both compounds significantly blunted the increase in 4-HNE seen in UC rats (UEP: 5.36 ± 0.43 μg/ml, P < 0.05).

DISCUSSION

This study addresses the effects of enalapril, an ACEI, and paricalcitol, a VDRA, alone or in combination, on the progression of renal deterioration and renal oxidative stress in uremic rats. Previous studies have reported the beneficial effects of ACEIs on the progression of renal failure (17, 24, 31, 37, 43). Vitamin D and its analogs have also been shown to have renoprotective effects in experimentally induced renal failure (29, 35, 50) and in patients with chronic kidney disease (10, 23, 51). In a recent study, de Zeeuw et al. (9) treated a group of patients with type II diabetic nephropathy, who were already taking ACEIs or ARBs, with the specific VDRA paricalcitol, at a dose of 1 or 2 μg/day. The patients who receive the 2-μg dose had a significant decrease in proteinuria. This was in addition to any decrease in proteinuria that may have occurred due to ACEI or ARB therapy. In addition to the classic effects of active vitamin D compounds on mineralization, evidence supports a crucial role for calcitriol in the regulation of cell proliferation and differentiation and immune modulation (11).

In the current study, we show that treatment with enalapril, as expected, controlled BP in uremic rats. In addition, enalapril improved kidney function as seen by the lower serum Cr levels and higher Cr clearances seen in UE and UEP rats. Enalapril prevented proteinuria and glomerular sclerosis and markedly reduced interstitial infiltration of mononuclear cells into the kidney. Paricalcitol also ameliorated proteinuria and prevented glomerulosclerosis. This VDRA also reduced interstitial infiltration of mononuclear cells into the kidney. The combination of enalapril and paricalcitol was better than either treatment alone in preventing interstitial infiltration and the additive effect of both of these drugs might indicate that they act through different pathways. We have previously shown similar results with enalapril and paricalcitol treatment on proteinuria, glomerulosclerosis, and interstitial infiltration of mononuclear cells (35) and showed that these effects were due, at least in part, to the inhibition of the TGF-β1 pathway. The overexpression of TGF-β1 is known to be involved in the progression of renal disease, specifically matrix expansion, interstitial fibrosis, and glomerulosclerosis. In our earlier study, paricalcitol treatment, as well as treatment with enalapril, markedly reduced the expression of TGF-β1 as well as Smad2, a mediator of TGF-β1 signaling. Other studies have shown that treatment with calcitriol (47) or its analog 22-oxacalcitriol (29) also reduced TGF-β1 expression in the uremic rat model. In addition, the amelioration of experimentally induced kidney diseases by the use of anti-TGF-β1 antibodies has been shown (4, 27). Thus the TGF-β1 pathway is considered to be an important modulator of the progression of renal insufficiency. Active vitamin D compounds are known to have other anti-inflammatory effects in experimental kidney disease as well (2, 40, 45). Alborzi et al. (2) reported decreases in C-reactive protein in patients with chronic kidney disease, and suppression of IL-6 and TNF-α levels by calcitriol in patients has also been shown (45). The effect of paricalcitol could also be due, at least in part, to its effects on renin. Renin, the first and rate-limiting component of the RAAS, is a protease synthesized and secreted predominantly by the juxtaglomerular cells in the kidney. Vitamin D is known to be a negative regulator of renin gene expression and acts by inhibiting the transcription CREB, which is required for renin gene expression (56).

ANP is manufactured by myocytes and released in response to high BP. ANP can suppress RAAS and acts to reduce water and sodium loads on the circulatory system to reduce BP. Our data show that ANP mRNA increased 133-fold in untreated uremic rats in tandem with the increase in BP in these animals. The increase in ANP mRNA in paricalcitol-treated rats, while still marked, was only 70% that of untreated uremic animals. Furthermore, this apparent reduction in ANP gene expression in the presence of paricalcitol occurred without a significant reduction in BP. This suggests that paricalcitol may have direct effects on the myocyte. Importantly, the combination therapy of both enalapril and paricalcitol had a more marked effect and totally inhibited the rise in ANP. Several studies indicate that in addition to its effects on regulation of volume and BP, ANP can act to preserve renal function (7, 34). Koga et al. (25) showed in rats that ANP also has antioxidant effects as demonstrated by its ability to attenuate ROS levels in a renal ischemia-reperfusion (I/R) injury model. Therefore, it is possible that in the current study ANP mRNA may be increased as a protective mechanism.

It is well known that oxidative stress and inflammation are implicated in both renal and cardiovascular diseases. Oxidative stress can result from an excess of free radicals/ROS, a decrease in antioxidants, or a combination of both. Angiotensin II is known to induce oxidative stress by activating NADPH oxidase, which leads to the generation of superoxides (1, 57). Activation of NADPH oxidase not only causes inflammation but also leads to the generation of inflammatory cytokines through NF-κB activation (58). We determined NADPH oxidase by measuring the protein expression of p22phox and p22phox is one of two membrane-spanning subunits of NADPH oxidase and serves as an electron-transfer component (3). The marked increase in p22phox protein expression seen in untreated uremic rats appeared in conjunction with the increase in BP in this group. Paricalcitol alone inhibited p22phox protein expression, even though BP was elevated in this group. Treatment with enalapril also prevented the increase as did cotreatment with the two drugs. Recent studies from members of our group also showed amelioration by paricalcitol and enalapril of uremia-induced NADPH oxidase activity in rat cardiac tissue (20) and of apolipoprotein E knockout-induced NADPH oxidase activity in the mouse aorta (21). Since active vitamin D compounds have been shown to negatively regulate the RAAS (26, 56), the inhibition of p22phox by either enalapril or paricalcitol points to a role for the RAAS in the oxidative stress seen in the kidney in the current study.

NOS are enzymes that catalyze the production of nitric oxide (NO), an important cell-signaling molecule. Sustained levels of high NO result in tissue damage, whereas low levels of NO are necessary for various cell functions and to protect organs from ischemic damage. NO is known to occur at higher levels in an oxidative environment and is activated during inflammation by inflammatory cytokines (33). Our data showed a significant inhibition of uremia-induced inducible NOS by monotherapy with enal-
april or paricalcitol and that cotreatment with both compounds resulted in total inhibition of iNOS induction. This indicates that the inhibition of iNOS induction by these two compounds may involve different pathways. It has been shown that in hypertension, renal iNOS expression can be increased in response to an increase in ROS (54, 55). Broadbent et al. (6) demonstrated that increasing pressure in cultured proximal tubular epithelial cells can also lead to early iNOS induction. In addition, two other studies reported that iNOS activity is associated with significant reductions in glomerular filtration rate in two different models of renal injury (39, 46). In both cases, iNOS inhibition was associated with a significant improvement in glomerular filtration rate. eNOS is expressed in endothelial cells, including those of the kidney, consistent with its important role in maintaining endothelial function (16). Our data show that eNOS is markedly suppressed in untreated uremic rats. Treatment with paricalcitol prevents this decrease. This same effect was seen with enalapril alone or with cotreatment with the two drugs. Several studies underscore the importance of eNOS in preventing the development of renal disease. eNOS knockout mice were shown to develop congenital renal abnormalities (12) and more severe inflammation (19). Mendoza et al. (32) reported that eNOS deficiency accelerates renal injury and that this injury could be ameliorated by the administration of antioxidants. In addition, a lack of eNOS was shown to accelerate both glomerular and tubulointerstitial injury and loss of glomerular and peritubular capillaries (38). Clearly, our current findings underscore the importance of eNOS in maintaining healthy renal function and that paricalcitol and enalapril can promote eNOS expression.

GSH is a major endogenous antioxidant made by cells and is produced by the reduction of GSSG by the enzyme GSR. GSH neutralizes free radicals and ROS and maintains the active states of vitamins C and E (49). GSH plays a fundamental role in protein synthesis and DNA synthesis and repair as well as in many other functions (49, 53). Most every system in the body is dependent upon the state of the glutathione system. Decreases in GSH levels can impair a cell’s defense against ROS and result in oxidative injury (49, 53). We analyzed GSR activity and showed that it was increased in uremia, whereas treatment with either enalapril or paricalcitol inhibited the increase in GSR. We also analyzed the activity of GPx, the other enzyme in the glutathione redox cycle. GPx catalyzes the reduction of peroxides, including those formed during the production of GSH. We found that untreated uremia produced a significant increase in GPx activity but that enalapril or paricalcitol prevented this increase. When given together, these two compounds had a more marked effect. The increased GSR and GPx activities in untreated uremia may be a compensatory response to the oxidative cellular environment in untreated uremic rats.

We found additional evidence of a protective effect against oxidative stress with the combined therapy of enalapril and paricalcitol in showing that uremia increases the expression of HNE, a marker of oxidative stress, in the kidney and that the combined therapy of enalapril and paricalcitol significantly inhibits this increase. HNE is a natural byproduct of lipid peroxidation and is increased in oxidative stress as a consequence of increased lipid peroxidation. These results imply that there is an increase in lipid peroxidation in uremia and that combined therapy with enalapril and paricalcitol can inhibit this.

SODs scavenge superoxide by catalyzing the dismutation of superoxide to H2O2 and O2. Mn-SOD is mitochondrial, while CuZn-SOD is cytosolic (30). These two enzymes are considered to be the first line of defense against oxygen radicals in cells. Our data in kidney show that Mn-SOD protein expression was increased in untreated uremic animals. This increase may be a compensatory response for the disposal of excess superoxides generated by NADPH oxidase activation. Monotherapy with either enalapril or paricalcitol inhibited the increase in Mn-SOD protein expression. Mn-SOD is known to be induced in response to inflammatory cytokines such as TNF-α (15) and both enalapril and paricalcitol are known to have anti-inflammatory actions. On the other hand, CuZn-SOD protein expression was markedly reduced in uremic control rats. Both enalapril and paricalcitol, when given alone, inhibited this decrease in CuZn-SOD protein expression. Husain et al. (20) recently showed similar results with both Mn-SOD and CuZn- SOD in cardiac tissue from uremic rats treated with enalapril and/or paricalcitol. CuZn-SOD also has anti-inflammatory actions, but it is also known to be susceptible to inactivation by ROS (44), which is possibly why it is decreased in untreated uremic rats.

In summary, our data confirm that similar to enalapril, paricalcitol can suppress the progression of renal failure by ameliorating proteinuria, glomerulosclerosis, interstitial inflammation, oxidative stress, and myocardial ANP gene expression. The effects of paricalcitol may be amplified when an ACE inhibitor is added since cotreatment with both compounds seems to have an additive effect on ameliorating uremia-induced changes in iNOS and CuZn-SOD expression, peroxidase activity, and renal histomorphometry. As we have shown previously, the action of paricalcitol is probably, at least in part, via the TGF-β pathway, decreasing the expression of both TGF-β and Smad2. Combination therapy with ACEIs, or likely ARBs, and paricalcitol may represent a novel and beneficial therapeutic strategy for suppressing the progression of chronic kidney disease and ameliorating oxidative stress.

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DISCLOSURES

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REFERENCES


