Renoprotective effects of paricalcitol on gentamicin-induced kidney injury in rats

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Park JW, Bae EH, Kim IJ, Ma SK, Choi C, Lee J, Kim SW. Renoprotective effects of paricalcitol on gentamicin-induced kidney injury in rats. Am J Physiol Renal Physiol 298: F301–F313, 2010. First published November 25, 2009; doi:10.1152/ajprenal.00471.2009.—Vitamin D is thought to exert a protective effect on renal disease progression, but the underlying molecular mechanism remains unclear. We investigated whether paricalcitol ameliorates tubular dysfunction and fibrosis in gentamicin (GM)-induced renal injury. Two groups of rats were treated with GM (100 mg·kg−1·day−1), one of which was cotreated with paricalcitol (0.3 μg·kg−1·day−1) for 14 days and the other was not. The control group was treated with vehicle only. HK-2 cells were cultured with GM in the absence or presence of paricalcitol. Paricalcitol restored impaired renal function and the downregulated renal sodium transporters and aquaporin-1 expression caused by GM. ED-1-expressing monocyte/macrophage accumulation induced by GM was attenuated by paricalcitol treatment. Paricalcitol prevented upregulated inflammatory cytokines (TNF-α, IL-1β, INF-γ) and adhesion molecules (monocyte chemoattractant protein-1, ICAM-1, VCAM-1) induced by GM. In addition, paricalcitol effectively reversed TGF-β1-induced epithelial-to-mesenchymal transition (EMT) process and extracellular matrix accumulation in GM-induced nephropathy. Increased collagen deposition and fibrosis in GM-treated kidney were ameliorated by paricalcitol. Paricalcitol also attenuated the upregulated NF-κB and phosphorylated ERK1/2 expression in HK-2 cells cultured with GM. In conclusion, paricalcitol prevents GM-induced renal injury by inhibiting renal inflammation and fibrosis, the mechanism of which is the interruption of NF-κB/ERK signaling pathway and preservation of tubular epithelial integrity via inhibiting EMT process.

inflammation; fibrosis

Calcitriol, the physiologically active form of 1,25-dihydroxyvitamin D3, plays an important role not only in the establishment and maintenance of calcium metabolism but also in direct modulatory effects on inflammation, mesangial cell proliferation, and proximal tubular cell growth in the kidney (40–42). The active form of vitamin D mediates its biological effects by binding to the vitamin D receptor (VDR), which then translocates to the nuclei of target cells (3). It has been shown to have therapeutic potential in attenuating experimentally induced glomerular diseases (15, 21, 28). Although most of these studies focused on the effect of calcitriol or its analogs on glomerular damage, kidney tubules, including proximal tubule, are identified as another target of active vitamin D (3, 35). The suppressive effect of calcitriol on renal interstitial myofibroblasts indicates that calcitriol has a potential role in inhibiting the development of renal interstitial fibrosis (16). A recent study demonstrated the renoprotective effects of paricalcitol (19-nor-1,25-dihydroxyvitamin D2), an active, nonhypercalcemic vitamin D analog, in various experimental models (24, 34, 36).

Aminoglycoside antibiotics continue to be widely used to treat serious gram-negative infections. One of their main side effects is nephrotoxicity, manifested by nonoliguric renal failure with a progressive increase of serum creatinine levels. The specificity of gentamicin (GM) for renal toxicity is apparently related to its preferential accumulation in the renal proximal convoluted tubules, which may induce tubulointerstitial inflammation (1, 29). Inflammatory and fibrogenic responses to GM were associated with increases in transforming growth factor (TGF)-β1, endothelin, and angiotensin II levels, implying their involvement in the progression of tubulointerstitial nephritis. In addition, formation of reactive oxygen species (ROS), such as superoxide, has been shown to increase with GM treatment (6). This evolution of ROS would stimulate the activation or expression of proinflammatory mediators, including nuclear factor (NF)-κB, leukocyte adhesion molecules, mitogen-activated protein kinases (MAPKs), and TGF-β1 (37), which contribute to progressive kidney damage induced by GM.

In the progressive renal disease including GM-induced nephropathy, renal inflammation, characterized by the infiltration of inflammatory cells such as monocytes/macrophages and myofibroblasts, is known to be an imperative pathological process in the evolution of chronic kidney disease (CKD) (31, 33). Infiltrated macrophages in renal tubular interstitium can release proinflammatory, chemoattractive cytokines, thereby leading to the formation of a vicious self-accumulation cycle. Furthermore, infiltrated macrophages in the inflamed kidney can produce profibrotic cytokines such as TGF-β1, which has a crucial role in progressive renal fibrosis through a tubular epithelial-to-mesenchymal transition (EMT) process beyond its property on extracellular matrix (ECM) accumulation (2, 17). It is now clear that progression of renal insufficiency is closely correlated to the degree of renal interstitial fibrosis (20). Myofibroblasts play a central role in progressive renal fibrosis, and one of the origins of these cells is derived from EMT of renal proximal tubular cells into myofibroblasts. Through EMT, renal proximal tubular cells have been shown to contribute to renal interstitial fibrosis (17). Hence, inhibition of renal inflammation may be therapeutically effective, resulting in an amelioration of renal fibrotic lesion.

Proximal tubular epithelial cells are reported to play a crucial role in producing cytokines, chemokines, and cell adhesion molecules for the recruitment, retention, and activa-
Table 1. Changes in renal functional parameters

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>GM</th>
<th>GM + Pari</th>
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<tbody>
<tr>
<td>n</td>
<td>8</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>UO, ml/day</td>
<td>17.6 ± 3.6</td>
<td>27.0 ± 3.0*</td>
<td>21.2 ± 11.1‡</td>
</tr>
<tr>
<td>PCr, mg/dl</td>
<td>0.06 ± 0.05</td>
<td>0.79 ± 0.10*</td>
<td>0.61 ± 0.11‡</td>
</tr>
<tr>
<td>Co, ml/min</td>
<td>0.80 ± 0.09</td>
<td>0.56 ± 0.04*</td>
<td>0.61 ± 0.06</td>
</tr>
<tr>
<td>PUA, mcg/l</td>
<td>141.5 ± 0.4</td>
<td>143.3 ± 0.9</td>
<td>143.1 ± 0.6</td>
</tr>
<tr>
<td>FEpos, %</td>
<td>1.29 ± 0.16</td>
<td>1.43 ± 0.15*</td>
<td>1.03 ± 0.19‡</td>
</tr>
<tr>
<td>Uosmol, mosmol/kgH2O</td>
<td>1442.9 ± 363.9</td>
<td>708.8 ± 216.4*</td>
<td>902.1 ± 310.8</td>
</tr>
<tr>
<td>Pposmol, mosmol/kgH2O</td>
<td>296.1 ± 3.4</td>
<td>306.6 ± 2.3*</td>
<td>308.6 ± 3.1</td>
</tr>
<tr>
<td>U/P posmol</td>
<td>4.89 ± 1.25</td>
<td>3.22 ± 0.69*</td>
<td>3.31 ± 0.71</td>
</tr>
<tr>
<td>PUA, mg/dl</td>
<td>0.09 ± 0.06</td>
<td>0.28 ± 0.09</td>
<td>0.51 ± 0.12</td>
</tr>
<tr>
<td>PUA, mg/dl</td>
<td>0.15 ± 0.07</td>
<td>0.16 ± 0.10</td>
<td>0.15 ± 0.07</td>
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<tr>
<td>Values are means ± SE; n = number of rats. GM, gentamicin; Pari, paricalcitol; UO, urine output; PCr, plasma creatinine; Co, creatinine clearance; PUA, plasma sodium; FEpos, fractional excretion of sodium into urine; Uosmol, urine osmolality; Pposmol, plasma osmolality; PCr, plasma total calcium; PUA, plasma inorganic phosphorus. *P &lt; 0.05; ‡P &lt; 0.01 compared with control. §P &lt; 0.05; †P &lt; 0.01 compared with GM-treated rats.</td>
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SDS-polyacrylamide gel electrophoresis was performed on 9 or 12% polyacrylamide gels. The proteins were transferred by gel electrophoresis (Mini Protean II; Bio-Rad, Hercules, CA) onto nitrocellulose membranes (Hybond ECL RPN3032D; Amersham Pharmacia Biotech, Little Chalfont, UK). The blots were blocked with 5% milk in PBS-T (80 mM Na2HPO4, 20 mM NaH2PO4, 100 mM NaCl, and 0.1% Tween 20, pH 7.5) for 1 h and then incubated overnight at 4°C with primary antibodies, followed by incubation with secondary anti-rabbit, anti-mouse, or anti-goat horseradish peroxidase-conjugated antibodies. The labeling was visualized using an enhanced chemiluminescence system.

Immunohistochemistry. A perfusion needle was inserted in the abdominal aorta, and the vena cava was cut to establish an outlet. Blood was flushed from the kidney with cold PBS (pH 7.4) for 15 s before switching to cold 3% paraformaldehyde in 0.1 M cacodylate buffer (pH 7.4) for 3 min. The kidney was removed, sectioned into 2- to 3-mm transverse sections, and immersion-fixed for 1 h, followed by three 10-min washes in 0.1 M cacodylate buffer (pH 7.4). The tissue was dehydrated in a graded ethanol series and left overnight in xylene. After the tissue was embedded in paraffin, 2-μm sections were made on a rotary microtome (Leica Microsystems, Herlev, Denmark). Immunoperoxidase labeling was performed as previously described (12).

Primary antibodies. Affinity-purified rabbit polyclonal antibodies were used against Na+/K+-ATPase α1-subunit (polyclonal; Upstate Biotechnology, Lake Placid, NY), type 3 Na+/H+ exchanger (NHE3; polyclonal; Alpha Diagnostic, San Antonio, TX), aquaporin-1 (AQP1; polyclonal; Alomone Laboratories, Jerusalem, Israel), IκBα (polyclonal; Santa Cruz Biotechnology, Santa Cruz, CA), phosphorylated NF-κB p65 (p65 NF-κB; Ser536; monoclonal; Cell Signaling Technology, Danvers, MA), histone H3 (polyclonal; Cell Signaling Tech-
nology), phosphorylated extracellular signal-regulated kinases 1/2 (pERK 1/2; monoclonal; Cell Signaling Technology), total ERK1/2 (polyclonal; Cell Signaling Technology), and TGF-β1 (polyclonal; Santa Cruz Biotechnology). Anti-mouse antibodies were used against inducible nitric oxide synthase (iNOS; monoclonal; BD Transduction Laboratories, Lexington, KY), ED-1 (a monoclonal marker for macrophages; Santa Cruz Biotechnology), E-cadherin (monoclonal; BD Transduction Laboratories), α-smooth muscle actin (α-SMA; monoclonal; Sigma Chemical), and fibronectin (monoclonal; Santa Cruz Biotechnology). Anti-goat antibody was used against connective tissue growth factor (CTGF; polyclonal; Santa Cruz Biotechnology).

Real-time PCR. Renal cortex was homogenized in Trizol reagent (Invitrogen, Carlsbad, CA). RNA was extracted with chloroform, precipitated with isopropanol, washed with 75% ethanol, and then dissolved in distilled water. The mRNA expression of inflammatory cytokines and adhesion molecules was determined by real-time PCR. cDNA was made by reverse transcribing 5 μg of total RNA using oligo(dT) priming and SuperScript reverse transcriptase.

Fig. 3. Effects on renal sodium transporters and aquaporin-1 (AQP1) expression. Semiquantitative immunoblotting of Na⁺/K⁺-ATPase α1-subunit (A), type 3 Na⁺/H⁺ exchanger (NHE3; B), and AQP1 (C) in the cortex/outer stripe of outer medulla (OSOM). The expression of sodium transporters and AQP1 was significantly decreased in the GM group compared with the control group. It was recovered by paricalcitol administration. Values are means ± SE of 4 rats. *P < 0.05 compared with control. †P < 0.05 compared with GM-treated rats.

Fig. 4. Immunoperoxidase microscopy of NHE3 and AQP1 in the cortex. Decreased immunolabeling of NHE3 and AQP1 is visible in GM-treated rats, but this was restored by paricalcitol cotreatment (magnification, ×200).
tase II (Invitrogen). cDNA was quantified using the SmartCycler II system (Cepheid, Sunnyvale, CA), and SYBR green was used for detection. Each PCR reaction was done in 10 μM forward primer, 10 μM reverse primer, 2× SYBR green Premix Ex Taq (Takara Bio, Seto, Japan), 0.5 μl of cDNA, and H2O to bring the final volume to 20 μl. Relative levels of mRNA were determined by real-time PCR using a Rotor-Gene 3000 detector system (Corbette Research, Mortlake, New South Wales, Australia). (See Table 2 for primer sequences.)

The PCR was performed according to the following steps: 1) 95°C for 5 min, 2) 95°C for 20 s, 3) 58–62°C for 20 s (optimized for each primer pair), 4) 72°C for 30 s, and 5) 85°C for 6 s to detect SYBR green. Steps 2–5 were repeated for an additional 64 cycles, and at the end of the last cycle, temperature was increased from 60 to 95°C to produce a melt curve. Data from the reaction were collected and analyzed with the Corbett Research Software. The comparative critical threshold (Ct) values from quadruplicate measurements were used to calculate the gene expression, with normalization to GAPDH as an internal control (18). Melting curve analysis was performed to enhance specificity of the amplification reaction.

Statistical analysis. Results are means ± SE. Multiple comparisons among the groups were made by one-way ANOVA and post hoc Tukey’s honestly significant difference test. P values <0.05 were considered significant.

RESULTS

Functional parameters. Table 1 represents changes of renal functional parameters. GM treatment resulted in increased plasma creatinine level and urine output compared with controls. Fractional excretion of sodium (FENa) was increased, and urine osmolality was decreased. Paricalcitol ameliorated increased plasma creatinine level, FENa, and urine output in GM-treated rats, whereas urine osmolality was not changed. Plasma total Ca2+ levels showed no significant differences among control, GM-, and GM plus paricalcitol-treated rats. In addition, plasma inorganic phosphorus levels were comparable between control and GM-treated rats, whereas paricalcitol treatment revealed increased inorganic phosphorus levels compared with GM-treated rats.

Effects of paricalcitol on accumulation of GM in renal tubular cells. We measured GM concentration in renal cortex and medulla treated with GM or GM plus paricalcitol in rats. We also measured GM concentration in HK-2 cells treated with GM or GM plus paricalcitol. There was no significant difference in GM concentration between GM and GM plus paricalcitol in HK-2 cells and in vivo (Fig. 1). Therefore, the
effect of paricalcitol on GM-treated rat proximal tubules in our study was not due to the competitive inhibition of GM accumulation in renal tubular cells.

**Effects on morphological changes of renal proximal tubules.** Hematoxylin and eosin (H&E) staining and CD10 (a marker of proximal convoluted tubule) immunohistochemical staining of renal cortex in control rats and rats treated with GM and GM plus paricalcitol was performed (Fig. 2). These stains revealed the tubular dilatation, blebs, and denuded cells in the proximal tubules in GM-treated rats compared with those in control rats. Paricalcitol improved these changes.

**Effects on sodium transporters and AQP1 expression.** We demonstrated the effect of GM with or without paricalcitol on the expression of proximal tubular transporters. The protein expression of \( \text{Na}^+\text{-K}^+\)-ATPase \( \alpha_1 \)-subunit, NHE3, and AQP1 was significantly decreased in GM-treated rats compared with controls, and this was counteracted by paricalcitol (Fig. 3). Immunohistochemistry confirmed a decreased NHE3 and AQP1 labeling in proximal tubules in GM-treated rats that was prevented by paricalcitol (Fig. 4). In vitro data also showed similar results. HK-2 cells were incubated with GM (1.0 mg/ml) for 24 h, and some of those cells were pretreated for 3 h with paricalcitol (0.2 ng/ml) before GM administration. Semiquantitative immunoblotting showed decreased expression of NHE3 and AQP1, whereas paricalcitol pretreatment reversed the downregulation of NHE3 and AQP1 in HK-2 cells (Fig. 5).

**Effects on inflammatory cell infiltration, inflammatory cytokines, and adhesion molecules.** To evaluate the effect of paricalcitol on infiltration of inflammatory cells, we analyzed the protein expression of ED-1 and the infiltration of ED-1-positive macrophages in renal tissue (Fig. 6, A and B). The protein expression of ED-1 in cortex/OSOM was significantly increased in GM-treated rats compared with controls, and this was ameliorated by paricalcitol (Fig. 6A). Immunohistochemistry showed that the infiltration of ED-1-positive macrophages was increased in GM-treated rats compared with controls. Paricalcitol cotreatment reduced inflammatory cell infiltration in GM-treated kidneys (Fig. 6B). The expression of iNOS, which is known to be synthesized during inflammation in renal disease, was increased in cortex/OSOM of GM-treated rats compared with control, and this was restored by paricalcitol.

![Fig. 6](http://ajprenal.physiology.org/)

**Fig. 6.** Effects on inflammatory cell infiltration and inducible nitric oxide synthase (iNOS) expression. **A**: semiquantitative immunoblotting of ED-1 expression. Inflammatory cell infiltration is determined by immunohistochemical staining of ED-1, a specific marker of macrophages in the cortex/OSOM. **B**: the infiltration of ED-1-expressing cells was significantly increased in GM-treated rats and lessened by paricalcitol treatment (magnification, \( \times 200 \)). Arrows indicate the infiltrated ED-1-positive cells. Bar graphs represent the quantitation of ED-1-positive cells in the cortex/OSOM. **C**: semiquantitative immunoblotting of iNOS in the cortex/OSOM. The expression of iNOS was increased in GM challenge compared with controls, and it was recovered by paricalcitol coadministration. Values are means ± SE of 4 rats. *\( P < 0.05 \); **\( P < 0.01 \) compared with control. †\( P < 0.05 \); ‡\( P < 0.01 \) compared with GM-treated rats.
controls, and this was counteracted by paricalcitol (Fig. 8). We also investigated the expression of TNF-α, IL-1β, and IFN-γ, which are key inflammatory cytokines produced by the infiltrated inflammatory cells. As presented in Fig. 8, GM showed higher mRNA expression of TNF-α, IL-1β, and IFN-γ in the kidney, whereas these changes were attenuated with paricalcitol cotreatment. Certain chemokines and adhesion molecules such as monocyte chemoattractant protein (MCP)-1, ICAM-1 and VCAM-1, which can activate, recruit, or transmigrate inflammatory cells into the site of renal injury, were also demonstrated. mRNA expression of MCP-1, ICAM-1, and VCAM-1 in the kidney was increased by GM compared with controls, and this was counteracted by paricalcitol (Fig. 8).

Effect on NF-κB expression. Figure 9A showed the changes of NF-κB p65 subunit level in nuclear extracts of renal tubular HK-2 cells incubated with GM (1.0 mg/ml). The expression of p65 subunits of NF-κB started to increase 1 h after GM exposure. Cytoplasmic total IkBα expression began to decrease at 2 h and increased progressively afterward. NF-κB expression was also determined in HK-2 cells that were pretreated with paricalcitol (0.1 and 0.2 ng/ml) for 3 h following GM (1.0 mg/ml) exposure for 6 h. The expression of p65 subunits of NF-κB was higher in GM-treated than in controls, whereas these increases induced by GM were attenuated with paricalcitol pretreatment (Fig. 9B).

Effect on pERK1/2 expression. When HK-2 cells were incubated with GM (0.5 mg/ml), pERK1/2 antibody binding started to increase 30 min after GM exposure and remained elevated for the whole period of observation (Fig. 10A). pERK expression was also determined in HK-2 cells pretreated with paricalcitol (0.1 and 0.2 ng/ml) for 2 h before GM administration. pERK overexpression induced by GM was significantly repressed by paricalcitol pretreatment (Fig. 10B).

Effect on TGF-β1 expression and fibrosis. Semiquantitative immunoblotting revealed that the expression of TGF-β1, an important profibrotic molecule derived by infiltrating inflammatory cells, was significantly increased in the cortex/OSOM of GM-treated rats, an increase that was markedly attenuated by paricalcitol (Fig. 11A). We also examined the expression of epithelial adhesion receptor E-cadherin and α-SMA, the molecular marker of myofibroblasts. GM induced a suppression of E-cadherin (Fig. 11B) and a dramatic induction of α-SMA (Fig. 11C), a shift that is in agreement with tubular EMT. On the other hand, paricalcitol preserved E-cadherin expression and inhibited α-SMA induction in GM-treated rats along with the TGF-β1 downregulation.

In addition, in HK-2 cells, GM treatment induced increased expression of fibronectin and CTGF compared with controls. Paricalcitol pretreatment also reduced their expression induced by GM (Fig. 12). In relation to these changes, Masson’s trichrome stain demonstrated that GM resulted in increased deposition of collagen and fibrosis in the kidney. These changes were ameliorated by paricalcitol (Fig. 13).

**DISCUSSION**

GM treatment increased serum creatinine levels along with urinary concentration defect. Accordingly, the expression of AQP1, Na⁺-K⁺-ATPase ζ-subunit, and NHE3 was decreased in the kidney. Immunoperoxidase microscopy of AQP1 and NHE3 also showed a pronounced decrease of its labeling in the proximal tubule. Moreover, paricalcitol prevented the dysregulation of these transporters and improved the renal function. Thus paricalcitol treatment may be of significant value in protecting GM-induced impaired renal tubule function.

25-(OH) vitamin D₃ and vitamin D binding protein filtered through the glomeruli are taken up by megalin into the proximal tubular cell, and megalin-dependent pathway can modulate the binding and endocytosis of polybasic drugs such as aminoglycoside (25, 26). Thus it may be possible that paricalcitol inhibits GM accumulation in proximal tubule by competition for GM binding to megalin. The present study, however, revealed that there was no significant difference in GM concentration between GM and GM plus paricalcitol in HK-2 cells and in vivo. Therefore, the beneficial effect of paricalcitol on GM-treated rat proximal tubule was not attributed to the competitive inhibition of paricalcitol for GM accumulation in renal tubular cells.

The molecular mechanisms by which the expression of renal sodium transporters and AQP1 water channel is decreased in the kidneys in GM-induced kidney injury are unknown. In the present study, the expression of NHE3 and AQP1 as examined by Western blotting and immunohistochemistry was reduced in the proximal tubule, and tubular dilatation, blebs, and denuded cells were seen in the proximal tubules from the GM-treated kidneys. This raises a possibility that GM treatment causes generalized cell dysfunction and protein malmetabolism associated with structural changes in the proximal tubule, which
may cause downregulation of NHE3 and AQP1. Other secondary mechanisms, however, may also contribute to the decreased expression of NHE3 and AQP1. A recent study (27) demonstrated that lipopolysaccharide (LPS)-induced inflammation downregulated the expression of renal sodium transporters and AQP water channels. In addition, in cortical collecting duct cells, exogenous injection of TNF-α, IL-1β, or IFN-γ also decreased renal tubular function and inhibited the expression of renal sodium transporters (32). We showed that the expression of renal proinflammatory markers such as TNF-α, IL-1β, and IFN-γ was increased in GM-induced kidney injury, which was reversed by paricalcitol. These findings suggest that the restoration of AQP1 and sodium transporters by paricalcitol may in part be ascribed to quenching inflammatory responses.

Inflammatory cells release proinflammatory chemokines, thereby leading to the formation of a vicious self-accumulation circle. Not surprisingly, decline of renal function in CKD often correlates to the extent of inflammation (19). In the present study, GM-treated rats displayed a marked increase in monocyte/macrophage infiltration into the renal cortex/medulla, as indicated by the large number of ED-1-positive cells in the interstitium. These findings are consistent with previous observations demonstrating that GM can cause an increase in monocyte/macrophage populations in the kidney (9). GM increased expression of proinflammatory markers such as TNF-α, IL-1β, IFN-γ, and iNOS. These inflammatory molecules participate in the pathogenesis of tubulointerstitial impairment via the promotion of leukocyte attraction and adhesion to inflamed renal tubular cells. The expression of cell surface adhesion molecules such as MCP-1, ICAM-1, and VCAM-1, which are highly specific chemotactic factors for macrophages, was increased in GM-treated kidney. These findings indicate the inflammatory process has a significant role in the pathogenesis of GM-induced renal injury. Furthermore, we showed that paricalcitol significantly reduced the infiltration of ED-1-expressing macrophages in the kidney and decreased renal expression of proinflammatory cytokines and cell surface adhesion molecules induced by GM. These findings suggest that paricalcitol can attenuate the resultant kidney damage by suppression of cell surface adhesion molecules as well as inflammatory cytokines.

NF-κB is thought to be a key transcription factor underlying renal inflammatory process by regulating gene expression of cytokines, chemokines, and adhesion molecules in progressive renal diseases. In unilateral ureteral obstruction (UOO) and the GM rat model, NF-κB was activated, followed by UOO or GM administration, and blockade of NF-κB activation reduced apoptosis and interstitial fibrosis (23, 39). NF-κB is released from an inhibitory subunit IkB and translocates into the nucleus, where it promotes the transcriptional activation of target genes (10). In the present study, the expression of nuclear p65 subunits of NF-κB was increased after GM treatment in HK-2 cells, which suggests that GM induced NF-κB activation and...
translocation via IκB-α degradation. Importantly, paricalcitol prevented the NF-κB activation induced by GM. A recent experiment (36) also suggested that paricalcitol is able to repress the NF-κB-mediated gene transcription in inflamed renal tubular epithelium. In that study, paricalcitol induced VDR binding to the p65 subunit of NF-κB and prevented it from interacting with cis-acting DNA element, thereby sequestering its ability to transactivate the transcription of its targeted genes (36). Hence, it seems reasonable to assume that paricalcitol may exert its immunomodulatory action in GM-induced renal injury by inhibiting NF-κB signaling pathway.

Multiple possible intracellular mechanisms are known to regulate inflammation and fibrosis cascades in diseased kidney. TGF-β1 is a key molecule in these processes, and a comprehensive survey indicates that in tubular epithelial cells it is capable of activating several signal transduction pathways such as MAPKs (5). MAPKs are fundamental regulators of most immune cell functions, including proliferation, differentiation, survival and apoptosis, chemotraction, and production of inflammatory mediators. They use three parallel signal transduction pathways such as ERK, c-Jun NH2-terminal kinase, and p38. One of these, ERK signaling, was shown to be activated by TGF-β in mesangial cells (13). TGF-β increases ERK activity and the expression of fibronectin in rat mesangial cells (14). In addition, a specific ERK inhibitor treatment prevented the TGF-β-induced fibronectin expression. In consideration with these results, ERK pathway may play an important role in TGF-β-mediated ECM production. Others (22) also have suggested that activation of the ERK pathway also plays a critical role in the proliferation of tubular epithelial and myofibroblast-like cells. ERK expression is one of those processes that can directly and indirectly involve the TGF-β1-mediated regulation of genes with functional roles in cell-matrix interactions, cell motility, and endocytosis. This is consistent with functional inhibition of TGF-β1-induced disassembly of adherens junctions and cell motility in the presence of ERK inhibitor (8, 43). In addition, ERK might be involved the VCAM-1 overexpression induced by TNF-α in experimental study, and ERK pathway may contribute to macrophage and lymphocyte infiltration in inflamed kidney (11). We also observed that ERK1/2 activation was induced within several hours after GM administration to HK-2 cells, and this increase was attenuated by paricalcitol. Since these changes coincide with the initiation of the EMT process in vivo and the overexpression of CTGF and fibronectin in vitro induced by TGF-β1, it appears that ERK signaling pathway evoked by GM would be expected to promote the TGF-β1-activated EMT process and ECM accumulation in kidney tubule cells.

Tubular EMT is a process in which renal tubular cells lose their epithelial phenotype, leading to generation of myofibroblasts in diseased kidney. Myofibroblast is a morphological
intermediate between fibroblast and smooth muscles. Like fibroblasts, it has the ability to produce and secrete ECM components such as collagen and fibronectin, and it also can express $\alpha$-SMA like smooth muscle cells. After TGF-$\beta$ stimulation, the expression of E-cadherin is downregulated. Furthermore, myofibroblasts are differentiated, which is characterized by increased expression of $\alpha$-SMA and fibronectin (17). We found EMT process induced by GM treatment. E-cadherin was downregulated, whereas $\alpha$-SMA expression was upregulated, in GM-treated HK-2 cells. It is interesting that paricalcitol preserved E-cadherin expression and inhibited $\alpha$-SMA induction in GM-induced renal injury. This suggests that paricalcitol may specifically target tubular EMT, a key event in the pathogenesis of renal interstitial fibrosis. Accordingly, the expression of fibronectin and CTGF was reduced by paricalcitol. Masson’s trichrome stain confirmed the attenuation of collagen deposition and fibrosis in GM-treated rat kidney by paricalcitol.

In conclusion, paricalcitol resulted in attenuation of GM-induced renal fibrosis, which was related to the attenuation of inflammatory processes. The possible mechanism involving these anti-inflammatory or antifibrotic effects of paricalcitol may be the interruption of NF-$\kappa$B and ERK signaling pathways.

GRANTS
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DISCLOSURES
No conflicts of interest are declared by the authors.

REFERENCES
Fig. 11. Effect on transforming growth factor (TGF)-β1 expression and epithelial-to-mesenchymal transition (EMT) process. Semiquantitative immunoblotting of TGF-β1 (A), the epithelial adhesion receptor E-cadherin (B), and α-smooth muscle actin (SMA; C), a molecular marker of myofibroblasts, was performed. Renal tubular EMT is known to be induced by TGF-β1 overexpression. TGF-β1 was significantly increased in the cortex/OSOM of GM-treated rats, which was markedly attenuated by paricalcitol (A). GM also induced suppression of E-cadherin (B) and induction of α-SMA (C), a shift that is in agreement with tubular EMT. Paricalcitol treatment prohibited the EMT processes through suppression of TGF-β1 induced by GM. Values are means ± SE. *P < 0.05; **P < 0.01 compared with control. ‡P < 0.01 compared with GM-treated rats.

Fig. 12. Semiquantitative immunoblotting of fibronectin (A) and connective tissue growth factor (CTGF; B) in HK-2 cells incubated with GM in the absence and presence of paricalcitol. Values are means ± SE of 3 independent experiments performed in duplicate. **P < 0.01 compared with control. ‡P < 0.01 compared with GM-treated HK-2 cells.
EFFECT OF PARICALCITOL ON GM NEPHROPATHY

Fig. 13. Masson’s trichome stain in the kidney. Collagen deposition (blue) increased in GM-treated rats but was restored in rats with paricalcitol cotreatment (magnification, ×100).


