ABSTRACT

Background. The effect of paricalcitol on renal ischemia–reperfusion injury (IRI) has not been investigated. We examined whether paricalcitol is effective in preventing inflammation in a mouse model of IRI, and evaluated the cyclooxygenase-2 (COX-2) and prostaglandin E2 (PGE2) pathways as a protective mechanism of paricalcitol.

Methods. Paricalcitol (0.3 μg/kg) was administered to male C57BL/6 mice 24 h before IRI. Bilateral kidneys were subjected to 23 min of ischemia, and mice were killed 72 h after IRI. The effects of paricalcitol on renal IRI were evaluated in terms of

Pretreatment with paricalcitol attenuates inflammation in ischemia–reperfusion injury via the up-regulation of cyclooxygenase-2 and prostaglandin E2

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Keywords: cyclooxygenase-2, inflammation, ischemia reperfusion, paricalcitol, prostaglandin E2
renal function, tubular necrosis, apoptotic cell death, inflammatory cell infiltration and inflammatory cytokines. The effects of paricalcitol on COX-2, PGE2 and its receptors were investigated.

**Results.** Paricalcitol pretreatment improved renal function (decreased blood urea nitrogen and serum creatinine levels), tubular necrosis and apoptotic cell death in IRI-mice kidneys. The infiltration of inflammatory cells (T cells and macrophages), and the production of proinflammatory cytokines (RANTES, tumor necrosis factor-α, interleukin-1β and interferon-γ) were reduced in paricalcitol-treated mice with IRI. Paricalcitol up-regulated COX-2 expression, PGE2 synthesis and mRNA expression of receptor subtype EP4 in post-ischemic renal tissue. The cotreatment of a selective COX-2 inhibitor with paricalcitol restored functional injury and tubular necrosis in paricalcitol-treated mice with IRI.

**Conclusions.** Our study demonstrates that paricalcitol pretreatment prevents renal IRI via the inhibition of renal inflammation, and the up-regulation of COX-2 and PGE2 is one of the protective mechanisms of paricalcitol in renal IRI.

**INTRODUCTION**

Renal ischemia–reperfusion injury (IRI) is a significant cause of acute kidney injury. Several studies have been introduced to provide a basis for pathophysiology, and post-ischemic kidneys are known to be a target of the systemic immune system as well as local inflammation [1–3]. The infiltration of macrophages and T cells were characterized after ischemia, as this has an imperative pathological process in the evolution of IRI. These inflammatory cells in the renal tubular interstitium can release proinflammatory and chemoattractive cytokines, thereby leading to the formation of a self-accumulation cycle [1–5]. Therefore, inhibition of inflammatory cell infiltration and inflammatory cytokines has become a critical target in ameliorating renal IRI [6].

Paricalcitol (19-nor-1,25-dihydroxyvitamin D2) is an active vitamin D analog that shows similar biological activity to a previous vitamin D2 analog, but has fewer adverse effects [7, 8]. Recently, several investigators have proposed that paricalcitol is renoprotective through anti-inflammatory action in diverse experimental models, and that its immunomodulatory action affects many types of immune cells [9–13]. In addition, vitamin D regulates cyclooxygenase (COX)-2 expression and prostaglandin E2 (PGE2) synthesis, which plays a major role in the pathogenesis of renal inflammation after IRI [14–17]. Prostanoid receptor expression was also affected by vitamin D in prostate and breast cancer cells [18, 19].

Whether paricalcitol has renoprotective effects in ischemic kidneys has not been investigated, and the mechanisms of its protective action are unclear in the renal IRI model. The purpose of this study was to investigate whether pretreatment with paricalcitol protects against renal injury in a mouse model of IRI, and whether paricalcitol is effective in preventing inflammation after IRI. Furthermore, we evaluated the COX-2 and PGE2 pathway as a protective mechanism of paricalcitol.

**MATERIALS AND METHODS**

**Animals and drugs**

The experimental protocol used in this study was approved by the animal experiments ethics committee of our institution. Male C57BL/6J mice, weighing ∼22 to 24 g, were housed under a 12-h light–dark cycle, and food and water were freely available. Paricalcitol was provided by Abbott Laboratories (North Chicago, IL, USA) and celecoxib, a selective COX-2 inhibitor, was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Paricalcitol 0.3 μg/kg was injected i.p. and celecoxib 100 mg/kg was administered via gavage 24 h before surgery [12, 13, 20]. The control and sham group received the same volume of sterile distilled water.

**Experimental protocol**

Six groups of mice were used in this experiment (sham, sham + paricalcitol, IRI, IRI + paricalcitol, IRI + celecoxib, IRI + paricalcitol + celecoxib), and each group consisted of eight mice. Mice were anesthetized with zoletil (30 mg/kg) and xylazine (10 mg/kg). The kidneys were exposed by a midline incision and both renal pedicles were occluded for 23 min with non-traumatic microaneurysm clamps. The core body temperature was maintained by a homeothermic pad. After clamp removal, the abdomen was closed. Sham-operated mice received an identical surgical procedure, except for the occlusion of renal pedicles.

The mice were killed at 72 h after ischemia and tissue specimens were collected. The removed kidneys were kept at ∼70°C for semiquantitative immunoblotting, kept in RNAlater® Soln (Ambion, Inc., Austin, TX, USA) for the assay of mRNA expression and preserved as paraffin-embedded blocks for histological analysis and immunohistochemical staining.

**Functional studies**

Renal function was evaluated using blood drawn from the lateral tail vein before injury (0 h) and at 24 and 72 h after injury. Blood urea nitrogen (BUN), serum creatinine, calcium and phosphorus levels were measured using an IDEXX VetTest® Chemistry Analyzer (IDEXX Laboratories, Inc., Westbrook, ME, USA).

**Hematocrit level measurement**

The blood was collected into a capillary containing anticoagulant at Day 3. The capillaries were centrifuged for 5 min at 12 000 rpm and the hematocrit level was calculated as a percent of packed cell volume to the total.

**Mean arterial blood pressure measurement**

Mice were anesthetized and body temperature was maintained by homeothermic pad. The left common carotid artery was cannulated by a portex bore polythene tube (inner diameter 0.28 mm, Smiths Medical International Ltd., Kent, England) and was connected to a pressure transducer (Harvard
Apparatus, Holliston, MA, USA) for the measurement of mean arterial blood pressure (MAP), which was displayed on a data acquisition system (Biopac Systems, Inc., Santa Barbara, CA, USA).

**Histological examination**

Kidney tissues were fixed in 10% formalin buffer for 24 h and then dehydrated in graded ethanol series and xylene. Samples were embedded in paraffin and sectioned into 3-mm-thick sections. Hematoxylin and eosin staining was performed for acute tubular necrosis scoring. The cortex and outer stripe of the outer medulla were assessed and at least 20 fields were reviewed for each slide. Markers of tubular damage were scored by calculating the percentage of tubules in the corticomedullary junction that displayed cell necrosis, loss of brush border, cast formation and tubular dilation, as follows: 0, none; 1, ≤10%; 2, 11–25%; 3, 26–50%; 4, 51–75% and 5, ≥76%.

**Immunohistochemistry**

After deparaffinization, sections were applied with autoclaving for antigen retrieval and the next steps were followed according to the manufacturer’s instruction for Streptavidin Biotin Universal Detection System (Immunotech, Marseille, France). Anti-CD3 antibody (Santa Cruz Biotechnology), anti-F4/80 antibody (Abcam, Cambridge, UK) and anti-CD40 antibody (Santa Cruz Biotechnology) were used as the primary antibody diluted in Antibody Diluent Buffer (Covance, Princeton, NJ, USA). For coloration, an AEC Chromogen Kit (Immunotech, Marseille, France) and hematoxylin were used. The apoptotic nuclei were identified with deoxynucleotidyl transferase biotin-dUTP nick end labeling (TUNEL) staining using the ApopTag Plus Peroxidase Kit (Millipore, Billerica, MA, USA). The numbers of positive cells were quantified per high-power field (HPF) of each kidney, and at least 20 fields were reviewed for each slide.

**Western blot**

Kidney tissues were homogenized using TissueLyser II (Qiagen, Hilden, Germany) in RIPA buffer (Elpis Biotech., Daejeon, Korea) containing protease inhibitor cocktail tablets (Roche Diagnostics GmbH, Mannheim, Germany). Homogenates were centrifuged at 13 000 rpm for 15 min at 4°C. Equal amounts of protein samples were resolved by SDS-polyacrylamide gel electrophoresis and then electroblotted onto a nitrocellulose membrane (GE Healthcare Bio-Sciences, Piscataway, NJ, USA). The membrane was blocked for 1 h in TBST (10 mM Tri-CL, 150 mM NaCl, pH 8.0, 0.05% tween-20) containing 5% non-fat powdered milk. The membranes were incubated with primary antibodies directed against COX-2 (Cell Signaling Technology, Beverly, MA, USA), Bax (Cell Signaling Technology), Bcl-2 (Cell Signaling Technology, Beverly, MA, USA), Bcl-x (Cell Signaling Technology) or vitamin D receptor (VDR, Santa Cruz Biotechnology) containing 3% bovine serum albumin in TBST. They were then incubated with horseradish peroxidase-conjugated anti-rabbit IgG (Invitrogen, Carlsbad, CA, USA) or anti-mouse IgG (Invitrogen). Positive bands were detected and analyzed by chemiluminescence technology using the ChemiDoc™ XRS+ (Bio-Rad Laboratories). As a loading control, each membrane was probed with β-actin (Cell Signaling Technology), origin recognition complex subunit 2 (ORC2, Santa Cruz Biotechnology).

**Real-time reverse transcript PCR**

Total RNA was prepared from kidney tissues using a NucleoSpin® RNAII kit (Macherey-Nagel, Düren, Germany). cDNA was synthesized from 1 μg RNA using Reverse Transcriptase Premix (Elpis Biotech, Daejeon, Korea). After reverse transcription of the RNA, cDNA was used as a template in PCR reactions using gene-specific primer pairs (Table 1). cDNA was amplified in a Power SYBR® Green PCR Master Mix (Applied Biosystems, Warrington, UK). Quantitative real-time PCR was performed by ABI 7500 FAST (Applied Biosystems, Foster City, CA, USA). The relative levels of mRNA were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

**Enzyme immunoassay**

Whole kidneys were homogenized in ProPrep™ Protein Extraction Solution (iNtRON Biotechnology, Seongnam, Korea) and then centrifuged for 10 min at 12 000 rpm. Equal amounts of protein were applied with PGE2 ELISA (Cayman Chemical Co., Ann Arbor, MI, USA). The optical densities of

<table>
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<th>Table 1. Primer sequences for real-time PCR</th>
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<tr>
<td><strong>Gene</strong></td>
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<td>RANTES</td>
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<td></td>
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<td>EP4</td>
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<td>GAPDH</td>
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Fwd, forward; Rev, reverse; TNF-α, tumor necrosis factor-α; IFN-γ, interferon-γ; MCP-1, monocyte chemoattractant protein-1; IL, interleukin; PGDH, hydroxyprostaglandin dehydrogenase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
the samples were determined at 450 nm in a microplate reader (Bio-Rad Laboratories).

**Statistical analysis**

Data are expressed as the mean ± standard error of the mean. Differences between the two groups were determined using Student’s t-test or Mann–Whitney U test, as appropriate. Multiple comparisons were performed using one-way analysis of variance and Tukey’s post hoc test. Statistical analysis was performed using SPSS software version 16.0 (SPSS, Inc., Chicago, IL, USA). A P value < 0.05 was considered significant.

**RESULTS**

**Paricalcitol pretreatment restores VDR expression after IRI**

We examined the expression of VDR after paricalcitol pretreatment (Figure 1). Semiquantitative immunoblotting revealed that VDR was highly expressed in paricalcitol-treated mice after a sham operation. IRI caused significant suppression of VDR expression. However, paricalcitol treatment largely restored VDR expression after IRI, compared with IRI only (0.64 ± 0.06-fold versus 0.85 ± 0.02-fold, *P* < 0.05).

**Paricalcitol pretreatment improves renal function after IRI**

The paricalcitol pretreatment significantly improved renal function at Days 1 and 3 after IRI, compared with the values of mice without paricalcitol pretreatment (Figure 2): BUN (195.6 ± 14.5 mg/dL versus 73.6 ± 13.0 mg/dL at Day 3, *P* < 0.05) and serum creatinine (0.70 ± 0.15 mg/dL versus 0.23 ± 0.02 mg/dL at Day 3, *P* < 0.05).

**Paricalcitol pretreatment decreases tubular necrosis and apoptosis**

Histological examination of sections indicated greater tubular necrosis in the outer medulla of kidneys of ischemic mice compared with that of sham-operated mice with paricalcitol pretreatment (Figure 3A). Quantitative analysis of tubular necrosis showed a decrease in the necrosis score of paricalcitol-treated mice compared with those of mice with IRI only (4.4 ± 0.3 versus 3.1 ± 0.3, *P* < 0.05).

The number of TUNEL-positive cells was greater in the kidneys of mice with IRI than in sham-operated mice with paricalcitol pretreatment (Figure 3B). TUNEL-positive cells were significantly attenuated with paricalcitol pretreatment in mice with IRI (10.3 ± 0.76/HPF versus 2.3 ± 0.4/HPF, *P* < 0.05). Compared with sham-operated mice with paricalcitol pretreatment, the Bax levels were significantly increased in mice with IRI alone (Figure 3C). The paricalcitol pretreatment significantly attenuated this pro-apoptotic marker in mice with IRI (1.76 ± 0.09-fold versus 1.16 ± 0.26-fold, *P* < 0.05). The expression of anti-apoptotic protein Bcl-2 was lower in mice with IRI alone than in sham-operated mice with paricalcitol pretreatment (0.27 ± 0.03-fold versus 0.80 ± 0.11-fold, *P* < 0.05).
Paricalcitol pretreatment decreases inflammatory cell infiltration

In sham-operated mice with paricalcitol pretreatment, few CD40 antigens were expressed in tubular epithelial cells (Figure 4). CD40 expression in the tubular cells was significantly increased after IRI. However, paricalcitol pretreatment effectively reduced CD40 antigen expression in mice with IRI (61.2 ± 6.3/HPF versus 5.0 ± 1.8/HPF, P < 0.05). IRI instigated significant infiltration of the F4/80 antigen-positive cells in the renal interstitium, but paricalcitol treatment markedly inhibited the infiltration of F4/80 antigen-positive cells in mice with IRI (13.0 ± 2.9/HPF versus 6.0 ± 0.6/HPF, P < 0.05). Similarly, administration of paricalcitol substantially reduced CD3 + T-cell infiltration after IRI (6.7 ± 3.3/HPF versus 1.7 ± 3.3/HPF, P < 0.05).

Paricalcitol pretreatment decreases inflammatory cytokines

We examined the expression of RANTES and tumor necrosis factor (TNF)-α which are key cytokines that induce peritubular infiltration of T cells and macrophages (Figure 5A and B). RT–PCR results revealed that a ~4-fold induction of RANTES mRNA and a 3.5-fold induction of TNF-α were observed in IRI mice without paricalcitol pretreatment. These RANTES and TNF-α inductions were significantly reversed by paricalcitol pretreatment in mice with IRI (1.79 ± 0.29-fold and 1.08 ± 0.32-fold, respectively, all P < 0.05 versus IRI group).

We further investigated interferon (IFN)-γ, interleukin (IL)-1β and monocyte chemoattractant protein (MCP-1) (Figure 5C–E). The mRNA expression of IFN-γ and IL-1β was significantly increased after IRI (all P < 0.05 versus sham + paricalcitol group). Paricalcitol pretreatment significantly inhibited mRNA expression of these inflammatory cytokines in mice with IRI (all P < 0.05 versus IRI group). MCP-1 was significantly increased in IRI mice, but paricalcitol seemed to have little effect on MCP-1 mRNA expression in mice with IRI.

Paricalcitol pretreatment up-regulates COX-2 and PGE2 synthesis

Semiquantitative immunoblotting demonstrated that COX-2 expression was increased by paricalcitol in sham-operated mice (1.00 ± 0.11-fold versus 1.83 ± 0.08-fold, P < 0.05) (Figure 6A). IRI alone also increased COX-2 expression compared with sham-operated mice without paricalcitol treatment, and paricalcitol further enhanced COX-2 expression in ischemic mice (1.56 ± 0.07-fold versus 2.09 ± 0.16-fold, P < 0.05).

mRNA expression of 15-hydroxyprostaglandin dehydrogenase (15-PGDH), which is the enzyme that catalyzes the conversion of PGE2 to a less bioactive form, was strongly produced in mice with IRI only (Figure 6B). However, the expression of 15-PGDH decreased in paricalcitol-treated mice with IRI (7.97 ± 1.47-fold versus 1.16 ± 0.45-fold, P < 0.05). In sham-operated mice, paricalcitol pretreatment significantly increased renal PGE2 concentrations (23.9 ± 1.8 pg/mg protein versus 50.7 ± 0.8 pg/mg protein, P < 0.05) (Figure 6C). PGE2 concentrations significantly increased after IRI compared with sham-operated mice without paricalcitol treatment. Paricalcitol-treated mice with IRI had increased PGE2 concentrations significantly after IRI (all P < 0.05 versus sham + paricalcitol group). Paricalcitol pretreatment significantly inhibited mRNA expression of these inflammatory cytokines in mice with IRI (all P < 0.05 versus IRI group). MCP-1 was significantly increased in IRI mice, but paricalcitol seemed to have little effect on MCP-1 mRNA expression in mice with IRI.

Table 2. The effects of paricalcitol on serum calcium, phosphate, hematocrit level and mean arterial blood pressure

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>Sham + Pari</th>
<th>IRI</th>
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<tr>
<td>Serum calcium level (mg/dL)</td>
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<tr>
<td>Day 0</td>
<td>8.0 ± 0.1</td>
<td>8.6 ± 0.2</td>
<td>8.0 ± 0.1</td>
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<td>Day 1</td>
<td>8.1 ± 0.2</td>
<td>8.5 ± 0.1</td>
<td>9.2 ± 0.5</td>
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<tr>
<td>Day 3</td>
<td>8.6 ± 0.4</td>
<td>7.9 ± 0.3</td>
<td>7.9 ± 0.3</td>
<td>9.1 ± 0.3</td>
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<td>Serum phosphate level (mg/dL)</td>
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<tr>
<td>Day 0</td>
<td>7.9 ± 0.1</td>
<td>8.0 ± 0.1</td>
<td>7.9 ± 0.1</td>
<td>8.0 ± 0.1</td>
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<tr>
<td>Day 1</td>
<td>6.8 ± 0.2</td>
<td>7.7 ± 0.2</td>
<td>18.8 ± 1.5*</td>
<td>9.6 ± 0.1*</td>
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<tr>
<td>Day 3</td>
<td>6.8 ± 0.2</td>
<td>7.6 ± 0.3</td>
<td>12.7 ± 1.9</td>
<td>8.7 ± 1.0</td>
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<tr>
<td>Hematocrit level (%)</td>
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<tr>
<td>Day 3</td>
<td>47.2 ± 0.7</td>
<td>48.3 ± 0.9</td>
<td>47.4 ± 1.2</td>
<td>47.7 ± 0.8</td>
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<td>Mean arterial blood pressure (mmHg)</td>
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<tr>
<td>Day 3</td>
<td>71.6 ± 2.0</td>
<td>75.1 ± 2.0</td>
<td>72.1 ± 2.2</td>
<td>72.8 ± 4.2</td>
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Pari, paricalcitol; IRI, ischemia–reperfusion injury. Values are means ± SEM.  
*P < 0.05 versus sham.  
**P < 0.05 versus sham + Pari.  
***P < 0.05 versus IRI.
compared with mice with IRI only (34.9 ± 2.8 pg/mg protein versus 47.9 ± 4.5 pg/mg protein, \( P < 0.05 \)).

**Paricalcitol pretreatment up-regulates PGE2 receptor subtype EP4**

As shown in Figure 7A, induction of EP2 mRNA expression was markedly higher in mice with IRI than in sham-operated mice without paricalcitol pretreatment (1.00 ± 0.48-fold versus 4.49 ± 0.52-fold, \( P < 0.05 \)). However, there was little effect of paricalcitol pretreatment on EP2 expression in both sham-operated and ischemic mice. Administration of paricalcitol substantially increased EP4 mRNA expression in sham-operated mice (1.00 ± 0.70-fold versus 7.08 ± 2.98-fold, \( P < 0.05 \)) (Figure 7B). Ischemic mice also showed a significant increment of EP4 expression compared with sham-operated controls without paricalcitol pretreatment. Paricalcitol pretreatment further increased the EP4 mRNA expression in post-ischemic mice (4.38 ± 0.84-fold versus 10.11 ± 1.09-fold, \( P < 0.05 \)).

**Celecoxib offset the renoprotective effects of paricalcitol after IRI**

The BUN and serum creatinine levels were 214.5 ± 12.1 mg/dL and 1.00 ± 0.08 mg/dL (Day 3), respectively, in celecoxib-treated mice after IRI, and it was similar to those of mice with IRI alone (all \( P > 0.05 \), Figure 8A). The celecoxib cotreatment with paricalcitol significantly restored the injury to a greater extent compared with that of IRI mice with paricalcitol pretreatment only: BUN (166 ± 28.2 mg/dL versus 66.1 ± 4.5 mg/dL at Day 3, \( P < 0.05 \)) and serum creatinine (1.08 ± 0.11 mg/dL versus 0.46 ± 0.06 mg/dL at Day 3, \( P < 0.05 \)). Histological examination of kidney sections indicated greater tubular necrosis in the mice kidney with celecoxib cotreatment than in the IRI mice kidney with paricalcitol pretreatment only (Figure 8B). The quantitative tubular necrosis score of celecoxib-cotreated mice kidney was significantly higher than that of the mice kidney with paricalcitol pretreatment only (4.3 ± 0.3 versus 2.2 ± 0.2, \( P < 0.05 \)).

**DISCUSSION**

The results of our study clearly demonstrate that paricalcitol pretreatment attenuated ischemic acute kidney injury, determined by improved renal function, histopathology, decreased inflammatory cell infiltration and inflammatory cytokines. As a potential renoprotective mechanism of paricalcitol, we evaluated the PGE2 pathway and found that paricalcitol treatment up-regulated COX-2 enzyme expression, renal PGE2 levels and the EP4 receptor. The renoprotective effect of paricalcitol after IRI was offset with selective COX-2 blockade. This finding provides evidence that COX-2 plays a renoprotective role in paricalcitol-treated mice with IRI.

It is well known that apoptosis and necrosis have been proposed as basic cell-death mechanisms in the kidney of

**FIGURE 3:** Effects of paricalcitol on tubular necrosis and apoptosis. (A) The acute tubular necrosis score in hematoxylin and eosin stained kidney sections was significantly improved by paricalcitol pretreatment. Original magnification, ×200. (B) The number of TUNEL-positive cells was reduced in the paricalcitol-treated kidney after IRI. Original magnification, ×400. (C) Paricalcitol treatment inhibited the Bax protein and increased Bcl-2 protein levels determined by the western blot test. *\( P < 0.05 \) versus IRI, †\( P < 0.05 \) versus Sham + Pari. Pari, paricalcitol; IRI, ischemia–reperfusion injury; ATN, acute tubular necrosis.
ischemic mice [21, 22]. In the present study, paricalcitol pre-
treatment improved not only renal function, but also the histo-
pathology, demonstrated by decreased tubular necrosis and
apoptotic cell death. On a molecular basis, paricalcitol pre-
treatment decreased levels of Bax and increased Bcl-2 protein
expression. These findings are consistent with previous reports
that paricalcitol prevents cisplatin- or ureteral obstruction-
induced renal injury by suppressing apoptosis, and we found
that paricalcitol also exerts a protective mechanism against
IRI-induced cell death [23, 24].

The protective mechanism of paricalcitol against cell death
in IRI may be multifactorial. In this study, we focused on the
anti-inflammatory effect of paricalcitol because inflammation
is the major injury mechanism of renal IRI [25, 26]. We evalu-
ated the mRNA expression of RANTES, which is a critical step
to inducing the peritubular infiltration of T cells and macro-
phages [12, 27]. In this study, paricalcitol inhibited RANTES
expression and this was accompanied by a decreased infiltration
of T cells and macrophages. In addition, paricalcitol pretreat-
ment significantly inhibited mRNA expression of TNF-α,
which stimulates tubular epithelial cells to produce RANTES
[28]. These findings suggest that paricalcitol effectively blocks
renal inflammation. On the other hand, MCP-1 mRNA was not
inhibited significantly by paricalcitol in this study. These
findings might be associated with a gene-specific effect of paricalcitol or with the protective role of MCP-1 in IRI [12, 29, 30].

It is well known that increased CD40 expression on tubular epithelial cells results in strongly increased production of RANTES, and this effect is augmented by IL-1 and IFN-γ [31, 32]. In our study, we demonstrated that paricalcitol remarkably reduced CD40 expression and mRNA expression of IL-1β and IFN-γ in mice with IRI. These findings suggest that paricalcitol reduces renal inflammation of IRI through the downregulated immunological function of tubular epithelial cells.

However, tubular cell death is not completely protected by paricalcitol in post-ischemic mice. Therefore, the anti-inflammatory effect of paricalcitol could be driven not only from direct inhibition on inflammatory cell infiltration and cytokine production, but also from decreased cell death.

Several experimental models demonstrated that up-regulation of COX-2 and PGE2 inhibited T-cell and macrophage infiltration, thereby leading to reduced inflammation [33, 34]. Furthermore, the activation of EP4 improved renal function in a model of acute kidney injury, and exerted its protective effects...
through the resistance to apoptosis and increased cell survival signaling [35–37]. We demonstrated that paricalcitol pretreatment effectively up-regulated the COX-2, PGE2 and EP4 levels, and that selective COX-2 blockade offset the renoprotective effects of paricalcitol after IRI. These findings suggest that up-regulation of the COX-2 and PGE2 pathway plays a critical role in the protective effects of paricalcitol.

The major synthetic enzyme for PGE2 (COX-2) and the catalyzing enzyme for PGE2 (15-PGDH) were evaluated to find out the effects of paricalcitol on PGE2 synthesis. We found that paricalcitol pretreatment increased the expression of COX-2 and decreased that of 15-PGDH. As a result, paricalcitol treatment increased the levels of PGE2 in kidney tissue. These findings are contrary to previous studies using prostate cancer and breast cancer cells, which demonstrated that calcitriol induced a decrement of COX-2 expression and PGE2 synthesis [18, 19]. We presume that these conflicting results could be driven from the tissue-specific action of vitamin D, and this opposite action of vitamin D in COX-2 modulation was also found in keratinocytes [38].

The results of our study clearly demonstrate the protective effect of paricalcitol, but other influential factors should be considered. First, we investigated the MAP to clarify the hemodynamic effects of paricalcitol-induced COX-2 modulation. However, paricalcitol pretreatment did not affect the MAP in either sham-operated or post-ischemic mice. Second, we measured the hematocrit level to investigate the dehydration element and found that the hematocrit was not changed. Thirdly, calcitriol (1,25-dihydroxyvitamin D3) also has a protective effect against IRI, which is already used in various clinical fields [39]. In this study, we demonstrated that paricalcitol requires a lower number of injections (single versus multiple) and causes fewer side effects (hypercalcemia and hyperphosphatemia), compared with calcitriol. Therefore, paricalcitol could offer a clinically applicable strategy to overcome the limitation of vitamin D in renal IRI.

In conclusion, our study demonstrated that paricalcitol attenuates renal IRI. This protective effect is associated with the inhibition of renal inflammatory cell infiltration, and decreased proinflammatory cytokines. Furthermore, the up-regulation of COX-2 and PGE2 synthesis by paricalcitol is one of the protective mechanisms of paricalcitol treatment.

**ACKNOWLEDGEMENTS**

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CONFLICT OF INTEREST STATEMENT

None declared.

REFERENCES

15. Feitoza CQ, Câmara NO, Pinheiro HS et al. Cyclooxygenase 1 and/or 2 blockade ameliorates the renal tissue damage triggered by ischemia and reperfusion injury. Int Immunopharmacol 2005; 5: 79–84
29. Vaddi K, Newton RC. Comparison of biological responses of human monocytes and THP-1 cells to chemokines of the inter- 

ORIGINAL ARTICLE

P aricalcitol attenuates renal ischemia–reperfusion injury
22-Oxacalcitriol prevents progression of endothelial dysfunction through antioxidative effects in rats with type 2 diabetes and early-stage nephropathy

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Background. Vitamin D deficiency is associated with endothelial dysfunction in type 2 diabetes patients, but the effectiveness of vitamin D supplementation remains controversial. We assessed whether 22-oxacalcitriol (OCT) could prevent endothelial dysfunction in type 2 diabetes mellitus (DM) rats.

Methods. DM rats with early-stage nephropathy were treated for 10 weeks with OCT (0.2 μg/kg) three times per week or by an implanted insulin pellet. Endothelial dysfunction was assessed by femoral flow-mediated dilation (FMD).

Results. Insulin significantly improved FMD as blood glucose levels normalized. OCT also improved FMD without hypercalcemia or hyperphosphatemia and without affecting blood glucose or blood pressure. In femoral arteries, OCT significantly suppressed the elevated expression of p22phox, a nicotinamide adenine dinucleotide phosphate (NADPH) oxidase subunit, and improved the endothelial nitric oxide synthase (eNOS) dimer-to-monomer ratio. In cultured endothelial cells, OCT significantly suppressed HG-induced p22phox expression and improved eNOS uncoupling as was observed in the in vivo study.

Conclusion. In DM rats, OCT improved endothelial dysfunction, at least in part, by suppressing ROS generation through

References:

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