Rho Kinase Inhibition by Fasudil Attenuates Cyclosporine-Induced Kidney Injury

Jeong Woo Park, Cheon Hoon Park, In Jin Kim, Eun Hui Bae, Seong Kwon Ma, Jong Un Lee, and Soo Wan Kim

Departments of Internal Medicine (J.W.P., E.H.B., S.K.M., S.W.K.) and Physiology (C.H.P., I.J.K., J.U.L.), Chonnam National University Medical School, Gwangju, South Korea

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ABSTRACT

It has been shown that the inhibition of the Rho/Rho kinase (ROCK) pathway prevents tubulointerstitial fibrosis and ameliorates renal function in various progressive renal disorders. The present study was to determine whether fasudil, a ROCK inhibitor, has a protective effect on cyclosporine A (CsA)-induced nephropathy. Male Sprague-Dawley rats were treated with CsA (n = 10, 20 mg·kg⁻¹·day⁻¹ s.c.), CsA + fasudil (n = 10, 3 mg·kg⁻¹·day⁻¹ i.p.), or vehicle alone (n = 10) for 28 days. Fasudil cotreatment ameliorated CsA-induced changes and restored renal function. CsA decreased the expression of endothelial nitric-oxide synthase and increased inducible nitric-oxide synthase/3-nitrotyrosine in the kidney. Accordingly, there was infiltration of inflammatory cells and up-regulation of inflammatory cytokines. Fasudil also significantly suppressed the expression of transforming growth factor-β1, Smad signaling, and subsequent epithelial-to-mesenchymal processes. In addition, fasudil augmented p27kipl expression and decreased the number of proliferating cell nuclear antigen-positive cells. In another series of experiments using HK-2 cells in culture, fasudil also suppressed CsA-induced increases in mitogen-activated protein kinase phosphorylation. CsA-induced expression of p53, the degree of which was attenuated by fasudil in association with decreases of proapoptotic markers such as Bad, Bax, and total/cleaved caspase-3. These results suggest that inhibition of the Rho/ROCK pathway attenuates CsA-induced nephropathy through the suppression of the induction of inflammatory, apoptotic, and fibrogenic factors, along with inhibition of Smad, mitogen-activated protein kinases, and nitric oxide signaling pathways.

Introduction

The introduction of cyclosporine A (CsA) has greatly improved morbidity and mortality in organ transplant patients. In the kidney, however, it may cause considerable adverse effects characterized by arteriolar hyalinosis and striped cortical interstitial fibrosis (Shihab, 1996; Gaston, 2009; Nae-sens et al., 2009). Although the mechanisms underlying nephrotoxicity have not been completely defined, there is evidence suggesting tubulointerstitial inflammation and fibrosis. Local vasoconstriction associated with the production of angiotensin II may cause ischemic injury to poorly perfused areas of the cortex and corticomedullary junction (Navarro-Antolín et al., 2001), resulting in an inflammatory response and the accumulation of reactive oxygen species (ROS) (Nae-sens et al., 2009). In addition, CsA may directly activate apoptotic processes, thus contributing to tubular cell death (Servais et al., 2008).

Rho kinase (ROK)is a Rho protein effector that phosphorylates downstream targets including myosin light chain and myosin light chain phosphatase (MYPT1) and modulates actin cytoskeletal organization and endothelial barrier func-
tion (Riento and Ridley, 2003). ROCK also participates in cell adhesion, migration, proliferation, cytokine activation, and migration of inflammatory cells (Riento and Ridley, 2003). Several recent studies suggest that ROCK may play an important role in renal fibrosis by enhancing signaling pathways including nuclear factor-κB (NF-κB) and transforming growth factor-β (TGF-β) (Sharpe and Hendry, 2003; Sun et al., 2006). Furthermore, inhibition of the Rho/ROCK pathway increases the levels of cyclin-dependent kinase (CDK) inhibitors such as p27kip1, subsequently inhibiting cell proliferation in progressive renal damage (Kanda et al., 2003). The protective effect of ROCK inhibitors has been demonstrated in a variety of renal injury models, including unilateral ureteral obstruction (Nagatoya et al., 2002), malignant hypertension (Ishikawa et al., 2006), and glomerulosclerosis in Dahl salt-sensitive hypertension (Nishikimi et al., 2004). The present study was to examine the protective efficacy of fasudil (1-(5-isoquinolinylsulfonyl)homopiperazine dihydrochloride, HA-1077 dihydrochloride, fasudil dihydrochloride), a ROCK inhibitor, in CsA-induced nephropathy.

Materials and Methods

Animals. Male Sprague-Dawley rats weighing 180 to 200 g were used. The animal protocol was approved by the Institutional Animal Care and Use Committee of Chonnam National University Medical School (Gwangju, South Korea).

Experimental Protocols. Rats were maintained in individual metabolic cages for the last 3 days of the experiment to allow urine collection. They were given a low-salt diet (0.05% sodium; Ziegler Brothers, Gardner, MA) and tap water ad libitum. CsA (Novartis, Basel, Switzerland) was dissolved in olive oil to achieve a concentration of 20 mg/ml. Rats were divided into three groups and were given injections of the vehicle alone (n = 10), CsA alone (n = 10, 20 mg·kg⁻¹·day⁻¹ i.p.; Sigma-Aldrich, St. Louis, MO) for 28 days. With this low dose, fasudil does not affect systemic blood pressure (BP) in rats with progressive renal disease (Kanda et al., 2003). Systolic BP was measured using an automated tail-cuff method under a conscious state. The rats were anesthetized with isoflurane, and blood samples were collected from the inferior vena cava. The right kidney was then rapidly removed, dissected into the cortex/outer stripe of outer medulla (OSOM), and processed for semiquantitative immunoblotting. The left kidney was fixed via retrograde perfusion for immunohistochemistry. A second set of rats was used for real-time polymerase chain reaction (PCR) assays. The rats were decapitated when they were conscious, and their kidneys were harvested and stored at −70°C for later assay.

HK-2 Cells. HK-2 (American Type Culture Collection, Manassas, VA) human renal proximal tubular epithelial cells were cultured. Cells were passaged every 3 to 4 days in 100-mm dishes containing combined Dulbecco’s modified Eagle’s medium and Ham’s F-12 medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 mg/ml streptomycin (Sigma-Aldrich). The cells then were incubated in a humidified atmosphere of 5% CO₂ and 95% air at 37°C for 24 h and subcultured until 70 to 80% confluence. HK-2 cells were plated onto 60-mm dishes in medium containing 10% FBS and incubated for 24 h, after which they were transferred to Dulbecco’s modified Eagle’s medium and Ham’s F-12 medium with 2% FBS and incubated for an additional 16 h. The cells then were treated with CsA (10 μM), either with or without fasudil (10 μM). The control cells were treated with a buffer solution alone. Another set of HK-2 cells was treated with fasudil alone to ascertain the proper effect of fasudil (10 μM) on HK-2 cells.

Histological Analysis. For perfusion fixation, a perfusion needle was inserted into the abdominal aorta, and the vena cava was cut to establish an outlet. Blood was flushed from the kidney with ice-cold phosphate-buffered saline (PBS) (pH 7.4) for 15 s before switching to ice-cold 3% paraformaldehyde in PBS (pH 7.4) for 3 min. The kidney was removed and cut into 2- to 3-mm-thick transverse sections, which were immersion-fixed for 1 h, followed by three 10-min washes in PBS. The tissue was dehydrated in a graded ethanol series and incubated in xylene overnight. After the tissue was embedded in paraffin, 2-μm-thick sections were cut with a rotary microtome and stained with hematoxylin and eosin (H&E) to assess histological tissue injury. Tubulointerstitial lesion indexes were determined using a semiquantitative scoring system (Gadola et al., 2004). We also used vimentin staining to visualize the epithelial-to-mesenchymal transition (EMT) process. Immunoperoxidase labeling was conducted as described previously (Kim et al., 2004).

Semiquantitative Immunoblotting. Tissues obtained from dissected cortex/OSOM were homogenized in ice-cold isolation solution containing 0.3 M sucrose, 25 mM mидazole, 1 mM EDTA, 8.5 μM leupeptin, and 1 mM phenylmethylsulfonyl fluoride (pH 7.2). The homogenates were centrifuged at 1000 × g for 15 min at 4°C to remove whole cells, nuclei, and mitochondria, and the total protein concentration was measured. All of the samples were adjusted to the same final protein concentration using isolation solution, solubilized at 65°C for 15 min in SDS-containing sample buffer, and stored at −20°C. To confirm equal protein loading, an initial gel was stained with Coomassie Blue. SDS-polyacrylamide gel electrophoresis was run on 9 or 12% polyacrylamide gels. The proteins were electrophoretically transferred onto nitrocellulose membranes using a Mini Protean II apparatus (Bio-Rad Laboratories, Hercules, CA). The blots were blocked with 5% milk in PBS-Tween 20 (80 mM Na₂HPO₄, 20 mM NaH₂PO₄, 100 mM NaCl, and 0.1% Tween 20 (pH 7.5) for 1 h, incubated overnight at 4°C with primary antibodies, and incubated with secondary anti-rabbit, anti-mouse, or anti-goat horseradish peroxidase-conjugated antibodies thereafter. The immunoblots were then visualized using an enhanced chemiluminescence system.

Primary Antibodies. The following antibodies were used: affinity-purified anti-mouse antibodies against ROCK-α (BD Biosciences Transduction Laboratories, Lexington, KY), endothelial nitric-oxide synthase (eNOS) (BD Biosciences Transduction Laboratories), inducible nitric-oxide synthase (iNOS) (BD Biosciences Transduction Laboratories), N.M., not measured.

Table 1

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>CsA</th>
<th>CsA + Fasudil</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Systolic BP (mm Hg)</td>
<td>125.20 ± 2.00</td>
<td>116.90 ± 2.00</td>
<td>107.40 ± 1.40</td>
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<tr>
<td>FCr (mg/dl)</td>
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<td>0.50 ± 0.04***</td>
<td>0.35 ± 0.02***</td>
</tr>
<tr>
<td>Ccr (ml/min)</td>
<td>2.52 ± 0.25</td>
<td>1.19 ± 0.10†</td>
<td>1.90 ± 0.95**</td>
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<tr>
<td>Uosmol (mOsm/kg H₂O)</td>
<td>2052.00 ± 134.50</td>
<td>854.00 ± 30.66***</td>
<td>131.00 ± 74.41†</td>
</tr>
<tr>
<td>UAE (mg/g Cr)</td>
<td>4.25 ± 0.53</td>
<td>12.68 ± 1.08***</td>
<td>8.23 ± 0.41†</td>
</tr>
<tr>
<td>CsA Cmin (ng/ml)</td>
<td>N.M.</td>
<td>2401.00 ± 4.39</td>
<td>2294.00 ± 28.61</td>
</tr>
</tbody>
</table>

BP, blood pressure; Fcr, plasma creatinine; Ccr, creatinine clearance; Uosmol, urine osmolality; UAE, urine albumin excretion; CsA Cmin, cyclosporine plasma trough level; N.M., not measured.

*†, p < 0.01; **, p < 0.001 compared with control. ††, p < 0.05; †††, p < 0.01 compared with CsA-treated rats.
Laboratories), ED-1 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), E-cadherin (BD Biosciences Transduction Laboratories), α-smooth muscle actin (α-SMA) (Sigma-Aldrich), fibronectin (Santa Cruz Biotechnology, Inc.), p21 (BD Biosciences Pharmingen, San Diego, CA), p27kip1 (BD Biosciences Transduction Laboratories), proliferating cell nuclear antigen (PCNA) (Cell Signaling Technology, Danvers, MA), and cyclin E (Cell Signaling Technology); anti-rabbit antibodies against p-MYPT1 (Cell Signaling Technology), 3-nitrotyrosine (Millipore, Billerica, MA), phosphorylated NF-κB p65 subunit (P-p65) (Cell Signaling Technology), inhibitor of NF-κB α (IκB-α) (Santa Cruz Biotechnology, Inc.), TGF-β1 (Santa Cruz Biotechnol-

Fig. 1. Expression of ROCK in the renal cortex. ROCK-α expression was increased by CsA treatment, which was counteracted by fasudil. The expression of p-MYPT1, which can reflect ROCK activity, was increased by CsA, which was attenuated by fasudil. Data are means ± S.E.M. (n = 10). *** p < 0.001 versus control. †, p < 0.05; ††, p < 0.01 versus CsA-treated rats.

Fig. 2. Expression of eNOS, iNOS, 3-nitrotyrosine, and NF-κB. A, expression of eNOS decreased, whereas that of iNOS increased, in CsA-treated rats, both of which were markedly attenuated by fasudil cotreatment. The 3-nitrotyrosine expression increased in CsA-treated rats but was reversed by fasudil. Data are means ± S.E.M. (n = 10). †††, p < 0.001 versus control. †, p < 0.05; ††, p < 0.01 versus CsA-treated rats.

B, immunohistochemical staining for ED-1 in the renal cortex (magnification, 200×). ED-1 expression was significantly increased in CsA-treated rat kidneys, the magnitude of which was attenuated by fasudil treatment. Arrows indicate infiltrated ED-1-positive cells.

Fig. 3. Expression of ED-1. A, semiquantitative immunoblotting of ED-1. Data are means ± S.E.M. (n = 10). *** p < 0.001 versus control. †††, p < 0.001 versus CsA-treated rats. B, immunohistochemical staining for ED-1 in the renal cortex (magnification, 200×). ED-1 expression was significantly increased in CsA-treated rat kidneys, the magnitude of which was attenuated by fasudil treatment. Arrows indicate infiltrated ED-1-positive cells.

Fig. 4. Effects of fasudil on proinflammatory cytokines and adhesion molecules. Proinflammatory cytokines (tumor necrosis factor-α, interferon-γ, and interleukin-1β) and adhesion molecules (monocyte chemoattractant protein-1, intercellular adhesion molecule-1, and vascular cell adhesion molecule-1) were significantly elevated in CsA-treated rat kidneys but were suppressed by fasudil. Data are means ± S.E.M. (n = 10). **, p < 0.01; ††, p < 0.001 versus control. †, p < 0.05; ††, p < 0.01 versus CsA-treated rats.
ogy, Inc.), Smad-2/3 (Cell Signaling Technology), Smad-4 (Cell Signaling Technology), Smad-6 (Cell Signaling Technology), phosphorylated extracellular signal-regulated kinase 1/2 (p-ERK1/2) (Cell Signaling Technology), phosphorylated c-Jun N-terminal kinase (p-JNK) (Cell Signaling Technology), phosphorylated p38 (P-p38) (Cell Signaling Technology), p53 (Cell Signaling Technology), phosphorylated p53 (P-p53) (Cell Signaling Technology), phosphorylated Bad (Cell Signaling Technology), phosphorylated p38 (P-p38) (Cell Signaling Technology), p53 (Cell Signaling Technology), phosphorylated p53 (P-p53) (Cell Signaling Technology), Bad (Cell Signaling Technology), phosphorylated Bad (Cell Signaling Technology), Bax (Cell Signaling Technology), total caspase-3 (Cell Signaling Technology), cleaved caspase-3 (Cell Signaling Technology), B-cell lymphoma 2 (Bcl-2) (Cell Signaling Technology), and CDK2 (Cell Signaling Technology); and anti-goat antibodies against connective tissue growth factor (CTGF) (Santa Cruz Biotechnology, Inc.) and vimentin (Santa Cruz Biotechnology, Inc.).

Real-Time PCR. The 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. First-strand cDNA was made by reverse-transcribing 5–H9262g aliquots of total RNA using oligo(dT) primers and Superscript II reverse transcriptase (Invitrogen). cDNA was quantified using the SmartCycler II System (Cepheid, Sunnyvale, CA), and SYBR Green was used for detection. PCR was performed using 10 ^{2}M each of forward and reverse primers, 2 ^{10}/H11003SYBR Green Premix Ex Taq (Takara, Kyoto, Japan), 0.5 ^{10}/H9262l of cDNA, and H_{2}O to bring the final volume to 20 ^{10}/H9262l. The relative levels of mRNA were determined by real-time PCR using a Rotor-Gene 3000 Detectors System (QIAGEN, Hilden, Germany). The primer sequences are listed in Supplemental Table 1. The PCR amplification was performed as described previously (Park et al., 2010). Data from the reaction were collected and analyzed with appropriate software from QIAGEN. The comparative critical threshold (Ct) values from quadruple measurements were used to calculate gene expression, with normalization against glyceraldehyde-3-phosphate dehydrogenase as an internal control (Livak and Schmittgen, 2001). Melting curve analysis was performed to enhance the specificity of the amplification reaction.

Terminal Deoxynucleotidyl Transferase dUTP Nick-End Labeling Assay. Apoptosis was detected using the ApoTag Plus Peroxidase In situ Apoptosis Detection Kit (Millipore Bioscience Research Agents, Temecula, CA), according to the manufacturer’s protocol. Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL)-positive cells were counted in cortical tubular cells in 10 fields per slide (magnification, 100 x).

Statistical Analysis. Values were expressed as mean ± S.E.M. Multiple comparisons among the three groups (control, CsA alone, and CsA + fasudil) were performed using one-way analysis of variance and the post hoc Tukey’s honestly significant difference test. For an additional set of experiments (Fig. 12) comparing control versus fasudil treatment, an unpaired t test was used. Differences with values of p < 0.05 were considered significant.

Fig. 5. Expression of TGF-β1, Smad, and MAPKs. A, in CsA-treated rat kidneys, the expression of TGF-β1 was up-regulated. An increase in total Smad-2/3 expression in CsA-treated kidneys was accompanied by an increase in Smad-4 and a decrease in the inhibitory Smad-6. These CsA-induced changes were restored by fasudil cotreatment. Data are means ± S.E.M. (n = 10). B, expression of MAPKs in HK-2 cells. The phosphorylation of ERK1/2, JNK, and p38 increased by CsA treatment (10 ^{5}, 3 h), the degree of which was attenuated by fasudil pretreatment (10 ^{5}, 1 h). Data are means ± S.E.M. of three independent experiments performed in duplicate. *, p < 0.05; **, p < 0.01; †, p < 0.001 versus control. †, p < 0.05; ††, p < 0.01; †††, p < 0.001 versus CsA-treated rats and HK-2 cells.
Results

Renal Function. CsA increased plasma creatinine levels and decreased creatinine clearance. Accordingly, urine osmolality decreased, and urine albumin excretion increased. Fasudil ameliorated the changes induced by CsA without affecting systolic BP or mean blood CsA concentrations (Table 1).

ROCK Expression and MYPT1 Phosphorylation. ROCK-α was significantly up-regulated by CsA treatment, which was ameliorated by fasudil (Fig. 1). MYPT1 phosphorylation has been used to evaluate ROCK activity in the kidney (Riento and Ridley, 2003; Gien et al., 2008). The expression of p-MYPT1 was increased by CsA treatment, indicating increased ROCK activity, which was again attenuated by fasudil.

Nitric Oxide System and NF-κB Expression. Figure 2A shows the expression of NO system components. After CsA treatment, the expression of eNOS decreased, whereas that of iNOS increased in the cortex/OsOM. The expression of 3-nitrotyrosine, a marker of NO-dependent oxidative stress, also increased. Cotreatment with fasudil restored the expression of eNOS and prevented the increase in iNOS and 3-nitrotyrosine expression. The transcription factor NF-κB is known to regulate the expression of many important genes involved in the development of inflammation, apoptosis, and cell proliferation (Magnani et al., 2000; Rangan et al., 2009). In resting cells, NF-κB dimers are bound to IκB proteins, and the degradation of IκB releases free NF-κB, which is translocated into the nucleus and binds to the promoters of inflammatory genes to trigger transcription (Magnani et al., 2000). In the present study, total cytoplasmic IκB-α expression in HK-2 cells was not significantly affected by CsA. However, the expression of P-p65 in the nuclear extracts was increased but was prevented by fasudil (Fig. 2B).

Inflammatory Cell Infiltration and Inflammatory Cytokine Expression. The expression of ED-1 protein, a marker for mononuclear phagocytes, significantly increased in CsA-treated rat kidneys, the degree of which was attenuated by cotreatment with fasudil (Fig. 3A).

Fig. 6. Effects of fasudil on EMT and fibrosis. A, expression of E-cadherin and α-SMA by semiquantitative immunoblotting. CsA treatment decreased the expression of E-cadherin and increased that of α-SMA, which were prevented or attenuated by fasudil cotreatment. Data are means ± S.E.M. (n = 10). B, immunohistochemical staining also revealed decreased E-cadherin expression (arrows) in renal tubules in CsA-treated rat kidneys. α-SMA staining (arrowheads) was strongly positive in renal interstitium in CsA-treated rat kidneys but was ameliorated by fasudil (magnification, 200×). C, expression of fibronectin and CTGF in HK-2 cells. Pretreatment with fasudil (10 μM, 1 h) effectively inhibited the up-regulation of fibronectin and CTGF induced by CsA (10 μM, 24 h). Data are means ± S.E.M. of three independent experiments performed in duplicate. *, p < 0.01; **, p < 0.01; ††, p < 0.001 versus control. †, p < 0.05; ††, p < 0.01 versus CsA-treated rats or HK-2 cells.
chemical staining also revealed that fasudil abrogated the increase of ED-1-positive inflammatory cell infiltration (Fig. 3B). CsA induced an up-regulation of tumor necrosis factor-α, interferon-γ, and interleukin-1β, the magnitude of which was attenuated by cotreatment with fasudil. CsA also induced an up-regulation of monocyte chemotactic protein-1, intercellular adhesion molecule-1, and vascular cell adhesion molecule-1, which was attenuated by fasudil (Fig. 4).

**TGF-β1, Smad, and Mitogen-Activated Protein Kinase Signaling.** The expression of TGF-β1 increased significantly in the cortex/OSOM of CsA-treated rats but was markedly attenuated by cotreatment with fasudil. We also evaluated the expression of Smad proteins, which are known as principal intracellular components of the TGF-β signaling pathway. In CsA-treated rats, total Smad-2/3 increased along with an increase of Smad-4 and a decrease of Smad-6. The CsA-induced changes were reversed by fasudil cotreatment (Fig. 5A). We further used an in vitro model to investigate the effects of CsA and fasudil on mitogen-activated protein kinase (MAPK) signaling. CsA increased the expression of p-ERK1/2, which was suppressed by pretreatment with fasudil. p-JNK and P-p38, known promoters of apoptosis, also increased in HK-2 cells treated with CsA and were attenuated by pretreatment with fasudil (Fig. 5B).

**EMT Process.** In HK-2 cells, CsA decreased the expression of E-cadherin and increased that of α-SMA, both of which were markedly attenuated by fasudil (Fig. 6A). Immunohistochemical staining also showed that CsA treatment decreased E-cadherin expression in renal tubular cells. α-SMA staining was strongly positive in renal interstitium in CsA-treated rat kidneys. Fasudil effectively ameliorated these changes (Fig. 6B). CsA increased the expression of fibronectin and CTGF, which was prevented by fasudil (Fig. 6C).

**Morphological Changes.** In the proximal tubule in CsA-treated rats, there appeared to be variable sizes of tubular vaculization and atrophy associated with sparse interstitial infiltration of mononuclear cells, which was prevented by cotreatment with fasudil (Fig. 7A). Tubulointerstitial damage was significantly increased in CsA-treated rats, the magnitude of which was also attenuated by fasudil (Fig. 7B). Vimentin staining showed CsA-induced EMT, which was prevented by fasudil (Fig. 7A).

**Renal Tubular Cell Apoptosis and Proliferation.** The expression of pro- and antiapoptotic markers was determined along with the TUNEL assay. CsA-treated rats showed increased expression of P-p53, which can initiate apoptotic processes. Accordingly, the expression of p21 was increased in CsA-treated rats but was attenuated by fasudil (Fig. 8). Likewise, fasudil attenuated CsA-induced increases in the proapoptotic markers Bad in rats and Bax and total/cleaved caspase-3 in HK-2 cells (Fig. 9). However, CsA decreased the expression of Bcl-2, an antiapoptotic protein, in tubular cells, which was restored by fasudil. The number of TUNEL-positive cells was increased in CsA-treated kidneys but was significantly restored by fasudil (Fig. 10).

The expression of p27kip1 was increased by CsA and was further augmented by fasudil. CsA also up-regulated PCNA expression, which was ameliorated by fasudil (Fig. 11A).

Immunohistochemical staining also showed an increased number of PCNA-positive cells in CsA-treated kidneys, which was significantly reduced by fasudil (data not shown). Likewise, in HK-2 cells, the expression of p27kip1 was augmented by fasudil pretreatment, whereas that of PCNA was decreased. CsA up-regulated the expression of CDK2/cyclin E, which was reduced by fasudil pretreatment (Fig. 11B).

**Proper Effect of Fasudil on HK-2 Cells.** In another set of experiments, we compared the effects of fasudil alone in HK-2 cells. Fasudil alone was without effects on the expression of p-ERK1/2, p-JNK, p-p38, Bax, Bcl-2, fibronectin, and CTGF (Fig. 12).
Discussion

The present study demonstrated that ROCK inhibition by fasudil exerted a renoprotective action, without affecting whole-blood CsA concentrations or systemic BP. It was also shown that fasudil effectively reduced CsA-induced increases in chemokines, iNOS, adhesion molecules, and inflammatory cell infiltration. The protective effects of fasudil may be multifactorial involving regulatory effects on inflammation, activities of the NO system, TGF-β1 signaling pathways, and apoptosis.

Chronic CsA administration differentially affects nitric-oxide synthase isoforms and NO production (Bobadilla et al., 1998). CsA decreases eNOS-mediated NO production, leading to endothelium-dependent NO-mediated renal vasodilation (Kou et al., 2002). CsA-induced up-regulation of iNOS is associated with increased free radical formation and superoxide production. By forming peroxynitrite, superoxides have been shown to decrease the bioavailability of NO, vasodilation, and glomerular filtration rates (Navarro-Antolín et
3-Nitrotyrosine is a product of tyrosine nitration by the oxidant peroxynitrite, an increase of which is a marker of NO-dependent oxidative stress (Eiserich et al., 1998). We observed CsA-induced decreases in eNOS expression, which were effectively reversed by fasudil. In contrast, CsA-induced iNOS up-regulation was prevented by fasudil. CsA-treated rats also exhibited elevated levels of 3-nitrotyrosine expression, which were decreased by fasudil. These findings suggest that the ROS/NO systems play a role in the pathogenesis of CsA-induced renal injury, in which an altered ROCK activity is involved.

Oxidative stress also leads to NF-κB activation in response to CsA, which in turn activates genes that trigger inflammation (Nishikimi et al., 2004). We observed that CsA increases iNOS expression and nuclear translocation of the p65 NF-κB subunit. Furthermore, being in agreement with previous studies (Nagatoya et al., 2002; Kanda et al., 2003), our results showed that fasudil effectively reduced CsA-induced increases in inflammatory cell infiltration and inflammatory cytokine expression. Because fasudil inhibits ROS generation (Nishikimi et al., 2004), it may not only directly decrease the expression of inflammatory cytokines and adhesion molecules but also indirectly inhibit inflammatory cell infiltration by inhibiting ROS generation.

Among others, TGF-β-mediated activation of Smad signaling plays an important role in EMT (Böttinger and Bitzer, 2002). Previous investigators have shown that ROCK inhibitors decrease the expression of TGF-β mRNA and suppress both extracellular matrix accumulation and α-SMA expression (Nishikimi et al., 2004, 2007; Wu et al., 2010). The present study showed that cotreatment with fasudil ameliorated CsA-induced overexpression of TGF-β1. It was also demonstrated that CsA induced overexpression of Smad-2/3 and Smad-4 while it decreased the expression of inhibitory Smad-6. Fasudil reversed these changes, thereby preserving E-cadherin expression and inhibiting α-SMA induction. Vimentin staining confirmed the inhibitory action of fasudil in the CsA-induced EMT process.

We also evaluated the MAPK signal transduction pathway, which is known to be activated by TGF-β1 (Böttinger and Bitzer, 2002). In CsA-induced nephropathy, ERK signaling may be involved in CsA-induced EMT processes and ROS generation (Cantley, 2002; Masaki et al., 2003). A recent study has suggested a critical role for p38 MAPK in CsA-induced Smad activation, in which type II TGF-β receptor and TGF-β play a role (Akol et al., 2008). JNK signaling is also known to be associated with TGF-β1-induced EMT processes (Chan and Riches, 2001; Ma et al., 2007). We observed that CsA modulated the expression of MAPKs. CsA induced phosphorylation of ERK1/2, JNK, and p38 in HK-2 cells, which was attenuated by fasudil. These changes coincided with the expression of proapoptotic, profibrotic, and EMT markers in HK-2 cells and in vivo. It is suggested that the MAPK signaling pathway activated by TGF-β1 mediates CsA-induced renal apoptosis and fibrosis, which are effectively inhibited by fasudil.

DNA damage is an early event in CsA-induced nephropathy (Naesens et al., 2009). Exposure to CsA can reduce telomere length, increase p53 phosphorylation, and then cause p21 up-regulation (Jennings et al., 2007), which is a negative regulator of p53-dependent apoptosis (Maddika et al., 2007). Induction of p21 by CsA may represent a feedback mechanism to control p53 activity during the apoptotic process. In the present study, the expression of p53 was significantly elevated in CsA-treated rats, and there were increases of proapoptotic markers such as Bad, Bax, and total/cleaved caspase-3 in HK-2 cells. These changes were effectively reversed by fasudil. CsA treatment increased the number of TUNEL-positive cells, which was also reversed by fasudil cotreatment. Therefore, our results indicate that the fasudil-
induced protective effects were partly due to antiapoptotic actions, such as the down-regulation of p53 expression. Unlike the expression of p21, that of p27^{kip1} was augmented by fasudil cotreatment. p27^{kip1} expression is known to be regulated by TGF-β1 and various other stimuli independent of p53 expression, and its up-regulation may reduce cell proliferation (Nourse et al., 1994). It is well known that renal cell proliferation and matrix expansion are crucial in the development of glomerulosclerosis and tubulointerstitial fibrosis (Kliem et al., 1996). Previous studies have suggested that the expression of active Rho decreases and ROCK inhibition increases the p27^{kip1} protein levels (Hu et al., 1999; Vidal et al., 2002; Croft and Olson, 2006). In angiotensin II-mediated vascular injury, fasudil reduced vascular smooth muscle cell proliferation and inflammatory cell migration by up-regulating p27^{kip1} (Kanda et al., 2005). In addition, PCNA, a processivity factor of DNA polymerase α-positive cells, has been associated with low p27^{kip1} expression levels (Kanda et al., 2003). Our study showed that fasudil augmented p27^{kip1} expression. Likewise, fasudil reduced the CsA-mediated increase of PCNA-positive cells, suggesting that fasudil-induced renoprotective effects included p27^{kip1} up-regulation and subsequent inhibition of cell proliferation/macrophage recruitment. In HK-2 cells, up-regulation of CDK2/cyclin E was induced by CsA but was prevented by fasudil pretreatment, indicating an antiproliferative effect of fasudil. It was shown that the activation of Rho/Rock was closely associated with both apoptotic and proliferative pathways in renal injury.

In summary, fasudil has renoprotective effects through the suppression of inflammatory, apoptotic, and fibrogenic factors. The underlying mechanisms may include inhibition of ROS/NO, Smad, and MAPK signaling pathways. Inhibitors of the Rho/Rock pathway may offer significant potential as therapeutic interventions for CsA-induced nephropathy.

**Authorship Contributions**

**Participant in research design:** J.W. Park and S.W. Kim.

**Conducted experiments:** J.W. Park, C.H. Park, and I.J. Kim.

**Performed data analysis:** J.W. Park and S.W. Kim.

**Wrote or contributed to the writing of the manuscript:** J.W. Park, C.H. Park, Bae, Ma, Lee, and S.W. Kim.

**References**


**Address correspondence to:** Dr. Soo Wan Kim, Department of Internal Medicine, Chonnam National University Medical School, H 501-757, South Korea. E-mail: skimw@chonnam.ac.kr
Rho-kinase Inhibition by Fasudil Attenuates Cyclosporine-induced Kidney Injury

Jeong Woo Park, Cheon Hoon Park, In Jin Kim, Eun Hui Bae, Seong Kwon Ma, JongUn Lee, Soo Wan Kim

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**Supplemental Table 1.** Primer sequences for real-time PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence</th>
<th>Size, bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-α</td>
<td>Fwd : CTTCAGCTCCACAGAGAA GAACTG C 298</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Rev: CACGATCATGTTGGACAACCTGCTC C</td>
<td></td>
</tr>
<tr>
<td>IL-1β</td>
<td>Fwd: TGATGTTCCATTAGACAGC 378</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Rev: GAGGTGCTGATGTACCAGTT</td>
<td></td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Fwd : AACCAGGCCATCAGCAACAACA 214</td>
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<tr>
<td></td>
<td>Rev: ACCGACTCCTTTTCCGCTTCCT</td>
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</tr>
<tr>
<td>MCP-1</td>
<td>Fwd : AGCCCCAGAAACCAGCCAAC 196</td>
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<tr>
<td></td>
<td>Rev: GCCGACTCATGGGATCATCTTT</td>
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<tr>
<td>ICAM-1</td>
<td>Fwd : GCCCGAGGATCACAAGAAGAC 186</td>
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<tr>
<td></td>
<td>Rev: CCTTTGAGCTGGCTGTAAGAGT</td>
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<tr>
<td>VCAM-1</td>
<td>Fwd : GGGGGCAAGTCCGTTCTGA 158</td>
<td></td>
</tr>
</tbody>
</table>
Rev: GGGGGCCACTGAATTGAATCTC

GAPDH Fwd: GCCAAAAGGGTCATCATCTC 229

Rev: GGCCATCCACAGTCTTCT

Abbreviations: TNF-α, tumor necrosis factor-α; interleukin-1β, IL-1β; IFN-γ, interferon-γ; MCP-1, monocyte chemoattractant protein-1; ICAM-1, intercellular adhesion molecule-1; VCAM-1, vascular cell adhesion molecule-1; Fwd, forward; Rev, reverse.