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

Departments of Internal Medicine (JWP, EHB, SKM, SWK) and Physiology (CHP, IJK, JUL), Chonnam National University Medical School, Gwangju 501-757, Korea
Running Title page

a. Running Title: Rho-kinase inhibition in CsA nephrotoxicity

b. Address Correspondence to; Soo Wan Kim, M.D. and Ph.D., Department of Internal Medicine, Chonnam National University Medical School, 8 Hakdong, Gwangju 501-757, Korea; E-mail: skimw@chonnam.ac.kr; Tel.: +82-62-2206271; Fax: +82-62-2258578

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d. Abbreviations; ROCK, Rho-kinase; CsA, cyclosporine A; MAPK, mitogen-activated protein kinase; MLC, myosin light chain; MYPT1, MLC phosphatase; NF-κB, nuclear factor-kappa B; TGF-β1, transforming growth factor-β1; CDK, cyclin-dependent kinase; EMT, epithelial-to-mesenchymal transition; eNOS, endothelial nitric oxide synthase; iNOS, inducible nitric oxide synthase; PCNA, proliferating cell nuclear antigen; α-SMA, α-smooth muscle actin; p-MYPT1, phosphorylated MYPT1; p-ERK1/2, phosphorylated extracellular signal-regulated kinase1/2; p-JNK, phosphorylated c-Jun N-terminal kinase; P-p38, phosphorylated p38; P-p53, phosphorylated p53; p-Bad, phosphorylated Bad; CTGF, connective tissue growth factor; TNF-α, tumor necrosis factor-α; IFN-γ, interferon-γ; IL-1β, interleukin-1β; MCP-1, monocyte chemoattractant protein-1; ICAM-1, intercellular adhesion molecule-1; VCAM-1, vascular cell adhesion molecule-1; ROS, reactive oxygen species; ECM, extracellular matrix.

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Abstract

It has been shown that an inhibition of Rho/Rho-kinase (ROCK) pathway prevents tubulointerstitial fibrosis and ameliorates renal function in various progressive renal disorders. The present study was aimed to determine whether fasudil, a ROCK inhibitor, has a protective effect on cyclosporine (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, along with a restoration of the renal function. CsA decreased the expression of endothelial nitric oxide synthase (NOS) and increased inducible NOS/3-nitrotyrosine in the kidney. Accordingly, there were infiltration of inflammatory cells and upregulation 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 p27\(^{kip1}\) expression and decreased the number of PCNA-positive cells. In another series of experiment using HK-2 cells in culture, fasudil also suppressed the CsA-induced increases in mitogen-activated protein kinase (MAPK) phosphorylation. CsA induced an expression of p53, the degree of which was attenuated by fasudil in association with decreases of pro-apoptotic markers such as Bad, Bax, and total/cleaved caspase-3. These results suggest that an inhibition of Rho/ROCK pathway attenuate CsA-induced nephropathy through suppressing the induction of inflammatory, apoptotic, and fibrogenic factors, along with inhibition of Smad, MAPKs,
and nitric oxide signaling pathways.
Introduction

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

Rho-kinase (ROCK) is a Rho-protein effector that phosphorylates downstream targets including myosin light chain (MLC) and MLC phosphatase (MYPT1), and modulates actin cytoskeletal organization and endothelial barrier function (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 (NFκB) and transforming growth factor-β (TGFβ) (Sharpe and Hendry, 2003; Sun et al., 2006). Furthermore, an inhibition of the Rho/ROCK pathway increases the levels of cyclin-dependent kinase (CDK) inhibitors such as
P27\textsuperscript{kip1}, subsequently inhibiting cell proliferation in progressive renal damage (Kanda et al., 2003).

The protective effect of Rho-kinase 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 aimed at examining a protective efficacy of fasudil (1-(5-Isoquinolinylsulfonyl)homopiperazine dihydrochloride, HA-1077 dihydrochloride, Fasudil dihydrochloride), a ROCK-inhibitor, in CsA-induced nephropathy.
Methods

Animals. Male Sprague-Dawley rats weighing 180–200 g were used. The animal protocol was approved by the Institutional Animal Care and Use Committee of Chonnam National University Medical School (Gwangju, 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, PA, USA) and tap water ad libitum. CsA (Novartis Pharma, Basel, Switzerland) was dissolved in olive oil to achieve a concentration of 20 mg.ml⁻¹. Rats were divided into 3 groups and injected with the vehicle alone (N = 10), CsA alone (N = 10, 20 mg.kg⁻¹.day⁻¹, s.c.), or CsA+fasudil (N = 10, 3 mg.kg⁻¹.day⁻¹, i.p.; Sigma-Aldrich Co., St Louis, MO, USA) 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 (ATCC, Manassas, VA, USA), human renal proximal tubular epithelial cells, were cultured. Cells were passaged every 3–4 days in 100-mm dishes containing combined Dulbecco’s modified Eagle’s media (DMEM) and Hams F-12 medium (Sigma) supplemented with 10% fetal bovine serum (FBS), 100 U.ml⁻¹ penicillin, and 100 mg.ml⁻¹ streptomycin (Sigma). The cells were then incubated in a humidified atmosphere of 5% CO₂ and 95% air at 37°C for 24 h, and sub-cultured until 70–80% confluence. HK-2 cells were plated onto 60-mm dishes in medium containing 10% FBS and incubated for 24 h, following which they were transferred to DMEM-F12 medium with 2% FBS and incubated for an additional 16 h. The cells were then 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 cold phosphate-buffered saline (PBS; pH 7.4) for 15 s before switching to 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 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 imidazole, 1 mM ethylenediamine tetraacetic acid, 8.5 μM leupeptin, and 1 mM phenylmethylsulfonfyl 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 samples were adjusted to the same final protein concentration using isolation solution, solubilized at 65°C for 15 min in sodium dodecyl sulfate (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 Bio-Rad Mini Protean II apparatus (Bio-Rad, Hercules, CA, USA). The blots were blocked with 5% milk in PBS-T (80 mM Na₂HPO₄, 20 mM NaH₂PO₄, 100 mM NaCl, and 0.1% Tween-20 at 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.
Primary antibodies. The following antibodies were used: affinity-purified anti-mouse antibodies against ROCK-α (BD Transduction Laboratories, San Jose, CA, USA), endothelial nitric oxide synthase (eNOS, BD Transduction), inducible nitric oxide synthase (iNOS, BD Transduction), ED-1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), E-cadherin (BD Transduction), α-SMA (Sigma), fibronectin (Santa Cruz), p21 (BD Pharmingen, San Diego, CA, USA), p27kip1 (BD Transduction), proliferating cell nuclear antigen (PCNA, Cell Signaling Technology, Beverly, MA, USA), and cyclin E (Cell signaling); anti-rabbit antibodies against p-MYPT1 (Cell Signaling), 3-nitrotyrosine (Upstate Biotechnology, Lake Placid, NY, USA), phosphorylated nuclear factor-kappa B (NF-κB) p65 subunit (P-p65, Cell Signaling), IκB-α (Santa Cruz), TGF-β1 (Santa Cruz), Smad-2/3 (Cell Signaling), Smad-4 (Cell Signaling), Smad-6 (Cell Signaling), phosphorylated extracellular signal-regulated kinase1/2 (p-ERK1/2, Cell Signaling), phosphorylated c-Jun N-terminal kinase (p-JNK, Cell Signaling), phosphorylated p38 (P-p38, Cell Signaling), p53 (Cell Signaling), phosphorylated p53 (P-p53, Cell Signaling), Bad (Cell Signaling), phosphorylated Bad (p-Bad, Cell Signaling), Bax (Cell Signaling), total caspase-3 (Cell Signaling), cleaved caspase-3 (Cell Signaling), Bcl-2 (Cell Signaling), and CDK2 (Cell Signaling); and anti-goat antibodies against connective tissue growth factor (CTGF, Santa Cruz) and vimentin (Santa Cruz).

Real-time PCR. The renal cortex was homogenized in TRIZol reagent (Invitrogen, Carlsbad, CA,
USA). 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-μg aliquots of total RNA using oligo(dT) primers and Superscript II reverse transcriptase (Invitrogen). cDNA was quantified using the Smart Cycler II System (Cepheid, Sunnyvale, CA, USA), and SYBR Green was used for detection. PCR was performed using 10 μM each of forward and reverse primers, 2× SYBR Green Premix Ex Taq (Takara Bio, Seta, Japan), 0.5 μl of cDNA, and H2O to bring the final volume to 20 μl. The relative levels of mRNA were determined by real-time PCR using a Rotor-Gene™ 3000 Detector System (Corbett Research, New South Wales, Australia). 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 Corbett Research. The comparative critical threshold (Ct) values from quadruple measurements were used to calculate gene expression, with normalization against GAPDH as an internal control (Livak and Schmittgen, 2001). Melting curve analysis was performed to enhance the specificity of the amplification reaction.

**TUNEL assay.** Apoptosis was detected using the ApopTag Plus Peroxidase *In situ* Apoptosis Detection Kit (Chemicon International, Temecula, CA, USA), according to the manufacturer’s protocol. TUNEL-positive cells were counted in cortical tubular cells in 10 fields per slide (at 100× magnification).
Statistical analysis. Values were expressed as mean ± standard error of the mean (SEM). Multiple comparisons among the 3 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 (Figure 12) comparing control versus fasudil treatment, an unpaired t-test was used. Differences with values of $p < 0.05$ were considered significant.
Results

Renal function

CsA increased plasma creatinine (P_{Cr}) levels, along with a decreased creatinine clearance (C_{Cr}). Accordingly, urine osmolality (U_{Osm}) decreased and urine albumin excretion (UAE) 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 upregulated by the CsA-treatment, which was ameliorated by fasudil (Figure 1). MYPT1 phosphorylation has been used to evaluate ROCK activity in the kidney (Gien et al., 2008; Riento and Ridley, 2003). The expression of p-MYPT1 was increased by the CsA-treatment, indicating an increased ROCK activity; which was again attenuated by fasudil.

Nitric oxide system and NF-κB expression

Figure 2A shows the expression of nitric oxide (NO) system components. Following the 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 IκBα expression in HK-2 cells was not significantly affected by CsA. However, the expression of P-p65 in the nuclear extracts was increased, which was prevented by fasudil (Figure 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, of which degree was attenuated by the cotreatment with fasudil (Figure 3A). Immunohistochemical staining also revealed that fasudil abrogated the increase of ED-1-positive inflammatory cell infiltration (Figure 3B). CsA induced an upregulation of tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ), and interleukin-1β (IL-1β), the magnitude of which was attenuated by the cotreatment with fasudil. CsA also induced an upregulation of monocyte chemoattractant protein-1 (MCP-1), intercellular adhesion molecule-1 (ICAM-1), and vascular cell adhesion molecule-1 (VCAM-1), which was attenuated by fasudil (Figure 4).
TGF-β1, Smad, and MAPK signaling

The expression of TGF-β1 increased significantly in the cortex/OSOM of CsA-treated rats, which was markedly attenuated by the 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 (Figure 5A). We further used an in vitro model to investigate the effects of CsA and fasudil on MAPK signaling. CsA increased the expression of p-ERK1/2, which was suppressed by the pretreatment with fasudil. p-JNK and P-p38, known promoters of apoptosis, also increased in HK-2 cells treated with CsA, which was attenuated by the pretreatment with fasudil (Figure 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 (Figure 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 (Figure 6B). CsA increased the expression of fibronectin and CTGF, which was prevented by fasudil (Figure 6C).
Morphological changes

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

Renal tubular cell apoptosis and proliferation

The expression of pro- and anti-apoptotic markers was determined along with TUNEL assay. CsA-treated rats showed an increased expression of P-p53, which can initiate apoptotic processes. Accordingly, the expression of p21 was increased in CsA-treated rats, which was attenuated by fasudil (Figure 8). Likewise, fasudil attenuated CsA-induced increases in the pro-apoptotic markers Bad in rats and Bax and total/cleaved caspase-3 in HK-2 cells (Figure 9). On the other hand, CsA decreased the expression of Bcl-2, an anti-apoptotic protein, in tubular cells, which was restored by fasudil. The number of TUNEL-positive cells was increased in CsA-treated kidneys, which was significantly restored by fasudil (Figure 10).

The expression of p27kip1 was increased by CsA, which was further augmented by fasudil. CsA also upregulated PCNA expression, which was ameliorated by fasudil (Figure 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). Similarly, in HK-2 cells, the expression of p27\(^{kip1}\) was augmented by fasudil pretreatment, while that of PCNA was decreased. CsA upregulated the expression of CDK2/cyclin E, which was reduced by fasudil pretreatment (Figure 11B).

**Proper effect of fasudil on HK-2 cells**

In another set of experiment, we compared the effects of fasudil alone in HK-2 cells. Fasudil alone was without effects on the expression of p-ERK 1/2, p-JNK, P-p38, Bax, Bcl-2, fibronectin and CTGF (Figure 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, the activities of NO system, TGF-β1 signaling pathways, and apoptosis.

Chronic CsA administration differentially affects NOS isoforms and NO production (Bobbadilla et al., 1998). CsA decreases eNOS-mediated NO production, leading to endothelium-dependent NO-mediated renal vasodilatation (Kou et al., 2002). CsA-induced upregulation 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 al., 2001). Nitrotyrosine is a product of tyrosine nitration by the oxidant peroxynitrite, of which increase is a marker of NO-dependent oxidative stress (Eiserich et al., 1998). We observed CsA-induced decreases in the eNOS expression, which was effectively reversed by fasudil. In contrast, CsA-induced iNOS upregulation was prevented by fasudil. CsA-treated rats also exhibited elevated levels of 3-nitrotyrosine expression, which was 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 (Kanda et al., 2003; Nagatoya et al., 2002), our results showed that fasudil effectively reduced CsA-induced increases in inflammatory cell infiltration and inflammatory cytokine expression. Since 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 (ECM) accumulation and α-SMA expression (Nishikimi et al., 2004; Nishikimi et al., 2007; Wu et al., 2010). The present study showed that cotreatment with fasudil ameliorated the CsA-induced overexpression of TGF-β1. It was also demonstrated that the CsA induced an overexpression of Samd-2/3 and -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 of p38 MAPK in CsA-induced Smad activation, in which Type II TGF-β-receptor and TGF-β play a role (Akool 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, c-JNK, and p38 in HK-2 cells, which was attenuated by fasudil. These changes coincided with the expression of pro-apoptotic, pro-fibrotic, and EMT markers in HK-2 cells and in vivo. It is suggested that the MAPK signaling pathway activated by TGF-β1 mediate CsA-induced renal apoptosis and fibrosis, which is effectively inhibited by fasudil.

DNA damage is an early event in CsA-induced nephropathy (Naesens et al., 2009). An exposure to CsA can reduce telomere length, increase p53 phosphorylation, and then cause p21 upregulation (Jennings et al., 2007) which is a negative regulator of p53-dependent apoptosis (Maddika et al., 2007). An 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 pro-apoptotic markers such as Bad, Bax, and total/cleaved caspase-3 levels in HK-2 cells. These changes were effectively reversed by fasudil. CsA
treatment increased the number of TUNEL-positive cells, which was also reversed with fasudil cotreatment. Therefore, our results indicate that the fasudil-induced protective effects were partly due to anti-apoptotic actions such as the downregulation of p53 expression.

Unlike the expression of p21, that of p27\textsuperscript{kip1} was augmented by fasudil cotreatment. p27\textsuperscript{kip1} expression is known to be regulated by TGF-\textbeta1 and various other stimuli independent of p53 expression, and its upregulation may reduce the 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\textsuperscript{kip1} protein levels (Croft and Olson, 2006; Hu et al., 1999; Vidal et al., 2002). In angiotensin II-mediated vascular injury, fasudil reduced vascular smooth muscle cell proliferation and inflammatory cell migration by upregulating p27\textsuperscript{kip1} (Kanda et al., 2005). Additionally, PCNA, a processivity factor of DNA polymerase \alpha-positive cells, has been associated with low p27\textsuperscript{kip1} expression levels (Kanda et al., 2003). Our study showed that fasudil augmented p27\textsuperscript{kip1} expression. Likewise, fasudil reduced the CsA-mediated increase of PCNA-positive cells, suggesting that fasudil-induced renoprotective effects included p27\textsuperscript{kip1} upregulation and subsequent inhibition of cell proliferation/macrophage recruitment. In HK-2 cells, an upregulation of CDK2/cyclin E was induced by CsA, which was prevented by fasudil pretreatment, indicating an anti-proliferative effect of fasudil. It was shown that an activation
of Rho/ROCK was closely associated with both apoptotic and proliferative pathways in renal injury.

In summary, fasudil has renoprotective effects through suppression of inflammatory, apoptotic,
and fibrogenic factors. The underlying mechanisms may include inhibition of ROS/NO, Samds, and
MAPKs signaling pathways. Inhibitors of Rho/ROCK pathway may offer a significant potential in
therapeutic intervention of CsA-induced nephropathy.
Authorship contributions

Participated in research design; JW Park, SW Kim

Conduced experiments; JW Park, CH Park, IJ Kim

Contributed new reagents or analytic tools; None

Performed data analysis; JW Park, SW Kim

Wrote or contributed to the writing of the manuscript; JW Park, CH Park, EH Bae, SK Ma, JU Lee,

SW Kim

Others: None
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Footnotes

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Please send reprint request to Prof. Soo Wan Kim, Department of Internal Medicine, Chonnam National University Medical School, 8 Hakdong, Gwangju, 501-757, Korea; E-mail: skimw@chonnam.ac.kr
Legends for figures

Figure 1. Expression of ROCK in renal cortex. The ROCK-α expression increased by CsA treatment, which was counteracted by fasudil. The expression of p-MYPT1, which can reflect the ROCK activity, increased by CsA; which was attenuated by fasudil. Data are means ± SEM (N = 10).

***p < 0.001 vs. control. †p < 0.05; ††p < 0.01 vs. CsA-treated rats.

Figure 2. Expressions of eNOS, iNOS, 3-nitrotyrosine and NF-κB. (A) The 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, which was reversed by fasudil. Data are means ± SEM (N = 10). (B) Expression of phosphorylated NF-κB p65 subunit (P-p65) and total IκBα in HK-2 cells. The expression of P-p65 increased by treatment with CsA (10 µM, 30 min), which was effectively suppressed by pretreatment with fasudil (10 µM, 1 h). The total IκBα expression did not differ between the control and CsA-treated HK-2 cells with or without pretreatment with fasudil. Data are means ± SEM of 3 independent experiments performed in duplicate. *p < 0.05; ***p < 0.001 vs. control. †p < 0.05; ††p < 0.01; †††p < 0.001 vs. CsA-treated rats or HK-2 cells.
Figure 3. Expression of ED-1. (A) Semiquantitative immunoblotting of ED-1. Data are means ± SEM (N = 10). *** p < 0.001 vs. control. ††† p < 0.001 vs. CsA-treated rats. (B) Immunohistochemical staining for ED-1 in the renal cortex/OSOM (200× magnification). 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.

Figure 4. Effects of fasudil on proinflammatory cytokines and adhesion molecules.

Proinflammatory cytokines (TNF-α, IFN-γ, and IL-1β) and adhesion molecules (MCP-1, ICAM-1, and VCAM-1) were significantly elevated in CsA-treated rat kidneys, which was suppressed by fasudil. Data are means ± SEM (N = 10). ** p < 0.01; *** p < 0.001 vs. control. † p < 0.05; †† p < 0.01 vs. CsA-treated rats.

Figure 5. Expressions of TGF-β1, Smad, and MAPKs. (A) In CsA-treated rat kidneys, the expression of TGF-β1 was upregulated. An increase in total Smad-2/3 expression in CsA-treated kidney 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 ± SEM (N= 10). (B) The expression of MAPKs in HK-2 cells. The phosphorylation of ERK1/2, c-JNK, and p38 increased by CsA treatment (10 µM, 3 h), the degree of which was attenuated by fasudil pretreatment (10 µM, 1
h). Data are means ± SEM of three independent experiments performed in duplicates. *p < 0.05; **p < 0.01; ***p < 0.001 vs. control. †p < 0.05; ††p < 0.01; †††p < 0.001 vs. CsA-treated rats and HK-2 cells.

Figure 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 ± SEM (N = 10). (B) Immunohistochemical staining also revealed a 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, which was ameliorated by fasudil (200× magnification). (C) Expression of fibronectin and CTGF in HK-2 cells. Pretreatment with fasudil (10 µM, 1 h) effectively inhibited the upregulation of fibronectin and CTGF induced by CsA (10 µM, 24 h). Data are means ± SEM of 3 independent experiments performed in duplicates. *p < 0.01; **p < 0.01; ***p < 0.001 vs. control. †p < 0.05; ††p < 0.01 vs. CsA-treated rats or HK-2 cells.

Figure 7. H & E and vimentin staining in rat kidneys. (A) H & E staining revealed a tubular atrophy associated with tubular vacuoles in the proximal tubules in CsA-treated rats. Interstitial infiltration of inflammatory cells (arrows) was also observed. These changes were countered by
fasudil. Staining with vimentin (arrowheads) demonstrated that CsA treatment increased the EMT process in kidneys, which was attenuated by concomitant treatment with fasudil (200× magnification).

(B) Tubulointerstitial damage was quantified by image analysis. The amount of tubulointerstitial damage increased with CsA, which was attenuated by fasudil cotreatment. Data are the means ± SEM (N = 5). ***p < 0.001 vs. control. ††p < 0.01 vs. CsA-treated rats.

Figure 8. Expressions of P-p53 and p21. CsA induced p53 phosphorylation (P-p53), which can initiate apoptotic process. Fasudil effectively suppressed the CsA-induced P-p53 expression. Fasudil cotreatment also attenuated the overexpression of p21 induced by CsA. Data are means ± SEM (N = 10). **p < 0.01; ***p < 0.001 vs. control. †p < 0.05 vs. CsA-treated rats.

Figure 9. Expressions of apoptotic markers in the cortex/OSOM in rat kidneys and HK-2 cells.

(A) The expression of p-Bad increased in CsA-treated rats, the degree of which was reduced by fasudil cotreatment. (B) HK-2 cells were pretreated with fasudil (10 µM) for 1 h before incubation with CsA (10 µM, 24 h). Semiquantitative immunoblotting of CsA-treated HK-2 cells indicated that the Bax and total/cleaved caspase-3 levels increased and the Bcl-2 level decreased. Fasudil pretreatment effectively ameliorated these changes. Data are means ± SEM (N = 10). *p < 0.05; **p < 0.01 vs. control. †p < 0.05; ††p < 0.01 vs. CsA-treated rats or HK-2 cells.
Figure 10. Effects of fasudil on apoptosis. The TUNEL assay represents that CsA-mediated increase in apoptosis, in which fasudil cotreatment reduced the number of TUNEL-positive cells (arrows) (N = 5, 100× magnification). *** \( p < 0.001 \) vs. control. ††† \( p < 0.001 \) vs. CsA-treated HK-2 cells or rats.

Figure 11. Effects of fasudil on the expressions of p27\(^{kip1}\), CDK2, cyclin E, and PCNA. (A) The expression of p27\(^{kip1}\) increased with CsA, the degree of which was augmented by fasudil. The expression of PCNA was also elevated; however, of which degree was reduced by fasudil. Data are means ± SEM (N = 10). (B) In HK-2 cells, the upregulation of p27\(^{kip1}\) was augmented by fasudil, whereas that of PCNA, CDK2 and cyclin E was attenuated by fasudil. Data are means ± SEM of 3 independent experiments performed in duplicates. * \( p < 0.05 \); *** \( p < 0.001 \) vs. control. † \( p < 0.05 \); †† \( p < 0.01 \); ††† \( p < 0.001 \) vs. CsA-treated rats or HK-2 cells.

Figure 12. Effects of fasudil alone in HK-2 cells. The expression of p-ERK 1/2, p-JNK, P-p38, Bax, Bcl-2, fibronectin and CTGF was not affected by fasudil alone.
# Table 1. Renal functional parameters

<table>
<thead>
<tr>
<th></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 (mmHg)</td>
<td>125.20 ±2.00</td>
<td>116.90 ±2.00</td>
<td>107.40±1.40</td>
</tr>
<tr>
<td>PCr (mg.dl⁻¹)</td>
<td>0.25±0.02</td>
<td>0.50±0.04 ***</td>
<td>0.35±0.02 †</td>
</tr>
<tr>
<td>CCr (ml.min⁻¹)</td>
<td>2.52±0.25</td>
<td>1.19±0.10 **</td>
<td>1.90±0.95 ††</td>
</tr>
<tr>
<td>U Osm (mOsm.kg⁻¹ H₂O)</td>
<td>2,052.00±134.50</td>
<td>854.00±30.66 ***</td>
<td>1131.00±74.41 †</td>
</tr>
<tr>
<td>UAE (mg.g⁻¹ Cr)</td>
<td>4.25±0.53</td>
<td>12.68±1.08 ***</td>
<td>8.23±0.41 †</td>
</tr>
<tr>
<td>CsA Cₘᵲ (ng.ml⁻¹)</td>
<td>N.M.</td>
<td>2,401.00±4.39</td>
<td>2,294.00±28.61</td>
</tr>
</tbody>
</table>

Abbreviations: BP, blood pressure; PCr, plasma creatinine; CCr, creatinine clearance; U Osm, urine osmolality; UAE, urine albumin excretion; CsA Cₘᵲ, cyclosporin 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. Values are expressed as the mean ± SEM.
Figure 1

![Western Blot Diagram]

**ROCK-α (Fraction of control)***

- Control
- CsA
- CsA+Fasudil

**p-MYPT1 (Fraction of control)***

- Control
- CsA
- CsA+Fasudil

**Statistical Significance**

- ***p < 0.001
- †p < 0.05
- ††p < 0.01
Figure 2

A

<table>
<thead>
<tr>
<th>Control</th>
<th>CsA</th>
<th>CsA+Fasudil</th>
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<tbody>
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<tr>
<td>130 kDa</td>
<td></td>
<td></td>
</tr>
<tr>
<td>55 kDa</td>
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- eNOS
- iNOS
- 3-nitrotyrosine

B

<table>
<thead>
<tr>
<th>Control (10 μM)</th>
<th>CsA (10 μM) + Fasudil (10 μM)</th>
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<tbody>
<tr>
<td>65 kDa P-p65</td>
<td></td>
</tr>
<tr>
<td>39 kDa IkBα</td>
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</tr>
<tr>
<td>43 kDa β actin</td>
<td></td>
</tr>
</tbody>
</table>

- P-p65
- IkBα

Bar graphs showing the fraction of control values for each group.
Figure 3

A

![Western blot analysis of ED-1 expression.](image)

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<tr>
<th>Control</th>
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<th>CsA+Fasudil</th>
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<tbody>
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<td>110 kDa</td>
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<tr>
<td>ED-1</td>
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B

![Immunohistochemical staining of ED-1.](image)

<table>
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<tr>
<th>Control</th>
<th>CsA</th>
<th>CsA+Fasudil</th>
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<tbody>
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Figure 5

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<td>62 kDa</td>
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***

<table>
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<tr>
<th>(Fraction of control)</th>
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<tbody>
<tr>
<td>Control</td>
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<table>
<thead>
<tr>
<th>Smad-2/3</th>
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††

<table>
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††

B

<table>
<thead>
<tr>
<th>Control</th>
<th>CsA (10 µM)</th>
<th>CsA (10 µM) + Fasudil (10 µM)</th>
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<tbody>
<tr>
<td>44 kDa</td>
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<td>42 kDa</td>
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<td>43 kDa</td>
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<table>
<thead>
<tr>
<th>p-ERK1/2</th>
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<tr>
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<tr>
<th>P-p38</th>
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**

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<tbody>
<tr>
<td>Control</td>
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†
Figure 8

The figure shows the effects of CsA and CsA+Fasudil on the expression of p53, P-p53, p21, and the ratio of these proteins to the control. The graphs indicate that CsA induces a significant increase in p53 and P-p53, while CsA+Fasudil further enhances these effects. The blot and bar charts are consistent with these findings.

- **Control**, **CsA**, and **CsA+Fasudil** conditions are compared.
- The protein bands for P-p53, p53, and p21 are shown in the blots.
- The bar charts display the fraction of control for each protein under the different treatment conditions.
- The statistical significance is indicated by the symbols: **,** **,** and **,** for **p53**, P-p53, and p21, respectively.

The data suggests that CsA and CsA+Fasudil have a synergistic effect on the expression of these proteins, with CsA+Fasudil showing the most significant increase in p53 and P-p53 compared to control.
Figure 10

Control  CsA  CsA+Fasudil

TUNEL-positive cell
/field

Control  CsA  CsA+Fasudil

***

****
Figure 11

A

Control    CsA    CsA+Fasudil

27 kDa     p27kip1

36 kDa     PCNA

B

Control    CsA (10 μM) + Fasudil (10 μM)

27 kDa     p27kip1

33 kDa     CDK2

48 kDa     Cyclin E

36 kDa     PCNA

Bars with statistical significance:
- * p < 0.05
- ** p < 0.01
- *** p < 0.001
- **** p < 0.0001

Bars with trends:
- † p < 0.1
- ‡ p < 0.05
- § p < 0.1
**Figure 12**

**Western Blot Analysis**

- **Control** vs **Fasudil (10 μM)**
  - **p-ERK1/2**
  - **p-JNK**
  - **P-p38**
  - **β-actin**
  - **Bax**
  - **Bcl-2**
  - **Fibronectin**
  - **CTGF**

A bar chart showing the fraction of control for each protein under control and fasudil conditions.

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