Protective effects of Rho kinase inhibitor fasudil on rats with chronic kidney disease

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The purpose of this study was to examine the long-term therapeutic effects of the Rho-kinase inhibitor fasudil on the spontaneously hypercholesterolemic (SHC) rat, which is a good model of CKD with continuous severe proteinuria (28). Furthermore, the effects of fasudil were compared with those of the ARB olmesartan (OL), while special attention was paid to macrophages efficiently produce inflammatory cytokines (23), while M2 macrophages are generally involved in immunoregulation and immunosupression by generating anti-inflammatory cytokines such as IL-10 (22, 30). In accordance with this general concept, it has been found that M2 macrophages are increased, whereas M1 macrophages are decreased in the restoration process of renal interstitial fibrosis in mice with unilateral ureteral obstructions (UUO) (18). Thus fasudil seems to be a potent proteinuria-reducing agent against renal disease.

Macrophages can be divided into two subclasses based on their activation condition: classically activated (M1) macrophages and alternatively activated (M2) macrophages (8, 23, 30). M1 macrophages efficiently produce inflammatory cytokines (23), while M2 macrophages are generally involved in immunoregulation and immunosupression by generating anti-inflammatory cytokines such as IL-10 (22, 30). In accordance with this general concept, it has been found that M2 macrophages are increased, whereas M1 macrophages are decreased in the restoration process of renal interstitial fibrosis in mice with unilateral ureteral obstructions (UUO) (18). Thus M2 macrophages have been shown to play an important role in repairing various organ damage (18, 23). However, there are no reports describing the effect of the Rho-kinase inhibitor on the phenotype of macrophages.

The incidence and prevalence of renal failure from chronic kidney disease (CKD) are increasing internationally, outcomes are poor, and costs are high. CKD is a serious public health problem. The major outcomes of CKD include progression to renal failure and development of cardiovascular disease (CVD) (19). Strategies to improve outcomes will require a global effort directed at CKD. Proteinuria is not only a capital sign of renal failure and development of cardiovascular disease (CVD), and death (12). Moreover, decreases in proteinuria have been related to improvements in both cardiovascular and renal outcomes, and strategies that reduce proteinuria are considered beneficial and crucial (1, 5). For now, the standard and sole medication against CKD is the renin-angiotensin system (RAS) inhibitor (3, 17, 32), angiotensin-converting enzyme (ACE) inhibitor, and angiotensin receptor blocker (ARB). Although RAS inhibitors have the significant renoprotective effect, not a few CKD patients undergoing RAS inhibitor treatment still progress to end-stage renal failure. Thus CKD patients are in great need of an alternative therapeutic strategy.

Rho A/Rho-associated kinase (ROCKs) play important roles in renal pathophysiology (15). Rho A is a member of the Ras superfamily of small GTP-binding proteins, and ROCKs, its downstream effectors, have a variety of biological functions (13, 15, 20). In several models of hypertensive renal injuries, fasudil has been found to prevent renal injuries by reducing expression of extracellular matrix genes, oxidative stress, and macrophage infiltration (10, 11, 25). Furthermore, fasudil decreased proteinuria through recovery of nephrin expression in rats with puromycin aminonucleotide (PAN)-induced nephrosis (33).

Thus fasudil seems to be a potent proteinuria-reducing agent against renal disease.

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Ethical Committee of the National Defense Medical College. The rats were given water and standard chow ad libitum.

We initiated baseline studies in 8-wk-old male SHC rats. Oral administration of drugs was started at 8 wk old and was continued for 24 wk. SHC rats were randomly allocated to the following six treatment groups (n = 7 each): 1) the vehicle-treated group (Ve), in which rats were treated with vehicle (0.1% sodium bicarbonate and 0.1% potassium bicarbonate) alone; 2) the low-dose fasudil-treated group (FL), in which rats were treated with low doses of fasudil (30 mg·kg⁻¹·day⁻¹); 3) the high-dose fasudil-treated group (FH), in which rats were treated with high doses of fasudil (100 mg·kg⁻¹·day⁻¹); 4) the OL-treated group (OL), in which rats were treated with OL (5 mg·kg⁻¹·day⁻¹); 5) the combination of low-dose fasudil- and OL-treated group (CL), in which rats were treated with low doses of fasudil and OL; 6) the combination of high-dose fasudil- and OL-treated group (CH), in which rats were treated with high doses of fasudil and OL. SD rats that were treated with vehicle alone for 24 wk (n = 7) served as the healthy control. OL was obtained from Daiichi Sankyo (Tokyo, Japan), and fasudil was obtained from Asahi Kasei (Tokyo, Japan).

Systolic blood pressure was measured by tail-cuff plethysmography at 8, 16, 24, and 32 wk old. Urine samples were collected for 24 h in metabolic cages at 8, 16, 24, and 32 wk old. Rats were euthanized by exsanguination under deep anesthesia with an intraperitoneal injection of pentobarbital sodium. Serum creatinine, blood urea nitrogen, total cholesterol, triglyceride, albumin, and urinary protein levels were measured with the standard methods. Whole kidneys were harvested, weighed, decapsulated, and cut into several pieces that were either fixed in 10% formalin and embedded in paraffin for histological analysis or stored at −80°C for subsequent studies.

Renal Immunoperoxidase Staining and Immunofluorescence Staining

Immunoperoxidase staining. Myofibroblasts in 3-μm-thick sections of formalin-fixed paraffin-embedded tissues were evaluated by immunoperoxidase staining for α-smooth muscle actin (α-SMA) using an anti-human α-SMA antibody (Dako, Carpinteria, CA). Direct immunoperoxidase staining was performed with the Enhanced Polymer One-step Staining reagent (Dako), as described previously (27).

Immunofluorescence staining. Indirect immunofluorescence (IF) staining for ED-1 (monocyte/macrophage marker), CD206 (mannose receptor and a type-1 transmembrane glycoprotein that mediates endocytosis and phagocytosis; M2 macrophage marker) (8), nephrin (a structural component of podocyte slit diaphragm) (2, 21), fibronectin, and collagen III was performed on 5-μm cryostat sections. The primary antibodies were mouse anti-ED-1 antibody (Serotec, Oxford, UK), rabbit anti-CD206 antibody (Abcam, Tokyo, Japan), guinea pig anti-nephrin antibody (Progen, Heidelberg, Germany), rabbit anti-fibronectin antibody (Sigma, St. Louis, MO), and rabbit anti-collagen III antibody (Acris Antibodies, Atlanta, GA). The secondary antibodies were Alexa Fluor (AF) 488-conjugated anti-mouse IgG, AF594-conjugated anti-rabbit IgG, AF594-conjugated anti-guinea pig IgG, and AF488-conjugated anti-rabbit IgG. To evaluate the podocyte cytoskeleton, fluorescence staining for F-actin was performed by using labeled phalloidin (4, 7, 35). After fixation with 4% paraformaldehyde, sections were incubated with AF488-conjugated phalloidin (Invitrogen, Carlsbad, CA), washed, and mounted.

Electron Microscopy

Tissue for electron microscopy (EM) was retrieved from paraffin blocks and subjected to deparaffinization and post fixation in 2% paraformaldehyde, 2.5% glutaraldehyde, and 1% osmium tetroxide. Sections were stained with uranyl acetate and lead citrate and examined with a JEOL 1010 transmission electron microscope with a Bioscan digital imaging system (Gatan, Pleasanton, CA).

Evaluation of Renal Fibrosis

We evaluated the renal fibrosis level histologically by picrosirius red staining and biochemically by total collagen assay. Picrosirius red staining that detects collagen fibrils was performed as described previously (18, 28). Sections of paraffin-embedded tissue (3 μm thick) were deparaffined, hydrated, and incubated overnight in picrosirius red solution (1% sirius red in saturated picric acid). After immersion in 0.01 N HCl for 2 min, the slides were dehydrated, mounted, and examined by polarized light microscopy.

For the total collagen assay, the hydroxyproline concentration was chemically measured in hydrolysates of precisely weighed frozen kidney samples, as described previously (26, 34). Total collagen was assumed to contain 12.7% hydroxyproline by weight. The results are expressed as micrograms collagen per milligram kidney weight.

Real-Time RT-PCR

The extraction of total RNA and subsequent real-time RT-PCR were performed as previously described (26, 34). We used primer/probe sets of...
Table 1. Physiological parameters in spontaneously hypercholesterolemic (SHC) rats

<table>
<thead>
<tr>
<th></th>
<th>n = 7</th>
<th>Weight, g</th>
<th>Kidney Weight, g</th>
<th>Total Cholesterol, mg/dl</th>
<th>Triglyceride, mg/dl</th>
<th>Serum Albumin, g/dl</th>
<th>BUN, mg/dl</th>
<th>Serum Creatinine, mg/dl</th>
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<tr>
<td>Control</td>
<td>678 ± 13</td>
<td>1.52 ± 0.03 **</td>
<td>83 ± 4.7 **</td>
<td>154 ± 21 **</td>
<td>4.0 ± 0.1§ *</td>
<td>22 ± 0.8</td>
<td>0.47 ± 0.01</td>
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<tr>
<td>Ve</td>
<td>560 ± 9.1§‡</td>
<td>1.76 ± 0.06</td>
<td>232 ± 16</td>
<td>351 ± 21</td>
<td>3.6 ± 0.1</td>
<td>23 ± 2.0</td>
<td>0.52 ± 0.03</td>
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<tr>
<td>FL</td>
<td>574 ± 15§‡</td>
<td>1.68 ± 0.05§</td>
<td>196 ± 12§‡</td>
<td>247 ± 25§‡</td>
<td>3.7 ± 0.1§</td>
<td>22 ± 0.8</td>
<td>0.55 ± 0.02</td>
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<tr>
<td>FH</td>
<td>459 ± 13§</td>
<td>1.45 ± 0.04 ***</td>
<td>153 ± 12 **‡</td>
<td>114 ± 7.5***</td>
<td>3.9 ± 0.1§</td>
<td>18 ± 0.6</td>
<td>0.61 ± 0.04</td>
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<tr>
<td>OL</td>
<td>583 ± 13§‡</td>
<td>1.63 ± 0.04</td>
<td>169 ± 11§‡</td>
<td>269 ± 28§‡</td>
<td>3.9 ± 0.1§</td>
<td>22 ± 0.3</td>
<td>0.48 ± 0.01</td>
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<td>CL</td>
<td>581 ± 8.7§‡</td>
<td>1.66 ± 0.04§</td>
<td>144 ± 4.2§‡</td>
<td>181 ± 13§‡</td>
<td>4.0 ± 0.1§</td>
<td>19 ± 0.9</td>
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<tr>
<td>CH</td>
<td>464 ± 16§</td>
<td>1.44 ± 0.05 **</td>
<td>107 ± 8.3 **</td>
<td>69 ± 3.8 **</td>
<td>4.6 ± 0.1 **</td>
<td>22 ± 1.7</td>
<td>0.60 ± 0.06</td>
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Values are means ± SE. BUN, blood urea nitrogen; control: healthy control (Sprague-Dawley rats) group; Ve, vehicle-treated group; FL, low-dose fasudil (30 mg·kg⁻¹·day⁻¹)-treated group; FH, high-dose fasudil (100 mg·kg⁻¹·day⁻¹)-treated group; OL, olmesartan (5 mg·kg⁻¹·day⁻¹)-treated group; CL, combination of low-dose fasudil (30 mg·kg⁻¹·day⁻¹) and olmesartan (5 mg·kg⁻¹·day⁻¹)-treated group; CH, combination of high-dose fasudil (100 mg·kg⁻¹·day⁻¹) and olmesartan (5 mg·kg⁻¹·day⁻¹)-treated group. *P < 0.05, **P < 0.01 vs. Ve group. ‡P < 0.05 vs. control group. §P < 0.05 vs. CH group. ψP < 0.05 vs. FL group. ψψP < 0.05 vs. FH group.

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Fig. 3. Immunofluorescence (IF) staining for nephrin (A–C), fluorescence staining for F-actin (D–F), and electron microscopy (EM; G–I) in SHC rats. Groups are defined as in Fig. 1. Representative photomicrographs (original magnification: ×200) showing IF staining for nephrin [labeled with Alexa Fluor (AF) 594-conjugated antibody] in kidney slices of the control group (A), Ve group (B), and CH group (C), and fluorescence staining for F-actin (with AF488-labeled phalloidin) in kidney slices of the control group (D), Ve group (E), and CH group (F) are shown. Representative EM photomicrographs (original magnification: ×12,500) in kidney slices of the control group (G), Ve group (H), and CH group (I) are also shown. Red arrows indicate glomerular foot processes.
Biotechnology, Santa Cruz, CA). The protein samples were extracted from the renal cortex by homogenization in a lysis buffer: 20 mM Tris·HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM sodium vanadate, 1%NP-40, and a proteinase inhibitor cocktail (Upstate, Lake Placid, NY) or nephrin (Progen) and horseradish peroxidase-conjugated secondary antibodies, and they were developed with an enhanced chemiluminescence Western blotting detection reagent, ECL (Amersham). Equal loading and transfer of samples were confirmed by pico Sirius red staining in kidney slices of the control group (A) and phosphorilated myosin phosphatase target subunit (MYPT)-1 (B) in SHC rats. Photographs are representative rearranged images from noncontiguous lanes of original blots. Noncontiguous portions are indicated by dividing lines. Levels of protein expression for nephrin and phosphorylated MYPT-1 (all values normalized to β-actin protein expression) are expressed in arbitrary units as means ± SE. Groups are defined as in Fig. 1. *P < 0.05, **P < 0.01 vs. Ve group.

Western Blotting

Western blotting for phosphorylated myosin phosphatase target subunit 1 (MYPT)-1 and nephrin were performed to assess the intrarenal activation of the Rho/Rho-kinase pathway and the state of the glomerular filtration barrier (2, 21). The protein samples were extracted from the renal cortex by homogenization in a lysis buffer; 20 mM Tris·HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM sodium vanadate, 1% NP-40, and a proteinase inhibitor cocktail (Sigma). Samples were denatured for 3 min at 98°C, put in individual wells (50 μg of protein/well), electrophoresed on a 10% polyacrylamide gel (Bio-Rad Laboratories), and electrophoretically transferred onto a nitrocellulose membrane. The membrane was stained as described previously (26, 34) using primary antibodies for phosphorylated MYPT-1 (Upstate, Lake Placid, NY) or nephrin (Progen) and horseradish peroxidase-conjugated secondary antibodies, and they were developed with an enhanced chemiluminescence Western blotting detection reagent, ECL plus (Amersham). Equal loading and transfer of samples were confirmed and adjusted by the β-actin protein level, which was assessed by using a horseradish peroxidase-conjugated anti-β-actin antibody (Santa Cruz Biotechnology, Santa Cruz, CA).

Statistical Analysis

Data are shown as means ± SE. We used one-way analysis of variance followed by Tukey’s post hoc analysis for statistical analyses. A P value <0.05 was considered significant. All statistical analyses were performed with JMP software (SAS Institute, Cary, NC).

RESULTS

Blood Pressure

At 8 wk, there were no significant differences in systolic blood pressure among the six groups, i.e., Ve, FL, FH, OL, CL, and CH. Throughout the study, systolic blood pressure was significantly higher in the Ve group than it was in the control group (SD rats were treated with vehicle alone), and it was the same in the FL and FH groups. The OL-treated (OL, CL, and CH) groups showed significantly lower systolic blood pressure compared with the Ve group from 16 to 32 wk old (Fig. 1).

Urinary Protein Excretion

There were no significant differences in 24-h urinary protein excretion among the groups at 8 wk old (Fig. 2). At 32 wk old, the urinary protein level in the Ve group (344 ± 47 mg/day) was prominently higher than in the control group (9.7 ± 0.8 mg/day), while the levels in the FH (130 ± 11 mg/day), OL (204 ± 35 mg/day), CL (130 ± 19 mg/day), and CH (16 ± 4.7 mg/day) groups were significantly lower than in the Ve group. The urinary protein level in the CH group was similar to that of the control group throughout the study.

Physiological Parameters

The body weights of the high-dose fasudil groups, FH and CH, were significantly lower than those of the other groups (Table 1). The kidney weights of the Ve group were significantly greater, while those of the high-dose fasudil groups, FH
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and CH, were significantly lower than that of the control group. Total cholesterol and triglyceride levels in the Ve group were significantly greater than in the control group, while those of the FH, CL, and CH groups were significantly lower than in the Ve group. The serum albumin levels in the Ve group were significantly lower than in the control group, while those of the CL and CH groups were significantly higher than in the Ve group. There were no significant differences in BUN or serum Cr level among the groups.

**IF Staining for Nephrin, Fluorescence Staining for F-Actin, and EM Observation**

In the control tissue, global continuous stainings of nephrin and F-actin were observed in glomerular podocytes. However, these stainings were focally lost or weakened in the Ve group. In the CH group, glomerular staining for these molecules was not focally lost, but rather was global and continuous. The EM observation showed that the foot processes of glomerular podocytes were diffusely effaced in the Ve group, but they were preserved in the CH group (Fig. 3).

**Western Blotting for Nephrin**

The protein expression for nephrin significantly decreased in the Ve group compared with the control group, and it significantly recovered in the CH group (Fig. 4A).

**Phosphorylated MYPT-1 Levels**

Renal phosphorylated MYPT-1 protein levels in the FL, FH, OL, CL, and CH groups were significantly lower than that of the Ve group (Fig. 4B).

**Degree of Renal Fibrosis**

The picrosirius red staining showed notable interstitial fibrosis in the Ve group compared with that of the control group, and the interstitial fibrosis was obviously attenuated in the CH group (Fig. 5, A–C). IF staining revealed a similar tendency for ECM components, i.e., for fibronectin (Fig. 5, D–F) and collagen III (Fig. 5, G–I). The total collagen content was significantly increased in the Ve group compared with the control group, whereas the increase was significantly attenuated in the CH group (Fig. 5J).

**Immunostaining for Myofibroblasts and Macrophages**

Interstitial α-SMA-positive cells were rare in the control group. Interstitial α-SMA-positive cells were prominent in the Ve group, whereas the increase in the numbers of these cells was significantly attenuated in the FL, FH, OL, CL, and CH groups (Fig. 6).

Similarly, interstitial ED-1-positive cells were prominent in the Ve group, whereas the increase in these cells was significantly attenuated in the FL, FH, OL, CL, and CH groups (Fig. 7).

There were significantly fewer interstitial CD206 (M2 macrophages marker)-positive cells in the control group compared with the other groups. There were no significant differences in the numbers of interstitial CD206-positive cells in the six treatment groups (Fig. 8).

**Gene Expressions of CD68, CD80, and CCL3**

To evaluate the phenotype of the macrophages, renal mRNA levels for CD68, CD80, and CCL3 were further analyzed by using real-time RT-PCR. CD68 was used to assess the total monocyte and macrophage level in renal tissues. CD80 and
CCL3 were evaluated as molecules preferentially expressed in M1 macrophages (24, 30). Compared with the control group, the CD68, CD80, and CCL3 mRNA levels relative to the GAPDH mRNA level were higher in the Ve group but significantly lower in the CH group (Fig. 9).

**Gene Expression of CD206 (M2 Macrophage Marker)**

To evaluate the M2 macrophages, we analyzed the levels of renal CD206 mRNA (8). Despite the prominent decrease in infiltrating monocytes and macrophages, CD206 mRNA ex-

![Image](https://example.com/image1.png)

**Fig. 7. Levels of macrophages in SHC rats.** Representative photomicrographs (original magnification: ×200) showing IF staining (labeled with AF488-conjugated antibody) for ED-1, a macrophage marker, in kidney slices of the control group (A), Ve group (B), and CH group (C). D: number of positive cells for ED-1 in renal cortex slices. Values are means ± SE. Groups are defined as in Fig. 1. **P < 0.01 vs. Ve group. ‡P < 0.05 vs. control group. §P < 0.05 vs. CH group. ¶P < 0.05 vs. FL.

![Image](https://example.com/image2.png)

**Fig. 8. Levels of M2 macrophages in SHC rats.** Representative photomicrographs (original magnification: ×200) showing IF staining (labeled with AF594-conjugated antibody) for CD206, the mannose receptor, M2 macrophage marker, in kidney slices of the control group (A), Ve group (B), and CH group (C). D: number of positive cells for CD206 in renal cortex slices. Values are means ± SE. Groups are defined as in Fig. 1. ‡P < 0.05 vs. control group.
pression did not significantly change in any of the treatment groups. Compared with the other treatment groups, the CH group had significantly greater relative expression of CD206 on macrophages (M2), as assessed by the ratio of the CD206 mRNA to CD68 mRNA levels (Fig. 10).

**DISCUSSION**

Although the renoprotective effects of RAS inhibitors against CKD are widely acknowledged (3, 17, 32), their effects are limited and many CKD patients with the treatments still progress to end-stage renal failure. Therefore, there is a compelling need for an alternative therapy. To investigate the possibility of an alternative therapeutic strategy, we chose the SHC rat as the model of CKD (9, 16, 28) and fasudil as a potent new therapeutic option.

The SHC rat was originally developed as a hypercholesterolemic rat with high plasma cholesterol levels. However, these rats were found to have renal lesions with nephrotic-range proteinuria. In male SHC rats, continuous proteinuria with histological changes mimicking focal segmental glomerulosclerosis develops spontaneously from 8 wk of age and results in decreased kidney function 8–9 mo later and death (16, 28, 36). For this reason, we decided to treat rats from 8 wk old to 32 wk old. Rodriguez-Iturbe et al. (31) recently showed that long-term treatment of SHC rats with OL significantly reduced proteinuria and exerted prominent functional and histological renoprotective effects.

We selected fasudil as a drug for CKD treatment, because its renoprotective effects have recently been reported in various models of renal disease. The protection mechanisms include inhibition of macrophage infiltration, cell proliferation, oxidative stress, expression of extracellular matrix genes, as well as urinary protein reduction (10, 11, 25). Moreover, intravenous injection of fasudil has been clinically used for treating cerebral vasospasm for several years. There have been few reports on the adverse effects of fasudil, and most of them could be due to pharmacological effects of fasudil such as induction of hypotension. We did not observe any adverse effects such as the hypotension in the fasudil treatment group of this study. Therefore, we think that fasudil could be administered to CKD patients. Moreover, we administered fasudil via the oral route. Western blotting showed that the phosphorylated MYPT-1 protein level, which is a marker of Rho A/ROCK activation, was significantly suppressed in the fasudil-treated group compared with the Ve group, thereby confirming the bioavailability and effectiveness of orally administered fasudil in renal tissue. We also observed a significant reduction in phosphorylated MYPT-1 in OL-treated rats. This result is in keeping with the

![Fig. 9. Gene expressions for CD68, CD80, and CCL3 in SHC rats. Levels of mRNA expression for CD68 (A), CD80 (B), and CCL3 (C). All values were normalized to GAPDH mRNA expression. Levels of mRNA in the control group were considered as 1. All of the results are expressed in arbitrary units as means ± SE. Groups are defined as in Fig. 1. **P < 0.01 vs. Ve group. ‡P < 0.05 vs. control group. §P < 0.05 vs. CH.](http://ajprenal.physiology.org/)

![Fig. 10. Gene expressions for CD206 in SHC rats. Levels of mRNA expression for CD206 (A), the mannose receptor M2 macrophage marker (value normalized to GAPDH mRNA expression). B: ratio of CD206 mRNA to CD68 mRNA. Levels of mRNA in the control group were considered as 1. All of the results are expressed in arbitrary units as means ± SE. Groups are defined as in Fig. 1. **P < 0.01 vs. Ve group. ‡P < 0.05 vs. control group. §P < 0.05 vs. CH.](http://ajprenal.physiology.org/)
monkeys. The dose for oral LD50 in rats was reported to be toxic in proteinuria and suppression of renal interstitial fibrosis.

Proteinuria is not only a principal sign of kidney disease but it is also a marker of CKD progression (12). Reduction in proteinuria is associated with improvements in cardiovascular and renal outcomes (1, 5). This study showed that the urinary protein excretion level in the CH group decreased to the same level as the control group. In SHC rats, the combination of fasudil and OL had an additive proteinuria-decreasing effect compared with monotherapy of fasudil or OL. In contrast to OL, fasudil did not alter the blood pressure level. Therefore, we suspect that the combination of the blood pressure-lowering effect of OL and the blood pressure-independent antiproteinuric effect of fasudil might have resulted in the additive proteinuria-decreasing effect of these drugs. The antiproteinuric effects that were independent of blood pressure level suggest that fasudil protects the glomerular filtration barrier. In this context, we investigated IF staining for nephrin and fluorescence staining for F-actin and found that these stainings were focally diminished in the Vg group and restored in the CH group. EM images showed that the foot process was preserved in the CH group. Moreover, Western blotting revealed a significant reduction in renal nephrin expression in the Vg group and its restoration due to the combination of high-dose fasudil and OL. Thus modulation of nephrin by fasudil and OL is found to be associated with protection of the integrity of the actin cytoskeleton in podocytes. These data are in line with recent evidence from podocytes in vivo on the relationship between Rho activation, abnormal distribution of F-actin, and downregulation of nephrin expression (29). Indeed, the stability of the actin cytoskeleton of kidney podocytes has been reported to be associated with the antiproteinuric effect (7).

Interstitial fibrosis is regarded as the final common pathway which determines the long-term prognosis and the degree of renal functional impairment of CKD patients (6). We have recently reported that repair of renal interstitial fibrosis is associated with alteration of the phenotype of macrophages. We demonstrated that M2 macrophages increased, while M1 macrophages decreased, in the repair process of renal fibrosis induced by UUO (18). Many studies have reported that fasudil prevents macrophage infiltration in several models of renal disease (10, 11, 13, 25), and our results are consistent with these reports. In this study, we observed not only a decrease in total macrophages but also a parallel decrease in M1 macrophages and a relative increase in M2 macrophages. We speculate that the alteration of macrophage phenotypes may account for the attenuation of renal fibrosis in the treatment involving the combination of fasudil and OL.

Regarding possible adverse effects, we observed significant weight losses in the high-dose fasudil-treated group. We are not sure whether weight loss is a beneficial effect (an antitubal action) or a harmful one (a toxic action). Oral-dose toxicity studies on fasudil were conducted in mice, rats, and monkeys. The dose for oral LD50 in rats was reported to be over 300 mg·kg⁻¹·day⁻¹, and under that condition adverse effects such as low activity, ptosis, and hypotension were observed (14). However, the dose we used was only 100 mg·kg⁻¹·day⁻¹, and we did not observe any of these symptoms. A future investigation is warranted of the mortality rate of SHC rats that have undergone fasudil treatment over a longer observation period.

In conclusion, oral administration of fasudil showed significant reductions in both proteinuria and renal fibrosis in CKD model rats with massive proteinuria. The decrease in proteinuria was independent of blood pressure level and may be associated with protection of podocyte integrity, whereas attenuation of renal fibrosis was associated with an alteration in the density and phenotype of macrophages. These results indicate that a combination of fasudil and ARB may have therapeutic value in the treatment of CKD patients.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

REFERENCES


