Long-Term Administration of Rho-Kinase Inhibitor Ameliorates Renal Damage in Malignant Hypertensive Rats

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Abstract—We have shown recently that fasudil, a Rho-kinase inhibitor, has renoprotective effects in salt-sensitive hypertensive rats. We hypothesized that activation of Rho-kinase is involved in the pathogenesis of glomerulosclerosis in malignant hypertensive rats. To test this hypothesis, we studied the following 4 groups: control Wistar–Kyoto rats, untreated deoxycorticosterone-acetate salt spontaneously hypertensive rats (DOCA-SHR), low-dose fasudil-treated DOCA-SHR, and high-dose fasudil-treated DOCA-SHR. After 3 weeks of treatment, the effects of fasudil were examined. DOCA-SHR was characterized by increased blood pressure (BP); increased kidney weight; decreased renal function; increased proteinuria; abnormal histological findings; increased monocyte/macrophage infiltration; increased urinary 8-isoprostan levels; increased gene expression of collagen I, collagen III, transforming growth factor-β, and reduced nicotinamide-adenine dinucleotide phosphate oxidase subunits (p40phox, p47phox, and p67phox); and decreased gene expression of endothelial NO synthase (eNOS) in the renal cortex as compared with Wistar–Kyoto rats. Long-term high-dose fasudil treatment significantly improved renal function and histological findings without changing BP, as compared with untreated DOCA-SHR. Interestingly, long-term fasudil treatment significantly decreased monocyte/macrophage infiltration and urinary 8-isoprostan excretion, in association with decreased mRNA levels of transforming growth factor-β, collagen I, collagen III, and NADPH oxidase subunits (p40phox, p47phox, and p67phox), and increased mRNA levels of eNOS in the renal cortex. Long-term low-dose fasudil treatment tended to improve these variables slightly but did not affect most of them significantly. Our results suggest that long-term fasudil treatment provides renoprotective effects independent of BP-lowering activity. These renoprotective effects are associated with inhibition of extracellular matrix gene expression, monocyte/macrophage infiltration, oxidative stress, and upregulation of eNOS gene expression. (Hypertension. 2006;47:1075-1083.)

Key Words: kinase ■ hypertension, malignant ■ oxidative stress ■ extracellular matrix ■ glomerulosclerosis ■ deoxycorticosterone ■ transforming growth factors

Rho, a small GTP-binding protein, is known to function as a molecular switch in various cellular functions, including regulation of calcium ion sensitivity in smooth muscle cells, formation of stress fibers and focal adhesions, regulation of cytokines after nuclear division, and regulation of the G1 to S phases of the cell cycle. Among known Rho effectors, Rho-associated Rho-kinase is best characterized. The cellular functions and signal transduction of Rho/Rho-kinase have been extensively studied; however, their in vivo functions remain poorly understood. Uehata et al reported that Y-27632, a synthetic compound, is a specific inhibitor of Rho kinase. This compound is a very powerful tool for elucidating the roles of the Rho/Rho-kinase pathway in vitro and in vivo. Because Rho is known to modulate the Ca2+ sensitization of vascular smooth muscle cells and is thought to act by inhibiting myosin phosphatase activity, the effect of Rho on the tonus of blood vessels and the role of Rho in the pathogenesis of arteriosclerosis have been intensively investigated.4 However, few studies have investigated the role of Rho in renal disease. Previous studies showed that the Rho-kinase inhibitor Y-27632 or fasudil significantly attenuated the tubulointerstitial fibrosis induced by unilateral ureteral obstruction in murine kidney.5,6 However, studies investigating the role of the Rho/Rho-kinase pathway in hypertensive glomerular sclerosis are scant. We very recently reported that the Rho-kinase inhibitor fasudil attenuated glomerulosclerosis in salt-induced hypertensive rats,7 suggesting that the Rho/Rho-kinase pathway may be involved in the pathogenesis of glomerulosclerosis in salt-sensitive hypertensive rats. Other investigators also reported beneficial effects of Rho-kinase inhibitor treatment on nephropathy in subtotally ne-
phrectomized spontaneously hypertensive rats. However, whether Rho/Rho-kinase is involved in the pathogenesis of glomerulosclerosis in malignant hypertensive rats remains unknown. In the present study, we hypothesized that the actions of the Rho/Rho-kinase pathway are partly responsible for the progression of glomerulosclerosis in malignant hypertensive rats. To test this hypothesis, we investigated the effects of long-term administration of fasudil on the development of glomerular sclerosis in deoxycorticosterone acetate–salt spontaneously hypertensive rats (DOCA-SHR). A secondary objective was to investigate potential mechanisms of drug action. Rho plays an important role in cell migration. In addition, angiotensin II infusion causes Rho-kinase activation and NADPH oxidase subunit mRNA expression in rat aorta, both of which are significantly suppressed by the Rho-kinase inhibitor fasudil. Moreover, Rho/Rho-kinase pathway is involved in the regulation of endothelial NO synthase (eNOS) expression. We, therefore, assessed potential mechanisms responsible for these actions.

Methods
This study was approved by our institutional animal care committee, and all of the procedures were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Experimental Animals and Design
Nine-week-old male Wistar–Kyoto rats (WKYs, n = 10) and SHR (Clea Japan, Tokyo, Japan, n = 23) were studied. DOCA-SHRs were used as a malignant hypertensive rat model in this study, because this model has extensive end organ damage, including malignant nephrosclerosis. DOCA (100 mg/kg per week) was subcutaneously injected, and 1% NaCl drinking water was started at the age of 9 weeks. DOCA-SHRs were randomly divided into the following 3 groups: a low-dose fasudil treatment group (30 mg/kg per day, n = 8), a high-dose fasudil treatment group (100 mg/kg per day, n = 9), and an untreated group (n = 6). Fasudil was administered in the drinking water. After oral administration, fasudil is metabolized to hydroxyfasudil, a major active metabolite of fasudil that specifically inhibits Rho-kinase. Recent studies have demonstrated that the levels of hydroxyfasudil in plasma after 4 weeks of oral treatment with fasudil (30 to 100 mg/kg per day) are within the specific therapeutic ranges of Rho-kinase inhibitor. Fasudil has been shown by kinase assay to selectively inhibit Rho-kinase activity. Therefore, fasudil is a relatively selective inhibitor for Rho-kinase.

Urine Collection
Twenty-four-hour urine samples were collected from rats in metabolic cages after 3 weeks of fasudil treatment. Urinary protein and creatinine levels were measured as reported previously. Creatinine clearance (CCr) was calculated using standard formulae.

Hemodynamic Measurement and Blood Sampling
Systolic blood pressure (BP) was measured every week by the tail-cuff method, as reported previously. After 3 weeks of administration of fasudil, hemodynamic studies (direct measurement of BP and heart rate) were performed with the animals under anesthesia, as reported previously. Blood was obtained from the carotid artery, and the plasma was stored at −80°C until assay. Immediately after the heart was arrested by the injection of 2 mmol of KCl, the right kidney was removed, weighed, and postfixed in 10% neutral-buffered formalin. The left kidney was removed, weighed, separated into the cortex and medulla, frozen in liquid nitrogen, and stored at −80°C until the RT-PCR analysis.

Telemetry BP
We implanted a telemetry BP system (TA11PA-C40 telemetry system, Data Sciences International) as described previously in 7- to 8-week-old SHRs (n = 9) and started DOCA salt and fasudil treatment 2 weeks after operation (n = 3 in each group). We serially measured telemetry BP (3 rats in each group) at 10 to 11, 11 to 12, and 12 to 13 weeks, respectively. Mean 24-hour systolic BP was obtained every week. After the telemetry study, renal histological examinations were performed.

Renal Morphology
The right kidney was excised and immersed in neutralized formalin for histological examination. Histological examination was performed by 2 observers in a blinded fashion. For semiquantitative evaluation, glomerular injury scores (GISs) and arteriolar injury score were determined as reported previously. The area of fibrotic lesions in the cortical interstitium (fibrosis area) was determined on sections stained by Masson’s trichrome method to stain the collagen fibers (stained in blue), using a computer-aided manipulator program as described previously.

Immunohistochemical Analysis
Immunohistochemical analysis using an antibody against ED-1 was performed as reported previously. Quantification of ED-1 was performed in 30 consecutive glomerular cross-sections and 20 high-power fields of tubulointerstitium without any glomerulus from 5 independent rat kidneys in each group. ED-1–positive cells were counted by computer-aided planimetry, using the Scion Image software. Then, images were digitized, captured with a charge-coupled device (CCD) camera connected to a personal computer, and scanned into Photoshop. To detect the localization of increased transforming growth factor (TGF)-β, immunohistochemical analysis was performed by methods reported previously.

Hormonal Analysis
The plasma–renin concentration (PRC) and plasma aldosterone concentration were measured by radioimmunoassay as reported previously. Measurement of 8-Isoprostane We measured urinary 8-isoprostane levels by enzyme immunoassay as an indicator of oxidative stress as reported previously.

Quantification of mRNA by RT-PCR
All of the procedures used for mRNA extraction, cDNA synthesis, and PCR are described in detail in previous reports. The number of PCR cycles for the 13 genes examined were as follows: TGF-β, 29; collagen I, 29; collagen III, 28; RhoA, 25; RhoB, 27; Rho-kinase α, 28; p22phox, 33; gp91phox, 31; p40phox, 34; p47phox, 33; p67phox, 34; eNOS, 30; and GAPDH, 22. For each of these numbers of PCR cycles, RT-PCR was performed in the linear range of the reaction. Quantification of each species of mRNA was performed as reported previously.

Statistical Analysis
Statistical comparisons among the 4 groups were carried out by ANOVA. If appropriate, the data were compared with the use of Bonferroni post-hoc test for multiple comparisons. P < 0.05 was considered to indicate statistical significance. Statistical analysis was performed using STATVIEW, version 5 (Abacus Concepts), or STATA, version 8 (STATA Corp).

Results
Physiological Profiles
The changes in systolic BP as measured by the tail-cuff method in the 4 groups are presented in Figure 1A. The systolic BP in 9-week-old DOCA-SHRs was already higher than that in WKYs, and it gradually increased and reached a
maximum after \( \approx 11 \) weeks. Long-term low-dose or high-dose fasudil treatment did not change the systolic BP in the DOCA-SHR groups.

Long-term fasudil treatment also did not change the systolic BP at any stage as measured by the telemetry system (10 to 11 weeks: untreated DOCA-SHR, 211±13 mm Hg; high-dose fasudil-treated DOCA-SHR, 215±24; low-dose fasudil-treated DOCA-SHR, 217±12; 11 to 12 weeks: untreated DOCA-SHR, 247±10 mm Hg; high-dose fasudil-treated DOCA-SHR, 246±21; low-dose fasudil-treated DOCA-SHR, 243±12; 12 to 13 weeks, untreated DOCA-SHR, 247±15 mm Hg; high-dose fasudil-treated DOCA-SHR, 243±15; low-dose fasudil-treated DOCA-SHR, 244±16). There were no significant differences in averaged systolic BP levels among the 3 groups. Figure 1B shows the whole-day averaged systolic BP in 3 groups at 11 to 12 weeks of age. Interestingly, there was no circadian variation of BP in any rat groups.

The physiological profiles of the 4 experimental groups are summarized in the Table. Body weight (BW) was significantly lower in the DOCA-SHR groups than in WKYs. Long-term low-dose or high-dose fasudil treatment did not change the BW in DOCA-SHRs. In contrast, untreated DOCA-SHRs had higher kidney weight/BW, left and right ventricular weight/BW, and urine volume as compared with WKYs. There were no differences in systolic aortic pressure (by direct method) among the 3 groups. Long-term high-dose fasudil treatment in DOCA-SHRs significantly decreased kidney/BW, left and right ventricular weight/BW, and urine volume, without changing BP. Long-term low-dose fasudil treatment decreased these levels slightly but not significantly.

**Renal Parameters**

Renal parameters are shown in Figure 2A through 2C. The CCr was significantly decreased in untreated DOCA-SHRs,
and serum creatinine level and urinary protein excretion were significantly increased as compared with WKYs (Figure 2A through 2C). Long-term fasudil treatment dose-dependently reduced urinary protein excretion, and long-term high-dose fasudil treatment significantly decreased the serum creatinine level and increased Ccr in DOCA-SHRs; however, these variables still differed significantly between WKYs and fasudil-treated DOCA-SHRs (Figure 2A through 2C).
Hormonal Responses to Long-Term Fasudil Treatment

PRC and plasma aldosterone levels are shown in Figure 2D and 2E. PRC and plasma aldosterone levels were significantly elevated in DOCA-SHRs as compared with WKYs. Long-term fasudil treatment did not change the PRC or aldosterone level.

Renal Pathological Findings

The morphological appearance of glomeruli, vessels, and interstitium was considered normal in WKY (Figure 3A, 3E, and 3I). Histological examination of the kidney in DOCA-SHR revealed severe glomerulosclerosis, marked thickening of the vascular wall, and tubulointerstitial changes with inflammatory cell infiltration (Figure 3B, 3F, and 3J). Long-term low- and high-dose fasudil treatment attenuated these glomerular, vascular, and interstitial changes (Figure 3C, 3D, 3G, 3H, 3K, and 3L). The GIS at the subcapsular and juxtamedullary cortex was markedly higher in untreated DOCA-SHRs than in WKYs (Figure 3M and 3N). Long-term high-dose, but not low-dose, fasudil treatment significantly attenuated these changes. The arterial injury score and fibrosis area were also markedly higher in untreated DOCA-SHRs than in WKYs (Figure 3O and 3P). Long-term high-dose, but not low-dose, fasudil treatment significantly attenuated these changes. However, there were still significant differences in these variables between WKYs and high-dose fasudil-treated DOCA-SHRs. Similar observations were obtained in the telemetered group (data were not included in the analysis).

Immunohistochemical Analysis

ED-1-positive cells (monocytes/macrophages) in the cortical interstitium (Figure 3Q through 3T) and in the glomerulus (Figure 3R through 3V) are shown. ED-1-positive cells are stained brown. The number of ED-1-positive cells in the cortical interstitium is shown in Y. (original magnification: A through D, ×200; E, ×100; F, ×100; G, ×100; H, ×200; I through L, ×100; M through T, ×200; U through X, ×200) Data are expressed as mean±SD. Number of rats was as follows: WKY, n=4; untreated DOCA-SHR, n=8; low-dose fasudil-treated DOCA-SHR, n=6; high-dose fasudil-treated DOCA-SHR, n=8; WKY, **P<0.01 vs WKY, ***P<0.001 vs WKY, †P<0.05 vs D-SHR, ††P<0.01 vs D-SHR, #P<0.05 vs low-dose fasudil-treated D-SHR.
(Figure 3U through 3X) are shown. The number of ED1–positive cells in the interstitium was significantly increased in DOCA-SHRs (Figure 3Y). Long-term high-dose, but not low-dose, fasudil treatment significantly decreased the number of ED1–positive cells. However, there were still significant differences between WKYs and fasudil-treated DOCA-SHRs.

TGF-β was expressed in the cytoplasm of perivascular fibroblasts and proximal, distal, and collecting tubular epithelial cells; furthermore, TGF-β was globally expressed in sclerotic glomeruli. Long-term fasudil treatment attenuated the TGF-β–positive cells (data not shown).

**Gene Expression Levels of TGF-β, Collagen I, III, RhoA, RhoB, and Rho-kinase α**

The mRNA levels of TGF-β, collagen I, and collagen III relative to the GAPDH mRNA levels in the renal cortex were all increased in DOCA-SHRs as compared with WKYs (Figure 4A through 4C). Long-term fasudil treatment dose-dependently reduced the mRNA levels of collagen III, and long-term high-dose fasudil treatment significantly decreased collagen I and TGF-β; however, there were still differences in the mRNA levels of these variables between WKYs and fasudil-treated DOCA-SHRs.

The mRNA levels of Rho-kinase α and RhoB relative to the GAPDH mRNA levels in the renal cortex were significantly increased in DOCA-SHRs as compared with WKYs (Figure 5A and 5B), whereas there was no significant difference in the mRNA levels of RhoA between DOCA-SHRs and WKYs (data not shown). Long-term high-dose fasudil treatment significantly decreased the mRNA levels of Rho-kinase α and RhoB relative to the GAPDH mRNA levels.

**Urinary Excretion of 8-Isoprostane**

Figure 5C shows the urinary excretion of 8-isoprostane as an indicator of oxidative stress. The excretion of 8-isoprostane was significantly increased in DOCA-SHRs as compared with WKYs. Treatment with high-dose fasudil, but not low-dose fasudil, significantly decreased 8-isoprostane excretion.

**Gene Expression Levels of NADPH Subunits and eNOS in the Renal Cortex**

As shown in Figure 6A through 6F, the mRNA expression levels of p40phox, p47phox, and p67phox in the renal cortex were higher, and the mRNA expression level of eNOS was lower in untreated DOCA-SHRs than in WKYs. High-dose fasudil treatment significantly decreased the mRNA expression levels of p40phox and p47phox. In addition, low- and high-dose fasudil treatment significantly increased the mRNA expression of eNOS in DOCA-SHRs. In contrast, fasudil treatment slightly decreased the mRNA expression of p67phox, but the difference did not reach the level of statistical significance. There were no differences in mRNA expression of p22phox or gp91phox between WKYs and untreated DOCA-SHRs.

**Discussion**

The main new findings of this study are that DOCA-SHRs show evidence of induction of the Rho/Rho-kinase pathway associated with renal dysfunction, enhanced expression of NADPH subunit genes (p40phox, p47phox, and p67phox), and extracellular matrix genes (TGF-β, collagen I, and collagen III); and increased macrophage/monocyte infiltration, increased 8-isoprostane excretion, and decreased eNOS mRNA expression. All of these changes are attenuated by long-term high-dose fasudil treatment.

In the present study, we showed that long-term fasudil treatment significantly decreased the mRNA expression of TGF-β, collagen I, and collagen III in the renal cortex. Previous studies showed that TGF-β signaling is regulated by Rho. Furthermore, the induction of connective growth...
factor by TGF-β was markedly decreased by Rho-kinase inhibitor.24 These findings suggest that the Rho/Rho-kinase pathway plays an important role in the TGF-β–collagen cascade. Accumulating evidence indicates that TGF-β plays an important role in the pathogenesis of glomerulosclerosis, arteriolar sclerosis, and tubulointerstitial fibrosis.5,7,25 Inhibition of TGF-β by neutralizing antibody in vivo prevents glomerular matrix expansion in rats with experimental glomerulonephritis, and transfection of the TGF-β gene into the rat kidney in vivo induces glomerulosclerosis with increased glomerular collagen accumulation. Furthermore, TGF-β, collagen I, and collagen III mRNAs have been reported to be elevated in various experimental renal diseases, including glomerulonephritis, diabetic nephropathy, and Adriamycin-induced nephropathy.25 Thus, a TGF-β-collagen cascade may play a central role in the development of glomerulosclerosis and tubulointerstitial fibrosis. In fact, previous studies demonstrated that long-term treatment with Rho-kinase inhibitor significantly decreased TGF-β and collagen gene expressions,5,7 which is consistent with our findings. Thus, one of the possible mechanisms of the renoprotective effect of fasudil might be suppression of TGF-β and subsequent collagen mRNA expression.

Rho/Rho-kinase is involved in many aspects of cell motility, from smooth-muscle contraction to cell migration.8,26 In the present study we showed that the infiltration of ED-1–positive cells was increased around the glomerulus and tubulointerstitial space in the kidneys of malignant hypertensive rats and that long-term fasudil treatment significantly decreased the infiltration of ED-1–positive cells. Recent studies showed that Rho-kinase inhibitor inhibits macrophage-monocyte infiltration in the tubulointerstitial space5,6 and thereby ameliorates the tubulointerstitial fibrosis in the unilateral ureteral obstruction mouse and rat models, suggesting the importance of Rho-kinase in macrophage/monocyte infiltration. Macrophages/monocytes secrete various cytokines and growth factors, including TGF-β, which stimulate the synthesis of extracellular matrix proteins by neighboring myofibroblasts and/or mesangial cells, leading to interstitial fibrosis.27,28 In the present study, we demonstrated that the TGF-β mRNA expression level was significantly reduced in the fasudil treatment group. Therefore, the inhibitory effect of fasudil on macrophage/monocyte infiltration may, in part, be responsible for the beneficial renoprotective effects of fasudil treatment.

The pathophysiology of hypertensive glomerulosclerosis has been shown to be related to oxidative stress, leading to enhanced expression of TGF-β and collagen.28,29 In the present study, the expression of NADPH oxidase subunits, such as p47phox, p40phox, and p67phox, was increased in the renal cortex. A recent study showed that p47phox and p67phox expression was enhanced in the kidneys of SHRs, with prominent expression in the glomerulus, renal vasculature, thin limb of the loop of Henle, macula densa cells, distal convoluted tubules, and collecting ducts.30 In addition, angiotensin II stimulates NADPH oxidase activity and expression of p22phox and p67phox in vascular smooth muscle cells, and its activation is mediated via the Rho/Rho-kinase pathway.10 Furthermore, recent studies have shown that hypertension causes oxidative stress in the blood vessels and produces superoxide by NADPH oxidase in endothelial and vascular smooth muscle cells.30 In this malignant hypertensive model, PRC is increased, and renal cortical angiotensin II levels and the levels of the intrarenal renin–angiotensin system are also increased as compared with those in WKYs.12 Thus, increased BP, per se, and/or activated circulating and

Figure 5. Effects of long-term fasudil treatment on mRNA expression of Rho kinase α (ROCK-α) and RhoB and urinary excretion of 8-isoprostane. (Top) representative ethidium bromide-stained agarose gels of RT-PCR products for ROCK-α, RhoB, and GAPDH. (Bottom) renal cortical tissue quantitative analysis of ROCK-α (A) and RhoB (B) mRNA levels normalized by GAPDH mRNA levels and urinary excretion of 8-isoprostane (C). Data are expressed as mean±SD. Number of rats was 5 to 7 in all groups in RT-PCR analysis and 6 to 9 in all groups in urinary excretion of 8-isoprostane levels. *P<0.05 vs WKY, **P<0.01 vs WKY, †P<0.05 vs D-SHR, #P<0.05 vs low-dose fasudil-treated D-SHR.
intrarenal renal renin–angiotensin systems might stimulate the expression of NADPH oxidase subunits. It is possible that fasudil attenuates these phenomena by inhibiting the intracellular signaling cascade, independent of the BP-lowering mechanism.

In the present study, we measured urinary excretion of 8-isoprostane, a noninvasive index of oxidative stress. A recently discovered series of prostaglandin F2-like compounds, 8-isoprostane, are produced in vivo nonenzymatically by free-radical catalyzed peroxidation of arachidonic acid in cell membranes and in circulating low-density lipoprotein. In the present study we found that 8-isoprostane had increased in DOCA-SHRs and decreased after long-term fasudil treatment. Thus, systemic oxidative stress is increased in this malignant hypertensive model, and long-term fasudil treatment significantly decreased such stress.

Recent studies have shown that eNOS expression is regulated by the Rho/Rho-kinase pathway. Hydroxy-fasudil upregulated the expression of eNOS in cultured endothelial cells. Furthermore, a number of studies showed that NO synthesis is reduced in chronic renal disease in both humans and animals, suggesting that the availability of NO in the kidney is an important factor defining the rate of progression of injury in renal disorders. In the present study we showed that eNOS was downregulated in DOCA-SHRs and that long-term fasudil administration upregulated the mRNA level of eNOS. Thus, beneficial effects of fasudil may be in part because of the upregulation of eNOS.

Our study also showed that the mRNA levels of RhoB and Rho-kinase α in the renal cortex were significantly increased in DOCA-SHRs as compared with WKYs. However, we cannot exclude the possibility that the inhibition of other Rho-kinases by fasudil may be involved in its renoprotective effects. Additional studies are required to evaluate Rho-kinase activity in individual cells or tissues.

In conclusion, our study showed that activation of the Rho/Rho-kinase pathway is related to the pathophysiology of
DOCA-SHRs and that long-term fasudil treatment has renoprotective effects in this malignant hypertension model. The mechanism of the renoprotective effect of fasudil may be a result of a combination of factors, including a reduction in the TGF-β–collagen cascade, control of inflammation, reduction in oxidative stress, and upregulation of eNOS gene expression.

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