Molecular Mechanisms and Therapeutic Strategies of Chronic Renal Injury: Renoprotective Effect of Rho-Kinase Inhibitor in Hypertensive Glomerulosclerosis

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Abstract. Among the GTP-binding proteins, Rho is known to function as a molecular switch in various cellular functions. Among the Rho effectors, the cellular function and signal transduction of Rho-kinase have been extensively studied. However, information about its in vivo functions is still limited. With the recent development of a specific Rho-kinase inhibitor such as Y-27632 and fasudil, the understanding of the role of the Rho/Rho-kinase pathway in vitro and in vivo has advanced. However, to date, there have been few studies investigating the role of Rho-kinase in renal disease. Recent studies have shown that Rho-kinase inhibitor significantly attenuated the tubulointerstitial fibrosis in kidney induced by unilateral ureteral obstruction. However, there have been few studies investigating the role of the Rho/Rho-kinase pathway in hypertensive glomerular sclerosis. In this review, we described the role of the Rho/Rho-kinase pathway in the progression of renal glomerulosclerosis in several forms of hypertensive rats. Our results suggest that chronic inhibition of the Rho-kinase pathway may be a new therapeutic approach for hypertensive glomerulosclerosis. Our results also suggest that the mechanism of the renoprotective effect of Rho-kinase inhibitor is partly mediated via inhibition of extracellular matrix gene expression, monocytes/macrophages infiltration, oxidative stress, and upregulation of eNOS gene expression.

Keywords: fasudil, Rho-kinase, hypertension, transforming growth factor, glomerulosclerosis

Introduction

Recent advances in molecular biology have revealed the important physiological roles of small GTP-binding proteins, such as Rho family members. At least 10 members of the Rho family (isoforms A – E and G) have been identified in mammals. Rho is known to function as a molecular switch in various cellular functions, including formation of stress fibers and focal adhesions, regulation of calcium ion sensitivity in smooth muscle cells, regulation of cytokines following nuclear division, and regulation of G1 to S cycle progression (1, 2). Among the Rho effectors, Rho-associated Rho-kinase is the best characterized, as a results of studies in the mid 1990s. The cellular function and signal transduction of Rho have been extensively studied, but information about its in vivo functions is still limited. Uchata et al. reported a synthetic compound named Y-27632 as a specific inhibitor of Rho-kinase (3). This drug is a very powerful tool elucidating the role 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, there have been few studies investigating the role of Rho in renal disease. A recent study showed that Y-27632 significantly attenuated the tubulointerstitial fibrosis in mouse kidney induced by unilateral ureteral obstruction (5). This observation was further supported by a study using a Rho-kinase inhibitor,
fasudil (6). These findings suggest that the Rho/Rho-kinase pathway plays an important role in the pathogenesis of tubulointerstitial fibrosis. However, there have been few studies investigating the role of the Rho/Rho-kinase pathway in hypertensive glomerulosclerosis.

We and other investigators recently reported that Rho-kinase inhibitor has a renoprotective effect in hypertensive glomerulosclerosis in rats. In this review, we described the role of the Rho/Rho-kinase pathway in the progression of renal glomerulosclerosis in several forms of hypertensive rats. We also attempted to describe the mechanism involved.

**Rho-kinase inhibitor in salt-sensitive hypertension**

First, we investigated the effect of a Rho-kinase inhibitor, fasudil, on the progression of glomerulosclerosis in Dahl salt-sensitive (DS) rats (7). DS rats at the age of 6 weeks were fed a diet containing high-salt (8% NaCl). At 11 weeks of age, the rats were randomly divided into two groups: fasudil treatment group (30 mg/kg per day) and untreated group. After seven weeks of treatment, we measured urinary parameters, hemodynamic measurements, hormonal parameters, histological findings, the gene expression levels, and transforming growth factor (TGF)-β protein levels. Dahl salt-resistant (DR) rats were used a control.

The changes of systolic blood pressure measured by the tail-cuff method in the DR rats, untreated DS rats, and fasudil-treated DS rats are presented in Fig. 1. The systolic blood pressure in 7-week-old DS rats was already higher than that in DR rats, and it was gradually increased and reached a maximum after around 13 weeks. The 7-week treatment with fasudil did not affect the blood pressure of DS rats. The systolic blood pressure remained unaltered in all DR rats. Body weight (BW) was significantly lower in DS rats than in DR rats. In contrast, DS rats had higher kidney weight/BW compared with DR rats. Long-term fasudil therapy in the DS rats significantly increased BW and significantly decreased kidney weight/BW; however, there were still significant differences of these parameters between DR rats and fasudil-treated DS rats.

Regarding renal function parameters, Ccr was markedly decreased in DS rats compared with DR rats. In contrast, urinary protein excretion, serum creatinine, and BUN levels were significantly increased in DS rats compared with DR rats. Chronic fasudil treatment significantly increased Ccr in DS rats. In addition, chronic fasudil therapy significantly reduced urinary protein excretion, serum creatinine, and BUN levels. However, there were still significant differences of these parameters between DR rats and fasudil-treated DS rats.

The renal histological appearance in the DS rats revealed severe afferent arteriolar sclerosis, hyalinosis with luminal encroachment, and periarterial fibrosis with inflammatory cells infiltration. Severe segmental and global glomerular sclerosis, interlobular arteriolar sclerosis with severe perivascular fibrosis, interstitial fibrosis with atrophic and dilated tubules, and tubular cast were also observed. Chronic fasudil treatment attenuated partially these arteriolar, glomerular, arterial, and tubulo-interstitial changes. The afferent arteriolar injury scores (AIS) were markedly higher in untreated DS rats than in DR rats. Chronic fasudil treatment significantly attenuated AIS. The subcapsular glomerular injury scores (GIS) and juxtamedullary cortex GIS were markedly higher in untreated DS rats than in DR rats. Chronic fasudil treatment caused significant amelioration of these changes, especially in the glomeruli at the subcapsular level.

The RhoB/GAPDH, Rho-kinaseα/GAPDH, and Rho-kinaseβ/GAPDH mRNA levels in the renal cortex was significantly increased in DS rats compared to DR rats, whereas there was no significant difference in the RhoA/GAPDH mRNA level or RhoC/GAPDH mRNA level between DS rats and DR rats at 18 weeks. Chronic fasudil treatment did not change the mRNA levels of RhoA, RhoB, RhoC, Rho-kinaseα, or Rho-kinaseβ. In contrast, the collagen I/GAPDH mRNA level,
collagen III/GAPDH mRNA level, and TGF-β/GAPDH mRNA level in the renal cortex were all increased in DS rats compared to DR rats. Chronic fasudil treatment significantly reduced the mRNA levels of collagen I/GAPDH, collagen III/GAPDH, and TGF-β/GAPDH; however, there were still differences of these mRNA levels between DR rats and fasudil-treated DS rats.

Western blot analysis revealed that the TGF-β/actin protein level was also significantly increased in DS rats compared to DR rats. Chronic fasudil treatment significantly reduced the protein level of TGF-β/actin; however, there were still differences of the TGF-β/actin protein levels between DR rats and fasudil-treated DS rats (Fig. 2).

This study was designed to clarify whether the Rho/Rho-kinase pathway is involved in the process of hypertensive glomerulosclerosis in DS rats and to assess the therapeutic effect of fasudil, a specific Rho/Rho-kinase inhibitor, on the development of hypertensive glomerulosclerosis. In DS rats, the mRNA expression of RhoB, Rho-kinaseα, and Rho-kinaseβ, but not RhoA or RhoC, was upregulated in the kidney. In addition, glomerular sclerosis, arteriolar sclerosis, and tubulointerstitial fibrosis in DS rats were partly attenuated by chronic fasudil administration, which also caused suppression of TGF-β, collagen I, and collagen III mRNA expression.

Previous studies showed that TGF-β signaling is regulated by Rho (8). Accumulating evidence indicates that TGF-β plays an important role in the pathogenesis of glomerulosclerosis, arteriolar sclerosis, and tubulointerstitial fibrosis (9). Thus, a TGF-β-collagen cascade may play a central role in the development of glomerulosclerosis and tubulointerstitial fibrosis. Our present data show that the TGF-β mRNA expression and protein level were enhanced in the kidneys of DS rats and that these changes in DS rats were attenuated by treatment with fasudil, which also caused a reduction of urinary protein excretion and improvement of kidney function. These results suggest that the effect of fasudil treatment might partly be due to suppression of TGF-β expression.

Thus, this study indicated that the Rho/Rho-kinase pathway is partly responsible for the pathogenesis of glomerular sclerosis, renal arteriosclerosis, and interstitial fibrosis in DS rats and that the improvement of these changes by long-term administration of fasudil may be mediated, in part, by the TGF-β cascade. The potential usefulness of Rho-kinase inhibitors for other renal diseases and the precise mechanism of the renoprotective effects of this Rho-kinase inhibitor remain to be examined in future studies.

Rho-kinase inhibitor in malignant hypertension induced by DOCA-salt SHR

As we described above, we very recently reported that the Rho-kinase inhibitor fasudil attenuated glomerulosclerosis in salt-induced hypertensive rats (7), suggesting that Rho-kinase inhibitor may be involved in the pathogenesis of glomerulosclerosis in salt-sensitive hypertensive rats. Other investigators also reported the beneficial effect of Rho-kinase inhibitor on nephropathy in subtotally nephrectomized spontaneously hypertensive rats (10). However, whether Rho/Rho-kinase is involved in the pathogenesis of glomerulosclerosis in malignant hypertensive rats remains unknown.

Therefore, next 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 Rho-kinase inhibitor on the development of glomerular sclerosis in deoxycorticosterone acetate-salt spontaneously hypertensive rats (DOCA-SHR) (11). A secondary objective was to investigate potential mechanisms of drug action. Rho plays an important role in cell migration (12). In addition, recent studies have shown the antioxidative effect of fasudil (13). Moreover, the Rho/Rho-kinase pathway is involved in the regulation of endothelial nitric oxide synthase (eNOS) expression (14). Therefore, we attempted to assess the potential mechanisms of its action focusing on these regards.

DOCA (100 mg/kg per week) was subcutaneously injected in SHR and 1% NaCl drinking water was started.

![Fig. 2](image-url) Effects of chronic fasudil treatment on protein level of TGF-β in renal cortex. Upper: Representative Western blotting of TGF-β and actin. Lower: Densitometry values (in arbitrary densitometry units) of TGF-β protein on Western blots. Data represent the mean ± S.D. *P<0.05 vs DR, **P<0.01 vs DR, †P<0.05 vs DS. Modified from Ref. 7.
at the age of 9 weeks. DOCA-salt SHR were randomly divided into the following three groups: low-dose fasudil treatment group (30 mg/kg per day), high-dose fasudil treatment group (100 mg/kg per day), and untreated group. After three weeks of treatment, we evaluated the hemodynamics, renal function, renal morphology (GIS), immunohistochemical analysis (ED-1 positive cell counting), hormone levels, urinary 8-isoprostane levels, and the gene expression levels. Wistar Kyoto (WKY) rats were used as a control.

The systolic blood pressure was already higher in 9-week-old DOCA-SHR than that in WKY, and it was gradually increased and reached a maximum around 11 weeks. Long-term low-dose or high-dose fasudil treatment did not change the systolic blood pressure in the DOCA-SHR groups, as the same effects of fasudil on the blood pressure was shown in DS rats.

In the physiological profiles, BW was significantly lower in DOCA-SHR groups than in WKY. Long-term low-dose or high-dose fasudil treatment did not change the BW in DOCA-SHR. In contrast, untreated DOCA-SHR had higher kidney weight/BW, mean arterial pressure, and urine volume compared with WKY. Long-term high dose of fasudil treatment in DOCA-SHR significantly decreased kidney/BW and urine volume without changing blood pressure.

In renal parameters, urinary protein excretion and serum creatinine level were significantly increased and CCr was significantly decreased in untreated DOCA-SHR compared with WKY. Long-term fasudil treatment dose-dependently reduced urinary protein excretion and chronic high dose fasudil treatment significantly decreased serum creatinine level and increased CCr in DOCA-SHR; however, there were still significant differences of these parameters between WKY and fasudil-treated DOCA-SHR.

The renal histological appearance in DOCA-SHR revealed severe glomerulosclerosis and periarterial fibrosis with inflammatory cell infiltration. Long-term fasudil treatment attenuated these glomerular and inflammatory changes. Chronic fasudil treatment attenuated the glomerular and inflammatory changes. The GIS was markedly higher in untreated DOCA-salt SHR than in WKY. Chronic high-dose fasudil treatment significantly attenuated these changes. However, there were still significant differences of these parameters between WKY and fasudil-treated DOCA-SHR.

ED-1 positive cells are shown in Fig. 3 (A – D). We used ED-1 antibody to evaluate the migration of monocytes/macrophages. ED-1 positive cells were stained brown and the number of ED-1 positive cells was found to be significantly increased in DOCA-salt SHR (Fig. 3). Long-term fasudil treatment significantly decreased the ED-1 positive cells. However, there were still significant differences between WKY and fasudil-treated DOCA-salt SHR.

The mRNA levels of collagen I, collagen III, and TGF-β relative to the GAPDH mRNA levels in the renal cortex were all increased in DOCA-salt SHR compared with WKY. Long-term fasudil treatment dose-dependently reduced the mRNA levels of collagen III and long-term high-dose fasudil treatment significantly decreased serum creatinine level and increased CCr in DOCA-SHR; however, there were still significant differences of these parameters between WKY and fasudil-treated DOCA-SHR.
decreased collagen I and TGF-β; however, there were still differences of these mRNA levels between WKY and fasudil-treated DOCA-salt SHR.

Figure 4 shows the urinary excretion of 8-isoprostane as an indicator of oxidative stress. The excretion of 8-isoprostane was significantly increased in DOCA-salt SHR compared with WKY. High-dose fasudil treatment significantly decreased it; however, there were still significant differences between WKY and fasudil-treated DOCA-SHR.

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-SHR than in WKY. High-dose fasudil treatment significantly decreased the mRNA expression levels of p40phox and p47phox, but not p67phox. In addition, low-and high-dose fasudil treatment significantly increased the mRNA expression of eNOS in DOCA-SHR. In contrast, fasudil treatment tended to decrease the mRNA expression of p67 phox, but the decrease did not reach the level of statistical difference. There were still significant differences in the mRNA expression of p40, p47, and eNOS between WKY and fasudil-treated DOCA-SHR. There were no differences in mRNA expression of p22phox or gp91phox between WKY and untreated DOCA-SHR.

In this study, long-term fasudil treatment significantly decreased the mRNA expression of TGF-β, collagen I, and collagen III in the renal cortex. The 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 a Rho-kinase inhibitor significantly decreased TGF-β and collagen gene expression (5, 7). Thus, one of the possible mechanisms of the renoprotective effect of fasudil is suppression of TGF-β and subsequent collagen mRNA expression (8).

Rho/Rho-kinase is involved in many aspects of cell motility, from smooth-muscle contraction to cell migration (10, 15). In this 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. Macrophages/monocytes secrete various cytokines and growth factors, including TGF-β (16).

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 (16, 17). 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 SHR (18). In addition, angiotensin II stimulates NADPH oxidase activity and expression of p22phox and p67phox in vascular smooth muscle cells via the Rho/Rho-kinase pathway (13). Thus, increased blood pressure per se and/or activated circulating and intrarenal renal renin-angiotensin system might stimulate the expression of NADPH oxidase subunits, and it is possible that fasudil attenuates this by inhibiting the intracellular signaling cascade independent of the blood pressure-lowering mechanism.

In addition, we measured urinary excretion of 8-isoprostane, a noninvasive index of oxidative stress (19). A recently discovered series of prostaglandin F2-like compounds, 8-isoprostane, are produced in vivo non-enzymatically by free radical catalyzed peroxidation of arachidonic acid in cell membranes and in circulating LDL (19). In the present study we observed that 8-isoprostane was increased in DOCA-SHR and that it was decreased after the long-term fasudil treatment. Thus, systemic oxidative stress is increased in this malignant hypertensive model, and long-term fasudil treatment significantly decreased it.

Recent studies have shown that eNOS expression is regulated by the Rho/Rho-kinase pathway. Hydroxyfasudil upregulated the expression of eNOS in cultured endothelial cells (14). Furthermore, a number of studies showed that nitric oxide synthesis is reduced in chronic renal disease in both humans and animals (20), suggest-
Renoprotective Effect of Rho-Kinase Inhibitor

In that the availability of nitric oxide in the kidney is an important factor in defining the rate of progression of the injury in renal disorders. In the present study we showed that eNOS was downregulated in DOCA-SHR and that long-term fasudil administration upregulated the mRNA level of eNOS. Thus, beneficial effects of fasudil may be in part due to the upregulation of eNOS.

These results suggest that activation of the Rho/Rho-kinase pathway is related to the pathophysiology of DOCA-SHR, 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 reduction in TGF-β-collagen cascade, control of inflammation, reduction of oxidative stress, and upregulation of eNOS gene expression.

**Rho-kinase inhibitor in severe hypertension**

As we described above, Rho-kinase inhibitor fasudil attenuated glomerulosclerosis in salt-induced hypertensive rats and malignant hypertension induced by DOCA-salt SHR. These results suggest that the Rho-kinase pathway may be involved in the pathogenesis of glomerulosclerosis in salt-sensitive hypertension and malignant hypertension and that Rho-kinase inhibitor may be a new therapeutic approach for the prevention of hypertensive glomerulosclerosis in certain forms of hypertension. However, whether Rho/Rho-kinase is involved in the pathogenesis of glomerulosclerosis in other hypertensive rats remains unknown. This study was designed to examine: 1) whether Rho/Rho-kinase pathway is involved in the other form of hypertensive glomerulosclerosis and 2) the effects of fasudil on the survival.

Severe hypertension causes structural and functional modifications in the cardiorenovascular system associated with an increase in mortality and morbidity. Stroke-prone spontaneously hypertensive rats (SHR-SP) are an experimental model of severe hypertension in which the animals develop severe cerebral and renal dysfunction/damage and die of stroke (21). SHR-SP at six week of age were fed a low-salt diet (0.12% NaCl) during a 2-week acclimatization period. At the end of this period, all rats were fed a high-salt diet (8% NaCl). SHR-SP were randomly divided into the following three groups: low-dose fasudil treatment group (40 mg/kg per day), high-dose fasudil treatment group (80 mg/kg per day), and untreated group. After eight weeks of treatment, we evaluated the blood pressure, physiological profiles, and survival by the Kaplan-Meier method. Wistar Kyoto (WKY) rats were used as a control.

The systolic blood pressure in 8-week-old SHR-SP was already higher than that in WKY, and it was gradually increased and reached a maximum after around 13 – 15 weeks. Long-term low-dose or high-dose fasudil treatment did not change the systolic blood pressure in the SHR-SP groups, as we described the same effects in DS rat and DOCA-salt SHR.

In the physiological profiles, BW was significantly lower in SHR-SP groups than in WKY. Long-term high dose fasudil treatment significantly increased the BW in SHR-SP. Untreated SHR-SP had higher kidney weight/BW and LV weight/BW compared with WKY. Long-term high dose of fasudil treatment in SHR-SP significantly decreased kidney weight/BW and LV weight/BW without changing blood pressure.

Survival rate was analyzed after the start of fasudil treatment. All SHR-SP treated with saline died between 80 and 110 days after treatment. Kaplan-Meier survival analysis showed that long-term high-dose fasudil treatment significantly prolonged the survival time of SHR-SP ($P<0.01$).

These three studies suggest that the renal Rho/Rho-kinase system is activated in various forms of hypertension and that the long-term Rho-kinase inhibitor fasudil treatment provides renoprotective effects and improves survival independent of blood pressure-lowering activity. Our results also suggest that the mechanism of the renoprotective effect of Rho-kinase inhibitor is partly mediated via inhibition of extracellular matrix gene expression, monocytes/macrophages infiltration, oxidative stress, and upregulation of eNOS gene expression. Our working hypothesis is shown in Fig. 5.

Thus, chronic inhibition of the Rho-kinase pathway may be a new therapeutic approach for the treatment of

**Possible Mechanism of Beneficial Effects of Fasudil on Hypertensive Glomerulosclerosis**

![Fig. 5. Working hypothesis of renoprotective effect of Rho-kinase inhibitor in hypertensive glomerulosclerosis.](image-url)
hypertensive glomerulosclerosis. Supporting data from large clinical trials, however, are required before such recommendations can be used in the clinical setting.

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