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Fasudil attenuates interstitial fibrosis in rat kidneys with unilateral ureteral obstruction

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Abstract

This study was designed to investigate possible effects of the Rho-kinase inhibitor, fasudil, on the progression of renal failure in rats with unilateral ureteral obstruction. The renal failure markers monitored were the extent of renal interstitial fibrosis and that of macrophage infiltration. In kidneys with unilateral ureteral obstruction, interstitial fibrosis was observed, using Sirius-Red staining, on day 16 after unilateral ureteral obstruction. Macrophage infiltration was observed by immunohistochemistry, using the antibody, ED1. Interstitial fibrosis and macrophage infiltration were significantly attenuated in fasudil-treated animals. The migration of monocytes in vitro elicited by *N*-formyl-methionyl-leucyl-phenylalanine was potently inhibited by fasudil and its active metabolite, hydroxyfasudil. These results suggest that inhibition of Rho-kinase produces a reduction of macrophage infiltration and represents a new therapeutic strategy for renal fibrosis, a major factor in the progression to end-stage renal failure.

Keywords: Unilateral ureteral obstruction; Renal interstitial fibrosis; Macrophage; Rho-kinase; Fasudil

1. Introduction

The final step in the process of chronic renal failure is renal interstitial fibrosis. To induce renal damage leading to tubulointerstitial fibrosis, chronic unilateral obstruction was used in various species to serve as a well-characterized experimental model (Sommer et al., 1999; Ludewig et al., 2000; Moriyama et al., 2001). The molecular and cellular mechanisms of renal interstitial fibrosis are now beginning to be elucidated (Guo et al., 1999; Hruska et al., 2000). One of the possible mechanisms has been identified as the inflammatory process, in which infiltrating macrophages play a major role as a source of inflammatory mediators. Inhibiting macrophage infiltration may reduce the production of such inflammatory mediators and may, therefore, present an option in the prevention of progressive renal fibrosis leading to end-stage renal failure (Anders et al., 2002).

Rho-kinase contributes to the reorganization of the actin cytoskeleton and to the formation of stress fibers, and is thought to be one of the critical elements involved in a variety of cytoskeleton-dependent cell functions such as cell migration (Niggli, 1999).

Fasudil is a protein kinase inhibitor that has shown clinical effectiveness in patients with subarachnoid hemorrhage (Shibuya et al., 1992), and that was launched for clinical use after subarachnoid hemorrhage in Japan. Studies in animal models show fasudil to be promising in the treatment of stroke and angina (Asano et al., 1991; Satoh et al., 2001a,b; Utsunomiya et al., 2001). Fasudil and its active metabolite, hydroxyfasudil, inhibit Rho-kinase more effectively than they inhibit other protein kinases; e.g., protein kinase C, or myosin light chain kinase (Shimokawa et al., 1999; Shimokawa, 2002; Davies et al., 2000; Nagumo et al., 2000). Using chemoattractants in vitro, we previously showed that neutrophil chemotaxis was inhibited by fasudil and hydroxyfasudil (Satoh et al., 1999a), as was in vivo neutrophil and macrophage infiltration (Satoh et al., 1999a, 2001a; Miyata et al., 2000; Ikegaki et al., 2001).

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Nagatoya et al. (2002) reported that pretreatment with Y-27632, another Rho-kinase inhibitor, from 2 days before unilateral ureteral obstruction until the day of killing prevented tubulointerstitial fibrosis in mouse kidneys with unilateral ureteral obstruction. In the present study, we investigated if the inhibition of Rho-kinase with fasudil or hydroxyfasudil would reduce macrophage infiltration into the kidneys and renal fibrosis after unilateral ureteral obstruction. We endeavored to determine the therapeutic potential of fasudil in renal failure since it is currently the only Rho-kinase inhibitor approved for clinical use to our knowledge. We, therefore, tried to ascertain whether renal fibrosis could be attenuated by fasudil treatment in the post-obstructed kidneys.

2. Materials and methods

2.1. Unilateral ureteral obstruction rat model

Male Sprague–Dawley rats (5 weeks old) were used. Rats were anesthetized with ether and underwent left unilateral ureteral ligation on day 1. The first intraperitoneal administration of fasudil (3 or 10 mg/kg) or saline was on day 2. Administration of fasudil or saline once daily was continued until day 15. On day 16, the rats were anesthetized with ether, and obstructed and contralateral kidneys were removed, sliced transversely, and fixed in buffered formalin. Sirius-Red staining was performed to evaluate the extent of fibrosis. The area of fibrosis was quantified using a computerized image analysis system, and was expressed as a percentage of the slice of the kidney. To identify macrophages infiltrated into the kidneys, kidney slices were immunostained using antibodies against rat monocyte/macrophage (ED1). From each histology sample, we collected 5 non-overlapping microscopic fields ($\times$ 200) and the number of macrophages per mm^2 was calculated from these.

2.2. Monocyte preparation and chemotaxis

Monocytes were isolated from heparinized peripheral blood of healthy human volunteers by density gradient centrifugation. Chemotaxis of monocytes was measured using a 96-well Boyden chamber in which a 5-μm pore-sized filter separates the upper and lower chambers. Monocyte suspensions (5×10^6 cells/ml) were preincubated for 5 min with fasudil or hydroxyfasudil, then monocytes (10^6 cells per well) were placed in the upper well and, across the membrane filter, 10^{-7} M *N*-formyl-methionyl-leucyl-phenylalanine (fMLP) was placed in the lower well. Incubation was carried out for 90 min. The number of monocytes responding to the chemotactic stimulus and appearing on the opposite side of the filter was determined using Diff-Quik (International Reagents, Kobe) staining. Migrated monocytes were quantified by measuring specific light absorbance (600 nm) using a densitometer.

2.3. Statistics

Values are expressed as means ± S.E.M. Statistical analysis of data was done with Student's *t*-test, Student's *t*-test for paired samples, or one-way analysis of variance (ANOVA) followed by Dunnett's test. *P* values of 0.05 or less were considered to indicate a significant difference.

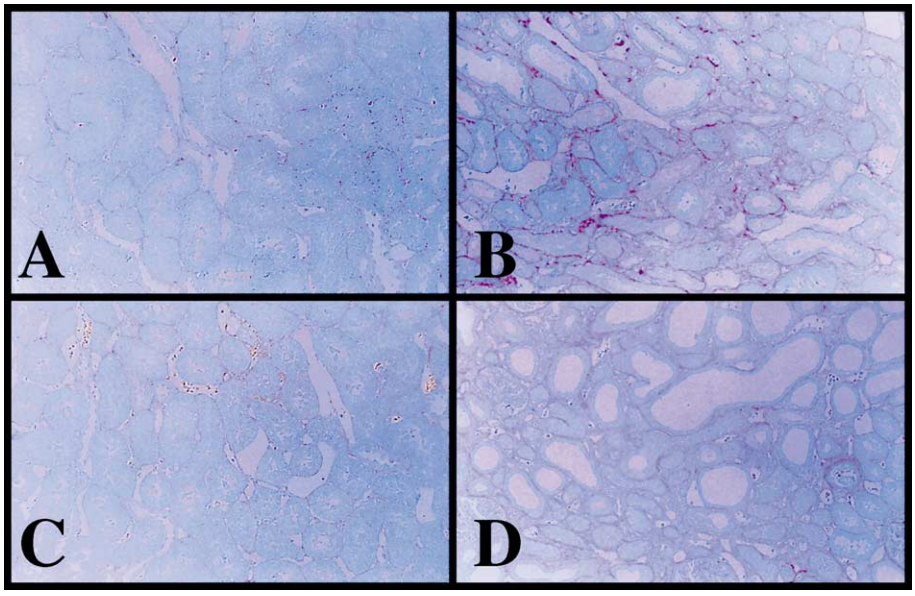

Fig. 1. Photomicrographs of kidneys on day 16 after unilateral ureteral obstruction in rats (Sirius-Red staining × 200). (A) Contralateral kidney of rat treated with saline; (B) obstructed kidney of rat treated with saline; (C) contralateral kidney of rat treated with fasudil (10 mg/kg); (D) obstructed kidney of rat treated with fasudil (10 mg/kg). Kidney with unilateral ureteral obstruction showed interstitial fibrosis. The interstitial fibrosis was attenuated in fasudil-treated rats.

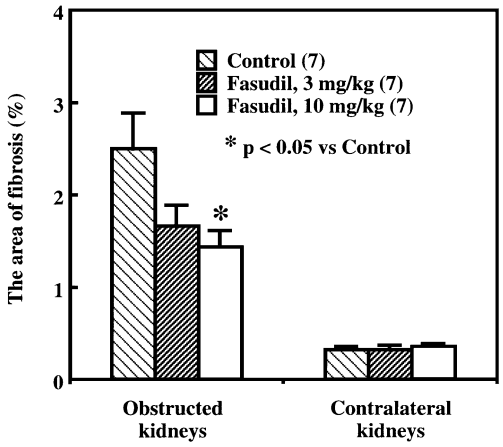

Fig. 2. Effect of fasudil on the progression of interstitial fibrosis in kidneys on day 16 after unilateral ureteral obstruction in rats. Each column represents the mean ± S.E.M. of the number of experiments shown in parentheses. The asterisks indicate a significant difference from the control (*$P < 0.05$: Dunnett's test).

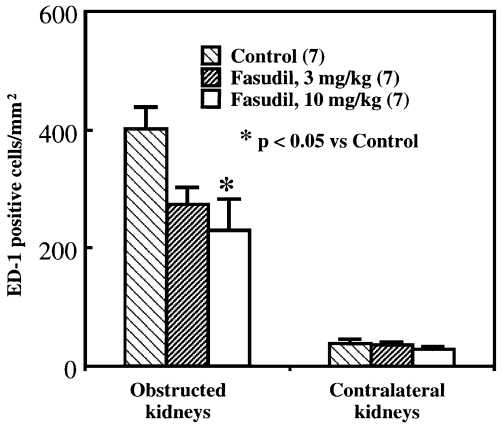

Fig. 4. Inhibitory effect of fasudil on the macrophage infiltration into obstructed kidneys on day 16 after unilateral ureteral obstruction in rats. Each column represents the mean ± S.E.M. of the number of experiments shown in parentheses. The asterisks indicate a significant difference from the control (*$P < 0.05$: Dunnett's test).

2.4. Drugs

The drugs used were fasudil and hydroxyfasudil (Asahi Kasei, Tokyo) and anti-ED1 (Serotec, Tokyo).

3. Results

3.1. Effects on renal interstitial fibrosis

On day 16 after unilateral ureteral obstruction, interstitial fibrotic lesions were found in obstructed kidneys (Fig. 1). The percent area of interstitial fibrosis in obstructed kidneys (control: 2.5 ± 0.4%) was significantly greater than that in contralateral kidneys (0.3 ± 0.0%) (Fig. 2). Fasudil at 3 and 10 mg/kg dose dependently inhibited the development of interstitial fibrosis. The mean area of fibrosis in kidneys of rats treated with fasudil 10 mg/kg (1.4 ± 0.2%, $P < 0.05$) was significantly smaller than that of obstructed kidneys of control rats (Fig. 2).

3.2. Effects on macrophage infiltration into the kidneys

The infiltration of ED1-positive macrophages into obstructed kidneys on day 16 after unilateral ureteral

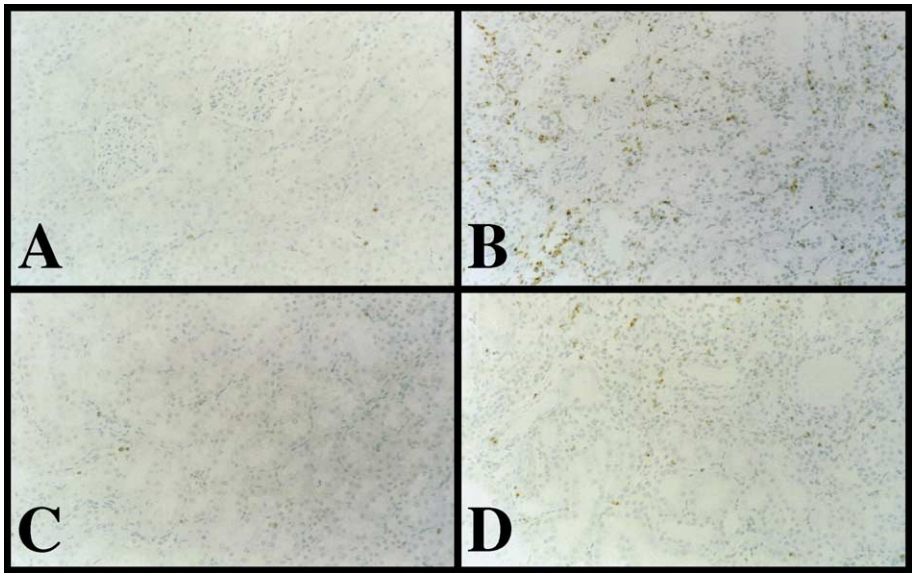

Fig. 3. Photomicrographs of the kidneys on day 16 after unilateral ureteral obstruction in rats (immunohistochemical staining of monocytes/macrophages with antibody ED1 × 200). (A) Contralateral kidney of rat treated with saline; (B) obstructed kidney of rat treated with saline; (C) contralateral kidney of rat treated with fasudil (10 mg/kg); (D) obstructed kidney of rat treated with fasudil (10 mg/kg). Kidney with unilateral ureteral obstruction showed macrophage infiltration. The macrophage infiltration was attenuated in fasudil-treated rats.

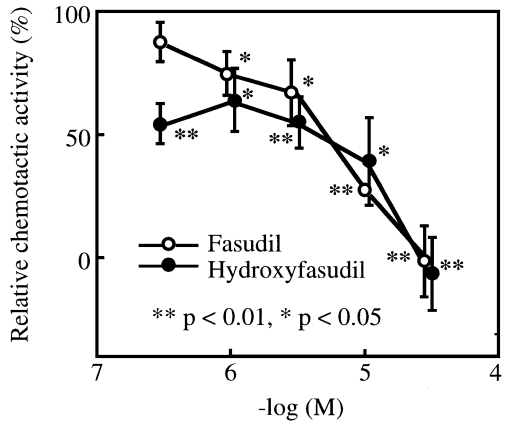

Fig. 5. Dose–response effect of fasudil or hydroxyfasudil on monocyte chemotaxis induced by fMLP (10^{-7} M). Each data point represents the mean ± S.E.M. of six to seven experiments. The chemotactic activity of fMLP-stimulated monocytes (control) was defined as 100%. The asterisks indicate a significant difference from control (Student's *t*-test for paired samples).

obstruction is shown in Fig. 3. In the obstructed kidneys, 400 ± 37 macrophages/mm^2 were observed, but in the contralateral kidneys no marked macrophage infiltration (39 ± 8 macrophages/mm^2) was detected (Fig. 4). Fasudil at 3 and 10 mg/kg dose dependently inhibited macrophage infiltration (Fig. 4).

3.3. Effects on monocyte chemotaxis in vitro

When monocyte chemotaxis was elicited with chemoattractant fMLP, a marked migration response was observed. Fig. 5 shows the effects of fasudil or hydroxyfasudil on the migration elicited by the optimum concentration of fMLP (10^{-7} M). The migration of monocytes was dose dependently inhibited by fasudil or hydroxyfasudil. The lowest concentration of fasudil and hydroxyfasudil at which significant inhibition of the chemotaxis induced by fMLP occurred was 1 and 0.3 μM, respectively.

4. Discussion

Previous studies have shown that unilateral ureteral obstruction in rats resulted in renal interstitial fibrosis and macrophage infiltration into the obstructed kidneys (Takeda et al., 2000; Sato et al., 2001); these morphological changes could also be observed in the present study. The mechanism of initiation and subsequent progression of fibrosis is still far from completely understood. Macrophages are thought to play a central role in the fibroproliferative response. Fibrogenic growth factors such as transforming growth factor-β, platelet-derived growth factor and basic fibroblast growth factor, most of which might be released by infiltrating macrophages, may be important in the pathogenesis of fibrosis in several tissues (Nagaoka et al., 1990; Henke et al., 1993; Diamond et al., 1994). Eddy (2001) summarized the possible mechanism by which interstitial macrophages promote renal fibrosis. She suggested that macrophages synthesize and secrete fibrogenic factors that contribute to recruitment of interstitial myofibroblasts and facilitate the activation of resident interstitial fibroblasts. Interstitial myofibroblasts and fibroblasts are the primary source of the extracellular matrix proteins in the interstitium that cause fibrosis.

Recent publications have been providing evidence suggesting the importance of Rho-kinase in monocyte migration. We previously reported that fasudil inhibits the infiltration of neutrophils and macrophages in vivo (Satoh et al., 1999a,b; Ikegaki et al., 2001), and that fasudil and hydroxyfasudil, both of which have potent inhibitory activity for Rho-kinase, inhibit the migration of neutrophils in vitro (Satoh et al., 1999a). It has also been hypothesized that Rho-kinase contributes to the reorganization of the actin cytoskeleton and formation of stress fibers, and Rho-kinase is one of the critical elements involved in a variety of cytoskeleton-dependent cell functions such as cell migration (Uehata et al., 1997; Maekawa et al., 1999). Other publications point to Rho-kinase involvement in controlling the development of polarity and migration of neutrophils (Niggli, 1999). Additionally we reported that fasudil or hydroxyfasudil inhibit neutrophil chemotaxis to fMLP; an effect observed with monocytes in the present study. Our results now demonstrated that fasudil and hydroxyfasudil also have a direct inhibitory effect on monocyte migration in vitro. All these results together suggest that the inhibitory action of fasudil and hydroxyfasudil on monocyte migration occurs through the inhibition of the Rho-kinase pathway.

The lowest concentration of fasudil and hydroxyfasudil at which we could observe significant inhibition of neutrophil chemotaxis induced by various chemoattractants, including fMLP, was 3–30 and 1–3 μM, respectively (Satoh et al., 1999a). In the present study, the lowest concentration of fasudil and hydroxyfasudil giving a significant inhibition of the monocyte chemotaxis induced by fMLP was 1 and 0.3 μM, respectively. Fasudil and hydroxyfasudil were more potent to inhibit monocyte chemotaxis than to inhibit neutrophil chemotaxis.

In rats, the maximum plasma concentration of fasudil and hydroxyfasudil after intraperitoneal administration of fasudil at 10 mg/kg was approximately 15 and 6 μM, respectively (Satoh et al., 2001a). These maximum concentrations were more than the minimum effective concentration of each needed for inhibition of monocyte migration in vitro; i.e., 1 μM for fasudil and 0.3 μM for hydroxyfasudil. The potency of fasudil to attenuate the development of interstitial fibrosis and macrophage infiltration was similar at both doses. The results observed so far suggest that in vivo inhibition of macrophage infiltration into the obstructed kidneys by fasudil is mediated—at least in part—through a direct inhibitory effect on monocyte migration. Such an effect seems to indicate that fasudil would make an effective inhibitor in the development of renal interstitial fibrosis to the extent to

which it limits macrophage infiltration. However, additional research may be required to define the mechanisms of fasudil for attenuation of renal fibrosis; for example, the effect of fasudil on renal fibroblast proliferation.

Fasudil was launched in Japan for clinical use in subarachnoid hemorrhage. Intraperitoneal administration of fasudil (1–10 mg/kg) in rats improved neurological function in ischemia-induced brain damage (Satoh et al., 1996; Toshima et al., 2000). Fasudil (10 mg/kg), which was administered from day 2 after unilateral ureteral obstruction until day 15, attenuated interstitial fibrosis and macrophage infiltration in rat kidneys with unilateral ureteral obstruction. The dose (10 mg/kg, i.p.) for systemic treatment with fasudil in this study was similar to that described for in vivo experiments in cerebral ischemia animal models; these results raise the possibility of a new effective therapy with fasudil in patients with renal failure. In the present study, fasudil was administered intraperitoneally. We previously reported that fasudil administered orally prevented coronary and myocardial damage in animal models (Ikegaki et al., 2001; Satoh et al., 2001b). In rats, the maximum plasma concentration of hydroxyfasudil after oral administration of fasudil, 10 mg/kg, was approximately 7 μM (Ikegaki et al., 2001), more than the minimum effective concentration of hydroxyfasudil needed for inhibition of monocyte migration in vitro (0.3 μM). These results suggest that oral administration of fasudil may also be effective in prevention of progressive renal failure.

In summary, fasudil and its active metabolite, hydroxyfasudil, have a direct inhibitory effect on monocyte migration in vitro. Treatment with fasudil reduces macrophage infiltration and renal fibrosis in kidneys with unilateral ureteral obstruction. These results suggest that inhibition of Rho-kinase produces a reduction of macrophage infiltration and represents a new therapeutic strategy for renal fibrosis, a major factor in the progression to end-stage renal failure.

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