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Possible Involvement of Rho/Rho-kinase Pathway in Platelet-derived Growth Factor BB-induced Expression of Transforming Growth Factor-β in Cultured Mesangial Cells

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ABSTRACT

Background/Aims : In the pathogenesis of diabetic nephropathy, expression of transforming growth factor (TGF)-β in mesangial cells plays a critical role ; however, the precise mechanism of upregulation of TGF-β remains unknown. This study aimed to investigate the involvement of the Rho/Rho-kinase pathway in platelet-derived growth factor (PDGF)-BB-induced expression of TGF-β, using cultured mesangial cells.

Methods : Primary cultured rat mesangial cells were stimulated by PDGF-BB. Membrane-bound RhoA and activities of extracellular signal-regulated kinase (ERK) were evaluated with immunoblot analysis. Expression of TGF-β was determined with Northern blot analysis.

Results : After stimulation with PDGF-BB, levels of RhoA, ERK, and TGF-β were significantly increased in mesangial cells. PDGF-BB-induced ERK and TGF-β were suppressed by a hydroxymethyl glutaryl coenzyme A reductase inhibitor (cerivastatin) and by a geranylgeranyltransferase inhibitor. The inhibitory effect of cerivastatin was reversed by the addition of geranylgeranyl pyrophosphate, suggesting involvement of RhoA in the PDGF-BB-induced activation of ERK and expression of TGF-β. A specific inhibitor of Rho-kinase also suppressed PDGF-BB-induced activation of ERK and expression of TGF-β.

Conclusion : The present study strongly suggests that the PDGF-BB-induced expression of TGF-β in the mesangial cells is mediated by activation of the Rho/Rho-kinase pathway.

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Key words : diabetes, nephropathy, Rho, Rho-kinase, platelet-derived growth factor, transforming growth factor-β

INTRODUCTION

Over the last decade, the incidence of end-stage renal disease due to diabetic nephropathy has been increasing throughout the world. End-stage renal disease has a high mortality rate in patients with diabetes. Progress in research on the pathogenesis of diabetic nephropathy has been hampered by the many factors contributing to the progression of kidney diseases, including hyperglycemia, hypertension, and hyperlipidemia, all of which can emerge simultaneously in any patient with diabetes. Nevertheless, recent advances in molecular biology have revealed that overexpression of the transforming growth factor

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(TGF)-β in glomeruli plays a critical role in the development of diabetic nephropathy[1]. Many investigations have focused on the mechanism whereby TGF-β is up-regulated in the diabetic kidney, which is still unknown.

Small guanosine triphosphatase (GTPase) proteins (small G proteins), including Ras, Rho, Rab, and Ran, which are monomeric proteins with a low molecular mass of 20 to 40 kDa, elicit a variety of important intracellular signals. The posttranslational lipid modifications (prenylation) of small G proteins are necessary for the translocation of inactive small G proteins from the cytosol to the plasma membrane, where these proteins are activated. Prenylation is the covalent linkage of isoprenoids, either the farnesyl or geranylgeranyl group, both of which are intermediates in the cholesterol biosynthetic pathway, to the cysteine residue located at the carboxyl-terminus of small G proteins[2]. Recently, it has been suggested that 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) reductase inhibitors (or statins), potent inhibitors of cholesterol biosynthesis, may confer renoprotection in various kidney diseases, including diabetic nephropathy independent of their lipid-lowering effects[3,4]. Moreover, a series of investigations have demonstrated that statins suppress translocation of small G proteins from the cytosol to the plasma membrane by inhibiting prenylation, which can produce the non-cholesterol dependent (pleiotropic) effects of statins[5]. In turn, these findings indicate profound involvement of small G proteins in the pathogenesis of diabetic nephropathy.

Among small G proteins, Rho and its novel effector, Rho-kinase/ROK/ROCK, regulate various cellular functions including cell contraction, migration, and proliferation[6]. Previously, we have reported that Rho/Rho-kinase pathway is upregulated in the cortex of rats with streptozotocin-induced diabetes, which is suppressed by statins, and that the Rho-kinase inhibitor, fasudil, has a preventive effect on the development of diabetic nephropathy via suppression of TGF-β and NAD(P)H oxidase in the diabetic kidney[7]. Involvement of Rho/Rho-kinase pathway in the pathogenesis of diabetic nephropathy is also suggested in the rodent model of type 2 diabetes[8]. However, the mechanism by which the Rho/Rho-kinase pathway is upregulated in the diabetic milieu remains unknown.

Platelet-derived growth factor (PDGF)-BB, a potent mitogenic cytokine, is upregulated in the glomeruli of the diabetic kidney[9,10] and plays an important role in the pathogenesis of diabetic nephropathy by enhancing the production of extracellular matrix proteins and the proliferation of mesangial cells.

On the basis of these observations, we hypothesized that PDGF-BB might be responsible for activation of the Rho/Rho-kinase pathway resulting in excessive synthesis of TGF-β in glomeruli. Hence, this study aimed to investigate contribution of the Rho/Rho-kinase pathway to the mechanism of PDGF-BB-induced expression of TGF-β in cultured mesangial cells and to clarify the possibility that Rho/Rho-kinase pathway might be a target molecule for statins in the treatment of diabetic nephropathy.

MATERIALS AND METHODS

1. *Materials*

The following reagents were obtained: PDGF-BB (PeproTech EC, Ltd, London, England); cerivastatin (Bayer AG, Wuppertal, Germany); a Rho-kinase specific inhibitor, Y27632 (Mitsubishi Pharmaceutical Industries, Saitama, Japan); geranylgeranyl pyrophosphate (GGPP) (Sigma-Aldrich, St. Louis, MO, USA), and farnesyl pyrophosphate (FPP) (Sigma-Aldrich), a selective inhibitor of farnesyltransferase, FTI277 (Calbiochem, Bad Soden, Germany); a selective inhibitor of geranylgeranyltransferase, GGTI286 (Calbiochem); monoclonal antibodies against RhoA, H-Ras, and K-Ras (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA); a p44/42 MAP kinase assay kit (Cell Signaling Technology Inc., Beverly, MA, USA); a peroxidase-conjugated anti-mouse secondary antibody (Amersham, Braunschweig, Germany); and the ECL Western blotting detection system (Amersham).

2. *Cell culture*

Mesangial cells were isolated from the glomeruli

of male Wistar rats (CLEA, Tokyo, Japan) with a sieving method as described elsewhere[11], and maintained in Dulbecco's modified Eagle medium (DMEM ; Nissui, Tokyo, Japan) supplemented with 10% fetal bovine serum, 50 μg/ml piperacillin Na, 100 μg/ml streptomycin sulfate, 5 μg/ml insulin, and 2 μg/ml amphotericin B. Cultures were kept at 37°C in a 5% CO_2 atmosphere. For passage, confluent cells were washed with HEPES buffer (Nissui), removed with trypsin/EDTA, and plated in DMEM. The experiments in this study were performed on the cells between the 5th and 7th passages, and the medium was exchanged for serum-free DMEM with a low glucose level (5.6 mM of D-glucose). All procedures were performed according to the guidelines for Animal Experiments at The Jikei University School of Medicine.

3. Assay for extracellular signal-regulated kinase

The activity of extracellular signal-regulated kinase (ERK1/2) was measured with an p44/42 MAP kinase assay kit, according to the manufacturer's protocol. After stimulation with PDGF-BB for 10 minutes, mesangial cells were rinsed with ice-cold phosphate-buffered saline and scraped in lysis buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na3VO4, 1 μg/ml leupeptin, and 1 mM PMSF). The lysate was sonicated and centrifuged at 10,000 g at 4°C for 10 minutes. The supernatant was used for the assay of ERK1/2. The protein content of the supernatant was determined with method of Lowry et al[12]. The samples were incubated at 4°C with a monoclonal antibody for ERK 1/2 overnight and centrifuged. The immunoprecipitate was incubated with Elk-1 fusion protein as a substrate in the presence of ATP and kinase buffer (25 mM Tris, pH 7.5, 5 mM β-glycerophosphate, 2 mM DTT, 0.1 mM Na3VO4 and 10 mM MgCl2). The phosphorylation of Elk-1 at Ser 383 was immunoblotted using an anti-phospho-Elk-1 antibody and was visualized with an enhanced chemoluminescence (ECL) detection system. The densitometry analysis was performed using Light-Capture II and a CS image analyzer (ATTO, Tokyo, Japan).

4. Northern blot analysis

For Northern blot analysis, mesangial cells were lysed with TRI Reagent (Sigma-Aldrich). Total RNA was extracted with a single-step method, using phenol and chloroform/isoamyl alcohol. Twenty micrograms of total RNA was electrophoresed in 1% agarose gel with 2.2 mM formaldehyde and transferred overnight onto a nylon membrane (Amersham, Tokyo, Japan). The cDNA probes used in this study were rat TGF-β and rat PDGF-B (GeneDetect, Bradenton, FL, USA). The cDNAs were labeled with α-[^{32}P] dCTP (3,000 Ci/mmol, NEN, Kanagawa, Japan) using a random primer extension kit (TaKaRa, Shiga, Japan) or γ-[^{32}P] ATP (7,000 Ci/mmol ; NEN) using a 5′-end labeling procedure (TaKaRa). Each hybridized band was detected with the Imaging Analyzer BAS2000 (Fuji Photo Film, Kanagawa, Japan) and quantitated as a relative ratio.

5. Assay for small G proteins RhoA, H-Ras and K-Ras in membrane fractions

To evaluate the activation of RhoA, H-Ras, and K-Ras, we examined changes in the membrane-bound fractions of these proteins via immunoblot analysis[2]. Mesangial cells were scrapped in lysis buffer (250 mM sucrose, 10 nM EGTA, 2 mM EDTA, 20 nM Tris-HCl, pH 7.5, 1 mM PMSF, 1 μg/ml leupeptin, and 1 μg/ml aprotinin) and broken down by sonication. The samples were centrifuged at 100,000 g at 4°C for 45 minutes to obtain a membrane fraction in the precipitate. The membrane fraction was resuspended in a lysis buffer. For Western blot analysis, 150 μg of the membrane protein was applied to 15% sodium dodecylsulfate-polyacrylamide gel electrophoresis. Immunoblotting was accomplished with monoclonal antibodies against RhoA, Ha-Ras, and K-Ras. Immunodetection was performed with a peroxidase-conjugated anti-mouse secondary antibody and detected with the ECL detection system.

6. Statistical analysis

Data are expressed as mean±S.E. Statistical significance was evaluated using Student's *t*-test or one-way analysis of variance followed by Tukey's posthoc test for multiple comparisons. The values

were considered to be statistically significant at $p < 0.05$.

RESULTS

1. The effect of PDGF-BB on membrane-bound small G proteins in mesangial cells

To clarify the effect of PDGF-BB on activation of small G proteins, we examined time-course of changes in membrane-bound RhoA, H-Ras, and K-Ras after stimulation with PDGF-BB. Mesangial cells in serum-free DMEM were stimulated with 20 ng/ml of PDGF-BB and harvested at 0, 5, 10, 15, 30, and 60 minutes. RhoA increased significantly after 30 to 60 minutes compared with before stimulation, whereas changes in K-Ras and H-Ras were not significant during the study period (Fig. 1).

2. The effect of a statin and prenylation inhibitors on PDGF-BB-induced translocation of RhoA

To confirm whether the activation of RhoA depends on geranylgeranylation and not farnesylation, we evaluated the effect of statins on membrane-bound RhoA, studying the recovery of statin-induced suppression of RhoA by supplementation of the isoprenoids FPP and GGPP (Fig. 2A). To assess the effect of statins on membrane-bound RhoA, the mesangial cells were preincubated with 2 μM cerivastatin for 30 minutes, followed by stimulation with 20 ng/ml PDGF-BB for 60 minutes. PDGF-BB increased membrane-bound RhoA in the mesangial cells, and pretreatment with cerivastatin completely suppressed the PDGF-BB-induced increase in membrane-bound RhoA. This inhibition on RhoA was fully recovered by the simultaneous addition of 10 μM GGPP but not of 10 μM FPP. We also examined the effect of prenylation inhibitors, a farnesyltransferase inhibitor (FTI-277) and a geranylgeranyltransferase inhibitor (GGTI-286), on PDGF-BB-induced translocation of RhoA (Fig. 2B). The mesangial cells were preincubated for 30 minutes with 2 μM of cerivastatin, 5 μM of FTI-277, or 5 μM of GGTI-286 before the addition of PDGF-BB. GGTI-286 suppressed the PDGF-BB-induced increase in membrane-bound RhoA to a similar extent to cerivastatin; however, FTI-277 had no effect. These data indicate that the PDGF-BB-induced translocation of RhoA to plasma membrane in mesangial cells depends on geranylgeranylation and not farnesylation.

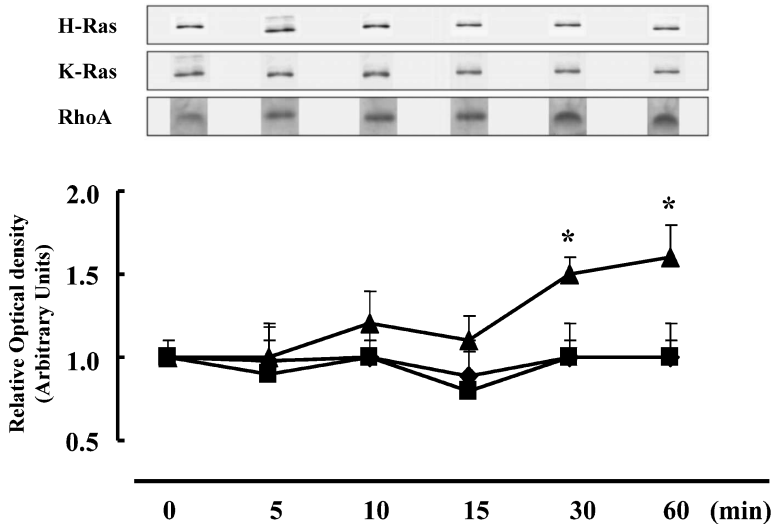

Fig. 1. The time-course of changes in membrane-bound RhoA, H-Ras, and K-Ras after stimulation with PDGF-BB.
After stimulation with 20 ng/ml of PDGF-BB, the changes in membrane-bound RhoA (▲), H-Ras (◆), and K-Ras (■) were evaluated in the membrane fractions of mesangial cells by means of immunoblot analysis. Data are expressed as the fold-increase over that before stimulation and means±S.E. of 3 independent experiments. *$p < 0.05$ vs. 0 minutes

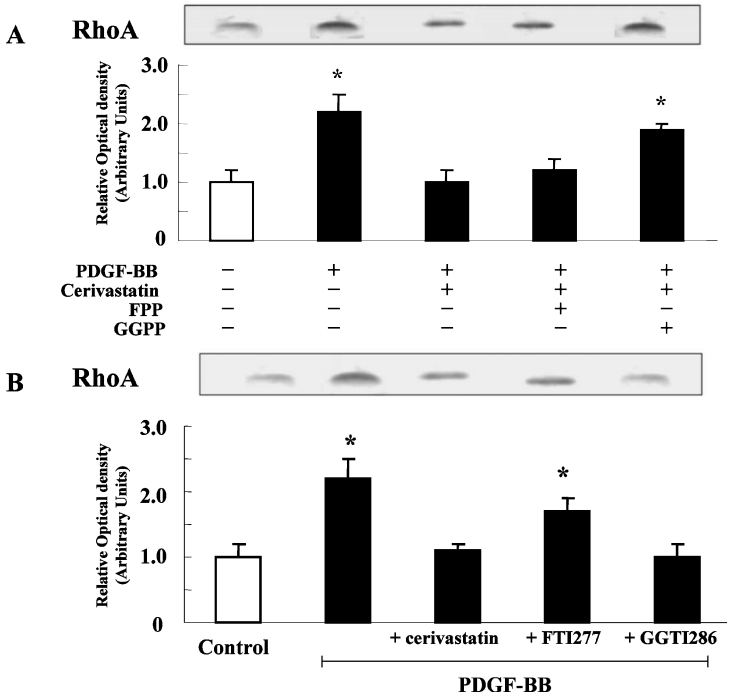

Fig. 2. Effect of PDGF-BB on membrane-bound RhoA and modulation by a statin and prenylation inhibitors. (A) Mesangial cells were pretreated with 2 μM of cerivastatin, cerivastatin plus 10 μM of FPP, or cerivastatin plus 10 μM of GGPP and stimulated with 20 ng/ml of PDGF-BB for 60 minutes. Membrane-bound RhoA was evaluated with immunoblot analysis in membrane fractions. (B) Effects of pretreatment with 2 μM of cerivastatin, 5 μM of FTI277, and 5 μM of GGTI286 were compared after stimulation of PDGF-BB. Data are expressed as the fold-increase versus control and means±S.E. of 3 independent experiments. *$p < 0.05$ vs. control.

3. The effect of a statin, prenylation inhibitors and Rho-kinase inhibitor on PDGF-BB-induced activation of ERK1/2

To investigate involvement of RhoA in the activation of ERK1/2, we evaluated the effect of statins on PDGF-induced activation of ERK1/2 and the recovery of statin-induced suppression of ERK1/2 by supplementation with either FPP or GGPP (Fig. 3A). In the absence of statins, stimulation with 20 ng/ml of PDGF-BB for 10 minutes induced maximal activation of ERK1/2. When the mesangial cells were preincubated with 2 μM of cerivastatin 30 minutes before the addition of PDGF-BB, cerivastatin completely suppressed the PDGF-BB-induced activation of ERK1/2, which was recovered by simultaneous addition of 10 μM GGPP, but not of 10 μM FFP.

We also examined the effects of prenylation inhibitors and Y27632, a specific inhibitor of Rho-kinase, on PDGF-BB-induced activation of ERK1/2 in mesangial cells (Fig. 3B). The mesangial cells were preincubated with 2 μM of cerivastatin, 5 μM of FTI-277, 5 μM of GGTI-286, or 10 μM of Y27632 for 30 minutes before the addition of PDGF-BB. GGTI-286 suppressed the PDGF-BB-induced activation of ERK1/2 to a similar extent to cerivastatin ; however, the introduction of FTI-277 showed less effect. Y27632 suppressed PDGF-BB-induced activation of ERK1/2 in mesangial cells. These data indicate that the Rho/Rho-kinase pathway is involved in the PDGF-BB induced activation of ERK1/2.

4. The effect of a statin, prenylation inhibitor, and Rho kinase inhibitor on PDGF-BB-induced expression of TGF-β

To investigate involvement of the Rho/Rho-kinase pathway in the expression of TGF-β in mesangial cells, we evaluated the effect of statins on PDGF-BB-induced expression of TGF-β mRNA and the

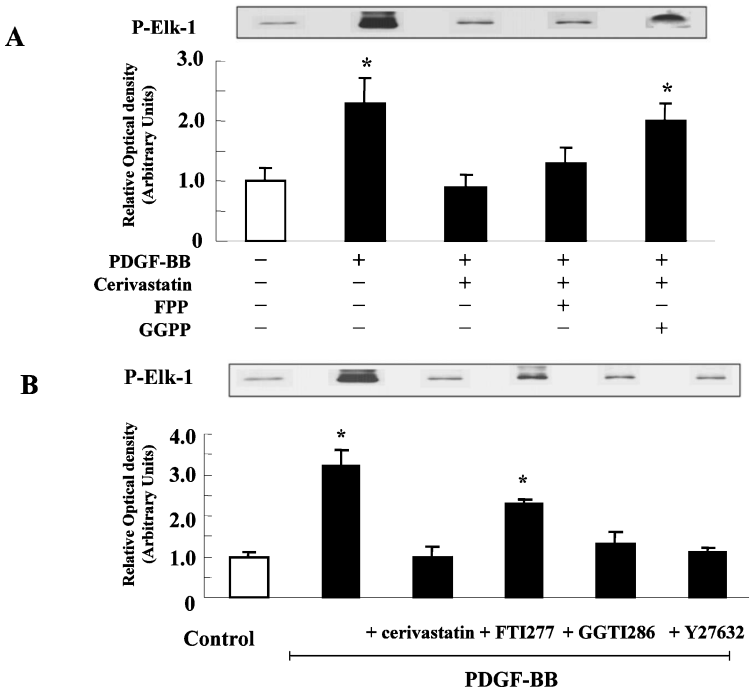

Fig. 3. Effect of PDGF-BB on activity of ERK1/2 and modulation by a statin and prenylation inhibitors.
(A) Mesangial cells were pretreated with 2 μM of cerivastatin, cerivastatin plus 10 μM of FPP, or cerivastatin plus 10 μM of GGPP, and stimulated with 20 ng/ml of PDGF-BB for 10 minutes. Activity of ERK1/2 was determined by evaluating phosphorylated Elk-1 with immunoblot analysis. (B) Effects of pretreatment with 2 μM of cerivastatin, 5 μM of FTI277, 5 μM of GGTI286, and 10 μM of Y-27632 were compared after stimulation of PDGF-BB. Data are expressed as the fold-increase versus control and means±S.E. of 3 independent experiments. *$p<0.05$ vs. control.

recovery of statin-induced suppression of TGF-β mRNA by supplementation with either FPP or GGPP (Fig. 4A). In the absence of statins, stimulation with 20 ng/ml of PDGF-BB for 6 hours induced expression of TGF-β mRNA. When mesangial cells were preincubated with 2 μM of cerivastatin 30 minutes before the addition of PDGF-BB, cerivastatin suppressed PDGF-BB-induced expression of TGF-β mRNA, which had been fully recovered by the simultaneous addition of 10 μM of GGPP, but not of 10 μM of FPP.

We also examined the effect of prenylation inhibitors and Y27632 on PDGF-BB-induced expression of TGF-β mRNA (Fig. 4B). Mesangial cells were preincubated with 2 μM of cerivastatin, 5 μM of FTI-277, 5 μM of GGTI-286, or 10 μM of Y27632 for 30 minutes before the addition of PDGF-BB. GGTI-286 suppressed PDGF-BB-induced expression of TGF-β mRNA to a greater extent than did FTI-277. Furthermore, Y27632 also suppressed PDGF-BB-induced expression of TGF-β mRNA in mesangial cells.

DISCUSSION

RhoA binds and subsequently activates Rho-kinase, a member of a family of serine/threonine kinases[2]. Rho-kinase is believed to be involved in cytoskeletal organization, gene expression, and transformations in the cells of various species[6]. Recent investigations have revealed that Rho-kinase contributes to the development of cardiovascular disease[6,13], participating in the thrombin-stimulated proliferation of vascular smooth muscle cells (VSMCs)[14] and in the oxidized low-density lipoprotein-induced contraction of endothelial cells[15]. In addition, fasudil, a specific inhibitor of Rho-kinase, prevents progression of coronary neointimal formation after stent implantation[16]. Other recent investigations have reported participation of the Rho/Rho-kinase pathway to the develop-

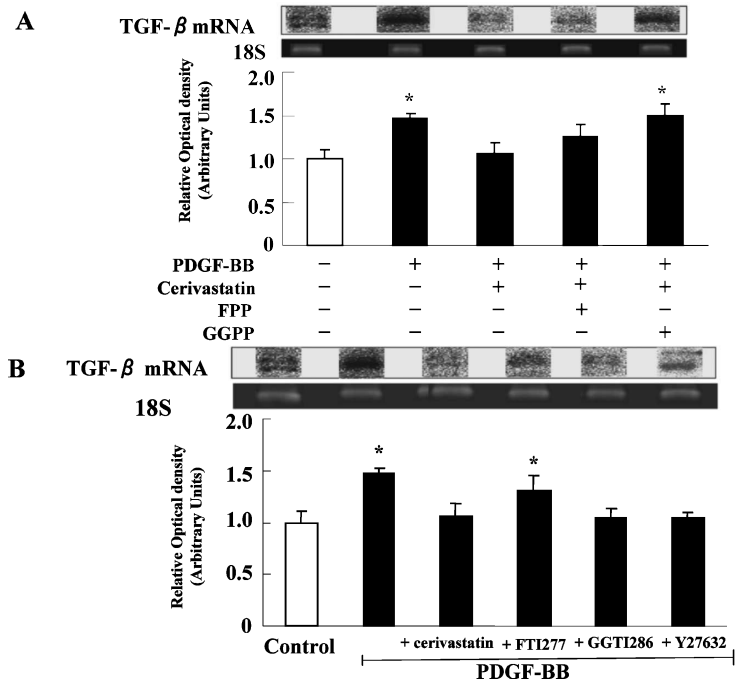

Fig. 4. Effect of PDGF-BB on expression of TGF-β mRNA and modulation by a statin and prenylation inhibitors. (A) Mesangial cells were pretreated with 2 μM of cerivastatin, cerivastatin plus 10 μM of FPP, or cerivastatin plus 10 μM of GGPP and stimulated with 20 ng/ml of PDGF-BB for 6 hours. Mesangial cells were then harvested for Northern blot analysis of TGF-β mRNA. (B) Effects of pretreatment with 2 μM of cerivastatin, 5 μM of FTI277, 5 μM of GGTI286, and 10 μM of Y-27632 were compared after stimulation of PDGF-BB. Data are expressed as the fold-increase versus control and means±S.E. of 3 independent experiments. *$p<0.05$ vs. control.

ment of kidney diseases, using Dahl salt-sensitive rats[17], nephrectomized spontaneously hypertensive rats[18], and ureter-ligated rats[19] as animal models. More recently, we have demonstrated that upregulation of the Rho/Rho-kinase pathway plays a role in the development of diabetic nephropathy[7]. However, the pathological mechanism in which this pathway might be involved remains to be determined. The present study was performed to investigate the contribution of the Rho/Rho-kinase pathway to PDGF-BB-induced expression of TGF-β in mesangial cells.

PDGF-BB increased membrane-bound RhoA in mesangial cells after 30 to 60 minutes of exposure, indicating that PDGF-BB enhances translocation of RhoA from the cytosol to the plasma membrane (Fig. 1). In contrast, changes in membrane-bound H-Ras and K-Ras were insignificant. These minimal changes in membrane-bound Ras do not necessarily indicate an inability of PDGF-BB to activate Ras in mesangial cells because the activity of Ras GTPase was not evaluated in our experiments. We are not able to exclude the possibility that PDGF-BB might activate Ras GTPase with a small change in membrane-bound protein. Translocation of small G proteins to the plasma membrane is facilitated through post-translational modification with covalent binding of isoprenoids to the C-terminus, or via prenylation. In principle, farnesylation, the addition of FPP, is characteristic of the Ras family and geranylgeranylation, the addition of GGPP, is characteristic of the Rho family[2]. The present study confirmed that PDGF-BB-induced increase in membrane-bound RhoA is completely suppressed by statins and that translocation of RhoA in mesangial cells is facilitated via geranylgeranylation (Fig. 2A, B). One of our previous investigations revealed that ERK1/2 is activated in the downstream portion of the Rho/Rho-kinase pathway and is involved in PDGF-BB-induced proliferation of VSMCs[13]. In the present study, PDGF-BB also caused activation of ERK1/2 in mesangial cells,

which was dependent on geranylgeranylation of a small G protein, most likely RhoA (Fig. 3A, B). This similarity between VSMCs and mesangial cells is interesting because both cell types share properties that originate from smooth muscle cells. As confirmation of the contribution of the Rho/Rho-kinase pathway to the activation of ERK1/2, Rho-kinase was inhibited by its specific inhibitor, Y27632. To be exact, Y27632 suppressed PDGF-BB-induced activation of ERK1/2, indicating involvement of the Rho/Rho-kinase pathway in the activation of ERK1/2 in mesangial cells. In addition, PDGF-BB-induced expression of TGF-β mRNA was also dependent on geranylgeranylated small G protein and was suppressed by Y27632 (Fig. 4A, B). These findings indicate that the Rho/Rho-kinase pathway is involved in PDGF-BB-induced expression of TGF-β in mesangial cells. The present study did not confirm a linkage between ERK1/2 and the expression of TGF-β in mesangial cells. However, ERK1/2, an important member of the mitogen-activated protein kinase family, plays a critical role in the upregulation of TGF-β in the diabetic kidney[1,20]. Therefore, a reasonable supposition is that Rho/Rho-kinase pathway enhances the expression of TGF-β via activation of ERK1/2 in mesangial cells.

The precise relationship between ERK1/2 and the Rho/Rho-kinase pathway remains controversial. Several studies have failed to prove the contribution of the Rho/Rho-kinase pathway to the activation of ERK1/2[21,22]. In contrast, others studies, including ours, have found that the Rho/Rho-kinase pathway is involved in the activation of ERK1/2[13,23]. In the mechanism of the proliferation of fibroblasts induced by mechanical stimuli, the Rho/Rho-kinase pathway serves as an upstream regulator of ERK1/2 activation[24]. Lysophosphatidic acid, a potent inducer of the Rho/Rho-kinase pathway, also induces activation of ERK1/2, via its receptor coupled to a heterotrimeric G protein[25]. These conflicting findings may depend on difference in the cell types and stimulants used. Moreover, there is no evidence that Rho/Rho-kinase can activate ERK1/2 as a direct substrate. In this regard, 2 recent studies have shown that the Rho/Rho-kinase pathway enhances translocation of ERK1/2 from the cytosol to the nucleus and promotes gene transcription[26,27]. To our knowledge, our present study is the first to characterize the role played by PDGF-BB in the activation of the Rho/Rho-kinase pathway in mesangial cells, which might be responsible for excessive synthesis of extracellular matrix. However, to clarify the underlying mechanism of the broad contribution of the Rho/Rho-kinase pathway to the pathogenesis of kidney disease, more detailed investigations are required.

In summary, on the basis of our findings, we conclude that the Rho/Rho-kinase pathway contributes to PDGF-BB-induced expression of TGF-β in mesangial cells, possibly via activation of ERK1/2. We have previously reported that the Rho/Rho-kinase pathway is activated in the retinas of diabetic rats and that the Rho-kinase inhibitor fasudil attenuates upregulation of vascular endothelial growth factor in the diabetic retina[28]. The present study not only indicates a contribution of the Rho/Rho-kinase pathway to the pathogenesis of kidney diseases, including diabetic nephropathy, but also supports the possibility that this pathway could be a new molecular target for pharmaceutical treatment of diabetic vascular complications.

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