UltraRapid Communication

Peroxisome Proliferator-Activated Receptor γ Ligands Inhibit Rho/Rho Kinase Pathway by Inducing Protein Tyrosine Phosphatase SHP-2

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Abstract—Although peroxisome proliferator-activated receptor γ (PPARγ) ligands have an antihypertensive effect in vivo, the precise mechanism has not been fully elucidated. We examined their effects on Rho/Rho kinase pathway, a key regulator of vascular tone. In cultured rat aortic smooth muscle cells (RASMC), Rho kinase stimulated by angiotensin II was suppressed by the pretreatment with pioglitazone and troglitazone, and these effects were explained by the inhibition of the Rho translocation to the cell membrane. We evaluated the role of Vav, a GTP/GDP exchange factor upregulating Rho kinase activity, and Src homology region 2–containing protein tyrosine phosphatase-2 (SHP-2), a protein tyrosine phosphatase that dephosphorylated Vav and subsequently inactivated Rho kinase. Both pioglitazone and troglitazone upregulated SHP-2, particularly in the cytosolic fraction, and the SHP-2-bound Vav, and reduced the phosphorylation of Vav. Furthermore, 4-week treatment with pioglitazone lowered systolic blood pressure in spontaneously hypertensive rats (SHR) and suppressed the Rho/Rho kinase activity in aortic tissues isolated from SHR. Consistently, the expression of SHP-2 was upregulated in vascular tissues from pioglitazone-treated SHR. The phosphorylated Vav was increased in SHR, compared with that in normotensive Wistar–Kyoto rats (WKY), which was mitigated by pioglitazone. Finally, both basal and angiotensin II–stimulated levels of Rho kinase activity were greater in RASMC from SHR than those from WKY, and the enhanced Rho kinase activity was blocked by pioglitazone or troglitazone in both strains. Collectively, PPARγ ligands inhibit the Rho/Rho kinase pathway through upregulation of cytosolic SHP-2 expression and inactivation of Vav, and may contribute to the hemodynamic, in addition to metabolic, action in hypertensive metabolic syndrome. The full text of this article is available online at http://circres.ahajournals.org. (Circ Res. 2004;95:e45–e55.)

Key Words: PPARγ, Rho ▪ Rho kinase ▪ SHP-2 ▪ hypertension ▪ Vav ▪ pioglitazone ▪ troglitazone

The peroxisome proliferator-activated receptor γ (PPARγ) is a member of the nuclear receptor family that heterodimerizes with retinoic acid-X receptors and is rendered transcriptionally active by binding to a specific DNA sequence element termed PPAR response element. Substantial evidence has accrued that PPARγ is expressed in all major cells of vascular beds and is activated not only by natural ligands such as free fatty acids and eicosanoids, but also by the insulin-sensitizing, thiazolidinedione-class agents, including troglitazone (TRO), ciglitazone, rosiglitazone, and pioglitazone (PIO). The expression of PPARγ in vascular wall cells suggests its potential role in vascular disease. PPARγ ligands exert depressor action in various models of experimental hypertension, including Zucker fatty rats, obese diabetic rats, diet-induced hypertensive rat, and spontaneously hypertensive rats (SHR), as well as in obese insulin-resistant human subjects. Although several mechanisms have been suggested regarding antihypertensive effects of PPARγ ligands, the precise mechanism by which this class of agent reduces blood pressure remains fully undetermined.

The small GTPase Rho, a member of the Rho subfamily of the Ras superfamily of monomeric GTPases, constitutes an important modulator of vascular smooth muscle contraction. Rho and its downstream effector Rho-associated kinase (Rho kinase) have been shown to regulate the phosphorylation level of myosin light chain of myosin II and contribute to agonist-induced Ca²⁺ sensitization in vascular smooth muscle contraction as an important common vasomotor regulatory factor. Consistently, this pathway participates in the pathogenesis of hypertension and has also been suggested to be linked to insulin resistance. Of note, Rho exhibits both GDP/GTP-binding activity and functions as a molecular switch, cycling between a GDP-bound inactive state (GDP-Rho) and a GTP-bound active state (GTP-Rho). In the resting state, the GDP-Rho dissociation inhibitor binds to GDP-Rho and extracts GDP-Rho from the membrane to the
cytosol. Under the stimulated condition, GDP-Rho is converted to GTP-Rho through the action of guanine nucleotide exchange factors (GEF), among which Vav proteins act as GTP/GDP exchangers for RhoA.14 Recently, it has been demonstrated that the protein tyrosine phosphatase (PTPase), Src homology region 2–containing protein tyrosine phosphatase-2 (SHP-2), previously described as SH2-containing PTPase, regulates Rho activity in vitro,15,16 and the perturbation of SHP-2 activity by a variety of genetic manipulations results in enhanced levels of active RhoA.15 These effects are attributed to the SHP-2–induced GEF dephosphorylation16 and the subsequent suppression of the conversion to GTP-Rho.

Although several mechanisms have been suggested regarding antihypertensive effects of PPARγ ligands,3–7 their effects on the Rho/Rho kinase pathway have not been examined. We therefore hypothesize that PPARγ ligands might affect vascular tone by interfering with the Rho/Rho kinase pathway. In this study, we demonstrated that both PIO and TRO inhibited the Rho/Rho kinase pathway through the upregulation of cytosolic SHP-2 expression in rat vascular smooth muscle cells (VSMCs). The increased SHP-2 binds to Vav protein, retains this protein complex in the cytosolic fraction, and subsequently elicits dephosphorylation and inactivation of Vav protein. Consistent with this in vitro observation, these PPARγ ligands lowered the blood pressure and downregulated the Rho activity in the vascular tissue of SHR. These data provide a novel mechanism of antihypertensive effects of PPARγ ligands and may offer more extensive implication in the treatment of insulin resistance and hypertension.

Materials and Methods

Cell Culture and Experimental Protocols

Rat aortic smooth muscle cells (RASMC) were prepared from thoracic aorta of 2- to 3-month-old Sprague-Dawley rats, 6-week-old SHR, and 6-week-old Wistar–Kyoto rats (WKY) by using the explant technique. The cells were cultured in Dulbecco modified Eagle medium (Invitrogen, Carlsbad, Calif) containing 10% fetal bovine serum (Irvine Scientific, Santa Ana, Calif), 100 U/mL penicillin, 100 mg/mL streptomycin, and 200 mmol/L L-glutamine. For all experiments, early passaged (passage 5 to 8) RASMC were grown 60% to 70% confluence and made quiescent by serum

Figure 1. PPARγ ligands inhibit Ang II–induced Rho/Rho kinase activity in rat aortic smooth muscle cells. A, The treatment with Ang II markedly increased the phospho-MYPT1 level (as a marker for Rho kinase activity). Both pioglitazone (a) and troglitazone (b) inhibited the Ang II–stimulated Rho kinase activity in a dose-dependent manner (top). Densitometric analysis of immunoblots is shown as fold-induction of quiescent cells after being normalized with expression level of total MYPT1 (bottom, n = 4). B, PPARγ ligands block the translocation of RhoA into the membrane fraction induced by Ang II. Five-minute exposure to Ang II caused upregulation of RhoA in the membrane (a) fraction, but not cytosolic (b) fraction, and this effect was suppressed by the pretreatment with pioglitazone (left panel) and troglitazone (right panel). Densitometric analysis of immunoblots is shown as fold-induction of quiescent cells after normalized with expression level of total Rho A in the same cell lysates (n = 4). C, PPARγ ligands attenuate the GTP-binding to Rho induced by Ang II. D, PPARγ ligands at the concentrations of 10 to 100 nmol/L had no effects on the expression of the Ang II type I receptor. Each blot was a representative result of 4 independent experiments. Results are presented as mean ± SEM (n = 4). #P < 0.05 vs quiescent cells; *P < 0.05 vs Ang II; **P < 0.01 vs Ang II.
starvation (0.4%, fetal bovine serum) for 24 hours. PPARγ ligands, PIO (kindly provided by Takeda Pharmaceuticals, Tokyo) and TRO (kindly provided by Sankyo Pharmaceuticals, Tokyo) were added 12 hours before the addition of angiotensin II (Ang II) (100 nmol/L; Sigma, St Louis, Mo) or platelet-derived growth factor (PDGF) (20 ng/mL; Sigma). Cell lysates were then obtained 6 hours or 5 minutes after the treatment with Ang II or PDGF.

Subcellular Fractionation
RASMC were harvested in 100 μL lysis buffer containing 20 mmol/L of Tris-HCl, 250 mmol/L of sucrose, and PMSF, as well as aprotinin and leupeptin (10 g/mL each) 5 minutes after the Ang II treatment. After 3 cycles of freeze and thaw, samples were centrifuged at 250 g at 4 °C for 5 minutes. The supernatant was then centrifuged at 100 000 g for 30 minutes at 4 °C. The supernatant was saved as cytosolic fraction. The pellet was dissolved in lysis buffer containing 1% Triton X-100 and saved as membrane fraction. The protein of each fraction was subjected to SDS-PAGE and immunoblotting.

Immunoblotting
Immunoblotting was performed as described previously using specific antibodies against phospho-myosin phosphatase target subunit-1 (MYPT1; Upstate Biochemistry, Lake Placid, NY), total MYPT1 (Santa Cruz Biotechnology, Santa Cruz, Calif), RhoA (Upstate), SHP-2 (Santa Cruz), phospho-Vav (Santa Cruz), Vav (Upstate), β-actin (Sigma), and Ang II type I receptor (Santa Cruz). Samples for vascular tissue were obtained by homogenizing tissues in lysis buffer as described previously.17 Immunoreactive bands were detected using an ECL detection kit (Amersham Biosciences, Uppsala, Sweden).

Rho Activity
The activity of Rho was measured by Rho Activation Assay Kit according to the manufacturer’s instructions (Upstate). Briefly, RASMC were lysed in RIPA buffer (50 mmol/L Tris-HCl, pH 7.4, 150 mmol/L NaCl, 1% NP-40, 2.5% Na-deoxycholate, 1 mmol/L EDTA, 1 μg/mL PMSF, 1 μg/mL aprotinin, 1 μg/mL leupeptin, 1 μg/mL pepstatin, 1 mmol/L Na3VO4, 1 mmol/L NaF). The same amount of total protein was incubated with GST-RBD (Rho-binding domain of rhotekin) to precipitate GTP-bound RhoA. Precipitated GTP-bound RhoA were resolved on SDS-PAGE and immunoblotted using monoclonal antibody against RhoA (Santa Cruz).

In Vivo Role of Rho/Rho Kinase as a Target for a PPARγ Ligand
Six-week-old male SHR and WKY weighing 130 to 140 grams were used. They were fed a standard rat chow (15 g/d, 0.38% sodium, 0.97% potassium, and 25.1% protein; Nippon Clea, Tokyo) with or without PIO (160 mg/kg per day) and were allowed free access to tap water throughout the experimental protocols. Rats were assigned to 4 groups: group 1, SHR (n=5); group 2, SHR given PIO (n=5); group 3, WKY (n=5); and group 4, WKY given PIO (n=5). PIO was administered by adding the drug to chow. Systolic blood pressure was measured by tail-cuff method every week. At week 5, the rats in each group were decapitated and abdominal aortas were collected. Phospho-MYPT1 expressions, SHP-2, and phospho-Vav in the aortic vascular tissue of SHR and WKY were determined by
Western blotting. All experiments were performed in accordance with the animal experimentation guideline of Keio University School of Medicine.

**Immunohistochemistry**

Vascular tissue samples were obtained from SHR, WKY, and PIO-treated SHR. They were fixed using 10% formaldehyde. Histological samples were embedded in paraffin and 4-μm sections were used for immunohistochemical analysis. After the quenching of the endogenous peroxidase activity, sections were incubated with the primary antibody against SHP-2 at 1:800 dilutions and phospho-Vav at 1:800 for 1 hour at room temperature. The slides were incubated with appropriate secondary antibodies for 30 minutes. The sections were rinsed in phosphate-buffered saline, and incubated in 0.02% 3,3’-diaminobenzidine tetrahydrochloride.

**Statistics**

Data are expressed as mean±SEM. Data were analyzed by 1-way or 2-way ANOVA as appropriate, followed by Bonferroni multiple comparison post hoc test. P<0.05 was considered statistically significant.

**Results**

**PPARγ Ligands Inhibit Ang II–Induced Activation of Rho Kinase**

To examine whether PPARγ ligands suppressed the Rho/Rho kinase activity, phosphorylated levels of MYPT1, one of the substrates of Rho-kinase, were examined in RASMC, with the use of phospho-MYPT1 levels at the inhibitory site (Thr696) as a marker for Rho kinase activity. Stimulation with Ang II (100 nmol/L) significantly increased the level of MYPT1 phosphorylation (3.13±0.30-fold induction, P<0.05 versus quiescent cells, n=4; Figure 1A). Pretreatment with PIO for 12 hours attenuated the Ang II–induced Rho kinase activation in a dose-dependent manner (10 nmol/L, 35.7±5.5% inhibition, P<0.05, n=4; 100 nmol/L, 64.3±7.6% inhibition, P<0.01 versus Ang II, n=4). Similarly, TRO markedly inhibited the Rho kinase activity (10 nmol/L, 36.5±6.8% inhibition, P<0.05, n=4; 100 nmol/L, 55.4±8.4% inhibition, P<0.01 versus Ang II, n=4).

In the quiescent state, Rho binds to GDP and resides in the cytosol. On activation, GDP-Rho is converted to GTP-Rho...
through the action of GEF, and GTP-Rho partially translocates from the cytosol to the membrane fraction. To confirm the inhibitory action of PPARγ ligands on the Ang II–induced Rho activation, we examined the RhoA expression in membrane and cytosol fractions. As shown in Figure 1B, Ang II (100 nmol/L) elevated the level of RhoA in the membrane fraction (3.14 ± 0.58-fold induction, \( P < 0.05 \) versus quiescent cells, \( n = 6 \)). The pretreatment with PIO reduced the RhoA activity in the membrane fraction in a dose-dependent manner (10 nmol/L, 62.4 ± 7.4% inhibition; 100 nmol/L, 86.8 ± 6.9% inhibition, both \( P < 0.05 \) versus Ang II, \( n = 4 \)). Similar effects were observed with TRO, with a significant inhibition attained at 100 nmol/L TRO (92.4 ± 5.9% inhibition, \( P < 0.05 \) versus Ang II, \( n = 4 \)). The RhoA level in the cytosol fraction was not altered by pretreatment of either ligand.

We next examined the effects of PPARγ ligands on the binding status of Rho with GTP. As shown in Figure 1C, Ang II increased the levels of GTP-bound Rho, as assessed by a pull-down assay using the Rho-binding domain of rhotekin, which interacted only with GTP-bound Rho. The upregulation of GTP-bound Rho was suppressed by the 12-hour pretreatment with both PIO and TRO. In concert, PPARγ ligands blocked the Rho/Rho kinase pathway by their inhibitory effects on the GTP–GDP exchange reaction and the membrane translocation of Rho.

In VSMCs, PPARγ ligands have been shown to downregulate Ang II type I receptors, which could explain the inhibitory actions of PIO and TRO on Rho/Rho kinase in this experiment. We therefore examined the expression levels of Ang II type I receptors in PIO-treated or TRO-treated RASMC. As shown in Figure 1D, the expression levels of Ang II type I receptors were unaltered by either ligand at concentrations of 10 to 100 nmol/L.

**PPARγ Ligands Induce Protein Tyrosine Phosphatase of SHP-2 in Cytosolic Fraction**

We examined the effects of PPARγ ligands on the expression of SHP-2, a factor that has been shown to regulate the RhoA activity. As illustrated in Figure 2A, the protein expression of SHP-2 in total cell lysate was upregulated by both PIO (10 nmol/L, 2.2 ± 0.6-fold, \( P < 0.05 \), \( n = 4 \)); 100 nmol/L, 2.4 ± 0.3 fold induction, \( P < 0.01 \) versus quiescent cells, \( n = 4 \)) and TRO (10 nmol/L, 1.7 ± 0.2-fold, \( P < 0.05 \), \( n = 4 \)); 100 nmol/L, 1.8 ± 0.3 fold induction, \( P < 0.01 \) versus quiescent cells, \( n = 4 \)).

We further evaluated the fractional (ie, cytosol versus membrane) changes in SHP-2 expression by the treatment...
with PPARγ ligands. Both PIO and TRO (100 nmol/L) prominently upregulated the SHP-2 expression in the cytosolic fraction by 4.8±0.2-fold and 2.8±0.1-fold, respectively (P<0.01 versus quiescent cells, n=4) (Figure 2B). The levels of membrane-associated SHP-2, however, were not altered by either ligand (data not shown).

**PPARγ Ligands Enhance the Protein Interaction Between SHP-2 and Vav Protein**

We evaluated the interaction between SHP-2 and Vav in RASMC by immunoprecipitation with antibody against SHP-2, followed by immunoblotting with antibody against Vav. The treatment with PIO and TRO (10 and 100 nmol/L) increased the interaction between SHP-2 and Vav (Figure 3A, a). In contrast, PIO or TRO failed to alter the level of SHP-2–bound p190Rho GTPase activating protein (p190RhoGAP), which is reported to act as a negative regulator for Rho by converting active GTP-Rho to inactive GDP-Rho (Figure 3A, b).

We next evaluated the phosphorylation of Vav using phospho-specific antibody against Vav. As shown in Figure 3B, Ang II stimulated tyrosine phosphorylation of Vav by 2.2±0.3-fold, and the Ang II–induced tyrosine phosphorylation of Vav was inhibited by PIO (10 nmol/L, 25.3±5.8% inhibition; 100 nmol/L, 83.7±3.6% inhibition, P<0.05 versus Ang II, n=4) and TRO (10 nmol/L, 49.6±2.9% inhibition; 100 nmol/L, 94.0±2.7% inhibition, P<0.05 versus Ang II, n=4) in a dose-dependent manner.

**Effects of PPARγ Ligands on PDGF-Induced Rho Kinase Activation in RASMC**

Rho kinase is activated not only by the stimulation of G-protein coupled receptor (eg, Ang II receptor) but also by the tyrosine kinase receptor activation (eg, PDGF receptor). Given that PPARγ ligands block Rho/Rho kinase pathway through inhibitory effects on Vav activation, PPARγ ligands can also inhibit PDGF-induced Rho kinase activation, because Vav is activated by receptor tyrosine kinase. As shown in Figure 4A, both PIO and TRO blocked PDGF-induced Rho kinase activation (PIO, 25.5±5.8% and 86.8±10% inhibition for 10 and 100 nmol/L, respectively, P<0.05 versus PDGF, n=4; TRO, 49.0±3.9% and 89.0±3.3% inhibition for 10 and 100 nmol/L, respectively, P<0.05 versus PDGF, n=4). Consistently, PDGF-induced tyrosine phosphorylation of Vav was attenuated by the pretreatment with PIO (10 nmol/L, 36.3±3.7% inhibition; 100 nmol/L, 59.6±1.2% inhibition, P<0.01 versus PDGF, n=4) or TRO (10 nmol/L, 16.9±3.6% inhibition; 100 nmol/L, 49.4±2.1% inhibition, P<0.05 versus PDGF, n=4). Collectively, PPARγ ligands block the Rho/Rho kinase pathway by the upregulation of cytosolic SHP-2 expression and the inactivation of Vav, which serves to function as GEF for Rho (Figure 5).

**PPARγ Ligand Lowers Blood Pressure and Attenuates Rho Kinase Activation in SHR**

To confirm these in vitro effects by PPARγ ligands, we treated the hypertensive rats with PIO. The treatment with PIO markedly reduced the blood pressure in SHR (Figure 6A). Thus, the blood pressure in SHR treated with PIO was markedly lower than that in SHR at 9 (P<0.01), 10 (P<0.01), and 11 weeks (P<0.05); after 4 weeks of the PIO treatment, the blood pressure nearly reached the level observed in WKY. In contrast, PIO had no effects on blood pressure in WKY.

Whether the PPARγ ligands suppressed the Rho kinase activity was evaluated in the aortic vascular tissue of SHR, with the use of phospho-MYPT1 levels as a marker for Rho kinase activity. In the vascular tissue of 11-week-old SHR, phospho-MYPT1 was increased by 4.1±0.9-fold (versus WKY, P<0.01, n=6), compared with that in age-matched WKY (Figure 6B). The 4-week treatment with PIO significantly attenuated the increased expression of phospho-MYPT1 in SHR (46.4±9.2% inhibition versus SHR, P<0.05, n=5). In contrast, PIO had no effect on the activity of Rho kinase in the vascular tissue of WKY.

**PPARγ Ligand Upregulates SHP-2 and Attenuates Phosphorylation of Vav in the Vascular Tissues in SHR**

To verify the mechanism for antihypertensive effects of PPARγ ligands, we further examined the expression of SHP-2 and phosphorylation levels of Vav in the vasculature of SHR. Immunoblotting against SHP-2 demonstrated that PIO treatment upregulated SHP-2 levels in the aortic tissue (3.2±0.7-fold induction versus WKY; P<0.01, n=5; Figure 7A, a and d). Immunoblotting against tyrosine-phosphorylated Vav showed that tyrosine phosphorylation of Vav was...
increased in the vascular tissues in SHR (2.9 ± 0.7-fold induction versus WKY; \( P < 0.05, n = 5 \); Figure 7A, b and e), which was attenuated by treatment with PIO (48.8 ± 9.0% inhibition versus SHR, \( P < 0.05, n = 5 \); Figure 7A, b and e). We also conducted immunohistochemical analysis for expression levels of SHP-2 and phospho-Vav within the vascular tissues. The staining level of SHP-2 was upregulated in the VSMC in PIO-treated SHR as compared with that in SHR or WKY (Figure 7B, a through c). The staining of phospho-Vav was upregulated in vascular tissues of SHR in comparison with that of WKY (Figure 7B, d and e). The treatment with PIO partially attenuated the increased staining level in the vascular tissues of SHR (Figure 7B, e and f).

**Effects of PPAR\(\gamma\) Ligands on Ang II–Induced Rho Kinase Activation in WKY and SHR**

Finally, we investigated the effects of PPAR\(\gamma\) ligands on Ang II–induced Rho kinase activation in WKY and SHR. Basal levels of Rho kinase activity as assayed by phospho-MYPT1 were higher in SHR than those in WKY (Figure 8, lanes 1 and 5). Ang II enhanced the Rho/Rho kinase activity in VSMCs from both WKY and SHR, which was higher in SHR than in WKY (lanes 2 and 6). The pretreatment with PIO or TRO markedly attenuated the Ang II–stimulated Rho/Rho kinase in both WKY (lanes 3 and 4) and SHR (lanes 7 and 8). Of note, in SHR, both PIO and TRO mitigated the Rho/Rho kinase activity to the level of WKY (lanes 7 and 8 versus lanes 3 and 4).

**Discussion**

In this study, we examined the effects of PPAR\(\gamma\) on the Rho/Rho kinase pathway in the cultured VSMCs. Thus, under the Ang II–stimulated condition whereby Rho kinase is augmented, PPAR\(\gamma\) ligands caused the upregulation of SHP-2 (a protein tyrosine phosphatase) and the dephosphorylation of Vav protein, which subsequently decreased the GTP-bound Rho and Rho kinase activity. Furthermore, we found that PIO reduced systemic blood pressure and suppressed the elevated Rho kinase activity in the vascular tissue of SHR. It has been reported that PPAR\(\gamma\) ligands stimulate the protein tyrosine phosphatase activity in VSMCs. In the present study, we have identified for the first time to our knowledge that protein tyrosine phosphatase SHP-2 represents a candidate of target molecules for PPAR\(\gamma\) ligands and have provided evidence that the stimulated SHP-2 suppresses the Rho kinase activity and therefore would modify vascular tone.

The cellular signaling mechanism linking the PPAR\(\gamma\) and the Rho/Rho kinase pathway remains undetermined. We have recently demonstrated that the treatment with PPAR\(\gamma\) ligands increases the activity of protein tyrosine phosphatase in RASMS, which subsequently inactivates protein kinase \(C\)\(\delta\) and inhibits mitogenic induction of p21\(^{Cip1}\).\(^{17}\) SHP-2 is a widely expressed cytoplasmic protein tyrosine phosphatase and regulates cell signaling triggered by various cytokines or growth factors.\(^{23}\) Furthermore, it has been shown that suppression of SHP-2 activity by a variety of genetic manipula-
tions augments RhoA activity, and SHP-2 is suggested to regulate RhoA activity in vitro. We therefore examined whether PPARγ ligands affected the SHP-2 expression and found that both PIO and TRO upregulated the SHP-2 expression in a dose-dependent manner (Figure 2A). Thus, the upregulation of SHP-2 by PPARγ would inhibit the Rho kinase pathway and may contribute to antihypertensive effects of PPARγ ligands. However, it has also been reported that SHP-2 acts positively to modulate the strength of signals emanating from some ligand-activated receptor protein tyrosine kinases such as PDGF receptors and FGF receptors, although PPARγ ligands have been shown to block the proliferation and migration of various cells induced by PDGF or FGF. In the present study, we demonstrated that the SHP-2 expression in cytosolic fraction was increased by Ang II, whereas the expression of SHP-2 recruited to the cell membrane was unaltered (Figure 2B). It is therefore reasonable to speculate that cytosolic rather than membrane-bound SHP-2 plays an important role in the beneficial action (e.g., inhibition of Rho kinase and cell migration) of this factor. This premise, however, requires further investigations.

The present study did not reveal the mechanism of upregulation of SHP-2 by PPARγ ligands, nor have there been reports indicating the presence of the PPAR response element within the promoter region of SHP-2 on chromosome 11 of rat genome. Recently, it has been demonstrated that nitric oxide upregulates SHP-2 and suppresses Rho/Rho kinase activity. Because PPARγ ligands stimulate nitric oxide synthesis in VSMCs, nitric oxide may serve to act as a mediator conveying signals from PPARγ ligands to SHP-2.

In the present study, we further evaluated the mechanism of the SHP-2–induced Rho inhibition. One possible mechanism of these effects is that SHP-2 suppresses the activity of GEF by dephosphorylating the tyrosine residue of GEF. The present study has demonstrated that both PIO and TRO increase the interaction between SHP-2 and Vav (Figure 3A, a) and reduce the phospho-Vav level augmented by Ang II (Figure 3B). Because Vav is expressed in the cytosol, the enhanced interaction between SHP-2 and Vav would result in a negative impact on the subsequent signaling event, including the Rho kinase activation. Although the mechanisms of this tethering effect of PPARγ have not been elucidated, integrin may regulate the recruitment of SHP-2 to the extent that PPARγ ligands downregulate integrin expression. Thus, PPARγ ligands might inhibit the membrane translocation of SHP-2 through modulating the expression of some isoform of integrin.
Several lines of studies have witnessed that the Rho kinase requires GTP binding for its activation, the process of which is mediated partially by GEF. Although there are >40 GEF known, we have focused on Vav as a candidate of GEF for Rho A because its catalytic activity is modulated by direct tyrosine phosphorylation, and Vav is physically associated with SHP-2 in mitogenic stimulation of hematopoietic cells. Furthermore, one isoform of Vav proteins, Vav2, has been reported to modulate Rho activity through the interaction of SHP-2. Thus, Kodama et al reported that a dominant-negative mutant of SHP-2 markedly increased the formation of stress fibers and focal adhesions in MDCK cells, and this augmented action was blocked by a dominant-negative mutant of Vav2. Although they did not evaluate the change in phosphorylated levels of Vav2 by transfection of dominant-negative SHP-2, we did demonstrate that PPARγ ligands decreased the phospho-Vav levels stimulated by Ang II (Figure 3B) and PDGF (Figure 4B). Therefore, in VSMCs, a phosphorylated level of Vav is regulated by SHP-2, and this regulation plays a pivotal role in the inhibitory effect of PPARγ ligands on Rho activation. Conversely, p190RhoGAP that acts as a negative regulator for Rho has also been reported to interact with SHP-2, and its activity is regulated by tyrosine phosphorylation. In this current study, however, either PIO or TRO had no effect on levels of p190RhoGAP bound to SHP-2 (Figure 3A, b).

Another possible mechanism for the inactivation of Ang II–induced Rho/Rho kinase pathway by PPARγ ligands is their effects on the expression of Ang II type I receptors. PPARγ ligands have been shown to downregulate the expression of Ang II type I receptors in RASMC. At the concentration used in this experiment (10 to 100 nmol/L), however, we found that PPARγ ligands had no effects at concentrations <1 μmol/L. Moreover, we have shown the inhibitory effects of PPARγ on PDGF-induced Rho/Rho kinase activation (Figure 4). In concert, it appears that the effect of PPARγ on the Rho/Rho kinase pathway is mediated mainly by the action independent of Ang II receptor levels in VSMCs at least at physiological concentrations.

Although it is well-established that the PPARγ ligand is capable of reducing the blood pressure, the precise mechanism for its antihypertensive action remains fully undetermined. Rosiglitazone has been reported to have calcium antagonistic actions, which may directly inhibit voltage-dependent calcium channels. PPARγ ligands also stimulate nitric oxide release from endothelial cells, and may thus contribute to the amelioration of hypertension. Alternatively, PPARγ ligands reduce endogenous production of endothelin-I and have beneficial effects in endothelin-dependent hypertension such as DOCA-salt rats. In the present study, we have demonstrated that PIO reduces the blood pressure in SHR (Figure 6A). More importantly,
PPARγ ligands downregulated the activated Rho/Rho kinase in the vascular tissues from SHR (Figure 6B), which was accompanied by a decrease in phospho-Vav and an increase in SHP-2 (Figure 7A). Furthermore, both PIO and TRO suppressed the Rho/Rho kinase activity during Ang II stimulation in VSMCs form SHR (Figure 8). Because Rho kinase constitutes a critical determinant of the vascular tone as a final common pathway in downstream signaling and is activated in various models of hypertension independent of the cause of hypertension, it is surmised that the Rho/Rho kinase pathway is a plausible target for PPARγ ligands. Collectively, it is strongly suggested that multiple mechanisms participate in the reduction in the blood pressure by PPARγ.

Role of Rho kinase in hypertension merits comments. As shown in Figure 6B, the Rho/Rho kinase pathway is activated in SHR as compared with that in WKY, a finding consistent with the recent report by Moriki et al. Furthermore, the previous in vivo demonstration that a Rho kinase inhibitor (Y-27632) reduces systemic blood pressure in SHR but not WKY endorses the formulation that the Rho/Rho kinase pathway is activated in SHR. Our current finding that PIO lowers blood pressure in SHR but has no antihypertensive effect in normotensive animals therefore parallels the effect on the Rho kinase activity (Figure 6). In this regard, the Rho/Rho kinase pathway exerts cell-proliferative action, and VSMCs from SHR is reported to grow faster than that from WKY. Several molecules, including EGF receptors and PDGF receptors, are supposed to be attributed to these phenotypic abnormalities, and Vav is activated by receptor tyrosine kinases on stimulation of these receptors. It is therefore suggested that enhanced Rho/Rho kinase activity after the stimulation of these receptors in SHR are attributable to the abnormal characteristics of VSMCs from SHR. Collectively, the novel mechanism for abnormalities in the vasculature and elevated systemic pressure may facilitate the progression of vascular injury in genetically hypertensive subjects, which could be ameliorated by beneficial actions of PPARγ ligands.

Clinical implication of the Rho inhibition by PPARγ ligands merits comment. The Rho/Rho kinase pathway participates in a variety of cellular functions in both muscle and nonmuscle cells, including stress-fiber formation, cell differentiation, migration, and proliferation. The inhibition of this pathway by PPARγ ligands therefore would provide novel information on the mechanism for various biological effects. Thus, PPARγ ligands inhibit migration of VSMCs and monocytes, which may result from their effects on the Rho/Rho kinase signaling. Moreover, PPARγ ligands ameliorate the insulin signaling impaired by TNFα and free fatty acid. In this regard, activation of the Rho/Rho kinase pathway induces serine phosphorylation of IRS-1 and inhibits insulin signaling in cultured VSMCs. We also found that pharmacological blockade of the Rho/Rho kinase pathway ameliorated insulin resistance in obese Zucker rats (unpublished observation). PPARγ ligands may therefore improve insulin signaling, at least in part, through the blockade of the Rho/Rho kinase signaling activated by various cytokines and growth factors such as TNFα or Ang II. Furthermore, the modulation of cytosolic SHP-2 expression and phosphorylation status of Vav by PPARγ ligands can explain some other effects of PPARγ ligands, including the inhibition of the transcriptional activity of nuclear factor of activated T cells (NFAT) in T cells and STAT-1 pathway, which constitutes a part of the anti-inflammatory processes.

In conclusion, we have demonstrated that PIO exerts a profound hemodynamic action in SHR but not in WKY. In this type of hypertensive animal in which Rho kinase is activated, PIO markedly attenuates the Rho kinase activity in the vascular tissue. Furthermore, PPARγ ligands inhibit the Rho/Rho kinase pathway through the upregulation of SHP-2 and dephosphorylation of Rho regulator, Vav. These effects of PPARγ ligands may provide novel information on the mechanisms for pleiotropic biological functions of these agents in vascular and nonvascular tissues and could expand clinical usefulness of these agents in metabolic syndrome.

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References
Peroxisome Proliferator-Activated Receptor γ Ligands Inhibit Rho/Rho Kinase Pathway by Inducing Protein Tyrosine Phosphatase SHP-2
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