**Peroxisome Proliferator-activated Receptor γ Ligands Inhibit Mitogenic Induction of p21Cip1 by Modulating the Protein Kinase Cδ Pathway in Vascular Smooth Muscle Cells**

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Vascular smooth muscle cells (VSMC) in the normal vessel wall proliferate at a very low frequency. Injury to the endothelium results in the release of mitogens that stimulate quiescent, G0/G1-arrested VSMC to reenter the cell cycle, replicate DNA, and divide. VSMC hyperplasia is a key event in the formation of restenotic and atherosclerotic lesions in the vasculature (1, 2). Cyclin-dependent kinases (CDKs) are serine-threonine protein kinases that regulate cell cycle progression after forming complexes with and being activated by cyclins (3). Mitogenic activation of cyclin D/CDK4, cyclin D/CDK6, and cyclin E/CDK2 during the G1 phase results in phosphorylation of retinoblastoma gene (Rb) proteins. In its hyperphosphorylated state, Rb functions as a gatekeeper for the G1 → S transition by binding and sequestering E2F, a transcription factor that induces the expression of a battery of genes that encode the enzymatic machinery for S phase DNA synthesis. Cyclin-dependent kinase inhibitors (CDKIs) can negatively regulate the mitogen-induced cascade of G1 events by inhibiting CDK activity and preventing Rb phosphorylation (3, 4).

Consistent with the view that CDKIs are major negative regulators of the cell cycle, overexpression of Cip/Kip family members (p21Cip1, p27Kip1) blocks the G1 exit in VSMC and other cell types (5, 6). Data emerging from recent studies, however, suggest that CDKIs p21Cip1 and p27Kip1 can also function as positive regulators during the G1 phase as assembly factors to promote formation of cyclin/CDK holoenzyme complexes (3, 7). Such a role for CDKIs may explicate apparent paradoxical observations that p21Cip1 is frequently up-regulated after mitogenic stimulation of quiescent cells (8, 9). In rat VSMC cell lines, prevention of mitogen-induced p21Cip1 expression by antisense oligodeoxynucleotides attenuated both DNA synthesis and cell proliferation (10). We recently reported that ligands for the nuclear receptor peroxisome proliferator-activated receptor gamma (PPARγ) inhibited Rb phosphorylation and G1 → S transition in rat aortic VSMC (11). Interestingly, the antiproliferative effects of PPARγ ligands were accompanied by an inhibition of the induction of p21Cip1 by platelet-derived growth factor (PDGF) plus insulin. This inhibition of the mitogenic induction of p21Cip1 by PPARγ ligands may be a
mechanism contributing to the arrest of VSMC in the G1 phase.

The protein kinase C (PKC) pathway plays a pivotal role in regulating p21\(^{Cip1}\) expression, and it can be divided into three groups on structural and functional bases (12): the conventional (c)PKC isozymes \(\alpha, \beta, \) and \(\gamma\), which are dependent on calcium, diacylglycerol, and phospholipid for activity; the novel (n)PKC isozymes \(\delta, \epsilon, \theta, \) and \(\gamma\), which are calcium-independent; and the atypical (a)PKC isozymes \(\xi, \) and \(\tau\), which are independent of both calcium and diacylglycerol. Control of p21\(^{Cip1}\) levels by PKC signaling appears to be complex, involving transcriptional (13) and post-transcriptional mechanisms (14). Several previous studies have demonstrated that PPAR\(\gamma\) ligands can inhibit signaling through PKC pathways (15, 16). In the present study, we investigated whether PPAR\(\gamma\) ligands inhibit the up-regulation of p21\(^{Cip1}\) during G1 in VSMC through effects on PKC. We report that PPAR\(\gamma\) ligands attenuated mitogen-induced p21\(^{Cip1}\) expression at the post-translational level by enhancing protein-tyrosine-phosphatase (PTPase) activity and decreasing PKC\(\xi\) phosphorylation and activity.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Treatment with Growth Factor and Reagents**—Rat aortic smooth muscle cells (RASMC) were prepared from thoracic aorta of 2–3-month-old Sprague-Dawley rats by using the explant technique. The cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% FBS (Irvine Scientific, Santa Ana, CA), 100 units/ml penicillin, 100 mg/ml streptomycin, and 200 mM l-glutamine. The cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% FBS were infected with Aedes aegypti virus (AMV) and the recombinant adenovirus encoding human PKCr\(\xi\) (Ad-PKCr\(\xi\)) at the multiplicity of infection of 100 pfu/cell. Following the infection, the cells were incubated in maintenance medium for 24 h. The medium was then replaced with fresh medium containing 10% FBS, and the cells were allowed to grow for 7 days. The cells were harvested at 7 days post-infection (dpi) for analysis.

**Measurement of Total PTPase Activity**—RASMC were harvested at the indicated time after the stimulation with PDGF. Cells were lysed in phosphate-free buffer (20 mM Tris-HCl, pH 7.4, 100 mM NaCl, 0.5% Triton X-100, and 1 mg/ml bovine serum albumin) using a sonicator. The PTPase activity in the whole cell lysate was measured by a nonradioactive photometric enzyme immunosassay kit (Roche Molecular Biochemicals, Indianapolis) as described previously (20). The reactions were performed directly in the wells of a microtiter plate with 100 mM biotinylated tyrosine-phosphorylated peptide substrate bound to the streptavidin matrix. The addition of 100 \(\mu\)M vanadate to quench the reactions, the fractions of unmetabolized substrate in the reactions were determined immunochromically using peroxidase-conjugated anti-phosphotyrosine antibodies. The development of color was monitored at 405 nm in a microtiter plate reader. (Sigma).

**RESULTS**

**PPAR\(\gamma\) Ligands Attenuate the Mitogenic Induction of p21\(^{Cip1}\) at a Post-transcriptional Level**—PPAR\(\gamma\) ligands attenuated the induction of p21\(^{Cip1}\) in quiescent RASMC induced to enter the S phase by stimulation with the competence factor PDGF (0.67 nM) and the progression factor insulin (1 \(\mu\)M) (11). Insulin at these supraphysiologic concentrations likely also induces mitogenic signaling through the insulin-like growth factor I pathway. To investigate the mechanism of this regulation more precisely, we analyzed the kinetics of p21\(^{Cip1}\) induction by mitogens. In quiescent RASMC, p21\(^{Cip1}\) protein expression was almost undetectable but was induced as early as 2 h after mitogenic stimulation with PDGF plus insulin (P+1) (Fig. 1A, P+1). Protein levels of p21\(^{Cip1}\) continued to increase up to 24 h after treatment. Pretreatment of PPAR\(\gamma\) ligands TRO and RSG had no effect on the early phase induction of p21\(^{Cip1}\) (2–6 h). In marked contrast, accumulation of p21\(^{Cip1}\) protein at later stages of the mitogenic response (12–24 h) was attenuated significantly by these ligands (24 h; TRO, 67 ± 8.5% inhibition; RSG, 62 ± 6.2% inhibition; p < 0.05 versus P+1, n = 3, Fig. 1A, and Ab). To investigate whether these effects were exerted via regulation of p21\(^{Cip1}\) mRNA, we analyzed the kinetics of mRNA induction by Northern blotting. Consistent with the protein induction, p21\(^{Cip1}\) mRNA levels were induced as early as 1 h after mitogenic stimulation with P+1 (Fig. 1B, P+1). No further induction of p21\(^{Cip1}\) mRNA was observed between 12 and 24 h despite sustained protein induction during this period. TRO and RSG had no effect on p21\(^{Cip1}\) mRNA expression (Fig. 1B,
versus P inhibition; GF 109203X, 51.0
also enhanced the degradation of p21 Cip1 protein with an esti-
imated half-life of 2.1 h (Fig. 2, A). The PKC
ligands, TRO (P 1+TRO) and RSG (P 1+RSG). In Northern
blotting, the bands for Chinese hamster ovary B (CHO-B) were used to
assess equal loading of samples. Panel Ab, densitometric analysis is
shown as x-fold induction over levels in quiescent RASMC. The results
presented are the mean ± S.E.; *p < 0.05 versus P 1+I, n = 3.
P 1+TRO and P 1+RSG). These data suggest that PPARγ ligands attenuated mitogenic induction of p21Cip1 at a post-
transcriptional level by decreasing accumulation of p21Cip1 protein.

Mitogenic Induction of p21Cip1 Is Dependent on a PKC Path-
way, Likely PKCδ—One mechanism identified which regulates mitogenic induction of p21Cip1 involves the PKC pathway. We
therefore explored the possible involvement of the PKC pathway
in mitogen-induced p21Cip1 expression and its inhibition by PPARγ ligands. Pretreatment with the PKC specific inhibi-
tors calphostin C and GF 109203X attenuated the mitogen-
induced expression of p21Cip1 (24 h; calphostin C, 53.8 ± 9.6% inhibition; GF 109203X, 51.0 ± 8.7% inhibition; both p < 0.01
group of 10 nM) was added 12 h after the addition of mitogen, and at the indicated time after
cycloheximide administration, steady-state levels of p21 Cip1 protein
were determined by immunoblotting (panel A). Panel B, the intensity of
bands was quantified and plotted as log% of the expression at 0 h. Data
represent the means of three independent experiments.

PPARγ Ligands Regulate p21Cip1 in VSMC—The effect of PDGF and insulin on the PKCδ-depend-
ent induction of p21Cip1 was checked separately because they
function as a competence factor and progression factor, respec-
tively, for entry into the S phase. TRO and RSG inhibited the
protein induction of p21Cip1 by PDGF, whereas the induction by
insulin was small and not affected by either ligand (Fig. 4, A
and B). TRO was more potent than RSG in inhibiting PDGF-
duced p21Cip1 expression. Unlike RSG, TRO is a bifunctional
ligand for PPARγ and contains an α-tocopherol moiety confer-
ing a potential antioxidant activity that could contribute to its
effect on p21Cip1. PDGF-induced expression of p21Cip1 was also
potently inhibited by the PKCδ inhibitor rottlerin, although insulin-induced p21Cip1 expression was not affected (Fig. 4, A and B). Although TRO, RSG, and rottlerin inhibited PDGF-dependent induction of p21Cip1 to a similar extent, this does not necessarily indicate a common mechanism of action. These results, however, do identify PKCδ as a potential target for the effects of PPARγ ligands on p21Cip1.

**PPARγ Ligands Attenuate PDGF-induced Enzymatic Activity of PKCδ**—To explore further the potential relationship among PPARγ ligands, PKCδ, and p21Cip1 expression, we examined the effects of PPARγ ligands on PKCδ activity after stimulation of VSMC with either PDGF or insulin. First, we checked the kinetics of activation of PKCδ in response to stimulation with PDGF because it was reported recently that PDGF initiated two distinct phases (acute and sustained) of PKC activity in HepG2 cells (21). We measured specific activity for PKCδ by using anti-PKCδ antibody and by eliminating Ca2+ from the reaction solution in immunocomplex kinase assay. As early as 5 min after the stimulation with PDGF, PKCδ activity transiently increased by 3.6-fold. After this initial surge, we observed a smaller sustained peak 12–18 h after the stimulation (~2.5-fold induction, Fig. 5A). Because our data suggested that the regulation of p21Cip1 expression decreased at a post-transcriptional level 12 h and later after mitogenic stimulation (Fig. 1), we focused on the late phase activity of PKCδ (Fig. 5B). PPARγ ligands TRO and RSG attenuated PDGF-induced PKCδ activity (TRO, 97.2 ± 7.2% inhibition; RSG, 67.6 ± 7.9% inhibition; both p < 0.01 versus PDGF alone, n = 4). Importantly, insulin did not induce PKCδ activity at 12 h after stimulation, and PPARγ ligands had no effect on the PKCδ activity at this time point. This result is consistent with our data that the induction of p21Cip1 by insulin was not affected with either PPARγ ligand (Fig. 4). Taken together, these data identified the PDGF → PKCδ pathway as a putative target for PPARγ ligand regulation of p21Cip1 expression.

**Overexpression of PKCδ Reversed the Inhibitory Effects of PPARγ Ligands on Mitogenic Induction of p21Cip1**—To confirm that inhibition of PKCδ activity by PPARγ ligands results in the attenuation of the mitogenic induction of p21Cip1, we examined the effects of overexpressing PKCδ on their inhibitory effects. RASMC infected with 20 pfu/cell of adenovirus harboring wild-type PKCδ gene expressed high levels of PKCδ compared with control RASMC infected with 20 pfu/cell of an adenovirus harboring the lacZ gene as assayed by immunoblotting (Fig. 6A). RASMC overexpressing PKCδ exhibited an ~5-fold increase in enzymatic activity in response to PDGF compared with cells infected with adenovirus expressing lacZ (Fig. 6B). Overexpression of PKCδ prevented PPARγ ligand-mediated down-regulation of p21Cip1 expression (Fig. 6C).

**PPARγ Ligands and a Src Kinase Inhibitor Attenuate PDGF-induced Tyrosine Phosphorylation of PKCδ**—Activation of the PDGF or epidermal growth factor receptors induces tyrosine phosphorylation of PKCδ (22–25). In some systems, tyrosine phosphorylation of PKCδ induces serine/threonine kinase activity of PKCδ. We therefore examined tyrosine phosphorylation of PKCδ in PDGF-treated RASMC and the impact of PPARγ ligands on this process. To detect tyrosine-phosphorylated PKCδ, total cell lysates were immunoprecipitated with anti-PKCδ antibody. Consistent with previous reports (24, 25), PDGF induced tyrosine phosphorylation of PKCδ with biphasic kinetics (5 min, 4.2-fold; 18 h, 2.1-fold induction) similar to that seen for PKCδ activity (Fig. 7A). Because PPARγ ligands inhibited mitogen-induced p21Cip1 expression at 12–24 h, we focused on the late phase phosphorylation of PKCδ. As shown in Fig. 7B, 18 h after the stimulation by PDGF, phosphorylation of PKCδ was enhanced, and this increased phosphorylation was attenuated by TRO and RSG. Phosphorylation of PKCδ was also blocked by the Src kinase inhibitor PP2, but not by the inactive analog PP3, consistent with the previous observation that PDGF induces tyrosine phosphorylation of PKCδ through the activation of Src kinase (24, 25) (Fig. 7B). To assess the functional role of c-Src-mediated phosphorylation of PKCδ, we also examined the effects of PP2 on the activity of PKCδ. As shown in Fig. 7C, PP2 but not PP3 inhibited the enzyme activity of PKCδ (PP2, 94.6 ± 10% inhibition; p < 0.01 versus PDGF alone, n = 4). Similarly, PP2 but not PP3 attenuated the PDGF-induced p21Cip1 expression (PP2, 96.3 ± 7.2% inhibition; p < 0.01 versus PDGF alone, n = 4, Fig. 7D). Phosphorylation of c-Src at pY416 within the catalytic domain leads to its activation (26). PDGF induced c-Src phosphorylation at pY416 at 5 min. The Src inhibitor PP2 completely blocked this phosphorylation, whereas the inactive analog PP3 had no effect. Neither TRO nor RSG at 10 μM affected PDGF-induced phosphorylation of c-Src at pY416 (Fig. 7E). Collectively, these results suggest that PDGF induces tyrosine phosphorylation of PKCδ via a Src-dependent pathway, which leads...
to the increased enzyme activity of PKCδ and the elevated expression of p21Cip1. Moreover, PPARγ ligands likely inhibit the activity of PKCδ by attenuating the tyrosine phosphorylation of PKCδ downstream of c-Src phosphorylation and activation.

**PTPase Inhibitors Reversed the Effects of PPARγ Ligands in RASMC**—Dephosphorylation of PKCδ by PPARγ ligands could result from PPARγ regulation of PTPase activity. To test this hypothesis, we first checked the effect of a PTPase inhibitor on the regulation of p21<sup>Cip1</sup> by PPARγ ligands. Specific PTPase inhibitors, sodium orthovanadate (Na<sub>3</sub>VO<sub>4</sub>) and dephostatin, were administered 6 h after the stimulation of PDGF. The inhibitory effect of TRO and RSG on PDGF-induced expression of p21<sup>Cip1</sup> was totally reversed by the addition of both PTPase inhibitors (Fig. 8A). Neither inhibitor had any effect on p21<sup>Cip1</sup> expression in quiescent RASMC (data not shown). To confirm this hypothesis further, we examined whether PTPase inhibitors could also reverse the inhibitory effects of PPARγ ligands on the PDGF-induced tyrosine phosphorylation of PKCδ and PKCγ activity. Both PTPase inhibitors blocked the effect of PPARγ ligands to decrease tyrosine phosphorylation of PKCδ (Fig. 8B) and inhibit its activity (Fig. 8C). PPARγ ligand-mediated activation of PTPase provides an attractive mechanism for their effect to attenuate PKCδ tyrosine phosphorylation and kinase activity of PKCδ, accelerate p21<sup>Cip1</sup> turnover, and decrease cellular concentration of p21<sup>Cip1</sup>.

**PPARγ Ligands Increase PTPase Activity in RASMC**—Finally, we examined the effect of PPARγ ligands on PTPase activity in PDGF-stimulated RASMC. As shown in Fig. 8B, PDGF had no significant effect on PTPase activity in RASMC total cell lysates. PPARγ ligands, however, significantly enhanced PTPase activity 12 and 18 h after stimulation with PDGF (12 h: TRO, 2.6 ± 0.3-fold, p < 0.01 versus PDGF alone n = 3; RSG, 1.9 ± 0.1-fold, p < 0.05 versus PDGF alone, n = 3; 18 h: TRO, 2.6 ± 0.1-fold; RSG, 2.1 ± 0.6-fold; both p < 0.05 versus PDGF alone, n = 3) (Fig. 9). These data support a mechanism by which PPARγ ligands activate PTPase to attenuate tyrosine phosphorylation and enzymatic activity of PKCδ, resulting in the destabilization and reduced expression of p21<sup>Cip1</sup> protein.

**FIG. 7. PPARγ ligands and Src kinase inhibitor attenuate PDGF-induced tyrosine phosphorylation of PKCδ.** Panel A, time course of tyrosine phosphorylation of PKCδ after the stimulation with 0.67 nm PDGF. Tyrosine-phosphorylated PKCδ was analyzed as described under “Experimental Procedures.” The results presented are the mean ± S.E.; **p < 0.01, *p < 0.05 versus activity at time 0.** Panel B, quiescent RASMC were preincubated with 10 μM TRO, 10 μM RSG, 10 μM selective Src inhibitor PP2, 10 μM inactive analog PP3, and dimethyl sulfoxide (control) for 30 min prior to the addition of PDGF. After 12 h, tyrosine-phosphorylated (upper panel) and total (lower panel) PKCδ were analyzed as described under “Experimental Procedures.” Panel C, quiescent RASMC were incubated with 10 μM PP2, 10 μM PP3 for 30 min prior to the addition of PDGF. After 12 h, the enzyme activity of PKCδ was analyzed. The results presented are the mean ± S.E.; **p < 0.01 versus PDGF-stimulated RASMC.** Panel D, quiescent RASMC were incubated with PP2, PP3, and dimethyl sulfoxide (control) for 30 min prior to the addition of PDGF. After 24 h, 30 μg of whole cell protein was assayed by immunoblotting using anti-p21<sup>Cip1</sup> antibody.

**Discussion**

The CDKI p21<sup>Cip1</sup> is a classical negative regulator of G<sub>1</sub> → S progression through its well described activity to inhibit cyclin-CDK holoenzymes that phosphorylate Rb to permit movement past the G<sub>1</sub> restriction point (4). Several recent studies, however, have suggested that p21<sup>Cip1</sup> can also function at a low concentration as a positive regulator during G<sub>1</sub> by promoting the assembly of stable cyclin-CDK complexes (3, 7). We have shown previously that PPARγ ligands inhibit G<sub>1</sub> → S progression of RASMC by attenuating Rb hyperphosphorylation and inhibiting cyclin D-dependent kinase activity (11). Inhibition of cyclin D-dependent kinase by PPARγ ligands occurred in the absence of an effect on cyclin D expression but was associated with an elevation in p27<sup>Kip1</sup> and Rb. We hypothesized that PPARγ ligands shifted the balance of CDKIs and cyclin-CDKs in G<sub>1</sub> to favor formation of enzymatically inactive cyclin D-CDK4/p27<sup>Kip1</sup> and cyclin D-CDK6/p27<sup>Kip1</sup> trimers. PPARγ ligands, however, also suppressed mitogen-in-
PPARγ Ligands Regulate p21<sup>Cip1</sup> in VSMC

Control of p21<sup>Cip1</sup> levels by PKC signaling could involve transcriptional, post-transcriptional, translational, and/or post-translational mechanisms (12). In the present study, we demonstrated that activation of PKCδ by mitogens stabilized p21<sup>Cip1</sup> protein against proteolytic degradation. The half-life of p21<sup>Cip1</sup> protein in mitogen-stimulated RASMC was 3.6 h, which decreased to ~2.1 h in the presence of the PKCδ-selective inhibitor rottlerin. Post-transcriptional regulation of p21<sup>Cip1</sup> by PKCδ is probably the main mechanism underlying its up-regulation by mitogens because pharmacological blockade of PKCδ signaling had no effect on p21<sup>Cip1</sup> mRNA levels. The precise mechanism by which PKCδ stabilizes p21<sup>Cip1</sup> protein remains to be elucidated, but it could involve interactions with the ubiquitin-proteasome pathway that triggers its degradation. Interestingly, there have been several reports that PKC activation can inhibit protein ubiquitination (28, 29). Entry of p21<sup>Cip1</sup> into the ubiquitin-proteasome pathway is also controlled by its interaction with proliferating cell nuclear antigen to form complexes that are resistant to degradation (30). Formation of p21<sup>Cip1</sup>-proliferating cell nuclear antigen complexes is negatively regulated by phosphorylation of p21<sup>Cip1</sup> at Ser-146 in its C terminus. Our data suggest that PKCδ signaling positively regulates p21<sup>Cip1</sup> by increasing its stability so that phosphorylation of Ser-146 is probably the main mechanism underlying p21<sup>Cip1</sup> stabilization during PKCδ signaling.

PPARγ ligands also blocked the mitogenic induction of p21<sup>Cip1</sup> by inhibiting PKCδ signaling and accelerating its degradation. Although insulin increased p21<sup>Cip1</sup> protein levels, neither PPARγ ligands nor pharmacological blockade of PKCδ signaling impacted this process. In marked contrast, both treatments potently attenuated PDGF-induced p21<sup>Cip1</sup> accumulation. Furthermore, PDGF had an acute and sustained effect of increasing PKCδ activity, which was attenuated by PPARγ ligands. In a variety of cell types, PKCδ is phosphorylated on the tyrosine residue in response to PDGF. PKCδ is also phosphorylated by various nonreceptor tyrosine kinases such as Fyn (22, 23), and c-Src (23), and growth factor receptors (22, 23). More recently, Krounfeld <i>et al.</i> (25) reported that Src-related kinases mediate PDGF-stimulated tyrosine phosphorylation of PKCδ in C6 glioma cells. Our data also support a role for Src in PKCδ tyrosine phosphorylation in PDGF-stimulated RASMC.

Engagement of tyrosine kinase growth factor receptors by mitogens results in Src-dependent tyrosine phosphorylation of PKCδ which is associated with an increased enzymatic activity and its translocation from the cytosol to the membrane fractions (31). For the PDGF receptors, increased intracellular hydrogen peroxide may be the second messenger for biological effects (32), and hydrogen peroxide has been shown to trigger the phosphorylation of PKCδ which increased its enzymatic activity (33). Such a mechanism may explain the rapid increase in PKCδ activity we observed as early as 5 min after stimulation of RASMC with PDGF. Even at much later times after PDGF stimulation, a good correlation persisted between the kinetics for PKCδ enzymatic activity and tyrosine phosphorylation. Both PPARγ ligands and the specific Src kinase inhibitor PP2 attenuated PDGF-stimulated tyrosine phosphorylation
and activity of PKCδ consistent with these two events being functionally related.

What is the molecular mechanism(s) responsible for the inhibition of PKCδ tyrosine phosphorylation and activity by PPARγ ligands? Decreased tyrosine phosphorylation of PKCδ could result from either an inhibitory effect of PPARγ ligands on tyrosine kinases, such as c-Src, or through a stimulatory effect on PTPases that act on PKCδ. PPARγ ligands had no effect on PDGF-induced tyrosine phosphorylation and activation of c-Src. Tyrosine phosphatase inhibitors, however, abrogated both the inhibition of PKCδ activity and down-regulation of p21<sup>Cip1</sup> by PPARγ ligands, which supports increased PTPase activity as a more likely mechanism of action (see Fig. 10 depicting proposed mechanism). Our finding that TRO and RSG increased PTPase activity in RASM is in accord with previous studies showing a similar effect by nuclear receptors other than PPARγ (34). Which specific PTPase(s) mediates the PPARγ-dependent decrease in PKCδ tyrosine phosphorylation? Regulation of PKCδ phosphorylation by phosphatases is poorly understood. Parekh et al. (35) have recently shown that PTEN, a dual specificity tyrosine, lipid phosphatase, inhibits PKCδ activity by decreasing phosphorylation at Thr-505 and Ser-662. Site-directed mutagenesis and overexpression of PTEN devoid of lipid phosphatase activity revealed that regulation of PKCδ resulted from PTEN's phosphoinositide-3-phosphatase activity on phosphatidylinositol 3,4,5-trisphosphate, a second messenger in the phosphoinositide-3-kinase signaling pathway. Possible contributions of the tyrosine phosphatase activity in PTEN, however, have not been explored fully. Interestingly, we have observed a 2-3-fold induction of PTEN expression by PPARγ ligands in RASM. Additional studies are required to ascertain whether there is a relationship between PTEN and PPARγ regulation of PKCδ as well as the identification of the specific PTPase(s) induced by PPARγ and their involvement in dephosphorylating PKCδ.

If the inhibition of PKCδ-dependent up-regulation of p21<sup>Cip1</sup> levels by PPARγ ligands contributes to their antiproliferative effect on VSMC, then both PKCδ and p21<sup>Cip1</sup> should function as positive regulators. In the proliferation and migration of VSMC. In vivo studies have shown whether activation of PKCδ signaling with rottlerin markedly increases p27<sup>Kip1</sup>-CDKI levels, which would prevent G<sub>1</sub> → S progression, support the studies implicating a role for PKCδ in promoting VSMC proliferation.

Similarly, Assender et al. (36) showed that endothelin-1-induced mitogenicity in VSMC was related to the sustained activation of PKCδ. In contrast, in a VSMC cell line stably overexpressing PKCδ, Fukumoto et al. (37) observed an inhibition of VSMC proliferation. These discrepant findings might be explained by the type of VSMC used: primary cells versus established cell line, or by the specific experimental conditions employed: activation of endogenous PKCδ by physiological concentrations of mitogen versus overexpression of PKCδ. Our data showing that inhibition of PKCδ signaling with rottlerin markedly increases p27<sup>Kip1</sup>-CDKI levels, which would prevent G<sub>1</sub> → S progression, support the studies implicating a role for PKCδ in promoting VSMC proliferation.

The role of p21<sup>Cip1</sup> in VSMC proliferation has also recently become controversial through studies suggesting that it can function as either a positive or negative regulator of growth depending on its cellular concentration (7). After arterial injury, p21<sup>Cip1</sup> is up-regulated, and its presence in the neointima correlated inversely with intimal VSMC proliferation (38). Moreover, Nabel and colleagues (5) have shown that adenoviral delivery and expression of p21<sup>Cip1</sup> into balloon-injured porcine arteries markedly attenuated intimal hyperplasia. Tanner et al. (39) also observed that p21<sup>Cip1</sup> in VSMC or human atherosclerotic lesions was most abundant in more advanced lesions in regions of low proliferation. Thus, in vivo studies favor an inhibitory role for p21<sup>Cip1</sup> in controlling VSMC proliferation.

If p21<sup>Cip1</sup> does function in vivo as a negative cell cycle regulator, then what is the physiological relevance of its down-regulation by PPARγ ligands in RASM? Up-regulation of p21<sup>Cip1</sup> by survival factors such as insulin-like growth factor I is required for effective suppression of apoptosis stimuli (40). PDGF is a potent survival factor for many cell types, including VSMC (41). Inhibition of PDGF-stimulated p21<sup>Cip1</sup> expression by PPARγ ligands, therefore, may block survival signaling and promote apoptosis. In fact, PPARγ ligands induce apoptosis in VSMC and tumor cells (42, 43). It will be of interest to determine whether PPARγ ligand inhibition of mitogenic and survival signaling pathways both contribute to their in vivo effect to attenuate restenotic and atherosclerotic lesions.

In summary, we report a novel mechanism for PPARγ ligands to accelerate p21<sup>Cip1</sup> turnover by inhibiting PKCδ signaling. Given the prominent role for p21<sup>Cip1</sup> in VSMC proliferation and apoptosis, this may partially explain the efficacy of PPARγ ligands in the treatment of proliferative vascular disease (44).

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