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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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The cyclin-dependent kinase inhibitor p21Cip1 is up-regulated in response to mitogenic stimulation in various cells. PPARγ ligands troglitazone (TRO, 10 μM) and rosiglitazone (RSG, 10 μM) attenuated the induction of p21Cip1 protein by platelet-derived growth factor (PDGF) and insulin without affecting cognate mRNA levels in rat aortic smooth muscle cells (RASMC). The protein kinase Cδ (PKCδ) inhibitor rottlerin also blocked the induction of p21Cip1 protein, whereas the conventional PKC isotype inhibitor Gö 6976 had no effect. Kinetic studies using the protein synthesis inhibitor cycloheximide showed that TRO, RSG, and rottlerin shortened the half-life of p21Cip1 protein. TRO, RSG, and rottlerin inhibited PDGF-induced expression of p21Cip1, but they did not affect insulin-induced expression of p21Cip1. Both ligands inhibited PKCδ enzymatic activity in PDGF-stimulated RASMC but not in insulin-stimulated cells. Adeno-virus-mediated overexpression of PKCδ rescued the down-regulation of p21Cip1 expression both by TRO and RSG in PDGF-treated RASMC. These data suggested that the PKCδ pathway plays a critical role in PDGF-induced expression of p21Cip1 in RASMC and may be the potential target for PPARγ ligand effects. Src kinase-dependent tyrosine phosphorylation of PKCδ was decreased substantially by TRO and RSG. Tyrosine phosphorylation and activation of c-Src in response to PDGF were unaffected by either PPARγ ligand. Protein-tyrosine-phosphatase inhibitors sodium orthovanadate and dephostatin prevented PPARγ ligand effects on PKCδ tyrosine phosphorylation and enzymatic activity. Both inhibitors also reversed PPARγ ligand effects on p21Cip1 expression in PDGF-treated RASMC. PPARγ ligands enhanced protein-tyrosine-phosphatase activity in RASMC, which may be the mechanism for decreased PKCδ tyrosine phosphorylation and activity. PPARγ ligands regulate p21Cip1 at a post-translational level by blocking PKCδ signaling and accelerating p21Cip1 turnover.

Vascular smooth muscle cells (VSMC)1 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 serinethreonine 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. Cyclindependent 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 anti-sense 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

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The abbreviations used are: VSMC, vascular smooth muscle cells; CDK, cyclin-dependent kinase; CDKI, cyclin-dependent kinase inhibitor; Rb, retinoblastoma; PPARγ, peroxisome proliferator-activated receptor gamma; PDGF, platelet-derived growth factor; PKC, protein kinase C; PTase, protein-tyrosine-phosphatase; RASMC, rat aortic smooth muscle cells; FBS, fetal bovine serum; TRO, troglitazone; RSG, rosiglitazone; PP2 and PP3, protein phosphatases 2 and 3, respectively; pfu, plaque-forming units; FTEN, phosphatase and tensin homolog deleted from chromosome 10.

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mechanism contributing to the arrest of VSMC in the G1 phase. The protein kinase C (PKC) pathway plays a pivotal role in regulating p21Cip1 expression, and it can be divided into three groups on structural and functional bases (12): the conventional (c)PKC isoenzymes α, β, and γ, which are dependent on calcium, diacylglycerol, and phospholipid for activity; the novel (n)PKC isoenzymes δ, ε, θ, and η, which are calcium-independent; and the atypical (a)PKC isoenzymes ζ and τ, which are independent of both calcium and diacylglycerol. Control of p21Cip1 levels by PKC signaling appears to be complex, involving transcriptional (13) and post-transcriptional mechanisms (14). Several previous studies have demonstrated that PPARγ ligands can inhibit signaling through PKC pathways (15, 16).

In the present study, we investigated whether PPARγ ligands inhibit the up-regulation of p21Cip1 during G1 in VSMC through effects on PKC. We report that PPARγ ligands attenuated mitogen-induced p21Cip1 expression at the post-translational level by enhancing protein-tyrosine-phosphatase (PTPase) activity and decreasing PKC8 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 (Irvin Scientific, Santa Ana, CA), 100 units/ml penicillin, 100 mg/ml streptomycin, and 200 μM l-glutamine. For all experiments, early pasaged (passages 5–8) RASMC were grown to 60–70% confluence and made quiescent by serum starvation (0.4%, FBS) for at least 24 h. Unless specified otherwise, PPARγ ligands and pharmacologic inhibitors were added 30 min before the addition of mitogens, human recombinant PDGF-BB (Sigma), and/or insulin (Eli Lilly, Indianapolis) at a final concentration of 0.67 ng/20 μg/ml and 1 μM, respectively. For all data shown, each individual experiment was performed using an independent preparation of RASMC. Troglitzatone (TRO) was kindly provided by Parke Davis (Ann Arbor, MI). Rosiglitazone (RSG) was a generous gift from GlaxoSmithKline (King of Prussia, PA). PKC inhibitors, GF 109203X, calphostin C, Gö 6976, and rottlerin were purchased from Biomol (Plymouth Meeting, PA). Cycloheximide was from Sigma. The selective Src family protein-tyrosine kinase inhibitor, PP2 (17), the inactive analog, PP3, as well as the PTPase inhibitors sodium orthovanadate and dephostatin were obtained from Calbiochem.

*Adenoviral Infection of RASMC—* Adenovirus encoding mouse PKC8 (Ax-PKC8), driven by a modified chicken β-actin promoter was constructed as reported previously (18). Recombinant type 5 adenovirus expression vector was generated using the Adeno-X Expression System (CLONTECH Laboratories, Inc. Palo Alto, CA). RASMC in Dulbecco’s modified Eagle’s medium containing 10% FBS were infected with Ax-PKC8 or Ax-LacZ at 20 pfu/cell. 48 h after infection, RASMC were made quiescent by serum starvation (0.4% FBS) for 24 h and stimulated with PDGF in the presence or absence of PPARγ ligands. 24 h after the stimulation with PDGF, total cell lysates were obtained and analyzed by Western blotting using antibody against nPKC8 (Santa Cruz). Infection efficiency (>90%) was established by staining for β-galactosidase activity using a β-galactosidase staining kit (Invitrogen, Carlsbad, CA).

*Western Blotting—* Western blotting was performed as described previously (11) using specific antibodies against nPKC8, p21Cip1 (6246, Santa Cruz) and phosphorylated Src (pY416, Upstate Biochemistry, Lake Placid, NY) at a 1:200 concentration. Immunoreactive bands were detected using an ECL detection kit (Amersham Biosciences, Inc.).

*Immunoprecipitations—* RASMC (in a 100-mm plate) were lysed in 1 ml of ice-cold RIPA buffer (1× phosphate-buffered saline, pH 7.4, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml leupeptin and aprotinin). Cell lysates were incubated with agarose-conjugated anti-phosphotyrosine antibody (Santa Cruz), which recognized all phosphotyrosines nonspecifically. The immune complexes were analyzed by immunoblotting with anti-PKC8 antibody to detect tyrosine-phosphorylated PKC8.

*Isolation and Analysis of RNA—* Total RNA was isolated using TRiZol reagent (Life Technologies, Inc.). 25 μg of total RNA was used for Northern blotting. The probe for p21Cip1 was radiolabeled with [α-32P]dCTP using the Rediprime II random prime labeling system (Amersham Biosciences, Inc.). Blots were also probed with Chinese hamster ovary B cDNA to assess equal loading of samples.

*PKC8 Activity Assay—* RASMC (in 100-mm plates) were harvested in 1 ml of ice-cold RIPA buffer and homogenized by repeated aspiration through a 21-gauge needle. Cell debris was removed by centrifugation at 10,000 × g for 10 min at 4 °C. After precipitating the lysate by adding 0.25 μg of normal rabbit IgG together with 20 μl of resuspended protein G-agarose (Santa Cruz), 1 μg of anti-PKCS antibody was added to the supernatant. After incubation for 1 h at 4 °C, 20 μl of protein G-agarose was added and incubated at 4 °C overnight with gentle rocking. To measure the background activity of whole cell lyses, one sample from the incubation mixture. The immunoprecipitates were collected by centrifugation at 10,000 × g for 5 min at 4 °C and washed four times with 1× phosphate-buffered saline. The pellet was suspended in 50 μl of phosphate-buffered saline and stored in ice before kinase assay. PKC8 activity was measured using the protein kinase C enzyme assay (Amersham Biosciences, Inc.) as described before (19). 25 μl of assay mixture (50 mM Tris-HCl, pH 7.5, 0.05% sodium azide, 2 mM l-α-photophosphatidylserine, 6 μg/ml phosphol 12-meristato 13-aceta, 7.5 mM dithiothreitol, and 225 μM PKC substrate peptide) without calcium chloride, 25 μl of ATP buffer (50 mM Tris-HCl, pH 7.5, 0.05% sodium azide, 150 mM ATP, 45 mM magnesium acetate), and 0.2 μM of [γ-32P]ATP were added to each immunoprecipitated sample and incubated for 20 min at room temperature. The reaction was terminated and 30 μl of stop solution (500 mM CuCl2, 10 μM PMSF, 0.5 μg/ml aprotinin) were added to separate phosphorylated peptide. The papers were washed with 5% acetic acid twice and then were transferred to scintillation vials for 32P counting. The presence of PKC8 in the immunoprecipitate was confirmed by Western blotting.

**RESULTS**

*PPARγ Ligands Attenuate the Mitogenic Induction of p21Cip1 at a Post-transcriptional Level—* PPARγ ligands attenuated the induction of p21Cip1 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 μM) (11). Insulin at these supraphysiological concentrations likely also induces mitogenic signaling through the insulin-like growth factor 1 pathway. To investigate the mechanism of this regulation more precisely, we analyzed the kinetics of p21Cip1 induction by mitogens. In quiescent RASMC, p21Cip1 protein expression was almost undetectable but was induced as early as 2 h after mitogenic stimulation with PDGF plus insulin (P+I) (Fig. 1A, P+I). Protein levels of p21Cip1 continued to increase up to 24 h after treatment. Pretreatment of PPARγ ligands TRO and RSG had no effect on the early phase induction of p21Cip1 (2–6 h). In marked contrast, accumulation of p21Cip1 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+I, n = 3, Fig. 1, Ab, and Ab).

To investigate whether these effects were exerted via regulation of p21Cip1 mRNA, we analyzed the kinetics of mRNA induction by Northern blotting. Consistent with the protein induction, p21Cip1 mRNA levels were induced as early as 1 h after mitogenic stimulation with P+I (Fig. 1B, P+I). No further induction of p21Cip1 mRNA was observed between 12 and 24 h despite sustained protein induction during this period. TRO and RSG had no effect on p21Cip1 mRNA expression (Fig. 1B,
P inhibiton; GF 109203X, 51.0/H11006 also enhanced the degradation of p21 Cip1 protein with an estimated half-life of 1.5 h for TRO and 1.6 h for RSG (both were preincubated with 10 nM TRO and 10 nM RSG for 30 min prior to the addition of 0.67 nM PDGF plus 1 mM insulin (P=I). Panel A, time-dependent induction of protein (panel a) and mRNA (panel b) of p21Cip1 by the stimulation with PDGF plus insulin (P+I) and the effects of PPARγ ligands, TRO (P+I+TRO) and RSG (P+I+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+I, n = 3.

Mitogenic Induction of p21Cip1 Is Dependent on a PKC Pathway, 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 inhibitors 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 versus P-I, n = 4, Fig. 2A). Because GF 109203X and calphostin C can inhibit both conventional and novel PKCs, we attempted to identify which PKC isomorph(s) was required for the mitogenic induction of p21Cip1. Inhibition of conventional PKC with Gö 6976 had no effect on mitogenic induction of p21Cip1 (Fig. 2A). The PKCδ-specific inhibitor rottlerin markedly suppressed p21Cip1 induction in a dose-dependent manner (2.5 μM; 61 ± 9.8% inhibition, p < 0.01 versus P+I, n = 4; Fig. 2A and B). We also examined the effect of rottlerin on the kinetics of p21Cip1 induction. Similar to the effects of PPARγ ligands, accumulation of p21Cip1 protein at later times after stimulation was attenuated by rottlerin. Rottlerin had no effect on p21Cip1 mRNA expression (data not shown).

PPARγ Ligands and PKCδ Inhibitor Decrease the Stability of p21Cip1 Protein in Mitogen-stimulated RASMC—The stability of p21Cip1 protein was examined in mitogen-stimulated RASMC. After treatment of cells with PDGF and insulin for 12 h, cells were cultured with 10 μg/ml cycloheximide to inhibit de novo protein synthesis. At the indicated time, steady-state levels of p21Cip1 protein were determined by immunoblotting (Fig. 3A). The half-life of p21Cip1 protein in mitogen-stimulated cells was estimated at 3.6 h (n = 3, Fig. 3B). In contrast, in cells treated with PPARγ ligands, the half-life of p21Cip1 was shortened to 1.5 h for TRO and 1.6 h for RSG (both n = 3). Rottlerin also enhanced the degradation of p21Cip1 protein with an estimated half-life of 2.1 h (n = 3).

PPARγ Ligands Block p21Cip1 Induction by PDGF, Not with Insulin—The effect of PDGF and insulin on the PKCδ-depend-
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 PKCδ 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 posttranscriptional level 12 h and later after mitogenic stimulation (Fig. 1), we focused on late phase activity of PKCδ. As shown in 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 followed by immunoblotting 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 in 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 p21Cip1 by PPARγ ligands. Specific PTPase inhibitors, sodium orthovanadate (Na3VO4) and dephostatin, were administered 6 h after the stimulation of PDGF. The inhibitory effect of TRO and RSG on PDGF-induced expression of p21Cip1 was totally reversed by the addition of both PTPase inhibitors (Fig. 8A). Neither inhibitor had any effect on p21Cip1 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 PKCδ 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 p21Cip1 turnover, and decrease cellular concentration of p21Cip1.

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, 3.0 ± 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 p21Cip1 protein.

FIG. 6. Overexpression of PKCδ reverses the inhibitory effects of PPARγ ligands on the mitogenic induction of p21Cip1 in RASMC. RASMC were infected with adenovirus harboring the PKCδ gene at 20 pfu/cell. 48 h after infection, cells were starved and stimulated with PDGF in the presence or absence of PPARγ ligands (10 μM TRO and RSG). As a control, RASMC were infected with adenovirus harboring the lacZ gene at 20 pfu/cell. Panel A, a high level of expression of PKCδ was confirmed by immunoblotting using anti-PKCD antibody compared with lacZ-infected cells. Panel B, a high level of PKCδ activity was confirmed after the stimulation with PDGF in PKCδ-overexpressed cells compared with lacZ-infected cells. Panel C, 24 h after the stimulation with PDGF, 30 μg of whole cell protein was assayed by immunoblotting using anti-p21Cip1 antibody.

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 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-p21Cip1 antibody. The autoradiogram is representative of three separate experiments. Panel E, same as in panel B except that RASMC were stimulated with PDGF for 9 min, and immunoblots were probed with antibody specific for pY416 in c-Src.

DISCUSSION

The CDKI p21Cip1 is a classical negative regulator of G1 → S progression through its well described activity to inhibit cyclin-CDK holoenzymes that phosphorylate Rb to permit movement past the G1 restriction point (4). Several recent studies, however, have suggested that p21Cip1 can also function at a low concentration as a positive regulator during G1 by promoting the assembly of stable cyclin-CDK complexes (3, 7). We have shown previously that PPARγ ligands inhibit G1 → 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 p27Kip1 CDKI levels. We hypothesized that PPARγ ligands shifted the balance of CDKIs and cyclin-CDKs in G1 to favor formation of enzymatically inactive cyclin D-CDK4/2p27Kip1 and cyclin D-CDK6/p27Kip1 trimers. PPARγ ligands, however, also suppressed mitogen-in-
stimulated RASMC plus TRO or RSG.

Experimental Procedures

The molecular basis for their regulation of this CDK1.

S progression, the present study was undertaken to elucidate

PPARγ Ligands Regulate p21Cip1 in VSMC

PPARγ ligands also blocked the mitogenic induction of p21Cip1 by inhibiting PKCδ signaling and accelerating its degradation. Although insulin increased p21Cip1 protein levels, neither PPARγ ligands nor pharmacological blockade of PKCδ signaling impacted this process. In marked contrast, both treatments potently attenuated PDGF-induced p21Cip1 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 et al. (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 growth factor receptors, increased intracellular hydrogen peroxide may be the second messenger for biological effects (32), and hydrogen peroxide has been shown to trigger 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 RASMC 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 RASMC. 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 dephosphorylation of 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 of VSMC growth. Conflicting studies have emerged concerning the role of PKCδ in VSMC proliferation. Leng et al. (27) suggested that activation of PKCδ by translocation to the particulate fraction played a positive role in the proliferation and migration of VSMC in response to PDGF. 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 over-expressing PKCδ, Fukumoto et al. (37) observed an inhibition of VSMC proliferation. These discrepant observations 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 rotterlin 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 RASMC? 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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Peroxisome Proliferator-activated Receptor γ Ligands Inhibit Mitogenic Induction of p21 Cip1 by Modulating the Protein Kinase Cδ Pathway in Vascular Smooth Muscle Cells

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