Peroxisome Proliferator-activated Receptor δ Is Up-regulated during Vascular Lesion Formation and Promotes Post-confluent Cell Proliferation in Vascular Smooth Muscle Cells*

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Jifeng Zhang, Mingui Fu, Xiaojun Zhu, Yan Xiao, Yongshan Mou, Hui Zheng, Mukaila A. Akinbami, Qian Wang, and Yuqing E. Chen†

From the Cardiovascular Research Institute, Morehouse School of Medicine, Atlanta, Georgia 30310

Although peroxisome proliferator-activated receptor (PPAR) δ is widely expressed in many tissues, the role of PPARδ is poorly understood. In this study, we report that PPARδ was up-regulated in vascular smooth muscle cells (VSMC) during vascular lesion formation. By using Northern blot analysis, we demonstrated that PPARδ was increased by 3–4-fold in VSMC treated with platelet-derived growth factor (PDGF) (20 ng/ml). In addition, PDGF-induced PPARδ mRNA expression neither needs de novo protein synthesis nor affects the stability of PPARδ mRNA in VSMC. Preincubation of VSMC with phosphatidylinositol 3-kinase inhibitor (LY294002, 50 μmol/liter) or infection of VSMC with an adenovirus carrying the gene for a dominant negative form of Akt abrogated PDGF-induced PPARδ mRNA expression, suggesting that phosphatidylinositol 3-kinase/Akt signaling pathway is involved in the regulation of PDGF-induced PPARδ mRNA expression in VSMC. To explore the role of PPARδ in VSMC, we generated rat vascular smooth muscle cells (A7r5) stably overexpressing PPARδ and the control green fluorescent protein. Overexpression of PPARδ in VSMC increased post-confluent cell proliferation by increasing the cyclin A and CDK2 as well as decreasing p57kip2. Taken together, the results suggest that PPARδ plays an important role in the pathology of diseases associated with VSMC proliferation, such as primary atherosclerosis and restenosis.

The peroxisome proliferator-activated receptors (PPARs) including α, β/δ, and γ are members of the superfamily of nuclear receptors. The general structural features of the family include a central DNA-binding domain and a carboxyl-terminal domain that mediates ligand binding, dimerization, and transactivation functions. PPARs function as a heterodimer with retinoid X receptors, another member of this family, to bind to the PPAR-responsive element, a DR1 element, which is a direct repeat of two similar hexanucleotide (5'-AGGTCA-3') half-sites separated by one nucleotide on its target genes (1). In the presence of both PPAR- and retinoid X receptor-specific ligands, this type of interaction confers synergistic activation of target genes (1).

PPARδ is highly expressed in the liver, muscle, kidney, and heart, where it stimulates the β-oxidative degradation of fatty acids. PPARγ is most abundantly expressed in fat cells, large intestine, and cells of the monocyte lineage (2). PPARγ has been linked to adipocyte differentiation and insulin sensitivity. Both PPARα and PPARγ are expressed in monocytes/macrophages, endothelial cells, and vascular smooth muscle cells (VSMC) in both medial and intimal layers. Activation of PPARs has been found to inhibit VSMC production of inflammatory factors (3), although activation of PPARγ has been reported to decrease both VSMC proliferation (4) and matrix production after vascular injury (5). Therefore, PPARα and PPARγ are emerging as important determinants of vascular function and structure (6–8).

Although PPARδ (also known as PPARδ and NUC-1) is widely expressed in many tissues, the physiological or pathophysiological roles of PPARδ are unclear. With no connection to important clinical manifestations, along with the lack of marketed PPARδ-specific ligands, the research to define PPARδ function has been hampered for many years. However, PPARδ has recently been linked to colon cancer proliferation (9–11), preadipocyte proliferation (12, 13), macrophage lipid accumulation (14), and embryo implantation (15). Interestingly, prostacyclin (PGI2), which is the natural ligand of PPARδ, is the characteristic prostanooid released by vascular endothelial and smooth muscle cells in response to stimulation by cytokines such as tumor necrosis factor α (16). Taken together, we hypothesize that PPARδ plays an important role in VSMC proliferation.

VSMC proliferation is the key component of vascular proliferative diseases including atherosclerosis, restenosis, and vein-graft failure (17, 18). Cytokines and growth factors such as tumor necrosis factor α and PDGF participate in these processes (19). In eukaryotic cells, the commitment to divide is made in the G1 phase of the cell cycle in response to various stimuli, including growth factors. The D- and E-type cyclins in combination with cyclin-dependent kinases (CDKs) regulate passage through the G1 phase (20, 21). Overexpression of cyclin D or E can shorten G1 phase (22–24), suggesting that the cyclin...
family is critical for progression through G1 phase. In this report, we document that PPARδ is up-regulated in VSMC during vascular lesion formation, and we demonstrate that PDGF stimulation increases PPARδ expression by 3–4-fold in VSMC. We also show that overexpression of PPARδ in VSMC promotes the proliferation of confluent cells by increasing the cyclin A and CDK2 but decreased p57kip2.

**EXPERIMENTAL PROCEDURES**

**Materials**—Platelet-derived growth factor (PDGF) BB, actinomycin D, and cycloheximide—were purchased from Sigma (St. Louis, MO). LD2894002, SB202190, and U0126 were obtained from Biomol Research Laboratories (Plymouth Meeting, PA). Cell culture medium and phosphate-buffered saline were purchased from Invitrogen.

**Cell Culture and Stimulation**—The rat aortic smooth muscle cells (RASMCS) were prepared as described previously (25). The RASMCS were confirmed by a smooth muscle actin immunochemical staining using anti-α-actin kit (Dako). Passage 6–10 RASMCS were used in DMEM/F-12 containing 10% FBS, 100 units/ml penicillin, 100 μg/ml streptomycin, and 200 μM L-glutamine. Human aortic smooth muscle cells were purchased from BioWhittaker (San Diego, CA). The actinomycin D was added to the cells after PDGF-BB stimulation for 6 h. The cycloheximide was added to the cells at the same time with PDGF-BB. The rat vascular smooth muscle cell line A7r5 was purchased from ATCC (catalog number CRL-1444, Manassas, VA).

**Balloon Injury and Immunohistochemical Staining**—Male Sprague-Dawley rat weighing 280–300 g were purchased from Tacomin Farms (Germantown, NY). Balloon-catheter injury was induced when rats were under ketamine (90 mg/kg) and xylazine (5 mg/kg) anesthesia. The left common carotid artery wall was injured with an embolectomy balloon catheter (2F Fogarty, Edwards Life Sciences, Memphis, TN) to induce neointimal formation as described previously, and the right common carotid artery was used as control (26). Animals were killed with an overdose of pentobarbital (120 mg/kg) and subjected to whole body perfusion with 4% paraformaldehyde at 7, 14, and 28 days after injury. The carotid arteries were removed, cut into cross-sectional segments, and embedded in paraffin. Sections 5 μm thick (n = 5 per animal) were immunohistochemically stained with a polyclonal antibody (Santa Cruz Biotechnology, 1:500 dilution) against PPARδ. The sections were counterstained with hematoxylin. The image was displayed in a high resolution monitor and digitized by using a video frame grabber (PCVISION Plus, Imaging Technology) on an IBM-compatible computer.

**RNA Isolation and Northern Blot Analysis**—Twenty μg of total RNA, isolated from each condition by using acid-guanidinium thiocyanate, was subjected to electrophoresis through 1% formaldehyde-agarose gels. After transferring to nylon membranes (Bio-Rad), the RNA was cross-linked to the membrane by a UV cross-linker (Bio-Rad). 32P-Labeled cDNA probes were generated by using the random primer labeling system (Invitrogen). Blots were pre-hybridized, hybridized, and washed once with 1× SSC at 65 °C for 30 min and once with 0.1× SSC, 1.0% SDS (w/v) at 65 °C for 15 min. The lane loading differences were normalized using the GAPDH.

**Western Blot Analysis**—Fifty μg of total cell lysate isolated from each condition was subjected to SDS-PAGE and electrotransferred to nitrocellulose membrane (Bio-Rad). After blocking in 20 mM Tris-HCl, pH 7.6, containing 150 mM NaCl, 0.1% Tween 20, and 5% (w/v) non-fat dry milk, blots were incubated for 1 h at 4 °C with specific antibodies (Santa Cruz Biotechnology) against PPARδ (sc-7197), cyclin A (sc-596), cyclin E (sc-481), Cd23 (sc-163), p57kip2 (sc-1040), or actin (sc-1616). The blots were then incubated with horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology). Immunoreactivity was visualized by using the enhanced chemiluminescence detection system (ECL, Amersham Biosciences) according to the manufacturer’s instructions.

**Quantitative RT-PCR**—The expression levels of PPARδ mRNA in rat carotid arteries were quantitated by the quantitative real-time RT-PCR strategy (Roche LightCycler PCR system, Roche Molecular Biochemicals). Sham-operated and balloon-injured rat carotid arteries at 7, 14, and 28 days after surgery were harvested. The adventitia of artery was removed from the medial layer by gross forces dissection. Pooled samples (n = 4) for each group were used for RNA preparation. Two primers, ratPapu (5′-cagctagaaccacctgctc3′, nt 867–892) and ratPilow2 (5′-ggcagcagctgcttttggt-3′, nt 1170 to 1146) corresponding to rat PPARδ cDNA (GenBank accession number NM_013141) were used for quantitative PCR. The PCR results were normalized by GAPDH.

**Adenovirus Preparation and Infection**—An adenovirus carrying the gene for a dominant negative form of Akt (ad-AktDN) was obtained from Dr. Ogawa (27). The ad-AktDN was amplified as described previously (28). In this study, VSMCs were infected with adenovirus vectors encoding the reporters. The cells were subjected to experiments 24–48 h after infection.

**Construction of PPARδ-A7r5 Stable Cells**—The mouse PPARδ cDNA was a gift from Dr. Grimaldi (29). Establishment of stable transfectants of A7r5 cells expressing PPARδ/GFP and GFP alone was performed by using the retroviral bicistronic expression vector pMX-IRES-GFP as described previously (30). Briefly, the PPARδ cDNA was inserted into the upstream of the encephalomyocarditis virus internal ribosomal entry sequence (IRES) which drives a GFP gene in the retroviral vector pMX. We infected 5 × 10^5 A7r5 cells with ~2 × 10^6 virus supernatant in the presence of 4 μg/ml of Polybrene for 4 h. Forty eight hours after the infection, the cells were sorted by a FACScan (BD Pharmingen) according to their GFP levels. A homogeneous population of PPARδ-A7r5 stable cells was used for stable transfection. At the same time, we generated the control A7r5 cells expressing GFP.

**Cell Number Determination and Cell Cycle Distribution**—To investigate the growth rate and cell cycle distribution between PPARδ-A7r5 and GFP-A7r5 cells, the cells were plated in 6-well plates at a density of 1.6 × 10^5 cells per well in DMEM containing 10% FBS. The medium was replaced every other day. The number of cells was determined by using the Coulter counter (model ZM, Coulter Electronics Ltd., Inc., Hialeas, FL) at different time intervals after seeding and was averaged for four wells.

Flow cytometry was performed to analyze cell cycle distribution between PPARδ-A7r5 and GFP-A7r5 cells. Briefly, the post-confluent cells were trypsinized, centrifuged at 1500 rpm for 5 min, washed with phosphate-buffered saline and treated with 20 μg/ml RNase A and 0.2% Triton X-100 for 30 min at 37 °C. The cell DNA was then stained with 100 μg/ml propidium iodide for 30 min at 4 °C and covered with aluminum foil. Samples were analyzed for DNA content by using a standard method on a FACScan (BD Pharmingen FACS System). DNA histogram analysis was performed using the CellQuest software (BD Pharmingen).

**Statistical Analysis**—Each experiment was repeated a minimum of three times. Statistical analyses were performed by analysis of variance and unpaired 2-tailed Student's t test. Data are presented as means ± S.E. The value for p < 0.05 was considered significant.

**RESULTS**

**PPARδ Is Expressed in Vascular Smooth Muscle Cells**—To determine whether PPARδ is expressed in VSMC, we examined the expression levels of PPARδ in both human and rat aortic smooth muscle cells. By using both Northern blot and Western blot analyses, we demonstrated that PPARδ is expressed in both human and rat VSMC (data not shown). These data document that PPARδ is expressed in vascular smooth muscle cells as described previously (31).

**PPARδ Is Up-regulated during Vascular Lesion Formation after Balloon Injury of Rat Carotid Artery**—To explore the role of PPARδ in VSMC, we compared the PPARδ expression levels between normal and injured vessels using the balloon-injured carotid artery model. Immunohistochemical results showed that neointima formation in this model was associated with a significant increase in PPARδ expression (Fig. 1B) compared with control (Fig. 1A).

To define the expression level of PPARδ in injured artery, we performed quantitative RT-PCR experiments using RNA samples isolated from sham-operated and balloon-injured rat carotid arteries. As shown in Fig. 1C, the level of PPARδ mRNA expression in balloon-injured rat carotid arteries was ~3.1-fold higher than that in sham-operated rat carotid arteries at 14 days after balloon injury. In addition, the PPARδ mRNA levels were increased 1.7- and 2.1-fold at 7 and 28 days after injury,
increase of PPAR expression. To investigate whether PDGF is responsible for the reactive staining for PPAR expression, we examined the half-life of PPAR mRNA in RASMC. Northern blot analyses were performed with the addition of actinomycin D (5 \( \mu \)g/ml) after 6 h of PDGF stimulation. As shown in Fig. 2A, the expression of PPAR mRNA was up-regulated in a time- and dose-dependent manner. A significant increase was observed at a PDGF concentration as low as 5 ng/ml, whereas maximal increases were obtained at a concentration of 10 ng/ml. These results reveal that PDGF can activate PPAR expression in VSMC.

PDGF induces PPAR expression in a time- and dose-dependent manner in VSMC—It was very interesting to document that PPARs are up-regulated during vascular lesion formation. To investigate whether PDGF is responsible for the increase of PPAR expression in the neointima, RASMCs were treated with 20 ng/ml PDGF for 0, 0.5, 2, 6, 12, 24, 48, and 72 h. Northern blot analysis showed that the levels of PPAR mRNA increased at 2 h, reached a peak at 6 h, and remained above the control level at least for 24 h after PDGF stimulation (Fig. 2A).

To determine whether PDGF-induced PPAR mRNA expression was documented at 6 h of PDGF stimulation. As shown in Fig. 2B, the expression of PPAR mRNA was up-regulated in a dose-dependent manner. A significant increase was observed at a PDGF concentration as low as 5 ng/ml, whereas maximal increases were obtained at a concentration of 10 ng/ml. These results reveal that PDGF can activate PPAR expression in VSMC.

The effect of PDGF on PPAR protein levels was also assessed by Western blot analysis. RASMCs were treated with 20 ng/ml PDGF for 0, 0.5, 2, 6, 12, 24, and 48 h. Two bands around 52–55 kDa were detected by anti-PPAR antibody. The upper band, which may be caused by PDGF-induced phosphorylation of PPAR, was increased as early as 0.5 h and reached a peak at 24 h. The lower band was first decreased at 0.5 and 2 h and then increased at 12 h and reached a peak at 24 h. These results demonstrate that PDGF induces PPAR protein in a time-dependent manner in RASMC. In parallel experiments, similar results were observed in human aortic vascular smooth muscle cells (data not shown) by both Northern blot and Western blot analyses.

PDGF-induced PPAR mRNA expression does not affect the stability of PPAR mRNA in VSMC—to evaluate whether PPAR mRNA stability contributes to PDGF-induced PPAR mRNA levels were analyzed by Northern blot analyses. The values were normalized by GAPDH, and the control was assigned an arbitrary value of 1. C, Western blot analysis of PPAR protein levels. The cells were treated with 20 ng/ml PDGF for different times in A. Fifty \( \mu \)g of the cell lysate was used for the analysis. Values were normalized by actin level. Three independent experiments showed similar results.

3B, the protein translation inhibitor, cycloheximide, did not alter PPAR mRNA levels after 24 h of PDGF stimulation, suggesting that PDGF-induced PPAR mRNA expression does not require de novo protein synthesis.

PDGF induces PPAR expression by PI3-kinase/Akt Signaling Pathway in VSMC—to investigate the signaling pathways mediating PDGF-induced PPAR expression, we initially focused on defining the roles of the PI3-kinase, MEK/ERK, and p38 mitogen-activated protein kinase. RASMCs were treated with 20 ng/ml PDGF for 6 h after pretreatment with LY294002, a PI3-kinase inhibitor (50 \( \mu \)M); SB202190, a p38 kinase inhibitor (25 \( \mu \)M); or U0126, a MEK inhibitor (10 \( \mu \)M) for 30 min. As shown in Fig. 4, LY294002 completely blocked the effect of PDGF \((p < 0.01)\). Although inhibition of p38 mitogen-activated protein kinase significantly attenuated the effect of PDGF-induced PPAR expression by 83 ± 9.5\% \((p < 0.01)\), SB202190 alone reduced the basic level of PPAR expression in VSMC by...
However, inhibition of MEK increased PDGF-induced PPARγ mRNA expression by 44.8% (p < 0.01).

To define further whether the PI3-kinase/Akt signaling pathway mediates PDGF-induced PPARγ expression in VSMC, we selectively blocked this signaling pathway by using ad-AktDN (an adenovirus carrying the gene for a dominant negative form of Akt). Blockade of the PI3-kinase/Akt pathway effectively prevented PDGF-induced PPARγ gene expression in RASMC (Fig. 5). However, PPARγ expression was not affected by the control adenovirus (Ad-GFP) infection in RASMC (data not shown). Taken together, these results provided the first evidence that PDGF-induced PPARγ gene expression is regulated by a PI3-kinase/Akt-dependent pathway.

To confirm further the involvement of Akt in PDGF-induced PPARγ expression in VSMC, we examined the PPARγ protein levels in the rat vascular smooth muscle cell lines (A7r5) that were stably transfected with a constitutively active Akt.
control cells stably transfected with a GFP construct. We found that PPARδ in Akt-A7r5 was easily detected by Western blot analysis but was undetectable in the control GFP-A7r5 (data not shown). These results further confirmed that Akt is involved in the regulation of PPARδ expression in VSMC.

Construction of PPARδ-A7r5 Cells—We were intrigued by the initial observation that the rat embryonic aorta A7r5 clonal VSMC line failed to express PPARδ by either Northern blot or Western blot analyses. This serendipitous finding of PPARδ expression between A7r5 cells and RASMC provided us with the opportunity to examine PPARδ-induced alterations in VSMC gene expression. To reconstitute PPARδ expression in the A7r5 cells, retroviral expression vectors were used (Fig. 6A). The transfected cells had an ~4-kb transcript that contains a chimeric PPARδ-GFP mRNA (Fig. 6B) that was translated into two separate proteins, GFP and PPARδ (Fig. 6C). A homogeneous population of PPARδ-A7r5 stable cells or the control A7r5 cells expressing GFP isolated by FACS was used for this study. There was no difference in size or morphology between PPARδ-A7r5 and GFP-A7r5 cells (data not shown). This in vitro cell model system enabled us to define the role of PPARδ in VSMC.

Overexpression of PPARδ in VSMC Promotes Post-confluent Cell Proliferation—To investigate the effect of PPARδ overexpression in VSMC, we first examined the rate of cell growth in both PPARδ-A7r5 and GFP-A7r5 cells by determining cell numbers. Although the growth rate of PPARδ-A7r5 cells was similar to that of GFP-A7r5 cells before confluence, the PPARδ-A7r5 cells grew significantly faster than GFP-A7r5 cells after confluence (Fig. 7A). Furthermore, the cell cycle distribution in PPARδ-A7r5 and GFP-A7r5 cells was determined by flow cytometry analysis when the cells were quiescent and post-confluent. The percentage of S phase cells in PPARδ-A7r5 was ~3.2-fold more than that in GFP-A7r5 (Fig. 7, B–D). We also examined the rate of apoptosis induced by serum withdrawal in both PPARδ-A7r5 and GFP-A7r5 cells by both nuclear morphology and FACS analysis. There was no significant difference between PPARδ-A7r5 and GFP-A7r5 cells (data not shown). Taken together, these results suggest that PPARδ in VSMC is involved in cell proliferation.

To investigate further the molecular basis that PPARδ promotes VSMC growth, we analyzed the cell cycle proteins in both PPARδ-A7r5 and GFP-A7r5 cells by Western blot analysis when the cells were quiescent and post-confluence (Fig. 7, A7r5 cells grew significantly faster than GFP-A7r5 cells after confluence). Interestingly, the level of CDK2 inhibitory protein, p27kip1, was significantly higher in PPARδ-A7r5 cells than in GFP-A7r5 cells after confluence, but there was no change in cyclin E levels between the two cell lines.

DISCUSSION

It is postulated that pathological changes in vessel structures are induced in part by transcription factors that govern cell growth, death, differentiation, inflammation, and matrix production. PPARα and PPARγ are members of a family of ligand-activated nuclear transcriptional factors that are emerging as important determinants of vascular function and structure. Activation of PPARα has been found to inhibit VSMC production of inflammatory factors (3). Recent studies have shown that the expression of PPARγ was up-regulated in intimal VSMC (4), and activation of PPARγ has been found to decrease both VSMC proliferation (32) and matrix production after vascular injury (5). Although it has been well documented that PPARα and PPARγ are important determinants of vascular function and structure, the role of PPARδ in vasculature is poorly understood.

In the present studies, we document that PPARδ is expressed in VSMC and up-regulated by 1.7-, 3.1-, and 2.1-fold in rat carotid artery at 7, 14, and 28 days after injury, respectively. Interestingly, Adams et al. (31) showed that PPARδ expression was up-regulated by 2.6-fold and was highest 4 h after injury, compared with control level, returned to the base line by 24 h, and did not change for 1 week, suggesting there is a biphasic increase in PPARδ after vessel injury. A detailed time course of PPARδ expression after vessel injury is currently ongoing to determine this contrasting finding in the two studies. In addition, further studies are required to understand the mechanism of this interesting phenomenon.

Vasculoproliferative disorders such as primary atherosclerosis, restenosis, and vein-graft failure are characterized by the accumulation of intimal smooth muscle cell proliferation, migration, and extracellular matrix deposition (17, 18). Cytokines and growth factors such as PDGF participate in these processes. We postulate that PDGF may up-regulate PPARδ expression in VSMC. Indeed, we documented that PDGF induced PPARδ expression in a time- and dose-dependent manner in VSMC. This further suggests that PPARδ is involved in VSMC proliferation during vascular lesion formation.

PDGF is an important regulator that mediates the aberrant behavior of VSMC in the pathogenesis of vascular diseases. PDGF binding to its receptor on VSMC can activate several signaling pathways including p58-, MEK1/ERK-, and PI3-kinase-mediated pathways, which transduce the signals into nucleus and stimulate the proliferation and migration of VSMC (33, 34). In the current study, we demonstrated that PDGF-induced PPARδ mRNA expression was most likely because of an induction of transcription rather than altering the stability of PPARδ mRNA because the addition of PDGF failed to change the degradation rates of PPARδ mRNA in VSMC. In addition, PDGF-induced PPARδ mRNA expression did not require de novo protein synthesis because the addition of protein synthesis inhibitor in VSMC did not abrogate PDGF-induced PPARδ expression. Taken together, the data suggest that there may be PDGF-response elements in the PPARδ promoter.

The PPARδ gene is composed of 9 exons spanning more than 85 kb on chromosome 6p21.2 (35). To date, little is known about PPARδ transcriptional regulation. The only report on the tran-
scriptional regulation of PPARδ gene revealed that there are two putative β-catenin/Tcf-4-binding sites located on the promoter (9). The up-regulation of PPARδ mediated by β-catenin/Tcf-4 was identified as one of the mechanisms involved in the initiation of colorectal tumors. Obviously, studying the transcriptional regulation of the PPARδ gene such as systematic deletion mapping of PDGF-response elements in the PPARδ promoter will not only help explain PPARδ gene regulation but also provide new insights that will define the role of PPARδ in vasculoproliferative disorders, diabetes, and cancer. Although this is beyond the scope of the present study, we have successfully cloned an ~5.5-kb human PPARδ gene promoter, and studies are underway to determine the potential PDGF-response elements in the PPARδ promoter.

We have shown that inhibition of PI3-kinase abrogates the effect of PDGF-induced PPARδ expression in VSMC, although the pharmacological probe used was relatively selective and the results were verified using adenoviral vector with a dominant negative mutant Akt construct. The level of PPARδ in Akt-A7r5 cells stably transfected with a constitutively active Akt construct was significantly increased.2 Taken together, these results provide the first definitive evidence that PPARδ gene expression is regulated by a PI3-kinase/Akt signaling pathway.

However, the MEK1 inhibitor increased PDGF-induced PPARδ mRNA expression. Further studies are required to clarify whether activation of the MEK1/ERK signaling pathway inhib-

FIG. 7. PPARδ promotes the G1 → S progression in VSMC. A, overexpression of PPARδ in VSMC promotes cell proliferation after confluence. PPARδ-A7r5 and control GFP-A7r5 cells were plated in 6-well plates at a density of 1.6 × 10⁵ cells/well. They were grown in DMEM/F-12 (Invitrogen) medium supplemented with 10% of fetal bovine serum. At defined time intervals, they were trypsinized and counted in a Coulter counter (model ZM, Coulter Electronics Ltd., FL). B and C show the representative DNA histograms for the quiescent and post-confluent VSMCs. The GFP-A7r5 or PPARδ-A7r5 cells were seeded in 6-well plates at a density of 5 × 10⁵ cells/well. The cells were grown to confluence in DMEM/F-12 (Invitrogen) medium supplemented with 10% FBS. To make the cell quiescence, growth medium was removed and replaced with Opti-MEM (Invitrogen) for 48 h. 1 × 10⁶ cells were analyzed by flow cytometry. D, mean percentage values of cells in S phase.

FIG. 8. Overexpression of PPARδ in VSMC alters cell cycle regulatory proteins. PPARδ-A7r5 and the control GFP-A7r5 cells were plated in the T25 flask at a density of 5 × 10⁵ cells/flask. They were grown in DMEM/F-12 (Invitrogen) medium supplemented with 10% fetal bovine serum. At defined stages as indicated, the cells were harvested, and 50 μg of protein extract was immunoblotted with the indicated antibodies. The relative values were normalized by actin. Three independent experiments showed similar results.

/* J. Zhang, M. Fu, X. Zhu, Y. E. Chen, unpublished data. */
its PDGF-induced PPARδ expression and to determine precisely the relationship between PI3-kinase/Akt and MEK1/ERK signaling pathways in the regulation of PDGF-induced PPARδ expression in VSMC.

Activation of MEK1/ERK signaling pathway could induced PPARγ phosphorylation, resulting in a down-regulation of PPARγ activity (36–38). In contrast, PPARα phosphorylation induced by MEK1/ERK pathway could enhance the PPARα activity (39). Interestingly, our data suggested that PDGF induced not only PPARδ expression but also PPARδ phosphorylation. More experiments are required to confirm PPARδ phosphorylation and to elucidate its function.

Although we have demonstrated that PDGF-induced PPARδ gene expression was mediated by the PI3-kinase/Akt-dependent pathway, it remained to be determined whether the PPARδ target genes are activated following PDGF stimulation in VSMC. However, it is currently not practical to resolve this issue because the PPARδ gene targets within VSMC have not been defined. To understand the role of PPARδ in vasculature, it would be necessary to define globally the PPARδ target genes in VSMC. Our laboratory is currently using a DNA microarray analysis to approach this challenge.

For many years, the lack of PPARδ-specific ligands has hampered efforts to define PPARδ function. It has been reported that carbaprostacyclin (cPGI), a stable analog of PGI2, is a PPARδ ligand (40). cPGI is a synthetic ligand that is structurally different from the endogenous PGI2. And questions have been raised whether cPGI itself can act as a bona fide ligand for PPARδ. However, testing the ability of PGI2 to activate PPARδ in VSMC is difficult because of the inherent instability of this compound. In neutral or acidic buffers, PGI2 is rapidly hydrolyzed (30–120 s) to 6-keto prostaglandin F1α (10). To resolve this problem, there is a need to create an experimental model in which PGI2 is functional as the endogenous ligand for PPARδ. Because PGI2 is the major prostanoi released by both endothelial cells (41) and VSMC (16) including A7r5 (42), we postulate that PPARδ-A7r5 may be a useful cell model to test the PPARδ function in VSMC. It is important to note that a recent intriguing report has identified the first high affinity PPARδ ligand, GW501516, with an EC50 = 1.2 ± 0.1 nM and >1,000-fold selective for PPARδ over other subtypes (43). This will definitely spur new interest in the study of PPARδ function.

Recent reports (10, 11) showed that PPARδ promotes colon cancer proliferation and preadipocyte proliferation (12, 13). In the present study, we demonstrate that PDGF induces PPARδ expression, and PPARδ is up-regulated in neointima during vascular lesion formation. We hypothesize that overexpression of PPARδ in VSMC is a sufficient condition to increase cell proliferation. Our results showed significantly faster growth rate in PPARδ-A7r5 cells than the control GFP-A7r5 cells after confluence and no differences in growth rate between PPARδ-A7r5 and GFP-A7r5 before cell confluence. This observation is consistent with a recent report (13) that PPARδ promotes post-confluent cell proliferation in 3T3 fibroblasts. In addition, our data suggest that A7r5 can generate enough endogenous PPARδ activators with comparable affinity to cPGI2, because the addition of cPGI2 into PPARδ-A7r5 cells failed to alter the growth rate.

VSMC proliferation is the major component of vasculoproliferative disorders. Vascular injury results in the release of growth factors and cytokines that stimulate quiescent, G0/G1 arrested VSMC to enter the cell cycle. Cell cycle progression is dependent on the expression and activation of specific enzymes, termed cyclin-dependent kinases (Cdks), which form complexes with their regulatory subunits, the cyclins. Activation of cyclin D + CDK4, cyclin D + CDK6, and cyclin E/A + CDK2 during G1 phase results in G1 → S transition. Moreover, cyclin-dependent kinase inhibitors (p27kip1, p21cip1, and p16ink4a) are major negative regulators of the cell cycle by binding to and inhibiting the activation of CDK-cyclin complexes. In this study, we demonstrated that cyclin A and CDK2 in PPARδ-A7r5 were significantly higher than that in GFP-A7r5. Consistent with this change, the level of p27kip1 was significantly lower in PPARδ-A7r5 than that in GFP-A7r5 cells after confluence. These changes could result in post-confluent VSMC proliferation through PPARδ overexpression. Interestingly, the level of CDK inhibitory protein, p21cip1, was significantly higher in PPARδ-A7r5 than in GFP-A7r5 cells after confluence. Our results are consistent with recent studies (44, 45) that suggested that p21cip1 and p27kip1 of CDK function as positive regulators during the G1 phase and as assembly factors to promote formation of cyclin-CDK holoenzyme complexes.

In summary, we report that PPARδ is expressed in VSMC and up-regulated in neointima during vascular lesion formation. In addition, we demonstrate that PDGF-induced PPARδ gene expression is mediated by PI3-kinase/Akt-dependent pathway. Overexpression of PPARδ in VSMC promotes the post-confluent cell proliferation. Taken together, our results suggest that PPARδ plays an important role in the modulation of vasculoproliferative disorders such as primary atherosclerosis, restenosis, and vein-graft failure.

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