Intimal Smooth Muscle Cells as a Target for Peroxisome Proliferator-Activated Receptor-\(\gamma\) Ligand Therapy

David Bishop-Bailey, Timothy Hla, Timothy D. Warner

Abstract—Activation of the nuclear receptor/transcription factor, peroxisome proliferator-activated receptor \(\gamma\) (PPAR\(\gamma\)), is a newly defined target for limiting vascular pathologies. PPAR\(\gamma\) is expressed in human and animal models of vascular disease, with particularly high levels being present in the cells of the neointimal microenvironment. In the present study, we show that intimal smooth muscle cells in vitro contain higher amounts of functional PPAR\(\gamma\) than medial smooth muscle cells. The PPAR\(\gamma\) ligand rosiglitazone more potently induced CD36 expression at low concentrations, and cell death by apoptosis at higher concentrations in intimal compared with medial smooth muscle cells. Intimal smooth muscle cells also contained high levels of cyclooxygenase-2 protein, and released a more diverse and larger amount of eicosanoids on arachidonic acid stimulation. Furthermore, when exogenous arachidonic acid was added, PPAR reporter gene activation was induced in a cyclooxygenase inhibitor–sensitive manner, an effect that correlated with an increase in CD36 expression. In summary, intimal smooth muscle cells contain functionally higher levels of PPAR\(\gamma\), PPAR\(\gamma\) ligands have high- and low-potency targets in vascular smooth muscle cells, and cyclooxygenase can serve as a source of potential endogenous PPAR ligands. Intimal vascular smooth muscle cells therefore represent a potentially important target for the antiproliferative, and antiatherosclerotic actions of PPAR\(\gamma\) ligands. (Circ Res. 2002;91:210-217.)

Key Words: peroxisome proliferator-activated receptor-\(\gamma\) ■ cyclooxygenase-2 ■ intimal smooth muscle cells ■ atherosclerosis ■ restenosis

Peroxisome proliferator-activated receptors (PPARs) are a family of 3 nuclear receptors: \(\alpha\) (NR1C1), \(\beta/\delta\) (also referred to as NUC1 or NR1C2), and \(\gamma\) (NR1C3), which heterodimerize with the retinoid X receptors (RXRs).\(^1\) PPAR\(\gamma\) is found primarily in adipose tissue, where it plays a critical role in the differentiation of preadipocytes into adipocytes.\(^2,3\) PPAR\(\gamma\) can be activated by a number of ligands,\(^3,4\) including the antidiabetic thiazolidinediones (PPAR\(\gamma\) selective) ligands, and a number of eicosanoids, including 12-hydroxyeicosatetraenoic acid, 15-hydroxyeicosatetraenoic acid, 13-hydroxoyctadecadienoic acid, and the cyclooxygenase (COX) products, prostaglandin (PG) \(A_1\), \(P G A_2\), \(P G I_2\), and \(P GD_2\); and the dehydrogen product of \(P GD_2\), 15-deoxy-\(\Delta^{12,14}\), \(P G J_2\) (15d-PGJ\(_2\)), oxidized low-density lipoprotein, and oxidized alkyl phospholipids.\(^3,5\)

PPAR\(\gamma\) is expressed in vascular smooth muscle cells (SMCs),\(^6-8\) endothelial cells,\(^9,10\) monocytes/macrophages,\(^11,12\) and TH lymphocytes.\(^13,14\) Notably, PPAR\(\gamma\) is highly expressed in human\(^15\) and murine atherosclerotic lesions,\(^16\) as well as in the neointimal SMC layer of a rat arteries after balloon angioplasty damage.\(^17\) In contrast, the expression of PPAR\(\gamma\) in the medial SMC layer remains relatively low.\(^17\) PPAR\(\gamma\) ligands inhibit inflammatory cell responses\(^11-14\); they inhibit the proliferation and migration of vascular smooth muscle cells in vitro,\(^7,8\) atherosclerotic lesion development,\(^18\) and neointimal formation in vivo,\(^17\) supporting a role for these receptors in the response to injury of the blood vessel wall.

Vascular smooth muscle cells exist in culture in different stable phenotypes. Most commonly utilized in culture is the “adult” medial spindle shaped SMC that grows typically with “hill and valley” morphology. However, probably of more relevance to the study of pathology and proliferative events in the blood vessel wall are the epithelial or “\(n\)” SMC; cell types that have not been extensively studied in lipid signaling. These developmental SMC phenotypes can be isolated from neonatal rat aorta, but are re-expressed in the adult after vascular injury, where they form the neointima SMC layer.\(^19-25\) These intimal SMC phenotypes differ from adult medial SMCs not only in morphology, but also in their ability to grow in plasma-derived serum, in their expression of PDGF-B, CYP1A1, elastin and osteopontin,\(^19,20,25\) plasminogen activator,\(^19\) cellular retinal-binding protein-1, cytokeratin 8,\(^24\) and in their relative low expression of \(\alpha_1\) (I) collagen and PDGF \(\alpha\)-receptor.\(^26\)

As the in vivo evidence for the expression of PPAR\(\gamma\) in restenosis clearly links high expression to the newly formed...
neointima, we performed an in vitro analysis of intimal and medial SMC lines. The intimal SMCs “constitutively” express higher levels of PPARγ and inducible cyclooxygenase (COX-2), and have exaggerated responses to PPARγ ligands. These results indicate that the effects of PPARγ ligands are selective toward intimal SMC compared with adult medial SMC responses, and correlate to the higher levels of protein expressed.

Materials and Methods

Materials

15d-PGJ2, arachidonic acid, COX-1, and COX-2 antisera were from Cayman Chemical Company; pEGFP-N1 was from Clontech; PPARγ antisera for Western blots, cigitazone, and WY14643 were from Biomol; 3H-PGE2 and 14C-arachidonic acid were from Amer sham Pharmacia Biotech; apoptosis ELISA, IL-1β, D-luciferin, ATP, DTT, and tricine were from Roche; Effectene was from Qiagen; Trizol reagent, DMEM and anti-biotic/mycotics were from Life Technologies; Western blot luminescent detection reagents, PPARγ-blocking peptide, antisera against PPARγ, RXRα, CD36, and HRP-conjugated anti-goat, anti-rabbit, and anti-mouse IgG were from Santa Cruz; FITC conjugated anti-goat IgG was from Cappell and HRP-conjugated anti-goat, anti-rabbit, and anti-mouse IgG were from Dako. pSV-β-galactosidase, and RT-PCR enzymes were from Promega; Rosglitazone, GW0072 and pACo.G.Luc were generous gifts from Dr Steven Smith (Glaxo SmithKline, Harlow, UK), Dr Timothy Willson (Glaxo SmithKline, Research Triangle, NC), and Dr Ruth Roberts (AstraZeneca, Macclesfield, UK), respectively; all other reagents were from Sigma-Aldrich.

Cell Culture

Polycellular rat aortic vascular SMC lines WKY12-22, and WKY3m-22, WKY3m-26, and pac-1 were a gift from Dr David Han (University of Washington, Seattle). Primary rat aortic SMCs (RASMCs) were grown from explants or aorta derived from male Wistar rats as previously described,28 and used between passage 3 to 5. Primary intimal SMCs derived from balloonized Wistar rats were a generous gift from Professor Giulio Gabbiani (University of Geneva, Switzerland). All cells were propagated in DMEM, containing 10% FBS, and supplemented with antibiotic/mycotic mix. All experiments were performed in serum-free medium, after cells had gone through an initial period of growth arrest for 24 house.

Western Blot

Western blot analysis using specific antibodies for COX-1 (1:1000) and COX-2 (1:1000) was performed as previously described.29 Similar experiments were performed for the determination of PPARγ (1:1000), RXRα (1:200) and β-actin. (1:1000)

Immunofluorescence

Immunofluorescent staining using specific antibodies for PPARγ (1:50), or CD36 (1:100) was as previously described.9,30 All images were taken after a 10-second exposure so that direct comparisons could be made to indicate changes in the intensity of staining.

RT-PCR

Total RNA was prepared using Trizol reagent according the manufacturer’s recommended protocol and converted to cDNA by standard methods. The rat CD36 primers (583 bp)31 were 5'-CAACAGCCCT- TATCAAAGAGT-3' and 5'-GCACACATACAGCTATCAG-3'. Rat glyceraldehyde-3-phosphate dehydrogenase (G3PDH) was chosen as a control. G3PDH (452 bp)32 primers were 5'-ACCAGAGTC- CATGCCATCAG-3' and 5'-TCCACACCTGTTGCTGTA-3'. Initial denaturing was done at 94°C for 3 minutes followed by 26 cycles (CD36 in intimal SMCs), 35 cycles (CD36 in medial SMCs), or 30 cycles (G3PDH both cell types) followed by 10 minutes at 72°C. For CD36, each cycle consisted of 30 seconds at 94°C, 30 seconds at 54°C, and 45 seconds at 72°C. For G3PDH, each cycle consisted of 35 seconds at 94°C, 35 seconds at 58°C, and 45 seconds at 72°C. PCR products were size fractionated with a 2% agarose gel and the bands visualized with ethidium bromide. Each PCR reaction resulted in a single band at the appropriate bp size. In parallel reactions where M-MLV RT was omitted, no bands were visible (data not shown).

Reporter Gene Activation

PPAR activation was measured as previously described9,30 using the PPRE of the rat acyl CoA oxidase promoter, linked to drive expression of luciferase (pACoG.Luc). Reporter gene activation was measured in a polyclonal selection of WKY12-22 cells stably expressing pACoG.Luc (WKY12-22-ACoG.Luc). WKY12-22 in 10-cm dishes were transfected with Effectene overnight using 1 μg pACoG.Luc, and 0.5 μg pEGFP-N1, which contains a neomycin resistance cassette. Clones were then selected in 0.5 μg/dL G418 sulfate and pooled. Treatments were performed in 12-well plates. Cells were lysed with 200 μL of distilled H2O for 10 to 15 minutes and luciferase activity was measured in 100 μL of lysates, according to the manufacturer’s recommended protocol (Promega).

Viability, Proliferation, and Apoptosis Assays

Cell morphology was assessed by phase contrast using a Zeiss Axiowert TV100 microscope, and pictures taken using a SPOT II digital camera (Diagnostic Systems). Cell viability was measured after 48 hours of drug treatment using the MTT (3-[4,5- dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay.9 Apoptosis was assessed at 24 hours after drug treatment by either nuclear morphology using Hoechst 33258,9 or by commercially available ELISA that detects cytoplasmic histone-associated DNA complexes.

COX-2 Activity

The spectrum of prostanoiod release from SMCs was initially characterized by thin-layer chromatography using 14C-labeled arachidonic acid.32 Individual PGE2 and PGD2, levels were measured in the conditioned culture supernatants by RIA28,29 or by commercially available ELISA, respectively.

Results

Differential Expression of PPARγ in an Intimal Compared With Medial Vascular Smooth Muscle Cells

Adult medial cells WKY3m-22, WKY3m-26, pac-1, and primary RASMCs displayed a weak expression of PPARγ throughout the cell (Figure 1). The only exception to this was in primary RASMCs where a small population of the cells (<10%) had PPARγ in the nucleus, a finding that may indicate a greater heterogeneity of cell phenotypes in this population. The intimal SMC cell line WKY12-22 and primary rat intimal SMCs (iSMCs) contained a weak cytoplasmic expression, but displayed a strong nuclear expression of PPARγ (Figure 1). This nuclear expression was also confirmed using confocal microscopy (data not shown) (see online Figure 1, which can be found in the online data supplement available at http://www.circresaha.org). In the absence of primary antibody (Figure 1), or when primary antibody was preabsorbed with blocking peptide (data not shown) (online Figure 1), no specific staining was observed.

Because WKY12-22 and WKY3m-22 cells are intimal and medial phenotype cells from identical genetic backgrounds, and have the advantage over primary cultures of being stable cell lines, these SMCs were used as a model system to further confirm and compare these findings at the level of expression and activity.
Differential Expression of COX Enzymes, PPARγ, and RXRα in Intimal and Medial Vascular Smooth Muscle Cells

Expression of PPARγ, its heterodimer binding partner, RXRα, COX-1, and COX-2 were determined by Western blotting. Adult medial (WKY3m-22) SMCs contained COX-1, and low but detectable levels of COX-2, PPARγ, and RXRα. In contrast, intimal SMCs (WKY12-22), contained no detectable COX-1, but significantly higher levels of COX-2, PPARγ, and RXRα (Figure 2A). Both cells contained similar levels of β-actin. Consistent with these findings, Northern blot analysis revealed that mRNA for PPARγ is higher in WKY12-22 compared with WKY3m-22 SMC (data not shown) (online Figure 1). These results indicate that the WKY12-22 intimal SMC has higher levels of PPARγ and RXRα in the neointima formed in vivo after balloon angioplasty.

Prostaglandin Release From Vascular Smooth Muscle Cells

We assessed the capacity of intimal and adult medial SMCs in culture to synthesize and release PGs from 14C labeled arachidonic acid using thin layer chromatography. Intimal SMCs released higher amounts of a broad range of PGs than adult medial SMC (Figure 2B), results that were confirmed by ELISA for PGD2 (WKY3m-22, 4±1 pg/dL per µg; WKY12-22, 12±3 pg/dL per µg protein; n=9; P<0.05) and radioimmunoassay for PGE2 (WKY3m-22, 4±2 pg/dL per µg; WKY12-22, 96±9 pg/dL per µg protein; P<0.05). Similarly, basal and arachidonic acid–stimulated PGE2 release was far greater in primary iSMCs than RASMCs (data not shown) (online Figure 2).

PPARγ Ligands More Potently Induce Intimal SMC Responses

The effects of PPARγ ligands were tested on the expression of CD36 and viability of intimal and adult medial SMC lines

Figure 1. Differential expression of PPARγ in rat medial and intimal SMCs. Fluorescent micrographs (>1000) show the cellular expression of PPARγ in (a) primary medial RASMC, (b) primary intimal SMC, (c) WKY3m-22 adult medial SMC, (d) WKY12-22 intimal SMC, (e) WKY3m-26 adult medial SMC, and (g) pac-1 adult medial SMC. f, Control lack of specific staining in WKY12-22 intimal SMC observed when primary antibody is omitted. White arrows indicate the strong nuclear staining of PPARγ observed in intimal SMC types.

Figure 2. Differential expression of PPARγ and COX enzymes in WKY3m-22 and WKY12-22 SMC. A, Expression of PPARγ, its binding partner RXRα, COX-1, COX-2, and β-actin were measured by Western blot analysis in samples of equal protein from medial (M: WKY3m-22) and intimal (I: WKY12-22) SMCs. Data represent n=3 experiments. B, Thin-layer chromatography radiograph showing the capacity and spectrum of prostanoids (AA indicates arachidonic acid; HETEs; PGD2, PGE2, PGF2α, and 6-k (6-keto)-PGF1α (the hydrolysis product of PGI2)), released by medial (M: WKY3m-22) and intimal SMC (I: WKY12-22) in response to 14C-arachidonic acid (15 µmol/L; 30 minutes).
Figure 3. Low concentrations of the PPARγ ligand rosiglitazone more potently induce CD36 expression in intimal than medial SMCs. A, Fluorescent micrographs (×1000) showing the rosigli-
tazone (Rosi; 0.3 and 1 μmol/L) induced cellular expression of CD36 in intimal (WKY12-22) and medial (WKY3m-22) SMCs for 48 hours. 2°Ab panels show the antibody control when experi-
ments were performed as per protocol with the exception that the primary antibody was omitted. Weak cellular staining for CD36 is observed in untreated cells. Multiple pictures were taken for each treatment, and data shown are representative of n=8 from 3 experimental days. B, Semiquantitative RT-PCR showing the relative ratio of CD36 mRNA to constitutive G3DPH mRNA in intimal (filled bars, WKY12-22) and medial (open bars, WKY3m-22) SMCs treated with rosiglitazone (Rosi; 0.1, 0.3 and 1 μmol/L) for 6 hours. RT-PCR was performed as described in Materials and Methods section. Bands were analyzed using UTHSCSA Image Tool v.3 (n=3). *P<0.05 between control and rosiglitazone treated cells by 1-sample t test.

in culture. CD36 is a bona fide target for PPARγ, as ligands are unable to induce CD36 expression in PPARγ−/− knockout cells.34,35 In contrast, PPARγ ligands induce growth arrest38 and/or apoptosis9 in vascular cells, although the molecular targets linking PPARγ to these growth inhibitory pathways in vascular cells are ill-defined.

Rosiglitazone induced concentration-dependent expression of CD36 protein (Figure 3A) and mRNA (Figure 3B) more potently in intimal (WKY12-22) than medial (WKY3m-22) SMCs. Concentrations of rosiglitazone above 1 μmol/L had no further significant effect on the induction of CD36 (data not shown). Similarly, rosiglitazone (Figure 4A), 15d-PGJ2, and ciglitazone (data not shown) (online Figure 3) induced concentration-dependent cell death more potently in intimal (WKY12-22) than medial (WKY3m-22) SMCs. To test that supra-pharmacological concentrations of rosiglitazone induced cell death via PPARγ, we used the partial agonist GW0072. Identical to the previously reported agonist and antagonist concentrations,36 GW0072 induced concentration-dependent cell death at agonist levels (>1 μmol/L; Figure 4B), but completely inhibited rosiglitazone induced cell death at antagonist concentrations (<1 μmol/L; Figure 4B). The PPARγ ligand induced cell death was via apoptosis, indicated by cytoplasmic rounding, nuclear condensation (data not shown), and an increase in the presence of cytoplasmic histone-associated DNA fragments, an early marker of cellular apoptosis. After 48 hours treatment, in intimal (WKY12-
22) SMC rosiglitazone (100 μmol/L) induced a 5.30±0.03-fold (n=3), ciglitazone (10 μmol/L) a 3.34±0.78-fold (n=6), and 15d-PGJ2 (3 μmol/L) a 7.15±4.65-fold (n=6) enrichment of cytoplasmic histone-associated DNA fragments relative to control.

In contrast to effects on CD36 expression where rosiglita-
zone acts at nmol/L, rosiglitazone induces apoptosis at μmol/L supra-pharmacological concentrations. These results show that the difference in protein levels of PPARγ is clearly associated with potency of the ligands to both express CD36 and induce apoptosis.

Cyclooxygenase Mediates PPAR Activation in Intimal Smooth Muscle Cells

Using WKY12-22 cells stably expressing a PPAR reporter gene, we assessed whether activation of COX resulted in a subsequent activation of PPARs. Arachidonic acid (0 to 10 μmol/L) gave a significant increase in PPAR (Figure 5A). This concentration of arachidonic acid had no effect on cell
mRNA to constitutive G3DPH mRNA in intimal (WKY12-22) SMCs treated for 48 hours. 2°Ab panels show the antibody control when experiments were performed as per protocol with the exception that the primary antibody was omitted. Multiple pictures were taken for each treatment, and data shown are representative of n = 6 from 3 experimental days.

Discussion

In the present study, we show that intimal SMCs contain significantly more active PPARγ than corresponding medial phenotype SMCs. These in vitro findings directly correlate with the pattern of both intimal SMC and PPARγ expression found in human and animal vascular lesions. Clearly, this suggests that drugs selective for PPARγ could be targeted to these cells with the aim of reducing lesion development.

In atherosclerosis, there is increased PPARγ expression throughout intimal and neointimal SMC layers.8,15,16 In rat models of restenosis after balloon injury, intimal thickening is due to SMC remodeling, with few inflammatory cell components. PPARγ in these studies is expressed exclusively in this neointimal vascular smooth muscle layer.17 Because the neointimal lesion here is considered primarily due to the proliferation of the intimal vascular SMC,19–26 we suggest that the increase in PPARγ expression seen in these lesions is at least due in part to the re-expression and proliferation of these cells. These findings are not without precedent as a number of in vitro markers for intimal SMC, such as plasminogen activator,21 cellular retinal binding protein-1, morpholino or viability (data not shown). When COX activity was blocked by piroxicam (10 μmol/L), an NSAID that does not directly interact with PPARs,37 arachidonic acid was unable to induce PPAR responses, indicating a COX product(s) as the PPAR activator. Moreover, arachidonic acid at concentrations that activated the PPRE (0 to 10 μmol/L) induced CD36 (Figure 5B). Expression of CD36 alone, or induced by rosiglitazone or arachidonic acid was inhibited by GW0072 at an antagonist concentration (Figure 6). Furthermore, GW0072 was also able to inhibit the PPAR reporter gene activity (given as relative light units/mg protein) in the absence (filled bars) or presence (unfilled bars) of piroxicam (10 μmol/L). WKY12-22-ACO-Luc is a polyclonal intimal SMC cell line that stably expresses the rat acyl CoA oxidase PPRE linked to drive luciferase expression (see Materials and Methods). PPAR activation is measured by the increase in luciferase activity (relative light units/mg protein) induced by rosiglitazone or arachidonic acid was inhibited by GW0072 at an antagonist concentration (Figure 6). Further-
cytokeratin 8,24 and osteopontin,19,20,25 are also found highly expressed in the neointima in vivo. With respect to PPARγ expression and activation it would, therefore, also seem that WKY12-22 SMCs and primary iSMCs are representative of cells present in the intimal environment in vivo.

Although, a number of stable characteristics of intimal smooth muscle cells in vivo are maintained in vitro, mediators such as M-CSF, GM-CSF, oxidized LDL, or PPARγ ligands themselves can also increase the expression of PPARγ.15 The effect of these mediators cannot, therefore, be discounted when one considers the levels of in vivo expression of the receptor. However, at least in terms of restenosis after angioplasty, where the intimal smooth muscle seem almost exclusively to form the neointima, it is clear that if an increase in expression of PPARγ does occur due to these stimuli, it is also selectively occurring in intimal SMCs.

A number of studies have investigated the effects of PPARγ ligands on adult medial SMC in vitro. These have indicated that PPARγ ligands can cause inhibition of adult SMC migration and proliferation7,8 by inducing cell cycle arrest at the G1-S interface.38 PPARγ ligands also inhibit angiotsin II type 1 receptor expression,39 upregulate CD36,40 and type II secretory PLA2,41 and decrease MMP-9 expression.7 Although ligands have many effects in vitro, the overall effect of PPARγ ligand therapy in animal models of vascular damage and inflammation does appear to lead to beneficial responses.18 Our study therefore used a simple model system to investigate the pharmacological differences between the different intimal and medial SMC phenotypes. PPARγ ligands were more effective both at inducing CD36 expression and causing apoptosis in intimal compared with medial SMCs.

All experiments were performed in the absence of serum, or other mitogens used to activate SMCs, and under these conditions SMCs directly undergo apoptosis when treated with high concentrations of PPARγ ligands. In SMCs activated by PGDF and insulin, PPARγ ligands induce cell cycle arrest with little evidence of apoptosis.38 In our experiments, SMCs were already in a quiescent state and rapidly under went apoptosis when treated with PPARγ ligands, although cyclin-dependent kinase inhibitor p21 induction may precede this apoptosis (unpublished observation, 2000). Although the exact mechanisms for the beneficial effects of PPARγ ligands’ ability to inhibit atherosclerosis and restenosis in vivo is not known, our results suggest that the high levels of PPARγ found in vascular lesion sites could provide a pharmacological differences that could be exploited by selective ligands.

Interestingly, rosiglitazone was a very weak inducer of cell death. These results are in contrast to its effect on CD36 expression, where rosiglitazone is 100 to 1000 times more potent. The use of cells that are genetically PPARγ deficient,24,35 clearly demonstrate that PPARγ ligands can have antiinflammatory and antiproliferative properties independent of PPARγ. The use of high concentrations of thiazolidinediones in this respect has therefore been controversial. However, our data support the contention that the actions of suprapharmacological concentrations of rosiglitazone on apoptosis are still PPARγ dependent, and indicate that there are concentration-dependent targets for rosiglitazone within SMCs. Theoretically, this is possible either by specificity of the activated PPARγ for different target PPREs,22–45 or by the concentration-dependent recruitment of different coactivators.46,47 In this respect, each PPARγ ligand to a varying degree may have selective PPARγ modulator (SPRM) activity on different targets, an acronym used to describe N-(9-fluorenylmethoxycarbonyl)-L-Leu, a PPARγ agonist that acts as an insulin sensitizer at a greater potency than it causes adipogenesis.47

Like PPARγ, COX-2 was expressed in higher amounts in intimal SMCs than in medial SMCs, whereas the opposite expression pattern was observed for COX-1. This COX was active, because the WKY12-22 cells released greater amounts of PGs under basal conditions, or when directly activated by 14C-arachidonic acid. Furthermore, COX-2–like PPARγ is highly expressed in human atherosclerotic lesions,48,49 animal models of vascular damage,50 and particularly at high levels in the neointima after balloon angioplasty in the rat.33 Vascular SMCs release large amounts of PGE2,29,31 from COX-2, and in the present study, we show that in vitro they are capable of releasing a wide variety of other arachidonic acid products including PGD2. COX products can both activate and inhibit PPARγ. The dehydrogenation products of PGD and PGE series prostaglandins, the PGI, and PGA series cyclopentanone prostaglandins, respectively, can serve as PPAR ligands,3,4 whereas PGF2α, can inhibit PPARγ activity via a MAP kinase–induced phosphorylation, mediated by activation of its cell surface FP receptor.52 When intimal cells were treated with the COX substrate arachidonic acid, PPAR reporter gene was activated and CD36 was induced. A COX product does appear to be serving as a PPAR ligand, as the arachidonic acid reporter gene activation was inhibited by the COX-inhibitor piroxicam, a NSAID with no reported PPARγ activity.57 Similar to rosiglitazone, the arachidonic acid induced reporter gene activation and CD36 expression was inhibited by GW0072 at antagonist concentrations.

In summary, developmental/intimal SMCs contains more active PPARγ and COX-2 than corresponding adult/medial SMC lines. Consequently, PPARγ ligands, which may include endogenous COX products, are more potent at producing functional PPARγ-mediated responses in intimal SMCs. Because there is increasing evidence that intimal SMCs play an important role in the pathology of vascular diseases, these intimal SMCs may represent a therapeutic target for PPARγ ligand therapy, and may provide a particularly good in vitro model system to study the ways in which PPARγ ligands exert antiatherosclerotic and antirestenotic effects.

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FIGURE 1. **Differential expression of PPARγ in rat medial and intimal SMC.** *A*, Confocal fluorescent micrographs of PPARγ in medial (WKY3m-22) and intimal (WKY12-22) SMC. Lower panels show control staining for PPARγ where procedures were identical to top panels with the exception that primary antibody was initially pre-absorbed with blocking peptide (1:50) for 1h. n=4; *B*, Representative poly A+ Northern blot showing PPARγ in medial (M; WKY3m-22) and intimal (I; WKY12-22) SMC, compared to the levels of control protein GAPDH.
FIGURE 2. Arachidonic acid more potently induces PGE2 release in primary intimal over primary medial SMC. Shows the release of PGE$_2$, measured by radioimmunoassay, in to the culture supernatant by primary intimal (closed squares; iSMC) and medial (open squares; RASMC) SMC cell lines treated with arachidonic acid (0-3µmol/L) for 48h. Data represents the mean ± S.E. mean of n=9 from 3 separate experiments.
FIGURE. 3. High concentrations of the PPARγ ligands more potently induces cell death in intimal over medial SMC. Shows the change in cell viability of intimal (closed squares; WKY12-22) and medial (open squares; WKY3m-22) SMC cell lines treated with A, 15d-PGJ2 (0-10μmol/L) or B, ciglitazone (0-100μmol/L). Data represents the mean ± S.E. mean of n=9-21 from 7 separate experiments.