Role for Peroxisome Proliferator-Activated Receptor α in Oxidized Phospholipid–Induced Synthesis of Monocyte Chemotactic Protein-1 and Interleukin-8 by Endothelial Cells

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Abstract—The attraction, binding, and entry of monocytes into the vessel wall play an important role in atherogenesis. We have previously shown that minimally oxidized/modified LDL (MM-LDL), a pathogenically relevant lipoprotein, can activate human aortic endothelial cells (HAECs) to produce monocyte chemotactic activators. In the present study, we demonstrate that MM-LDL and oxidation products of 1-palmitoyl-2-arachidonyl-sn-glycero-3-phosphocholine (PAPC) activate endothelial cells to synthesize monocyte chemotactic protein-1 (MCP-1) and interleukin-8 (IL-8). Several lines of evidence suggest that this activation is mediated by the lipid-dependent transcription factor peroxisome proliferator-activated receptor α (PPARα), the most abundant member of the PPAR family in HAECs. Treatment of transfected CV-1 cells demonstrated activation of the PPARα ligand-binding domain by MM-LDL, Ox-PAPC, or its component phospholipids, 1-palmitoyl-2-oxovalaroyl-sn-glycero-phosphocholine and 1-palmitoyl-2-glutaroyl-sn-glycero-phosphocholine; these lipids also activated a consensus peroxisome proliferator-activated receptor response element (PPRE) in transfected HAECs. Furthermore, activation of PPARα with synthetic ligand Wy14,643 stimulates the synthesis of IL-8 and MCP-1 by HAECs. By contrast, troglitazone, a PPARγ agonist, decreased the levels of IL-8 and MCP-1. Finally, we demonstrate that unlike wild-type endothelial cells, endothelial cells derived from PPARα-null mice do not produce MCP-1/JE in response to Ox-PAPC and MM-LDL. Together, these data demonstrate a proinflammatory role for PPARα in mediation of the activation of endothelial cells to produce monocyte chemotactic activity in response to oxidized phospholipids and lipoproteins. (Circ Res. 2000;87:516-521.)

Key Words: atherosclerosis • lipoproteins • phospholipids • interleukins • monocyte chemotactic protein-1 • endothelium

The migration of monocytes into the vessel wall is fundamental to the pathogenesis of atherosclerosis. There is considerable evidence that oxidized lipids play an important role in this recruitment. Our laboratory demonstrated that the treatment of human aortic endothelial cells (HAECs) with minimally oxidized/modified LDL (MM-LDL) can stimulate monocyte endothelial interactions by increasing synthesis of specific monocyte adhesion molecules and monocyte activators. The levels of these induced molecules are all elevated in fatty streak lesions. Oxidized 1-palmitoyl-2-arachidonyl-sn-glycero-3-phosphocholine (Ox-PAPC) and 3 of its component phospholipids, 1-palmitoyl-2-oxovalaroyl-sn-glycero-phosphocholine (POVPC), 1-palmitoyl-2-glutaroyl-sn-glycero-phosphocholine (PGPC), and 1-palmitoyl-2-(5,6-epoxyisoprostane E2)-sn-glycero-3-phosphocholine (PEIPPC), were found to play a major role in the activation of endothelial cells by MM-LDL. These compounds are increased in atherosclerotic lesions, and antibodies that recognize them are detected in apoE-null mice, suggesting their in vivo relevance. Our group has demonstrated that the treatment of endothelial cells with MM-LDL, Ox-PAPC, and POVPC leads to an increase in cAMP levels and that this increase (inhibitable by H-89) was critical to the induction of monocyte binding.

In the present study, we examined the mechanism by which MM-LDL and bioactive phospholipids stimulate endothelial production of monocyte activators (chemotactic factors), monocyte chemotactic protein-1 (MCP-1), and interleukin-8 (IL-8). We hypothesized that peroxisome proliferator-activated receptors (PPARs), a group of lipid-activated transcription factors, may play a role in this stimulation. PPARs are transcription factors that bind to regulatory regions of target genes. We investigated the role of PPARα in the activation of HAECs by MM-LDL or Ox-PAPC.

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genes and activate transcription in response to the binding of lipid-like molecules.\textsuperscript{8–11} Three PPAR family members are known: α, γ, and δ. The function of the ubiquitously expressed PPARδ is not yet clear. Both PPARα and PPARγ have been linked to signaling by lipids and inflammatory mediators. We present evidence to suggest that PPARα may play an important role in mediation of the induction of monocyte chemotactic factors by oxidized phospholipids.

Materials and Methods

Preparation of Cells and Lipids

CV-1 cells were obtained from American Type Culture Collection. Human aortic endothelial cells (HAECs) were isolated and maintained as described previously and used at passages 4 to 8.\textsuperscript{12} PPARα-null mice on a C57B16/J background were generated as described.\textsuperscript{13} Endothelial cells from the thoracic aorta of mice (MAECs) were isolated and cultured (passages 2 to 4) as described previously; purity was >95%.\textsuperscript{14} Human monocytes were isolated and cultured (passages 4 to 8).\textsuperscript{15} CV-1 cells were obtained from American Type Culture Collection.

Preparation of Cells and Lipids

Methods.4–6 POVPC was prepared through the ozonolysis of PGPC, and PEIPC were prepared according to previously described methods.\textsuperscript{4–6} Ox-PAPC, Ox-POVPC, and Ox-PEIPC were prepared in parallel.\textsuperscript{4–6}

Measurement of Levels of PPARα mRNA With TaqMan Real-Time RT-PCR

Total cell RNA was collected from HAECs and human monocytes and treated with DNase, and PPARα and PPARγ were amplified with TaqMan real-time RT-PCR as described previously.\textsuperscript{16} Human PPARα was amplified with sense primer 5'-CTCTTTTGTTGGCTGCTATC-3' and antisense primer 5'-GGGAGGTTGTAGCAGCATGTTG-3'. PPARγ was amplified with sense primer 5'-GAAGAGGCTCTTAACCTCCT-3' and antisense primer 5'-GAACTCCTATGTAATCAGAACG-3'. PPARα reaction produced a 106-bp PCR product, and PPARγ reaction produced an 80-bp PCR product. The amplification reaction also contained the following TaqMan probes (100 nmol/L each): PPARα probe 5'-ATCGTCTGGCGGTCTTAAACGTAG-(FAM)-3' and PPARγ probe 5'-TCCTCCAGACAGACATCTTCAATGGCCA-(FAM)-3'. All reactions were coamplified with human GAPDH probes and primers obtained from Perkin–Elmer Biosystems.

Measurement of Peroxisome Proliferator-Activated Receptor Response Element (PPRE) and PPAR Activation

To determine whether oxidized phospholipids activate PPAR in HAECs, cells were transfected with PPRE-TK-LUC construct\textsuperscript{18} (0.5 μg/well) and pCMX-β-galactosidase (0.5 μg/well) with Superfect Transfection Reagent (Qiagen). Approximately 15% transfection efficiency was determined with a luciferase assay. The luciferase units were normalized with the corresponding β-galactosidase activity. To compare the activation of PPARα, PPARγ, and PPARδ by oxidized lipoproteins and lipids, we used a ligand activation assay with a chimeric receptor that contains the ligand-binding domain of PPARα, PPARγ, or PPARδ fused to the DNA binding domain of the yeast transcriptional activator GAL4 as described previously.\textsuperscript{18,19}

Chemokine Assays

To measure chemokine levels in the conditioned medium, cells were preincubated for 16 hours with M199 supplemented with 0.8 mg/mL human lipoprotein-deficient serum (HAECs) or DMEM that contained 1% FBS (MAECs); they were then treated for 4 hours with lipids, Wy14,643 (Chemyn or BIOMOL), or troglitazone (Sankyo). In some experiments, HAECs were pretreated for 30 minutes with 2.5 μmol/L H-89 before the addition of the oxidized phospholipids. Wy14,643 and troglitazone contained <0.001 pg/mL LPS. Medium was used to assay levels of IL-8 or MCP-1 (HAECs) or MCP-1/JE (MAECs) with QuantiBek kits (R and D Systems). RNA was harvested from HAECs for measurement of MCP-1 and IL-8 mRNA levels with RiboQuant Multi-Probe RNase protection kit (PharMingen).

Results

Quantification of PPARs in HAECs

The levels of PPARα and PPARγ mRNA in HAECs and human monocytes were determined with TaqMan real-time RT-PCR. PPARα was found to be the most abundantly expressed PPAR in HAECs. The ratio of α to γ was 5.06. By contrast, the ratio of α to γ in monocytes was 0.043. We conclude that although both receptors are present HAECs, it is likely that PPARα is the dominant receptor.

Oxidized Phospholipids and PPARα Agonist

Increase and PPARγ Agonist Decreases the Production of IL-8 and MCP-1 by HAECs: Effects of Oxidized Phospholipids Are Not Inhibited by H-89

Previous studies from our laboratory have demonstrated that the treatment of endothelial cells with MM-LDL increased the level of monocyte chemotactic activity in the medium and increased synthesis of MCP-1.\textsuperscript{20} We now show that the oxidized phospholipids from MM-LDL, Ox-PAPC, POVPC, and PGIC increase both IL-8 (Figure 1A) and MCP-1 (Figure 1B) protein synthesis (levels of IL-8 and MCP-1 in medium from Ox-PAPC–treated cells averaged 7 and 1 ng/mL, respectively). Unlike the induction of monocyte binding, the cAMP pathway was not involved in the induction of IL-8 or MCP-1 synthesis, because protein levels were not different in cells pretreated for 30 minutes with 2.5 μmol/L H-89 (increase in IL-8: Ox-PAPC 620%±40%; Ox-PAPC+H-89 601%±35%; increase in MCP-1: Ox-PAPC 213%±10%, Ox-PAPC+H-89 181%±13%). To test the hypothesis that PPARα was involved in the increased synthesis of IL-8 and MCP-1 effects of Wy14,643 and troglitazone were also examined. HAECs were treated for 4 hours with 20 μmol/L Wy14,643 (a level shown to be a PPARα specific\textsuperscript{21}) or 20 μmol/L troglitazone (a level shown to be PPARγ specific). Wy14,643 significantly increased the level of IL-8 (Figure 1A) and MCP-1 (Figure 1B). Wy14,643 increased chemokine production at concentrations from 5 to 10 μmol/L in separate experiments. In contrast, troglitazone decreased IL-8 (Figure 1A) and MCP-1 (Figure 2B) synthesis. Troglitazone also strongly inhibited the ability of Ox-PAPC to increase IL-8 synthesis, whereas Wy14,643 had no significant effect (Figure 1C). Troglitazone displayed a similar inhibitory effect on MCP-1 synthesis (data not shown). In a separate experiment, in which MCP-1 and IL-8 protein levels were increased ~100% and ~200%, respectively, mRNA (after 4 hours of treatment) for MCP-1 and IL-8 was also significantly increased by Ox-PAPC (MCP-1 40%, IL-8 80%) as determined with RNase protection assay. Pretreatment of HAEC with actinomycin D completely abolished the increases in MCP-1 and IL-8 (data not shown). We conclude that oxidized phospholipids and...
Wy14,643 increase the levels of IL-8 and MCP-1 mRNA and protein, whereas a PPAR γ agonist decreases the levels. Ox-PAPC, the most active lipid, was not toxic to the cells at 50 μg/mL as measured by the amount of 14C released from endothelial cells containing 14C-labeled ATP. This method has been shown to provide an early measure of toxicity. 22,23

Oxidized Phospholipids Activate PPARs

To determine whether oxidized phospholipids can activate PPAR-dependent signaling in endothelial cells, HAECs were transfected with a reporter plasmid containing a consensus PPRE upstream of a luciferase gene. Wy14,643, as well as Ox-PAPC, PGPC, and POVP, activated endogenous PPARs to induce the transcription of the PPRE-luciferase reporter gene (Figure 2). To determine which PPAR was activated, CV-1 monkey kidney fibroblasts were transiently transfected with the GAL4-PPAR α, -PPAR γ, or -PPAR δ expression vectors and UAS-luciferase reporter and assayed for response to modified lipoproteins and oxidized phospholipids. MM-LDL in contrast to native LDL activated the PPAR α ligand-binding domain, and most of this activity could be attributed to the oxidized phospholipid (PL) present in the lipoprotein; activity was not found in fatty acids or neutral lipids (Figure 3). Ox-PAPC, the most active MM-LDL component, but not native PAPC, dose dependently activated the PPAR α tran-
To identify the specific bioactive lipid responsible for activation, we tested the effects of 2 components of Ox-PAPC, namely POVPC and PGPC in the reporter assay. As shown in Figure 4B, both POVPC and PGPC activated PPARα at concentrations of 1 to 5 μg/mL (1 to 7 μmol/L). Neither MM-LDL, Ox-PAPC, phospholipid components of MM-LDL, or POVPC significantly activated PPARγ. However, PGPC activated PPARγ ∼2-fold at the highest concentration used compared with the 8-fold increase in PPARα activation. None of the compounds activated PPARδ.

**Decreased Response to Ox-PAPC and MM-LDL by Aortic Endothelial Cells From PPARα-Null Mice**

To test directly the role of PPARα in the induction of MCP-1/JE by oxidized phospholipids, aortic endothelial cell cultures from PPARα-null mice and wild-type mice on the C57Bl6/J background were exposed to LPS, Ox-PAPC, or MM-LDL for 4 hours. The levels of MCP-1/JE were measured with ELISA (Figure 5). Although there was considerable variation between the wild-type and PPARα-null mice, the mean increases in response to LPS were 1006% and 1136% above control, respectively, and were not significantly different in the 2 strains. In response to MM-LDL, wild-type cells showed dramatic increases in JE, ranging from 700% to 2500%, whereas PPARα-null endothelial cells did not respond to the MM-LDL and actually showed a small decrease. Native LDL did not induce MCP-1/JE production by either strain of mice (data not shown). In response to Ox-PAPC, PPARα-null MAEC displayed a mean decrease in MCP-1/JE (∼37%), compared with a mean increase of 77% in cultures from wild-type endothelial cells. These experiments suggest that PPARα-dependent signaling has an important role in maintenance of the basal expression level of MCP-1/JE and mediation of the increase in MCP-1/JE in response to Ox-PAPC and MM-LDL.

**Discussion**

These results provide evidence that PPARα plays an important role in the effects of MM-LDL, and its component oxidized phospholipids on endothelial synthesis of monocyte activators. The increased production of MCP-1 and IL-8 was not inhibited by H-89 pretreatment, suggesting that the cAMP pathway was not involved. Several lines of evidence from the current study support a role for the PPARα pathway. PPARα has previously been reported to be present in endothelial cells. Quantification of mRNA levels revealed that unlike monocytes, PPARα is expressed at higher levels than PPARγ in HAECs. Our studies demonstrate that ligand activation of PPARα by Wy14,643 in HAECs leads to increased production of monocyte chemotactic factors MCP-1 and IL-8 protein (Figures 1A and 1B) and mRNA. We have shown that oxidized phospholipids activate a PPRE reporter transfected into HAECs and activate PPARα in CV-1 cells (Figures 2, 3, and 4). The most direct evidence for a role for PPARα in the action of Ox-PAPC was the finding that...
induction of MCP-1/IE synthesis in response to MM-LDL or Ox-PAPC was essentially abolished in PPARα-null aortic endothelial cells (Figure 5). Thus, our studies definitively demonstrate a role for PPARα in the induction of MCP-1/IE by Ox-PAPC and MM-LDL in mouse aortic endothelial cells. They suggest (because of PPRE activation and Wy14,643-induced increases) that PPARα also plays a role in MCP-1 and IL-8 induction by oxidized phospholipids in HAECs. Interestingly, the PPARγ activator troglitazone inhibited basal IL-8 synthesis as well as that stimulated by Ox-PAPC (Figure 1C). This is similar to the findings of Su et al.26 which showed that troglitazone and other known PPARγ ligands inhibit the induction of MCP-1 and IL-8 in colonic epithelial cells. In vivo studies have shown that PPARα and PPARγ agonists exert differing effects on metabolism.27 Furthermore, knockouts of PPARα and PPARγ have very different phenotypes.13,28

Based on previous studies, there are several possible mechanisms by which these bioactive phospholipids could stimulate PPARα activation. The oxidized phospholipids (Ox-PL) could serve as ligands for PPARs. However, it is unlikely that oxidized phospholipids would move to the nucleus and bind to the PPAR receptor. A second possibility is that hydrolytic products of the bioactive phospholipids might activate PPARs. However, hydrolytic products of Ox-PAPC did not increase MCP-1 or IL-8 or activate PPARα- or PPARγ-dependent transcriptional activity (data not shown). Based on past studies, we suggest that Ox-PL activates a second messenger pathway, such as the lipoxigenase (LO) pathway, producing PPAR ligands. Our group and others have shown that LO products can activate PPARs and are ligands for both α and γ.10,29–32 Our group has shown that the treatment of HAECs with MM-LDL increases the production of LO products and that inhibition of this pathway blocks the induction of monocyte binding.33 This second messenger system would represent a novel lipid activation pathway. In this pathway, an oxidized phospholipid would induce the generation of endogenous lipid ligand for PPARs through binding to a surface receptor and subsequent activation of 12/15-LO. Studies with 12/15-LO–null animals suggest an important role for this molecule in atherogenesis.34 It is also possible that the oxidized phospholipids generate an as-yet-unidentified, high-affinity ligand through the activation of a different pathway. The existence of a ligand-independent activation pathway for PPARα also cannot be excluded at this point.

Although the present study demonstrates a proinflammatory effect of PPARα activation, previous studies from our group and others have reported anti-inflammatory roles of PPARs in cytokine- or LPS-induced activation. These studies have demonstrated inhibition of endothelin-1, human vascular cell adhesion molecule-1 and vascular smooth muscle cell activation, as well as the induction of interleukin-6 in response to PPAR activation.25 Some of our group has shown inhibition of LPS-induced vascular cell adhesion molecule expression by Wy14,643 in HAECs.25 Here we report specific proinflammatory effects of PPARα activation in the absence of cytokines or LPS. The actions of PPARα agonists in HAEC are thus similar to our previously reported effects of MM-LDL and oxidized phospholipids on specific inflammatory responses. These agents are proinflammatory when added to cells in the absence of cytokines and LPS but inhibit several proinflammatory effects of LPS and tumor necrosis factor.6 The behavior of PPARα in endothelial cells is similar to that of PPARγ in macrophages, which also depends on cell context. PPARγ ligands have been documented to suppress cytokine gene expression in activated macrophages36,37 but to induce gene expression in nonactivated monocytes.10,30 We thus hypothesize that the transcription factors assembled on the promoter are different in the presence and the absence of cytokines and LPS.

It is important to consider the implications of our studies for the use of PPARα agonists such as drugs (fibrates) in the treatment of atherosclerosis. Systemic treatment results in the simultaneous activation of many PPARα pathways. The overall effect of PPARα agonists on atherosclerosis has been shown to be beneficial, probably because of their effects on lipid levels. However, this does not preclude a role for PPARα in mediation of the induction of inflammatory genes in the vessel wall in response to endogenous ligands present in atherosclerotic lesions. This latter effect of PPARα agonist may, in particular context, have clinical significance; and interference in this pathway could, in some settings, be therapeutic.

In summary, the present study demonstrates that PPARα plays a role in mediation of the effects of oxidized phospholipids on endothelial cell synthesis of monocyte activators MCP-1 and IL-8. In separate studies, we have observed that the levels of Ox-PAPC products in aortas of animals with atherosclerotic lesions are 2- to 10-fold more than required to increase MCP-1 and IL-8,38 which supports their in vivo relevance to atherosclerosis. Thus, our group, in the present and past studies, has identified several mechanisms by which PPAR activators may be proinflammatory and potentially proatherogenic, as summarized in Figure 6. Others have identified settings in which the activation of PPARα may be anti-inflammatory. Taken together, results from the present and past studies from our group and others suggest that the role of PPARα activation may differ in different inflammatory settings or may involve multiple signaling pathways.
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