Oxidized low-density lipoprotein and peroxisome-proliferator-activated receptor α down-regulate platelet-activating-factor receptor expression in human macrophages

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Regulation of the expression of platelet-activating factor (PAF) receptor by atherogenic lipoproteins might contribute to atherogenesis. We show that progressive oxidation of low-density lipoprotein (LDL) gradually inhibits PAF receptor expression on the macrophage cell surface. We tested the effect of oxidized LDL (oxLDL) on PAF receptor expression in human monocytes that do not contain peroxisome-proliferator-activated receptor γ (PPAR γ), a nuclear receptor activated by oxLDL. OxLDL decreased by 50% ($P \leq 0.001$) and by 29% ($P \leq 0.05$) the binding of PAF and the expression of PAF receptor mRNA respectively. Next we demonstrated that progressive oxidation of LDLs significantly activated PPAR α -dependent transcription in transfected mouse aortic endothelial cells. Finally we demonstrated, in mature macrophages, that fenofibrate (20 μ M), a specific PPAR α agonist, but not the specific PPAR γ agonist BRL49653 (20 nM), significantly decreased both PAF binding

and PAF receptor mRNA expression, by 65% and 40% ($P \leq 0.001$) respectively. Additionally, another PPAR α agonist, Wy14,643, decreased PAF receptor promoter activity by 70% ($P \leq 0.05$) in transfected THP-1 cells, suggesting the involvement of the proximal promoter region (–980 to –500) containing a series of four nuclear factor (NF)- κ B motifs. Thus PPAR α might be involved in the down-regulation of PAF receptor gene expression by oxLDLs in human monocytes/macrophages. The oxidation of one or more lipid components of LDLs might result in the formation of natural activators of PPAR α . It is hypothesized that such activators might modulate inflammation and apoptosis upon atherogenesis by decreasing the expression of PAF receptor.

Key words: atherosclerosis, inflammation, thiobarbituric-acid-reactive substances.

INTRODUCTION

Human monocyte-derived macrophages and macrophage-derived foam cells have a key role in the formation of atherosclerotic plaques [1]. The accumulation of macrophages follows the adhesion of monocytes to the endothelium and their subsequent transmigration into the arterial wall. These highly active cells synthesize numerous proinflammatory substances, including platelet-activating factor (PAF) [2], a potent phospholipid mediator of inflammation, thrombosis, apoptosis and anaphylaxis that has been extracted from human atherosclerotic plaques [3]. PAF is produced in small amounts upon the oxidation of only defined subspecies of low-density lipoproteins (LDLs) [4] and might have an important role in early atherogenesis by contributing to inflammation [5]. PAF autocrine and paracrine effects are mediated via a specific cell-surface receptor that had initially been cloned from guinea-pig lung [6].

The PAF receptor is a member of the seven-transmembrane-domain receptor family coupled to G-proteins [6,7], leading to the activation of various second messenger systems, including phospholipases A₂, C and D [8] and the mitogen-activated-

protein kinase cascade [9]. The PAF receptor mRNA has been detected in several tissues and in circulating cells such as monocytes [10,11]; its transcription is driven from two distinct promoters [12], indicating a potential variability in PAF receptor gene regulation in different tissues. In monocyte/macrophages, promoter-1-directed transcription predominates [13]. Promoter-1 contains four nuclear factor (NF)- κ B [14] and one Spl consensus binding sites, a myeloid specific sequence and a sequence similar to the 'Initiator' sequence that controls 50–60% of the basal PAF receptor expression in both U937 and THP-1 cell lines [15]. We have demonstrated that oxidized LDLs (oxLDLs) down-regulate the constitutive expression of the PAF receptor gene in human macrophages *in vitro* [13] and *in vivo* in arterial wall [16], although the transcriptional mechanism of this inhibition has not been established.

A key feature of atherogenesis involves the entry of LDLs into the arterial intima, where they are modified by oxidation [17]. The retention of oxLDLs in the extracellular matrix and their uptake by macrophages lead to intracellular cholesterol accumulation and foam-cell formation. Macrophages recognize and internalize various forms of oxLDL via multiple receptors

Abbreviations used: HODE, hydroxyoctadecadienoic acid; LDL, low-density lipoprotein; MAE, mouse aortic endothelial; NF, nuclear factor; oxLDL, oxidized low-density lipoprotein; PAF, platelet-activating factor; PPAR, peroxisome-proliferator-activated receptor; PPARE, peroxisome-proliferator-responsive element; TBARS, thiobarbituric-acid-reactive substances; TNF, tumour necrosis factor.

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[18], including scavenger receptors SRA I and SRA II, CD36, a member of the SRB family as well as SR-BI/CLA-I, and Fc receptors. Monocytes and monocyte-like cell lines express substantial levels of SRA receptors but only after differentiation into macrophages [19]; in contrast, CD36 is expressed in both monocytes and macrophages [18] and contributes to the uptake of oxLDL-containing fatty acids. Additionally, Nagy et al. [20] showed that CD36 is involved in the uptake of oxLDL-containing ligands for peroxisome proliferator-activated receptor γ (PPAR γ) and principally two major oxidized metabolites of linoleic acid, namely 9- and 13-hydroxyoctadecadienoic acid (9-HODE and 13-HODE).

PPARs are transcription factors belonging to the nuclear receptor family [21] that are important in lipid and glucose metabolism and thus modulate the onset of human metabolic diseases such as dyslipidaemia and type II diabetes. PPARs are ligand-dependent transcription factors that, on dimerization with the 9-*cis*-retinoic acid receptor, bind to specific response elements termed peroxisome-proliferator-response elements (PPRE), thus regulating the expression of target genes [22]. PPAR α and PPAR γ mediate the action of hypolipidaemic fibrates [23] and the anti-diabetic thiazolidinediones [24] respectively. Natural prostaglandin J_2 derivatives [25] and 9(*S*)-HODE and 13(*S*)-HODE [20] are ligands for PPAR γ , whereas eicosanoids such as leukotriene B_4 [26] and 8(*S*)-hydroxyeicosatetraenoic acid [23,27] are natural ligands for PPAR α . Activators of PPARs not only regulate plasma cholesterol and triacylglycerol concentrations but might also exert an activity at the level of the vascular wall. Indeed, the PPAR α activator fenofibrate decreases human atherosclerotic plaque formation [28]. Additionally, it has been postulated that both PPAR α and PPAR γ might interfere with atherogenesis, in part by exerting anti-inflammatory activities [29–31]. *In vivo*, PPAR α knock-out mice exhibit a prolonged response to inflammatory stimuli [26]. Finally, PPAR γ might have a role in foam-cell formation by promoting the differentiation of monocytes into macrophages followed by an enhanced uptake of oxLDL [20,32].

The aim of the present study was to evaluate the effect of oxLDL and oxidized lipids such as 9(*S*)-HODE and 13(*S*)-HODE on the expression of the PAF receptor in human monocytes and monocyte-derived macrophages and to study the role of PPARs therein. We show that PPAR α , but not PPAR γ , is implicated in the down-regulation of PAF receptor gene expression by oxLDL.

MATERIALS AND METHODS

Materials

Buffy coats for monocyte isolation were obtained from the Broussais Hospital Transfusion Center (Paris, France). RPMI-1640 culture medium and PBS were supplied by BioWhittaker. Pools of human sera (100–150 donors) for use in cell culture media were supplied by ATGC (Noisy-le-Grand, France). Nutridoma HU medium was supplied by Boehringer Mannheim (Meylan, France). RNA Plus, phenol and oligonucleotides were from Bioprobe System (Montreuil-sous-Bois, France). Dyna-Zyme™ thermostable DNA polymerase was obtained from Finnzymes OY (Espoo, Finland). PAF $C_{18:0}$ (1-*O*-octadecyl-2-*O*-acetyl-*sn*-glycero-3-phosphocholine) was supplied by France Biochem (Meudon, France). The labelled nucleotides [α - 32 P]dCTP (3000 Ci/mmol) and [3 H]octadecyl-9,10-PAF (100 or 160 Ci/mmol) were purchased from Dupont de Nemours Division NEN (Les Ulis, France). Superscript RNase H reverse transcriptase was from Gibco-BRL (Bethesda Research Laboratories, Bethesda, MD, U.S.A.). Prostaglandin J_2 was from Sigma (St

Louis, MO, U.S.A.) and fenofibrate was from Laboratoires Fournier (Daix, France). The chromogenic *Limulus* amoebocyte lysate assay for lipopolysaccharide (catalogue no. 822734) was purchased from Biogenic (Maurin, France). The assay kits for lactic dehydrogenase (catalogue no. 1442597) and the bicinchoninic acid reagents (catalogue no. 23223) were purchased from Roche Diagnostics and from Pierce (Interchim, France) respectively. Optiphas Hisafe3 was from Fisher Chemicals (Loughborough, Leics., U.K.). Wy14,643 was from Chemsyn (Lenexa, KS, U.S.A.). BRL49653 was provided by Dr Berthelon (Lipha, Lyon, France). The 9(*S*)-HODE (catalogue no. 38410) and 13(*S*)-HODE (catalogue no. 38610) were from Cayman Chemical (Ann Arbor, MI, U.S.A.), supplied in ethanol at -20°C .

Isolation and culture of human monocytes and monocyte-derived macrophages

Mononuclear cells were isolated from 1-day-old buffy coats of healthy normolipidaemic donors and were plated into 15 mm diam. plastic culture dishes, in RPMI medium containing gentamicin (40 $\mu\text{g}/\text{ml}$) and glutamine (0.05%) at a density of 10^6 cells per well for receptor binding assays, and into 35 mm diam. plastic culture dishes at 3×10^6 cells per well for RNA assays [13,33]. Monocytes were obtained after 1 h of adherence to the plastic dishes, followed by three washes with PBS. Monocyte monolayers were either used immediately or were further cultured in RPMI medium supplemented with human serum [10% (v/v)]; at day 12 of culture, monocyte-derived macrophages (denoted as macrophages) were washed three times with PBS and then incubated for defined periods with oxLDL (100 $\mu\text{g}/\text{ml}$ protein), with various compounds (indicated in each legend) or without treatment, in the same medium, except that human serum was replaced by Nutridoma HU [1% (v/v)]. All cell cultures and incubations were performed in a humidified 37°C incubator in air/ CO_2 (19:1). The viabilities of cells, measured by Trypan Blue exclusion and the release of lactic dehydrogenase into the medium, were similar in treated and untreated cells. In addition, cell protein content was not affected by treatment. Monocytes and macrophages were characterized by immunohistochemical methods [34].

Lipoprotein purification and chemical modifications

LDLs were isolated from normolipidaemic human plasma with sterilized material in a single-step discontinuous gradient in a Beckman NVT65 rotor [35]. Before chemical modification, LDLs were dialysed against 0.01 M PBS, pH 7.4. Copper-oxidized LDLs were prepared under sterile conditions by incubating 500 μg of LDL protein/ml with $2.5 \mu\text{M}$ CuCl_2 for various periods (0–24 h) at 37°C . At the end of the incubation period, oxidations were stopped by the addition of EDTA (3 mM). Before treatment of macrophages, oxLDLs were extensively dialysed at 4°C against PBS, pH 7.4, then against RPMI-1640 and subsequently filtered through a $0.22 \mu\text{m}$ pore-size filter (Millipore). Dialysed, untreated LDLs are denoted by $_{\text{cu}}$ LDL. The degree of lipid oxidation was estimated on the basis of the contents of thiobarbituric acid-reactive substances (TBARS) [36], 31 ± 3 nmol equiv. MDA/mg of LDL protein for $_{\text{ox}_{24\text{h}}}$ LDL compared with $_{\text{cu}}$ LDL (6 ± 2 nmol equiv. MDA/mg LDL protein) ($P \leq 0.001$, $n = 3$). Properties of LDL preparations oxidized for shorter periods are detailed in each Figure legend. The endotoxin content of LDL preparations was measured before addition to cell culture medium by use of the chromogenic *Limulus* amoebocyte lysate assay based on activation by endotoxin of an enzyme present in lysate that hydrolyses a synthetic

substrate containing paranitraniline; all LDL preparations contained less than 50 pg of endotoxin per 100 μ g of LDL protein.

Binding assays of [3 H]PAF to macrophages

Binding assays were performed directly in the culture dishes, at 20 °C for 30 min, in a final volume of 300 μ l with 4.2 mM Hepes/saline buffer containing 1.3 mM CaCl_2 , 1 mM MgCl_2 and 0.25% BSA at pH 7.4. In all experiments the binding assays were performed at a unique [3 H]PAF concentration of 1 nM as described previously by Stengel et al. [13]. Specific binding was determined as the total radioactivity bound minus the radioactivity bound in the presence of 1 μ M unlabelled PAF (non-specific binding). The binding reaction was terminated by rinsing (eight times) with cold Hepes/saline buffer, pH 7.4 (CaCl_2 , MgCl_2 and BSA omitted). The adherent cells were lysed in 0.5 ml of 0.1 M NaOH and then counted for their content of radioactivity in the presence of Optiphase Hisafe3.

RNA isolation, first-strand cDNA synthesis and PCR

Total RNA was isolated from adherent monocytes or from differentiated macrophages with RNA Plus. First-strand cDNA synthesis was performed with 5 μ g of total RNA in a total volume of 30 μ l and, after denaturation, reverse transcription was performed at 37 °C for 2 h in a total volume of 50 μ l as described [13]. Detection and quantification of human PAF receptor mRNA and actin mRNA were performed in the same tube by PCR in the presence of two specific oligonucleotides: (1) L1 and C1 corresponding to transcript 1 of the human PAF receptor (190 bp) [12] and (2) actin 1 and actin 2 from Clontech (838 bp). The final concentrations were 1 $\times$ Dynazyme buffer, 0.2 mM dNTP, 100 ng of oligonucleotides of each upstream and downstream primer, and 1 unit of Dynazyme DNA polymerase in the presence of [α - 32 P]dCTP, described previously [13]. Incubations were performed in an Omnigene Hyber thermocycler starting with a cycle at 94 °C for 5 min followed by 30 cycles of 30 s at 94 °C, 45 s at 59 °C and 2 min at 71.2 °C successively; these cycles were followed by one cycle for 7 min at 72 °C. The PCR products were analysed by fractionation of 20 μ l aliquots on a 2% (w/v) agarose Tris/acetate/EDTA gel and counting 32 P-labelled bands after slicing the gel.

Plasmids and cell transfection

Plasmid pGal5-TK-pGL3 was obtained by inserting five copies of the Gal4 DNA-binding site in front of the thymidine kinase promoter and luciferase reporter gene of the pTK-pGL3 plasmid. Plasmids pGal4-hPPAR α and pGal4-hPPAR γ were constructed by PCR amplification of the hPPAR α and hPPAR γ 2 DEF domains, with pSG5-hPPAR α and pSG5-hPPAR γ 2 as templates [37]. The resulting PCR products were cloned into pBD-Gal4 (Stratagene, La Jolla, CA, U.S.A.) and the chimaeras were subsequently subcloned into the pCDNA3 vector.

Mouse aortic endothelial (MAE) cells, grown to 50–60% confluence in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal calf serum, were transiently transfected with a lipid cationic technique (RPR 120535B; Rhône-Poulenc Rorer, Vitry, France) with reporter and expression plasmids. The latter permitted the production of a chimaeric protein between the Gal4 DNA-binding domain and the PPAR ligand-binding and transactivator domains. pGK- β -galactosidase expression plasmid (50 ng) was co-transfected as a control for transfection efficiency. At 2 h after transfection, medium was refreshed and supplemented with the various PPAR activators. After 48 h, cells were collected and the luciferase and β -galactosidase assays were

performed. Reporter gene activity is presented as fold induction over vehicle control.

The promoterless luciferase reporter gene plasmid, pGL3basic (Promega), was used to construct all PAF receptor promoter plasmids. For the p2.1Luc (–2100 to +37 bp of promoter 1) Raji DNA was used to amplify the sequence by PCR on the basis of the sequence (GenBank® accession number U11032) [15]. The p.98Luc (–980 to +37), p.5Luc construct (–500 to +37) and p.16Luc construct (–157 to +37) were prepared by PCR from the p2.1 sequence.

THP-1 cells (500 000 per well) were transfected with Superfect (Quiagen) as described by the manufacturer with various PAF receptor promoter constructs (2 μ g per well) and CMV- β Gal (0.5 μ g) as an internal control. At 12 h after transfection, THP-1 cells were refed with RPMI supplemented with 0.2% fetal calf serum in the presence of Wy14,643 (20 μ M) or vehicle [DMSO 0.1% (v/v)] and incubated for 24 h. After 48 h, cells were collected and the luciferase and β -galactosidase assays were performed as described above.

Statistical analysis

Data are expressed as means $\pm$ S.D. Differences were examined by the paired Student's *t* test. All experiments were performed at least three times with different cell preparations.

RESULTS

Effect of oxLDL on PAF receptor expression

We have demonstrated that oxLDLs down-regulate PAF receptor expression at the transcriptional level in monocyte-derived macrophages [13]. In the present study we investigated the impact of the degree of oxidation of LDLs on PAF receptor expression in macrophages. After a 6 h incubation with LDLs oxidized for 2 or 4 h respectively, expression of the PAF receptor was decreased by 28% and 45%, whereas highly oxidized LDLs (6 or 24 h) PAF binding more markedly decreased (by 62% and 70%; $P \leq 0.05$, $n = 3$) (Figure 1). Thus, progressive oxidation of LDLs (TBARS content above 15 nmol equiv. of malonyldialdehyde/mg of LDL protein) gradually inhibits PAF receptor expression on the macrophage cell surface (Figure 1). We also observed that neither native LDL ($97 \pm 6\%$) nor acetylated LDL ($87 \pm 14\%$) decreased PAF binding to macrophages, which indicated that modified LDL, in which the lysine residues of apoB100 had undergone derivatization, exerted no detectable effect of PAF receptor expression. We therefore postulated that a non-defined oxidized lipid component might be involved in the regulation of this receptor.

We observed that oxysterols (5 μ g/ml) such as 7 β -hydroxycholesterol or 25-hydroxycholesterol did not inhibit PAF binding to macrophages ($99 \pm 14\%$ and $110 \pm 13\%$ respectively). Thus we excluded a role of these oxysterols in PAF receptor regulation. In contrast, we examined the effect of two major oxidized metabolites of linoleic acid present in oxLDL, 9(S)-HODE and 13(S)-HODE, on PAF binding to macrophages. 9(S)-HODE and 13(S)-HODE (34 μ M) significantly inhibited PAF binding, by 60% ($P < 0.05$, $n = 3$) and 40% ($P < 0.01$, $n = 3$) respectively. In contrast, treatment of macrophages with linoleic acid (the precursor of these two oxidized fatty acids) did not diminish PAF binding (results not shown). These results suggest that oxidized metabolites of linoleic acid, i.e. 9(S)-HODE and 13(S)-HODE, might mediate the down-regulation of PAF receptor expression by oxLDLs. As these HODEs are potent agonists of PPAR α and PPAR γ in macrophages [20,38], they might be involved in the oxLDL-mediated effect on PAF receptor ex-

Figure 1 Impact of oxidation degree of LDL on PAF binding in mature macrophages

Mature human macrophages were treated for 6 h with LDL (100 μ g/ml) oxidized to different degrees for the determination of PAF binding (A). Binding of PAF to macrophages was expressed as a percentage of control. [3 H]PAF binding to control macrophages (100%), treated with $_{ox}$ LDL, was determined as 477 fmol/ μ g of protein. LDLs were oxidized by incubation at 37 $^{\circ}$ C at a concentration of 0.5 mg of LDL protein/ml with 2.5 μ M $CuCl_2$ for 0, 1, 2, 4, 6 or 24 h. TBARS contents were determined in (B). Values are means $\pm$ S.D. for three separate experiments, each performed in triplicate. * $P \leq 0.05$; ** $P \leq 0.01$ relative to control values.

Figure 2 Effect of highly oxidized LDL on PPAR α -dependent transcription in endothelial cells

MAE cells were transiently transfected with luciferase reporter plasmids (100 ng) driven by five copies of Gal4-binding site (Gal5TKpGL3-Luc) in the presence of the chimaeric receptor for PPAR α (Gal4-hPPAR α , 20 ng). Cells were incubated for 48 h in the presence of $_{ox_{24h}}$ LDLs at increasing concentrations from 0 to 100 μ g/ml protein. Reporter gene activity was determined as fold induction relative to the control. Values are means $\pm$ S.D. for three independent determinations. * $P \leq 0.05$; ** $P \leq 0.01$ relative to control values.

pression. Because human monocytes do not contain PPAR γ [39], we tested the effect of oxLDLs on PAF receptor expression in these cells. Both [3 H]PAF binding and mRNA expression were diminished, respectively by 50% ($P \leq 0.001$, $n = 3$) and 29% ($P \leq 0.05$, $n = 3$), after incubation of adherent monocytes for 6 h (for binding assay) or 4 h (for mRNA studies) with $_{ox_{24h}}$ LDL (100 μ g/ml). Similar preparations of oxLDL also diminished the PAF receptor expression in mature monocyte-derived macrophages [13], indicating that oxLDL might similarly down-regulate PAF receptor expression in both monocytes and monocyte-derived macrophages. Furthermore, the latter observation might suggest that PPAR γ is not involved in the regulation of the PAF receptor in these cells.

Figure 3 Impact of degree of oxidation of LDLs on PPAR α -dependent transcription

The activity of LDLs oxidized to different degrees (100 μ g/ml) on PPAR α -dependent transcription was determined with the transient transfection system described in Figure 5. LDLs were copper-oxidized *in vitro* from 0 to 24 h. Control indicates the absence of LDL treatment. Reporter gene activity was determined as fold induction relative to the control. Values are means $\pm$ S.D. for three independent determinations. * $P \leq 0.05$; ** $P \leq 0.01$ relative to control values.

Effect of oxLDL and oxidized lipids on PPAR-dependent transcription

To determine whether PPAR α were involved in the oxLDL-mediated regulation of PAF receptor expression, we first tested the hypothesis that oxLDLs might activate PPAR α -dependent transcription. MAE cells were transiently transfected with a chimaeric receptor for PPAR α and a plasmid containing five Gal4-binding sites linked to a luciferase reporter gene, and were subsequently incubated with increasing concentrations of $_{ox_{24h}}$ LDL (10–100 μ g/ml). Figure 2 shows that treatment of MAE cells with $_{ox_{24h}}$ LDL at concentrations of 50 and 100 μ g/ml activated PPAR α -dependent transcription 10-fold and 33-fold respectively. Next we compared the ability of LDL oxidized to different degrees (0–24 h) to activate PPAR α -dependent transcription (Figure 3). The degree of oxidation of LDL preparations

Figure 4 Effect of 9(S)-HODE and 13(S)-HODE on PPARα-dependent and PPARγ-dependent transcription

MAE cells were transiently transfected with luciferase reporter plasmids (100 ng) driven either by five copies of Gal4-binding site (Gal5TKpGL3-Luc) in the presence of the chimeric receptor for PPARα (Gal4-hPPARα, 20 ng), or by PPARγ (Gal4-h PPARγ, 20 ng). Cells were incubated for 48 h in the presence of 9(S)-HODE or 13(S)-HODE (68 μM). Specific agonists of PPARα, and PPARγ, Wy14,643 (50 μM) and BRL49653 (1 μM) respectively, were used as positive controls. Reporter gene activity was determined as fold induction relative to the solvent [0.1% (v/v) DMSO, control]. Values are means ± S.D. for three independent determinations. * $P \leq 0.05$; ** $P \leq 0.01$ relative to control values.

was estimated from the content of TBARS (5–28 nmol equiv. of malonyldialdehyde/mg of LDL protein). PPARα-dependent transcription increased on incubation with LDLs oxidized for 2 or 6 h (7 ± 1-fold and 9 ± 3-fold induction). Highly oxidized LDLs (18 and 24 h) markedly increased PPARα-dependent transcription (respectively 16 ± 5-fold and 26 ± 5-fold). These results indicate that oxLDLs activate PPARα, suggesting that PPARα might be involved in the diminished expression of the PAF receptor.

PAF binding was decreased by oxidized metabolites of linoleic acid, 9(S)-HODE and 13(S)-HODE (30 μM), which are ligands of PPARγ. We examined the effect of 9-HODE and 13-HODE on PPARα-dependent transcription. As shown in Figure 4, 9(S)-HODE, and to a smaller extent 13(S)-HODE, increased PPARα-dependent transcription (respectively 25 ± 4-fold and 11 ± 1-fold). In contrast, neither their precursor linoleic acid (71 μM) nor cholesterol, oxysterols (i.e. 7β-hydroxycholesterol and 25-hydroxycholesterol) or PAF (1 μM) was able to activate PPARα-dependent transcription (results not shown). Surprisingly, 9(S)-HODE and 13(S)-HODE were twice as potent in activating PPARα-dependent transcription (25 ± 4-fold and 11 ± 1-fold induction) than PPARγ-dependent transcription (11 ± 3-fold and 2 ± 0.01-fold induction). As a control, specific agonists of PPARs, BRL49653 (1 μM) for PPARγ and Wy14,643 (50 μM) for PPARα, led to a significant elevation of PPARγ- and PPARα-dependent transcription (Figure 4). Thus 9(S)-HODE and 13(S)-HODE, which are components of oxLDLs, activate PPARα at least as efficiently as PPARγ [20].

Table 1 PAF receptor expression in human macrophages treated with PPARα and PPARγ agonists

Mature human macrophages were treated with the PPARγ agonists prostaglandin-J₂ (PG-J₂) and BRL49653 for 48 h for [³H]PAF binding or for 24 h for mRNA expression, and with the PPARα agonist fenofibrate for 24 h for [³H]PAF binding or for 12 h for mRNA expression. Total mRNA was extracted and cellular PAF binding was assayed as described in the Materials and methods section. After normalization with actin, cDNA synthesis was expressed as a percentage of initial amount. Specific binding of PAF to macrophages was determined as the percentage inhibition relative to vehicle control. Values are means ± S.D. for three separate experiments, each performed in triplicate. * $P < 0.01$; ** $P < 0.001$ compared with control.

Treatment	[³ H]PAF binding (%)	mRNA expression (%)
Control	100	100
BRL49653 (20 nM)	70.3 ± 20	102.4 ± 19.5
PG-J ₂ (1 μM)	62.5 ± 11*	72.9 ± 16
ox _{24h} LDL (100 μg/ml)	36.5 ± 11**	60.9 ± 5*
Fenofibrate (20 μM)	34.8 ± 21*	56.8 ± 1.5**

Effect of PPAR agonists on human macrophage PAF receptor expression

To determine whether PPARs were involved in the regulation of the expression of the PAF receptor, we investigated the cellular effects of PPARα and PPARγ agonists. Macrophages treated for 24 h with fenofibrate (20 μM), a PPARα ligand, resulted in significant decreases in PAF binding (by 65%; $P \leq 0.01$) and PAF receptor mRNA expression (by 43%; $P \leq 0.001$) (Table 1). The latter effect was dose-dependent (results not shown) and similar to that of oxidized LDL (Table 1). Thus agonists of PPARα down-regulated PAF receptor gene expression in human macrophages. In contrast, PPARγ agonists showed no significant effect on PAF receptor gene expression; macrophages were treated for 48 h with either the prostanoid prostaglandin J₂ (1 μM), the precursor of 15-deoxy-Δ^{12,14}-prostaglandin J₂ or were treated with the specific anti-diabetic agent BRL49653 (20 nM). Treatment with prostaglandin J₂ significantly decreased specific PAF binding (by 40%; $P \leq 0.01$) but had no significant effect on PAF receptor mRNA expression. Treatment with BRL49653 did not significantly decrease PAF binding to macrophages and was without effect on PAF receptor mRNA expression. Thus agonists of PPARγ did not seem to be involved in regulation of the expression of the PAF receptor gene in human macrophages, even though PPARγ is expressed in mature human macrophages [39]. These findings suggest that prostaglandin might down-regulate PAF binding at the macrophage cell surface by a mechanism independent of PPARγ activation, as recently described for interleukin 6 and tumour necrosis factor (TNF)-α responses of macrophages to lipopolysaccharides [40]. Thus the transcriptional regulation of the PAF receptor gene might be specifically PPARα-dependent.

To determine whether PPARα-agonists were indeed involved in PAF receptor gene down-regulation, we performed transfection experiments in a monocytic cell line THP-1 with various PAF receptor promoter constructs bearing the luciferase reporter gene (Figure 5, upper panel). Figure 5 (lower panel) shows that Wy14,643 significantly decreased PAF receptor promoter p2.1Luc activity (by 70%; $P \leq 0.05$). A shorter construct (p.98Luc) showed a similar decrease (by 62%; $P \leq 0.05$) in promoter activity, suggesting that the sequence (−2100 to −980 bp) is not involved in PPARα-induced inhibition. In contrast, we did not observe any effect of Wy14,643 when much shorter constructs were used (p.5Luc and p.16Luc) devoid of four NF-κB [14], activator protein 1 and CCAAT-enhancer-binding protein β motifs ('MathInspector'; Figure 5, upper

Figure 5 Effect of Wy14,643 on PAF receptor expression in THP-1 cells

Upper panel: human PAF receptor promoter constructs with respect to the initiation start site (+1). Abbreviations: C/EBP β , CCAAT-enhancer-binding protein β ; AP1, activator protein 1; Inr, initiator. Lower panel: THP-1 cells were transiently transfected with luciferase reporter plasmids of various PAF receptor promoter constructs (2 μ g per well). At 12 h after transfection, cells were incubated for 24 h in the presence of PPAR α agonist (Wy14,643, 20 μ M, solid columns) or untreated [0.1% (v/v) DMSO, control, open columns]. Reporter gene activity was determined as relative activity units. Values are means and S.D. for three independent determinations. * $P \leq 0.05$ relative to control values; NS, not significant.

panel). The later result suggested that the promoter region (–980 to –500 bp) present in the p.98Luc construct was involved in the PPAR α -agonist-induced inhibition of PAF receptor expression. The sequence similar to the ‘initiator’ motif present in the PAF receptor promoter construct p.16Luc (indicated by +1 in Figure 5, upper panel), which recognizes the basal transcriptional machinery in TATA-less promoters, is probably not implicated in this mechanism.

DISCUSSION

Here we have shown for the first time that oxidized LDLs down-regulate the expression of the PAF receptor in both human monocytes and monocyte-derived macrophages, at least in part, via the activation of PPAR α . We have shown that specific PPAR α agonists decrease PAF receptor expression in mature macrophages and decrease PAF receptor promoter expression in a transfected monocytic cell line.

Modulation of the expression of the PAF receptor by atherogenic lipoproteins might contribute to atherogenesis by potentially decreasing the effects of PAF and its oxidized phosphatidylcholine ether analogues [5]. Indeed, PAF activates the recruitment and adhesion of circulating monocytes [41,42] and induces inflammation in arterial intima by stimulating the synthesis and secretion of cytokines such as TNF- α , interleukin 1 and interleukin 6 [2]. Previously, Rouis et al. [43] demonstrated *in vitro* that PAF might contribute to LDL oxidation by stimulating the production of active oxygen species by human monocyte-derived macrophages. Furthermore, these authors

demonstrated that PAF stimulates the production and secretion of elastase-type enzymes, which degrade extracellular matrix [33].

We demonstrated that oxLDL diminished the expression of the PAF receptor in monocytes and mature macrophages. Thus the effect of oxLDLs might be CD36-dependent, because monocytes express the CD36 receptor but not the classical scavenger receptor class A [18,19]. Additionally, acetylated LDL showed no effect on PAF receptor expression, suggesting that chemical modification of apoB100 is not involved in the inhibition exerted by oxLDLs. Therefore the active moiety of oxLDL might be an oxidized lipid or a derivative but not, as shown here, the oxysterols. In our study, progressive oxidation of LDLs gradually inhibited PAF binding to macrophages. Esterbauer et al. [44] demonstrated a temporal relationship between the degradation of polyunsaturated fatty acids (mainly linoleic acid and arachidonic acid) and the increase in malonyldialdehyde and other aldehydic lipid peroxidation products in LDLs. The oxidation of LDLs involves formation and release of lysophospholipids and oxidized fatty acids. *In vitro*, the lipid profile generated by copper-mediated oxidation is very similar to that of oxLDLs found in human atherosclerotic lesions [45,46]. Indeed, the presence of specific oxidized fatty acids, namely 9(*S*)-HODE and 13(*S*)-HODE, is correlated with lesion severity [47]. Biochemical analysis of LDL oxidized *in vitro* showed that 67% of oxidized linoleate products were indeed 9- and 13-derivatives [45]. In our study we showed that 9-HODE and 13-HODE decreased PAF binding to the macrophage cell surface; in contrast, no effect was observed on treatment of macrophages

with linoleic acid, the precursor of both compounds. However, other lipids produced by the oxidation of LDLs and phospholipase A₂-treated LDLs might mediate gene regulation by PPAR α , as described [38].

Nagy et al. [20] have shown that 9(*S*)-HODE and 13(*S*)-HODE, which are products of LDL oxidation, are ligands of PPAR γ . In the present study, however, we observed that oxLDLs diminished the expression of the PAF receptor in monocytes. Because PPAR γ is not expressed in human monocytes [39], we hypothesized that the oxidation of one or more lipid components of LDLs might result in the formation of effective activators of PPAR α , which is present in monocytes. We clearly demonstrated that oxLDLs activate PPAR α -dependent transcription in a murine cell line in a dose-dependent manner. We compared PPAR α -dependent and PPAR γ -dependent transcription in response to 9(*S*)-HODE and 13(*S*)-HODE and found that both compounds increased PPAR α -dependent transcription to a greater extent than PPAR γ . Thus 9(*S*)-HODE and 13(*S*)-HODE are not only ligands for PPAR γ [20] but might equally serve as activators for PPAR α . Our results are in accordance with recent studies that showed that conjugated linoleic acid is indeed a ligand and activator of PPAR α [48].

We demonstrated that PPAR α , which is transcriptionally active in differentiated macrophages [39] and is activated by specific PPAR α agonists, down-regulated PAF receptor expression in mature macrophages. Because the PAF receptor is implicated in inflammation and apoptosis [49], we therefore postulate that PPAR α might modulate inflammation and apoptosis by decreasing the expression of PAF receptor. In contrast, PPAR γ agonists did not significantly diminish the expression of PAF receptor, even though PPAR γ is transcriptionally active in our macrophage model [39]. Thus the transcriptional regulation of the PAF receptor gene might be specifically PPAR α -dependent. Such differential activities of PPAR α and PPAR γ in macrophages has not previously been reported. The PPAR α effects were possibly achieved through direct protein–protein interactions involving PPAR α itself and other transcription factors. In this context, it has been shown that PPAR α diminishes the activation of NF- κ B in transfected Cos-1 cells, leading to the inhibition of PMA-induced production of cyclo-oxygenase-2 [29].

The promoter of the PAF receptor gene does not contain a PPRE consensus sequence; no results supporting an interaction with PPARs have yet been reported. It remains unclear as to which mechanism is involved in the regulation of the expression of the PAF receptor. We have shown here that the PPAR α agonist, Wy14,643 markedly down-regulated the expression of PAF receptor promoter constructs transfected into THP-1 cells, suggesting that PPAR α might directly or indirectly down-regulate PAF receptor expression. Using deleted promoter constructs we showed that the region [–980 to –500] containing the series of four NF- κ B sites that have been implicated in mechanical stretch-induced up-regulation of PAF receptor expression in smooth-muscle cells [14] seemed necessary for the inhibitory effect of Wy14,643 in PAF receptor gene expression. Because a constitutive NF- κ B might be functionally active in monocyte/macrophages [50], PPAR α agonists might regulate PAF receptor expression by down-regulating NF- κ B transactivation activity, a mechanism that has been described recently [37]. Additionally, the activator protein 1 consensus site, which is present in the sequence of the promoter region (–980 to –500), might also be involved in the inhibitory effect of Wy14,643 in PAF receptor gene expression by the same mechanism [37]. Identification of the precise mechanism of regulation will require further studies in the promoter region [–1980 to –157].

In conclusion, we have demonstrated that PPAR α is certainly a good candidate for the down-regulation of PAF receptor gene expression by oxLDLs in human monocytes and macrophages. The oxidation of one or more lipid components in oxLDLs might result in the formation of natural activators of PPAR α . Oxidized fatty acids, and probably other lipid components of oxLDLs, might modulate inflammation and apoptosis by decreasing the cellular expression of PAF receptor. Thus PPAR α ligands generated from oxLDLs might modulate the progression of atherosclerotic plaque formation.

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