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Oxidized phospholipids inhibit cyclooxygenase-2 in human macrophages via nuclear factor-κB/IκB- and ERK2-dependent mechanisms

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Abstract

Objective: Oxidized low-density lipoproteins (ox-LDL) or their components suppress macrophage inflammatory response by down-regulating cytokine synthesis, nitric oxide synthase and inducible cyclooxygenase (Cox-2). This event is crucial for the pathophysiological process leading to the formation of atherosclerotic plaque. Our present study focused on the mechanisms through which oxidized phospholipids inhibit LPS-induced Cox-2 expression in human macrophages. **Methods:** Macrophages were incubated with a mixture of oxidized fragmented phospholipids (ox-PAPC), present in modified LDL, and then exposed to LPS. Cox-2 was evaluated in terms of protein levels, mRNA and activity. **Results:** Ox-PAPC dose-dependently inhibited Cox-2 protein, mRNA and activity by preventing NF-κB binding to DNA. This effect was consequent to alterations of the degradation pattern of IκBα. Moreover, ox-PAPC markedly prevented extracellular signal-regulated kinase (ERK2) activation, leading to Cox-2 expression, whereas activation of the transcription factor peroxisome proliferator-activated receptors (PPARs) was not influenced. **Conclusion:** ox-PAPC down-regulates LPS-induced Cox-2 expression in human macrophages by targeting both NF-κB/IκB and ERK2 pathways. An altered inflammatory response by macrophages within atheromata may contribute to the progression of atherosclerosis.

Keywords: Atherosclerosis; Infection/inflammation; Macrophages; Prostaglandins; Signal transduction

1. Introduction

Macrophages are significant cellular participants throughout the development of atherosclerosis, which presents features of an inflammatory disease [1]. These cells are engaged to ingest modified lipoproteins, e.g., ox-LDL, through a variety of scavenger receptors and to recognize oxidized phospholipids as markers of oxidized moieties, all events that ultimately lead to the generation of the foam cells.

Once activated by inflammatory stimuli, macrophages synthesize and release eicosanoids that are potent modulators of inflammation. The rate-limiting step in their synthesis is the enzyme cyclooxygenase (Cox), present in two isoforms. Cox-1, which is constitutive and detected in most human tissues, and the inducible Cox-2, selectively expressed by a variety of cells exposed to bacterial lipopolysaccharide (LPS) and cytokines [2]. In macrophages, Cox-2 induction by LPS is characterized by 'redundancy' both at the extranuclear signaling level and at the level of transcriptional activation [3]. Mitogen-activated protein kinase (MAP-kinase) and protein kinase C (PKC) are involved in Cox-2 expression by LPS [4,5]. These pathways act, in a coordinate way, with several

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transcription factors, among which the nuclear factor κ-B (NF-κB) [3,6]. NF-κB activation is controlled by the family of inhibitors of κB protein (IκB) which retain the transcription factor in the cytoplasm. Treatment of macrophages with inflammatory stimuli targets the degradation of IκBα and allows NF-κB to translocate into the nucleus [7]. NF-κB activation is also regulated by members of the peroxisome proliferator-activated receptors (PPARs) family, through a mechanism termed 'transrepression' [8–10]. Different isoforms of PPARs have been identified so far; in particular, PPARγ activation has been shown to down-regulate several inflammatory responses of monocytes and macrophages, among which is the production of inflammatory cytokines [8].

We have previously shown that ox-LDL and its phospholipid component as well, impairs Cox-2 in human macrophages, an effect mimicked by ox-PAPC, a surrogate for oxidized phospholipids obtained by autoxidation of 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine [11]. Ox-PAPC has been identified as a component of minimally oxidized LDL and its molecular structure and biological activities have been characterized [12,13].

In this study we have investigated the mechanisms through which ox-PAPC impairs Cox-2 in human macrophages.

2. Materials and methods

2.1. Reagents

Cell culture medium and reagents were from BioWhittaker. Lipopolysaccharide (*E. coli* 0114:B4) was from Difco Labs. Benzamidine hydrochloride, leupeptin hemisulfate, soybean trypsin inhibitor, sodium orthovanadate, 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine (PAPC), bovine serum albumin (fatty acid-free and low endotoxin), clofibrate, neutral red, 3-(4,5-dimethylthiazol-2-yl)2,5-di-phenyltetrazolium bromide (MTT) and bisphenol A diglycidyl ether (Der Resin 332, BADGE) were from Sigma. EDTA and NaF was from Carlo Erba. Electrophoresis reagents were from Pharmacia Biotech. Wy 14,643 and 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2) was from Biomol, 13-oxo-octadecadienoic acid (13-oxoODE) was from Cayman Chemicals and TRIzol Reagent from Life Technologies. BRL-49653 was a kind gift by Dr. S. Trowbridge (SmithKline Beecham Pharmaceuticals).

Ox-PAPC was prepared according to Watson by transferring 1 mg PAPC in 100 μl of chloroform to a clean 16×25-mm glass test tube and evaporating the solvent under nitrogen stream [12]. The lipid residue was allowed to oxidize on exposure to air for 48 h. Caution was taken to avoid LPS contamination that was checked by the Limulus amebocyte lysate assay test (BioWhittaker). Aliquots of ox-PAPC were stored at −80 °C under nitrogen and used within 1 week.

2.2. Monocyte culture

Venous blood from healthy donors was obtained in accordance with the principles outlined in the Declaration of Helsinki. Mononuclear cells were separated by Ficoll-Paque density gradient (Pharmacia Fine Chemicals), as described [11]. Monocytes were isolated from lymphocytes by adherence to six-well plates (2 h at 37 °C, 5% CO_2). Cell preparations were >90% monocytes, as determined by nonspecific esterase staining. Macrophages were obtained by culturing monocytes for 7 days at 37 °C in a 5% CO_2 humid atmosphere in M-199 containing 2 mM glutamine, 0.5% antibiotics and 10% human heat-inactivated AB serum. They were identified by the presence of CD68Ag detected by a specific monoclonal mouse anti-human macrophage antibody (Dako). The endotoxin content of all culture materials and reagents was measured with the Limulus assay. Unless otherwise specified, macrophages were incubated with various agents in serum-free medium containing 0.2% fatty acid-free bovine serum albumin for 2 h. Medium was then removed and macrophages were exposed for further 4 h to fresh medium containing LPS (1 μg/ml). This experimental protocol was adopted in order to avoid a direct interaction between LPS and ox-PAPC, since the formation of a stable complex LPS/ox-LDL has been reported [14]. Incubation time and LPS concentration were selected on the basis of results obtained in preliminary experiments. Macrophage viability was assessed by Neutral Red and MTT assay.

2.3. Cyclooxygenase activity

Macrophages were washed with Hanks' buffer (pH 7.4) containing 1 mg/ml BSA and incubated with the same buffer containing 10 μM sodium arachidonate. After 30 min, supernatants were harvested and the amount of TxB_2 released in the supernatant was measured by enzyme immunoassay (Cayman Chemicals), as previously described [11].

2.4. Cell extract and Western blot analysis

Whole cell lysates were prepared in RIPA buffer containing 5 mM sodium orthovanadate, 100 mM NaF, 5 μg/ml leupeptin, 5 μg/ml aprotinin, 2 mM pefabloc. Protein content was determined by the micro-bicinchoninic acid assay [15]. For cytosol extract preparation, macrophages were washed twice in ice-cold TBS, harvested in 100 μl buffer A (10 mM Hepes, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT) and incubated for 15 min on ice. Triton X-100 (0.4%) was added. Cytosols were separated from nuclei by centrifugation at 15 000×*g* for 1 min at 4 °C. SDS–PAGE was performed as previously described [11]. Membranes were subsequently incubated for 1 h at room temperature with monoclonal antibodies raised against Cox-2, mAb 29 (1/10 000) or Cox-1, mAb

10 and 11 (5 μg/ml), kindly donated by A. Habib, U 348, INSERM, Paris. Antibodies directed against the C-terminal PPARγ peptide (monoclonal, 1/500), against IκBα (polyclonal, 1/500), and against haem-oxygenase-1 (polyclonal, 1/2000) were from Santa Cruz Biotechnology. MAP-kinase immunoblotting was performed using antibodies (1/1000) against phosphospecific p44–p42 MAP-kinase (Thr202/Tyr204, monoclonal, New England Biolabs, Celbio) and p38-kinase ($pTpY^{180/182}$, polyclonal, Biosource International), as previously described [16]. Blots were incubated with donkey anti-mouse or anti-rabbit IgG conjugated with peroxidase (Jackson ImmunoResearch) at 1/5000 for 1 h at room temperature. Monoclonal antibody directed against β-actin (Sigma) was used as internal standard for control of protein load. ECL substrates (Amersham) were used to reveal positive bands that were visualized after exposure to HyperfilmTM ECL (Amersham).

2.5. RNA extraction and reverse transcriptase-polymerase chain reaction (RT-PCR)

Total cellular RNA was extracted from macrophages with TRIzol Reagent. Cox-2 mRNA levels were detected using a coupled reverse transcription-polymerase chain reaction (RT-PCR) performed using a GeneAmp RNA PCR kit (Perkin-Elmer) according to the manufacturer's instructions. To identify Cox-2 mRNA, exact primers were synthesized by an analytical RT-PCR procedure developed for human cyclooxygenase. Cox-2 primers were 5′-TTCAAATGAGATTGTGGAAAATTGCT-3′ (a 27-mer sense, oligonucleotide at position 573) and 5′-AGATCATCTCTGCCTGAGTATCTT-3′ (a 24-mer antisense oligonucleotide at position 878), giving rise to a 305-bp PCR product [17]. Primers were also synthesized to amplify the cDNA encoding GAPDH, a constitutively expressed gene, as control. GAPDH primers were 5′-CCACCCATGGCAAATTCCATGGCA-3′ (a 24-mer sense oligonucleotide at position 216) and 5′-TCTAGACGGCAGGTCAGGTCCACC-3′ (a 24-mer antisense oligonucleotide at position 809) giving rise to a 593-bp PCR product. RNA concentration was determined spectrophotometrically. Total cellular RNA from each sample (1 μg) was reverse transcribed at 37 °C for 1 h. The same amount of the resulting cDNA was used for amplification by specific primer for human Cox-2. PCR amplification was carried out, in the presence of 0.2 μM primers, using a thermal cycler GeneAmp PCR System 2400 (Perkin-Elmer). The following conditions were used: 30 cycles with denaturation at 94 °C for 1 min; primer annealing at 55 °C for 1 min: extension at 72 °C for 1 min. The amplification of human GAPDH was used as internal standard. Aliquots (10 μl) of amplified products were resolved by electrophoresis through a 2% agarose gel. Amplified cDNA bands were detected by ethidium bromide staining.

2.6. Nuclear extracts and electrophoretic mobility shift assay

Nuclear extracts were prepared from cell suspensions using a protocol according to Dignam et al. [18], slightly modified [19]. The nuclear pellet was washed with 1 ml buffer B (20 mM Hepes, pH 7.9, 400 mM NaCl, 25% glycerol, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM PMSF, and 0.5 mM DTT). Samples were vortexed for 30 min at 4 °C. Debris was removed by centrifugation at 13 000×*g* for 10 min. After supernatant collection, the protein content was determined and electrophoretic mobility shift assays were performed as follows: the synthetic single-stranded oligonucleotides (Eurogentec, Herstal, Belgium) containing the proximal and distal NF-κB sites were annealed with the complementary primers and radiolabeled with [^{32}P]dCTP (Amersham). The consensus sequences for NF-κB are underlined: distal NF-κB site (upstream, within −455 to 428 from the transcriptional start site) 5′-GGCGGGAGAGGGGATTCCCTGCGCCCCC-3′; proximal NF-κB site (downstream, within −232 to 205 from the transcriptional start site) 5′-CAGGAGAGTGGGGACTACCCCCTCTGCT-3′ [20]. Nuclear extracts (4 μg) were incubated for 20 min at room temperature with 100 000 cpm of ^{32}P-labeled double-strand oligonucleotides. Reactions were performed in binding buffer (18 μl) containing 60 mM KCl, 20 mM Hepes, 1 mM EDTA, 15 mM DTT, 12% glycerol and 4 μg poly(dI/dC) (Amersham). Protein–DNA complexes were separated from free DNA probe by electrophoresis through 5% nondenaturating acrylamide gels in 0.5×TBE (45 mM Tris base, 45 mM boric acid, 1 mM EDTA). Gels were dried and subjected to autoradiography. NF-κB specific bands were confirmed by competition with a 100-fold molar excess of an unlabeled NF-κB probe.

2.7. Transfection assays

CHO cells grown in F-12 medium were transiently transfected using a calcium phosphate precipitation method, as described by Sambrook et al. [21]. A reporter construct containing three copies of a consensus PPRE cloned upstream of the thymidine kinase-luciferase reporter (J_3-TK-LUC) was used [22]. The control vector pRSV-galactosidase (Promega) was cotransfected as a control for transfection efficiency. The cells were plated in six-well plates and transfected when 80–90% confluent, 3 h after a complete medium replacement. Cells were incubated with calcium phosphate-precipitated DNA (1 μg plasmid/well) for 5 h. After shock (2-min glycerol, 15%, v/v), fresh medium containing stimuli was added and cells were harvested 2 h later. Supernatants were removed and luciferase production was measured on cell lysates using a commercially available kit (Promega). After scraping, cells were transferred into Eppendorf tubes, vortexed, subjected to freezing and thawing and finally centrifuged at 12 000×

g for 15 s. Aliquots of supernatants (20 μl) were mixed with the luciferase substrate reagent and absorbancies were determined by a fluorimeter (Lumat LB 9501, Berthold). β-Galactosidase activity was determined by standard assay (Invitrogen). Luciferase data were corrected for β-galactosidase activity and are reported as percentage activity compared with control cells.

2.8. Statistical analysis

Data are reported as means±S.E.M. Computer-assisted statistical analyses used the ANOVA program, and probability values were calculated using Fisher's protected least-significant difference test. A value of $P<0.05$ was considered significant.

3. Results

3.1. Ox-PAPC inhibits LPS-induced Cox-2 in human macrophages

Macrophages differentiated from monocytes (7-day culture) did not express Cox-2, nor did they after incubation with ox-PAPC (5 μg/ml) for 4–24 h. In contrast, exposure to 1 μg/ml LPS for 4 h induced appreciable expression of Cox-2, recognized as a double band at 70 kDa. Ox-PAPC incubated for 2 h before LPS diminished the amount of Cox-2 in a concentration-dependent manner (Fig. 1A). The same extent of inhibition was observed when ox-PAPC was incubated longer (up to 24 h) before LPS addition (not shown). In contrast, ox-PAPC added concomitantly with, or 1 or 2 h after LPS did not affect Cox-2 (Fig. 1B). Subsequent experiments therefore were performed in macrophages exposed to ox-PAPC for 2 h prior to LPS. Ox-PAPC reduced Cox-2 activity, measured as thromboxane B_2 (TxB_2) released in the supernatant (Fig. 1C). The maximal concentration of ox-PAPC used throughout the study (10 μg/ml) has been reported to be present in 100 μg/ml ox-LDL [23].

Ox-PAPC (10 μg/ml) did not influence macrophage viability as assessed by Neutral Red (>90% macrophages were viable). A minor decrease of the cell number was observed by MTT test, which suggests a very limited, if any, toxicity.

Under these experimental conditions, the expression of Cox-1 was not influenced (not shown).

Cox-2 is characterized by the presence of a haem group.

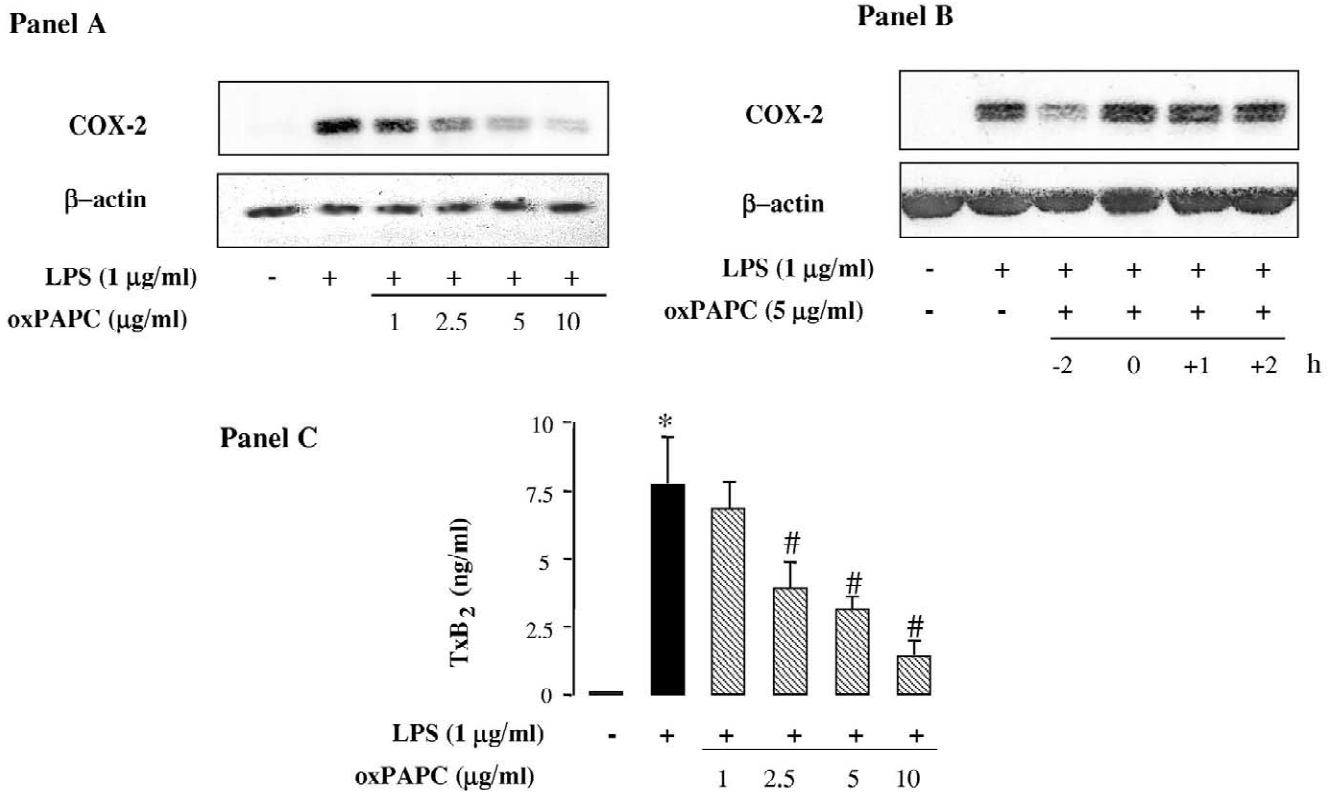


Fig. 1. Ox-PAPC concentration-dependently inhibits Cox-2 and thromboxane B_2 (TxB_2) formation in LPS-stimulated macrophages. (A) Macrophages were incubated with medium or with ox-PAPC (1–10 μg/ml) for 2 h. LPS (1 μg/ml) was added and incubated for further 4 h. (B) ox-PAPC (5 μg/ml) was added to macrophage culture 2 h before, simultaneously with or after (1 or 2 h) LPS. Cox-2 and β-actin were determined in macrophage lysates by Western blot analysis. (C) Cox-2 activity was measured in macrophage supernatants as TxB_2 formation, by EIA. Results are the means±S.E.M. of three independent experiments. *$P<0.01$ versus basal; #$P<0.01$ versus LPS.

The possibility that ox-PAPC impairs Cox-2 levels through an increased expression of the haem-degrading enzyme haem oxygenase-1 (HO-1) was therefore tested. Ox-PAPC was ineffective to induce HO-1, ruling out a role of this enzyme in the observed decrease of Cox-2 levels (not shown).

Reduction of Cox-2 protein levels was consequent to attenuation of Cox-2 mRNA levels, as assessed by RT-PCR. In contrast unoxidized PAPC did not affect Cox-2 mRNA (Fig. 2).

3.2. Ox-PAPC inhibits LPS-induced NF-κB activation in human macrophages by altering the degradation of IκBα

Ox-LDL are known to influence the expression of several LPS-induced inflammatory genes by interfering with NF-κB activation [24–26]. Nuclear extracts of macrophages exposed to LPS for 45 min formed a DNA–protein complex with the distal NF-κB sequence which was prevented if a 100-fold excess of unlabeled NF-κB probe has been added (Fig. 3A). No DNA–protein complex was observed when the proximal NF-κB sequence was used as a probe (data not shown). This is in agreement with results published by Barrios-Rodiles et al. in the same cell type [20].

Ox-PAPC concentration-dependently diminished the amount of the DNA–protein complex (Fig. 3A), which suggests that Cox-2 expression is impaired by the prevention of NF-κB translocation.

To further explore the mechanisms involved in the effect of ox-PAPC on NF-κB activation, the pattern of IκBα degradation was evaluated. LPS-treatment of macrophages resulted in a loss of immunoreactive IκBα protein, detected at 30 min from LPS addition (Fig. 3B). Macrophages, pretreated with ox-PAPC exhibited a reduced IκBα degradation (Fig. 3B). The effect was concentration-dependent (Fig. 3C).

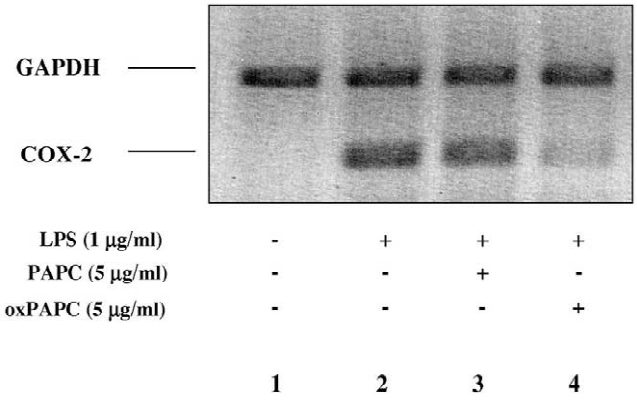


Fig. 2. Ox-PAPC decreases Cox-2 mRNA, PAPC does not. Macrophages were incubated with medium (lanes 1 and 2) or with PAPC (5 μg/ml), either oxidized (lane 4) or unoxidized (lane 3) for 2 h. LPS (1 μg/ml) was then added for 1 h. Total RNA was extracted and Cox-2 and GAPDH mRNA levels were estimated by RT-PCR. The sequences of the primer sets and oligonucleotide probes for Cox-2 and GAPDH are given in Section 2. Results are representative of three independent experiments.

3.3. Ox-PAPC inhibits LPS-induced p38 and ERK2 activation in human macrophages

NF-κB activation in LPS-stimulated macrophages is under the control of MAP-kinase [3]. To define the role of MAP-kinase cascade in Cox-2 induction by LPS, human macrophages were preincubated for 30 min with 25 μM PD098059, a specific inhibitor of ERK1/2 activity and with 10 μM SB203580, which is specific for p38 kinase. Macrophages were then exposed to LPS for further 4 h. LPS-induced Cox-2 was prevented by PD098059 but not by SB203580 (not shown), which suggests a pivotal role of ERK1/2 in LPS-induced Cox-2 expression in human macrophages.

The hypothesis that ox-PAPC interferes with MAP-kinase pathway was tested by a Western blot technique using antibodies specific for the phosphorylated forms of p38 and ERK1/2. Compared to unstimulated macrophages, bands corresponding to the phosphorylated isoform of p38 kinase and ERK2, respectively, were evident in macrophages exposed to LPS (Fig. 4). The band appeared within 10–20 min, with a peak at 30–40 min and values still above the basal level by 60 min (Fig. 4A). Ox-PAPC (5 μg/ml) almost completely reduced p38 and ERK2 phosphorylation (Fig. 4A). The effect of ox-PAPC on both kinases was concentration-dependent (Fig. 4B)

3.4. Effect of PPAR activators on LPS-induced Cox-2

Ox-PAPC represents a natural ligand of PPARα in human endothelial cells [27,28]. The presence of PPARα has been detected in human monocytes [29], whereas in monocytes differentiated into macrophages, the PPARγ isoform predominates [29]. The presence of PPARγ was detected in differentiated macrophages obtained in our culture condition (not shown). To investigate whether PPAR activation is involved in the NF-κB-mediated inhibition of Cox-2, we exposed macrophages for 2 h to a series of PPAR ligands. The synthetic PPARα ligand Wy 14,643 (100 μM) and clofibrate (500 μM), did not affect Cox-2 (Fig. 5A). Similarly, BRL-49653, a synthetic high affinity PPARγ ligand (Fig. 5B) did not affect Cox-2, even when incubated with macrophages for much longer time (up to 24 h) before LPS addition (not shown). The natural PPARγ ligand 13-oxoODE did inhibit Cox-2 expression (Fig. 5B), but only at 50 μg/ml, a concentration twice as high as that reported to activate PPARγ [30]. Another natural PPARγ ligand, 15d-PGJ_2 [31,32], concentration-dependently decreased Cox-2 protein (Fig. 5C).

Transient transfection experiments were carried out in CHO cells, which express PPARγ [33], using a reporter plasmid containing three copies of a consensus PPRE upstream of a luciferase reporter gene (J_3-TK-LUC). Ox-

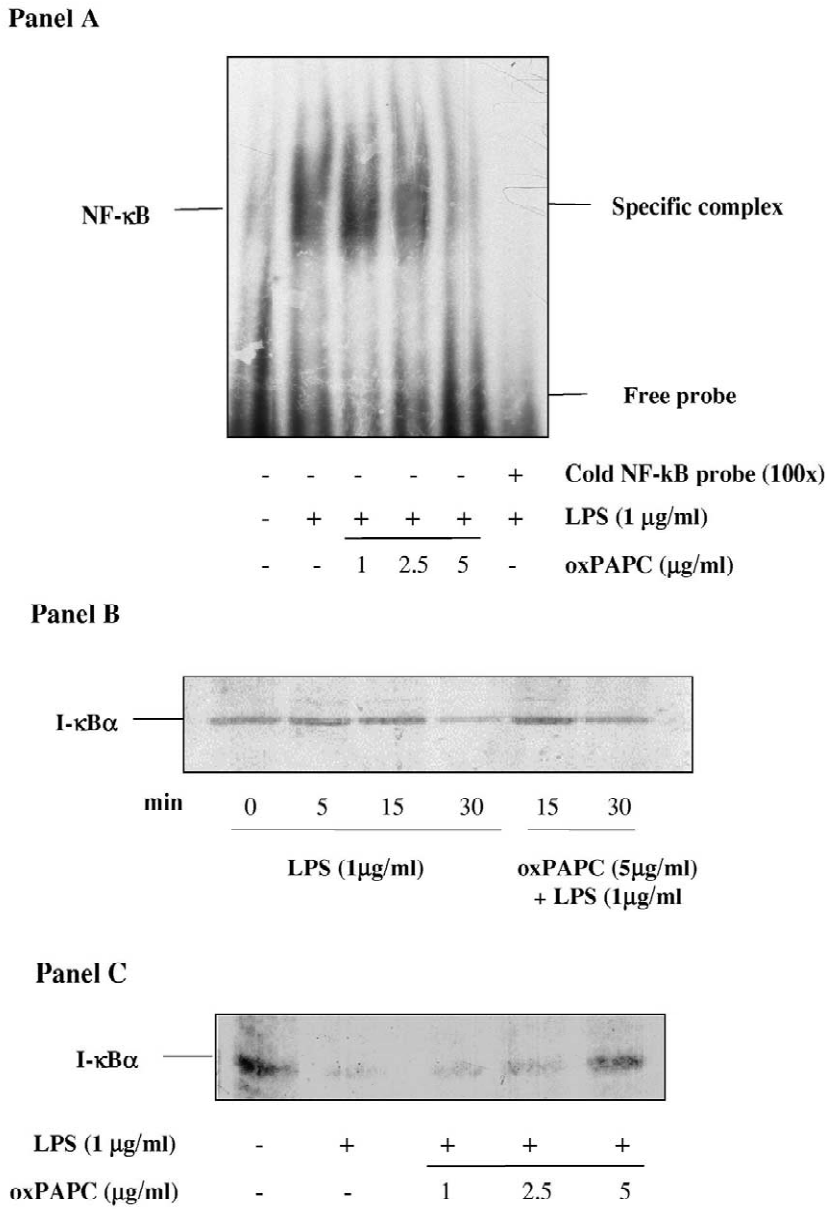


Fig. 3. Ox-PAPC inhibits LPS-induced NF-κB translocation and reduces IκBα degradation in human macrophages. (A) EMSAs for the distal NF-κB probe were performed on nuclear extracts of macrophages treated with medium alone (lanes 1 and 2) or with medium supplemented with ox-PAPC (1–5 μg/ml) (lanes 3–5). Macrophages were then exposed to LPS (1 μg/ml) for 45 min. Specificity of the binding was determined on cell extracts by competition, using a 100-fold excess of unlabeled distal probe (lane 6). Results are representative of three independent experiments. (B) Macrophages pretreated with ox-PAPC (5 μg/ml) or medium alone were stimulated with LPS for the indicated times. (C) Ox-PAPC concentration-dependently reduces IκBα degradation. Analysis of IκBα protein content was performed by Western blot on cytosolic extracts, as described in Section 2. Results are representative of three independent experiments.

PAPC (5 μg/ml, 2-h incubation) did not affect luciferase activity. In contrast, BRL-49653 (10 μM), Wy 14,643 (100 μM) and 15d-PGJ_2 (10 μM), increased PPRE-luciferase activity within 2 h (Fig. 6). These data together make it unlikely an effect of ox-PAPC on Cox-2 via PPAR activation. Moreover, BADGE, an antagonist of PPARγ ligands that prevents the transcriptional action of the receptor [34] did not affect the inhibition of Cox-2 exerted by ox-PAPC (data not shown).

4. Discussion

Oxidized phospholipids are important markers for recognition of oxidized moieties by macrophages and therefore represent potential modulators of the atherogenic process. Within this context, ox-PAPC provides a useful tool to investigate mechanisms of inflammation related to atherogenesis [35,36]. This mixture of fragmented phospholipid can be indeed formed ‘in vitro’ by free-radical

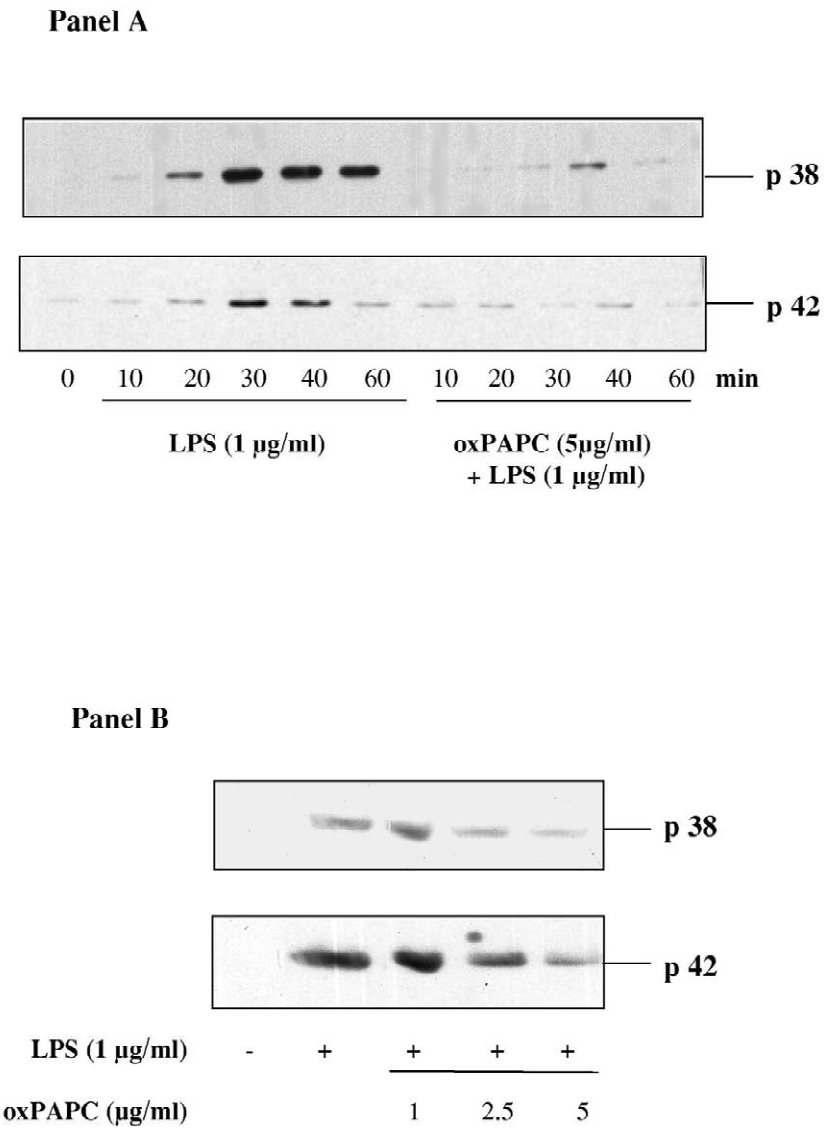
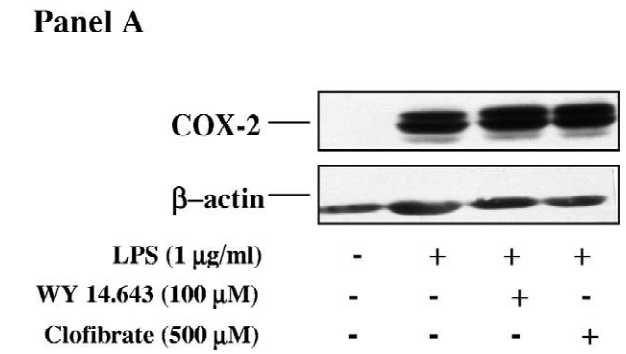


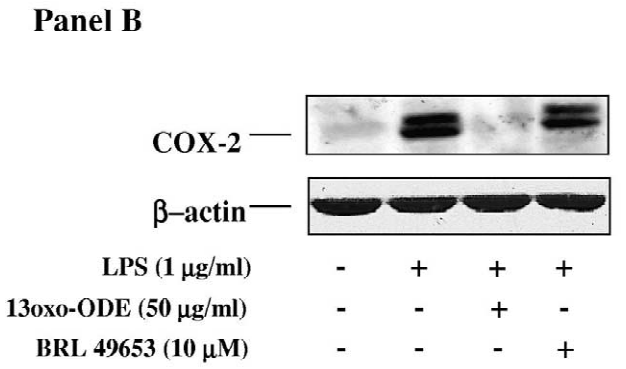
Fig. 4. Ox-PAPC inhibits LPS-induced MAP-kinase activation in human macrophages. (A) Macrophages, preincubated for 2 h with ox-PAPC (5 μg/ml) or with medium alone were exposed to LPS (1 μg/ml) for indicated times. (B) Ox-PAPC concentration-dependently inhibits MAP-kinase activation. MAP-kinase immunoblotting was performed using antibodies against phosphospecific p44–p42 (ERK1/2) and p38-kinase, as described in Section 2. Results are representative of three independent experiments.

treatment of unsaturated phospholipids and data from 'in vivo' studies indicate that levels of oxidized phospholipids are increased in fatty streak lesions from cholesterol fed rabbits [13]. In addition, increased plasma levels of fragmented phosphatidylcholine in response to various forms of oxidative stress have been reported in human and rat [37].

In this study we show that ox-PAPC quickly inhibits Cox-2 expression induced by LPS in macrophages by interfering with NF-κB/IκBα and MAP-kinase pathways. The capacity of ox-PAPC to target multiple steps involved in Cox-2 expression that act in a coordinate way to transduce signals from LPS to nucleus might explain the marked inhibitory effect observed. Inhibition of NF-κB activation by ox-PAPC is consequent to alteration of IκB degradation. This mechanism is identical to that described for ox-LDL in mononuclear phagocytes and considered as responsible for the inhibition of several inducible genes necessary for the inflammatory response [25].

Besides the inhibition of NF-κB binding to DNA, we show, for the first time, that ox-PAPC acts also by preventing LPS-induced activation of both p38 and ERK2. This finding suggests that oxidized phospholipids may act as potential modulators of a signal transduction pathway leading to the expression of a variety of genes associated with the inflammatory response. Interestingly, from our data obtained with specific inhibitors of MAP-kinases, it emerges that only ERK1/2 is involved in Cox-2 expression induced by LPS. This finding is in accordance with a recent report which addresses a role of ERK1/2 in Cox-2





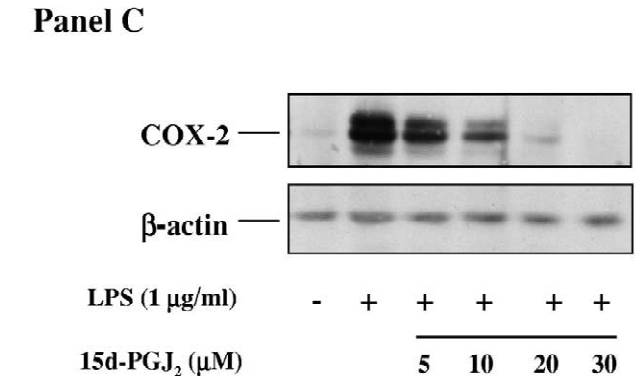


Fig. 5. Effect of PPAR ligands on LPS-induced expression of Cox-2 in human macrophages. Macrophages were incubated for 2 h in medium alone or in medium containing the PPARα ligands WY 14,643 (100 μM) or clofibrate (500 μM) (A) and the PPARγ ligands BRL 49653 (10 μM), 13 oxo-ODE (50 μg/ml) (B) or 15d-PGJ_2 (5–30 μM) (C). LPS (1 μg/ml) was then added, where indicated, for an additional 4 h. Cox-2 and β-actin proteins were determined in macrophage lysates as described in Fig. 1. This figure is representative of three independent experiments.

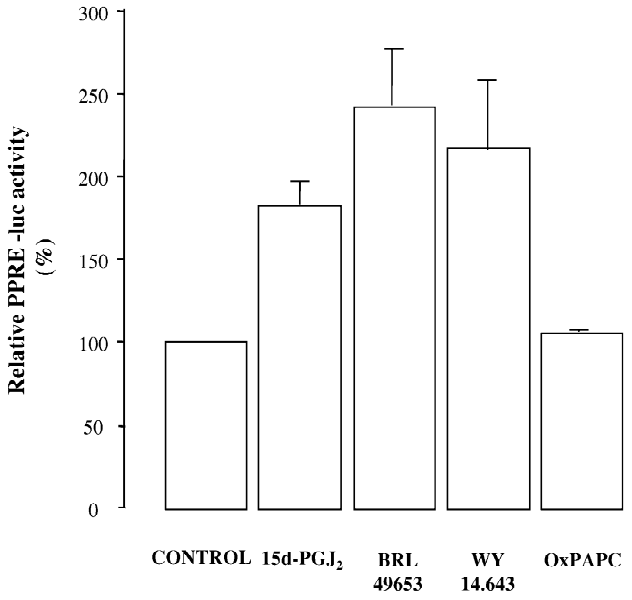


Fig. 6. Effect of ox-PAPC and PPAR agonists on PPRE activation in transiently transfected CHO cells. Transfections were performed in 80–90% confluent cells using a reporter plasmid containing three copies of a consensus PPRE upstream of a luciferase gene. The control vector pRSV-galactosidase was cotransfected as a control for transfection efficiency. Cells were treated for 2 h with ox-PAPC (5 μg/ml), BRL-49653 (10 μM), WY 14,643 (100 μM) and 15d-PGJ_2 (10 μM). Luciferase data were corrected for β-galactosidase activity and are reported as percentage activity compared with control cells. Values are the means±S.E.M. of five individual experiments.

transcription through the NF-κB site [3]. The observation that p38 and NF-κB activation are under the control of separate pathways, at least in LPS-activated macrophages [38], may explain the lack of effect of SB203580 on Cox-2, observed in our condition. Whether activation of ERK2 results in IκB phosphorylation which, in turn, inhibits NF-κB translocation is an attractive issue that deserves further investigation.

A role of ox-PAPC as a regulator of gene transcription has recently been proposed [27,28]. In particular, ox-PAPC exerts, via a PPARα-mediated mechanism, a proinflammatory effect in that it stimulates the synthesis of specific monocyte adhesion molecules and monocyte activators by endothelial cells [28]. Interestingly, the same authors described an anti-inflammatory effect of PPARα-activators in LPS-stimulated endothelial cells [39]. These apparently discrepant results allowed the conclusion that PPAR ligands behave differently depending upon the presence of an inflammatory stimulus in the experimental system.

Our data rule out a role of PPAR activation in Cox-2 inhibition by ox-PAPC. Cox-2 levels are negatively affected by the natural PPARγ ligands 13-oxoHODE and 15d-PGJ_2. 13-HODE, however, has been reported to act also as selective inhibitor of classical protein kinase C isoenzymes [40] and this signaling pathway is recognized as being critically involved in LPS-mediated induction of Cox-2 [5]. 15d-PGJ_2 has been recently shown to inhibit Cox-2 expression by PPARγ-independent mechanisms, by affecting multiple steps in NF-κB signaling pathways [41–43].

Though our study did not explore these aspects, it seems likely that such mechanisms are operative in our experimental conditions.

In conclusion, our data indicate that ox-PAPC down-regulates LPS-induced Cox-2 in macrophages by targeting both NF-κB/IκBα and ERK2 pathways.

It can be envisaged that in the sub-endothelial microenvironment, conditions of oxidative stress/antioxidant imbalance impair the capacity of macrophages to correctly contribute to the resolution of the inflammatory response. Indeed, the capacity of macrophages within lesions to be activated is crucial for the resolution of inflammation, and consequently an impaired prostaglandin formation in foam cells, coupled with a reduction of cytokine synthesis [44,45], may represent an important contributing factor in the formation and progression of fatty streaks.

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References

[1] Ross R. Atherosclerosis: an inflammatory disease. New Engl J Med 1999;340:115–126.
[2] Habib A, Creminon C, Frobert Y, Grassi J, Pradelles P, Maclouf J. Demonstration of an inducible cyclooxygenase in human endothelial cells using antibodies raised against the carboxyl-terminal region of the cyclooxygenase-2. J Biol Chem 1993;268:23448–23454.
[3] Mestre JR, Mackrell PJ, Rivadeneira DE, Stapleton PP, Tanabe T, Daly JM. Redundancy in the signaling pathways and promoter elements regulating cyclooxygenase-2 gene expression in endotoxin-treated macrophages/monocytic cells. J Biol Chem 2001;276:3977–3982.
[4] Hwang D, Jang BC, Yu G, Boudreau M. Expression of mitogen-inducible cyclooxygenase induced by lipopolysaccharide: mediation through both mitogen-activated protein kinase and NF-κB signaling pathways in macrophages. Biochem Pharmacol 1997;54:87–96.
[5] Giroux M, Descoteaux A. Cyclooxygenase-2 expression in macrophages: modulation by protein kinase C-α. J Immunol 2000;165:3985–3991.
[6] Inoue H, Tanabe T. Transcriptional role of the nuclear factor κB site in the induction by lipopolysaccharide and suppression by dexamethasone of cyclooxygenase-2 in U937 cells. Biochem Biophys Res Commun 1998;244:143–148.
[7] Baeuerle PA, Henkel T. Function and activation of NF-κB in the immune system. Annu Rev Immunol 1994;12:141–179.
[8] Jiang C, Ting AT, Seed B. PPAR-γ agonists inhibit production of monocyte inflammatory cytokines. Nature 1998;391:82–86.
[9] Ricote M, Li AC, Willson TM, Kelly CJ, Glass CK. The peroxisome proliferator-activated receptor-γ is a negative regulator of macrophage activation. Nature 1998;391:79–82.
[10] Staels B, Koenig W, Habib A, Merval R, Lebret M, Pineda Torra I, Delerive, Fadel PA, Chinetti G, Fruchart J-C, Najib J, Maclouf J, Tedgui A. Activation of human aortic smooth-muscle cells is inhibited by PPARα but not PPARγ activators. Nature 1998;393:790–793.
[11] Eligini S, Colli S, Basso F, Sironi L, Tremoli E. Oxidized low density lipoprotein suppresses expression of inducible cyclooxygenase in human macrophages. Arterioscler Thromb Vasc Biol 1999;19:1719–1725.
[12] Watson AD, Leitinger N, Navab M, Faull KF, Horkko S, Witztum JL, Palinski W, Schwenke D, Salomon RG, Sha W, Subbanagounder G, Fogelman AM, Berliner JA. Structural identification by mass spectrometry of oxidized phospholipids in minimally oxidized low density lipoprotein that induce monocyte/endothelial interactions and evidence for their presence in vivo. J Biol Chem 1997;272:13597–13607.
[13] Leitinger N, Watson AD, Hama SH, Ivandic B, Qiao J-H, Huber J, Faull KF, Grass DS, Navab M, Fogelman AM, de Beer FC, Lusis AJ, Berliner JA. Role of group II secretory phospholipase A2 in atherosclerosis 2. Potential involvement of biologically active oxidized phospholipids. Arterioscler Thromb Vasc Biol 1999;19:1291–1298.
[14] Van Lenten BJ, Fogelman AM, Haberland ME, Edwards PA. The role of lipoproteins and receptor-mediated endocytosis in the transport of bacterial lipopolysaccharide. Proc Natl Acad Sci USA 1986;83:2704–2708.
[15] Pierce J, Suelter CH. An evaluation of the Coomassie Brilliant Blue G-250 dye-binding method for quantitative protein determination. Anal Biochem 1977;81:478–480.
[16] Banfi C, Mussoni L, Risè P, Cattaneo MG, Vicentini L, Battaini F, Galli C, Tremoli E. Very low density lipoprotein-mediated signal transduction and plasminogen activator inhibitor type1in cultured HepG2 cells. Circ Res 1999;85:208–217.
[17] O'Neill GP, Ford-Hutchinson AW. Expression of mRNA for cyclooxygenase-1 and cyclooxygenase-2 in human tissues. FEBS Lett 1993;330:156–160.
[18] Dignam JD, Lebovitz RM, Roeder RG. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nucleic Acids Res 1983;11:1475–1488.
[19] Colli S, Eligini S, Lalli M, Camera M, Paoletti R, Tremoli E. Vastatins inhibit tissue factor in cultured human macrophages: a novel mechanism of protection against atherothrombosis. Arterioscler Thromb Vasc Biol 1997;17:265–272.
[20] Barrios-Rodiles M, Tiraloche G, Chader C. Lipopolysaccharide modulates cyclooxygenase-2 transcriptionally and posttranscriptionally in human macrophages independently from endogenous IL-1β and TNF-α. J Immunol 1999;163:963–969.
[21] Sambrook T, Fritsch EF, Maniatis T. In: Molecular cloning: a laboratory manual, Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press, 1989.
[22] Vu-Dac N, Chopin-Delannoy S, Gervois P, Bonnelye E, Martin G, Fruchart J-C, Laudet V, Staels B. The nuclear receptors peroxisome proliferator-activated receptor α and apolipoprotein A-I expression by fibrates. J Biol Chem 1998;273:25713–25720.
[23] Watson AD, Navab M, Hama SY, Sevanian A, Prescott SM, Stafforini DM, McIntyre TM, La Du BN, Fogelman AM, Berliner JA. Effect of platelet activating factor-acetylhydrolase on the formation and action of minimally oxidized low density lipoprotein. J Clin Invest 1995;95:774–782.
[24] Shackelford RE, Misra UK, Florine-Casteel K, Thai SF, Pizzo SV, Adams DO. Oxidized low density lipoprotein suppresses activation of NF-κB in macrophages via a pertussis toxine-sensitive signaling mechanism. J Biol Chem 1995;270:3475–3478.
[25] Hamilton TA, Major JA, Armstrong D, Tebo JM. Oxidized LDL modulates activation of NF-κB in mononuclear phagocytes by altering the degradation of Iκ-Bs. J Leukocyte Biol 1998;64:667–674.
[26] Ohlsson BG, Englund MCO, Karlsson ALK, Knutsen E, Erixon C, Skribeck H, Liu Y, Bondjers G, Wiklund O. Oxidized low density

lipoprotein inhibits lipopolysaccharide-induced binding of nuclear factor-κB to DNA and the subsequent expression of tumor necrosis factor-α and interleukin-1β in macrophages. J Clin Invest 1996;98:78–89.
[27] Delerive P, Furman C, Teissier E, Fruchart J-C, Duriez P, Staels B. Oxidized phospholipids activate PPARα in a phospholipase A2-dependent manner. FEBS Lett 2000;471:34–38.
[28] Lee H, Shi W, Tontonoz P, Wang S, Subbanagounder G, Hedrick CC, Hama S, Borromeo C, Evans RM, Berliner JA, Nagy L. Role for peroxisome proliferator activated receptor α in oxidized phospholipid-induced synthesis of monocyte chemotactic protein-1 and interleukin-8 by endothelial cells. Circ Res 2000;87:516–521.
[29] Chinetti G, Griglio GS, Antonucci M, Pineda Torra I, Delerive P, Majd Z, Fruchart J-C, Chapman J, Najib J, Staels B. Activation of proliferator-activated receptors α and γ induces apoptosis of human monocyte-derived macrophages. J Biol Chem 1998;273:25573–25580.
[30] Nagy L, Tontonoz P, Alvarez JGA, Chen H, Evans RM. Oxidized LDL regulates macrophage gene expression through ligand activation of PPARγ. Cell 1998;93:229–240.
[31] Forman BM, Tontonoz P, Chen J, Brun RP, Spiegelman BM, Evans RM. 15-Deoxy-$\Delta^{12,14}$-prostaglandin J_2 is a ligand for the adipocyte determination factor PPARγ. Cell 1995;83:803–812.
[32] Kliewer SA, Lenhard JM, Willson TM, Patel I, Morris DC, Lehmann JM. A prostaglandin J2 metabolite binds peroxisome proliferator-activated receptor γ and promotes adipocyte differentiation. Cell 1995;83:813–819.
[33] Wang M, Wise SC, Leff T, Su TZ. Troglitazone, an antidiabetic agent, inhibits cholesterol biosynthesis through a mechanism independent of peroxisome proliferator-activated receptorγ. Diabetes 1999;48:254–260.
[34] Wright HM, Clish CB, Mikami T, Hauser S, Yanagi K, Hiramatsu R, Serhan CN, Spiegelman BM. A synthetic antagonist for the peroxisome proliferator-activated receptor γ inhibits adipocyte differentiation. J Biol Chem 2000;275:1873–1877.
[35] Berliner J, Leitinger N, Watson A, Huber J, Fogelman A, Navab M. Oxidized lipids in atherogenesis: formation, destruction and action. Thromb Haemost 1997;78:195–199.
[36] Witztum J, Berliner JA. Oxidized phospholipids and isoprostanes in atherosclerosis. Curr Opin Lipidol 1998;9:441–448.
[37] Frey B, Haupt R, Alms S, Holzmann G, Konig T, Kern H, Kox W, Rustow B, Schlame M. Increase in fragmented phosphatidylcholine in blood plasma by oxidative stress. J Lipid Res 2000;41:1145–1153.
[38] Wesselborg S, Bauer MKA, Vogt M, Schimtz ML, Schulze-Osthoff K. Activation of transcription factor NF-kB and p38 mitogen-activated protein kinase is mediated by distinct and separate stress effector pathways. J Biol Chem 1997;272:12422–12429.
[39] Jackson SM, Parhami F, Xi X-P, Berliner JA, Hsueh WA, Law RE, Demer LL. Peroxisome proliferator-activated receptor activators target human endothelial cells to inhibit leukocyte-endothelial cell interaction. Arterioscler Thromb Vasc Biol 1999;9:2094–2104.
[40] Pongracz J, Lord JM. The lipoxygenase product 13-hydroxy-octadecadienoic acid (13-HODE) is a selective inhibitor of classical PKC isoenzymes. Biochem Biophys Res Commun 1999;256:269–272.
[41] Straus DS, Pascual G, Li M, Welch JS, Ricote M, Hsiang C-H, Sengchanthalangsy LL, Ghosh G, Glass CK. 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 inhibits multiple steps in the NF-κB signaling pathway. Proc Natl Acad Sci USA 2000;97:4844–4849.
[42] Rossi A, Kaphai P, Natoli G, Takahashi T, Chen Y, Karin M, Santoro MG. Anti-inflammatory cyclopentenone prostaglandins are direct inhibitors of IκB kinase. Nature 2000;403:103–108.
[43] Castrillo A, Diaz-Guerra MJM, Hortelano S, Martin-Sanz P, Bosca L. Inhibition of IκB kinase and IκB phosphorylation by 15-deoxy-$\Delta^{12,14}$-Prostaglandin J_2 in activated murine macrophages. Mol Cell Biol 2000;20:1692–1698.
[44] Fong LG, Fong TA, Cooper AD. Inhibition of lipopolysaccharide-induced interleukin 1β mRNA expression in mouse macrophages by oxidized low density lipoprotein. J Lipid Res 1991;32:1899–1910.
[45] Chung S-W, Kang B-Y, Kim S-H, Pak Y-K, Cho D, Trinchieri G, Kim T-S. Oxidized low density lipoprotein inhibits interleukin-12 production in lipopolysaccharide-activated mouse macrophages via direct interactions between peroxisome proliferator-activated receptor-γ and nuclear factor-κB. J Biol Chem 2000;42:32681–32687.