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# Oxidized Low Density Lipoprotein Suppresses Expression of Inducible Cyclooxygenase in Human Macrophages

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***Abstract***—Atherogenesis involves several aspects of chronic inflammation and wound healing. Indeed, the atheroma is considered a special case of tissue response to injury. Injurious stimuli may include lipoproteins trapped within lesions where protein and lipid moieties have undergone chemical modifications. We have studied the effect of oxidized low density lipoproteins (ox-LDL) on inducible cyclooxygenase (Cox-2) in human monocyte–derived macrophages exposed to bacterial lipopolysaccharide (LPS). Levels of both Cox-2 and constitutive cyclooxygenase (Cox-1) were assessed using Western blot analysis. Prior incubation of macrophages with ox-LDL resulted in a strong inhibition of Cox-2 induced by LPS, without effect on Cox-1. The inhibitory effect was dependent on ox-LDL concentration and its onset was early in time (already detectable 1 hour after macrophage exposure to ox-LDL). Native LDL, and other forms of modified LDL, were without effect. The inhibition was dependent on endocytosis of ox-LDL and could be reproduced using the lipid extract from ox-LDL. Lysophosphatidylcholine, 7$\beta$-hydroxycholesterol, and 7-oxocholesterol failed to mimic the inhibition, but oxidized arachidonic acid–containing phospholipids, produced by autoxidation of 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine, markedly inhibited Cox-2. The observation that ox-LDL downregulates Cox-2 in human macrophages may explain the fact that, within atheromata, the transformation of macrophages into foam cells results in attenuation of the inflammatory response, thus contributing to progression of atherogenesis. **(*Arterioscler Thromb Vasc Biol*. 1999;19:1719-1725.)**

**Key Words:** cyclooxygenase-2 ■ macrophages ■ oxidized lipoproteins ■ oxidized phospholipids ■ atherosclerosis

Monocyte-derived macrophages play a prominent role in the formation and progression of the atherosclerotic plaque, particularly after their transformation into foam cells.[1,2] In this scenario an important feature is the oxidized LDL (ox-LDL) present in the atherosclerotic lesion.[3] These lesion LDLs have many of the same physical and chemical properties as LDLs that have been oxidatively modified in vitro.[3]

The interaction of ox-LDL with macrophages is generally believed to be an early step in the process of their transformation into the foam cells composing the fatty streak, a primary histologic aspect of incipient atherosclerosis. Indeed, the accumulation of lipid within macrophages appears to be mediated by their selective recognition and uptake of ox-LDL.[4] In addition to being internalized by macrophages, ox-LDL affects many aspects of macrophage behavior in ways that appear to promote atherogenesis.[5–8]

Macrophages, when activated by inflammatory stimuli, synthesize and secrete various mediators (ie, proteases, active oxygen species, cytokines, prothrombotic substances, and eicosanoids), which cause the clinical manifestations and acute clinical complications of atherosclerosis.[9]

The eicosanoids derived from the metabolism of arachidonate have been extensively investigated because several studies have focused on their close relation to atherogenesis.[10–17] In macrophages, as well as in other cell types, arachidonate metabolites are synthesized by the cyclooxygenase enzyme, which is present in 2 isoforms. Cyclooxygenase-1 (Cox-1) is constitutively expressed, whereas the more recently described isoform, the mitogen-inducible cyclooxygenase (Cox-2), is usually absent from resting cells and is selectively expressed by macrophages exposed to lipopolysaccharide (LPS).[18] Distinct pools of arachidonic acid are available to Cox enzymes: 1 pool used by Cox-1 for physiological functions; the other released on cell activation and used as substrate by Cox-2.[19] In macrophages, Cox-2 expression appears to be mediated through both mitogen-activated protein kinase and nuclear factor-$\kappa$B (NF-$\kappa$B) signaling pathways.[20]

In mouse macrophages exposed to atherogenic lipoproteins and subsequently stimulated with various inflammatory stimuli a decreased production of eicosanoids such as prostacyclin and prostaglandin $E_2$ has been described, which indicates that the uptake of modified lipoproteins negatively affects some aspects of the proinflammatory potential of macrophages.[21,22] The link between the oxidative modification of LDL and an impairment of macrophage inflammatory response has been strengthened by data from Hamilton et al,[23,24] who elegantly demonstrated that ox-LDL suppresses several inducible genes

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necessary for the inflammatory response of macrophages. These data suggest a potent, direct connection between the oxidative modification of LDL and the failure of macrophages to correctly resolve the tissue damage within atherogenic lesions, which then leads to a chronic inflammatory status.

In this study we investigated the effect of native and modified LDL on Cox-2 enzyme in human monocyte–derived macrophages in culture. Our data show that ox-LDL inhibits Cox-2 enzyme and that this inhibition is caused by a marked attenuation of Cox-2 mRNA levels.

## Methods

### Reagents

Cell culture medium and reagents were purchased from BioWhittaker. Lipopolysaccharide (*Escherichia coli* 0114:B4) was from Difco Labs. Benzamidine hydrochloride, leupeptin hemisulphate, soybean trypsin inhibitor, sodium orthovanadate, fucoidin, cytochalasin B, chloroquine, phosphatidylcholine (PC), lysophosphatidylcholine (lysoPC) from egg yolk, 7-oxocholesterol, 7$\beta$-hydroxycholesterol, 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine (PAPC), monoclonal antibody anti-$\beta$-actin, BSA (fatty acid–free and low in endotoxin), and Fat Red 7B were from Sigma Chemical Co. EDTA and NaF were from Carlo Erba. Electrophoresis reagents were from Pharmacia Biotech. TRIzol Reagent was from Life Technologies. Peroxidase-conjugated anti-mouse IgG antibody was from Jackson ImmunoResearch. Monoclonal antibodies against Cox-2 (mAb 29) and Cox-1 (mAb 10 and 11) were kindly donated by Dr J. Maclouf (U 348, INSERM, Paris).

### Isolation of LDL and Phospholipids and Their Modifications

LDLs were isolated by sequential preparative ultracentrifugation of plasma from normolipidemic donors (containing 100 U/mL aprotinin and 10 mmol/L phenylmethylsulfonylfluoride) in a Beckman SW50Ti rotor at $d$=1.024 to 1.050 g/mL.[25] LDLs were extensively dialyzed against phosphate buffer (1.5 mmol/L $NaH_2PO_4$ and 3.5 mmol/L $Na_2HPO_4$, pH 7.5) at 4°C. Acetyl-LDL was obtained by diluting LDL with an equal volume of saturated sodium acetate followed by treatment with acetic anhydride.[26] Ox-LDL was obtained by dialysis against phosphate buffer supplemented with 10 $\mu$mol/L $CuSO_4$ at 4°C for 48 hours. Malondialdehyde-modified LDL (MDA-LDL) was prepared by incubating LDL for 3 hours at 37°C with 0.5 mol/L MDA at a constant ratio of 100 $\mu$L/mg of LDL. MDA was freshly generated from malonaldehyde bis(dimethylacetal) by acid hydrolysis at 37°C for 10 minutes.[27] The extent of LDL modification was assessed by electrophoretic mobility using 1% agarose. Protein contents of native and modified LDL were quantified using the Lowry method.[28] Native and modified LDL were sterilized by passage through 0.22 $\mu$m filters, stored in sterile tubes at 4°C, and used within 1 month. All preparations were tested for LPS contamination using the Limulus test. Only those preparations containing LPS levels <0.1 ng/mg protein were used for the study. Oxidized PAPC was obtained by transferring 1 mg PAPC in 100 $\mu$L of chloroform to a clean 16×125 mm glass test tube and evaporating the solvent under a stream of nitrogen. The lipid residue was allowed to autoxidize on exposure to air for 48 hours. Oxidized PAPC (ox-PAPC) was reduced by resuspending 250 $\mu$g of lipid residue in 0.5 mL of a 0.1 mol/L borate buffer (pH 8.0) and adding 10 $\mu$g of sodium borohydride. The lipids were instantly reduced.[29]

### Cell Culture and Incubation

Venous blood from healthy donors was anticoagulated with 3.8% sodium citrate, and mononuclear cells were then separated using Ficoll-Paque solution (Pharmacia Fine Chemicals) at 450*g* for 20 minutes. Mononuclear cells were washed with PBS containing EDTA (5 mmol/L) and suspended ($3\times10^6$/mL) in RPMI 1640 supplemented with 2 mmol/L glutamine, 0.5% penicillin-streptomycin-fungizone, and 10% human heat-inactivated AB serum. Monocytes were isolated from lymphocytes by adherence (2 hours at 37°C, 5% $CO_2$, humid atmosphere) to 6-well plates. 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 supplemented with 2 mmol/L glutamine, 0.5% antibiotics, and 10% human heat-inactivated AB serum; macrophages were identified by the presence of the CD68Ag, which was detected using a specific monoclonal mouse anti-human macrophage antibody (Dako). The endotoxin content of all culture materials and reagents was measured with the Limulus amebocyte lysate assay (BioWhittaker) and only those containing <3 pg/mL of endotoxin were used.

Macrophages were first incubated in serum-free medium containing 0.2% fatty acid–free BSA for times ranging from 1 to 24 hours with native or modified LDL. Medium was then removed and macrophages were exposed for an additional 4 hours to fresh medium containing LPS (1 $\mu$g/mL). Incubation times and LPS concentration were selected after preliminary experiments. Cell viability was >95% as determined by Neutral Red assay. To evaluate cyclooxygenase activity, cells were washed with Hanks' buffer (pH 7.4) containing 1 mg/mL BSA and incubated with the same buffer containing 10 $\mu$mol/L sodium arachidonate. After 30 minutes, supernatants were harvested and $TxB_2$ levels were quantified using enzyme immunoassay (EIA).

### Western Blot Analysis

After incubation, macrophages were lysed in buffer (20 mmol/L Tris-HCl [pH 6.8], 4% SDS, 20% glycerol, 1 mmol/L EDTA, 1 mmol/L benzamidine hydrochloride, 1 $\mu$g/mL leupeptin, 10 $\mu$g/mL soybean trypsin inhibitor, 1 mmol/L NaF, and 1 mmol/L orthovanadate). Protein content was quantified using the microbicinchoninic acid assay[30]; 40 $\mu$g of protein (reducing conditions) was used for analysis. SDS-PAGE analysis was performed using 7% and 3% acrylamide for the separating gel and stacking gel, respectively. Proteins were transferred onto nitrocellulose membranes (Sartorius AG) with a semidry transfer unit (Hoefer Scientific Instruments).[31] Transfer was performed in a 25 mmol/L Tris, 192 mmol/L glycine buffer (pH 8.3; containing 0.01% SDS and 15% methanol), for 2 hours at 200 mA. Blots, stained with 0.4% Ponceau Red in 0.3% trichloroacetic acid to visualize proteins, were saturated for 2 hours at room temperature with 5% fat-free dry milk in Tris-buffered saline (50 mmol/L Tris-HCl [pH 7.5], 250 mmol/L NaCl, and 0.1% Tween 20) and incubated with Cox-2 (1/10 000) or Cox-1 (5 $\mu$g/mL) mAbs for 1 hour at room temperature. Monoclonal antibody directed against $\beta$-actin was used as internal standard for control of protein load. Blots were incubated with donkey anti-mouse IgG conjugated with peroxidase at 1/5000 (0.1 mL/cm$^2$ for 1 hour at room temperature. ECL (Amersham) substrates were used according to the manufacturer's instructions to reveal positive bands. Bands were visualized after exposure to Hyperfilm ECL (Amersham).

### Detection of Cox-2 mRNA by RT-PCR

Total cellular RNA was extracted from macrophages with TRIzol Reagent; Cox-2 mRNA levels were determined using a coupled reverse transcription–polymerase chain reaction (RT-PCR), performed using a GeneAmp RNA PCR kit (Perkin-Elmer Corp) according to the manufacturer's instructions. To identify Cox-2 mRNA, exact primers were synthesized based on an analytical RT-PCR procedure developed for human cyclooxygenase. The Cox-2 primers were 5′-TTCAAATGAGATTGTGGAAAATTGCT-3′ (27mer sense oligonucleotide at position 573) and 5′-AGATCATCTCTGCCTGAGTATCTT-3′ (24mer antisense oligonucleotide at position 878), resulting in a 305-bp PCR product.[32] Primers were also synthesized to amplify the cDNA encoding GAPDH, a constitutively expressed gene, as a control. The GAPDH primers were 5′-CCACCCATGGCAAATTCCATGGCA-3′ (24mer sense oligonucleotide at position 216) and 5′-TCTAGACGGCAGGTCAGGTCCACC-3′ (24mer antisense oligonucleotide at position 809), resulting in a 593-bp PCR product. RNA concentration was determined spectrophotometrically; total cellular RNA samples from each (1 $\mu$g) sample were reverse transcribed at 42°C for 30 minutes, and the same amount of the resulting cDNA was used for amplifi-

cation by specific primers for human Cox-2 and human GAPDH. PCR amplification reaction was carried out in the presence of 0.2 μmol/L Cox-2 primers and 0.1 μmol/L GAPDH primers for 30 cycles with denaturation at 94°C for 1 minute, primer annealing at 55°C for 1 minute, and extension at 72°C for 1 minute, using a thermal cycler GeneAmp PCR System 2400 (Perkin Elmer Corp). Ten microliters of the amplified products was resolved by electrophoresis through a 2% agarose gel. Amplified cDNA bands were detected by ethidium bromide staining.

### Determination of Intracellular Lipids

Macrophage lipids were extracted with hexane/isopropanol (3:2, vol/vol), 1 mL per well for 30 minutes, repeated twice. Extracts from 2 wells were pooled and total cholesterol and triglycerides were quantified by enzymatic methods (Roche) and expressed as μg/mg of cell protein.

### Lipid Extraction From Native and Ox-LDL

Lipids were extracted from native and ox-LDL with chloroform/methanol (2:1 vol/vol) containing 5 μg/mL butylated hydroxytoluene.[33] The organic phase was dried under nitrogen and the content of extracts was determined by microgravimetry. The lipid residue was dissolved in ethanol and added to culture medium. Total phospholipids were isolated from the lipid extract by thin layer chromatography on silica-gel 60 HR plates (Merck) using hexane/diethyl ether/acetic acid (80:20:1 vol/vol/vol) as developing agents.[34] After brief exposure to iodine vapor, the zones containing total phospholipids were scraped off, dissolved in chloroform, and added to culture medium.

### Vesicle Preparation

To prevent micellar formation, PC/lysoPC vesicles were prepared using a 3:1 molar ratio of PC to lysoPC. A mixture of PC with 25 mol% of lysoPC was dissolved in chloroform and the solvent was completely removed under a stream of nitrogen. The phospholipids were dispersed in buffer (10 mmol/L Tris-HCl [pH 8.0], 150 mmol/L NaCl, 0.01% EDTA, and 0.006% $NaN_3$) containing 10 mg/mL cholate. The vesicles were dialyzed twice in buffer and once in saline.[35]

### Neutral Lipid Staining

Macrophages were fixed with 4% formalin (pH 7.0) for 3 hours at room temperature and stained for neutral lipids using a supersaturated solution of Fat Red 7B in 70% ethanol. Hematoxylin was used for nuclear staining, and stained cells were examined with phase contrast microscopy.

### Statistical Analysis

Data are reported as mean±SEM or as percentage of control for lipid measurements. Statistical analyses were performed using a paired Student's *t* test.

## Results

### Effect of Bacterial Lipopolysaccharide on Cox-2 Protein Expression in Human Macrophages

Macrophages incubated with medium alone did not express appreciable levels of Cox-2, whereas exposure to LPS (0.1 to 10 μg/mL) induced a concentration-dependent expression of Cox-2 protein, recognized by a specific mAb as a doublet at 70 kDa. Timecourse experiments showed that the maximum expression of the protein was reached within 4 hours and did not change over 24 hours of incubation (data not shown). The expression of protein detected 4 hours after LPS exposure was accompanied by the formation of $TXB_2$ from exogenous arachidonate (0.60±0.14 and 3.94±1.1 ng/mL in unstimulated and LPS-stimulated macrophages, respectively; n=7; $P$=0.02).

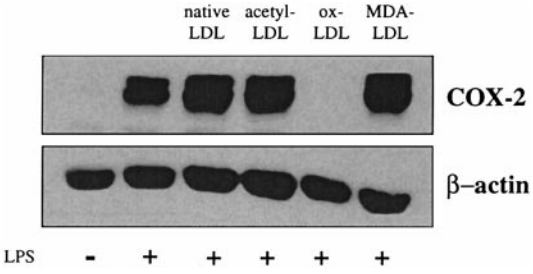


**Figure 1.** Effect of native and modified LDL on LPS-induced expression of Cox-2 protein in human macrophages. Macrophages were first incubated in medium alone or with 50 μg/mL of native LDL, acetyl-LDL, ox-LDL, or MDA-LDL. LPS (1 μg/mL) was then added for an additional 4 hours where indicated. For Cox-2 and β-actin proteins, solubilized proteins of macrophages were analyzed by Western blot using monoclonal Cox-2 and β-actin antibodies. This figure is representative of 5 experiments.

### Inhibition of Cox-2 Protein Synthesis by Ox-LDL

The exposure of unstimulated macrophages to medium for 24 hours did not induce Cox-2 expression, whereas stimulation with LPS did induce Cox-2 expression (Figure 1). By contrast, when macrophages were incubated with ox-LDL (50 μg/mL) for 2 to 24 hours, then stimulated with LPS for 4 hours, Cox-2 expression was completely inhibited (Figure 1). Neither native nor otherwise-modified LDL influenced LPS-induced Cox-2 expression (Figure 1). The inhibitory effect exerted by ox-LDL was concentration-dependent: it was appreciable at 25 μg/mL ox-LDL and was complete (>90%) at 50 μg/mL (Figure 2A). The effect was selective for Cox-2; levels of Cox-1 were unaffected (Figure 2B). The onset of the inhibitory effect was rapid: inhibition was nearly 50% after 1 hour and was complete after 2 hours (Figure 3). Ox-LDL reduced Cox-2 expression only when incubated with macrophages before the addition of LPS. Conversely, the incubation of cells with ox-LDL 1 hour after their exposure to LPS did not affect Cox-2 levels (data not shown). As a consequence of Cox-2 inhibition, a marked decrease of $TXB_2$

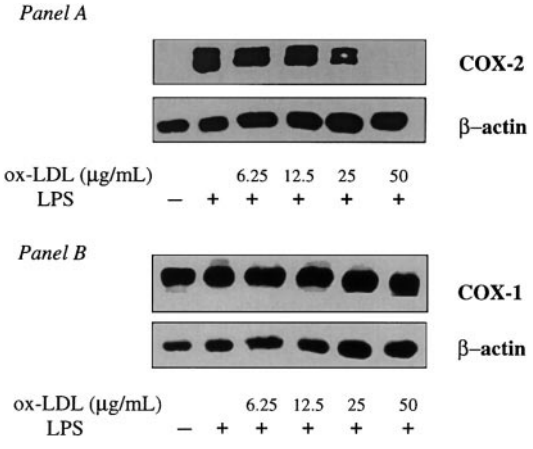


**Figure 2.** (A) The inhibition of macrophage Cox-2 protein expression by ox-LDL is concentration-dependent. Macrophages were first incubated in medium or with ox-LDL (6.25, 12.5, 25, and 50 μg/mL) then exposed to 1 μg/mL LPS where indicated. (B) Same as (A), but using Cox-1 immunodetection. For Cox-2, Cox-1, and β-actin proteins, solubilized proteins of macrophages were analyzed by Western blot using monoclonal Cox-2, Cox-1, and β-actin antibodies. This figure is representative of 2 to 4 experiments.

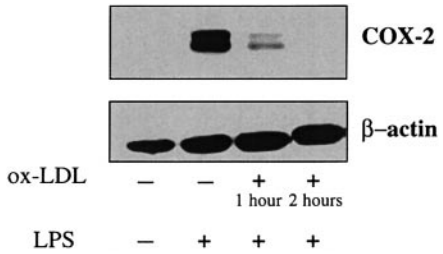


**Figure 3.** Onset of Cox-2 inhibition by ox-LDL in human macrophages. Macrophages were first incubated with medium alone or with 50 μg/mL ox-LDL for 1 and 2 hours. LPS (1 μg/mL) was then added where indicated. This figure is representative of 3 experiments.

production from exogenous substrate was detected (3.94±1.1 and 1.51±0.47 ng/mL in LPS-treated cells and in LPS-treated cells previously exposed to ox-LDL, respectively; n=7; $P=0.05$).

Because ox-LDL is toxic to a variety of cells in culture, cytotoxicity was measured to assess whether the inhibition of Cox-2 was caused by cell death. Exposure of macrophages to ox-LDL in concentrations up to 50 μg/mL for 24 hours did not affect cell viability as assessed by Neutral Red assay.

The inhibition of Cox-2 protein expression by ox-LDL was caused by attenuation of Cox-2 mRNA levels, as assessed by RT-PCR analysis. The exposure of macrophages to 1 μg/mL LPS for 1 hour was associated with Cox-2 mRNA accumulation. Macrophage exposure to 50 μg/mL ox-LDL for 2 hours completely prevented the induction of Cox-2 mRNA by LPS (Figure 4).

## Uptake of Lipoproteins, Endocytosis and Lysosomal Degradation Pathway

Native LDL did not affect intracellular total cholesterol, whereas both ox-LDL and acetyl-LDL (50 μg/mL) increased it to similar degrees; triglyceride levels were only marginally affected (data not shown).

The uptake of ox- and acetyl-LDL was confirmed by Fat Red staining. Human macrophages incubated for 24 hours with ox-LDL adopted a foam cell–like morphology, with the cytoplasm characterized by large accumulations of lipid droplets that stained with Fat Red and were visible by light microscopy (data not shown).

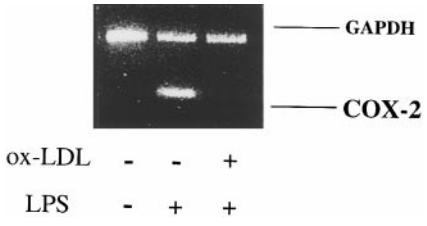


**Figure 4.** RT-PCR analysis of mRNA for Cox-2 in human macrophages exposed to ox-LDL. Macrophages were first incubated for 2 hours in medium alone or with 50 μg/mL ox-LDL. Medium alone or LPS (1 μg/mL) was then added for an additional hour. Total cellular RNA was extracted and Cox-2 and GAPDH mRNA levels were estimated by RT-PCR. GAPDH mRNA was used as an internal standard. The sequences of the primer sets and oligonucleotide probes for Cox-2 and GAPDH mRNA are shown in the Methods. This figure is representative of 2 experiments.

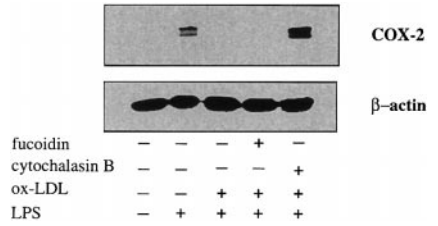


**Figure 5.** Endocytosis, but not binding to scavenger receptors, is a prerequisite for the inhibition of Cox-2 expression by ox-LDL. Macrophages were first incubated in medium only, 50 μg/mL ox-LDL, 30 μg/mL fucoidin plus 50 μg/mL ox-LDL, or 5 μg/mL cytochalasin B plus 50 μg/mL ox-LDL. LPS (1 μg/mL) was added where indicated. Western blotting was performed as for Figure 1. This figure is representative of 3 experiments.

We next investigated whether the receptor-mediated endocytosis was a prerequisite for the inhibitory effect of ox-LDL on Cox-2. Cells were preincubated for 15 minutes with fucoidin (30 μg/mL), a specific ligand for the scavenger receptor, or with cytochalasin B (1 to 5 μg/mL), which blocks endocytosis. Ox-LDL (50 μg/mL) was added in the presence of fucoidin or cytochalasin B for an additional 24 hours. The medium was removed and macrophages were exposed to LPS for 4 hours. The data indicate that the inhibitory effect of ox-LDL on Cox-2 induced by LPS was not prevented by the binding of the scavenger receptor (Figure 5). In contrast, the inhibition of endocytosis exerted by cytochalasin B resulted in the recovery of Cox-2 induced by LPS (Figure 5).

To assess whether the degradation pathway of endocytosed ox-LDL was relevant to the inhibition exerted on Cox-2, chloroquine (50 μmol/L), an inhibitor of lysosomal acid hydrolases, was added to culture medium containing ox-LDL. The effect of ox-LDL on Cox-2 was not changed by chloroquine (data not shown). These data suggest that the incorporation of ox-LDL particle by macrophages, but not its degradation, is a prerequisite for the inhibitory effect on Cox-2.

## Localization of the Inhibitory Component to the Lipid Fraction of Ox-LDL

To examine whether the inhibitory activity of ox-LDL was associated with the lipid component of the particle, we incubated macrophages with chloroform-methanol extracts of native and ox-LDL (equivalent to 25 to 50 μg LDL protein/mL of culture medium) before stimulation with LPS. The lipid extracts from ox-LDL inhibited the enzyme synthesis induced by LPS, whereas those from native LDL did not (Figure 6).

A number of oxidized lipid compounds (lysoPC, 7-oxocholesterol, and 7β-hydroxy-cholesterol), all identified in ox-LDL[36,37] as well as in atherosclerotic plaques,[3,38] were tested for their ability to influence Cox-2 at concentrations close to that reported to possess biological activity.[5,39,40] LysoPC (25 to 50 μg/mL), 7-oxocholesterol (5 μg/mL), and 7β-hydroxy-cholesterol (5 μg/mL) did not affect the expression of Cox-2 when added to culture medium up to 24 hours before macrophage exposure to LPS (data not shown).

Macrophages were then exposed to the phospholipid fractions isolated from native and ox-LDL (at levels equivalent to 50 μg LDL protein/mL of culture medium). Phospholipids extracted from ox-LDL, but not those extracted from native

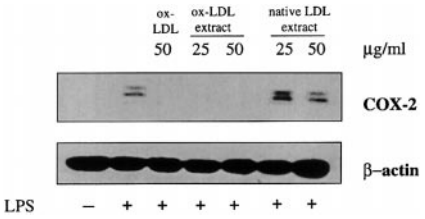


**Figure 6.** The inhibitory activity of ox-LDL is associated with the lipid component of the lipoprotein. Macrophages were first incubated with medium or with lipid extracts equivalent to 25 and 50 μg LDL protein/mL from ox-LDL and native LDL. The effect of lipid extracts was compared with that of 50 μg/mL ox-LDL. Cells were subsequently exposed to 1 μg/mL LPS. Western blotting was performed as for Figure 1. This figure is representative of 3 experiments.

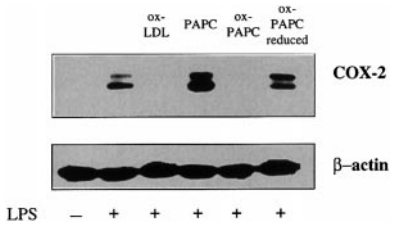


**Figure 8.** PAPC (1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine), a surrogate arachidonic acid–containing phospholipid, abolishes Cox-2 in its oxidized form only. Macrophages were first incubated with medium or with 5 μg/mL PAPC in its unoxidized form, its oxidized form (ox-PAPC), or its oxidized-reduced form (ox-PAPC reduced). The effect of ox-PAPC was compared with that of 50 μg/mL ox-LDL. LPS (1 μg/mL) was then added where indicated. Western blotting was performed as for Figure 1. This figure is representative of 5 experiments.

LDL, fully mimicked the inhibition of Cox-2 exerted by intact ox-LDL (Figure 7).

Among phospholipids generated during oxidative modification of LDL, Ox-PAPC, a mixture of oxidized arachidonic acid–containing phospholipids that is obtained by autoxidation of 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine, has been reported to be responsible for some biological activities of ox-LDL that are relevant in atherogenesis.[41] In addition, the evidence for the importance of these compounds in vivo is suggested by their presence in fatty streak lesions from cholesterol-fed rabbits.[29] Ox-PAPC, incubated for 2 hours with macrophages at 5 μg/mL, a concentration reported to be present in 50 μg/mL ox-LDL,[41] completely abolished the LPS induction of Cox-2, comparable to the effect exerted by ox-LDL in toto (Figure 8). PAPC in its nonoxidized form had no effect on Cox-2 (Figure 8), and reduction of ox-PAPC with sodium borohydride abolished the capacity of ox-PAPC to inhibit Cox-2 (Figure 8).

## Discussion

The data presented in this study indicate that ox-LDL, but not native or other modified forms of LDL (acetyl-LDL, MDA-LDL), at concentrations that are not cytotoxic, markedly inhibits Cox-2 protein and mRNA induced by LPS in human macrophages. The inhibitory effect of ox-LDL on Cox-2 was not mediated by intracellular cholesterol accumulation: cholesterol levels in macrophages exposed to ox-LDL were similar to those obtained with acetyl-LDL, which does not inhibit Cox-2. Preincubation of macrophages with fucoidin for 24 hours did not affect the inhibition by ox-LDL, suggesting that the recognition of the scavenger receptor by ox-LDL is not responsible for Cox-2 inhibition, at least in

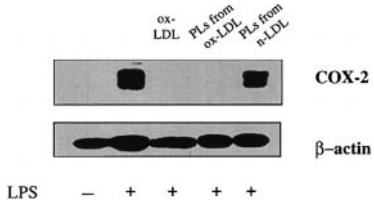


**Figure 7.** Phospholipids (PLs) extracted from ox-LDL but not those extracted from native LDL mimic the inhibitory effect of ox-LDL on Cox-2. Macrophages were exposed to medium or to the PL fraction isolated from ox-LDL and native LDL at levels equivalent to 50 μg LDL protein/mL of culture medium. The effect of PL extracts was compared with that of 50 μg/mL ox-LDL. LPS (1 μg/mL) was then added where indicated. Western blotting was performed as for Figure 1. This figure is representative of 2 experiments.

experimental conditions in which macrophages are exposed to ox-LDL for a relatively long time period. Moreover, macrophages possess several structures that function as scavenger receptors not only for modified lipoproteins but also for anionic phospholipids.[42,43] Thus, the interaction of ox-LDL with other novel classes of receptors may account for Cox-2 inhibition. Interestingly, Cox-2 was inhibited when macrophages were exposed to the lipid extract of ox-LDL. Among the lipid constituents that have been found in ox-LDL, lysoPC, 7-oxocholesterol, and 7β-hydroxy-cholesterol failed to mimic the inhibition of Cox-2 exerted by ox-LDL. In contrast, ox-PAPC, a surrogate mixture of oxidized arachidonic acid–containing phospholipids, abolished Cox-2 expression. Ox-PAPC (obtained by autoxidation of 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine) is a mixture of products, some of which have been identified in mildly oxidized-LDL,[41] and is considered to be responsible for the biological activity of mildly oxidized-LDL.[41] Two of these compounds have been further characterized in terms of molecular structure and biological activity.[29] In addition, the presence of these oxidized phospholipids in fatty streak lesions from cholesterol-fed rabbits has been documented,[29] which suggests that specific oxidized derivatives of arachidonic acid–containing phospholipids may be important initiators of atherogenesis. Interestingly, the hypothesis that phospholipids generated during oxidative modification of LDL may participate in atherosclerosis is supported by their biological activities, eg, the capacity to stimulate smooth muscle cell proliferation and to induce leukocyte activation.[44]

Oxidative modification of PAPC was essential for the inhibitory effect on Cox-2 because it was observed neither with the unoxidized mixture nor after reduction of ox-PAPC. The finding that the phospholipid fraction extracted from ox-LDL, but not that from native LDL, inhibits Cox-2 in a fashion fully comparable to that exerted by ox-LDL in toto indicates that a component(s) responsible for the impairment of Cox-2 is localized to the phospholipid fraction of ox-LDL, probably the arachidonic acid–containing phospholipids.

The observation that ox-LDL downregulates Cox-2 in human macrophages consistent with a number of reports[23,24,45–48] dealing with a suppressive effect of ox-LDL on inducible inflammatory genes in macrophages: in particular, inhibition of cytokine gene mRNAs as well as of inducible nitric oxide synthase and platelet-derived growth factor

B-chain. Thus it is clear that ox-LDL impairs the response of macrophages to inflammatory stimuli. Interestingly, in atheroma the magnitude of cytokine expression is diminished or lacking in regions proximal to the lesion core, where macrophages may accumulate greater amounts of oxidized lipids or may have been exposed to LDL that has been more extensively oxidized.[24]

Our data indicate that in macrophages exposed to ox-LDL the synthesis of eicosanoids is also impaired. Besides having a role in the inflammatory response, eicosanoids, in particular prostaglandin $E_2$ and prostacyclin, negatively affect release of macrophage colony stimulating factor (M-CSF) by monocytes[49,50] and by various cell types within the atheroma.[51] Considering the role of M-CSF in atherogenesis,[52] one could envision that ox-LDL, through the inhibition of eicosanoid synthesis, markedly enhances M-CSF availability within the atheroma. This condition, which favors macrophage proliferation and accumulation, ultimately accelerates the progression of atherogenesis.

The observation of a marked reduction of Cox-2 mRNA levels in LPS-stimulated macrophages exposed to ox-LDL suggests that ox-LDL affects either Cox-2 mRNA stabilization/degradation or that it operates at transcriptional level. Previous reports have found that ox-LDL inhibits selected genes and functions of macrophages through this latter mechanism: ox-LDL inhibits LPS-induced binding of the transcription factors NF-$\kappa$B and AP-1 to DNA.[40,53,54] These transcription factors are involved in the regulation of several genes induced in macrophages by inflammatory stimuli such as LPS,[55,56] and recently, the requirement of the activation of NF-$\kappa$B to induce the expression of Cox-2 in LPS-stimulated macrophages has been described.[20]

In conclusion, we showed that Cox-2 expression induced by LPS is suppressed by ox-LDL in human macrophages. This finding represents a further demonstration of a link between the oxidative modification of LDL and the dampening of the inflammatory potential of macrophages. The observed effect could be relevant in atheromata, where close contact between macrophages and oxidized lipids might ultimately result in the development of an impaired inflammatory response, together with a cell failure to repair tissue damage. This phenomenon may thus represent an important contributing feature in the conversion of the early atherosclerotic lesion to a late, less reversible lesion.

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