Stimulation of Phospholipase D Activity by Oxidized LDL in Mouse Peritoneal Macrophages

Antonio Gómez-Muñoz, Jason S. Martens, Urs P. Steinbrecher

Abstract—Oxidation of LDL is an important factor in the development of atherosclerosis. However, the mechanisms by which oxidized LDL exerts its atherogenic actions are poorly understood. In the present work, we show that oxidized LDL stimulates phospholipase D (PLD) activity in mouse peritoneal macrophages and that this effect increases with the degree of LDL oxidation. Oxidative modification of LDL results in the production of lipid peroxides and the conversion of phosphatidylcholine to lysophosphatidylcholine. Although we found that lysophosphatidylcholine alone activates PLD, the stimulation of this enzyme activity by oxidized LDL is independent of lysophosphatidylcholine formation. Also, 7-ketocholesterol, the major oxysterol in oxidized LDL, failed to stimulate PLD activity. To determine the mechanism(s) whereby oxidized LDL activates PLD, the possible involvements of protein kinase C and tyrosine phosphorylation were investigated. Pretreatment of macrophages with the protein kinase C inhibitor Ro-32-0432 or downregulation of protein kinase C activity by prolonged incubation with 100 nmol/L 4β-phorbol 12-myristate 13-acetate did not alter the stimulatory effect of oxidized LDL on PLD activation. However, oxidized LDL stimulated tyrosine phosphorylation of several macrophage proteins, and preincubation of the macrophages with genistein, a tyrosine kinase inhibitor, blocked the activation of PLD by oxidized LDL. In addition, pretreatment with orthovanadate, which inhibits tyrosine phosphatases, enhanced basal and oxidized LDL–stimulated PLD activity. Pretreatment of macrophages with pertussis toxin decreased the stimulatory effect of oxidized LDL, indicating that GTP-binding proteins may also be involved in the activation of PLD by oxidized LDL. We also found that the platelet-activating factor receptor antagonists WEB 2086 and L-659,989 inhibit the oxidized LDL stimulation of PLD, suggesting a role for platelet-activating factor receptor in this process. The stimulation of the PLD pathway by oxidized LDL may be of importance in atherogenesis, because PLD activation leads to generation of important second messengers such as phosphatidate, lysophosphatidate, and diacylglycerol, which are known to regulate many cellular functions. (Arterioscler Thromb Vasc Biol. 2000;20:135-143.)

Key Words: oxidized LDL ▪ phospholipase D ▪ lysophosphatidylcholine ▪ macrophages

Several lines of evidence implicate oxidized LDL in the pathogenesis of atherosclerosis. Oxidized LDL is present in atherosclerotic lesions, and in some animal models, antioxidant drugs can slow the progression of the disease.1 Oxidized LDL exhibits many potentially atherogenic actions in vitro, including direct chemoattractant activity for monocytes2-3; induction of monocyte chemotactic protein-1 secretion by endothelial cells and smooth muscle cells4; induction of genes for granulocyte colony–stimulating factor, macrophage colony–stimulating factor, and granulocyte-macrophage colony–stimulating factor5-6; and enhancement of endothelial expression of adhesion molecules, including vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule (ICAM-1), P-selectin, and a monocyte-specific adhesion molecule of the GRO family.7-10 In addition, oxidized LDL can stimulate the growth of macrophages and vascular smooth muscle cells.11-14 For many of these actions, neither the component of oxidized LDL that is responsible nor the signaling pathways that are involved have been defined. However, it is known that oxidized LDL has actions that affect a number of cell signaling pathways: it can increase intracellular calcium,15,16 modulate protein kinase C,17 stimulate phosphoinositide (PI) 3-kinase,18 and increase mitogen-activated protein kinase activities.19 Another important pathway that might be involved in some of these actions of oxidized LDL is the phospholipase D (PLD) pathway. PLD acts on phosphatidylcholine (PC) to release phosphatidic acid (PA), a biologically active molecule that has been implicated in the regulation of numerous cellular functions. For example, PA can stimulate phospholipase A2, leading to arachidonic acid release and eicosanoid production, and is also involved in the stimulation of cell proliferation.20 PA can be converted to lysopPA by the action of an A-type phospholipase activity, and both PA and...
lysoPA have chemotactic properties. Alternatively, PA can be converted to diacylglycerol (DAG) by phosphatidylinositol phosphohydrolase, thereby affecting a host of cellular functions through protein kinase C, including cell proliferation. Natarajan and colleagues found that in rabbit arterial smooth muscle cells, oxidized LDL induced DNA synthesis, and this was associated with stimulation of PLD activity. More recently, it was reported that the stimulation of proliferation of bovine aortic smooth muscle cells by oxidized LDL was accompanied by stimulation of sphingomyelinase (SMase) activity and production of ceramides. The proliferative action of oxidized LDL in these cells was mimicked by exogenous SMase or by cell-permeable ceramides, but not by exogenous PLD. This stimulation of growth by ceramide is unexpected, and in fact, in human endothelial cells the induction of the SMase pathway by oxidized LDL causes apoptosis through the generation of ceramide.

We previously found that oxidized LDL can stimulate the growth of macrophages and that this was partially related to signaling through PI 3-kinase. However, ~50% of the growth-stimulating effect of oxidized LDL could not be inhibited by PI 3-kinase inhibitors, suggesting that activation of other pathways might also be involved in cell proliferation. Accordingly, the present study was undertaken to determine whether oxidized LDL stimulates PLD and/or SMase activity in macrophages and to establish the mechanism(s) whereby such activation occurs.

**Materials**

**A23187, calphostin C, cholera toxin, daidzein, genistein, PC, sodium orthovanadate, pertussis toxin, 4β-phorbol 12-myristate 13-acetate (PMA), PMSF, phospholipase A2 (from *Crotalus atrox* venom), sphingomyelin (SM), SMase, staurosporin, and thapsigargin were purchased from Sigma Chemical Co. BAPTA-AM and chelerythrine were obtained from Calbiochem. C8- ceramide was from Matreya, Inc. 1-Palmitoyl 2-(6-[7-nitrobenzoxadiazoyl]amin) caproyl phosphotidylcholine (C8NBD-PC) and C8NBD-aminocaproic acid were obtained from Avanti Polar Lipids. L-659,989 was a gift from Dr William L. Henckler, Merck Research Laboratories (Rahway, NJ). LY294002 was obtained from BIOMOL Research Laboratories, Inc. WEB 2086 was provided by Boehringer Ingelheim. Other chemicals were the highest grade available from Fisher or WVR Canlab.

**Cell Culture**

CD-1 mice were obtained from the University of British Columbia Animal Care Center or from Charles River (Montreal, Quebec, Canada). Scavenger receptor class A type II (SR-AII) knockout mice were provided by Dr T. Kodama (University of Tokyo). The description of the construct and the phenotypic characterization in homozygous knockout mice have been reported elsewhere. Resident peritoneal macrophages were obtained from 8- to 10-week-old mice by peritoneal lavage with ice-cold Ca2+-free Dulbecco’s PBS. Cells were resuspended in DMEM supplemented with 10% FBS and gentamicin (50 mg/L). Pooled macrophages from 8 to 10 mice were resuspended in DMEM supplemented with 10% FBS and gentamicin (50 mg/L) and were then seeded at 10° cells/well in 6-well culture dishes. After 2 hours of incubation at 37°C, nonadherent cells were removed by gentle washing with DMEM and then incubated for 22 hours before use in experiments. Human arterial smooth muscle cells were obtained from a superior mesenteric artery biopsy obtained at the time of organ harvest for transplantation. Cells were cultured in DMEM with 10% FBS and were used between the fourth and seventh passages.

**Lipoprotein Isolation and Modification**

Normal human LDL (d=1.019 to 1.063) was isolated by sequential ultracentrifugation of EDTA-anticoagulated plasma. The concentration of EDTA in LDL preparations was reduced before oxidation by dialysis against Ca2+-free Dulbecco’s PBS containing 10 μmol/L EDTA. Standard conditions for LDL oxidation were 200 μmol/L LDL in Dulbecco’s PBS containing 5 μmol/L CuSO4, incubated at 37°C for the times indicated. The extent of LDL oxidation was controlled by addition of 100 μmol/L EDTA and 40 μmol/L butylated hydroxytoluene at various times, as indicated. Oxidized LDL was concentrated at ~1 mg/mL by use of ultrafiltration membrane cones (Centricon CF 25, Amicon), passed through a 0.45-μm filter, and stored at 4°C under sterile conditions. Oxidized LDL was used within 2 weeks of preparation. Acetylation of LDL was performed by sequential addition of acetic anhydride. This resulted in a 4-fold increase in electrophoretic mobility relative to native LDL on agarose gels and modification of 65% to 80% of lysine residues of apolipoprotein B. Phospholipase A1, digestion of LDL was performed by addition of 5 U of this enzyme in 0.2 mL of 0.1 mol/L Tris-HCl to 1.5 mg of native or acetylated LDL in 1 mL. Enzyme and incubation were then performed at 37°C for 2 hours. The reaction was then stopped by addition of 10 mmol/L EDTA followed by refrigeration. Acetyl-LDL was enriched with lysoPC by incubation of 6 or 10 mmol lysoPC with 100 μg of the lipoprotein for 1 hour at 37°C. Unbound lysoPC was removed by washing of the acetyl-LDL–lysoPC complex with Ca2+-free PBS in Centricon CF 25 ultrafiltration membrane cones. Under these conditions, ~90% of the added lysoPC was recovered in LDL.

**Assays**

PLD was determined on the basis of its transphosphatidylation activity, which leads to the production of [3H]phosphatidylethanol when cells containing the probe [3H]HPC are stimulated in the presence of ethanol. Ethanol was added at a final concentration of 1%. This concentration gave maximal formation of phosphatidylethanol, with no toxicity to cells. Macrophages were washed once with DMEM containing 0.1% BSA and then incubated for 3 hours with this medium containing 1 μCi of [3H]myristate/mL to rapidly label cell PC. The radioactive medium was then aspirated, and the cells were washed twice with nonradioactive DMEM containing 0.1% BSA. The macrophages were then washed once with ice-cold Ca2+-free PBS and scraped into 0.5 mL of methanol. The wells were washed with a further 0.5 mL of methanol, and the 2 methanol samples were combined and mixed with 0.5 mL of chloroform. Lipids were extracted by separation of phases with a further 0.5 mL of chloroform and 0.9 mL of a solution containing 2 mol/L KCl and 0.2 mol/L H3PO4. Chloroform phases were dried down under N2, and lipids were separated by thin-layer chromatography using Silica Gel 60–coated glass plates. The plates were developed for 50% of their length with chloroform/methanol/acetic acid (9:1:1 by volume) and then dried. They were then developed for their full length with petroleum ether, boiling point 40°C to 60°C/diethyl ether/acetic acid (60:40:1 by volume). The position of the lipids was identified after staining with I2 vapor by comparison with authentic standards. Radiolabeled lipids were quantified after being scraped from the plates by liquid scintillation counting.

The formation of [3H]ceramides was determined by scraping the ceramides from the same thin-layer plate as that used for isolating [3H]phosphatidylethanol, as indicated previously. The identity of the ceramides was confirmed by cochromatography with authentic long-chain ceramides. Similar studies on ceramide formation were performed after the cells had been labeled with 10 μCi [3H]palmitate/mL for 24 hours. The levels of [3H]SM were also determined from [3H]palmitate-labeled cells by developing the thin-layer plates in chloroform/methanol/acetic acid/formic acid/water (35:15:6:2:1 by volume) and quantifying the radioactive SM by liquid scintillation counting.
Oxidized LDL Stimulates PLD in Macrophages

The activity of platelet-activating factor (PAF)-acetylhydrolase was determined by use of the fluorescent substrate C$_6$NBD-PC, as reported previously. Briefly, 10-µg aliquots of LDL samples were incubated with 10 nmol of substrate in 1 mL Dulbecco’s PBS at 37°C for 10 minutes. Samples containing 5 nmol/L PMSF were preincubated for 60 minutes at 37°C before addition of substrate. Reactions were terminated by vortexing for 1 minute with 1.2 mL methanol and 1.2 mL chloroform. The mixtures were centrifuged at 2000g for 15 minutes to separate the phases, and the fluorescence of the aqueous layer was measured with a Turner 450 fluorometer at excitation 470 nm and emission 533 nm. The mass of fluorescent substrate hydrolyzed was calculated from a standard curve generated for the macrophages. These observations suggest that the stimulation of PLD by oxidized LDL is independent of generation of ceramide or its further metabolism. In fact, it seems unlikely that oxidized LDL would stimulate the generation of both ceramides.

Immunoblotting
Cultured macrophages were scraped from culture dishes, lysed with buffer containing 1% Triton X-100, and centrifuged to remove debris. Aliquots containing 40 µg protein were boiled in 10% SDS and then separated by electrophoresis on 10% PAGE gels. The gels were electroblotted onto nitrocellulose membranes, washed, and incubated with 1:4000 horseradish peroxidase–conjugated goat anti-phosphotyrosine mouse monoclonal antibody (Santa Cruz Biotechnology). Membranes were washed and incubated with 1:4000 horseradish peroxidase–conjugated goat anti-rabbit polyclonal antibody (Calbiochem). Bands were visualized with an enhanced chemiluminescence kit (Amersham).

Statistical Analysis
All experiments were performed in duplicate and were repeated at least twice. The significance of differences between means of control and experimental conditions was assessed with Student’s paired t test.

Results
Oxidized LDL Stimulates PLD Activity but Does Not Increase Ceramide Production
Figure 1 shows that oxidized LDL stimulates PLD activity in mouse peritoneal macrophages in a time- and concentration-dependent manner. Under optimal conditions (60 minutes of incubation with the lipoproteins at a final concentration of 60 µg/mL), the effect of oxidized LDL was much greater than that of native LDL or acetyl-LDL (Tables 1 and 2). In contrast to results reported with oxidized LDL in bovine smooth muscle cells and in cultured human umbilical vein endothelial cells, we failed to detect any generation of ceramides by oxidized LDL in macrophages (Figure 1) or in human aortic smooth muscle cells (data not shown). However, the possibility remained that oxidized LDL could activate SMase to generate ceramide but that this was then rapidly converted to sphingosine by ceramidase. Sphingosine, in turn, can be phosphorylated to sphingosine 1-phosphate by intracellular kinases, and both sphingosine and sphingosine 1-phosphate are potent stimulators of PLD. To rule out the possibility that activation of PLD by oxidized LDL was a result of SMase activity, macrophages were labeled with [3H]palmitate and then challenged with 60 µg/mL oxidized LDL. There was no change in the content of [3H]SM over a period of 60 minutes, confirming that there was no activation of SMase by oxidized LDL in these cells (data not shown). Furthermore, experiments in the presence of N-oleoyl ethanolamine (2 to 5 µmol/L), a ceramidase inhibitor, did not reveal any accumulation of ceramides that might have been synthesized de novo by the action of oxidized LDL. Concentrations >5 µmol/L of N-oleoyl ethanolamine were toxic for the macrophages. These observations suggest that the stimulation of PLD by oxidized LDL is independent of generation of ceramide or its further metabolism. In fact, it seems unlikely that oxidized LDL would stimulate the generation of both ceramides.

<table>
<thead>
<tr>
<th>Oxidation Time (h)</th>
<th>Relative PLD Activity</th>
<th>LysoPC Content (nmol/mg)</th>
<th>Protein (mg/mL)</th>
<th>Electrophoretic Mobility</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (native)</td>
<td>1.37 ± 0.11</td>
<td>14 ± 4</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>1.82 ± 0.12</td>
<td>98 ± 17</td>
<td>1.45 ± 0.05</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>2.30 ± 0.16</td>
<td>133 ± 12</td>
<td>2.05 ± 0.05</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>2.61 ± 0.06</td>
<td>188 ± 10</td>
<td>2.40 ± 0.10</td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>2.62 ± 0.05</td>
<td>258 ± 11</td>
<td>3.65 ± 0.05</td>
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Macrophages were labeled as described in Figure 1. They were then stimulated for 60 minutes with 60 µg/mL of LDL that had been oxidized by incubation for varying times with 5 µmol/L CuSO$_4$. The results for PLD activity were calculated as a percentage of the radioactivity present in [3H]phosphatidylethanolamine compared with that in total lipids and then expressed as the fold stimulation relative to incubations in the absence of LDL. The lysoPC content and electrophoretic mobility relative to native LDL of each lipoprotein were determined as described in the Methods. Values shown for PLD activity and lysoPC content are mean ± SEM of 3 independent experiments performed in duplicate, except for the relative PLD activity at time 0, where n = 7. Results for electrophoretic mobility represent mean ± range of 2 independent experiments.
and PA, because these are generally considered to be antagonistic signals.29,32 To verify that ceramide can indeed antagonize the stimulation of PLD by oxidized LDL in macrophages, the intracellular concentration of ceramide was increased by preincubation of the cells for 2.5 hours with the cell-permeable ceramide N-acetyl sphingosine (C2-ceramide) or by pretreatment with exogenous bacterial SMase. Figure 2 shows that as expected, the oxidized LDL–activated PLD was inhibited by increasing concentrations of C2-ceramide. Similar results were obtained by preincubating the macrophages with 200 mM/mL of bacterial SMase. Under these conditions, SMase reduced the oxidized LDL–stimulated PLD from 2.46±0.11-fold to 1.24±0.1-fold (mean±range of 2 independent experiments). The inhibition of oxidized LDL–stimulated PLD activity by cell-permeable ceramides was not caused by a physical interaction of the ceramides with the oxidized LDL particles in the incubation medium. Pretreatment of cells for 2.5 hours with 10 μmol/L C2-ceramide followed by its removal from the medium and subsequent addition of oxidized LDL still blocked the activation of PLD by oxidized LDL.

Some of the actions of oxidized LDL, such as interaction with scavenger receptors or the stimulation of macrophage growth, require extensive oxidation of LDL, whereas other actions, such as induction of GRO or MCP-1 expression, are seen only with mildly oxidized LDL.18,36 To define the extent of oxidation required for PLD activation, a series of oxidized LDLs that differed in extent of oxidation were prepared by varying the time of exposure to copper from 0 to 24 hours. As shown in Table 1, significant activation of PLD was seen after only 3 hours of oxidation and was nearly maximal after 6 hours. This indicates that products that are formed relatively early during copper oxidation of LDL are responsible for PLD activation. Furthermore, because LDL acquires ligand activity for scavenger receptors only after >10 hours of oxidation,26 these data suggest that internalization of LDL may not be required for activation of PLD.

Role of LysoPC in the Stimulation of PLD by Oxidized LDL

LysoPC is a major component of oxidized LDL and is thought to mediate several of its biological actions.3,7,8,11,37–40 In particular, it has been reported that lysoPC stimulates PLD activity in cultured endothelial cells.54 We first sought to determine whether lysoPC was also capable of activating PLD in macrophages simply by incubating cells with lysoPC in the absence of lipoproteins. Concentrations of lysoPC as low as 2 μmol/L increased PLD activity by 251±14% (mean±SEM of 3 independent experiments). In addition, treatment of native LDL or acetyl-LDL with phospholipase A2 enhanced their ability to activate PLD (Table 2). Because oxidized LDL contains numerous bioactive components, a further experiment was required to determine whether activation of PLD by oxidized LDL was entirely attributable to lysoPC. Accordingly, before oxidation, LDL was treated with PMSF to block PAF-acetylhydrolase, an LDL-associated serine esterase that converts oxidized PC to lysoPC during the oxidation of LDL.33 PMSF inhibited PAF-acetylhydrolase activity by 90±2% (mean±range of 2 independent experiments) and reduced the content of lysoPC in oxidized LDL from 260 to 57 nmol/mg protein after 24 hours of oxidation. Despite this 78% decrease in lysoPC content, PMSF-pretreated oxidized LDL stimulated PLD to an extent similar to that of standard oxidized LDL (Table 2). This suggests that the stimulation of PLD by oxidized LDL is independent of lysoPC formation. To rule out the possibility that the small residual content of lysoPC in the PMSF-pretreated oxidized LDL was sufficient to activate PLD, macrophages were incubated with 60 μg/mL of acetyl-LDL that was complexed to lysoPC

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**TABLE 2. Stimulation of PLD Activity by Modified LDLs**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Relative PLD Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1</td>
</tr>
<tr>
<td>Native LDL</td>
<td>1.33±0.06</td>
</tr>
<tr>
<td>PLA2-treated native LDL</td>
<td>2.35±0.16</td>
</tr>
<tr>
<td>Acetyl-LDL</td>
<td>1.39±0.13</td>
</tr>
<tr>
<td>PLA2-treated acetyl-LDL</td>
<td>2.14±0.11</td>
</tr>
<tr>
<td>Oxidized LDL</td>
<td>2.40±0.13</td>
</tr>
<tr>
<td>Oxidized PMSF-treated LDL</td>
<td>2.38±0.10</td>
</tr>
</tbody>
</table>

Macrophages were labeled as in Figure 1 and were then stimulated for 60 minutes with 60 μg/mL of modified LDLs as indicated. Native LDL and acetyl-LDL were digested with 5 U/mL of phospholipase A2 (PLA2) for 2 hours at 37°C. This converted >90% of the lipoprotein PC to lysoPC. The modified LDLs were then reisolated from PLA2 by repeated washes with PBS in ultracentrifugation membrane cones with a 100-kDa cutoff. To prevent the generation of lysoPC during oxidation, PAF-acetylhydrolase in LDL was inactivated by incubation at 37°C for 60 minutes with 5 mmol/L PMSF before LDL oxidation. This reduced the content of lysoPC in oxidized LDL from ~260 to 57 nmol/mg protein. Values for PLD activity were calculated as a percentage of the radioactivity present in [3H]phosphatidylethanol compared with that in total lipids and then expressed as the fold stimulation relative to control incubations without lipoproteins. Results are the mean±SEM of 3 independent experiments, except for values with PLA2-treated native LDL and PLA2-treated acetyl-LDL, which are mean±range of 2 independent experiments performed in duplicate.
at a concentration of 54 or 90 nmol/mg protein. The enrichment with lysoPC did not increase the activation of PLD by acetyl-LDL. However, treatment of macrophages with optimum concentrations of oxidized LDL after 60 minutes of incubation with 2 μmol/L lysoPC increased PLD activity from 2.44 ± 0.04-fold control with lysoPC alone to 3.23 ± 0.21-fold control (mean ± range of 2 independent experiments). Although the effects of oxidized LDL and lysoPC were not strictly additive, these results suggest that the 2 agonists may activate PLD through independent mechanisms.

Oxidized LDL contains high concentrations of oxysterols, and 7-ketocholesterol, the major oxysterol found in copper-oxidized LDL, has been reported to inhibit SMase activity. Addition of 7-ketocholesterol to the medium did not alter PLD activity in the macrophages, nor did it affect the ability of lysoPC to activate PLD (data not shown).

**Activation of PLD by Oxidized LDL in Macrophages Is Independent of Ca²⁺**

Oxidized LDL has been shown to increase intracellular Ca²⁺ concentrations. To determine whether the effect of oxidized LDL on PLD resulted from changes in Ca²⁺ concentrations, extracellular Ca²⁺ was chelated with 5 mmol/L EGTA. As expected, this concentration of EGTA blocked the activation of PLD by the ionophore A23187. However, there was no effect on the activation of PLD by oxidized LDL (data not shown). To rule out an effect of Ca²⁺ ions released from intracellular stores, macrophages were preincubated for 30 minutes with the intracellular Ca²⁺ chelator BAPTA-AM (5 to 20 μmol/L) before stimulation with oxidized LDL. BAPTA-AM has been shown not to alter basal PLD activity in smooth muscle cells. However, it dramatically stimulated basal PLD in the macrophages and therefore could not be used to evaluate a possible involvement of intracellular Ca²⁺ in these cells. Instead, the macrophages were pretreated for 20 or 40 minutes with 1 μmol/L thapsigargin, an intracellular Ca²⁺-ATPase inhibitor that depletes intracellular Ca²⁺ stores and blocks agonist-induced intracellular Ca²⁺ mobilization in murine peritoneal macrophages. Under these conditions, thapsigargin did not inhibit the stimulation of PLD by oxidized LDL in the macrophages (data not shown), suggesting that it is a Ca²⁺-independent process, in agreement with previous observations in smooth muscle cells.

**Stimulation of PLD by Oxidized LDL Is Protein Kinase C–Independent**

The role of protein kinase C in the stimulation of PLD by oxidized LDL was evaluated by use of the selective inhibitor Ro-32-0432. Other protein kinase C inhibitors such as staurosporin, calphostin C, and chelerythrine increased basal PLD activity and failed to block PMA-stimulated PLD activation in the macrophages. Therefore, these compounds could not be used to evaluate the role of protein kinase C in these cells. There are precedents for such unexpected responses to some protein kinase C inhibitors in some cell types, because staurosporin has recently been shown to stimulate basal PLD activity and to potentiate the PA mass formation induced by f-Met-Leu-Phe in human neutrophils, and calphostin C failed to inhibit protein kinase C–mediated PLD activation in human coronary endothelial cells. Pretreatment of macrophages with 1 μmol/L Ro-32-0432 for 30 minutes decreased the PMA-stimulated PLD activity from 16.5 ± 1.2- to 6.3 ± 0.9-fold (mean ± range of 2 independent experiments) in the macrophages. However, the oxidized LDL–induced PLD activation was not attenuated by this inhibitor (data not shown). The possible involvement of protein kinase C in oxidized LDL–induced PLD stimulation was further examined by downregulating protein kinase C by prolonged incubation (20 hours) with 100 nmol/L PMA. Under these conditions, the macrophages lost their sensitivity to stimulation of PLD by PMA, but the oxidized LDL–stimulated PLD was not changed significantly in 4 independent experiments performed in duplicate (data not shown). Therefore, these data suggest that the stimulation of PLD by oxidized LDL is not a protein kinase C–mediated effect.

**Involvement of GTP-Binding Proteins in Oxidized LDL–Induced PLD Stimulation**

Previous studies have shown that oxidized LDL can inhibit signaling pathways mediated by pertussis toxin–sensitive GTP-binding proteins (G proteins). To evaluate the possible involvement of G proteins in oxidized LDL–induced PLD activation, the macrophages were preincubated for 30 minutes with 1-μg/mL concentrations of cholera toxin, which stimulates Gs, or pertussis toxin, which inhibits Gi. Cholera toxin did not alter PLD activation by oxidized LDL significantly in 2 independent experiments. However, pertussis toxin caused a significant decrease (P < 0.05), from 2.42 ± 0.10- to 1.69 ± 0.14-fold (mean ± SEM of 5 independent experiments performed in duplicate), suggesting a possible role for Gi in this process.

**Stimulation of PLD by Oxidized LDL in Macrophages Involves Tyrosine Phosphorylation Processes**

To determine whether oxidized LDL increases protein tyrosine phosphorylation in mouse peritoneal macrophages, a crude cell extract was analyzed by anti-phosphotyrosine immunoblotting. As shown in Figure 3, oxidized LDL enhanced the phosphorylation of several proteins. The involve-

Figure 3. Phosphotyrosine immunoblot of macrophage lysates. Macrophages were incubated with or without 60 μg/mL oxidized LDL for 8 minutes, as indicated. Cell lysates (40 μg/lane) were then separated by SDS/PAGE and transferred to nitrocellulose membranes. Membranes were incubated with anti-phosphotyrosine antibody PY20 and visualized by enhanced chemiluminescence using a goat anti-mouse secondary antibody (left). The position of molecular weight markers (in kDa) is indicated at left. Right, Results of scanning densitometry of the exposed film. Error bars indicate the range of values from duplicate scans of a single gel. Similar results were obtained in other experiments with 4G10 phosphotyrosine antibody.
PAF receptor antagonists.14 PAF DNA in bovine coronary artery smooth muscle cells and that this effect can be blocked by PAF receptor antagonists. It has been shown that oxidized LDL stimulates the synthesis of protein tyrosine kinase inhibitor, and orthovanadate, an inhibitor of tyrosine kinases and phosphatases in the activation of PLD by oxidized LDL was examined by use of genistein, a specific inhibitor for the PAF receptor, because it appears to have a direct inhibitory effect on PLD.48 PLD activity was decreased from 2.76±0.24-fold to 1.97±0.22-fold (P<0.05) in 3 independent experiments. This effect appeared to be specific in that WEB 2086 did not block the activation of PLD by other agonists, including PMA, A23187, or lysoPA, which stimulated PLD by 19.42±1.60-, 6.13±0.41-, and 2.74±0.17-fold, respectively (mean±SEM, n=6). These results suggest a contributory role for PAF receptor activation in the stimulation of PLD by oxidized LDL. Another PAF receptor antagonist, L-659,989, was more potent than WEB 2086 in inhibiting the activation of PLD by oxidized LDL. However, L-659,989 may not be a specific inhibitor for the PAF receptor, because it appears to have a direct inhibitory effect on PLD.48

**Discussion**

The signal transduction mechanisms involved in cellular responses to oxidized LDL are poorly understood. The
biological activity of oxidized LDL in vitro appears to be dependent on the degree of LDL oxidation, because some of the effects of oxidized LDL (i.e., cytotoxicity and binding to scavenger receptors) require high degrees of oxidation, whereas others are seen only with mildly oxidized LDL. For example, mildly oxidized LDL induces the expression of growth factors, monocyte-specific adhesion molecules, monocyte chemotactic protein-1, oxidized LDL receptors, and tissue factor, whereas extensively oxidized LDL does not.\textsuperscript{36,51} This is an important consideration because mildly oxidized LDL has been demonstrated in human atherosclerotic lesions, whereas the existence of heavily oxidized LDL in lesions has not been established.\textsuperscript{52}

In the present work, we show that oxidized LDL stimulates PLD in murine peritoneal macrophages and that this effect can be elicited by either mildly or extensively oxidized LDL. PLD activation is of particular interest in cell signaling, because its product PA is an important second messenger that can control a variety of cellular events.\textsuperscript{23} As mentioned before, PA can give rise to the generation of lysoPA or DAG. Both lysoPA and DAG are also important second messengers that are involved in many cellular functions, including cell proliferation.\textsuperscript{22,23} Oxidized LDL stimulates growth of murine macrophages, and part of this effect might be mediated by the generation of PA by PLD. Paradoxically, it has been reported that the stimulation of smooth muscle cell proliferation by oxidized LDL was due to the production of ceramides.\textsuperscript{25} It seems unlikely that both PA and ceramides are generated by the same agonist, because they are antagonistic signals.\textsuperscript{29,32} The present results indicate that oxidized LDL does not induce ceramide accumulation in the macrophages. In fact, increasing the intracellular concentration of ceramide by preincubation with \( C_2 \)-ceramide or with exogenous SMase increased the intracellular concentration of this cation with the \( C_2 \) ionophore A23187 in mouse peritoneal macrophages\textsuperscript{55} or with calcitriol (the active metabolite of vitamin D\textsubscript{3}) in intact cells.\textsuperscript{56} It was recently reported that inhibitors of PI 3-kinase, such as LY294002 and wortmannin, inhibit the activation of these 2 enzymes by oxidized LDL.

The biochemical mechanisms for regulation of mammalian PLD are not completely understood. In intact cells, PLD activity has been shown to be controlled by both protein kinase C and tyrosine phosphorylation events.\textsuperscript{53} Although \( C_2 \) ions may not be required for PLD activity,\textsuperscript{54} elevation of the intracellular concentration of this cation with the \( C_2 \) ionophore A23187 in mouse peritoneal macrophages\textsuperscript{55} or with calcitriol (the active metabolite of vitamin D\textsubscript{3}) in rat skeletal muscle\textsuperscript{56} led to the activation of PLD. We found that PLD stimulation by oxidized LDL in murine macrophages is independent of protein kinase C and \( C_2 \) ions. However, PLD activation by oxidized LDL was blocked by the tyrosine kinase inhibitor genistein and was enhanced by orthovanadate (an inhibitor of tyrosine phosphatases), suggesting a role for tyrosine phosphorylation in this process. These results are consistent with previous observations in rabbit smooth muscle cells.\textsuperscript{24} It was recently reported that inhibitors of PI 3-kinase, such as LY294002 and wortmannin, inhibit the stem cell factor–induced PLD activation in porcine aortic endothelial cells,\textsuperscript{57} suggesting that PLD is a downstream effector of PI3-kinase in this system. However, pretreatment of macrophages with LY294002 did not inhibit the oxidized LDL–induced PLD activation (data not shown). These observations could be explained either by activation of PLD by oxidized LDL upstream of PI3-kinase or by independent activation of these 2 enzymes by oxidized LDL.

The existence of several mammalian PLD isoforms was recently reported.\textsuperscript{58} PLD1 has low basal activity and is activated by protein kinase C and small GTP-binding proteins, such as ADP-ribosylation factor, Rho, or Cdc42.\textsuperscript{54,58} By contrast, PLD2 is thought to be constitutively active and is not regulated by G proteins or protein kinase C.\textsuperscript{60,61} The activation of PLD1 can be blocked by cell-permeable ceramides by inhibiting the translocation to membranes of small GTP-binding proteins and protein kinases C-\( \alpha \) and -\( \beta_1 \).\textsuperscript{62} The finding that pertussis toxin and \( C_2 \)-ceramide blocked the ability of oxidized LDL to activate PLD would be compatible with an effect on the PLD1 isoform, but the apparent lack of involvement of protein kinase C is not consistent with properties of PLD1 in other cells. Our data cannot be explained by invoking any one of the different mammalian PLD isoforms that have been identified to date, but there are several recent reports of a PLD activity in mammalian cells that is inducible, yet independent of protein kinase C.\textsuperscript{63,64}

As mentioned above, oxidized LDL has been shown to be mitogenic for macrophages.\textsuperscript{11,13,18,65} It has been proposed that the active component responsible for macrophage proliferation is lysoPC and that growth stimulation requires the uptake of modified LDL containing lysoPC by the scavenger receptor.\textsuperscript{11} Although we found that lysoPC alone can stimulate PLD to an extent similar to that with oxidized LDL, the stimulation of PLD by oxidized LDL is independent of its lysoPC content, and it does not appear to require internalization of oxidized LDL. Hence, activation of PLD is probably not directly linked to the effect of lysoPC in modified LDL on growth stimulation. The mechanism by which lysoPC stimulates PLD in macrophages is unknown. However, it may be different from that of oxidized LDL, because the stimulation of PLD by optimum concentrations of oxidized LDL and lysoPC are additive. Furthermore, the activation of PLD by oxidized LDL in macrophages is independent of protein kinase C, whereas PLD activation by lysoPC in human coronary endothelial cells\textsuperscript{41} or in murine macrophages\textsuperscript{66} is to a large extent mediated by protein kinase C.

The components that are responsible for stimulation of PLD by oxidized LDL are unknown at present. Oxidized LDL contains phospholipids with PAF-like bioactivity, and these have been implicated in growth stimulation of vascular smooth muscle cells by oxidized LDL.\textsuperscript{14} Our finding that the PAF receptor antagonist WEB 2086 attenuated the stimulation of PLD by oxidized LDL in murine macrophages suggests that this effect involves the PAF receptor. However, the inhibition was only \( \approx 30\% \), so PAF receptor–independent mechanisms are probably involved as well. We also found that pertussis toxin decreased the stimulation of PLD by oxidized LDL to an extent similar to that by WEB 2086; this may be relevant, because some PAF-induced signals involve guanine nucleotide regulatory proteins.\textsuperscript{67}

In conclusion, we show in this report that both mildly and extensively oxidized LDLs stimulate PLD activity in murine peritoneal macrophages. This effect is independent of protein kinase C, \( C_2 \), or the formation of lysoPC during the oxidation process and apparently does not require internalization of the oxidized LDL particles or the presence of SR-AI/II. PLD stimulation by oxidized LDL in macrophages involves tyrosine phosphorylation, pertussis toxin–sensitive G proteins, and PAF receptor activation. In contrast to
findings reported in smooth muscle cells, oxidized LDL did not induce ceramide production in macrophages. Because oxidized LDL is involved in atherosclerosis, the present findings suggest a possible role for PLD in this disease and provide a possible mechanism for signaling by oxidized LDL in atherogenesis. Further work will be necessary to characterize the component(s) of oxidized LDL that are responsible for PLD activation and the effect of this activation on macrophage function.

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References


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