Lysophosphatidylcholine stimulates phospholipase D activity in mouse peritoneal macrophages

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Abstract Lysophosphatidylcholine (lysoPC) is a bioactive phospholipid that is involved in atherogenesis and inflammatory processes. However, the present understanding of mechanisms whereby lysophosphatidylcholine exerts its pathophysiological actions is incomplete. In the present work, we show that lysoPC stimulates phospholipase D (PLD) activity in mouse peritoneal macrophages. PLD activation leads to the generation of important second messengers such as phosphatidic acid, lysophosphatic acid, and diacylglycerol, all of which can regulate cellular responses involved in atherogenesis and inflammation. The activation of PLD by lysoPC was attenuated by down-regulation of protein kinase C activity with prolonged incubation with 100 nm of 4-phorbol 12-myristate 13-acetate (PMA). Preincubation of the macrophages with the tyrosine kinase inhibitor genistein also decreased the stimulation of PLD by lysoPC, while pretreatment with orthovanadate, which inhibits tyrosine phosphatases, enhanced basal and lysoPC-stimulated PLD activity. The activation of PLD by lysoPC was attenuated by the platelet activating factor (PAF) receptor antagonist WEB-2086, suggesting a role for PAF receptor activation in this process. Furthermore, acetylation of lysoPC substantially increased its potency in activating PLD, suggesting that a cellular metabolite of lysoPC such as 1-acyl 2-acetyl PC might be responsible for at least part of the effect of lysoPC on PLD. —Gómez-Muñoz, A., L. O’Brien, R. Hundal, and U. P. Steinbrecher. Lysophosphatidylcholine stimulates phospholipase D activity in mouse peritoneal macrophages. J. Lipid Res. 1999. 40: 988–993.

Supplementary key words lysophosphatidylcholine • phospholipase D • protein kinase C • macrophages

Lysophosphatidylcholine (lysoPC) is a bioactive phospholipid that is generated by the hydrolysis of PC by phospholipase A2 (PLA2) and is associated with a variety of physiologic and pathologic processes, including inflammation and atherosclerosis (1–4). LysoPC is a major component of oxidized LDL (5, 6) and several actions of oxidized LDL that promote foam cell formation have been ascribed to lysoPC. For example, lysoPC induces the expression of VCAM-1 by endothelial cells and this would favor recruitment of mononuclear leukocytes into the arterial intima (3). As well, it is mitogenic for vascular smooth muscle cells (7) and murine peritoneal macrophages (8). LysoPC has also been shown to induce the expression by endothelial cells of genes for several growth factors that are involved in atherogenesis (3, 4). More recently, lysoPC has been demonstrated to selectively activate the ICAM-1 promoter in human umbilical cord vein endothelial cells (9), and to inhibit the generation of endothelium-dependent relaxation factor and the expression of inducible nitric oxide synthase (10–13). In addition, lysoPC can induce the expression of cyclooxygenase-2 with consequent enhancement of prostacyclin synthesis by endothelial cells (14).

The mechanisms and signaling pathways that mediate these effects of lysoPC are incompletely understood. Recently, lysoPC has been shown to increase the intracellular concentration of cyclic-AMP in different cell types including human platelets (2), and by stimulating the activity of mitogen-activated protein kinases in rat vascular smooth muscle cells (15). LysoPC can also stimulate activator protein-1 and c-jun terminal kinase activity (16) and has been demonstrated to regulate the activity of protein kinase C (17–20). In addition, lysoPC causes intracellular Ca2+ mobilization in vascular smooth muscle cells and this has been linked to its mitogenic properties (21).

Another important signaling pathway that might be involved in some of the actions of lysoPC is the phospholipase D (PLD) pathway. Activation of PLD generates phosphatidic acid (PA), a well-known intermediate in several pathways of lipid metabolism, and an important regulator of a variety of cellular functions (22–24). Recently, PA has been shown to activate cytosolic PLA2 (25), leading to generation of arachidonic acid and of the platelet activating factor (PAF) precursor 2-alkenyl PC. Eicosanoids and PAF are of obvious importance in inflammatory reactions. In addition, PAF has been shown to stimulate PLD (26),

Abbreviations: DMEM, Dulbecco’s modified Eagle’s medium; LDL, low density lipoproteins; PA, phosphatidic acid; PAF, platelet activating factor; PBS, phosphate-buffered saline; PC, phosphatidylcholine; PI, phosphatidylinositol; PLA2, phospholipase A2; PLD, phospholipase D; PMA, 4-phorbol 12-myristate 13-acetate; SM, sphingomyelin; lysoPC, lysophosphatidylcholine.

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and incubated at 37°C. Lipids were extracted by separating phases with a further 0.5 ml of methanol. The wells were washed with a further 0.5 ml of methanol.

Materials

Calphostin C, cholera toxin, genistein, lysoPC, sodium orthovanadate, pertussis toxin, 4β-phorbol-12-myristate 13-acetate (PMA) and staurosporin were purchased from Sigma Chemical Co. (Mississauga, Ontario, Canada). C2-ceramide was from Matreya, Inc. (Pleasant Gap, PA). L-659,989 was a gift from Dr. John Chabala of Merck, Sharpe and Dohme Research (Rahway NJ). WEB-2086 was obtained from Boehringer Ingelheim (Ingelheim, Germany). Other chemicals were the highest grade available from Fisher or VWR Canlab (Edmonton, AB, Canada).

Cell culture

Murine resident peritoneal macrophages were obtained from female CD-1 mice (8–10 weeks old) by peritoneal lavage with ice-cold Ca2+-free Dulbecco’s PBS. Cells were resuspended in DMEM supplemented with 10% fetal bovine serum, and gentamicin (50 µg/ml). Cells were seeded at 105 cells/well on 6-well culture dishes and incubated at 37°C for 2 h in a humidified atmosphere of 5% CO2 in air. Non-adherent cells were then removed by gentle washing with DMEM medium and then incubated further for 22 h. All experiments were conducted in serum-free DMEM, unless indicated to the contrary.

Assay of PLD activity

PLD was determined on the basis of its transphosphatidylation activity, which leads to the production of [3H]phosphatidylethanol when cells containing [3H]phosphatidylcholine are incubated in the presence of ethanol (27). Macrophages were washed once with DMEM containing 0.1% BSA and then incubated for 3 h with this same medium containing 1 µCi of [3H]myristate/ ml to label cell phosphatidylcholine. The radioactive medium was then aspirated and the cells were washed twice with non-radioactive DMEM containing 0.1% BSA. The macrophages were incubated for a further 2.5 h in BSA- or serum-free DMEM. No intermediate washes were carried out along the procedure to prevent the burst of sphingolipids and diacylglycerol that occurs rapidly after changing the medium (28, 29). Ethanol (1% final concentration) was added 5 min prior to the addition of agonists. The macrophages were incubated for varying times, then washed once with ice-cold Ca2+-free PBS and extracted with chloroform–methanol–acetic acid as follows. Cells were scraped into 0.5 ml of methanol, and wells were washed with a further 0.5 ml of methanol. The two aliquots were combined and mixed with 0.5 ml of chloroform. Lipids were extracted by separating phases with a further 0.5 ml of chloroform and 0.9 ml of 2 M KCl and 0.2 M H3PO4. Chloroform phases were dried down under N2 and lipids were separated by thin-layer chromatography using Silica Gel 60 coated glass plates. TLC plates were developed for 50% of their lengths with chloroform–methanol–acetic acid 9:1:1 (v/v/v) and then dried. The plates were then redeveloped for their full length with petroleum ether–diethylether–acetic acid 60:40:1 (v/v/v). The position of the lipids was identified after staining with I2 vapor by comparison with authentic standards. Radioactive lipids were quantitated after scraping from the plates by liquid scintillation counting.

Measurement of ceramide production and sphingomyelin (SM) levels

[3H]labeled ceramides were determined by scraping the ceramides from the same thin-layer plate as that used for isolating [3H]phosphatidylethanol, as indicated previously (27). The identity of the ceramide was confirmed by cochromatography with authentic longchain ceramides. Similar studies were performed after labeling the cells with 10 µCi [3H]palmitate/ml for 24 h. The levels of [3H]sphingomyelin 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.

Other preparative and analytic techniques

To remove trace amounts of lysoPC, cells were digested with PLAP. A portion of the material that comigrated with PAF was digested with PLAP. A portion of the material that comigrated with PAF was then extracted and characterized by thin-layer chromatography using chloroform–methanol–acetic acid–formic acid–water 35:15:6:2:1 (by volume) and quantifying the radioactive SM by liquid scintillation counting.

RESULTS

LysoPC stimulates PLD activity in murine peritoneal macrophages

LysoPC stimulated PLD activity in mouse peritoneal macrophages in a manner that was time- and concentration-dependent. Maximal response was achieved after 60 min of incubation with 1–2 µg lysoPC/ml (Fig. 1). Concentrations of lysoPC higher than 5 µg/ml were toxic for the macrophages, as assessed by rounding, blebbing, and cell detachment when examined by phase contrast microscopy.

As mentioned above, lysoPC is a major component of oxidized LDL and as such, it has been suggested to be the mediator of many of its biological actions (3, 8, 20). Recently, it has been reported that oxidized LDL stimulates the proliferation of smooth muscle cells by a mechanism involving a rapid stimulation of SMase activity and subsequent generation of ceramides (30). However, it is unlikely that the stimulation of PLD by lysoPC in smooth muscle cells is mediated by ceramides because they are potent inhibitors of PLD activity (22, 31, 32). Nevertheless, the possibility exists that ceramides might still be generated by the action of lysoPC but rapidly converted to sphingosine by the action of ceramidase activity. Sphingosine, in turn, can be phosphorylated to sphingosine 1-phosphate by intracellular kinases, and both sphingosine and sphingosine 1-phosphate are potent stimulants of PLD (33). To rule out this possibility, we measured SM levels in [3H]palmitate prelabeled macrophages that were chal-
Macrophages were then preincubated with 1 μg/ml of lysoPC for various times (left panel) or with increasing concentrations of lysoPC for 60 min (right panel) without changing the medium, in the presence of 1% ethanol. [3H]phosphatidylethanol formation was determined by separating the lipids by thin-layer chromatography and processed as indicated in Materials and Methods. The results were calculated as a percentage of the radioactivity present in [3H]phosphatidylethanol compared to that in total lipids, and then expressed as the fold-stimulation relative to incubations in the absence of lysoPC. For control incubations, typical radioactivity measurements were 2500 dpm per dish in phosphatidylethanol and 400,000 dpm in total lipids. Results are the means ± SEM of three independent experiments.

Role of protein kinase C (PKC) in the stimulation of PLD by lysoPC

The possible involvement of PKC in the stimulation of PLD by lysoPC was evaluated by down-regulating PKC by prolonged incubations (20 h) with 100 nm PMA. Under these conditions, the macrophages lost their sensitivity to stimulation of PLD by PMA, and the activation of PLD by lysoPC was significantly decreased (Fig. 2). The role of PKC was evaluated further by using standard PKC inhibitors. Preincubation of macrophages with 1 μm Ro-32-0432 decreased (P < 0.01) the PMA-stimulated PLD activity from 15.72 ± 1.17-fold to 6.32 ± 1.05-fold (means ± SEM of four independent experiments) but surprisingly, it did not attenuate the lysoPC-induced PLD activation. This paradoxical result could be explained by the involvement of protein kinase C isoforms that are insensitive to Ro-32-0432 in the lysoPC-stimulation of PLD. Other protein kinase C inhibitors such as staurosporin, calphostin C, or chelerythrine were found to be unsuitable because they caused an increase in basal PLD activity in macrophages, and did not inhibit PMA-stimulated PLD activation (A. Gómez-Muñoz, unpublished results). There are precedents for such unexpected responses to protein kinase C inhibitors, as staurosporin has recently been shown to stimulate basal PLD activity and to potentiate the formation of PA by f-Met-Leu-Leu-Ph in human neutrophils (34). Furthermore, calphostin C failed to inhibit protein kinase C-mediated PLD activation in human coronary endothelial cells (35).

Stimulation of PLD by lysoPC in macrophages involves tyrosine phosphorylation processes

We have previously shown that oxidized LDL activates PLD in macrophages, and that this is inhibited by genistein (a tyrosine kinase inhibitor), and enhanced by orthovanadate, an inhibitor of tyrosine phosphatase activity (A. Gómez-Muñoz, and U. Steinbrecher, unpublished results). To determine whether the activation of PLD by lysoPC also involved tyrosine phosphorylation, macrophages were preincubated with 100 μm genistein for 30 min before exposure to lysoPC. This attenuated the activation of PLD (Fig. 3). Conversely, pretreating macrophages with 100 μm orthovanadate for 15 min increased the basal PLD activity.
Fig. 3. Effect of genistein and vanadate on lysoPC-induced PLD activation. Macrophages were treated as in Fig. 1 and they were preincubated for 30 min with or without 100 μM genistein (GEN) or 30 μM orthovanadate (VAN), as indicated. The cells were then stimulated with 1 μg/ml of lysoPC (LPC) and the incubations continued further for 60 min. Results are expressed as the fold-stimulation relative to incubations in the absence of any of the additions, and they are the means ± SEM of four independent experiments (*P < 0.01).

Stimulation of PLD by lysoPC involves activation of the PAF receptor

It has been reported that lysoPC causes intracellular Ca\(^{2+}\) mobilization in murine peritoneal macrophages via stimulation of the PAF receptor (36). To determine whether the stimulation of PLD by lysoPC also involves PAF receptor activation, macrophages were preincubated for 5 min with the PAF receptor antagonist WEB-2086 prior to stimulation with lysoPC. As shown in Fig. 4, WEB-2086 did not alter basal PLD, but it attenuated the stimulation of PLD by lysoPC. This effect appeared to be specific in that WEB-2086 did not significantly change the stimulation of PLD by other agonists including lysoPA (Fig. 4), PMA, or the Ca\(^{2+}\) ionophore A23187 (data not shown). These results suggest a role for PAF receptor activation in the stimulation of PLD by lysoPC. Another PAF receptor antagonist, L-659,989, was more potent than WEB-2086 in inhibiting the activation of PLD by lysoPC. However, we recently found that L-659,989 has a direct inhibitory effect on PLD in addition to its action as a PAF receptor antagonist (A. Gómez-Muñoz, W. S. Martens, and U. P. Steinbrecher, unpublished results), and so the results with WEB-2086 probably are a better indication of the relative importance of the PAF receptor in PLD activation by lysoPC.

To determine whether lysoPC itself, or a potential metabolite that more closely resembles PAF was responsible for PAF receptor activation, lysoPC was acetylated with acetic anhydride and the product (1-acyl, 2-acetyl PC) was tested for ability to activate lysoPC. Table 1 shows that 1-acyl, 2-acetyl PC was more potent than lysoPC in activating PLD, suggesting that at least part of the activation of PLD might be mediated by a cellular metabolite of lysoPC such as 1-acyl, 2-acetyl PC. To rule out the possibility that trace amounts of PAF contaminating lysoPC preparations were responsible for these results, lysoPC was treated with PLA\(_2\), reisolated, and tested for ability to activate PLD. Table 1 shows that there was no significant effect of this digestion on PLD activation.

Table 1. Acetylation of lysoPC increases its potency for activating PLD

<table>
<thead>
<tr>
<th>Agonist</th>
<th>Concentration nmol/ml</th>
<th>Relative PLD Activity</th>
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<tr>
<td>PAF</td>
<td>0.625</td>
<td>1.29 ± 0.32</td>
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<tr>
<td></td>
<td>0.25</td>
<td>1.62 ± 0.23(^{ab})</td>
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<tr>
<td></td>
<td>1.0</td>
<td>1.73 ± 0.34(^{a})</td>
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<tr>
<td>Acetyl-PC</td>
<td>0.0625</td>
<td>1.07 ± 0.12</td>
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<tr>
<td></td>
<td>0.25</td>
<td>1.38 ± 0.25</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>1.74 ± 0.04(^{ab})</td>
</tr>
<tr>
<td>LysoPC</td>
<td>0.25</td>
<td>1.01 ± 0.22</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>1.44 ± 0.31</td>
</tr>
<tr>
<td></td>
<td>4.0</td>
<td>1.77 ± 0.35(^{a})</td>
</tr>
<tr>
<td>PLA(_2)-lysoPC</td>
<td>1.0</td>
<td>1.37 ± 0.47</td>
</tr>
<tr>
<td></td>
<td>4.0</td>
<td>1.51 ± 0.11(^{a})</td>
</tr>
</tbody>
</table>

Macrophages were labeled as in Fig. 1 and then stimulated with the indicated concentration of PAF, 1-acyl 2-acetyl PC, or lysoPC. As well, cells were also treated with lysoPC that had been digested with PLA\(_2\) and reisolated by TLC to verify that the effect of lysoPC was not due to contamination with PAF. Results are expressed as mean ± SD (n = 3) of the fold-stimulation relative to control incubations without agonist. Similar results were obtained in two replicate experiments. Significance was assessed by two-tailed t-test.

\(^{a}\)P < 0.05 versus control.
\(^{ab}\)P < 0.05 versus lysoPC at the same concentration.
The PAF receptor belongs to the G-protein coupled receptor superfamily (37); therefore, we investigated the possible involvement of GTP-binding proteins in lysoPC-induced PLD activation. We found that pretreatment of macrophages for 30 min with 1 μg/ml of pertussis toxin decreased the stimulation of PLD by lysoPC from 2.59 ± 0.09-fold to 1.80 ± 0.20-fold (mean ± SEM of four independent experiments, P < 0.05). By contrast, pretreatment with 1 μg/ml of choler toxin did not significantly alter lysoPC-induced PLD activation in the macrophages. These data suggest a role for an inhibitory G-protein (Gi) in the activation of PLD by lysoPC.

**DISCUSSION**

LysoPC plays an important role in the establishment and progression of atherosclerosis, and in the proinflammatory effects of secretory PLA2 (4, 14, 38). Although many pathophysiological effects of lysoPC have been described, the molecular mechanism(s) by which this natural lysophospholipid can alter cell function remains unclear. In intact cells, PLD activity has been shown to be controlled by both protein kinase C and tyrosine phosphorylation events (39). We observed that the stimulation of PLD by lysoPC in the macrophages is attenuated by down-regulating protein kinase C with prolonged incubations with PMA. These results are consistent with previous observations in human coronary endothelial cells where it was demonstrated that the stimulation of PLD by lysoPC is a protein kinase C-mediated effect (35). Protein kinase C inhibitors also prevented some other effects of lysoPC including the inhibition of agonist-induced phosphatidylinositol hydrolysis and calcium transients, and the lysoPC-induced arachidonate release in cultured endothelial cells (40, 41). The stimulation of PLD by lysoPC that we observed in the macrophages was not completely blocked by down-regulation of protein kinase C. Consequently, we tested to see whether tyrosine phosphorylation could also be involved in this process. We found that pretreatment with genistein decreased lysoPC-induced PLD activity, and pretreatment with orthovanadate, which stimulates PLD activity, and pretreatment with genistein decreased lysoPC-induced PLD activity, and pretreatment with orthovanadate, which is known to activate PLD through inhibition of tyrosine phosphatases (42), enhanced both basal and lysoPC-stimulated PLD activation. Therefore, we concluded that the activation of PLD by lysoPC involves both protein kinase C and tyrosine phosphorylation events. Also, PLD activation by lysoPC was specifically attenuated by the PAF receptor antagonist WEB-2086, suggesting that at least part of this effect is mediated through stimulation of the PAF receptor. Acetylation of lysoPC increased its potency in activating PLD, suggesting that a cellular metabolite of lysoPC such as 1-acyl 2-acetyl PC may account for at least part of this effect. The activation of PLD by PAF has been demonstrated in several different cell types including mouse peritoneal macrophages (37). Interestingly, although Ca²⁺ ions are not involved in the regulation of PLD activity (39, 43), it has been reported that lysoPC transduces Ca²⁺ signaling via the PAF receptor in murine peritoneal macrophages (36).

The PAF receptor is a G-protein coupled receptor, and we have demonstrated that pertussis toxin attenuates the stimulation of PLD by lysoPC, suggesting a role for the inhibitory G-protein in this process. Although G₁₃ has been implicated in the activation of adenyl cyclase by lysoPC (2), pretreatment of macrophages with cholera toxin (which acts on G₁₃) had no effect on lysoPC-stimulated PLD activity. It has also been shown that inhibitors of PI3-kinase such as LY294002 and wortmannin inhibit the stem cell factor-induced activation of PLD in porcine aorta endothelial cells (44) suggesting that PLD may be a downstream effector of PI3-kinase. However, pretreatment of macrophages with LY294002 did not inhibit the lysoPC-induced PLD activation (data not shown). These observations could be explained either by activation of PLD by lysoPC upstream of PI3-kinase or by independent activation of these two enzymes by lysoPC.

As noted above, lysoPC has been proposed as the mediator of several of the biologic effects of oxidized LDL. However, two lines of evidence in the present study suggest that components other than lysoPC are responsible for the activation of PLD by oxidized LDL. First, we found that the effect of lysoPC on PLD was at least partly mediated by protein kinase C, whereas there is no evidence of protein kinase C involvement in the stimulation of PLD by oxidized LDL. Second, the effects of optimum concentrations of lysoPC and oxidized LDL on PLD were nearly additive, suggesting different mechanisms of action for these two agonists on PLD activation (A. Gómez-Muñoz, J. S. Martens, and U. P. Steinbrecher, unpublished results).

In conclusion, we have shown in this report that lysoPC stimulates PLD activity in murine peritoneal macrophages by a mechanism involving both protein kinase C activation and tyrosine phosphorylation, and that at least part of this effect is mediated by PAF receptor activation. LysoPC is proinflammatory (2, 41) and our findings highlight one possible mechanism for this, i.e., activation of cytosolic PLA₂ by PA, and the subsequent release of arachidonic acid and lyso PAF from phospholipids.

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