Lysophosphatidylcholine Inhibits Surface Receptor–Mediated Intracellular Signals in Endothelial Cells by a Pathway Involving Protein Kinase C Activation

Kiyotaka Kugiyaıma, Masamichi Ohgushi, Seigo Sugiyama, Toyoaki Murohara, Kohji Fukunaga, Eishichi Miyamoto, and Hirofumi Yasue

Lysophosphatidylcholine (lysoPC) transferred from oxidatively modified low density lipoprotein (Ox-LDL) to the endothelial surface membrane has been shown to produce a selective unresponsiveness to cell surface receptor–regulated endothelium-dependent relaxation (EDR) in the rabbit aorta. To determine its mechanism we examined the effects of lysoPC on endothelial surface receptor–mediated transmembrane signals. Incubation for 1 minute with palmitoyl lysoPC (5–10 μM) decreased thrombin (Th, 2 units/ml)– or histamine (His, 0.1 mM)–stimulated inositol 1,4,5-trisphosphate (IP₃) production in primary cultures of human umbilical vein endothelial cells (HUVECs). LysoPC also decreased Th- or His-induced intracellular calcium ([Ca²⁺]ᵢ, fura 2) elevation. Pretreatment with protein kinase C (PKC) inhibitors staurosporine (100 nM) or H-7 (50 μM) prevented the inhibitory actions of lysoPC, but HA-1004 had no effect. Incubation for 5 minutes with phorbol 12-myristate 13-acetate (PMA, 100 nM) produced the inhibitory actions on the Th- or His-induced intracellular signals, which closely mimic those exhibited by lysoPC. However, the inhibitory effect of lysoPC was lost in cells that were depleted of PKC by pretreatment for 24 hours with 100 nM PMA. Furthermore, incubation of the cells for 1 minute with lysoPC stimulated PKC activity in the membrane fraction. In organ chamber experiments with porcine coronary artery rings, pretreatment with staurosporine (20 nM) attenuated lysoPC-induced impairment of EDR in response to Th. These results indicate that lysoPC, which accumulates in Ox-LDL and atherosclerotic arterial walls, inhibits the early transmembrane signaling pathway in endothelial cells, and PKC activation could at least partially be involved in the negative regulation by lysoPC. These intracellular actions of lysoPC may play a role in the mechanism of the lysoPC-induced impairment of EDR in response to cell surface receptor–mediated stimulations. (Circulation Research 1992;71:1422–1428)

KEY WORDS • lysophosphatidylcholine • inositol 1,4,5-trisphosphate • cytosolic free calcium • endothelial cell • protein kinase C

It has been recently demonstrated that lysophosphatidylcholine (lysoPC), which accumulates in oxidatively modified low density lipoprotein (Ox-LDL), produces a selective unresponsiveness to receptor-regulated endothelium-dependent arterial relaxation (EDR) that mimics the condition observed in the early-onset atherosclerotic arteries.1–4 LysoPC in Ox-LDL impairs the EDR that is mediated by stimulations of endothelial surface receptors, whereas lysoPC generally has no effect on EDR induced by calcium ionophores that bypass receptor-dependent membrane regulation.1,2

Protein kinase C (PKC) mediates intracellular signal transduction in a variety of cellular functions that include cell proliferation, morphological transformation, modulation of gene expression, and many other functions.5–7 PKC is also known to exert inhibitory actions on agonist-induced inositol 1,4,5-trisphosphate (IP₃) formation and subsequent intracellular calcium mobilization.5,8–10 The inhibitory actions of activated PKC on receptor agonist–mediated early signal transduction have been shown in many different types of cells, including endothelial cells.10 Furthermore, a variety of lipids, including lysophospholipids, are capable of activating PKC.11 Therefore, the present study addressed the possibility that PKC activation could be involved in the mechanism of lysoPC-induced unresponsiveness to the endothelial surface receptor–mediated stimulations.

Materials and Methods

Cell Culture

Primary cultures of human umbilical vein endothelial cells (HUVECs) were obtained by collagenase diges-
tion and grown to confluence on gelatin-coated dishes in medium 199 with 15% fetal calf serum (FCS), endothelial growth factor, and antibiotics. After confluence was reached, the medium was replaced with medium 199 that contained 5% FCS and antibiotics without growth factor for 48 hours before the experiments. Only primary cultures of HUVECs were used for the experiments. Confluent cultures of HUVECs showed the typical cobblestone morphology, and almost all of those cells contained factor VIII-related antigen as determined by the use of indirect immunofluorescence.

Measurement of Inositol 1,4,5-Trisphosphate Production

The monolayers of cultured HUVECs on 60-mm Petri dishes at a density of 1 x 10⁶ cells/cm² were washed three times with Hanks' balanced salt solution (HBSS) and incubated with serum-free medium 199 that contained 10 mM LiCl for 20 minutes. The cells were then stimulated by thrombin (Th, 2 units/ml) or histamine (His, 0.1 mM) after preincubation with phospholipids for the appropriate times or with phosphorol 12-myristate 13-acetate (PMA) for 5 minutes (as indicated in the text and figure legends). In some experiments the cells were pretreated for 5 minutes with PKC inhibitors before the incubation with phospholipids or PMA. In the experiment that examined the effects of lysoPC on the agonist-induced IP₃ production in PKC-downregulated cells, confluent monolayers of cells were incubated for 24 hours with 100 nM PMA in medium 199 that contained 2% FCS. Then the culture medium was replaced with serum-free medium 199 that contained 10 mM LiCl after washing three times with serum-free medium 199; afterwards, the cells were incubated for 20 minutes at 37°C. Cells were then stimulated by Th after preincubation for 1 minute with or without lysoPC.

Stimulation by Th or His was terminated with aspiration of the solution and addition of 2.0 ml ice-cold 15% trichloroacetic acid (TCA) and followed by incubation for 30 minutes on ice. The cells were then removed from the dishes by scraping. After centrifugation of the mixture, the supernatant was extracted with diethyl ether to remove the TCA. IP₃ levels were determined by the use of a protein binding assay system (Amersham Intl., code TRK 1000) after adjustment of the pH to 7.3 by the addition of Tris buffer. In a preliminary experiment that examined the time courses of Th- or His-induced IP₃ production, peak increases of IP₃ were obtained within 20 seconds of the addition of Th or His. On the basis of these time course observations, the stimulations were terminated at 20 seconds after the admixture of Th or His.

Measurement of Cytosolic Free Calcium Elevation

The fluorescent calcium indicator fura 2 was used to monitor changes in cytosolic free calcium concentration in suspensions of primary cultured HUVECs. For this experiment, primary cultures of HUVECs were briefly exposed to 0.01% trypsin-EDTA to detach the cells. Trypsin was inactivated by the addition of a trypsin inhibitor and culture medium with 15% FCS immediately after cell detachment. The cells were then centrifuged, washed once with HBSS, resuspended in Krebs-Ringer HEPES solution (KRH, pH 7.4; composition [mM]: NaCl 128, KCl 5, CaCl₂ 2.7, MgSO₄ 1.2, Na₂HPO₄ 1, glucose 10, and HEPES 20) and followed by exposure to 4 µM fura 2-AM for 30 minutes at 37°C. The cells were washed three times with HBSS, resuspended in KRH (3 x 10⁶ cells/ml), and then transferred into a quartz cuvette. The suspension was stirred continuously and maintained at 37°C. Fluorescence signals were monitored on a Hitachi F-3000 fluorescence spectrophotometer (Hitachi, Tokyo) at excitation and emission wavelengths of 340 and 505 nm, respectively. The cells were then stimulated by Th (2 units/ml) or His (0.1 mM) after preincubation with phospholipids for the appropriate times or with PMA for 5 minutes (indicated in the text and figure legends). In some experiments, the cells were pretreated for 5 minutes with PKC inhibitors before the incubation with phospholipids or PMA.

$$[Ca^{2+}] = K_d (F - F_{min}) / (F_{max} - F)$$

where F is the fluorescence intensity from cells, Fₘₐₓ is the maximum fluorescence from lysed cells by the addition of 0.1% Triton X-100, and Fₘᵢₙᵢ is the minimum fluorescence after Ca²⁺ in the buffer is chelated with 10 mM EGTA. The value of Kₐ was 224 nM at 37°C for fura 2 according to Grynkiewicz et al.

Organ Chamber Experiments

The left anterior descending coronary arteries were isolated from Yorkshire pigs within 10 minutes after death. The arteries were cleaned of adherent connective tissues and cut into ring segments of 3-mm length. The rings were suspended by stainless steel hooks in the organ chambers, which were filled with Krebs' buffer (composition [mM]: NaCl 118, KCl 4.7, NaH₂PO₄ 1.2, MgSO₄ 1.2, CaCl₂ 2.0, NaHCO₃ 25, and glucose 10). This solution was aerated with 15% O₂-5% CO₂-80% N₂ (P₀₂≈100 mm Hg) and maintained at 37°C. During this procedure care was taken not to injure the luminal surface. The rings were then stretched to an optimum basal tension of 4 g, and the isometric tension was monitored by means of a force transducer (Minebea, Tokyo) and a polygraph machine (Nihon Kohden, Tokyo). After equilibration for 120 minutes, the contractile response to 60 mM KCl was first obtained and then followed by repeated washing. The rings were subsequently pretreated with 10 µM indomethacin for 30 minutes followed by the incubation with 10 µM palmitoyl lysoPC or dipalmitoyl phosphatidylcholine for an additional 30 minutes in the presence or absence of staurosporine (20 nM). Indomethacin remained in the bath solution during the incubations. After the incubation the rings were washed repeatedly and then contracted with prostaglandin F₂₀ (PGF₂₀, 30 µM) and tested with increasing concentrations of various vasoconstrictors as indicated. Vasorelaxation was expressed as the maximum percent change in the PGF₂₀-induced contractions.

Treatment and Fractionation of the Cells for Protein Kinase C Assay

Confluent cultures of HUVECs were quickly frozen by liquid nitrogen after washing three times with HBSS. Cells were removed by scraping and homogenated and resuspended in the HEPES buffer with protease inhibitors. Then the suspension was centrifuged (100,000 g for...
60 minutes) for separation into cytosolic and particulate fractions. The particulate fraction was extracted with 0.1% Triton X-100. In the experiments that examined downregulation of PKC, confluent cultures of HUVECs were pretreated for 24 hours with 100 nM PMA. Thereafter, the cells were quickly frozen after washing and fractionized as described above. In the experiments that examined the effects of lysoPC or PMA on PKC activity in intact cells, confluent cultures of the cells were washed three times with HBSS, and the incubation medium was replaced with serum-free medium 199. The cells were then incubated with various concentrations of lysoPC or PMA for 0.5, 1, 5, 10, or 30 minutes. After the incubation the medium was aspirated, and the cells were quickly frozen and fractionized as described above.

Assay of Protein Kinase C Activity

PKC activity in samples was assayed by measuring the incorporation of [32P] into the synthetic peptide PK substrate from [γ-32P]ATP. The standard reaction mixture for the assay of PKC activity contained 50 mM HEPES (pH 7.5), 10 mM magnesium acetate, 500 μM CaCl2, 0.1 mM [γ-32P]ATP, 3 μM mastoparan, 5 μM of a synthetic peptide inhibitor of cAMP kinase (PKI-tide), and 40 μM PKC substrate in a volume of 25 μL. The reactions were initiated by the addition of 5 μL of the samples from HUVECs to the reaction mixture. After incubation for 8 minutes at 30°C, 15-μL aliquots were spotted on phosphocellulose paper squares, and the radioactivity was counted. The standard reaction mixture that included 5 μg/ml diolein and 50 μg/ml phosphatidylserine was used when detecting phosphatidylserine-dependent phosphorylation of the substrate was assessed for the PKC activity in the cytosolic and soluble particulate fractions from the cells pretreated with lysoPC or PMA. The standard reaction mixture that included 50 μg/ml phosphatidylserine and varying concentrations of lysoPC or PMA was used for the examination of the direct effects of lysoPC or PMA on the activity of PKC purified from the cytosolic fraction of the untreated cells by means of a diethylaminoethyl-Sepharose column. Protein concentrations were determined by the method of Bradford, with bovine serum albumin as the standard.

Materials

All reagents for the cell culture were obtained from GIBCO. Radioactive materials came from Amersham Int'l., Tokyo; staurosporine from Kyowa Hakko Co. Ltd., Tokyo; H-7 and HA-1004 from Seikagaku Kagyo Co. Ltd., Tokyo; endothelial growth factor (bovine pituitary) from Collaborative Research, and PKC substrate from Bachem, Torrance, Calif. Other chemicals were obtained from Sigma Chemical Co., St. Louis, Mo. Phospholipids were used after sonication in HBSS. Staurosporine, fura-2-AM, PMA, or A23187 was dissolved in dimethyl sulfoxide (DMSO). Final concentrations of DMSO were less than 0.1% in the solution. Indomethacin solution was prepared in an equimolar (1-mM) concentration of Na2CO3.

Statistical Analysis

All values were expressed as mean±SEM. Statistical evaluation of the data was performed by Student’s t test for paired or unpaired observations. When more than two groups were compared analysis of variance was used. A value of p<0.05 was considered significant.

Results

Measurements of Inositol 1,4,5-Trisphosphate Formation and [Ca2+]i Elevation

As shown in Figures 1 and 2, preincubation for 1 minute with submicellar concentration of palmitoyl lysoPC decreased Th- or His-induced IP3 formation in monolayers of primary cultures of HUVECs in a dose-

![Figure 1](http://circres.ahajournals.org/content/circres/suppl/1992/6/1424/supplrendo.png)

**Figure 1.** Line graph showing effects of lysophosphatidylcholine (palmitoyl, lysoPC) or phosphatidylcholine (dipalmitoyl, PC) on thrombin (Th)-induced inositol 1,4,5-trisphosphate (IP3) production in human umbilical vein endothelial cells (HUVECs). Confluent monolayers of HUVECs were incubated for 20 minutes with serum-free medium-199 that contained 10 mM LiCl and then exposed to lysoPC or PC for 1 minute and followed by stimulation with Th (2 units/ml). The stimulation was terminated by aspiration of the solution and the addition of ice-cold trichloroacetic acid at 20 seconds after the initiation of Th-induced stimulation. Exposure to lysoPC significantly suppressed Th-induced IP3 production in a dose-dependent manner, whereas PC had no effect. *p<0.01 vs. PC (n=9–16).

![Figure 2](http://circres.ahajournals.org/content/circres/suppl/1992/6/1424/supplrendo.png)

**Figure 2.** Bar graph showing effects of lysophosphatidylcholine (lysoPC) with or without protein kinase C (PKC) inhibitors on thrombin (Th)- or histamine (His)-induced inositol 1,4,5-trisphosphate (IP3) production in human umbilical vein endothelial cells (HUVECs). Monolayers of HUVECs were pretreated for 5 minutes with staurosporine (STS, 100 nM), H7 (50 μM), or HA-1004 (50 μM) and followed by the addition of lysoPC (5 μM). At 1 minute after the addition of lysoPC, cells were stimulated by Th (2 units/ml) or His (0.1 mM). In cells pretreated for 5 minutes with STS or H7 but not HA-1004, the inhibitory effects of lysoPC on Th- or His-induced IP3 production were attenuated. *p<0.01 vs. lysoPC alone (n=10–16), +p<0.01 vs. control (no pretreatment).
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lysoPC also showed inhibitory actions in n=1 P3 formation and (STS, 100 nM), H7 (50

on thrombin

dependent manner. Preincubation for 1 minute with lysoPC (1–5 μM) also inhibited Th- or His-induced [Ca2+]i, elevation in suspensions of HUVECs, as shown in Figures 3 and 4. Incubation for 1 minute with stearoyl lysoPC also showed inhibitory actions on Th-induced IP3, formation and [Ca2+]i, elevation (IP3: control [no pretreatment, n=16], 51±3 versus 5 μM stearoyl lysoPC [n=6], 36±5 pmol/10^6 cells, p<0.01; peak [Ca2+]i: control [n=12], 201±13 versus 2 μM stearoyl lysoPC [n=9], 136±10 nM, p<0.01), whereas dipalmitoyl phosphatidylcholine had no significant effect on Th-induced IP3 production and [Ca2+]i, elevation (IP3: 49±3 pmol/10^6 cells [10 μM phosphatidylcholine, n=7]; peak [Ca2+]i: 196±11 nM [5 μM phosphatidylcholine, n=6]). In the experiment that examined the effects of various preincubation times with lysoPC on the Th-induced IP3 production and [Ca2+]i, elevation, the maximum inhibitions were obtained as early as 1 minute after the addition of lysoPC into the incubation mixture, and the inhibitions were sustained with the same magnitude for up to 30 minutes (the longest incubation time tested). Basal levels of IP3 in monolayers of HUVECs were not affected by the incubation with up to 10 μM palmitoyl lysoPC alone (control [no treatment, n=5], 4.2±0.3 versus 10 μM lysoPC [n=6], 4.1±0.2 pmol/10^6 cells, p=NS). However, the resting levels of [Ca2+]i in suspensions of HUVECs were increased by the incubation with lysoPC alone at higher concentrations than 5 μM (control [no treatment, n=8], 96±5 versus 5 μM lysoPC [n=8], 120±6 nM, p<0.01).

To evaluate the role of PKC activation in the inhibitory effects of lysoPC on agonist-induced IP3 production and [Ca2+]i, elevation, the effects of the PKC inhibitors staurosporine and H-7 were tested. As shown in Figures 2–4, pretreatment with staurosporine (100 nM) or H-7 (50 μM) significantly attenuated the inhibitory effects of lysoPC on Th- or His-induced intracellular signals. Neither staurosporine nor H-7 is a specific PKC inhibitor, but it also inhibits A and G kinases at higher concentrations.19,20 However, HA-1004 (50 μM), which has strong inhibitory actions on A and G kinases but only a weak action on PKC,20 did not attenuate the inhibitory actions of lysoPC on the agonist-induced intracellular signals (Figures 2 and 4). Preincubation for 5 minutes with PMA (100 nM), a specific PKC activator, inhibited Th- or His-induced IP3 formation and [Ca2+]i, elevation in HUVECs, and staurosporine or H-7 but not HA-1004 attenuated the inhibitory effects of PMA, as shown in Figure 5. However, preincubation for 1 minute with lysoPC had no significant influence on Th-induced IP3 production in the cells pretreated for 24 hours with 100 nM PMA (incubation with lysoPC [5 μM, n=8], 38±3 versus that without lysoPC [n=8], 41±2 pmol/10^6 cells, p=NS). The pretreatment with PKC inhibitors alone at the respective concentrations used in this study did not influence Th-induced IP3 production and [Ca2+]i, elevation (IP3: staurosporine, 52±3; H-7, 50±2; and HA-1004, 49±4 pmol/10^6 cells; n=5–8, p=NS compared with control [no pretreatment, n=16]; peak

Figure 3. Charts showing thrombin (Th)-induced [Ca2+]i, elevations in human umbilical vein endothelial cells (HUVECs). [Ca2+]i, was monitored with fura 2 fluorescence. Panel A: Th (2 units/ml) that transiently elevates [Ca2+]i, in HUVECs. Panel B: Effects of the exposure to lysophosphatidylcholine (lysoPC) on Th-induced [Ca2+]i, elevation. Lysophosphatidylcholine (lysoPC) (2 μM) was admixed into the cuvette and incubated for 1 minute before Th (2 units/ml) stimulation. Exposure to lysoPC inhibits Th-mediated [Ca2+]i, elevation without altering a resting level of [Ca2+]i, Panel C: The effect of pretreatment for 5 minutes with staurosporine (100 nM) on lysoPC (2 μM)-induced inhibition of [Ca2+]i, elevation in response to Th (2 units/ml). Staurosporine attenuates the inhibitory effect of lysoPC on Th-induced [Ca2+]i, elevation.

Figure 4. Bar graph showing effects of lysophosphatidylcholine (lysoPC) with or without protein kinase C (PKC) inhibitors on thrombin (Th)- or histamine (His)-induced [Ca2+]i, elevation in human umbilical vein endothelial cells (HUVECs). Suspensions of HUVECs were pretreated for 5 minutes with staurosporine (STS, 100 nM), H7 (50 μM), or HA-1004 (50 μM) in a quartz cuvette, followed by the addition of 2 μM lysoPC that is a subthreshold concentration for elevating the resting level of [Ca2+]i. Cells were then stimulated with Th (2 units/ml) or His (0.1 mM) at 1 minute after the addition of lysoPC. The inhibitory effects of lysoPC on Th- or His-induced [Ca2+]i, elevation were attenuated in cells pretreated with STS or H7 but not with HA-1004. *p<0.01 versus lysoPC alone (n=9–12). +p<0.01 versus control (no pretreatment).
TABLE 1. Arterial Relaxations in Response to Various Vasodilators

<table>
<thead>
<tr>
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<th>Thrombin (n=18)</th>
<th>A23187</th>
<th>Nitroglycerin (n=9)</th>
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<tbody>
<tr>
<td>Time control</td>
<td>98±3</td>
<td>96±7</td>
<td>108±8</td>
</tr>
<tr>
<td>LysoPC</td>
<td>43±5*</td>
<td>92±5</td>
<td>102±6</td>
</tr>
<tr>
<td>LysoPC and staurosporine</td>
<td>76±8*</td>
<td>94±9</td>
<td>106±7</td>
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Isolated rings from porcine coronary arteries were mounted for the monitoring of isometric tension and equilibrated in oxygenated Krebs buffer at 37°C. The coronary rings were then incubated with lysophosphatidylcholine (lysoPC) (10 μM) for 30 minutes in the presence or absence of staurosporine (20 nM). After the incubation the rings were washed repeatedly and contracted with prostaglandin F$_{2α}$ (PGF$_{2α}$) (30 μM) and then relaxed with various vasodilators as indicated. Relaxations are expressed as the maximum percent changes in the PGF$_{2α}$-induced contractions that were not influenced by the incubations. The incubation with lysoPC inhibited arteriolar relaxation in response to thrombin but not to A23187 and nitroglycerin. Coincubation with staurosporine attenuated the inhibitory effect of lysoPC on thrombin-induced arterial relaxation.

$p<0.01$ compared with time control, $t$ $p<0.01$ compared with lysoPC.

**response to Th (maximal relaxation; time control [n=18], 98±3% of precontraction versus staurosporine, [n=9] 96±9%; $p$=NS). Incubation with DMSO alone, as a drug vehicle of staurosporine, at a concentration of less than 0.1% in the bath solution did not influence the lysoPC-induced inhibition of vasorelaxation in response to Th (data not shown).**

**Protein Kinase C Assay**

As shown in Figure 6, the incubation of HUVEC monolayers in culture dishes with lysoPC (palmitoyl, 1 μM) caused a significant increase in diolene and phosphatidylserine-dependent PKC activity in the particulate fraction of the cells, with a maximum (by a 32% increase from the baseline value) at 1 minute after the addition of lysoPC into the culture medium, and the activity subsequently decreased to the baseline value. The PKC activity in the cytosolic fraction slightly decreased during the increase of the particulate PKC activity (a 5–13% decrease from the baseline value). In comparison, the incubation of the cells with PMA (100 nM) also caused an increase in PKC activity in the particulate fraction, with a maximum (a 67% increase from the baseline value) at 5 minutes after the addition of PMA into the medium, and at the same time the PKC activity in the cytosolic fraction decreased by 31% from the baseline value at 5 minutes.

Furthermore, lysoPC (palmitoyl, 1–5 μM) directly stimulated the activity of the PKC purified from HUVECs in the presence of phosphatidylserine (PKC activity; presence of 1 μM lysoPC [n=8], 826±48 versus absence of lysoPC [n=8], 634±52 pmol/mg protein per minute, $p<0.01$), whereas lysoPC (up to 10 μM) did not significantly stimulate the PKC activity in the absence of phosphatidylserine (presence of 1 μM lysoPC [n=8], 170±26 versus absence of lysoPC [n=8], 146±32 pmol/mg protein per minute, $p$=NS). The maximum lysoPC (1 μM)-stimulated activity of the purified PKC in the presence of phosphatidylserine was 28% of the maximum PMA (100 nM)-stimulated activity.

**[Ca$^{2+}$]:** staurosporine, 206±9; H-7, 203±11; HA-1004, 202±8 nM; n=7–9, $p$=NS compared with control [no pretreatment, n=12]. Basal levels of IP$_3$ and [Ca$^{2+}$] were not significantly changed by the pretreatment for 5 minutes with staurosporine, H-7, HA-1004, or PMA alone at the respective concentrations used in this study (data not shown). DMSO (as a drug vehicle) at a concentration of less than 0.1% in the final solution did not affect the inhibitory effects of lysoPC on Th-induced IP$_3$ production and [Ca$^{2+}$] elevation (data not shown).

**Organ Chamber Experiment**

After incubation with lysoPC (10 μM), but not phosphatidylcholine, relaxation of a precontracted artery in response to Th was inhibited, whereas relaxation in response to calcium ionophore A23187 was completely preserved, as shown in Table 1. Nitroglycerin, an endothelium-independent vasodilator, elicited complete relaxation after incubation with lysoPC or PMA. Coincubation with staurosporine (20 nM) attenuated the inhibitory effect of lysoPC on Th-induced vasorelaxation (Table 1). Contractions elicited by PGF$_{2α}$ were not significantly influenced by the incubations (time control, 89±11% of 60 mM KCl-induced contraction; lysoPC, 92±8%; lysoPC plus staurosporine, 86±10%; n=12–18, $p$=NS). Incubation for 30 minutes with staurosporine alone did not significantly affect vasorelaxation in response to Th (maximal relaxation; time control [n=18], 98±3% of precontraction versus staurosporine, [n=9] 96±9%; $p$=NS). Incubation with DMSO alone, as a drug vehicle of staurosporine, at a concentration of less than 0.1% in the bath solution did not influence the lysoPC-induced inhibition of vasorelaxation in response to Th (data not shown).

**FIGURE 5. Bar graphs showing effects of phorbol 12-myristate 13-acetate (PMA) with or without protein kinase C (PKC) inhibitors on thrombin (Th)-induced inositol 1,4,5-trisphosphate (IP$_3$) production and [Ca$^{2+}$] elevation in human umbilical vein endothelial cells (HUVECs).** HUVECs were pretreated for 5 minutes with staurosporine (STS, 100 nM), H-7 (50 μM), or HA-1004 (50 μM) followed by the addition of PMA (100 nM) and then incubated for an additional 5 minutes before Th (2 units/ml) stimulation. The incubation for 5 minutes with PMA inhibited Th-induced IP$_3$ production and [Ca$^{2+}$] elevation; however, the inhibitory effects of PMA were attenuated in cells pretreated with STS or H-7 but not HA-1004. *$p<0.01$ vs. PMA alone (n=9–13), +$p<0.01$ vs. control (no treatment).
This leads to the production of IP$_3$, and 1,2-diaclyglycerol. IP$_3$ may serve as a mediator of [Ca$^{2+}$], release from an intracellular store. Elevated [Ca$^{2+}$] leads to the generation of endothelium-derived relaxing factor.$^{24}$ Concomitant production of 1,2-diaclyglycerol stimulates PKC, which in turn inhibits continued or repeated activation of this transmembrane signaling system$^{5,8,9,22,25}$ either at the level of the receptor, G-proteins, or phospholipase C. The present study demonstrated that lysoPC inhibited Th- or His-induced IP$_3$ production and [Ca$^{2+}$], elevation in endothelial cells. These inhibitory effects of lysoPC on agonist-induced responses were attenuated by the potent PKC inhibitors staurosporine and H-7. Furthermore, the incubation with PMA for 5 minutes produced the inhibitory effects on the agonist-induced intracellular signals, which closely resembled those exhibited by lysoPC. However, the inhibitory effect of lysoPC was lost in the cells that were depleted of PKC by long-term preincubation with PMA. Furthermore, the present study demonstrated that the incubation of these cells with lysoPC activated PKC in the membrane fraction. These results obtained from the intracellular experiments indicate that lysoPC inhibits the early transmembrane signaling pathway in endothelial cells, and PKC activation could be partially involved in the negative regulation by lysoPC.

Present intracellular responses to lysoPC are compatible with the results obtained from the organ chamber experiments. It has been demonstrated in previous reports,$^{1,2}$ in which rabbit aortas were used, that lysoPC transferred from Ox-LDL to the endothelial surface membrane produced impairment of endothelial surface receptor-mediated EDR, which closely resembled those observed in early-onset atherosclerotic arteries. In the present study, in which we used porcine coronary arteries, we demonstrated that lysoPC impaired EDR in response to thrombin but not to A23187. Furthermore, staurosporine prevented the lysoPC-induced impairment of EDR. Thus, these results obtained from organ chamber experiments also indicate that PKC activation may be at least partially involved in the underlying mechanism of lysoPC-induced inhibition of EDR in response to endothelial surface receptor-mediated stimulations. Activated PKC has been shown to exert a negative feedback control on surface receptor-coupled IP$_3$ formation and subsequent [Ca$^{2+}$] mobilization in response to numerous agonists in many different types of cells.$^{5,8-10}$ This negative regulation by activated PKC may be responsible for the lysoPC-induced inhibitions observed in the present cellular and organ chamber experiments.

LysoPC is generated in several pathophysiological situations.$^{26-30}$ Concentrations of lysoPC are greatly increased in atherosclerotic arterial walls.$^{26,27}$ There are at least two sources of lysoPC found in atherosclerotic arterial walls. One source is Ox-LDL, which accumulates in atherosclerotic arterial walls.$^{31}$ LDL oxidation is associated with the hydrolysis of PC to lysoPC by the action of an intrinsic LDL-associated phospholipase A$_2$.$^{29}$ It has been previously demonstrated that the inhibitory activity of Ox-LDL on EDR is present in its lipid component, and most of it is attributable to lysoPC in Ox-LDL.$^{1,2}$ Another source of arterial lysoPC is the plasma in which the lecithin:cholesterol acyltransferase enzyme catalyzes lysoPC formation.$^{26,27}$ Lecithin:cho-

**Figure 6.** Line graph showing time course of protein kinase C (PKC) activity in the cytosolic and particulate fractions of intact human umbilical vein endothelial cells (HUVECs) treated by lysophosphatidylcholine (lysoPC) or phorbol 12-myristate 13-acetate (PMA). Confluent monolayers of HUVECs were washed three times with Hanks' balanced salt solution (HBSS), and the culture medium was replaced with serum-free medium 199. The cells were then incubated with lysoPC (1 μM) or PMA (100 nM) for 0.5, 5, 10, or 30 minutes. After the incubation the medium was aspirated and the cells quickly frozen. PKC activity in the cytosolic and particulate fractions from the cell homogenized was assayed by measuring diolen and phosphatidylserine-dependent phosphorylation of the substrate, as described in "Materials and Methods." Results are mean±SEM of the two separate experiments, and each one consisted of triplicate determinations.

**Discussion**

According to current concepts,$^{21-22}$ the binding of an agonist to a cell surface receptor, which is coupled by a G-protein to a specific phospholipase C, leads to the hydrolysis of phosphatidylinositol 4,5-bisphosphate.

**Table 2.** PKC Activity in HUVECs After Prolonged PMA Treatment

<table>
<thead>
<tr>
<th>PKC Activity in HUVECs</th>
<th>Control</th>
<th>PMA</th>
<th>p</th>
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<tr>
<td>Cytosol</td>
<td>605±22</td>
<td>86±8</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Particulate</td>
<td>96±6</td>
<td>12±4</td>
<td>&lt;0.01</td>
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</table>

PKC, protein kinase C; HUVECs, human umbilical vein endothelial cells; PMA, phorbol 12-myristate 13-acetate.

HUVECs incubated for 24 hours with medium 199 contained 2% fetal calf serum in the presence or absence of PMA (100 nM). Then cells were homogenized and centrifuged for separation into cytosolic and particulate fractions. PKC activity was directly assayed in both of the cytosolic and particulate fractions from homogenates of HUVECs. Diolein and phosphatidylserine-dependent phosphorylation of the substrate was assayed under the standard condition, except for the inclusion of diolein and phosphatidylserine. Results (pmol/mg protein per minute) are mean±SEM of the three separate experiments, and each experiment consisted of quadruplicate determinations.
lersterol acyltransferase activity has been shown to increase with hypercholesterolemia.\textsuperscript{26,27} The abundant lysoPC in atherosclerotic arteries is transferable to accessible membranous or macromolecular acceptors through the aqueous phase.\textsuperscript{32} In fact, previous reports\textsuperscript{1,2} have shown that lysoPC in Ox-LDL is transferred and incorporated into the endothelial surface membrane in an apoprotein-independent manner. Transferred lysoPC is slowly translocated to the inner plasma membrane and then partially metabolized.\textsuperscript{39} During transmembrane movement lysoPC could access and directly activate PKC in the membrane fraction, as shown in present experiments for PKC assay. However, the precise mechanism of lysoPC-induced PKC activation remains to be determined. Isozymes of lysoPC-inducible PKC in HUVECs, its endogenous substrates in HUVECs, and its activation process in the in vivo endothelium are now under investigation in our laboratory. It cannot be excluded that some metabolites of lysoPC may contribute to the lysoPC-induced inhibitors. However, our preliminary experiment, in which we used \textsuperscript{3H}-radiolabeled lysoPC, showed that a major metabolite of the incorporated lysoPC in HUVECs was phosphatidylcholine, which has been shown to be inactive in the endothelial responses to thrombin. At this moment we do not have any evidence that lysoPC may be metabolized to active substances, which could play a major role in the lysoPC-induced inhibitions.

PKC also plays crucial roles in the signal transduction that involves cellular proliferation and modulation of gene expression.\textsuperscript{5,7,9} Therefore, lysoPC in atherosclerotic arterial walls could activate PKC in a variety of cells in the artery, which may play a role in the mechanisms of their mitogenic changes and functional alterations in atherosclerotic arteries.

In conclusion, lysoPC, which is abundant in atherosclerotic arterial walls, inhibits surface receptor–mediated intracellular signals in human endothelial cells, and PKC activation could be at least partially involved in the negative regulation by lysoPC. These intracellular actions of lysoPC may play a role in the mechanism of lysoPC-induced unresponsiveness to cell surface receptor–regulated EDR, which closely mimics that observed in early-onset atherosclerotic arteries.

References

Lysophosphatidylcholine inhibits surface receptor-mediated intracellular signals in endothelial cells by a pathway involving protein kinase C activation.

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