Lysophosphatidylcholine Transcriptionally Induces Growth Factor Gene Expression in Cultured Human Endothelial Cells

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Abstract

Lysophosphatidylcholine (lyso-PC) is a major phospholipid component of atherogenic lipoproteins (e.g., oxidized LDL and β-VLDL) and also can be generated through the action of leukocyte-secreted phospholipase A_2 at sites of inflammation. We have previously reported that lyso-PC can activate cultured endothelia, resulting in the selective upregulation of adhesion molecules, such as vascular cell adhesion molecule-1 and intercellular adhesion molecule-1. In this study, we have found that lyso-PC increased steady state mRNA levels for two smooth muscle/fibroblast-directed growth factors, the A and B chains of PDGF and heparin-binding EGF-like protein (HB-EGF), in cultured human endothelial cells. Lyso-PC did not upregulate the expression of certain other inducible endothelial genes, including E-selectin, IL-8, or monocyte chemoattractant protein-1 in the same cells, in contrast to the coordinate pattern of activation typically observed with other stimuli, such as TNFα, bacterial endotoxin, or PMA. Nuclear runoff assays documented an increased transcriptional rate for the HB-EGF gene in lyso-PC–treated cells. Northern blot analyses, after actinomycin D treatment, further indicated that the increased amounts of mRNA for HB-EGF, PDGF A and B chains, and intercellular adhesion molecule-1 were not dependent upon message stabilization. We conclude that lyso-PC can induce growth factor gene expression in cultured endothelial cells and thus may contribute to the migration and proliferation of smooth muscle cells and fibroblasts in various response-to-injury settings in vivo. (*J. Clin. Invest.* 1994. 93:907–911.) Key words: atherosclerosis • inflammation • wound healing • smooth muscle • lipoproteins

Introduction

Nonadaptive alterations of vascular endothelial functions in response to various pathophysiologic stimuli have been implicated in the pathogenesis of atherosclerosis and other chronic inflammatory disease processes. These changes, which collectively have been termed endothelial dysfunction, include modulated expression of growth factors, hemostatic components, cytokines, chemoattractants, and endothelial leukocyte adhesion molecules (1–4). A growing body of evidence indicates that lysophosphatidylcholine (lyso-PC),[1] a polar phospholipid product potentially generated by secretory phospholipase A_2 in inflammatory lesions (5) and a prominent component of atherogenic lipoproteins such as oxidatively modified LDL and β-migrating VLDL (β-VLDL) (6, 7), can modulate gene expression in a variety of cell types including vascular endothelial cells (8, 9). We have recently shown that lyso-PC at nontoxic concentrations can selectively upregulate the expression of certain inducible endothelial leukocyte adhesion molecules, in particular vascular adhesion molecule-1 (VCAM-1), and intercellular adhesion molecule-1 (ICAM-1), in cultured human and rabbit endothelial cells, thus suggesting a potential role in leukocyte recruitment (8). In this study, we have examined the effect of lyso-PC on certain other endothelial-expressed genes relevant to atherosclerosis and inflammation, including growth factors and cytokines. We now report that the genes encoding PDGF A and B chains and heparin-binding EGF-like protein (HB-EGF), both potent vascular smooth muscle and fibroblast mitogens (4, 10, 11), appear to be transcriptionally induced by lyso-PC. Taken together, these observations suggest an important role for lyso-PC in modulating endothelial activation and dysfunction in atherosclerosis, inflammation, and wound healing.

Methods

Cells. Cultured human umbilical vein endothelial cells (HUVEC) were isolated and grown in medium 199 with 20% FBS (Gibco, Grand Island, NY), supplemented with 25 U/ml heparin (porcine intestinal; Sigma Immunochemicals, St. Louis, MO) and 12.5 μg/ml endothelial cell growth supplement (Collaborative Research Inc., Lexington, MA) as described previously (8). All experiments were performed in medium 199 containing 5% FBS, without heparin or endothelial cell growth supplement. Only second passage cells were used.

Reagents. Lyso-PC (palmitoyl, C16:0) was purchased from Avanti Polar Lipids (Birmingham, AL). Recombinant human TNFα was obtained from Biogen (Cambridge, MA), and actinomycin D was ob-

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1. *Abbreviations used in this paper:* HB-EGF, heparin-binding EGF-like protein; HUVEC, human umbilical vein endothelial cells; ICAM-1, intercellular adhesion molecule-1; lyso-PC, lysophosphatidylcholine; MCP-1, monocyte chemoattractant protein-1; VCAM-1, vascular cell adhesion molecule-1.

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tained from Calbiochem-Novabiochem Corp. (La Jolla, CA). All other reagents were from the previously identified sources (8).

Northern blot analysis. Total cellular RNA, isolated from HUVEC by acid-guanidinium phenol-chloroform method, was electrophoresed through 1% agarose gels containing formaldehyde and was transferred to nitrocellulose membranes. Northern blots were hybridized with cDNA probes for human ICAM-1, HB-EGF, and PDGF A and B chains labeled with [^{32}P]dCTP using random hexanucleotide primers. Some blots were also hybridized with human E-selectin, monocyte chemoattractant protein-1 (MCP-1)/JE, and IL-8 cDNA. A 1.3-kb XhoI fragment of human ICAM-1 (12), a 0.8-kb EcoRI/KpnI fragment of human HB-EGF (10), a SacII/EcoRI fragment of human PDGF A chain (13), a PstI/EcoRI fragment of human PDGF B chain (14), a 2.7-kb XbaI fragment of human E-selectin (15), a 0.8-kb XhoI fragment of human MCP-1/JE (16), and a 0.5-kb EcoRI fragment of human IL-8 cDNA (17) were used. Human HB-EGF cDNA was a generous gift from Dr. Michael Klagsbrun (Children's Hospital, Boston, MA), and human PDGF A and B chains, and E-selectin cDNA were provided by Dr. Tucker Collins (Vascular Research Division, Brigham and Women's Hospital). Human MCP-1/JE cDNA was kindly provided by Dr. Barry Rollins (Dana Farber Cancer Institute, Boston, MA), and human IL-8 cDNA was provided by Dr. Joffrey Baker (Genentech Inc., South San Francisco, CA).

Nuclear runoff assay. Nuclear runoff assay was performed as described previously (18). In brief, nuclei from 5–8 × 10^7 cells were extracted by NP-40 lysis followed by centrifugation. Nascent transcription in vitro was performed with [^{32}P]UTP (Dupont/New England Nuclear, Boston, MA) and other unlabeled nucleotides (Pharmacia LKB Biotechnology Inc., Piscataway, NJ) at 30°C for 30 min. Transcribed RNA was isolated by guanidinium thiocyanate–cesium chloride method, followed by denaturation with sodium hydroxide. Target cDNAs (5 μg in plasmid form) were denatured and immobilized onto nitrocellulose membranes using slot blot apparatus (Schleicher & Shuell, Inc., Keene, NH), and were hybridized with transcribed RNAs containing equal amounts of radioactivity (2–3 × 10^6 cpm) at 65°C for 24 h.

Results

Lyso-PC selectively upregulates gene expression of HB-EGF, and PDGF A and B chains. To examine the effect of lyso-PC on growth factor gene expression in HUVEC, we performed Northern blot analysis. We included 5% FBS in the culture medium to preserve the viability of the cultured cells during several hours of incubation with lyso-PC. Under these conditions, we did not detect any cytotoxicity up to at least 100 μM lyso-PC, as previously described (8). As seen in Fig. 1, the addition of 50 μM lyso-PC to 5% FBS containing media significantly increased (2.4-fold by densitometry) the amount of HB-EGF mRNA after a 1-h incubation. This effect of lyso-PC on HB-EGF mRNA upregulation was dose dependent up to 100 μM (7.5-fold increase at 100 μM) and was more remarkable than 1 μg/ml of LPS (2.1-fold increase), a maximally effective concentration of Gram-negative bacterial endotoxin. E-selectin mRNA was not detectable after the same treatments with lyso-PC (25–100 μM), although 1 μg/ml of LPS dramatically increased the amount of E-selectin mRNA, as previously reported (15) (Fig. 1).

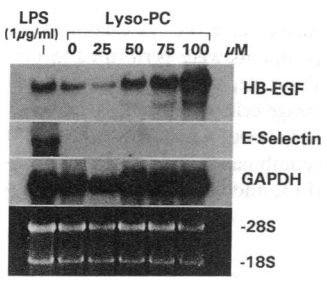

Figure 1. Dose–response of lyso-PC effect on HB-EGF mRNA levels. Confluent monolayers of HUVEC were treated with the indicated concentrations of lyso-PC or 1 μg/ml LPS in medium 199 with 5% FBS for 1 h. Total cellular RNA was isolated, and Northern blot analysis was performed as described in Methods. Blots were rehybridized with E-selectin and GAPDH cDNAs for comparison. Ethidium bromide staining of the gel also is illustrated. Each lane contained 15 μg of total RNA. One of two similar experiments is shown.

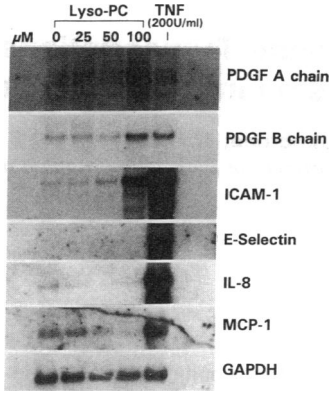

Figure 2. Dose–response of lyso-PC effect on PDGF A and B chains, ICAM-1, E-selectin, IL-8, MCP-1, and mRNA levels. Confluent monolayers of HUVEC were treated with the indicated concentrations of lyso-PC or 200 U/ml TNFα for 4 h. Total cellular RNA was isolated, and Northern blot analysis was performed as described in Methods. Blot was rehybridized with cDNA probes for each of the genes indicated. Each lane contained 15 μg of total RNA. Comparable data were obtained in several experiments.

We also examined the effect of lyso-PC on the genes encoding the A and B chains of PDGF, another potent smooth muscle mitogen. Lyso-PC treatment (100 μM) for 4 h significantly enhanced the levels of both PDGF A chain (2.5-fold) and PDGF B chain (3.8-fold) mRNAs. ICAM-1 mRNA was also similarly upregulated (9.4-fold), as reported previously by us (8); however neither E-selectin, IL-8, nor MCP-1 were induced by lyso-PC. The concentration threshold of the lyso-PC effect on PDGF A and B chains, and ICAM-1 expression appeared to be between 50 and 100 μM in the presence of 5% FBS (Fig. 2).

Lyso-PC upregulates HB-EGF, PDGF A and B chains, and ICAM-1 gene expression with different kinetics. To further characterize the pattern of endothelial activation by lyso-PC, we examined the time courses of upregulation of these lyso-PC–responsive genes (Fig. 3). Northern analysis clearly showed increased (10.2-fold) amounts of HB-EGF mRNA detectable as early as 1 h and sustained for at least 4 h. In contrast, mRNAs

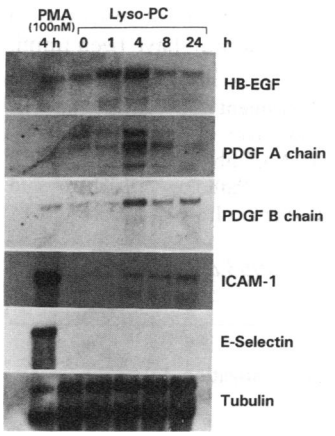

Figure 3. Time course of endothelial gene induction by lyso-PC. Confluent monolayers of HUVEC were treated with 100 μM lyso-PC (with 5% FBS) for the indicated times or with 100 μM PMA for 4 h. Northern blot analysis was performed as described in Methods. Blot was rehybridized with the various cDNAs indicated. Each lane contained 20 μg of total RNA. One of two similar experiments is shown.

for PDGF A and B chains were not significantly altered after 1 h, became clearly increased (8.7- and 8.6-fold, respectively) after 4 h, and declined by 8 h. As we have observed previously (8), ICAM-1 mRNA upregulation by lyso-PC was detected at 4 h and continued to increase gradually for up to 24 h. Also illustrated in Fig. 3, PMA, an activator of protein kinase C, elicited a pattern of endothelial gene upregulation at 4 h that appears distinct from that observed with lyso-PC, suggesting that mechanisms independent of PMA-regulatable protein kinase C may be involved in mediating the effect of lyso-PC.

Half-lives of mRNA for HB-EGF, PDGF A and B chains, and ICAM-1 were not significantly different in lyso-PC–and sham-treated cells. As an initial approach to exploring the mechanisms of endothelial gene upregulation by lyso-PC, we compared message half-lives in both sham- and lyso-PC–treated cells to test the hypothesis that lyso-PC might be acting to stabilize certain mRNAs. After endothelial monolayers were incubated with or without lyso-PC for 4 h, actinomycin D (5 μg/ml) was added to block new RNA synthesis, and then chased up to an additional 4 h. As shown in Fig. 4, mRNA levels for both ICAM-1, HB-EGF, and PDGF A chain were not significantly decreased during the 4-h actinomycin D chase even in the unstimulated (control) cells. Furthermore, the relative mRNA stabilities for each of these genes appeared to be comparable in control and lyso-PC–treated cells. Precise evaluation of the half-lives of PDGF A and B chain mRNAs is difficult because of the low levels of message for both of these genes present in control cells (Fig. 4); however, a progressive decrease in PDGF B chain mRNA and a modest decrease in PDGF A chain mRNA were observed in lyso-PC–treated cells after the addition of actinomycin D. Taken together, these results indicate that the increases in steady state mRNA levels for HB-EGF, ICAM-1, and PDGF A and B chains associated with lyso-PC treatment did not result primarily from mRNA stabilization.

Upregulation of HB-EGF, PDGF, and ICAM-1 depends upon de novo RNA synthesis. To examine the dependence of the lyso-PC–induced upregulation on new mRNA synthesis, we included actinomycin D in the culture medium 30 min before the addition of test stimuli, using TNFα as a positive control stimulus. Actinomycin D pretreatment essentially abolished the increased levels of mRNA for HB-EGF, PDGF A and B chains, and ICAM-1 elicited by lyso-PC or TNFα (Fig. 5). This strongly suggests that lyso-PC stimulates the transcription of the HB-EGF, PDGF A and B, and ICAM-1 genes, although it does not completely exclude the possibility that some newly transcribed mRNA, and subsequently translated protein, might affect their mRNA half-lives.

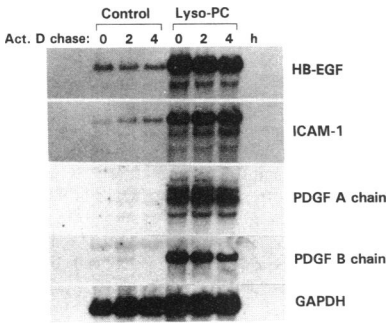

Figure 4. Stability of mRNA for HB-EGF, ICAM-1, and PDGF A and B chains. After HUVEC were treated with or without lyso-PC (100 μM) for 4 h (in medium 199 with 5% FBS), 5 μg/ml actinomycin D was added, and the cells were incubated for the indicated times. Total cellular RNA was extracted, and Northern blot analysis was performed using HB-EGF, ICAM-1, and both PDGF A B chain cDNAs. Each lane contained 20 μg of total RNA. One of three similar experiments is shown.

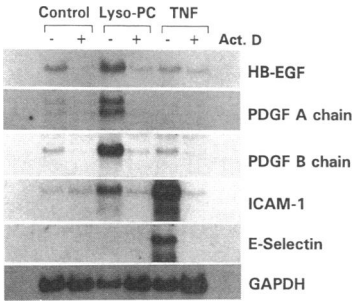

Figure 5. Dependence of endothelial gene induction on de novo RNA synthesis. HUVEC were treated with lyso-PC (100 μM), TNFα (200 U/ml), or media alone (*Control*) in the presence or absence of actinomycin D (5 μg/ml), for 4 h. Actinomycin D was added 30 min before lyso-PC and TNF. Northern blot analysis was performed as described in Methods. Each lane contained 15 μg of total RNA. One of two similar experiments is shown.

Lyso-PC increases transcriptional rate for HB-EGF gene. To directly assess the effect of lyso-PC on the transcriptional rate of the HB-EGF gene, we performed nuclear runoff assays using nuclei isolated from lyso-PC–and sham-treated cells. As shown in Fig. 6, nuclei extracted from lyso-PC–treated cells generated 7.6-fold more abundant radiolabeled mRNA for HB-EGF, thus documenting that lyso-PC can act to stimulate HB-EGF gene expression at the transcriptional level.

Discussion

Endothelial cells play a pivotal role in inflammation and atherogenesis through their inducible expression of various cytokines, chemoattractants, growth factors, and adhesion molecules (1, 4). We have previously shown that lyso-PC, a phospholipid increased in both atherosclerotic and inflammatory lesions, selectively upregulates functional VCAM-1 and ICAM-1, but not E-selectin, expression in cultured endothelial cells, suggesting a potential role for this agent in mononuclear leukocyte recruitment (8). Interestingly, this pattern of endothelial gene upregulation was qualitatively distinct from that elicited by the so-called septic triad (bacterial endotoxin, IL-1, TNF), thus indicating that this phospholipid (and perhaps related compounds) might be alternative or additive to the action of other locally generated endothelial-directed stimuli, such as vessel wall cytokines, bacterial products, and biomechanical forces (1–4, 17, 19–23). In this report, we provide evidence that lyso-PC can activate the transcription of endothelial genes encoding two key effector molecules relevant to smooth muscle and fibroblast growth, HB-EGF and PDGF, thus further supporting the potential pathophysiologic relevance of this agent to atherosclerosis, chronic inflammation, and wound healing.

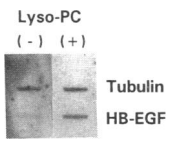

Figure 6. Induction of HB-EGF gene transcription. After HUVEC monolayers were treated with or without lyso-PC (100 μM) in medium 199 with 5% FBS for 1 h, nuclei were extracted, and nuclear runoff assays were performed as described in Methods. Transcribed RNA samples containing equal amounts of radioactivity were hybridized with HB-EGF and tubulin cDNAs immobilized on nitrocellulose membranes. One of two similar experiments is shown.

HB-EGF was originally described by Klagsbrun and co-workers (10, 11) as a macrophage-derived protein that binds to EGF receptors and is strongly mitogenic (potency comparable with PDGF) for fibroblast and vascular smooth muscle cells but not for endothelial cells. Cultured vascular endothelial cells subsequently were found to express HB-EGF, and the rate of transcription of this gene was increased in the presence of certain cytokines, such as TNFα (19). In a preliminary report (9), Nakano and co-workers have observed elevated mRNA levels for HB-EGF and enhanced secretion of mitogenic activity in human monocytes but not cultured human vascular smooth muscle in response to lyso-PC at concentrations comparable with those we have previously reported to be effective in cultured endothelium (8). In their study, however, the basal level of HB-EGF in freshly isolated blood monocytes was substantial, and the level of regulation of gene expression was not clearly defined. In our studies, human endothelial cells cultured under standard conditions expressed relatively low HB-EGF mRNA levels which were upregulated in a dose- and time-dependent fashion by nontoxic levels of lyso-PC. The mRNA levels induced by lyso-PC treatment exceeded those observed with maximally effective doses of Gram-negative bacterial endotoxin, TNFα, or PMA (Figs. 1, 3, and 5). The inhibition of lyso-PC induced HB-EGF mRNA by actinomycin D (Fig. 5) and the relatively stable half-life observed during both sham and lyso-PC treatment (Fig. 4) strongly suggest that de novo transcription rather than message stabilization was occurring. Direct evidence that lyso-PC acts to enhance the rate of HB-EGF gene transcription was obtained in nuclear runoff assays (Fig. 6). In preliminary experiments, cell surface–associated HB-EGF immunoreactive protein was increased significantly by treatment with lyso-PC for 6 h with similar dose-dependency (Kume, N., unpublished observation).

In addition to HB-EGF, lyso-PC also acted to upregulate the expression of the PDGF A and B chains, and ICAM-1 genes in a dose-dependent fashion (Fig. 2), apparently at the level of de novo transcription (Figs. 4 and 5), although with different kinetics (Fig. 3). In contrast, lyso-PC did not upregulate E-selectin, IL-8, or MCP-1 gene expression (Figs. 1–3). This qualitatively selective pattern of endothelial activation by lyso-PC is distinct from that elicited in the same endothelial cultures by TNFα, LPS, or PMA, thus suggesting that different inductive mechanisms might be involved. Specific transcriptional factors, such as AP-1 and nuclear factor-kB, have been implicated in the regulation of expression of growth factors, cytokines, and endothelial leukocyte adhesion molecules (18, 24–26). Further studies are required to define which transcriptional regulators are responsible for the unique pattern of growth factor and adhesion molecule expression induced by lyso-PC in vascular endothelium.

Migration and proliferation of smooth muscle cells in focal areas of the arterial intima are crucial steps in atherosclerotic lesion formation and appear to depend upon locally generated growth factors and cytokines (4). The data presented here establish that vascular endothelial cells can respond to lyso-PC, a component of oxidized LDL particles, with enhanced transcription of the genes encoding two potent smooth muscle–directed mitogens, HB-EGF and PDGF, and, further, that this stimulus appears to be more effective than cytokines and endotoxin in our in vitro system. Studies by others have demonstrated that lyso-PC can act as a selective monocyte chemoattractant (27), and we have previously reported that VCAM-1, a relatively selective mononuclear adhesion molecule, is also upregulated in cultured human and rabbit arterial endothelial cells by this agent. Given the potential for the local generation and sequestration of lyso-PC within LDL particles undergoing oxidative modification in the subendothelial space (or conceivably during transendothelial transport) (6, 7, 28), this polar phospholipid (and related compounds) may be playing multiple roles in the initiation and progression of atherosclerotic lesions. In the context of acute and chronic inflammatory reactions and related pathologic processes, the potential for generation of lyso-PC also exists through the action of leukocyte-secreted phospholipase A_2 (5), with similar implications for mononuclear leukocyte recruitment. In addition, in light of our findings on endothelial-generated HB-EGF and PDGF, local generation of lyso-PC may be playing a role in wound healing and fibroplasia. Further studies of the local production and cellular action of lyso-PC and related compounds may provide new insights into the molecular mechanisms of endothelial activation and its pathophysiologic consequences.

Acknowledgments

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