Lysophosphatidylcholine upregulates the level of heparin-binding epidermal growth factor-like growth factor mRNA in human monocytes

(atherosclerosis, oxidized low density lipoprotein/phospholipase A$_2$)

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ABSTRACT Lysophosphatidylcholine is increased in the plasma of hypercholesterolemic patients, is a component of oxidatively modified low-density lipoprotein, and, as such, may play an important role in atherosclerosis. Here we demonstrate that in human monocytes, lysophosphatidylcholine increases the level of mRNA encoding the heparin-binding epidermal growth factor-like growth factor (HB-EGF), a potent smooth muscle mitogen. Lysophosphatidylcholine treatment also enhances the release of heparin-binding mitogenic activity by these cells in culture. The anti-inflammatory glucocorticoid dexamethasone inhibits the upregulation of HB-EGF mRNA induced by either lysophosphatidylcholine or bacterial lipopolysaccharide in cultured monocytes. However, the responses induced by lysophosphatidylcholine and by lipopolysaccharide differ in their kinetics. In addition, the response to lysophosphatidylcholine is resistant to the action of cycloheximide, whereas the response to lipopolysaccharide is not, suggesting that the activation mechanisms induced by these two stimuli are different. Since a nuclear run-on assay showed no effect of lysophosphatidylcholine on the transcription of the HB-EGF gene, we speculate that lysophosphatidylcholine may increase the level of HB-EGF mRNA by altering the processing or degradation of primary or mature transcripts. Lysophosphatidylcholine enhancement of monocyte production of HB-EGF may represent an important result of the interactions among oxidized low-density lipoprotein and monocyte-derived macrophages and may play a role in initiation of smooth muscle proliferation in atherogenesis.

The advanced lesions of atherosclerosis result from intimal accumulation of smooth muscle cells (SMCs), monocytes/macrophages, connective tissue, and lipids. The increased number of SMCs within the intima results from the response of these cells to a number of growth-regulatory molecules that may be released by arterial cells or cells from the circulation. Since attachment of monocytes to the artery wall and monocyte infiltration into the intima are among the earliest events in the development of the lesions of atherosclerosis (1), monocytes/macrophages may be important early sources of substances that can induce chemotaxis and proliferation of both monocytes and SMCs.

One of the candidate monocyte/macrophage-derived growth factors for SMCs is a macrophage-derived mitogen, heparin-binding epidermal growth factor-like growth factor (HB-EGF), which stimulates the proliferation of fibroblasts and SMCs and migration of SMCs in vitro. Although the actions of HB-EGF appear to be mediated by binding of HB-EGF to the epidermal growth factor (EGF) receptor, HB-EGF is much more potent than EGF as a mitogen and chemotaxant for SMCs (2, 3). Increased expression of HB-EGF and the EGF receptor has been observed in human atherosclerotic lesions (J. Miyagawa, S. Higashiyama, S. Kawata, Y. Inui, S. Tamura, K. Yamamoto, M. Nishida, T. Nakamura, S. Yamashita, Y. Matsuzawa, and N. Taniguchi, personal communication).

Numerous epidemiologic studies have demonstrated that chronically elevated levels of plasma lipoproteins, particularly low-density lipoprotein (LDL) and very-low-density lipoprotein, are associated with an increased incidence of atherosclerosis (4). Intensive lipid-lowering therapy has been shown to enhance the regression of coronary artery disease (5). However, although hyperlipidemia is important in the development of atherosclerosis, the mechanisms which link hyperlipidemia and SMC proliferation remain to be clarified.

A large amount of data from experimental animals has implicated oxidized LDL as a principal lipid mediator of lesion formation in atherosclerosis (6). However, in vitro studies with oxidized LDL have demonstrated variability in biological activities among laboratories and different preparations (7). We therefore investigated whether the phospholipid lysophosphatidylcholine (lysoPC), a major component of oxidized LDL, activates monocytes/macrophages and stimulates their production of growth factors. Here we report that lysoPC elevates levels of HB-EGF mRNA and stimulates the release of heparin-binding mitogenic activity from human monocytes. We discuss possible mechanisms for the production of lysoPC in the lesions of atherosclerosis and the possible role of lysoPC in initiation of the proliferative response by SMCs.

MATERIALS AND METHODS

Preparation and Culture of Human Monocytes. Egg yolk lysoPC, synthetic palmitoyl and stearoyl lysoPC, cycloheximide, and dexamethasone were obtained from Sigma. Human monocytes were isolated from buffy coats obtained from the Puget Sound Blood Center. Buffy coats were diluted with endotoxin-free phosphate-buffered saline to 1 × 10^7 leukocytes and 3–4 × 10^6 erythrocytes per milliliter and 10-ml aliquots transferred to LeukoPrep tubes (Becton Dickinson). The tubes were spun at 1000 × g for 25 min at room temperature. The cell layer containing monocytes was collected and washed. Cell preparations with <90% monocytes were not used, and the preparations generally consisted of 92% monocytes, 6% lymphocytes, and 2% neutrophils. Monocytes (2 × 10^5 cells) were cultured in 12 ml of RPMI 1640 (BioWhittaker) containing 10% human plasma-derived

Abbreviations: SMC, smooth muscle cell; EGF, epidermal growth factor; HB-EGF, heparin-binding EGF-like growth factor; LDL, low-density lipoprotein; lysoPC, lysophosphatidylcholine; LPS, lipopolysaccharide; LCAT, lecithin–cholesterol acyltransferase.

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serum. The plasma-derived serum and RPMI 1640 had <1 endotoxin unit/ml (Limulus amebocyte lysate chromogenic assay BioWhittaker). To prevent attachment of the monocytes to plastic, the 10-cm dishes were coated with 2 ml of 12% Hydrone (Interferon Sciences, New Brunswick, NJ) in ethanol.

**Culture of SMCs.** Human aortic SMCs were isolated from newborn human thoracic aortas (8) and cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum. Cells were grown to confluence and changed to DMEM with 1% human plasma-derived serum (8) for 48 hr prior to experiments.

**Assay of Heparin-Binding Mitogenic Activity with Swiss Mouse 3T3 Cells.** Culture supernatants from 2 x 10⁷ monocytes were collected, diluted 2-fold in 0.05 M Tris-HCl (pH 7.4), and loaded onto 0.3-ml heparin-Sepharose columns (Pharmacia). After extensive washing of the columns with the same buffer containing 0.2 M NaCl, the samples were eluted with 2 ml of 0.05 M Tris-HCl, pH 7.5/3 M NaCl, desalted in Centricon-10 miniconcentrators (Amicon), and assayed for mitogenic activity on Swiss 3T3 cells (9).

**Radio-receptor Assay for EGF.** The radio-receptor assay was performed by simultaneous incubation of sample aliquots and [t-32P]dCTP by A-431 human epidermoid carcinoma cells (10).

**Northern Blotting.** Total cellular RNA was extracted (11), and samples (10 μg per lane) were run in 1% agarose/2.2 M formaldehyde gels and transferred to nylon membranes (Nylon, Schleicher & Schuell) (12). After UV crosslinking, reversible RNA staining with methylene blue was performed to check the amount and quality of RNA (13). After destaining, the membranes were subjected to hybridization (14) with probes labeled with [γ-32P]UTP (Amersham) by means of a random primer labeling system (Amersham). After hybridization, the membranes were washed with 2 x standard saline citrate (SSC)/0.1% SDS at room temperature and with 0.2 x SSC/0.1% SDS at 65°C prior to autoradiography. For reprobing, blots were stripped with two 30-min washes with 0.02 x SSC/0.1% SDS at 90°C. All blots were rehybridized with β-actin cDNA or glyceraldehyde-3-phosphate dehydrogenase cDNA probes to verify the mRNA load.

**Transcriptional Analysis.** A nuclear run-on assay was conducted (15) with some modifications. Monocytes (8 x 10⁷ cells per reaction mixture) were cultured for 3 or 24 hr in the presence or absence of 75 mM stearoyl lysoPC and then collected by centrifugation at 1000 x g for 10 min. Each cell pellet was suspended in 4 ml of lysis buffer [10 mM Tris-HCl, pH 7.4/10 mM NaCl/3 mM MgCl₂/0.5% Nonidet P-40/2.75 mM dithiothreitol with RNasin (Promega) at 20 units/ml], placed on ice for 5–8 min, and centrifuged at 270 x g for 5 min at 4°C. The supernatants were removed, and each pellet was suspended in 4 ml of lysis buffer without Nonidet P-40. Nuclei were spun down at 270 x g for 5 min at 4°C. The nuclear pellet from each sample was suspended in 200 μl of storage buffer [50 mM Tris-HCl, pH 8.3/40% (vol/vol) glycerol/5 mM MgCl₂/0.1 mM EDTA] and stored at -70°C until use. Each nuclear run-on reaction mixture (400 μl) contained 200 μl of nuclei, 0.625 mM ATP, 0.312 mM GTP, 0.312 mM CTP, 250 mM of [γ-32P]UTP (>3000 Ci/mmolute, New England Nuclear; 1 Ci = 37 GBq), 40 mM Tris-HCl (pH 8.3), 150 mM NaH₂Cl, 7.5 mM MgCl₂ and RNasin at 200 units/ml. This mixture was incubated for 30 min at 30°C. Radiolabeled RNA was precipitated by the same method used for Northern blotting, subjected to limited base hydrolysis (0.2 M NaOH on ice for 3 min), ethanol precipitated, and suspended in 1 ml of hybridization buffer at 10⁶ cpm/ml. Unlabeled cDNA probes (5 μg per blot) for hybridization with run-on products were denatured at 0.3 M NaOH at 65°C for 1 hr, dot blotted onto a Nytran membrane, hybridized for 45 hr and washed as described for Northern blotting.

**RESULTS**

LysoPC Upregulates mRNA Levels in Monocytes but not in SMCs. Freshly isolated human monocytes were incubated in suspension in Hydrone-coated dishes for 24 hr with various concentrations of egg lysoPC dissolved in ethanol. Expression of HB-EGF mRNA was then examined by RNA blotting. Incubation of monocytes with egg lysoPC at 12.5–75 μg/ml for 24 hr upregulated the level of HB-EGF mRNA (Fig. 1). In this experiment, 37.5 μg/ml was the optimal concentration of lysoPC for induction of HB-EGF mRNA (4.1-fold, from densitometric scans of the films that included a film from reprobing the same blots with β-actin cDNA to normalize for load differences). Although β-actin mRNA levels were not affected, there was some variation in the optimal concentration, as well as the degree of mRNA upregulation, between experiments (data not shown). This may be due in part to variable uptake and modification of lysoPC by the cells, which may alter the concentration of lysoPC in the medium. Neither lysoPC nor the diluent (ethanol) had any effect on the viability of the cells as measured by trypan blue exclusion.

Although HB-EGF was originally described as a monocyte/macrophage-derived growth factor, it is also produced by SMCs (16, 17). In contrast to monocytes, the HB-EGF mRNA level in SMCs was not affected by lysoPC (Fig. 1).

The Activity of LysoPC in Upregulation of HB-EGF mRNA Is Dependent on Its Fatty Acid Composition. The major fatty acids in mammalian phospholipids and in the egg yolk lysoPC used in our experiments are palmitic acid and stearic acid. We compared the activity of synthetic palmitoyl lysoPC and stearoyl lysoPC on the upregulation of HB-EGF mRNA. Fig. 2 is representative of the results of four experiments in which stearoyl lysoPC upregulated the mRNA level of HB-EGF but palmitoyl lysoPC failed to do so. This was observed with two different lots of the synthetic lysoPC.

Secrecion of HB-EGF-Like Activity from Monocytes Is Increased by LysoPC. To examine the release of growth-stimulatory molecules from monocytes, we used heparinase to fractionate the conditioned media of lysoPC-treated and control monocytes. Incubation of monocytes with lysoPC significantly increased the heparin-binding mitogenic activity in the conditioned medium, and the release of mitogenic activity was well correlated with the HB-EGF mRNA level (Fig. 3). Since, in addition to binding heparin, HB-EGF competes with [125I]-EGF for binding to the EGF receptor, we also assayed EGF competitive activity in the heparin-binding fraction from monocyte-conditioned media.

![FIG. 1. Effect of lysoPC on HB-EGF mRNA levels in human monocytes and arterial SMCs. Human monocytes or human arterial SMCs were incubated with various concentrations of egg lysoPC or with vehicle for 24 hr. HB-EGF mRNA levels were analyzed as described in Materials and Methods. No variations in β-actin mRNA levels were observed. These data are representative of four replicate experiments.](image-url)
The conditioned medium of monocytes incubated with lysoPC contained 12 times more heparin-binding EGF competitive activity than the conditioned medium of monocytes incubated without lysoPC (basal, 0.011–0.019 ng per 10⁶ cells; lysoPC-treated, 0.14–0.23 ng per 10⁶ cells).

**LysoPC and Lipopolysaccharide (LPS) Effects on HB-EGF mRNA Levels Appear to Be Stimulated via Distinct Signaling Mechanisms.** HB-EGF mRNA was also increased in human monocytes treated with LPS, a bacterial endotoxin (Fig. 4). To further characterize the mechanism of mRNA increase, we examined the effects of several agents on the upregulation of HB-EGF mRNA by either lysoPC or LPS. First, we examined the effect of dexamethasone, a synthetic glucocorticoid, on HB-EGF mRNA levels. Dexamethasone completely inhibited both lysoPC- and LPS-induced upregulation of HB-EGF mRNA (Fig. 4A). Consistent with these results, dexamethasone has also been shown to downregulate the expression of HB-EGF mRNA in both unstimulated and thrombin-stimulated cultured human SMCs (17). This suggests that dexamethasone inhibits increases in HB-EGF mRNA with distinct activation patterns in different cell types.

We next determined whether de novo protein synthesis was necessary for the upregulation of HB-EGF mRNA observed at 24 hr. Cycloheximide, an inhibitor of protein synthesis, did not inhibit the lysoPC enhancement of HB-EGF mRNA, whereas it completely blocked the LPS effect (Fig. 4B). The level of HB-EGF mRNA was also enhanced by interleukin 1β, and this effect was inhibited by cycloheximide (data not shown).

To further examine the different cycloheximide susceptibilities of the lysoPC and LPS effects, we studied the time course of regulation of HB-EGF mRNA levels in lysoPC- or LPS-stimulated monocytes (Fig. 5). The level of the HB-EGF mRNA was relatively high just after the preparation of monocytes (at 0 hr) and remained relatively high. However, in the absence of lysoPC or LPS, the level decreased further incubation. The level of HB-EGF mRNA immediately following isolation was variable among donors but did not appear to be due to endotoxin contamination of reagents, which were all monitored. In the presence of lysoPC, upregulation was observed at 3 hr and was maintained for at least 24 hr. Cycloheximide did not affect the time course of the lysoPC-induced upregulation (data not shown). On the other hand, in the LPS-stimulated monocytes, the mRNA level...
reached a maximum at 1 hr, decreased to a low level at 3 hr, and increased again after 8 hr. In the presence of cycloheximide, LPS induced the first peak but did not induce the second, indicating that the second increase in HB-EGF mRNA was dependent on de novo protein synthesis.

**Effect of LysoPC on Transcription of the HB-EGF Gene and Stability of HB-EGF mRNA.** To examine the effect of lysoPC on transcription of the HB-EGF gene, we performed nuclear run-on assays (Fig. 6). LysoPC did not enhance transcription at 3 hr or at 24 hr, whereas dexamethasone inhibited HB-EGF mRNA transcription.

We also examined the effect of lysoPC on mRNA stability in the presence of an inhibitor of RNA synthesis, actinomycin D (Fig. 7). LysoPC had no effect on the decrease of HB-EGF mRNA observed in the presence of actinomycin D.

**DISCUSSION**

LysoPC and HB-EGF as Potential Mediators of the Development of Atherosclerotic Lesions.** It has been reported that lysoPC is a chemoattractant for human monocytes (18) and that it enhances expression of the cell adhesion molecules VCAM-1 and ICAM-1 in cultured human umbilical vein endothelial cells (19). Such an effect could increase the adherence of monocytc cells to the endothelium if it occurred in vivo. The data reported here demonstrate that lysoPC stimulates monocytes to release EGF-like heparin-binding mitogenic activity and to upregulate mRNA of HB-EGF, a potent mitogen for SMCs.

Heparin-binding EGF is a member of a family of molecules structurally similar to EGF that include transforming growth factor α, amphiregulin, and the poxvirus growth factors (20). In addition to sharing structural similarities, these distinct gene products all bind with high affinity to the EGF receptor. However, HB-EGF stimulates SMC proliferation to a level comparable to that observed in response to platelet-derived growth factor and is much more potent than other members of the EGF family (3). This appears in part to be due to its ability to bind heparin, although this is also a property of amphiregulin, which binds the EGF receptor with a lower affinity than other EGF family members (21). The enhanced response to HB-EGF observed in SMCs appears to be mediated by a dual receptor system requiring interaction with both cell surface glycosaminoglycans and the EGF receptor (22), similar to the dual receptor system required for activity of basic fibroblast growth factor (23). HB-EGF may also have a distinct receptor in addition to the EGF receptor.

A recent survey of human thoracic and abdominal aortae obtained from individuals from 2 months to 86 years of age demonstrated regulated expression of HB-EGF protein (J. Miyagawa et al., personal communication). The medial SMCs of the aorta in neonates and infants constitutively synthesized HB-EGF protein, whereas HB-EGF-producing SMCs were rare in young and middle-aged adults. However, in

**FIG. 7.** Effect of lysoPC on degradation of HB-EGF mRNA. Human mononies were cultured for the indicated times with or without stearoyl lysoPC (37.5 µg/ml) in the presence of 1 mM actinomycin D. HB-EGF mRNA levels were analyzed. These data are representative of three replicate experiments.

in atherosclerotic plaques, marked production of HB-EGF protein was detected in both SMCs and macrophages. In addition, EGF receptors were detected on SMCs in the lesions, many of which were positive for proliferating-cell nuclear antigen, an index of cycling cells. Thus, the association of HB-EGF protein and the EGF receptor with lesions of atherosclerosis is consistent with a potential role for HB-EGF in SMC migration and proliferation, characteristic of these developing lesions.

**Mechanism of Upregulation of HB-EGF mRNA.** HB-EGF mRNA in monocytes can be upregulated by lysoPC as well as by LPS. However, the differences in time course and susceptibility to cycloheximide suggest that lysoPC and LPS upregulate HB-EGF mRNA via different mechanisms. Inhibition of the second peak of LPS-induced HB-EGF mRNA expression by cycloheximide suggests that the second response requires protein synthesis, whereas upregulation by lysoPC is independent of protein synthesis.

In evaluating the mechanism of lysoPC alteration of HB-EGF mRNA levels, we performed nuclear run-on assays to investigate transcriptional regulation. We could not detect enhancement of transcription of HB-EGF at 3 or 24 hr after stimulation with lysoPC, although we did detect downregulation by the glucocorticoid dexamethasone (Fig. 6). As noted above, lysoPC enhancement of HB-EGF mRNA levels is not dependent upon protein synthesis, nor is the stability of mature mRNA altered in the presence of the RNA synthesis inhibitor actinomycin D. It is possible that lysoPC may alter the processing or degradation of primary or mature HB-EGF mRNA, but this effect may be inhibited by actinomycin D. This lysoPC inhibition of HB-EGF mRNA degradation or processing would depend upon the stability of some form of RNA. Further experiments will be required to determine the nature of the RNA and whether this suggestion is correct.

**Formation of LysoPC in Atherosclerosis.** Hydrolysis of fatty acid on the sn-2 position of phospholipids is a key step in the formation of lysophospholipids. Lecithin–cholesterol acyltransferase (LCAT; phosphatidylcholine:sterol acyltransferase, EC 2.3.1.43) can hydrolyze the sn-2 fatty acid of phosphatidylcholine, transfer the fatty acid to cholesterol (24), and produce lysoPC. LCAT is present in plasma, and high-density lipoprotein enhances the catalytic activity of LCAT (25). Higher LCAT activity has been observed in the plasma of atherosclerotic patients, who also have higher concentrations of lysoPC (26), suggesting that LCAT may be a major factor in the production of lysoPC in these patients.

Oxidative modification of LDL has been proposed as a possible contributor to the development of atherosclerosis (6). In vitro, oxidized LDL stimulates expression of colonystimulating factors in endothelial cells (27) and acts as a chemoattractant for human monocytes (28). However, in vitro studies with oxidized LDL have demonstrated variability in biological activities among laboratories and different preparations (7). We have, therefore, focused these studies on a major component of oxidized LDL. Oxidation of LDL is a major source of phospholipids, especially in the plasma.
of hyperlipidemic individuals. During the oxidation of LDL, as much as 40% of the phosphatidylcholine of LDL may be converted to lysoPC (29).

The biological activities of lysoPC presented here and in other papers (18, 19) suggest that lysoPC may contribute to the atherogenic activities of oxidized LDL. It has been shown that apolipoprotein B in LDL possesses phospholipase A\textsubscript{2} activity. Thus, the increased lysoPC content of oxidized LDL may be due to the hydrolysis of phosphatidylcholine in LDL by the phospholipase A\textsubscript{2} activity of apolipoprotein B (30). Since LDL can be oxidatively modified by the cells in or around the lesions of atherosclerosis (31–33), and since oxidatively modified LDL is present in the lesions (34–37), lysoPC in oxidized LDL may be important in the activation of monocytes/macrophages in the lesions.

The Fatty Acid Chain Length of LysoPC Is Important. The signal transduction mechanisms responsible for the upregulation of HB-EGF mRNA in human monocytes by lysoPC may be an important issue to study. HB-EGF can act as a chemoattractant for human monocytes, and it has been postulated that generation of diacylglycerol, an activator of protein kinase C, through a mechanism involving lyso-phospholipase C\textsubscript{2} may be involved in lysoPC-induced chemotaxis (38). However, our preliminary experiments using a synthetic diacylglycerol did not demonstrate upregulation of HB-EGF mRNA in human monocytes (data not shown). This suggests, therefore, that, different from chemotaxis, HB-EGF mRNA induction may not involve protein kinase C.

Our data showed that a small difference in the fatty acid chain length of lysoPC caused a profound difference in potency of lysoPC in upregulating HB-EGF mRNA. Stearoyl lysoPC upregulated the mRNA, but palmitoyl lysoPC did not. Although the precise mechanism for the action of lysoPC is not clear, a small difference in the physical property of lysoPC appears to be important for upregulation of HB-EGF mRNA in monocytes. LysoPC also induces expression of an adhesion-molecule gene in human endothelial cells (19). In this case, however, palmitoyl lysoPC induces the expression of the adhesion molecule. This difference in effect of fatty acid chain length on these two cells may suggest that the molecular mechanism for HB-EGF induction in monocytes is different from the mechanism that induces adhesion-molecule expression in endothelial cells.

To further clarify the importance of lysoPC in vivo, it will be necessary to develop specific agents that inactivate lysoPC or inhibit LCAT and phospholipase A\textsubscript{2}. Such agents may be capable of altering the development of lesions of atherosclerosis as well as other inflammatory fibroproliferative responses.

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