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Lysophosphatidylcholine induces tPA gene expression through CRE-dependent mechanism

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Abstract

Lysophosphatidylcholine (lysoPC) is implicated in the development of atherosclerosis and certain autoimmune diseases, and is reported to induce tissue-type plasminogen activator (tPA) at the protein level in endothelial cells. This study was designed to investigate the effect of lysoPC on tPA gene expression and the underlying molecular mechanisms in cultured endothelial cells. LysoPC transiently induced the mRNA expression of tPA in endothelial cells. LysoPC also induced the mRNA expression of urokinase-type plasminogen activator (uPA), uPA receptor, and plasminogen activator inhibitor-1, but the kinetics were different from that of tPA. Promoter analysis revealed that the cyclic AMP-responsive element of the tPA gene (tPACRE) is required for lysoPC-induced tPA expression. Furthermore, an electrophoresis mobility shift assay showed that lysoPC increased the binding activity of CRE binding protein to tPACRE. These results indicated that lysoPC transcriptionally upregulated the gene expression of tPA in endothelial cells, at least in part, via tPACRE activation.

Keywords: Endothelial cells; Lysophosphatidylcholine; tPA; Gene expression; Plasminogen activation system; tPACRE

Lysophosphatidylcholine (lysoPC) is a bioactive phospholipid that is abundant in atherosclerotic lesions [1]. During the oxidation of low density lipoprotein (LDL), lysoPC is produced from the phosphatidylcholine of LDL by enzymatic activity of phospholipase A_2 [2], and was increased several fold in atherosclerotic vessels compared to their normal counterparts [1]. LysoPC is also produced in apoptotic cells, act as a chemoattractant for T cells, and is involved in the pathogenesis of systemic lupus erythematosus [3,4]. Previous reports have shown that lysoPC modulates multiple genes relevant to atherosclerosis and inflammation [5–11]. In cultured endothelial cells, it induces intercellular adhesion molecule-1 [5], vascular cell adhesion molecule-1 [5], cyclooxygenese (Cox)-2 [6], endothelial nitric oxide synthase (eNOS) [7], and platelet-derived growth factors [8], and inhibits interferon-γ-induced CXC chemokine expression in endothelial cells [11].

Tissue-type plasminogen activator (tPA) is mainly produced by the endothelial cells, and is involved in the degradation and removal of fibrin from the vascular bed by converting zymogen plasminogen to active plasmin [12,13]. Plasmin can also degrade a variety of extracellular matrix proteins directly or indirectly through the activation of certain matrix metalloproteinases [13]. Several clinical and in vivo studies have demonstrated the importance of tPA in pathophysiological conditions, such as angiogenesis, tumor growth, and atherosclerosis [13–19]. For example, tPA contributes to the invasion, angiogenesis, and growth of pancreatic cancer [14]. A high level of tPA is associated with the presence of heart diseases, a greater risk of myocardial infarction and stroke, and tPA expression and activity increases in human atherosclerotic lesions [15–18]. Furthermore, an in vivo study using tPA-deficient mice has demonstrated

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that tPA has proatherogenic properties [19]. The other members of the plasminogen activation system, urokinase-type plasminogen activator (uPA), uPA receptor (uPAR), and plasminogen activator inhibitor (PAI)-1, are also reported to be associated with tumor growth, angiogenesis, and atherosclerosis [13,18,20].

The human tPA gene is reported to be induced by several stimuli such as phorbol ester [21], growth factors [22], steroids [23], and polyphenolics [24]. Regarding transcriptional regulation, two *cis*-acting sites in the proximal promoter region of the tPA gene are well characterized; one is a cyclic AMP-responsive element-like element of tPA (tPACRE), and the other is referred to as the SP-1 binding site [21]. In endothelial cells, both the tPACRE and SP-1 sites are required for phorbol 12-myristate ester (PMA)-induced tPA gene expression [21]. PMA also induces tPA expression in HeLa cells, but suppresses that in HT-1080 fibrosarcoma cells [25]. The authors suggest that the differential binding of CREB and ATF-2 to tPACRE is responsible for this differential expression of tPA in these two cell lines, indicating the complex regulatory mechanism of this gene depending on the cell type and stimulation.

Although it is reported that lysoPC can increase the secretion of tPA in endothelial cells [26], the molecular mechanisms of lysoPC-induced tPA expression are unknown. Given the importance of the role of tPA in the pathogenesis of atherosclerosis, we systematically investigated the molecular mechanism of lysoPC-induced tPA expression in endothelial cells. We here report the involvement of tPACRE in the tPA gene expression induced by lysoPC.

Materials and methods

Materials. L-α-lysoPC (from egg phosphatidylcholine) was purchased from Avanti Polar Lipids. M199 medium, penicillin G, L-glutamine, and fetal calf serum (FCS) were from Gibco-BRL.

Cell culture. Human umbilical vein endothelial cells (HUVECs) were isolated from human umbilical cords using collagenase (Collaborative Research) and cultured in M199 medium supplemented with endothelial cell growth supplement (Sigma), heparin (from porcine intestinal mucosa; Sigma), antibiotics (100 U/ml penicillin G and 100 mg/ml streptomycin), L-glutamine, and 20% FCS. Confluent cells after 2–5 passages were used in this study. To stimulate HUVECs, M199 medium containing 5% FCS, L-glutamine, and antibiotics was used for all experiments.

Northern blot analysis. Northern blot analysis was performed as previously described [27]. To prepare cDNA probes for tPA, uPA, uPAR, and PAI-1, RT-PCR was carried out using total RNA extracted from HUVECs treated with 80 μM lysoPC for 8 h. The following primers were used for PCR: tPA upper 5′-CAGAGA TGAAAAAAGCAGA-3′ and tPA lower 5′-ACCCATTCCCAAAGT AGCAGT-3′, uPA upper 5′-CTCCAAAGGCAGCAATGAAC-3′ and uPA lower 5′-CTTGAGCGACCCAGGTAGAC-3′, uPAR upper 5′-TGGCCGGGCTGTCACCTATT-3′ and uPAR lower 5′-CTTGA GCGACCCAGGTAGAC-3′, and PAI-1 upper 5′-ACAAATCAGA CGGCAGCACT-3′ and PAI-1 lower 5′-TTGTCCCAGATG AAGGCGTC-3′. The length of the PCR products was 539, 678, 497, and 603 bp, respectively. A 0.9-kb *Hin*dIII/*Acc*I fragment of GAPDH cDNA, purchased from ATCC, was used to determine the RNA loading.

ELISA. Culture supernatants were collected and the concentration of human tPA was measured by sandwich ELISA using a Protein Detector ELISA Kit (AngioPharm, USA) according to the manufacturer's instructions. Optical densities were measured with a plate reader (EAR400, SLT-Lab instruments). The tPA concentration was calculated after preparing a standard curve using recombinant human tPA (AngioPharm, USA).

Preparation of tPA promoter/luciferase construct. A 2221 bp tPA promoter fragment containing a TATA-dependent transcription start site at +1 and TATA box (TATAAA) at positions −25 to −29, relative to the TATA-dependent transcription start site, was generated with PCR using genomic DNA as the template. The DNA fragment spanned from positions −2059 to +162 relative to the TATA-dependent transcription start site. The PCR was carried out, using KOD101 DNA polymerase (TOYOBO), with tPA promoter upper (5′-gatcggt accCCATTGTCACCTTATCAGCCTGCCC-3′) having a *Kpn*I site and tPA promoter lower (5′-gatcagatctTCCTCGCAGAGGTTTCTC TCCAGC-3′) having a *Bgl*II site at the 5′ end (underlined). The PCR product was cloned into a pGL3-basic expression vector (Promega) to give the plasmid tPA 2221 Luc. The orientation and vector insert boundaries were verified with sequence analysis.

Construction of tPA promoter deletion mutants. Deletion mutants containing a tPA promoter of various lengths were prepared from the tPA 2221 Luc plasmid by unidirectionally digesting the insert with exonuclease III (Promega) according to manufacturer's protocol. For the unidirectional deletion we digested the plasmid with *Blp*I, resulting in a 988 bp insert with a 5′ overhang. For protection of the vector DNA the plasmid was also digested with *Kpn*I to produce a 3′ overhang.

Site-directed mutagenesis in the tPA promoter. Selected base substitutions were made in the regions of the tPA promoter using a commercially available QuikChange Site-Directed Mutagenesis Kit (Stratagene), according to the manufacturer's protocol. The tPACRE, Sp-1 or both sites were mutated in a 608 bp tPA luciferase construct (tPA 608 Luc). In the tPACRE (ATGACATCACGGA) site, the underlined nucleotides were substituted to AGGCTATCACGGA. In the Sp-1 (GACCCCACCCCCTGC) site, the underlined nucleotides were mutated to GACCGGAAGCCCTGC. All constructs were sequenced to confirm the desired mutation.

Transient transfection assay. HUVECs were transiently cotransfected with tPA promoter/firefly luciferase construct, and a *Renilla* luciferase construct containing CMV promoter as an internal control (Promega). HUVECs on 12-well plates were transfected in duplicate with 0.3 μg DNA per well for 4 h using Lipofectamine Plus reagent (Invitrogen) according to the manufacturer's protocol. Cells were stimulated 48 h after transfection, harvested, and a dual luciferase assay (Promega) was performed.

Preparation of nuclear extract and electrophoretic mobility shift assay (EMSA). Nuclear extracts were prepared as described previously [28]. Double-stranded oligos corresponding to tPACRE (gatcATTCAATGACATCACGGCTGTG) of human tPA promoters were used as probes after being radiolabeled with [α^{32}P]dCTP (NEN) using a klenow fragment of DNA polymerase I. 100,000 cpm of the DNA probes were used for binding reactions with nuclear protein (10 μg). For the super-shift assay, nuclear extracts were incubated with 2 μg/ml of rabbit polyclonal antibodies directed to CREB and ATF-2 (Santa Cruz Biotechnology) 20 min before the addition of the probes. For the competition assay, a 50× molar excess of unlabeled double stranded tPACRE oligonucleotide was added simultaneously with the probe.

Statistical analysis. Differences were analyzed using one-way ANOVA and then by Fisher's test. A p value of <0.05 was regarded as significant.

Results

LysoPC induces tPA mRNA expression in endothelial cells

The results of Northern blotting showed that the basal expression of tPA mRNA was very small and that lysoPC induced the expression of tPA mRNA in HUVECs (Fig. 1A). Increased levels of tPA mRNA were observed at 4 h but not at 1 h, and they reached a peak at 8 h. The expression had declined nearly to the basal level at 24 h. Dose–response experiments revealed that the tPA mRNA expression induced by lysoPC was slight at 40 μM and increased in a dose-dependent manner up to 80 μM lysoPC (Fig. 1B).

We also investigated the effects of lysoPC on the mRNA expression of uPA, uPAR, and PAI-1 in HUVECs. As shown in Fig. 1C, the basal expression of uPA and uPAR mRNA was very small, similar to tPA, while that of PAI-1 was relatively high. LysoPC induced the mRNA expression of uPA, uPAR, and PAI -1 with similar kinetics: the expression was time-dependently increased up to 4 h and then gradually decreased (Fig. 1C). The kinetics of tPA mRNA induced by lysoPC was apparently different from those of the others (Fig. 1C).

LysoPC induces tPA protein secretion in endothelial cells

The secretion of tPA into the culture medium of HUVECs was analyzed with ELISA. Compared to basal secretion, lysoPC increased the tPA secretion 2, 5, and 14 times at 40, 60, and 80 μM, respectively (Fig. 2). These results were consistent with those of Northern blotting and a previous report [26].

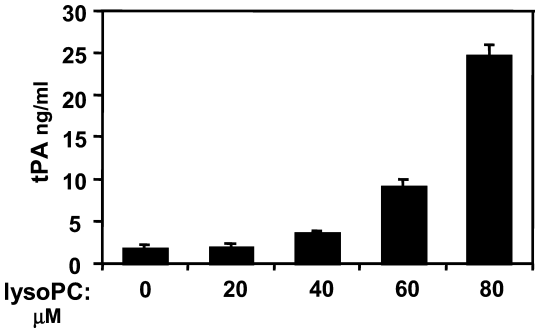

Fig. 2. LysoPC increases tPA protein secretion in HUVECs. HUVECs were treated for 16 h, with medium alone, or with the indicated concentrations of lysoPC. Culture supernatants were collected and subjected to sandwich ELISA to measure secreted tPA protein. The results are shown as means ± SE of four independent experiments.

tPA promoter region between −148 and −76 bp contains a lysoPC-responsive element

To examine whether lysoPC upregulates tPA gene expression at the transcriptional level, we prepared a luciferase construct containing 2221 bp of the tPA (tPA 2221 Luc) promoter spanning from −2059 to +162 relative to the TATA-dependent transcription start site, and co-transfected with the pRL-CMV plasmid to HUVECs. The promoter activity of the tPA promoter was significantly increased an average of 3.5-fold by lysoPC ($p < 0.0001$). We prepared a series of deletion mutants of the tPA promoter luciferase construct (tPA2221 Luc) (Fig. 3B). The extent of induction tended to decrease when the promoter region was deleted to −818 (tPA 980 Luc), but the difference did not reach significance ($p = 0.09$ vs. tPA 2221). Further deletion of this construct showed that the inducibility was significantly reduced to 1.3-fold ($p < 0.01$ vs. tPA 310 Luc,

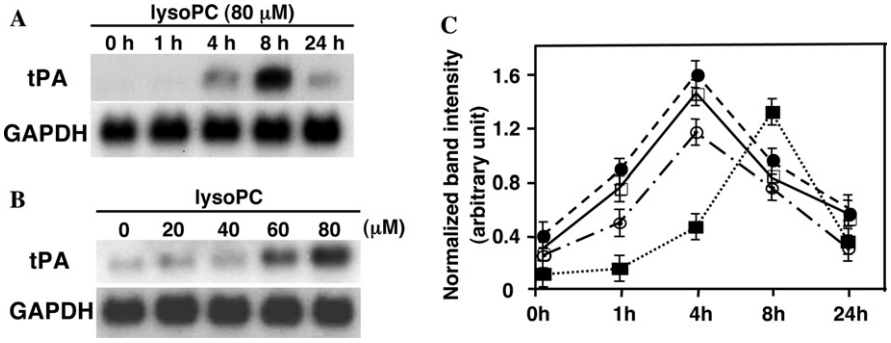

Fig. 1. LysoPC induces tPA, uPA, uPAR, and PAI-1 mRNA expression in HUVECs. (A,B) Confluent HUVEC monolayers were stimulated with lysoPC (80 μM) for the indicated time (A) or with the indicated dose of lysoPC (B). Total RNA was isolated, and the levels of tPA mRNA were analyzed by Northern blotting as described under Materials and methods. GAPDH mRNA served as a loading control. (C) HUVEC monolayers were stimulated with lysoPC (80 μM) for the indicated time. Levels of tPA, uPA, uPAR, and PAI-1 mRNA were evaluated by Northern blotting and densitometric analysis was performed. Signal intensities of mRNA were normalized with the corresponding GAPDH mRNA. The mean ± SE of the relative band intensities of tPA (■), uPA (○), uPAR (□), and PAI-1 (●) mRNA from four independent experiments is shown.

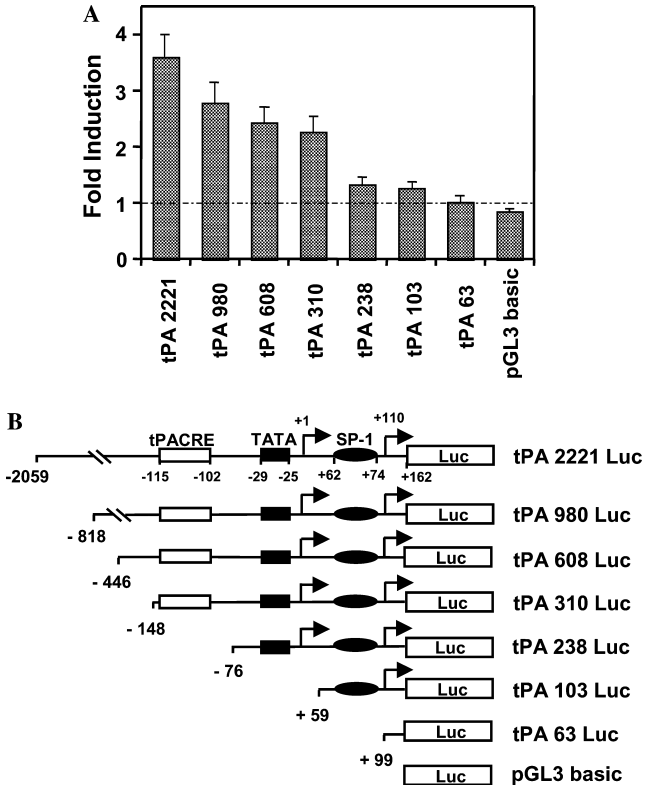

Fig. 3. LysoPC increases the promoter activity of tPA in HUVEC. (A) HUVECs were cotransfected with the tPA promoter luciferase constructs containing a tPA promoter of the indicated length (B) and pRL-CMV plasmids as an internal control. After recovery for 48 h, HUVECs were treated for 8 h, with medium alone, or 80 μM lysoPC, and the promoter activity was determined by dual-luciferase assay, as described under Materials and methods. The firefly luciferase activity of each construct was normalized to the *Renilla* luciferase activity of pRL-CMV. Results are expressed as means ± SE of the fold-induction compared to the medium-stimulated control from five to six individual experiments. (B) Schematic representation of tPA promoter constructs used in the tPA promoter activity experiments.

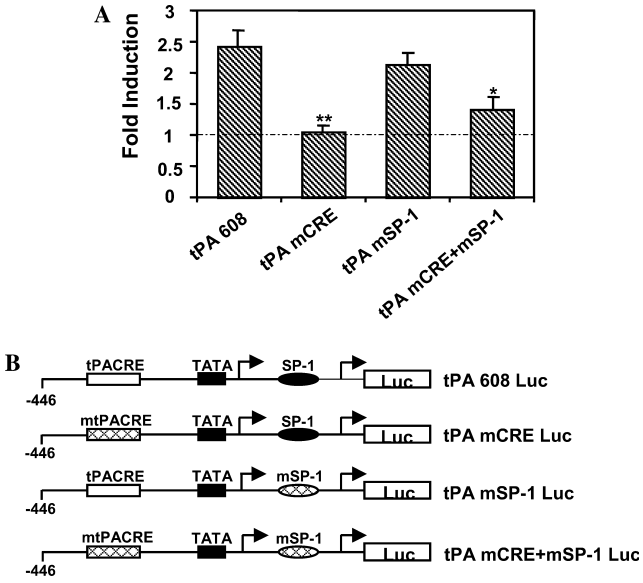

Fig. 4. Role of the tPACRE and SP-1 site in lysoPC-induced tPA promoter activity in HUVEC. (A) HUVECs were cotransfected with tPA 608 Luc, tPA mCRE Luc, tPA mSP-1 Luc, or tPA mCRE+mSP-1 Luc (B), with pRL-CMV plasmids as the internal control. After recovery for 48 h, HUVECs were treated for 8 h, with medium alone, or 80 μM lysoPC, and the promoter activity was determined by dual-luciferase assay, as described under Materials and methods. Results for each construct were normalized to the *Renilla* luciferase activity of the pRL-CMV plasmid. Data are shown as the fold induction. Results are expressed as means ± SE of four individual experiments. *$p < 0.05$ vs. tPA 608, **$p < 0.01$ vs. tPA 608. (B) Schematic representation of the tPA promoter constructs used in the mutational study in the promoter activity experiments.

and $p < 0.001$ vs. tPA 608 Luc) when the promoter region was deleted to −76 (tPA 238 Luc) (Fig. 3A). This result indicated that the region between −148 and −76 bp of the tPA promoter contained a lysoPC-responsive element. Regarding the basal expression of the tPA promoter activity, the plasmid (tPA 103 Luc) containing +59 to +162 bp of the tPA promoter showed an almost identical basal expression to tPA 2221 Luc, while the tPA promoter construct (tPA 63 Luc) containing +99 to +162 of the tPA promoter showed no basal expression (data not shown).

tPACRE is involved in lysoPC-induced tPA expression in endothelial cells

It was previously reported that the tPACRE and SP-1 site, spanning −115 to −102 and +62 to +74, respectively, are important for tPA expression in endothelial cells [29]. As shown in Fig. 3B, tPA 310 Luc contains both a tPACRE and SP-1 site, and tPA 238 Luc and tPA 103 Luc only contain an SP-1 site. Therefore, to investigate whether tPACRE is a lysoPC-responsive element, we mutated the tPACRE site (tPA mCRE Luc) or the SP-1 site (tPA mSP-1 Luc) or both (tPA mCRE+mSP-1 Luc) sites of the tPA 608 Luc plasmid (Fig. 4B). A promoter analysis study showed that mutation of tPACRE completely abolished the lysoPC-induced tPA promoter activity ($p < 0.01$ vs. tPA 608 Luc) (Fig. 4A). On the other hand, SP-1 mutation had no effect on the lysoPC inducibility (Fig. 4A). Similar to that of tPACRE mutation, mutation of both the tPACRE and SP-1 sites significantly reduced the lysoPC induced tPA promoter activity ($p < 0.05$) (Fig. 4A).

Effects of lysoPC on CREB and ATF-2 binding activity to the tPACRE element in the tPA promoter

Previous reports have shown that the tPACRE site of the tPA promoter is able to bind CREB and ATF-2, and that lysoPC can activate CREB in endothelial cells. Therefore, we performed EMSA to examine whether lysoPC increases the binding activity of CREB and ATF-2 to the tPACRE site. EMSA revealed two specific shifted bands (shifted band 1 and shifted band 2), and

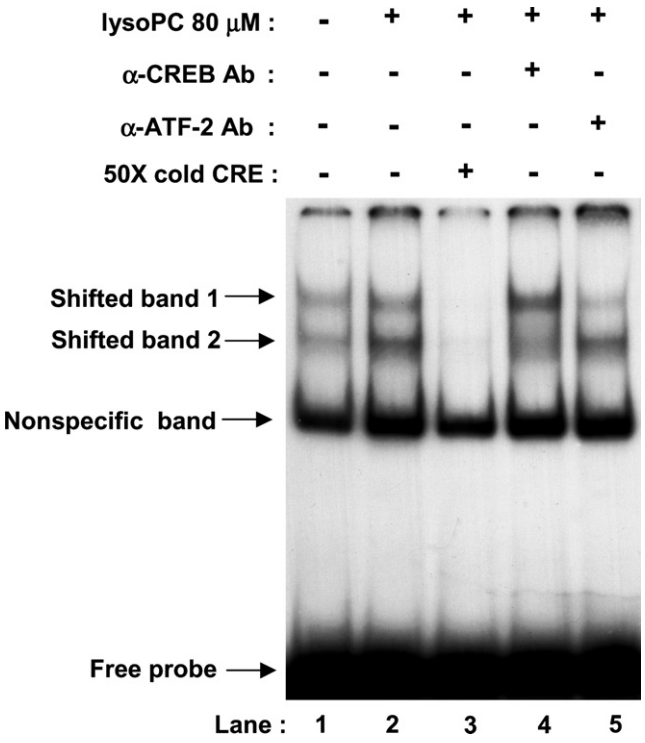

Fig. 5. LysoPC increases the CREB binding to tPACRE of the tPA promoter in HUVEC. Nuclear extracts were prepared from HUVECs treated with medium alone or lysoPC (80 μM) for 4 h. EMSA was performed using radiolabeled oligonucleotide probe corresponding to the tPACRE site of the human tPA promoter as described under Materials and methods. For the competition assay, a 50× molar excess of cold tPACRE was added along with the radiolabeled tPACRE oligonucleotide probe to the lysoPC stimulated sample (lane 3). For supershift analysis, nuclear extracts were incubated for 20 min with α-CREB antibody (lane 4), or α-ATF-2 antibody before adding the radiolabeled probe (lane 5).

their specificities were confirmed by a competition assay using a 50× molar excess of cold tPACRE oligonucleotide (Fig. 5, lane 3). LysoPC reproducibly increased the intensity of, at least, shifted band 2, and probably shifted band 1 (Fig. 5, lane 2). Supershift analysis revealed that the α-CREB antibody reduced the intensity of shifted band 2 and increased the intensity of shifted band 1 (compare lanes 2 and 4), suggesting that shifted band 2 contains CREB. Adding α-ATF-2 antibody reduced the intensity of shifted band 1, indicating that this band contains ATF-2 (compare lanes 2 and 5). Taken together, these results suggest that lysoPC increases the binding activity of CREB and probably ATF-2 to tPACRE.

Discussion

The present study demonstrated that lysoPC induced tPA protein and mRNA expression, and promoter activity in HUVECs. Furthermore, we observed that a CRE-like responsive element (tPACRE) in the tPA promoter is a lysoPC-responsive element. Previous reports have shown that tPACRE and an SP-1 binding site are important *cis*-elements in the tPA promoter in endothelial cells [21,29]. tPACRE can bind CREB, ATF-2, CREM, and c-jun, and the main protein that binds to the SP-1 site is SP-1 in HUVEC [21]. In this study, the promoter analysis of the tPA gene showed that tPACRE is required for lysoPC-induced tPA expression. We also showed that lysoPC increased the binding of CREB at tPACRE. LysoPC can activate CREB in endothelial cells through p38 and the ERK 1/2 pathway, and such activation is required for lysoPC-induced Cox-2 expression [6]. A similar signaling mechanism may be involved in the lysoPC-induced tPA expression in endothelial cells. LysoPC can also activate SP-1 and through that activation it induces eNOS in HUVEC [7]. In the present study, we showed that mutation of the SP-1 binding site has no effect on the lysoPC-induced tPA promoter activity, but tPA 238 Luc and tPA 103 Luc plasmids showed some induction due to lysoPC in deletion mutant experiments. Therefore, the possibility that the SP-1 site is also involved to a lesser extent than tPACRE in lysoPC-induced tPA expression cannot be excluded.

Deletion of the tPA promoter to +59 (tPA 103 Luc), which does not contain a TATA-dependent transcription start site, caused the promoter to become non-responsive to lysoPC, but a basal expression was maintained. It was previously reported that the tPA gene contains two transcription start sites, one is a TATA-dependent site and the other is a TATA-independent initiation site, which is 110 bp downstream of the TATA-dependent transcription start site [29]. The authors showed that endothelial cells predominantly use the TATA-independent transcription start site. Our result for tPA 103 Luc, which showed full basal transcriptional activity, indirectly supports that observation.

LysoPC induced uPA, uPAR, and PAI-1 mRNA expression in endothelial cells and showed similar kinetics, which differed from those of tPA. These results suggest that the molecular mechanisms of uPA, uPAR, and PAI-1 mRNA expression are different from that of tPA mRNA expression. The promoter regions of uPA, uPAR, and PAI-1 contain an AP-1 site and have been shown to be essential for their expression in various cell lines [30–33]. For example, the AP-1 site is required for uPA expression in the HepG2 cell line [30], PAI-1 expression in endothelial cells and smooth muscle cells [33], and PMA-induced uPAR expression in a colon cancer cell line [32]. Since lysoPC can activate AP-1 in endothelial cells [34], it may upregulate uPA, uPAR, and PAI-1 expression through the activation of AP-1. Moreover, NFκB activation is important for uPA expression in breast cancer cells, and TNFα-induced PAI-1 expression in endothelial cells [31,35]. LysoPC

activates NFκB in endothelial cells [36], through which it may influence uPA and PAI-1 expression.

The components of the plasminogen activation system are involved in several pathophysiological conditions, including tumor growth, angiogenesis, and atherosclerosis [13–20]. It is reported that both tPA and uPA are involved in the chemotaxis of smooth muscle cells [37]. Also, uPA is implicated in chemoattracting monocytes [38]. Since the accumulation of macrophages and smooth muscle cells is important for the development of atherosclerosis, lysoPC may influence the development of atherosclerosis by regulating the expression of plasminogen activators. Furthermore, extracellular matrix degradation is important for cell migration, angiogenesis, tumor growth, and invasion, and plasmin activated by plasminogen activators can degrade the extracellular matrix directly or indirectly through the activation of MMPs [13]. Therefore, by modulating the expression of members of the plasminogen activation system, lysoPC is able to regulate cell migration, angiogenesis, tumor growth, and invasion.

In conclusion, in the present study, we reported that lysoPC induced tPA, uPA, uPAR, and PAI-1 gene expression, and that tPACRE is involved in lysoPC-induced tPA expression in endothelial cells. Given the importance of the plasminogen activation system in atherosclerosis, inflammation, and tumor invasion, our study provides a new insight into the role of lysoPC in those pathological conditions.

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