Lysophosphatidylcholine phosphorylates CREB and activates the jun2TRE site of c-jun promoter in vascular endothelial cells

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Received 16 June 1999; received in revised form 22 July 1999

Abstract Lysophosphatidylcholine (lyso-PC), a polar phospholipid increased in atherogenic lipoproteins and atherosclerotic lesions, has been shown to induce transcription of a variety of endothelial genes relevant to atherogenesis. Lyso-PC has been shown to activate c-jun N-terminal kinase (JNK) and activator protein 1 (AP-1) and thereby stimulate transcription of the c-jun gene. Here we provide evidence that lyso-PC can phosphorylate cyclic AMP responsive element binding protein (CREB) and thereby activate the jun2 12-*O*-tetradecanoylphorbol 13-acetate response element (jun2TRE) site of the c-jun promoter, which appears to be the major molecular mechanism involved in lyso-PC-induced c-jun gene expression in cultured bovine aortic endothelial cells (BAEC). Transient transfection of BAEC with a 1.6-kbp c-jun promoter and luciferase reporter fusion gene resulted in a 12.9-fold increase in luciferase activity by lyso-PC treatment. Serial deletion mutation in c-jun promoter and luciferase reporter gene assay revealed that the 5′ promoter region between nucleotide numbers −268 and −127, which contains a jun2TRE binding sequence, was most crucial for lyso-PC-induced transcription. The 5′ promoter region between −76 and −27, which contains an AP-1 site, also affected lyso-PC-induced transcription of the c-jun gene. Point mutation in the jun2TRE site reduced lyso-PC-induced transcription of the c-jun promoter-luciferase fusion gene by a 70.3% decrease in c-jun promoter activity. Electrophoretic mobility shift assays showed increased binding of ^{32}P-labeled oligonucleotides with jun2TRE in nuclear extracts isolated from lyso-PC-treated BAEC, which was abolished or supershifted by anti-CREB antibody. Immunoblotting with anti-phosphorylated CREB antibody showed rapid phosphorylation of this protein after lyso-PC treatment. These results indicate that lyso-PC phosphorylates CREB, which was then bound to the jun2TRE site of the c-jun promoter and activated transcription. Activation of jun2TRE may play a key role in the transcriptional activation of c-jun as well as other endothelial genes depending upon these transcription factors.

Key words: Lysophosphatidylcholine; Phosphorylation; Jun2 12-*O*-tetradecanoylphorbol 13-acetate response element; Cyclic AMP responsive element binding protein; Activator protein 1

1. Introduction

Lysophosphatidylcholine (lyso-PC) is a prominent phospholipid component of atherogenic lipoproteins and atherosclerotic lesions [1–3]. Lyso-PC is also generated in wounds and inflammatory lesions by actions of extracellular phospholipase A activities [4]. Recent reports have shown that lyso-PC can induce expression of various genes [5–11] in cultured vascular endothelial cells and other cell types in addition to its inhibitory effects on endothelial-dependent vasorelaxation [12] and endothelial cell migration [13].

Lyso-PC has been shown to mobilize intracellular calcium [14], activate protein kinase C [15], disrupt a receptor-G protein coupling [16], elevate intracellular cyclic AMP [17], activate mitogen-activated protein kinases (MAP kinases), such as extracellular signal-regulated protein kinase (ERK) [18] and JNK [18,19], induce activator protein 1 (AP-1) DNA binding [20], activate NF-κB [20], and stimulate tyrosine phosphorylation of platelet endothelial cell adhesion molecule-1 (PECAM-1) [21] in cultured vascular endothelial cells. Furthermore, reagents that increase intracellular cyclic AMP, such as forskolin and dibutyryl cyclic AMP, suppress lyso-PC-induced expression of platelet-derived growth factor (PDGF)-B chain and intercellular cell adhesion molecule-1 (ICAM-1) [21].

C-Jun, as well as c-Fos, can be phosphorylated in response to biological stimuli and bind to AP-1 sites in promoters, thereby activating gene transcription [23]. Transcriptional regulation of the c-jun gene by ischemic reperfusion in rat kidney cells has been shown to depend upon the jun2 12-*O*-tetradecanoylphorbol 13-acetate response element (jun2TRE) site in the c-jun promoter [24]. To gain insight into the molecular mechanisms involved in lyso-PC-induced gene transcription, we have explored the transcriptional regulatory mechanisms of the c-jun gene as a model. Here, we provide evidence that lyso-PC-induced transcription of the c-jun gene depends mainly upon the jun2TRE site of its promoter. We furthermore show that lyso-PC can phosphorylate cyclic AMP response element binding protein (CREB), which is thereby bound to the jun2TRE site of the c-jun promoter.

2. Materials and methods

2.1. Reagents

Lysophosphatidylcholine (1-palmitoyl) was obtained from Avanti Polar Lipids. Antibodies directed to c-Jun (rabbit polyclonal IgG), JunB (goat polyclonal IgG), JunD (goat polyclonal IgG), c-Fos (goat polyclonal IgG), activating transcription factor 2 (ATF-2) (rabbit polyclonal IgG) and CREB (rabbit polyclonal IgG) were obtained from Santa Cruz Biotechnology. A polyclonal rabbit antibody specific for a phosphorylated (Ser-133) form of CREB (phospho-Ser133-CREB) was from Upstate Biotechnology. The gel shift assay system, pGL2 promoter vector, pRL-SV40 vector, dual-luciferase reporter assay system were obtained from Promega. [γ-^{32}P]ATP and [α-^{32}P]-dCTP were purchased from Amersham.

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PII: S0014-5793(99)01049-2

2.2. Cell culture

Bovine aortic endothelial cells (BAEC) were harvested from bovine aortas by scraping with a glass coverslip and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal calf serum (FCS), 100 U/ml of penicillin and 100 μg/ml of streptomycin, and were grown in an atmosphere of 95% air, 5% CO_2 at 37°C. Confluent BAEC with passage numbers between 5 and 20 were serum starved for 24 h and used for experiments.

2.3. Plasmid constructs

A 1590-bp fragment of the c-jun promoter was subcloned into promoterless pGL-2 luciferase plasmid. Unidirectional deletion mutants were made in the forward orientation promoter constructs: −730/+850 Jun-Luc, −268/+850 Jun-Luc, −127/+850 Jun-Luc, −76/+850 Jun-Luc and −26/+850 Jun-Luc were made. Each deleted double-stranded DNA fragment was subcloned into the *Sma*I/*Xho*I site of pGL2 promoter vector (Promega). Site-specific mutagenesis of the jun2TRE site was created by an overlapping PCR procedure [25,26]. The AP-1 site, −191 ATTACCTCATC −181, was changed to −191 TGGACCTCGAG −181 (mAP-1 Jun-Luc). The jun2TRE site, −71 TGACAT −66, was changed to −71 ATCCAC −66 (mjun2TRE Jun-Luc), as described by Stein et al. and Angel [27,28]. Each mutated double-stranded DNA fragment was subcloned into the *Mlu*I/*Bgl*II site of the pGL2 promoter vector. Nucleotide sequences of these mutant constructs were examined by the dideoxy chain termination method.

2.4. DNA transfection and luciferase assay

BAEC were transfected with 2μg of Jun-Luc and 50 ng of pRL-SV40 plasmid (Promega) by the lipofection method. Eight hours after transfection, cells were washed with PBS and replaced with DMEM with 1% FCS, and grown to confluence and near quiescence (3–4 days after transfection). After reaching confluence, BAEC were incubated for 2 h with lyso-PC (15 μM) in serum-free DMEM, subsequently incubated with DMEM in the absence of lyso-PC for an additional 6 h. Firefly and *Renilla* luciferase activities in BAEC lysates were measured using the dual-luciferase reporter assay system (Promega). Luciferase activities were normalized for protein concentrations of cell lysates. Transfection efficiencies of these plasmid constructs, which were evaluated by *Renilla* luciferase activities, were not significantly different between lyso-PC-treated and sham-treated BAEC.

2.5. Nuclear protein extraction

Nuclear protein extracts were prepared by the method of Sadowski and Gilman [29]. In brief, BAEC were washed twice with ice-cold PBS, once with PBS containing 1 mM Na_3VO_4 and 5 mM NaF (PBS+V+F), and once with hypotonic buffer which consisted of 20 mM HEPES, pH 7.9, 20 mM NaF, 1 mM Na_3VO_4, 1 mM $Na_4P_2O_7$, 1 mM EDTA, 1 mM ethylene glycol tetraacetic acid (EGTA), 1 mM DTT, 0.5 mM PMSF, 1 μg/ml of leupeptin, 4 kallikrein inhibitor units (KIU)/ml of aprotinin. Cells were then lysed with ice-cold hypotonic buffer supplemented with 0.2% Nonidet P-40 (NP-40). Cell lysates were centrifuged at maximum speed for 20 s, and the pelleted nuclei were resuspended in hypotonic buffer with 420 μM NaCl and 20% glycerol added and rocked gently for 30 min at 4°C. Soluble extracted nuclear proteins were isolated by centrifugation at maximum speed for 20 min, and the supernatant (nuclear extract) was stored at −80°C.

2.6. Gel shift assay

Gel shift assays were performed as described by Stein et al. [30]. In brief, nuclear extracts (10 μg of protein) were incubated on ice for 15 min with 2 μg of poly (dI·dC)·poly (dI·dC) in binding buffer (10 mM Tris, pH 7.9, 0.5 mM EDTA, 10% glycerol, 1 mM DTT, BSA) and then incubated with double-stranded ^{32}P-labeled oligonucleotides containing the jun2TRE binding sequence for 20 min at room temperature. The jun2TRE probe was made by annealing 5′-AGCTGCATTACCTCATCCC-3′ (−194 to −179 of the c-jun promoter underlined and core element in bold) and 5′-GATCGGGATGAGGTAATGCT-3′. The mutant jun2TRE by annealing 5′-AGCTAGCTGGACCTCGAGCC-3′ (core element in bold, mutated site underlined) and 5′-GGCTCGAGGTCCAGCTAGCT-3′ [24]. Nuclear extract-oligonucleotide mixtures were then subjected to electrophoresis through 5% (w/v) polyacrylamide gels containing 10% glycerol. Gels were dried, and analyzed by Fujix Bioimage Analyzer BAS2000 (Fuji Photo Film). Competition experiments were preincubating the extracts with unlabeled jun2TRE or mutated jun2TRE oligonucleotides for 5 min. Labeled oligonucleotide was then added for 20 min prior to electrophoresis. Antibody supershift assays were performed by adding the appropriate antibody to the extract after a 20-min preincubation of extract with labeled oligonucleotide. After 4 h at 4°C, samples were subjected to electrophoresis.

2.7. Western blot analysis

BAEC were washed twice with ice-cold PBS and lysed with lysis buffer containing 20 mM Tris (pH 7.4), 10 mM EDTA, 60 mM β-glycerophosphatase, 10 mM $MgCl_2$, 1.0% Triton X-100, 1 mM sodium vanadate, 1 mM Pefabloc SC, 1 μg/ml leupeptin and 0.1 U/ml aprotinin. Protein concentrations were determined by the Bradford method. Equal protein concentrations of the total cell lysates were subjected to SDS-polyacrylamide (8%) gel electrophoresis, and electroblotted onto nitrocellulose membranes (ECL, Amersham, UK). Membranes were incubated with optimal concentrations of a rabbit polyclonal antibody directed to phosphorylated CREB, and subsequently with peroxidase-conjugated anti-rabbit IgG (Amersham). Bands were visualized by ECL reagents (Amersham).

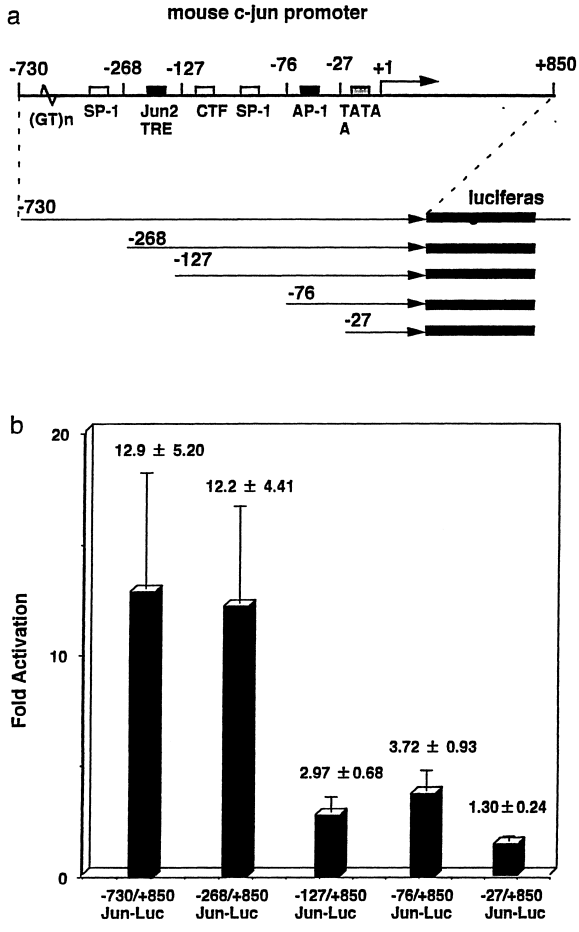

Fig. 1. Lyso-PC stimulates transcription of c-jun promoter-luciferase fusion genes. Fusion gene constructs consisting of the c-jun promoter linked to the luciferase gene, including serial deletion mutants, were constructed as indicated (A). BAECs were transiently transfected with these c-jun promoter-luciferase fusion gene constructs, and subsequently treated with or without lyso-PC (15 μM) for 2 h. After incubation without lyso-PC for an additional 6 h, the luciferase activities were measured. Each value (fold increase in luciferase activities in lyso-PC-treated BAEC as compared with BAEC treated without lyso-PC) is indicated as mean ± S.D. from six independent experiments.

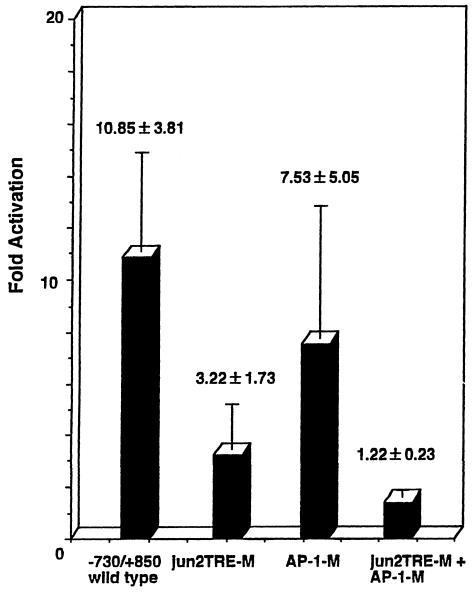

Fig. 2. Lyso-PC-induced transcription of the c-jun gene depends upon jun2TRE and AP-1 sites of the promoter. Fusion gene constructs that consist of the c-jun promoter linked to the luciferase gene, including point mutations in the jun2TRE and AP-1 sites, were constructed as described in Section 2. BAEC were transiently transfected with these c-jun promoter-luciferase fusion gene constructs, and subsequently treated with or without lyso-PC (15 μM) for 2 h. After incubation without lyso-PC for an additional 6 h, the luciferase activities were measured. Each value (fold increase in luciferase activities in lyso-PC-treated BAEC as compared with BAEC treated without lyso-PC) is indicated as mean ± S.D. from six independent experiments.

3. Results

3.1. The jun2TRE site in the c-jun promoter is crucial for lyso-PC-induced transcription of the c-jun gene

To gain insights into the molecular mechanisms involved in lyso-PC-induced transcription of the c-jun gene, a 1580-bp fragment (−730/+850) of the c-jun promoter, as well as those containing serial 5′ deletion mutations (Fig. 1A), was linked to a luciferase reporter gene and transiently transfected into BAEC. After treatment with or without lyso-PC, luciferase activities were measured. As shown in Fig. 1B, lyso-PC treatment resulted in 12.9 ± 5.20-fold and 12.2 ± 4.41-fold increases in luciferase activities in BAEC transfected with −730/+850 Jun-Luc (the full length 1580-bp c-jun promoter) and −268/+850 Jun-Luc, respectively. In contrast, −127/+850 Jun-Luc and −76/+850 Jun-Luc, which lack the jun2TRE site, showed 2.97 ± 0.68-fold and 3.72 ± 0.93-fold increases in lyso-PC-induced luciferase activities, respectively. −27/+850 Jun-Luc, which lacks both jun2TRE and AP-1 sites, did not show any significant transcriptional activation induced by lyso-PC (Fig. 1B).

Since jun2TRE and AP-1 binding sites are located in these lyso-PC responsive elements, we constructed c-jun promoter-luciferase fusion genes containing point mutations in jun2TRE and AP-1 sites. These constructs, designated

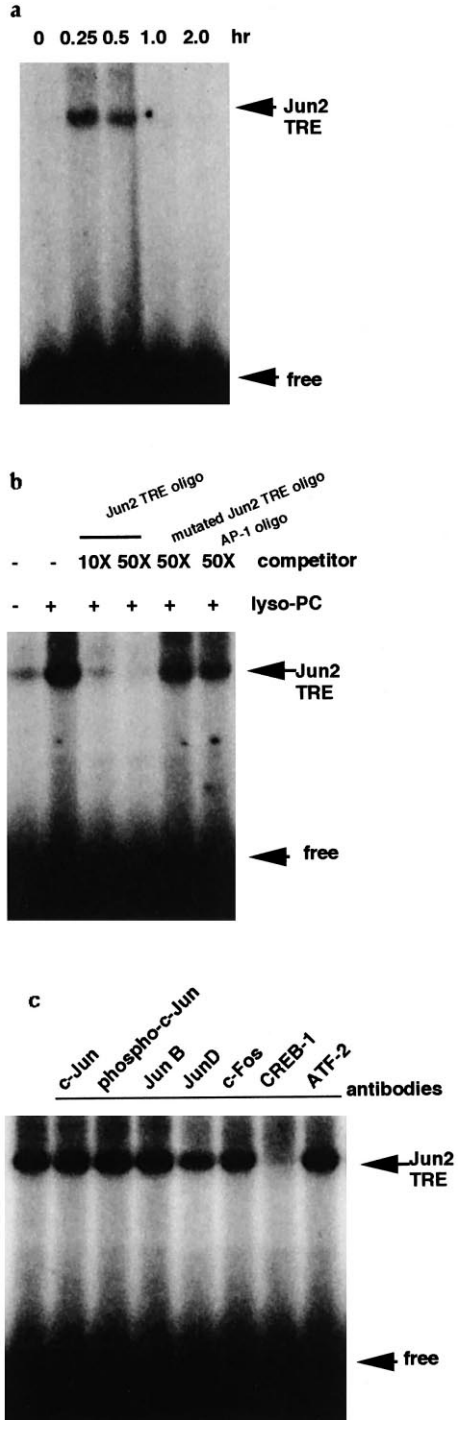

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Fig. 3. Lyso-PC stimulates CREB binding to the jun2TRE site. Gel shift assays were performed using nuclear extracts prepared from BAEC treated with 15 μM of lyso-PC for the indicated time periods (A). Nuclear extracts obtained from BAEC stimulated with 15 μM of lyso-PC for 15 min were incubated with radiolabeled jun2TRE binding oligonucleotide in the presence or absence (−) of 10-fold (10×) or 50-fold (50×) excess amounts of unlabeled jun2TRE oligonucleotide, or a 50-fold excess amount of unlabeled mutant jun2TRE oligonucleotide or AP-1 oligonucleotide (B). Nuclear extracts obtained from BAEC treated with 15 μM of lyso-PC for 15 min were preincubated with antibodies directed against c-Fos, c-Jun, phosphorylated c-Jun, JunB, JunD, ATF-2, CREB, or non-immune IgG, followed by incubation with radiolabeled jun2TRE oligonucleotide probe and gel electrophoresis, followed by electrophoresis and autoradiography (C). Jun2TRE binding is indicated by the upper arrow, and unbound oligonucleotide probe is indicated by the lower arrow. A representative figure from four independent experiments is shown.

jun2TRE-mut and AP-1-mut, resulted in 70.3% and 30.6% reductions in lyso-PC-induced luciferase activities, respectively (Fig. 2). A c-jun promoter-luciferase construct that contains point mutations in both the jun2TRE and AP-1 sites (jun2TRE-mut+AP-1-mut) exhibited a more potent inhibitory action (88.8% reduction) on lyso-PC-induced transcription (Fig. 2). These results demonstrate that both jun2TRE and AP-1 sites are essential for c-jun gene transcription induced by lyso-PC; however, the jun2TRE site appears to play a major role in this process when compared to the AP-1 site.

3.2. Nuclear extracts from lyso-PC-activated BAEC bind to jun2TRE sites

To examine whether certain transcription factors can bind to the jun2TRE site in the c-jun promoter in response to lyso-PC, gel shift assays were performed, using radiolabeled oligonucleotides with the jun2TRE sequence, on nuclear extracts isolated from lyso-PC-treated BAEC. As shown in Fig. 3A, lyso-PC induced jun2TRE shift bands in a time-dependent manner. Shift bands with jun2TRE oligonucleotides were detectable after 15 min, peaked at 30 min, and declined after 1 h. These shift bands elicited by lyso-PC appeared to be specific, because preincubation of the nuclear extracts with unlabeled jun2TRE oligonucleotides dose-dependently abolished jun2TRE shift bands (Fig. 3B).

To identify molecules that bind to the jun2TRE site in response to lyso-PC, nuclear extracts were preincubated with antibodies directed to known transcription factors, and subsequently gel shift assays were carried out. Preincubation with an antibody for CREB, but not for c-Jun, JunB, JunD, c-Fos or ATF-2, abolished the retarded band by jun2TRE oligonucleotides (Fig. 3C). Taken together, binding of CREB to the jun2TRE in the c-jun promoter is involved in lyso-PC-induced transcription of the c-jun gene.

3.3. Phosphorylation of CREB by lyso-PC

Previous studies have shown that Ser-133 of CREB can be phosphorylated and thereby bind to the cyclic AMP response element (CRE) of the chromogranin A promoter [31]. Therefore, we sought to define whether lyso-PC can phosphorylate CREB and thereby bind to the jun2TRE site. Immunoblotting with polyclonal antibodies directed to phospho-Ser133-CREB demonstrated that lyso-PC phosphorylates CREB in a time-dependent fashion (Fig. 4). Phosphorylation of CREB was detectable at 15 min after lyso-PC treatment, and remained for at least 2 h (Fig. 4). This time course of CREB phosphorylation by lyso-PC appears to be correlated with the time course of the DNA binding to the jun2TRE site (Fig. 3A).

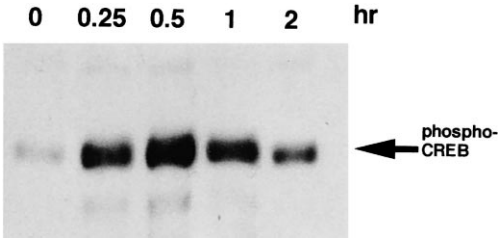

Fig. 4. Phosphorylation of CREB by lyso-PC. BAEC were treated with or without 15 μM of lyso-PC for the indicated time periods, and immunoblotting was performed using antibody directed to phosphorylated CREB.

4. Discussion

Lyso-PC, a bioactive phospholipid relevant to atherogenesis and inflammation, has been shown to stimulate transcription of a variety of genes [5–11]. We have demonstrated, for the first time, that lyso-PC can stimulate phosphorylation of CREB and transactivate the jun2TRE site of the c-jun promoter, which appears to be a major transcriptional regulatory mechanism in lyso-PC-induced c-jun gene transcription. In accordance with previous studies [20], lyso-PC also phosphorylates c-Jun and thereby activates the AP-1 site, which appears to be another transcriptional regulatory pathway involved in lyso-PC-induced transcription of the c-jun gene. These two pathways elicited by lyso-PC appear to be necessary for lyso-PC-induced transcription of c-jun, as shown by the promoter-reporter gene assay. These mechanisms may also be involved in other endothelial gene expression whose transcriptional regulation depends upon these two factors.

Although c-Jun and ATF-2 have been shown to bind to the jun2TRE site in the c-jun promoter in rat kidney cells after ischemia and reperfusion [24], our gel shift assays demonstrated that CREB, but not ATF-2 or c-Jun, could bind to the jun2TRE site in the c-jun promoter in lyso-PC-activated BAEC. A previous report by Fang et al. has shown that lyso-PC activates JNK, and thereby stimulates AP-1-dependent gene transcription in Rat-1 and HeLa cells [19]. Our studies also have shown that lyso-PC activates JNK, as well as ERK, in BAEC [19], which can subsequently stimulate phosphorylation of c-Jun.

Involvement of protein kinase C (PKC) has been implicated in lyso-PC-induced signal transduction [15]. Our previous studies, however, showed that neither lyso-PC-induced expression of ICAM-1 or PDGF-B chain, nor tyrosine phosphorylation of PECAM-1 depends upon PKC activation [21,22]. Furthermore, activation of JNK by lyso-PC appears to be independent of PKC [19,20]. Involvement of PKC in lyso-PC-induced phosphorylation of CREB remains to be determined; however, lyso-PC-induced phosphorylation of c-Jun, and subsequent transcriptional activation of the c-jun gene, as well as other jun and fos gene family members, may also be independent of PKC activation.

In addition to jun family genes, lyso-PC can also induce expression of c-fos in BAEC (data not shown) as well as in cultured vascular smooth muscle cells [32]. Phosphorylation of CREB, as well as activation of serum response factor (SRF), has been implicated in transcription activation of the c-fos gene induced by various biological stimuli [33,34]. Phosphorylation of CREB by lyso-PC, therefore, may be responsible for lyso-PC-induced transcription of c-fos. Furthermore, SRF might also be involved in c-fos gene expression elicited by lyso-PC. Further studies would be necessary to clarify the transcriptional regulatory mechanisms involved in lyso-PC-induced c-fos gene expression.

Which signal transduction cascades are involved in upstream of phosphorylation of c-Jun and CREB? Previous studies have shown that small GTP binding proteins, such as Rac/cdc42 [35] and Ras [36,37], and MAP kinase pathways, including JNK and ERK [36,38], are involved in upstream of c-Jun and CREB phosphorylation, respectively. p38 MAP kinase has also been implicated in upstream of both c-jun and CREB phosphorylation [39]. Since lyso-PC can activate JNK [18,19] and ERK [18], studies are in progress in our

laboratory to determine whether lyso-PC can active these small GTP binding proteins.

In summary, the present study provides evidence, for the first time, that lyso-PC can phosphorylate CREB, which consequently transactivates jun2TRE sites in the c-jun promoter. Lyso-PC, therefore, may also transcriptionally induce other endothelial genes relevant to atherogenesis and inflammation, whose transcriptional regulation depends upon these factors. Further studies with transcriptional regulation and upstream signal transduction cascades elicited by lyso-PC may provide new insights to understand the biological actions of this active phospholipid.

Acknowledgements: This work has been supported by Research Grants from the Ministry of Education, Science and Culture of Japan (08407026, 08670788, 09044293, 09281104, 09281103) and a Research Grant for Cardiovascular Diseases (A8-1) from the Ministry of Health and Welfare of Japan.

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