Involvement of the cAMP Response Element Binding Protein, CREB, and Cyclin D1 in LPA-Induced Proliferation of P19 Embryonic Carcinoma Cells

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Lysophosphatidic acid (LPA) is a lipid growth factor that induces proliferation of fibroblasts by activating the cAMP response element binding protein (CREB). Here, we further investigated whether LPA induces proliferation of P19 cells, a line of pluripotent embryonic carcinoma cells. 5'-Bromo-2-deoxyuridine incorporation and cell viability assays showed that LPA stimulated proliferation of P19 cells. Immunoblot experiments with P19 cells revealed that the mitogen activated protein kinases, including p-ERK, p38, pAKT, glycogen synthase kinase 3 β , and CREB were phosphorylated by treatment with 10 μ M LPA. LPA-induced phosphorylation of CREB was efficiently blocked by U0126 and H89, inhibitors of the MAP kinases ERK1/2 and mitogen- and stress-activated protein kinase 1, respectively. Involvement of cyclin D1 in LPA-induced P19 cell proliferation was verified by immunoblot analysis in combination with pharmacological inhibitor treatment. Furthermore, LPA up-regulated CRE-harboring cyclin D1 promoter activity, suggesting that CREB and cyclin D1 play significant roles in LPA-induced proliferation of P19 embryonic carcinoma cells.

INTRODUCTION

Lysophosphatidic acid (LPA; 1-acyl-2-hydroxy-sn-glycero-3-phosphate) is a lipid growth factor with diverse cellular functions, such as oncogenesis, brain development, wound healing, and immune functions by regulating various cell activities, including cell proliferation, differentiation, migration, and survival (Yang et al., 2005). LPA signals transduction through at least six specific cell membrane-bound G protein-coupled receptors, designated as LPA₁, LPA₂, LPA₃, LPA₄, LPA₅, and LPA₆ (Choi et al., 2010; Herr and Chun, 2007). LPA₁ is involved in neuronal differentiation in the developing cerebral cortex (Fukushima et al., 2007; Kingsbury et al., 2003). Binding of LPA to its cognate receptors elicits a variety of intracellular signaling cascades, including

stimulation of phospholipase C and D, inhibition of adenylate cyclase, and stimulation of small GTPase, mitogen-activated protein kinase (MAPK), and phosphoinositide 3-kinase (Herr and Chun, 2007). However, the signaling mechanism of LPA-induced cell proliferation in developing neural cells has not been elucidated. P19 cells are a multipotent carcinoma cell line derived from embryonic tissues that can grow continuously in serum-supplemented media. Retinoic acid effectively induces P19 cells into development as neurons, astroglia, and microglia cell types.

LPA rapidly stimulates cAMP response element-binding protein (CREB) phosphorylation at Ser133 in a time dependent manner. In addition, CREB is a substrate for several cellular kinases, including protein kinase A (Gonzalez and Montminy, 1989), protein kinase C (PKC) (Yamamoto et al., 1988), Akt (Du and Montminy, 1998), Ca²⁺/calmodulin-dependent protein kinases (Sheng et al., 1991), ERK/p90 ribosomal S6 kinase (p90RSK) (Xing et al., 1996), p38 MAPK/MAPKAP kinase-2 (Tan et al., 1996), and mitogen- and stress- activated protein kinase 1 (MSK-1) (Deak et al., 1998). Among these kinases, MSK-1 is an ERK- and p38 MAPK-activated kinase that is important for stress- and mitogen-induced CREB phosphorylation in fibroblasts and embryonic stem cells (Arthur and Cohen, 2000).

Cyclin D1 is a major regulator of cell cycle progression into the proliferative stage. Additionally, enhancing cyclin D1 expression is a hallmark of many cancers, particularly mammary carcinoma, in which > 50% of primary breast tumors show increased levels of the cyclin D1 protein (Sherr and Roberts, 1999). As a CRE resides 5' upstream of the mRNA start site of cyclin D1 and plays a key role in both basal and induced cyclin D1 expression (Beier et al., 1999), we were interested in whether activation of CREB might regulate cyclin D1 expression in LPA-stimulated P19 cells.

In this report, we hypothesized that LPA is capable of stimulating proliferation of P19 cells by activating CREB and cyclin D1 based on previous findings that various growth factor stimuli regulate CREB function and that LPA has lipid growth factor-

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like activity. Using the BrdU incorporation assay and phosphohistone H3 immunohistochemistry, we identified LPA-stimulated P19 cell proliferation. Immunoblot and pharmacological inhibitor experiments further revealed that several MAP kinases are activated by LPA. Involvement of cyclin D1 in the LPA-induced P19 cell proliferation signaling cascade was verified by measuring activity of the cyclin D1 promoter that exists as a CRE motif.

We provide evidence that LPA-induced P19 cell proliferation is necessary for up-regulation of cyclin D1 and that activation of ERK, p38 MAPK, and MSK-1 are responsible for CREB activation.

MATERIALS AND METHODS

Materials

LPA (1-oleoyl-2-hydroxy-sn-glycero-3-phosphate) and fatty acid-free bovine serum albumin were purchased from Sigma (USA). Antibodies to phospho-p38 MAPK, phospho-CREB, phospho-ERK1/2, phospho-AKT, phospho-MSK-1, and CREB were obtained from Cell Signaling Technology (USA). PD98059, SB203580 and wortmannin (Wt) were obtained from Tocris (USA). H89 was purchased from Biomol (Plymouth Meeting, USA). GF109203X was purchased from Calbiochem (USA). The luciferase assay system and the pGL3-basic plasmid were obtained from Promega (USA). Minimum essential medium alpha medium (α -MEM), fetal bovine serum (FBS) and bovine calf serum (BCS) were obtained from Gibco/BRL (USA). A cytotoxicity assay kit (CKK-8) was purchased from Dojindo Lab (USA). All other reagents were analytical grade or of the highest purity available.

Cell culture

P19 cells, derived from an embryonic terato-carcinoma induced in a C3H/He mouse, were grown in α -MEM supplemented with 7.5% BCS, 2.5% FBS, 100 units/ml penicillin, 100 μ g/ml streptomycin, 2 mM L-glutamine, and 2 mM sodium pyruvate in a humidified incubator with 5% CO₂ at 37°C. P19 cells were starved for LPA stimulation experiments by growing them in serum free α -MEM for 48 h. Treatment of the cells with various pharmacological inhibitors, followed by LPA stimulation, is described in the figure legends.

Cell viability assay

Cell viability was determined using a CKK-8 cytotoxicity assay kit, according to the manufacturer's protocol. Cells were plated in 96-wells to a density of 50-60% confluence. After a 48 h incubation in serum free α -MEM at 37°C, the cells were treated with LPA for 1 or 2 days. Cells were pretreated with pharmacological inhibitors (10 μ M U0126, 25 μ M PD98059, 10 μ M SB203580, 2 μ M GF109203, 25 μ M Wt, 25 μ M genistein, and 5 μ M H89) for 1 h. After treatment, CKK-8 (10 μ l) was added to each well and incubated for 3 h. A 96-well microtiter plate reader (Dynatech) was used to determine the absorbance at 450 nm. The mean concentration in each set of five wells was measured.

Western blot analysis

Cells were lysed in lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 1% Triton X-100, 1 mM Na₃VO₄, 5 mM NaF, and protease inhibitor cocktail). After a 30-min incubation on ice, the lysates were centrifuged (15,000 $\times$ g, 15 min). Supernatants were collected, and protein concentrations were determined by the Bradford assay (Bio-Rad, USA).

Equal amounts of protein were loaded and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride membranes (Millipore, USA), and blocked with 5% non-fat milk. The membranes were incubated in primary antibody overnight at 4°C, washed in TBST (10 mM Tris, 140 mM NaCl, 0.1% Tween 20, pH 7.6), incubated with the appropriate secondary antibody, and washed again in TBST. Bands were visualized by enhanced chemiluminescence and exposed to X-ray film.

Reverse transcription-polymerase chain reaction (RT-PCR) analysis

Total RNA was isolated from cells and tissue by the guanidine isothiocyanate method (Chomczynski and Sacchi, 1987), and cDNA was prepared using 1 μ g of total RNA and AMV reverse-transcriptase (Promega) in 20 μ l reaction mixtures in the presence of 2.5 μ M oligo (dT) primer and 20 μ M dNTP mixture for 60 min at 42°C. For PCR amplification, specific oligonucleotide primer pairs (0.5 μ M each) were incubated with 200 ng cDNA, 2 units of Taq polymerase (PerkinElmer, USA), 1 $\times$ Taq buffer [10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂], and 10 μ M dNTP in 20 μ l reaction mixtures. The sequences of primers used were as follows: *Lpar1* forward primer, 5'-TCTTCTGGG CCATTTTCAAC-3'; *Lpar1* reverse primer, 5'-TGCCTGAAGG TGGCGCTCAT-3'; *Lpar2* forward primer, 5'-CCTACCTCTT CCTCATGTTC-3'; *Lpar2* reverse primer, 5'-TAAAGGGTG GAGTCCATCAG-3'; *Lpar3* forward primer, 5'-AGTGTCAT ATGACAAGC-3'; *Lpar3* reverse primer, 5'-GAGATGTTGC AGAGGC-3'; *Lpar4* forward primer, 5'-TGAAGGCTTCTCC AAACGTGTCGT-3'; *Lpar4* reverse primer, 5'-GTTTCAGAGT GCAAGGCACAAGG-3'; β -actin forward primer, 5'-TGGAAAT CCTGTGGCATCCATGAAA-3'; β -actin reverse primer, 5'-TA AAACGCAGCTCAGTAACAGTCCG-3'. PCR cycling conditions for the *Lpar1*, 2, and 4 receptors were 95°C for 45 s, 55°C for 30 s, 72°C for 120 s; and for *Lpar3* they were 95°C for 45 s, 50°C for 30 s, 72°C for 120 s, for a total of 33 cycles.

Bromodeoxyuridine (BrdU) incorporation assay

P19 cells were grown on coverslips until the cells reached 50% confluence. The cells were further cultured with serum free α -MEM medium for 2 days and then treated with 10 μ M BrdU and LPA for 1 day. After fixation in 4% paraformaldehyde for 20 min, the cells were permeabilized with 0.5% Triton X-100 for 15 min. Cell nuclei were denatured with 2 N HCl for 10 min and washed three times with 0.1 M sodium borate (pH 8.5) in PBS. Following an incubation with 10% normal serum to block non-specific binding, the cells were incubated overnight at 4°C with mouse anti-BrdU antibody (1:200, Sigma) diluted in blocking solution. Then, the cells were washed three times with PBS and stained using AlexaFluor 488 conjugated to the appropriate secondary antibody (1:1000, Invitrogen, USA) at room temperature for 3 h in the dark. After air-drying, the slides were mounted using a Prolong Antifade kit (Molecular Probes, USA) and examined using a fluorescence microscope (Zeiss, Germany) with a digital camera attachment.

Immunocytochemistry

P19 cells were seeded in four-well chamber slides (Nalgene/Nunc, USA) at a density of 50-60% confluence and 37°C for 24 h. After a 24 h starvation in serum free α -MEM medium at 37°C, the cells were incubated for 24 h in serum free α -MEM medium with or without LPA. Before fixation, the cells were washed with ice-cold PBS (pH 7.4) twice. After washing, the cells were fixed in 4% paraformaldehyde and permeabilized with 0.5% Triton X-

Fig. 1. Lysophosphatidic acid (LPA)-induced P19 cell proliferation. LPA receptor gene expression was analyzed by reverse transcriptase polymerase chain reaction. Each cDNA from P19 cells was amplified using specific LPA primers. The β -actin amplification intensity was used as a relative loading control for cDNA quantity (A, B). P19 cells cultured in a 96-well plate in serum-free media were treated with vehicle or various concentrations of LPA for 24 h (C) or 48 h (D). Cell viability was determined with a CCK-8 kit. Two day serum-starved P19 cells were cultured further in the absence (E, G) or presence (F, H) of LPA (10 μ M) for 24 h. After chasing BrdU for 12 h, cells were immunolabeled with an anti-BrdU (E, F) or an anti-phospho-H3 antibody (G, H). *** p < 0.001, ** p < 0.001, * p < 0.05 vs. untreated control.

100 for 5 min at room temperature. Following three rinses in PBS, the cells were blocked with 5% bovine serum albumin in PBS (pH 7.4) for 1 h at room temperature and incubated with a primary antibody to phosphorylated histone H3 (1:200, Upstate, USA) overnight at 4°C and subsequently with AlexaFluor 568 conjugated to the appropriate secondary antibody (1:1000, Invitrogen) at room temperature for 3 h in the dark. Chamber slides were mounted using a Prolong Antifade kit. After air-drying, the slides were mounted with a Prolong Antifade kit and examined using a fluorescence microscope (Zeiss) with a digital camera attachment.

Luciferase reporter assay

P19 cells were cultured to 70-80% confluence for transfection experiments in 24-well plates. The cells were transfected with 0.8 μ g of the cyclin D1 reporter gene plus 0.2 μ g pRL-CMV (*Renilla* luciferase plasmid, Promega) using Lipofectamine 2000 (Invitrogen). After a 24-h serum starvation, the cells were stimulated with the agents for 24 h, rinsed twice with PBS, and lysed in 100 μ l of lysis buffer (Promega). Luciferase activity in the cell lysates was assayed using the Dual-luciferase Reporter Assay System (Promega) and Autolumat an Luminometer (lumet LB9507, Berthold).

Statistics

Results are presented as mean $\pm$ standard error from more than three independent experiments. Differences between groups were assessed with the paired *t*-test using Prism 4 software (GradPad Software Inc., USA).

RESULTS

LPA induces P19 cell proliferation

P19 is a line of pluripotent embryonic carcinoma cells that are capable of growing continuously in serum-supplemented media. A mitogen such as retinoic acid effectively induces several types of neural cells, including neurons, astroglia, and microglia. Here, we wondered whether LPA would have a proliferative effect on P19 cells. As shown in Fig. 1A, LPA treatment increased the number of P19 cells in a dose-dependent manner. After a 48 h treatment, cell numbers increased by more than 2.5-fold in 10 μ M LPA. LPA also increased BrdU incorporation (Figs. 1B and 1C) and histone H3 phosphorylation (Figs. 1D-1F), as revealed by immunocytochemistry with BrdU and histone H3 antisera. These data show that LPA induced P19 cell proliferation.

LPA activates components of the MAPK signaling pathway in P19 cells

To determine whether LPA-induced proliferation in P19 cells is mediated by MAPKs including ERK1/2, p38, and MSK-1, phospho-specific antibodies to ERK1/2 and p38 MAPK were used to determine changes in the phosphorylation status of these MAPK family members in P19 cells treated with 10 μ M LPA. As shown in Fig. 2A, LPA-induced ERK1/2 and p38 phosphorylation peaked at 2 min and returned to basal levels at 120 min after LPA treatment (Fig. 2A). We also examined whether LPA would induce CREB phosphorylation. As shown in Fig. 2B, CREB phosphorylation peaked at 5 min after LPA treatment and returned to basal levels. These results clearly demonstrate

Fig. 2. Phosphorylation of mitogen activated protein kinase (MAPK) components by lysophosphatidic acid (LPA). P19 cells were cultured in 60-mm dishes to near confluence and then starved in serum free α -MEM for 48 h. Cells were treated with LPA (10 μ M) for the indicated period of time. p-Akt, p-p38, p-ERK1/2, p-MSK-1, p-cAMP response element-binding protein (CREB), total ERK1/2, and total CREB were detected via immunoblot analysis.

Fig. 3. Inhibition of mitogen activated protein kinase (MAPK) prevented cell proliferation and cAMP response element-binding protein (CREB) phosphorylation. Serum starved P19 cells were stimulated with U0126 (10 μ M), SB203580 (10 μ M), wortmanin (25 μ M), genistein (25 μ M), GF (2 μ M), or H89 (20 μ M). (A) Cell viability was determined by the CCK-8 assay. (B) CREB phosphorylation level was determined by Western blotting.

that LPA rapidly activated phosphorylation of MAPK pathway components.

The MAPK signaling cascade is involved in LPA-induced P19 cell proliferation

To determine which specific MAPK is involved in LPA-induced P19 cell proliferation, various pharmacological inhibitors were tested 1 h before stimulation with 10 μ M LPA. As shown in Fig. 3A, LPA-induced P19 cell proliferation was strongly blocked by the phosphatidylinositol 3-kinase inhibitor Wt and by the MSK-1 inhibitor H89 and was partially blocked by the ERK1/2 inhibitor U0126 and the Akt inhibitor GF. These data suggest that MAPKs and Akt are involved, at least in part, in LPA-induced P19 cell proliferation.

Furthermore, to determine which MAPK is involved in LPA-induced CREB phosphorylation, we pretreated P19 cells with the specific kinase inhibitors Wt, H89, U0126, SB203580, and PD98059, 1 h before stimulation with 10 μ M LPA. As shown in Fig. 3B, LPA-stimulated CREB phosphorylation was dramatically abrogated when ERK1/2, p38, and MSK-1 were inhibited. However, CREB phosphorylation by LPA was not significantly down-regulated by Wt and GF, a PKC inhibitor (Fig. 3B).

Cyclin D1 is involved in LPA-stimulation of P19 cells through the Akt pathway

To assess whether cyclin D1, a nuclear protein, is required for cell cycle progression, and is involved in LPA-stimulated P19 cell proliferation, we treated the cells with LPA and measured

Fig. 4. Lysophosphatidic acid (LPA) increases cyclin D1 expression level and cyclin D1 promoter activity in P19 cells. (A) P19 cells were cultured in 60-mm dishes to near confluence and then starved with serum free α -MEM. Cells were treated with 10 μ M LPA for the indicated periods of time. Cells were pretreated in the absence (-) or in the presence (+) of 1 μ M wortmanin, 2 μ M GF, or 10 μ M H89 for 1 h. Thereafter, cells were treated without or with 10 μ M LPA for 2 h. Immunoblot analyses were performed with antibody to cyclin D1. (B) Cyclin D1 promoter reporter gene constructs (96 bp and 29 bp) were transiently transfected into P19 cells. Cells were starved in serum-free media for 24 h and then treated with 10 μ M LPA for 24 h prior to harvest, followed by measurement of relative luciferase activity.

the cyclin D1 protein level by immunoblot. As shown in Fig. 4A, the cyclin D1 protein level was maintained at a low level in the serum-free condition, however, it increased rapidly following treatment with 10 μ M LPA, reaching its maximal level in 2 h. LPA-stimulated cyclin D1 accumulation was inhibited by H89, an inhibitor of MSK-1, and Wt, a Akt inhibitor (Fig. 4A).

As the 5'-proximal promoter region of the cyclin D1 gene possesses a CRE, a CREB binding site, we reasoned that CREB could transcriptionally activate cyclin D1 in LPA-stimulated P19 cells (Li et al., 2003). To test this possibility, we transfected two cyclin D1 promoter-reporter constructs, one with a CRE and another without a CRE, into two separate groups of P19 cells, treated the cells with 10 μ M LPA, and measured relative luciferase activity. As shown in Fig. 4B, the cyclin D1 promoter that possessed one CRE at -52 bp from the transcription start site of the cyclin D1 gene was activated by LPA, whereas the cyclin D1 promoter-reporter construct that lacked the CRE failed to be activated by LPA. Specifically, luciferase activity of the construct with a CRE site (at position -52) increased 1.5-fold after LPA treatment compared with that in untreated controls.

DISCUSSION

LPA is a potent activator of CREB-dependent gene expression and stimulates proliferation in cancer cell lines. Our laboratory has demonstrated that LPA stimulates CREB through MSK-1 in fibroblasts (Lee et al., 2003). In the present study, LPA-stimulated CREB phosphorylation was inhibited when either ERK1/2 pathway or p38 MAPK inhibitors were tested. A further decrease in CREB phosphorylation was observed when both inhibitors were tested together. LPA-induced CREB activation was not completely lost by inhibiting both ERK1/2 and p38 MAPK. Thus, it is likely that there could be additional pathways other than ERK1/2 and p38 MAPK that are involved in LPA-induced CREB activation in P19 cells.

MSK-1 is a genuine CREB kinase and can be activated by MAPK p38. Additionally, the ERK- and p38 MAPK-activated kinases, MSK-1 and MSK-2, mediate stress-induced CREB phosphorylation in embryonic fibroblasts (Wiggin et al., 2002). We used a pharmacological inhibitor of cAMP-dependent kinase A that inhibits MSK-1 activity to identify the role of MSK

in CREB activation in P19 cells. As a result, H89 significantly inhibited LPA-stimulated CREB phosphorylation in a dose-dependent manner. These data suggest that LPA-stimulated activation of the ERK1/2 and p38 MAPK pathways stimulated CREB phosphorylation via the common downstream CREB kinase MSK-1.

LPA promoted proliferation of embryonic carcinoma P19 cells through the CREB pathway, regulated CREB phosphorylation, and had a mitogenic effect on P19 cells. Expression of cyclin D1 is a key step in mitogenic signaling, including established or potential binding sites for the transcription factors AP1, Ets-1, NF-kB, SP-1, TCF/LEF, Oct-1, ATF-2, and CREB (Tetsu and McCormick, 1999). To clarify the molecular mechanism of cyclin D1 expression during P19 cell growth, we investigated the binding of nuclear protein to the cyclin D1 gene promoter domain. The CRE, located upstream of the mRNA start site, has a key role inducing cyclin D1 expression. LPA increased transcriptional activity of the cyclin D1 reporter gene, an effect that was dependent upon the CRE. Overexpression of the cyclin D1 protein is found in many human cancers and is particularly linked to oncogenic transformation in mammary epithelium (Wang et al., 1994). Here, we showed that LPA is a potentially important activator regulating cyclin D1 expression in a cell line derived from a mouse embryonic carcinoma. Cyclin D1 includes a potential binding site for the transcription factor CREB, which is regulated by LPA. In addition, CREB is phosphorylated by several cellular kinases, including p90RSK and MSK-1. Among these CREB kinases, MSK-1 is activated by both ERK1/2 and p38 MAPK.

Other signaling component candidates for LPA-induced CREB phosphorylation might include PKC pathway members that lead to Ras activation and stimulation of MAPK activity (van Biesne et al., 1996). In addition, PKC members mediate p38 MAPK activation by a yet unidentified mechanism (Clerk et al., 1989). Another clue of the involvement of PKC in regulating cyclin D1 transcription is found in airway smooth muscle (Page et al., 2002). Our present data show that LPA-induced CREB phosphorylation was blocked by a PKC inhibitor, which consistently inhibited cyclin D1 activity. Therefore, PKC is involved in LPA signaling.

Interestingly, we found that GF, a PKC inhibitor, partially pre-

vented LPA-induced P19 cell proliferation and cyclin D1 protein expression. Some previous studies have demonstrated that glycogen synthase kinase 3 β (GSK-3 β) may play an important role in cell cycle arrest. In a future study, we will determine the relationship between GSK-3 β and cyclin D1, and the role of the PKC-dependent GSK-3 β signaling pathway in LPA-induced P19 cell proliferation.

In conclusion, we found that CREB was an important component of the transcriptional regulatory element necessary for activating cyclin D1 in LPA-stimulated P19 cells, and that CREB phosphorylation induced by MSK-1 was associated with up-regulation of P19 cell proliferation by LPA.

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