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Upregulation of endothelin-1 production by lysophosphatidic acid in rat aortic endothelial cells

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Abstract

Addition of lysophosphatidic acid (LPA) to rat aorta-derived endothelial cells significantly induced preproendothelin-1 (preproET-1) mRNA expression. PreproET-1 mRNA levels reached a plateau within 1 h after the addition of 0.5 μM LPA and declined after 2 h. The induction was superinduced by cycloheximide and was blocked by actinomycin D. Suramin, an LPA receptor antagonist, abolished the induction of preproET-1 mRNA by LPA. Protein kinase C inhibitors, H7 and bisindolylmaleimide, were able to block the induction. Transient transfection experiment revealed that the elevated preproET-1 mRNA was a result of the activation of ET-1 gene activity. Electrophoretic mobility shift assay revealed that LPA stimulated the binding of AP-1. The secreted level of ET-1 was elevated 2.3-fold after 12 h of stimulation with LPA. Our results suggest that the upregulation of preproET-1 by LPA may serve to augment and prolong the vasoconstriction action of LPA.

Keywords: Lysophosphatidic acid; Endothelin-1; Protein kinase C; Aortic endothelial cell; (Rat)

1. Introduction

Endothelin-1 (ET-1) is a very potent vasoconstrictor that has been initially isolated from the supernatant of cultured porcine endothelial cells [1]. The mRNA for preproET-1 is expressed in porcine aortic endothelial cells in vivo [2] and human umbilical vein endothelial cells in vitro [3]. Many physiological stimuli were found to increase ET-1 production and mRNA level, including thrombin [4], calcium ionophore [5], tumor promoter [5], insulin [6], bradykinin [7], transforming growth factor-β [8], and interleukin-1 [9]. We reported that angiotensin II stimulated ET-1 production in rat heart endothelial cells [10].

Lysophosphatidic acid (LPA) is a bioactive phospholipid that is released from activated platelets [11]. LPA has been reported to be involved in a number of biological processes, including mitogenesis, adhesion, and chemotaxis (for reviews, see [12–14]). LPA receptors have been cloned by several groups of investigators [15–17]. The receptor couples to distinct G proteins and stimulates the activation of Ras and Rho proteins via different pathways [12,13].

LPA was found to be the lipid component in soybean extract that was responsible for the vasopressor activity in rats [18]. LPA was also found to exert a vasopressor effect on spontaneously hypertensive rats and Wistar Kyoto rats [19]. These observations prompted us to examine whether the effect of LPA

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involves the induction of some other vasoconstriction agents. Our results show that exposure of rat aorta-derived endothelial cells (RAEC) to LPA is associated with a rapid induction of preproET-1 mRNA. The increase in the expression and release of ET-1 may augment or prolong the action of LPA.

2. Materials and methods

2.1. Cell culture and materials

RAEC cultures were provided by Diglio et al. [20]. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Life Technologies) containing 10% fetal bovine serum (FBS), 2 mM L-glutamine, and 25 μg/ml gentamycin at 37°C in a humidified atmosphere of 5% CO_2. Cells were serially subcultured by treatment with 0.05% Trypsin and used for experimentation between passages 12 and 20. LPA, salmon testes DNA, polydI·polydC, and H7 were obtained from Sigma (St. Louis, MO). [^{32}P]dCTP and [γ-^{32}P]ATP were obtained from ICN (Costa Mesa, CA). Ultraspec II reagent was obtained from Biotecx (Houston, TX). ITS was purchased from Collaborative Biomedical (Bedford, MA). Fetal bovine serum was obtained from Biofluids (Rockville, MD). T4 kinase and AP-1 consensus sequence were obtained from Promega (Madison, WI). Bisindolylmaleimide-HCl (BIM) and suramin were purchased from Calbiochem-Nova Biochem (La Jolla, CA). Lipofectin and Opti-MEM were purchased from Life Technologies (Grand Island, NY). Endothelin ELISA kit was purchased from Cayman (Ann Arbor, MI). All other reagents used were of the highest grade commercially available.

2.2. Northern blot analysis

Cells were seeded into 100 mm dishes containing DMEM and 10% FBS. Near confluent cells were changed to serum-free DMEM supplemented with ITS (1 μM insulin, 6.25 μg/ml transferrin and 6.25 ng/ml selenous acid) for 24 h before the addition of test agents. After the designated time, total RNA was extracted using Ultraspec II reagent. Fifteen μg of RNA was denatured and separated by electrophoresis on 1% agarose gel containing 2.2 M formaldehyde. Following transfer, the RNA was covalently bound to GeneScreen (NEN, Boston, MA) by UV-crosslinking. Hybridization and washing conditions were carried out according to the method of Church and Gilbert [21]. A rat ET-1 cDNA probe was generously provided by Dr. Tomoh Masaki and Dr. M. Yanagisawa (Kyoto University) [22]. The cDNA was labeled with [^{32}P]dCTP using a random primer kit from Amersham. The blots were exposed to Kodak XAR-5 films with an intensifying screen. The intensity of the bands was quantitated by a Digital Imaging System (Alpha Innotech). The experiments were repeated three to four times and representative data were shown.

2.3. ET-1 promoter activity

RAEC cultures in 60 mm plates were transfected with 4 μg of 4.4 kb ET-1 promoter coupled to chloramphenicol acetyltransferase (CAT) gene (provided by Dr. Mu-En Lee, Harvard Medical School) [23], pSV0CAT, or pSV2CAT. Transfection was carried out with 32 μg of lipofectin reagent in the presence of Opti-MEM for 6 h. The medium was changed to DMEM containing 10% FBS for 16 h. The cultures were then incubated with serum-free DMEM in the presence or absence of 0.5 μM LPA. After 36 h, cell lysates were prepared, and aliquots containing the same amount of protein were assayed for CAT activities as described by Gorman et al. [24]. CAT activities were quantitated by counting the radioactivity excised from the spots corresponding to chloramphenicol and acetylated chloramphenicol.

2.4. Electrophoretic mobility shift assay

Nuclear extracts from control and LPA-treated cells were prepared according to Andrews and Faller [25]. AP-1 consensus sequence was end-labeled with [γ-^{32}P]ATP and T4 kinase. Binding reaction (20 μl) containing 5 μg of nuclear extract, 1 μg testes DNA, 1 μg polydI·polydC in 10 mM HEPES (pH 7.9), 10% glycerol, 100 mM NaCl, 0.5 mM $MgCl_2$, 0.8 mM EDTA, 0.2 mM dithiothreitol and 0.08 mM phenylmethylsulfonyl fluoride, was preincubated at room temperature with or without unlabeled AP-1 for 15 min. ^{32}P-Labeled AP-1 (90 000 cpm) was then added to the reaction and incubated at room temper-

ature for another 20 min. The samples were resolved on native 5% polyacrylamide gel at 4°C with 0.5×TBE (1×TBE: 0.045 M Tris-borate, 1 mM EDTA). The gel was dried and analyzed by autoradiography.

2.5. Secreted levels of ET-1

RAEC cultures were plated in DMEM containing 10% FBS in 35 mm dishes. Near-confluent cells were maintained in serum-free DMEM with the addition of ITS for 24 h before the addition of 1 μM LPA for 12 h. ET-1 released into the medium was measured by a commercially available ELISA kit (Cayman) using a Bio-Tek model EL311 microplate reader (Winooski, VT). The experiment was performed four times.

2.6. Statistical analysis

Statistical analysis was made by the Student's *t*-test for comparison of any two sample means. Values are expressed as means ± S.E.M. *P*-values < 0.05 were considered statistically significant.

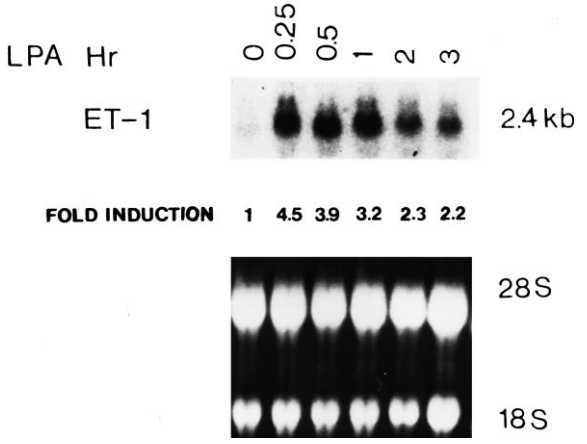

Fig. 1. Time course of preproET-1 mRNA induction in RAEC cultures by LPA. Near-confluent cultures were exposed to LPA (1 μM) for the times indicated. Northern blot analysis was performed as described in Section 2. Hybridization pattern of control cells (lane 1); cells treated with LPA for 15 min (lane 2); 30 min (lane 3); 1 h (lane 4); 2 h (lane 5); and 3 h (lane 6); are shown. Fold of induction was determined by densitometric scanning of preproET-1 mRNA band.

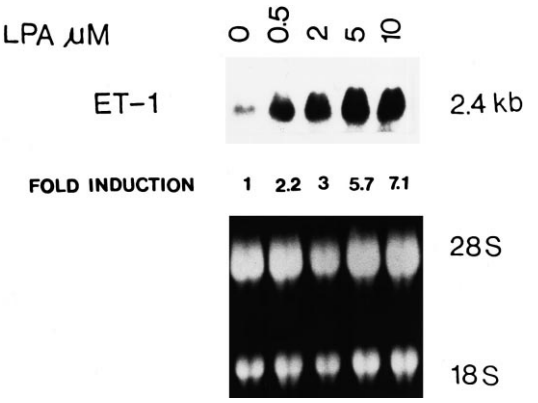

Fig. 2. Induction of preproET-1 mRNA as a function of LPA dose. Near-confluent RAEC cultures were treated with different concentrations of LPA for 1 h. Northern blot analysis was performed as described in Section 2. Hybridization patterns of control cells (lane 1); cells treated with 0.5 μM (lane 2); 2 μM (lane 3); 5 μM (lane 4); 10 μM LPA (lane 5); are shown. Fold of induction was determined by densitometric scanning of preproET-1 mRNA band.

3. Results

Steady-state levels of preproET-1 mRNA were determined in RAEC cultures by Northern blot analysis. A specific transcript of preproET-1 (2.4 kb) was observed. The basal level of preproET-1 mRNA was low (Fig. 1, lane 1). The mRNA level increased rapidly upon the addition of LPA. Levels of preproET-1

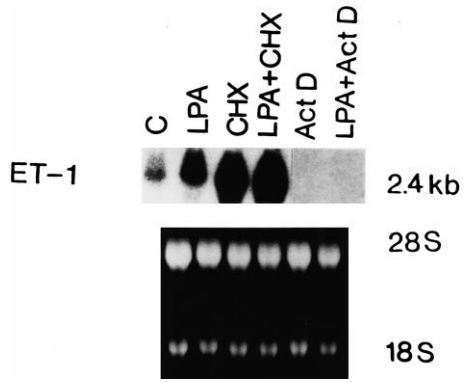

Fig. 3. Effect of cycloheximide (CHX) and actinomycin D (ActD) on the induction of preproET-1 mRNA by LPA. Near-confluent RAEC cultures were pretreated with CHX (20 μg/ml) or ActD (1 μg/ml) for 30 min before the addition of 1 μM LPA for 1 h. Northern blot analysis was performed as described in Section 2. Hybridization patterns of control cells (lane 1); cells treated with 1 μM LPA (lane 2); CHX (lane 3); LPA and CHX (lane 4); ActD (lane 5); LPA and ActD (lane 6); are shown.

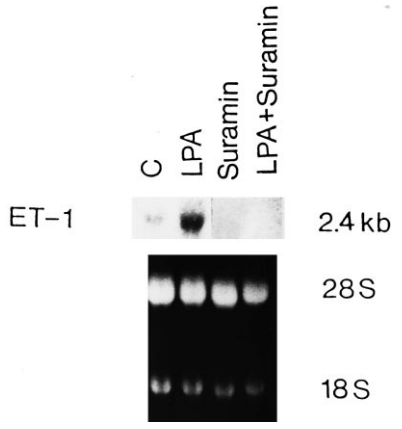

Fig. 4. Effect of suramin, an antagonist of LPA receptor, on the induction of preproET-1 mRNA. RAEC cultures were pretreated with 100 μM suramin for 1 h before the addition of LPA for 1 h. Northern blot analysis was performed as described in Section 2. Hybridization patterns of control cells (lane 1); cells treated with 1 μM LPA (lane 2); suramin (lane 3); LPA and suramin (lane 4); are shown.

mRNA increased 4.5-fold at 15 min (lane 2) and declined over 2 h (lane 5). As shown in Fig. 2, preproET-1 mRNA responded to LPA in a dose-dependent manner. When the cells were stimulated with 0.5 and 10 μM LPA, preproET-1 mRNA level increased from 2.2- to 7.1-fold.

The next series of experiments were performed to examine the mechanism of preproET-1 mRNA induction. Inhibitors of protein and RNA synthesis

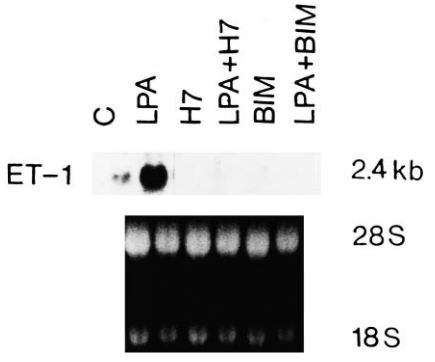

Fig. 5. Effect of PKC inhibitors on the induction of preproET-1 mRNA by LPA. Near-confluent RAEC cultures were pretreated with 5 μM H7 or 10 μM BIM for 30 min before the addition of 1 μM LPA for 1 h. Northern blot analysis was performed as described in Section 2. Hybridization patterns of control cells (lane 1); cells treated with 1 μM LPA (lane 2); H7 (lane 3); LPA and H7 (lane 4); BIM (lane 5); LPA and BIM (lane 6); are shown.

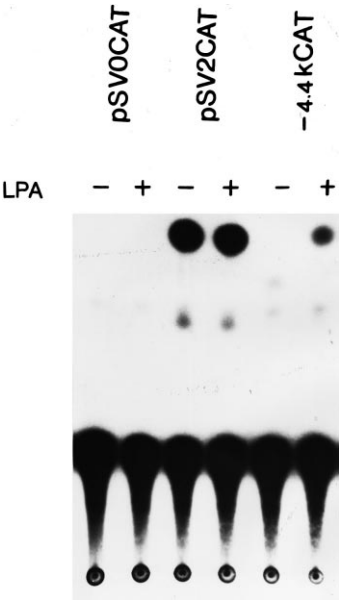

Fig. 6. Stimulation of ET-1 promoter activity by LPA. RAEC cultures in 60 mm plates were transfected with 4 μg of either pSV0CAT, pSV2CAT or ET-1 promoter (−4.4kCAT) and 32 μg of Lipofectin reagent for 6 h. The medium was then changed to DMEM in the presence or absence of 1 μM LPA for 36 h prior to harvest for CAT assays. CAT activity of cells transfected with pSV0CAT (lanes 1 and 2); pSV2CAT (lanes 3 and 4); and ET-1 promoter (lanes 5 and 6); are shown. Lanes 1, 3 and 5 represent the pattern in the absence of LPA; lanes 2, 4 and 6 represent the pattern in the presence of LPA.

were included singly or in combination with LPA. Cycloheximide was included to examine whether the induction of preproET-1 gene is a direct effect of LPA or whether intermediate protein synthesis is required. Cycloheximide alone induced preproET-1 mRNA (Fig. 3, lane 3). In the presence of LPA and cycloheximide (lane 4), the induction was higher than LPA alone (lane 2), implying that the induction does not require de novo protein synthesis. In the

Fig. 7. Activation of AP-1 binding by LPA. RAEC cultures were treated with or without 1 μM LPA for 10 min before the nuclear extracts were prepared for EMSA. Gel shift patterns of nuclear extract from control cells (lane 1); and cells treated with 1 μM LPA for 10 min (lane 2); are shown. Lanes 3 and 4 represent the binding pattern in the presence of 200-fold excess of unlabeled AP-1.

presence of actinomycin D, the induction was abolished (lane 6), suggesting that de novo RNA synthesis is required.

Suramin, a specific antagonist for LPA receptor [26], was included to examine if the effect of LPA is receptor-mediated. Fig. 4 shows that 100 μM suramin pretreatment for 1 h could block the induction (lane 4), indicating that the induction of preproET-1 mRNA is mediated by the LPA receptor.

One of the pathways of LPA is the activation of phospholipase C, which in turn stimulates the activity of protein kinase C (PKC) [14]. To study whether the induction of preproET-1 mRNA involves PKC, specific inhibitors of PKC were included. In this experiment, cells were pretreated with 5 μM H7 [27] or 10 μM bisindolylmaleimide (BIM) [28] for 30 min before the addition of LPA. Fig. 5 shows that both H7 and BIM could block the induction of preproET-1 mRNA (lanes 4 and 6), suggesting that the effect of LPA is mediated by a PKC-dependent pathway.

Expression of elevated ET-1 mRNA level after LPA treatment may be the result of increased stability of the message and/or activation of ET-1 gene activity. To test whether the transcription rate of ET-1 is altered by LPA, transfection experiments were carried out with ET-1 promoter coupled to the CAT gene in the absence or presence of LPA. Fig. 6 shows that LPA increased CAT activity of ET-1 promoter by four-fold (lanes 5 and 6). LPA had no effect on the CAT activities in cells transfected with pSV0CAT (lanes 1 and 2) or pSV2CAT (lanes 3 and 4).

In order to examine the involvement of transcription factor AP-1, EMSA was carried out on nuclear extracts prepared from RAEC cultures treated with or without LPA. Binding of AP-1 was activated after 10 min stimulation with LPA (Fig. 7, lane 2). The specificity of the assay was demonstrated by the disappearance of the band after the addition of excess unlabeled AP-1 (lanes 3 and 4).

The release of ET-1 in response to LPA was determined in the conditioned medium using an ELISA kit. The amount of ET-1 released from cells treated with LPA for 12 h was 2.3-fold that of the control cells (108 ± 11 vs 47 ± 7 pg per 10^4 cells, respectively, $P < 0.05$).

4. Discussion

The present study demonstrated that LPA acts on RAEC culture to induce preproET-1 mRNA in a dose- and time-dependent manner. The induction is mediated by LPA receptor and involves a PKC-dependent pathway. In addition, the increase of ET-1 mRNA correlated well with the release of ET-1 into the conditioned medium. It is noted that the induction of preproET-1 mRNA begins with 0.5 μM LPA. This concentration is close to the physiological level of LPA [11].

The level of preproET-1 mRNA is 'superinduced' in the presence of cycloheximide and LPA. PreproET-1 mRNA in the presence of cycloheximide alone is higher than the basal level. Yanagisawa et al. [1] reported the 3′-end of preproET-1 mRNA contains several 'AUUUA' motifs that confer mRNA instability.

Our data demonstrated that the induction of preproET-1 mRNA by LPA could be attributed partially to transcriptional activation. The promoter of the ET-1 gene has been shown to contain AP-1 sites [29,30]. Our EMSA results showed that AP-1 binding was activated after 10 min of stimulation with LPA. Other elements responsible for the transcriptional activation are under investigation.

LPA has been shown to activate a variety of second messenger pathways, including activation of PKC [14,31]. Yakubu et al. reported that the addition of factors produced by hemorrhaged blood (such as LPA, serotonin, and thromboxane receptor agonist U-46619), to piglet cerebral microvascular endothelial cells resulted in an enhanced ET-1 production. These authors concluded that ET-1, together with cerebral hematoma, could contribute to enhanced cerebral constriction [32]. In this study we demonstrate in RAEC cultures that LPA induces ET-1 production by transcriptional activation. The induction is mediated by a PKC-dependent pathway.

In conclusion, we observed that ET-1 level is elevated in LPA-treated RAEC cultures. The enhanced production of ET-1 may augment and prolong the action of LPA.

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