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Lysophosphatidic Acid Inhibits Melanocyte Proliferation *via* Cell Cycle Arrest

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Lysophosphatidic acid (LPA) is a well-known mitogen in various cell types. However, we found that LPA inhibits melanocyte proliferation. Thus, we further investigated the possible signaling pathways involved in melanocyte growth inhibition. We first examined the regulation of the three major subfamilies of mitogen-activated protein (MAP) kinases and of the Akt pathway by LPA. The activations of extracellular signal-regulated protein kinase (ERK) and c-Jun *N*-terminal kinase (JNK) were observed in concert with the inhibition of melanocyte proliferation by LPA, whereas p38 MAP kinase and Akt were not influenced by LPA. However, the specific inhibition of the ERK or JNK pathways by PD98059 or D-JNKI1, respectively, did not restore the antiproliferative effect. We next examined changes in the expression of cell cycle related proteins. LPA decreased cyclin D_1 and cyclin D_2 levels but increased $p21^{WAF1/CIP1}$ (p21) and $p27^{KIP1}$ (p27) levels, which are known inhibitors of cyclin-dependent kinase. Flow cytometric analysis showed the inhibition of DNA synthesis by a reduction in the S phase and an increase in the G_0/G_1 phase of the cell cycle. Our results suggest that LPA induces cell cycle arrest by regulating the expressions of cell cycle related proteins.

Key words: Lysophosphatidic acid, Proliferation, ERK/JNK, Cell cycle

INTRODUCTION

Lysophosphatidic acid (LPA) is one of the lipid growth factors, which mediates various biological activities including, proliferation, differentiation and survival (Karliner *et al.*, 2001; Mattingly *et al.*, 1999; Retzer and Essler, 2000; Ye *et al.*, 2002). It has been reported that LPA plays a role in inflammation and atherogenesis (Fueller *et al.*, 2003), and that it stimulates cell proliferation in cultured fibroblasts (Tigyi *et al.*, 1994), human keratinocytes (Piazza *et al.*, 1995), and human colon carcinoma cells (Shida *et al.*, 2003). Thus, it is generally believed that LPA is an important mediator of mitogenesis. However, it has also been reported that LPA potently inhibits the proliferation of Sp2 myeloma cells (Tigyi *et al.*, 1994). These findings demonstrate that the biological effects of LPA are cell type-dependent. However, the role of LPA in melanocytes has received little attention. Therefore, we investigated the effects of LPA on the proliferation of a mouse melanocyte cell line, Mel-Ab. To the best of our knowledge, this is the first study to examine the effects of LPA on melanocyte proliferation. Interestingly, we found that LPA inhibits melanocyte proliferation. Since the antiproliferative signaling mechanism of LPA is poorly understood, we further examined the signaling pathways related to cell proliferation.

The activation of the Ras-Raf-extracellular signal-regulated protein kinase (ERK) cascade plays a critical role in the mitogenic signaling of a number of growth factors (Blenis, 1993; Blumer and Johnson, 1994), and the Akt pathway has also been implicated in cell growth stimulation (Dufourny *et al.*, 1997; Kim *et al.*, 2004; Kim *et al.*, 2001). Furthermore, c-Jun *N*-terminal kinase (JNK) and p38 mitogen-activated protein (MAP) kinase are also known to regulate cell proliferation (Douziech *et al.*, 1999; Goss *et al.*, 2003). Therefore, we investigated whether these signaling pathways are involved in LPA-mediated cellular

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responses.

D-type cyclins like cyclin D_1 and cyclin D_2 are known to be important for cell cycle progression (Blagosklonny and Pardee, 2002; Prietzsch *et al.*, 2002). In contrast, the cyclin-dependent kinase inhibitors, $p21^{WAF1/CIP1}$ (p21) and $p27^{KIP1}$ (p27) cause cell cycle arrest (Gartel and Tyner, 2002; Tang *et al.*, 2002; Weiss and Randour, 2000). To explain the antiproliferative effect of LPA in terms of cell cycle regulation, we also studied changes in the expression of cell cycle related proteins.

MATERIALS AND METHODS

Materials

LPA was obtained from Avanti Polar Lipids (Alabaster, AL, USA), and fatty acid-free bovine serum albumin (BSA), 12-*O*-tetradecanoylphorbol-13-acetate (TPA) and cholera toxin (CT) from Sigma (St. Louis, MO, USA). The selective JNK inhibitor (D-JNKI1) and pertussis toxin (PTX) were obtained from Alexis (San Diego, CA, USA), PD98059 from Cell Signaling Technology (Beverly, MA, USA), antibodies recognizing phospho-specific Akt (Ser473, #9271S), phospho-specific ERK1/2 (Thr202/Tyr204, #9101S) and total (phosphorylated and non-phosphorylated) ERK1/2 (#9102) were purchased from Cell Signaling Technology. Antibodies recognizing phospho-specific JNK1/2 (Thr183/Tyr185, G-7, sc-6254), total JNK2 (D-2, sc-7345), p21 (sc-397), p27 (sc-528), cyclin D_1 (sc-6281), and actin (I-19) were obtained from Santa Cruz Biotech Inc. (Santa Cruz, CA, USA), and cyclin D_2 was purchased from Pharmingen (San Diego, CA, USA).

Cell cultures

The Mel-Ab cell line is a mouse-derived spontaneously immortalized melanocyte cell line, which produces large amounts of melanin (Dooley *et al.*, 1994). Mel-Ab cells were incubated in DMEM supplemented with 10% fetal bovine serum (FBS), 100 nM TPA, 1 nM CT, 50 μg/mL streptomycin, and 50 U/mL penicillin at 37°C in 5% CO_2.

Cell viability assay

Cell viability was determined by crystal violet assay (Dooley *et al.*, 1994). After incubating with LPA for 24 h, the culture medium was removed. Then, cells were stained with 0.1% crystal violet in 10% ethanol for 5 min at room temperature, and rinsed four times. The crystal violet retained by adherent cells was then extracted with 95% ethanol, and absorbance was determined at 590 nm using an ELISA reader (TECAN, Salzburg, Austria).

MTT dye-reduction assay for the determination of proliferation

Cells (1×10^4 cells/well), seeded into 24-well plates for 24 h, were incubated with LPA in DMEM containing 2% FBS for 72 h at 37°C in 5% CO_2. After adding 100 μL/well of MTT solution (5 mg/mL), the plates were incubated for another 4 h. Supernatants were then removed and the formazan crystals were solubilized in 1 mL of dimethylsulfoxide. Optical density was determined at 540 nm using an ELISA reader.

Western blot analysis

Cells were lysed in cell lysis buffer [62.5 mM Tris-HCl (pH 6.8), 2% SDS, 5% β-mercaptoethanol, 2 mM phenylmethylsulfonyl fluoride, protease inhibitors (Complete™, Roche, Mannheim, Germany), 1 mM Na_3VO_4, 50 mM NaF, and 10 mM EDTA]. Ten micrograms of protein per lane was separated by SDS-polyacrylamide gel electrophoresis and blotted onto PVDF membranes, which were saturated with 5% dried milk in Tris-buffered saline containing 0.4% Tween 20. Blots were incubated with the appropriate primary antibodies at a dilution of 1:1000, and then further incubated with horseradish peroxidase-conjugated secondary antibody. Bound antibodies were detected using an enhanced chemiluminescence plus kit (Amersham International, Little Chalfont, U.K.).

Cell cycle analysis

Cells were trypsinized, adjusted to 5×10^5 to 1×10^6 cells/tube, washed with ice-cold phosphate-buffered saline (PBS), and re-suspended in 2 mL of methanol. After incubation at 4°C for 1 h, the methanol was removed, and 100 μL of ribonuclease solution (10 mg/mL) was added to each test tube. The tubes were re-incubated at room temperature for 30 min. Five hundred microliters of the analysis solution (3.7 mg EDTA and 100 μL Triton X-100 per 100 mL PBS) and 100 μL propidium iodide solution (400 μg/mL) were then added. Samples were stored in the dark at 4°C and analyzed using a flow cytometer (FACSCalibur™, Becton Dickinson, San Jose, CA, USA). The data obtained were analyzed using a computer with specialized software (ModFit LT™ version 1.0, Verity Software House, Topsham, ME, USA). The proportion of cells in the G_0/G_1, S, G_2/M phases and apoptotic cells with low DNA contents were determined.

Statistics

Differences between results were assessed for significance using the Student's *t*-test.

RESULTS

Effects of LPA on Mel-Ab proliferation

Melanocyte proliferation was examined in the presence of 0.1-20 μM of LPA by MTT assay (Fig. 1A). LPA was found to inhibit the proliferation of Mel-Ab cells signifi-

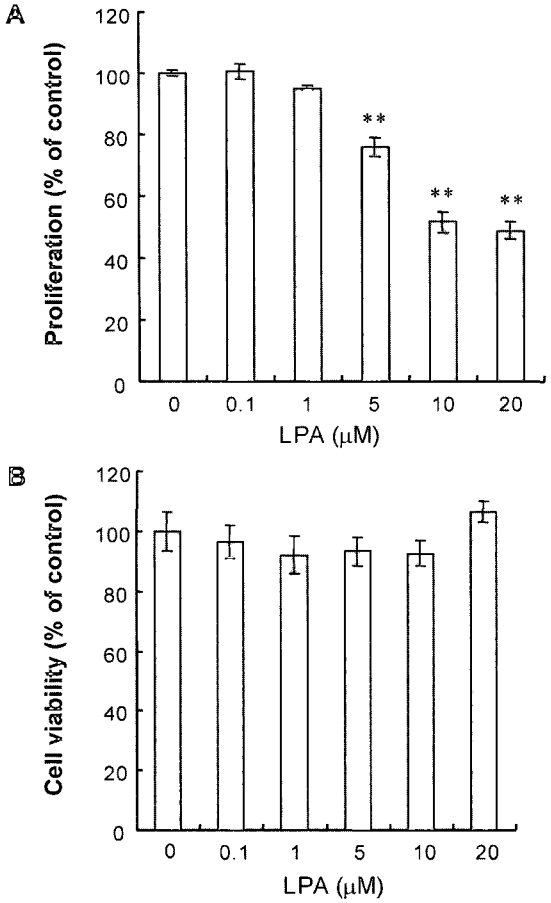

Fig. 1. Effects of LPA on the proliferation and viability of Mel-Ab cells. Cells were treated with various concentrations of LPA (0.1-20 μM), as described in "Materials and Methods". Cell proliferation was determined by MTT assay (A), and cell viability was examined by crystal violet assay (B). The values are means±SD of triplicate wells. **P<0.01 compared to control.

cantly. Proliferation was inhibited by ca. 50% at a concentration of 10 μM. Cell viability as determined by crystal violet assay, showed that LPA was not toxic to Mel-Ab cells at concentrations of 0.1-20 μM (Fig. 1B). Furthermore, apoptotic cells were also analyzed by flow cytometry, and LPA did not increase the percentage of apoptotic cells represented by sub-G_0/G_1 phase (data not shown).

Activation of ERK and JNK by LPA in Mel-Ab cells

To examine the signal transduction pathway by which LPA inhibits melanocyte proliferation, we studied the activation of the three major subfamilies of MAP kinases and of the Akt pathway. Fig. 2 shows the result of an ERK and JNK Western blot assay after treating with 10 μM of LPA in a time course experiment. We found that LPA stimulated the activation of ERK in Mel-Ab cells, and observed that LPA induced the phosphorylation of JNK 30 min after LPA treatment. However, we could not detect changes in p38 MAP kinase or Akt after LPA treatment

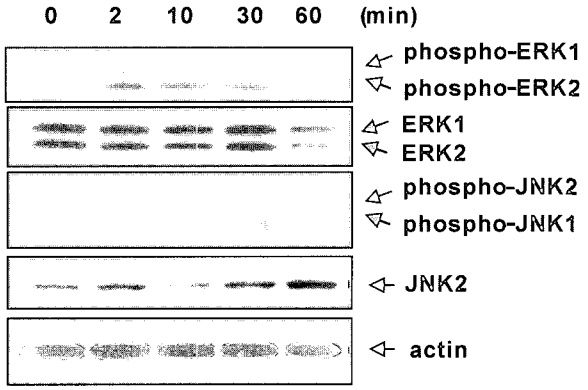

Fig. 2. Effects of LPA on the ERK and JNK signaling pathways. Mel-Ab cells were treated with 10 μM LPA for the indicated times. Whole cell lysates were then subjected to Western blot analysis with antibodies against phospho-specific ERK and phospho-specific JNK. Equal protein loadings were confirmed by reaction with phosphorylation-independent ERK, JNK, and actin antibodies.

(data not shown), indicating that these pathways are not related to LPA-induced cell growth inhibition.

Effects of PD98059, D-JNKI1 and pertussis toxin on the cell growth inhibition induced by LPA

Since LPA induced ERK and JNK activation, we also investigated the involvements of the ERK and the JNK signaling cascades in LPA-mediated growth inhibition by using specific inhibitors. Thus, LPA-treated melanocytes were cultured for 72 h in the presence of PD98059 (a specific ERK pathway inhibitor) or D-JNKI1 (a selective JNK inhibitor), and proliferation was measured by MTT assay.

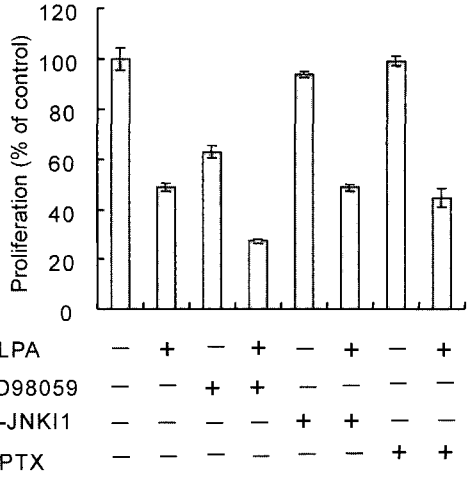

Fig. 3. Effects of PD98059, D-JNKI1 and PTX on the cell growth inhibition induced by LPA. After preincubation with or without 20 μM PD98095 for 1 h, 1 μM D-JNKI1 for 1 h, or 100 ng/mL PTX for 1 h, the cells were treated with 10 μM of LPA for 72 h, as described in "Materials and Methods". Cell proliferation was determined by MTT assay. The values are means±SD of triplicate wells.

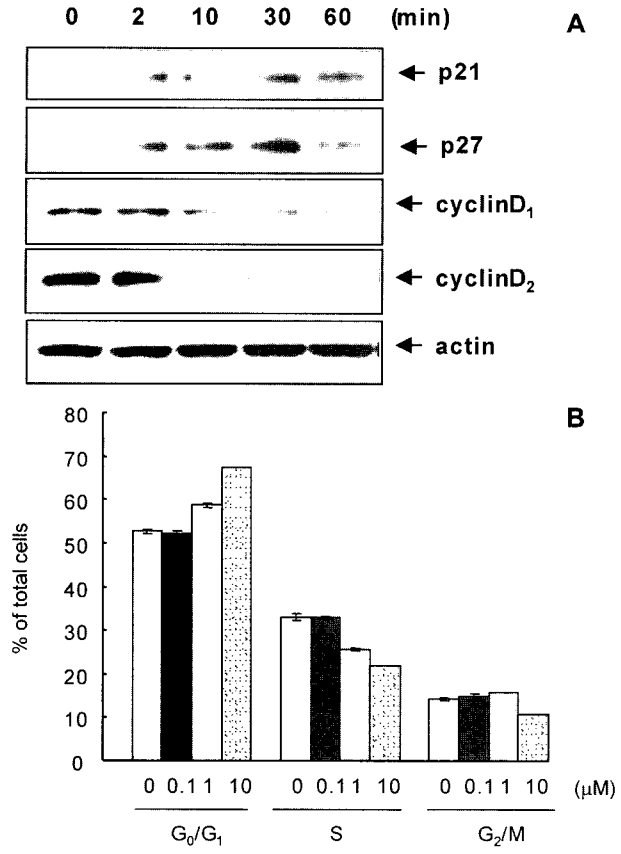

Fig. 4. Effects of LPA on the cell cycle of Mel-Ab cells. (A) Cells were treated with 10 μM LPA for the indicated times. Whole cell lysates were then subjected to Western blot analysis with antibodies against cyclin D_1, cyclin D_2, p21, and p27. Equal protein loadings were confirmed using anti-actin antibody. (B) Cells were treated with 0.1-10 μM LPA in 2% serum-containing media for 48 h. Cells were then analyzed by flow cytometry as described in "Materials and Methods".

As shown in Fig. 3, 20 μM LPA induced a 50% reduction in the MTT assay, as compared with the untreated control. In addition, our data shows that PD98059 alone inhibits cell proliferation, and subsequent treatment with LPA enhances the antiproliferative effect of PD98059. Furthermore, D-JNKI1 did not recover the growth inhibition by LPA, indicating that the JNK pathway is not involved in the antiproliferative effects of LPA on Mel-Ab cells. To determine the signaling mechanism, Mel-Ab cells were pretreated with 100 ng/mL of PTX for 1 h before adding LPA, but PTX did not reverse the antiproliferative effect of LPA. These results indicate that the antiproliferative effect of LPA is not mediated by G_i-protein coupled receptors.

Effects of LPA on the cell cycle of Mel-Ab cells

To investigate the mechanism of the antiproliferative effect of LPA, levels of cell cycle related proteins including cyclin D_1, cyclin D_2, p21, and p27 were examined by Western blot analysis. As shown in Fig. 4A, LPA treatment reduced the levels of cyclin D_1 and cyclin D_2 protein, but increased the levels of p21 and p27. Furthermore, the cell cycle changes were analyzed by flow cytometry. A dose-dependent decrease in the percentage of cells in the S phase and an increase in the G_0/G_1 phase were observed when cells were treated with LPA (Fig. 4B). These findings suggest that LPA induces cell cycle arrest and subsequent cell growth inhibition.

DISCUSSION

LPA is a broad-spectrum bioactive lipid messenger, and various studies have suggested that LPA is involved in cell proliferation, cell differentiation and apoptosis (Karliner *et al.*, 2001; Ye *et al.*, 2002). In addition, it is well known that LPA acts as a positive regulator of cell growth in the majority of cell (Mattingly *et al.*, 1999; Piazza *et al.*, 1995; Shida *et al.*, 2003). However, LPA also inhibits the growth of Sp2 myeloma cells by some unknown mechanism (Tigyi *et al.*, 1994). Despite our increasing knowledge on this topic, the actions of LPA in melanocytes are poorly understood. In this study, we found that LPA significantly inhibits Mel-Ab proliferation.

The ERK and Akt signaling pathways are known to play critical roles in cellular proliferation (Kiely *et al.*, 2002; Pebay *et al.*, 2001). Therefore, we tested the hypothesis that LPA regulates cell growth signaling *via* the ERK and/or the Akt pathways in melanocytes. In this study, Akt was not influenced by LPA treatment in Mel-Ab cells, indicating that the Akt pathway is not involved in the LPA-mediated antiproliferative effect. Although ERK is an important mitogenic signal (Davis, 1993), it has been proposed that prolonged activation of the ERK pathway may induce cell growth arrest (Alblas *et al.*, 1998). In Mel-Ab cells, we also found that LPA induced the prolonged activation of ERK and inhibited the proliferation of Mel-Ab cells. Therefore, it is feasible that the prolonged activation of ERK may induce growth inhibition in Mel-Ab cells. However, our results show that the inhibition of the ERK pathway by PD98059 enhanced cell growth inhibition by LPA. Because PD98059 (a specific ERK pathway inhibitor) can inhibit the transient and delayed activation of ERK, both growth factor-induced transient proliferative ERK activation and LPA-induced delayed ERK activation could have been simultaneously inhibited by PD98059. We also studied the effects of a JNK pathway inhibitor, since LPA also induced JNK activation. In our study, D-JNKI1 pretreatment did not abolish LPA-induced cell growth inhibition. These findings suggest that JNK activation by LPA is not related to the regulation of Mel-Ab cell proliferation.

It has been reported that LPA exerts many of its effects through three subtypes of cell surface receptors (i.e., EDG-2, EDG-4 and EDG-7) (Karliner *et al.*, 2001; Shida

et al., 2003). It is also known that EDG-2 and EDG-4 are coupled to PTX-sensitive $G\alpha_i$, whereas EDG-7 is coupled predominantly to a PTX-insensitive $G\alpha_q$ (An *et al.*, 1998; Bandoh *et al.*, 1999; Im *et al.*, 2000). Here, we demonstrate that the growth inhibition by LPA is not abolished by PTX treatment, indicating that LPA-induced growth inhibition may be mediated by PTX-insensitive EDG-7 receptor.

In the cell cycle, D-type cyclins are rate-limiting and critical factors for progression through the G_1 phase (Blagosklonny and Pardee, 2002; Prietzsch *et al.*, 2002). In addition, the cyclin-dependent kinase inhibitors, p21 and p27 play a critical role in cell cycle regulation. Hence, the induction of p21 and p27 may cause cell cycle arrest, resulting in cell growth inhibition (Gartel and Tyner, 2002; Tang *et al.*, 2002; Weiss and Randour, 2000). In the present study, LPA-induced growth inhibition was associated with cyclin D_1 and cyclin D_2 downregulation and p21 and p27 upregulation. Moreover, LPA induced a dose-dependent decrease in the percentage of cells in the S phase and an increase in the G_0/G_1 phase. These results explain that the growth inhibitory effect of LPA in melanocytes occurs *via* cell cycle arrest. In summary, our study demonstrates that LPA inhibits melanocyte proliferation, and these effects are the result of cell cycle arrest.

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