Lysophosphatidic acid increases the proliferation and migration of adipose-derived stem cells via the generation of reactive oxygen species

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Abstract. Phospholipid derivatives, such as lysophosphatidic acid (LPA), exhibit mitogenic effects on mesenchymal stem cells; however, the molecular mechanism underlying this stimulation has yet to be identified. The aims of the present study were as follows: To evaluate the stimulatory effects of LPA on the proliferation and migration of adipose-derived stem cells (ASCs); to study the association between reactive oxygen species (ROS) and LPA signaling in ASCs; and to investigate the microRNAs upregulated by LPA treatment in ASCs. The results of the present study demonstrated that LPA increased the proliferation and migration of ASCs, and acted as a mitogenic signal via extracellular signal-regulated kinases 1/2 and the phosphoinositide 3-kinase/Akt signaling pathways. The LPA1 receptor is highly expressed in ASCs, and pharmacological inhibition of it by Ki16425 significantly attenuated the proliferation and migration of ASCs. In addition, LPA treatment generated ROS via NADPH oxidase 4, and ROS were able to function as signaling molecules to increase the proliferation and migration of ASCs. The induction of ROS by LPA treatment also upregulated the expression of miR-210. A polymerase chain reaction array assay demonstrated that the expression levels of adrenomedullin and Serpine1 were increased following treatment with LPA. Furthermore, transfection with Serpine1-specific small interfering RNA attenuated the migration of ASCs. In conclusion, the present study is the first, to the best of our knowledge, to report that ROS generation and miR-210 expression are associated with the LPA-induced stimulation of ASCs, and that Serpine1 mediates the LPA-induced migration of ASCs. These results further suggest that LPA may be used for ASC stimulation during stem cell expansion.

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Introduction

Lysophosphatidic acid (LPA) is a phospholipid derivative that is synthesized by the removal of the choline group from lysophosphatidylcholine. LPA is an intermediate in the synthesis of phosphatidic acid and is able to act as a signaling molecule in various cell types (1-4). In particular, LPA acts as a potent mitogen via activation of high-affinity G protein-coupled receptors, such as EDG2 and EDG4 (5,6). Due to its ability to stimulate cell proliferation, aberrant LPA signaling has been linked to cancer progression, through numerous mechanisms. Dysregulation of the LPA receptors may lead to cellular hyperproliferation, which may in turn contribute to oncogenesis and metastasis (7-9). Signaling associated with LPA has also been reported to regulate the growth of fibroblasts, vascular smooth muscle cells, endothelial cells, and keratinocytes (10-13). In addition, the small GTPase Rho, a downstream molecule of the LPA receptor signal cascade, induces the formation of stress fibers and enhances cell migration (4). Therefore, LPA has been identified as a strong mitogenic factor. Recently, the molecular mechanisms underlying the mitogenic effects of LPA have been reported. Saunders *et al* (14) reported that LPA increases cell growth and inhibits apoptosis via the redox-dependent activation of extracellular signal-regulated kinases (ERK)-, Akt-, and nuclear factor-κB-dependent signaling pathways in ovarian cancer cells (14). Furthermore, the existence of an association between LPA signaling and reactive oxygen species (ROS)-mediated signaling has been suggested in other cell types (15-17).

In addition to cancer cells, numerous studies have reported that LPA exerts stimulatory effects on mesenchymal stem cells (MSCs). LPA protects MSCs against hypoxia and serum deprivation-induced apoptosis via the ERK12 and phosphoinositide 3-kinase (PI3K)/Akt signaling pathways, and induces the migration of human lung-resident MSCs via the β-catenin pathway (18,19). LPA also rescues MSCs from endoplasmic reticulum stress-associated apoptosis, induced by hypoxia and serum deprivation through the p38 pathway (20). In addition, LPA induces the osteoblastic differentiation of MSCs by increasing cyclic adenosine mono-phosphate and Ca^{2+} levels (21). LPA also affects the paracrine activity of MSCs, and stimulates secretion of vascular endothelial growth factor (VEGF) and stem cell-derived factor 1 (22). LPA promotes VEGF secretion by increasing the expression of 150 kDa oxygen-related proteins in MSCs (23). However, there are few studies that specifically address the molecular mechanisms underlying LPA-induced ASC proliferation and migration. Our previous studies demonstrated that ROS generation exerts stimulatory effects on ASC proliferation and migration (24-27); however, the association between ROS and LPA signaling in ASCs remains poorly understood. Therefore, the present study aimed to investigate the stimulatory effects of LPA on ASC proliferation and migration, and the association between ROS and LPA signaling in ASCs, as well as the microRNA (miR) expression in ASCs following LPA treatment.

Materials and methods

Cell culture and chemical inhibition. The sampling of human subcutaneous adipose tissue, isolation of ASCs, and characterization of ASCs with ASC-specific surface markers using flow cytometry were performed as previously described (28,29). Liposuction aspirates were obtained from a healthy female donor following the attainment of informed consent and approval from Bundang CHA Hospital (Seongnam, Korea; BD2011-152D). Human ASCs were cultured in α-minimum essential medium (Gibco Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Gibco Life Technologies) and 1% penicillin-streptomycin (Gibco Life Technologies) at 37˚C in a humidified atmosphere containing 5% CO_2.

For chemical inhibition studies, the ASCs were co-treated with 5 mM U0126 (ERK12 inhibitor; EMD Millipore, Billerica, MA, USA), 5 mM LY294002 (PI3KAkt inhibitor; EMD Millipore), 0.1-1 mM Ki16425 (Sigma-Aldrich, St. Louis, MO, USA) or oleoyl-L-α-lysophosphatidic acid sodium salt (Sigma-Aldrich). For the inhibition of ROS generation, the ASCs were treated with 100 μM NADPH oxidase (Nox) inhibitor diphenyleneiodonium chloride (DPI; Sigma-Aldrich).

Cell proliferation assay. ASCs were seeded at $7x10^3$ cells/well in 48-well plates. Following incubation with the LPA (1-50 μM) for 48 or 72 h, cell numbers were measured using the Cell Counting kit (CCK)-8 Assay (Dojindo Molecular Technologies, Inc., Rockville, MD, USA). Briefly, the medium in each well was replaced with medium containing the water-soluble tetrazolium salt WST-8 (10% vv). Following incubation for 2 h at 37˚C, absorbance was measured at 450 nm using a Sunrise™ Microplate Absorbance Reader (Tecan Group Ltd., Männedorf, Switzerland).

Cell migration assay. Cell migration was measured using a transwell chamber. The transwell inserts with an 8 μm 3422 pore polycarbonate membrane (Corning Life Sciences, Cambridge, MA, USA), were coated with 0.1% gelatin (Sigma-Aldrich) in phosphate-buffered saline (PBS) for 2 h at 37˚C. The ASCs were seeded at $1x10^4$ cells per 200 μl in the upper chamber and incubated for 2 h at 37˚C in an atmosphere containing 5% CO_2. The ASCs were allowed to migrate towards 500 μl medium containing 10 μM LPA in the lower chamber. Migration induced by serum-free medium was used as a negative control. Following 16 h incubation at 37˚C, the transwell inserts were fixed with ice-cold 100% methanol for 20 min and stained with 0.23% crystal violet (Sigma-Aldrich) in 2% ethanol for 30 min. Following further washing with distilled water, the non-migratory cells were removed by gently wiping the upper face of the transwell inserts with a cotton swab. Images of the stained cells were captured using a light microscope (CKX41; Olympus, Tokyo, Japan) and were analyzed using Image J 1.47 software (National Institutes of Health, Bethesda, MD, USA), in order to determine the number of cells that had migrated to the lower side of membrane.

Intracellular ROS staining. Intracellular ROS production was measured by flow cytometry using the ROS-sensitive dye 2',7'-dichlorofluorescin diacetate (DCF-DA; Molecular Probes Life Technologies, Carlsbad, CA, USA), as previously described (25,26,30). Briefly, the ASCs were seeded at $2.5x10^5$ cells/60 mm dish. The following day, the cells were incubated with 20 μM DCF-DA for 10 min at 37˚C, and treated with LPA for 20 min at 37˚C. Following incubation, the cells were trypsinized and resuspended in PBS. Green fluorescence intensity was measured using a BD FACSCalibur flow cytometer and analyzed using CellQuest Pro software (BD Biosciences, Franklin Lakes, NJ, USA).

Polymerase chain reaction (PCR) array and reverse transcription-quantitative PCR (RT-qPCR). Total RNA was isolated using the RNeasy kit (Qiagen, Inc., Valencia, CA, USA), and was reverse transcribed using a cDNA Synthesis kit (Promega Corporation, Madison, WI, USA), according to the manufacturer's instructions.

The synthesized cDNA was used in a PCR Array analysis using a CFX96 Real-Time PCR Detection system (Bio-Rad Laboratories, Inc., Hercules, CA, USA), according to the manufacturer's instructions. A PAHS-014ZD Human Signal Transduction Pathway Finder RT2 Profiler PCR Array (Qiagen, Inc.), which was composed of 84 transcripts representing 10 distinct signaling pathways, was used to identify the genes associated with signaling pathways that may be affected by LPA treatment. Differential gene expression and statistical analysis of the data were performed using PCR Array Data Analysis software (Qiagen, Inc.).

RT-qPCR was performed using a StepOne Plus Real-Time PCR system (Applied Biosystems Life Technologies, Foster City, CA, USA) using the following primers: Adrenomedullin

(ADM) forward, CTTATTCGGCCCCAGGACAT, and reverse, ACTGGTAGATCTGGTGTGCC; Serpine1 forward, CCGCCTCTTCCACAAATCAG, and reverse, AATGTT GGTGAGGGCAGAGA; LPA receptor 1 (LPAR1) forward, TCATCTGGACTATGGCCATC, and reverse, CAAGTT GAAAATGGCCCAGA; LPAR2 forward, CAATGCTGC TGTGTACTCTT, and reverse, TGGGCAGAGGATGTA TAGTG; LPAR3 forward, CTACAAGGACGAGGACATGT, and reverse, ATCCTCTATGTACTGGCTGC; LPAR4 forward, TCTGCAAGATCTCTGGAACT, and reverse, ACA CAATGGCAGAATTCCTC; LPAR5 forward, TTCTCC CGTGTCCTGACTA, and reverse, ACATGTACACGCTCA CCAC; LPAR6 forward, TTGTATGGGTGCATGTTCAG, and reverse, CAAGTCTGACATTGCCAAGT; and GAPDH forward, CGAGATCCCTCCAAAATCAA, and reverse, TGT GGTCATGAGTCCTTCCA. GAPDH served as a loading control. Thermal cycling over 40 cycles consisted of an initial denaturation at 95˚C for 10 min, then 95˚C for 15 sec, 60˚C for 1 min and 95˚C for 15 sec, and was terminated by a final extension at 60˚C for 1 min.

Measurement of the expression levels of miR-210. Total RNA was extracted using TRIzol® reagent (Invitrogen Life Technologies, Carlsbad, CA, USA), and 0.5 μg total RNA was reverse transcribed using a Mir-X miRNA First-Strand Synthesis kit (Clontech Laboratories, Inc., Mountain View, CA, USA) according to the manufacturer's instructions. The expression levels of miR-210 were measured by RT-qPCR using the miR-210 primer (CTGTGCGTGTGACAGCGG CTGA; Genolution Pharmaceuticals, Inc., Seoul, Korea) as previously described (24). U6 small nuclear RNA served as a loading control (Clontech Laboratories, Inc.).

Transfection with small interfering (si)RNA and miR-210 inhibitor. The Stealth RNAi™ siRNA specific to human Nox4 (sense, UUAUCCAACAAUCUCCUGGUUCUCC; and antisense, GGAGAACCAGGAGAUUGUUGGAUAA), the Silencer Select siRNA for human Serpine1 (ID# s10013), and the non-targeting negative control (scramble) siRNA (Silencer Select Negative Control#1 siRNA) were purchased from Invitrogen Life Technologies. The miR-210 inhibitor (hsa-miR210) and the miRNA inhibitor negative control were purchased from Genolution Pharmaceuticals, Inc. ASCs were seeded in 60 mm dishes at a density of $2x10^6$ with complete medium. The following day, confluent ASCs were maintained in serum-free medium without antibiotics for 2 h. Subsequently, the ASCs were transfected with 20 nM of each siRNA or miR-210 inhibitor using Lipofectamine® 2000 transfection reagent (Invitrogen Life Technologies), according to the manufacturer's instructions in 24-well plates or 60 mm dishes.

Western blotting. Proteins of ASCs were isolated using SDS sample buffer. The soluble protein concentration was determined using the Quick start Bradford 1X dye reagent (Bio-Rad Laboratories, Inc.). The western blot analysis was performed as previously described (25) using antibodies targeting p-ERK12 (1:2,000; cat. no. 9106; in 5% milk), ERK12 (1:4,000; cat. no. 9102; in 5% milk), p-Akt (1:1,000; cat. no. 9271; in 5% milk), and Akt (1:4,000; cat. no. 9272; in 5% milk). All antibodies were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Horseradish-peroxidase (HRP)-conjugated secondary mouse antibody (1:10,000) was purchased from Vector Laboratories, Inc. (Burlingame, CA, USA) and HRP-conjugated secondary rabbit antibody was purchased from Cell Signaling Technology, Inc. (1:10,000; cat. no. 7074).

Table I. LPAR expression in adipose-derived stem cells.

Gene	Ct-value
LPAR1	24.44±0.50
LPAR2	29.82±0.78
LPAR3	30.85±0.94
LPAR4	30.84±0.90
LPAR5	31.44±0.54
LPAR6	29.60±0.57
GAPDH	18.84±0.09

Ct, cycle threshold; LPAR, lysophophatidic acid receptor.

Statistical analysis. All data are presented as the mean ± standard deviation. Statistical analysis of the data was performed using Microsoft Excel software (Microsoft Corporation, Redmond, WA, USA). A Student's t-test was used to analyze the data. $P<0.05$ was considered to indicate a statistically significant difference.

Results

LPA increases the proliferation and migration of ASCs. The present study initially investigated whether LPA treatment increased the proliferation and migration of ASCs. As expected, 1-50 mM LPA significantly increased the proliferation (Fig. 1A) and migration (Fig. 1B) of ASCs in a dose-dependent manner. Since PI3K/Akt and mitogen-activated protein kinase (MAPK) signaling pathways have previously been reported to be associated with the mitogenic effects of LPA, the phosphorylation levels of Akt and ERK12 were measured following LPA treatment, and phosphorylation of both proteins increased in a time-dependent manner (Fig. 1C). In the inhibition study, pharmacological inhibition of PI3K/Akt by LY294002 significantly reduced the LPA-induced proliferation of ASCs (Fig. 1D). In addition, pharmacological inhibition of PI3K/Akt by LY294002, and of ERK by U0126 significantly reduced the LPA-induced migration of ASCs (Fig. 1E). These results suggest that LPA acts as a strong mitogenic factor in ASCs.

LPA mediates the mitogenic effects of ASCs via the LPA receptor. The expression levels of LPAR were measured in the ASCs (Table I), indicating that LPAR1 is highly expressed in ASCs, as compared with the other isoforms. To measure the involvement of LPAR in the mitogenic function of LPA, pharmacological inhibition of LPAR by Ki16425 was performed. Co-treatment with Ki16425 (0.1 and 1 mM) significantly

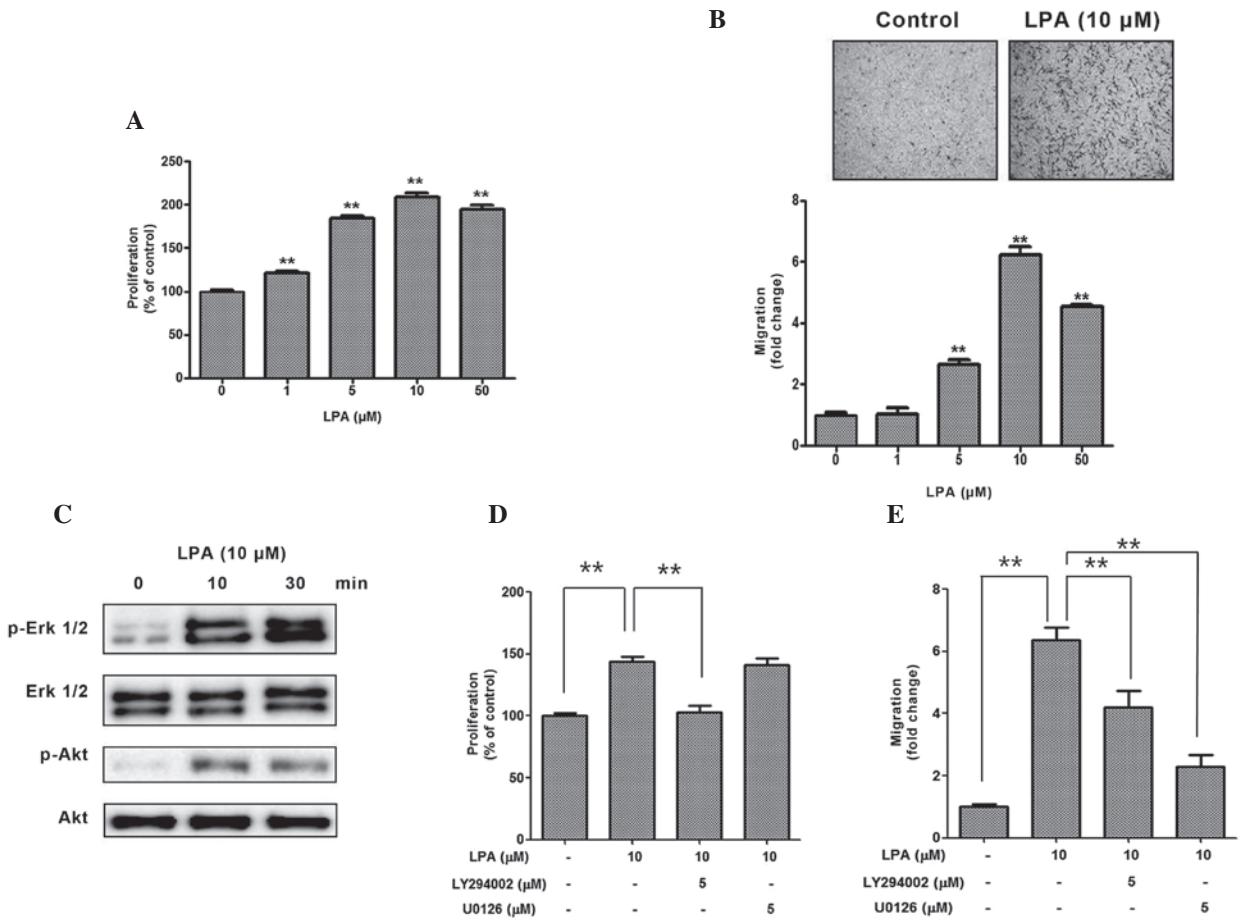

Figure 1. Lysophosphatidic acid (LPA) increases the proliferation and migration of adipose-derived stem cells (ASCs). (A and B) LPA (1-50 mM) significantly increased the (A) proliferation and (B) migration of ASCs, as measured by the Cell Counting kit (CCK)-8 Assay and transwell chambers, respectively. (C) Western blot analysis of the phosphorylation of Akt and extracellular signal-regulated kinases (ERK)1/2 showed that band density increased following LPA treatment. (D) Pharmacological inhibition of phophoinositide 3-kinase (PI3K)/Akt by LY294002 significantly reduced the LPA-induced proliferation of ASCs. (E) Pharmacologic inhibition of PI3K/Akt by LY294002 and of ERK by U0126 significantly reduced the LPA-induced migration of ASCs. Data are presented as the mean ± standard deviation. *P<0.05, and **P<0.01.

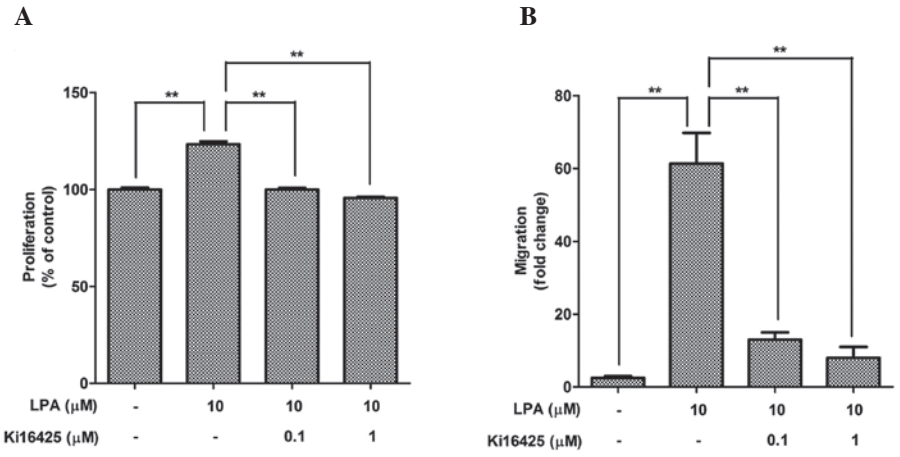

Figure 2. Lysophosphatidic acid (LPA) mediates the mitogenic effects of adipose-derived stem cells (ASCs) via LPA receptors (LPAR). Pharmacological inhibition of LPAR by Ki16425 (0.1 and 1 mM) significantly attenuated the LPA-induced (A) proliferation and (B) migration of ASCs. Data are presented as the mean ± standard deviation. **P<0.01.

attenuated LPA-induced proliferation (Fig. 2A) and migration (Fig. 2B) of ASCs.

LPA treatment induces ROS generation. The present study also investigated whether the LPA-induced proliferation

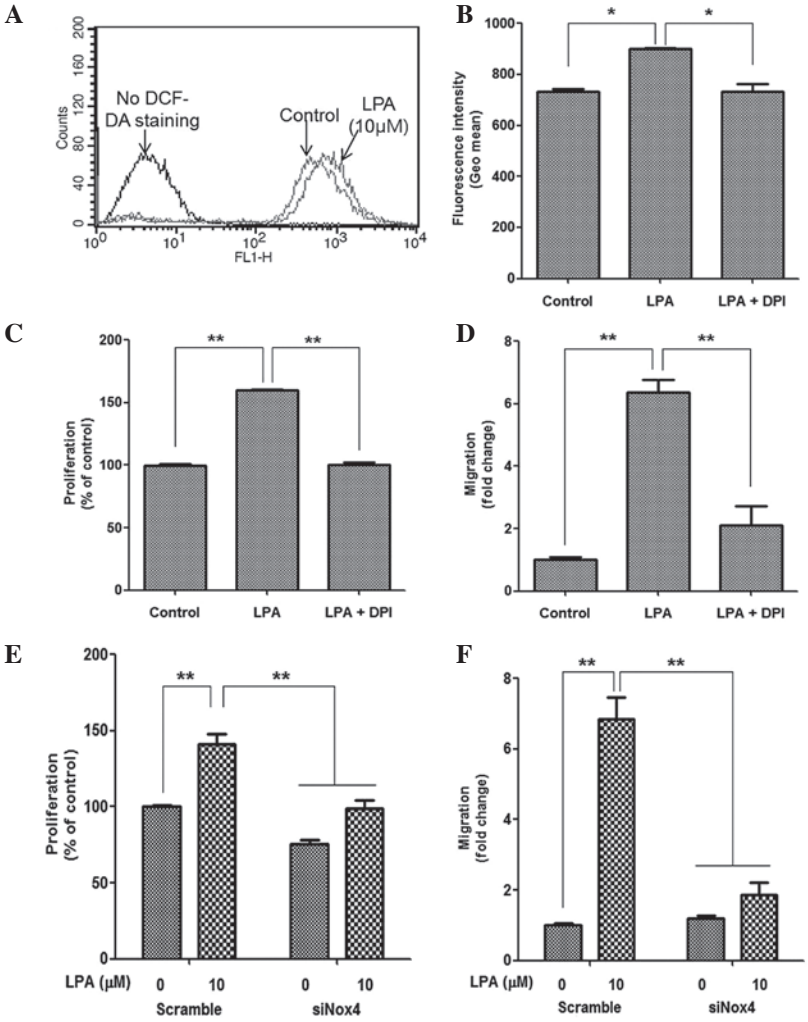

Figure 3. Lysophosphatidic acid (LPA) treatment induces reactive oxygen species generation. (A) LPA treatment significantly increased the fluorescence intensity of dichlorofluorescin diacetate (DCF-DA) assessed by flow cytometry. (B) Pharmacological inhibition of NADPH oxidase (Nox) by diphenyleneiodonium (DPI) significantly reduced the fluorescence intensity of DCF-DA. (C and D) DPI treatment significantly reduced the LPA-induced (C) proliferation and (D) migration of ASCs. (E and F) Downregulation of Nox4 by small interfering (si)RNA transfection significantly decreased the LPA-induced (E) proliferation and (F) migration of ASCs. Data are presented as the mean ± standard deviation. *P<0.05, **P<0.01.

and migration of ASCs was mediated by ROS generation. As expected, LPA treatment significantly increased the fluorescence intensity of DCF-DA, as determined by flow cytometric analysis (Fig. 3A); and pharmacological inhibition of Nox following treatment with DPI significantly attenuated the fluorescence intensity of DCF-DA (Fig. 3B). In addition, DPI treatment significantly reduced LPA-induced proliferation (Fig. 3C) and migration (Fig. 3D) of ASCs. As Nox4 is highly expressed in ASCs and primarily mediates ROS generation (26), downregulation of Nox4 expression in ASCs by siRNA transfection and Nox4 silencing significantly decreased LPA-induced cell proliferation (Fig. 3E) and migration (Fig. 3F). These results suggest that Nox4 may be associated with LPA-mediated ROS generation and downstream mitogenic effects in ASCs.

LPA increases miR-210 expression. Our previous study demonstrated that ROS generation induced by hypoxia, or chemicals, induced miR-210 expression and increased the proliferation and migration of ASCs (24); therefore, the present study investigated the involvement of miR-210 in the LPA-induced proliferation and migration of ASCs. As expected, LPA treatment increased miR-210 expression (Fig. 4A); however, the extent of upregulation was not as great as that induced by hypoxia and chemical ROS donors. In the inhibition study, miR-210 inhibitors significantly attenuated LPA-induced proliferation (Fig. 4B) and migration (Fig. 4C) of ASCs. These results suggest that miR-210 is involved in the LPA-induced proliferation and migration of ASCs.

LPA upregulates the expression of ADM and Serpine1. The human Signal Transduction Pathway Finder RT2 Profiler PCR Array was used to identify the LPA target genes that mediated the proliferation and migration of ASCs. A total of 4 h following LPA treatment, the expression levels of ADM increased 5.12-fold, as demonstrated by the PCR array (Fig. 5A). RT-qPCR analysis confirmed that the expression levels of ADM were upregulated ~6-fold (Fig. 5B). A total of 24 h following LPA treatment, the expression levels of Serpine1 increased 8.28-fold, as determined by the PCR array (Fig. 5C), and ~18-fold, as determined by the RT-qPCR (Fig. 5D). Therefore, the present study investigated whether ADM

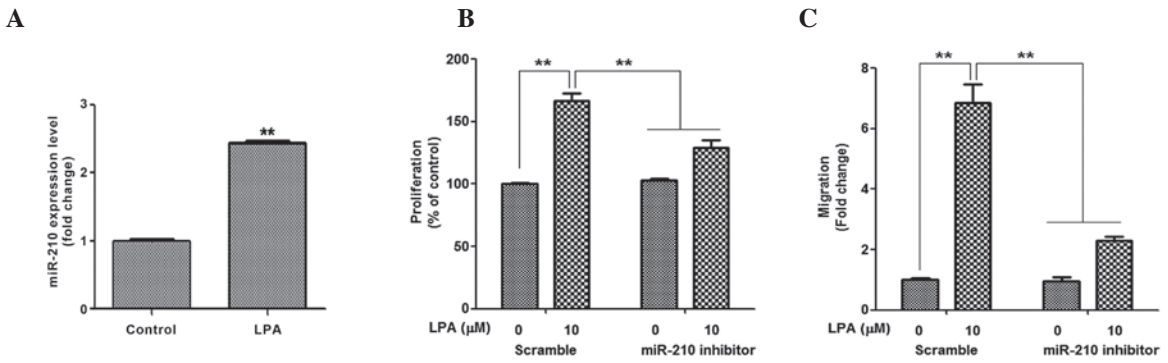

Figure 4. Lysophosphatidic acid (LPA) increases the expression levels of microRNA (miR)-210. (A) Reverse transcription-quantitative polymerase chain reaction analysis demonstrated that LPA treatment increased the expression levels of miR-210 in the adipose-derived stem cells (ASCs). Transfection with miR-210 inhibitor significantly attenuated the LPA-induced (B) proliferation and (C) migration of ASCs. Data are presented as the mean ± standard deviation. **P<0.01.

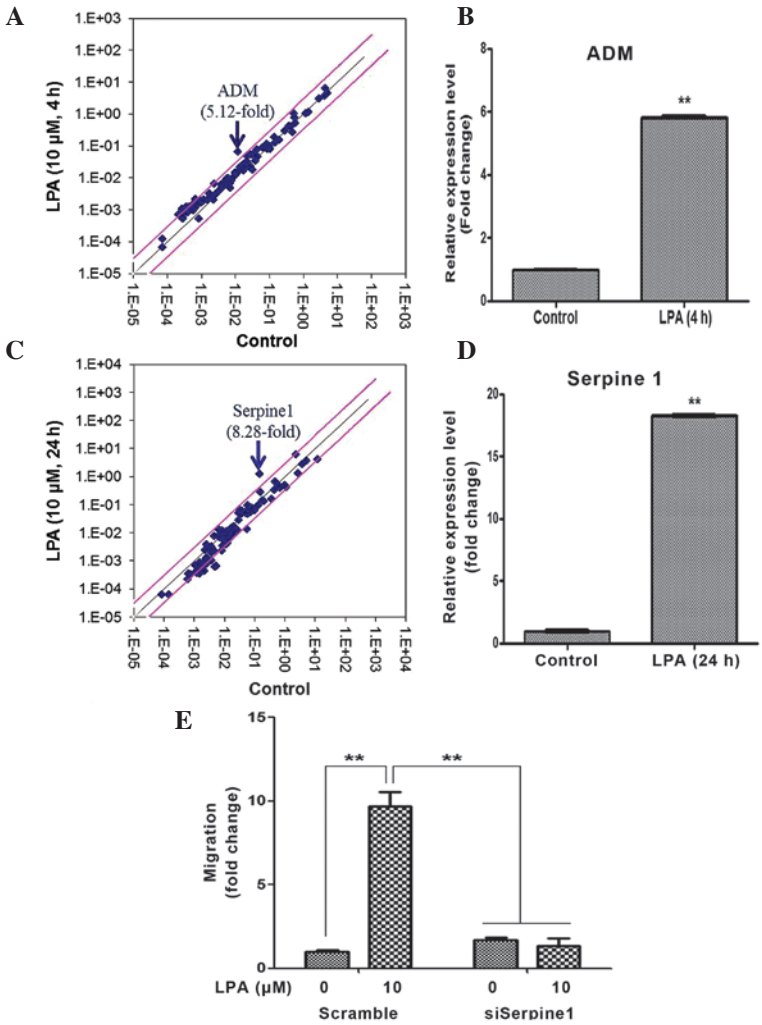

Figure 5. Lysophosphatidic acid (LPA) upregulates the expression levels of adrenomedullin (ADM) and Serpine1, and mediates the migration of adipose-derived stem cells (ASCs) via Serpine1. (A) Identification of the target genes of LPA that mediated the proliferation and migration of ASCs using a Human Signal Transduction Pathway Finder RT2 Profiler Polymerase Chain Reaction (PCR) Array. The expression levels of ADM significantly increased 4 h following LPA treatment. (B) Confirmation of the upregulation of ADM expression was provided by reverse transcription quantitative RT-q(PCR) analysis. (C) The expression levels of Serpine1 increased 24 h following LPA treatment, as determined by PCR array. (D) Increased Serpine1 expression as determined by RT-qPCR. (E) Transfection with Serpine1 small interfering (si)RNA significantly attenuated LPA-induced migration of ASCs. The pink lines indicate a 3-fold change in gene expression between the control and LPA treatment. Data are presented as the mean ± standard deviation. **P<0.01.

and/or Serpine1 mediated LPA-induced proliferation and migration of ASCs using siRNAs specific for ADM and Serpine1. Although silencing of ADM did not affect proliferation or migration (data not shown), transfection with Serpine1 siRNA significantly attenuated the LPA-induced migration of ASCs (Fig. 5E).

Discussion

The present study investigated the signal pathways and molecular mechanisms underlying LPA-induced proliferation and migration of ASCs. Previous studies have demonstrated that LPA is able to mediate the proliferation and migration of various cells, primarily via the ERK12 and PI3K/Akt signaling pathways (19,31,32). The results of the present study demonstrated that LPA may function as a mitogenic signal via LPARs, and the ERK12 and PI3K/Akt signaling pathways in ASCs. In addition to these signaling pathways, the present study demonstrated that LPA treatment induced ROS generation via Nox4, and that these ROS act as signaling molecules to increase the proliferation and migration of ASCs. Furthermore, upregulation of miR-210 expression by ROS may contribute to the increased proliferation and migration of ASCs, results which are concordant with those of a previous study (24). LPA also upregulated Serpine1 expression, which increased ASC migration.

As determined by PCR array, the expression levels of ADM and Serpine1 increased 4 and 24 h following LPA treatment, respectively. In addition, the results of the present study demonstrated that Serpine1 mediated LPA-induced migration of ASCs; however, no proliferation-associated genes were identified in the present study. Although the involvement of ADM in ASC proliferation was hypothesized, siRNA silencing of ADM did not inhibit the proliferation of ASCs. A previous study also reported that ADM did not increase, but rather inhibited, LPA-induced proliferation, and attenuated the stimulation of the MAPK pathway by LPA treatment in adventitial cells (33). Therefore, the mediator(s) of LPA-induced proliferation of ASCs have yet to be identified.

miRNAs exert their actions primarily at the post-transcriptional level, and their expression is known to be regulated by redox signaling (24,34-36). In our previous study, the expression levels of miR-210 were significantly increased via various ROS generators (hypoxia, antimycin, rotenone, and platelet-derived growth factor subunit B), and regulated the proliferation and migration of ASCs (24). The present study demonstrated that LPA-induced ROS generation upregulated miR-210 expression in ASCs, and increased the proliferation and migration of ASCs. However, the extent of miR-210 LPA-induced upregulation was lower, as compared with other ROS generators such as hypoxia. In addition, ROS generation by S1P did not induce miR-210 expression in ASCs (data not shown). Therefore, it is reasonable to assume that ROS generation is not a prerequisite for the upregulation of miR-210 expression in ASCs.

In conclusion, the present study demonstrated that LPA increases the proliferation and migration of ASCs via Nox4-induced ROS generation. The present study is the first, to the best of our knowledge, to demonstrate that miR-210 expression is associated with LPA-induced stimulation, and that Serpine1 mediates the LPA-induced migration of ASCs. Therefore, phospholipid derivatives, such as LPA and S1P, may be used to stimulate ASCs during stem cell expansion.

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