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Lysophosphatidic Acid Promotes Cell Invasion by Up-Regulating the Urokinase-Type Plasminogen Activator Receptor in Human Gastric Cancer Cells

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Abstract There is a strong correlation between the overexpression of urokinase-type plasminogen activator receptor (uPAR) and gastric cancer invasion. This study examined the effect of phospholipid lysophosphatidic acid (LPA) on uPAR expression in human gastric cancer AGS cells and the underlying signal transduction pathways. Treating human gastric AGS cells with LPA induced the expression of uPAR mRNA and promoter activity in both a time- and dose-dependent manner. Small interfering RNA targeting for LPA receptors, dominant negative Rho-family GTPase (RhoA, Rac1, and Cdc42) and an expression vector encoding a mutated c-jun (TAM67) partially blocked the LPA-induced uPAR expression. Site-directed mutagenesis and electrophoretic mobility shift studies showed that the transcription factors activation protein-1 (AP-1) and nuclear factor (NF)-κB are essential for the LPA-induced uPAR transcription. In addition, AGS cells treated with LPA showed enhanced invasion, which was partially abrogated by the uPAR-neutralizing antibodies and inhibitors of Rho kinase, JNK, and NF-κB. This suggests that LPA induces uPAR expression through the LPA receptors, Rho-family GTPase, JNK, AP-1 and NF-κB signaling pathways, which in turn stimulates the cell invasiveness of human gastric cancer AGS cells. J. Cell. Biochem. 104: 1102–1112, 2008.

Key words: LPA; uPAR; invasion; signaling; gastric cancer

Although the incidence and mortality of gastric cancer has decreased over the last few decades, gastric cancer is still the fourth most common cancer worldwide [Parkin et al., 2002]. Chemotherapy or radiation therapy does not significantly affect the length or quality of life of advanced gastric cancer patients due to local invasion and metastasis. The development of effective anti-invasive strategies for gastric cancer would be helpful in improving the treatment outcome.

Cancer invasion and metastasis are multifactorial processes that require the coordinated action of cell-secreted proteolytic enzymes and their inhibitors [Wei et al., 2007]. Urokinase-type plasminogen activator (uPA), its inhibitors, and uPA receptor (uPAR) form a complex proteolytic system that has been implicated in cancer invasion and metastasis. uPA is a serine protease with the ability to convert plasminogen to active plasmin [Lund et al., 2006]. Furthermore, the uPA-uPAR binding interaction can affect the cell motility, integrin function, and gene expression independently [Waltz et al., 2000]. In many cancers, uPAR expression is essential for the invasive and metastatic phenotype [Pulukuri et al., 2005]. The overexpression of uPAR increases the ability of cells to penetrate the barrier of the reconstituted basement membrane. On the other hand, blockade of uPAR through the expression of a catalytically inactive enzyme or an antisense uPAR cDNA decreases the invasiveness of the cells remarkably. In gastric cancers, increased levels of uPA and uPAR are essential for maintaining the invasive and metastatic phenotypes, and these increases are considered prognostically significant [Cho et al., 1997].

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Lysophosphatidic acid (LPA) is a natural phospholipid present in serum at concentrations of 1–10 μM with variable reports of plasma concentrations ranging from undetectable to 2 nM [Pages et al., 2001]. LPA is involved in a broad range of cellular responses including cell proliferation, cell adhesion, migration, smooth muscle cell contraction, platelet aggregation and survival [Van Leeuwen et al., 2003]. LPA is a ligand for the G-protein coupled receptors, and its cellular responses are mediated by at least three different G protein-coupled receptors, LPA1/Edg-2, LPA2/Edg-4, and LPA3/Edg-7 [Moolenaar, 1999].

During tumor progression, tumors are associated with local bleeding involving the activation of platelets. From the activated platelets, lysophospholipids are released and are subsequently converted to LPA by lysophospholipase [Aoki et al., 2002]. Therefore, LPA is believed to be abundant in tumors and regulates various tumorigenic processes such as invasion and metastasis. LPA has been shown to have a positive role in tumor progression such as in ovarian cancer, breast cancer, colon and gastric cancer [Li et al., 2005; Symowicz et al., 2005; Smicun et al., 2007]. However, there is little information on the mechanism of LPA-induced tumor invasion, particularly in gastric cancer.

In this study, it was found that LPA induces the expression of uPAR through multiple signaling pathways, which stimulates the cell invasiveness in human gastric cancer AGS cells.

MATERIALS AND METHODS

Cell Culture and Culture Conditions

Human gastric carcinoma AGS cells were obtained from the American Type Culture Collection (Manassas, VA). The cells were cultured with RPMI-1640 supplemented with 10% FBS and 1% penicillin-streptomycin at 37°C in an atmosphere containing 5% CO_2. In order to determine effects of LPA on uPAR expression, the cells harvested at various intervals and the level of uPAR mRNA was determined by Northern blot analysis. The role of the specific signaling pathways in the LPA-induced expression of uPAR was examined by pretreating the AGS cells with PD98059 (a MEK inhibitor; New England Biolabs, Beverly, MA), SP600125 (a JNK inhibitor; Calbiochem, La Jolla, CA), SB203580 (a specific P38 MAPK inhibitor; Calbiochem), BAY 11-7082 (a NF-κB inhibitor; Calbiochem), Wortmannin (a PI-3 kinase/AKT inhibitor; Calbiochem) and Y-27632 (a Rho kinase inhibitor; Calbiochem) for 1 h before exposure to LPA.

Northern Blot Analysis

The total RNA extraction and Northern blot hybridization were performed as previously described [Kim et al., 2005]. Each cDNA probe was radiolabeled with [α-^{32}P]deoxyribonucleoside triphosphate using the random-priming technique with the Rediprime labeling system (Amersham Corp., Van Nuys, CA). The probed nylon membranes were exposed to radiographic film (Life Technologies, Inc., Grand Island, NY).

Measurement of uPAR Promoter Activity

The transcriptional regulation of uPAR was examined using transient transfection with an uPAR promoter-luciferase reporter construct (pGL3-uPAR) as previously described [Kim et al., 2005]. The cotransfection studies were performed in the presence or absence of the AP-1 decoy oligodeoxynucleotides (ODNs) or dominant-negative mutants of RhoA (T19N), Rac1 (T17N), Cdc42 (T17N), and c-Jun (TAM67). The phosphorothioate double-stranded ODNs with the sequences against the AP-1 binding site (5′-CAG CTC AGA AGT CAC TTC-3′, 3′-GAA GTG ACT TCT GAG CTG-5′) were prepared (Genotech Corp., Yusung, Korea) and annealed (AP-1 decoy ODNs). The dominant-negative mutants of RhoA (T19N), Rac1 (T17N), Cdc42 (T17N), and c-Jun (TAM67) were kindly provided by Dr. Yasuhiko Horiguchi (Osaka University, Osaka, Japan) [Masuda et al., 2002] and Dr. M. J. Birrer (University of Helsinki, Helsinki, Finland) [Katabami et al., 2005], respectively. The importance of NF-κB and AP-1 in the induction of uPAR by LPA was examined by transfecting the AGS cells with pGL3-uPAR, in which the NF-κB and AP-1 site had been mutated.

Transient Transfection of NF-κB- and AP-1-Reporter

The NF-κB- and AP-1-reporter construct was purchased form Clontech (Palo Alto, CA). Once the cells had reached 60–70% confluence, they were washed with DMEM and incubated with DMEM without serum and antibiotics for 18 h. The cells were then transfected with 1 μg NF-κB- and AP-1-reporter containing the pGL3

vector using Lipofectamine 2000 (Invitrogen). In order to determine the effects of SP600125 and Y-27632 on NF-κB and AP-1 by LPA, the transfected cells were pretreated with 10 μM SP600125 and 10 μM Y-27632 for 1 h and incubated with 5 μM LPA for 2 h. After incubation, the cells were lysed and the luciferase activity was measured using a luminometer.

RT-PCR

The total RNA was extracted from the AGS cells using TRIzol reagent (Invitrogen). One microgram of the total RNA was used for first strand cDNA synthesis using random primers and Superscript reverse transcriptase (Invitrogen). The cDNA was subjected to PCR amplification with the primer sets for GAPDH, LPA_1, LPA_2, and LPA_3. The specific primers sequences are as follows: GAPDH sense, 5′-TTG TTG CCA TCA ATG ACC CC-3′, and GAPDH antisense, 5′-TGA CAA AGT GGT CGT TGA GG-3′ (836 bp); LPA1 sense, 5′-AAT CGA GAG GCA CAT TAC GG-3′, and LPA1 antisense, 5′-TGT GGA CAG CAC ACG TCT AG-3′ (432 bp); LPA2 sense, 5′-CAT CAT GCT TCC CGA GAA CG-3′, and LPA2 antisense, 5′-GGG CTT ACC AAG GAT ACG CAG-3′ (352 bp); LPA3 sense, 5′-AGG ATG CGG GTC CAT AGC AA-3′, and LPA3 antisense, 5′-GAT GAT GGG GTT CAC GAC GG-3′ (481 bp). The PCR conditions were as follows: denaturation at 94°C for 40 s, annealing at 58°C for 40 s and extension at 72°C for 40 s.

siRNA Transfection

The AGS cells were transiently transfected with the siRNA oligonucleotides using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's protocol. Starved AGS cells were transfected with 10–20 nM siRNAs for 5 h and stabilized for 48 h. The siRNA oligonucleotide sequences of LPA1, LPA2 and LPA3 are as follows: LPA1, 5′-GAAAUGAGCGCCACCUUUATT-3′; LPA2, 5′-GGUCAAUGCUGCUGUG UACTT-3′; LPA3, 5′-CAGCAGGAGUUACCUUGUUTT-3′.

Western Blot Analysis

The protein extraction and Western blot hybridization were performed as previously described [Kim et al., 2005]. The following antibodies were used: anti-phospho-Erk1/2, anti-phospho-JNK and anti-phospho-P38 MAPK (New England Biolabs). The total protein levels were assayed by washing the blotted membrane with a stripping solution (100 mM 2-mercaptoethanol, 2% SDS, and 62.5 mM Tris–HCl (pH 6.7)) for 30 min at 50°C, and then reprobing the membrane with the anti-β-actin mouse monoclonal antibody (Sigma Chemical Co., St. Louis, MO)

Extraction of Nuclear Proteins and Electrophoretic Mobility Shift Assay (EMSA)

The nuclear protein extraction and EMSA were performed as previously described [Kim et al., 2005]. In order to determine the effect of SP600125 and Y-27632on LPA-induced AP-1-DNA and NF-κB-DNA complex formation, the cells were pretreated with the inhibitors for 1 h before being exposed to LPA. the oligonucleotide containing the consensus sequence for AP-1 (5′-CGC TTG ATG AGT CAG CCG GAA-3′) and NF-κB (5′-AGT TGA GGG GAC TTT CCC AGG-3′) were end-labeled with [α-^{32}P]adenosine triphosphate (3,000 μCi/mmol; Amersham Pharmacia Biotech, Buckinghamshire, UK) using T4 polynucleotide kinase, purified in Microspin G-25 columns (Sigma) and used as the probe for EMSA. In competition studies, a 50-fold excess of unlabeled oligonucleotide was included in the reaction mixture along with the radiolabeled probe.

Matrigel Invasion Assay

The cell invasion assay was carried out using the BIOCOAT matrigel invasion chambers (Becton-Dickinson, Bedford, MA) according to the manufacturer's protocol. The AGS cells(10^5) in 300 μl were added to each chamber with 0–5 μM LPA and allowed to invade the matrigel for 24 h. The number of invading cells was counted using phase-contrast microscopy. In order to determine the effect of the anti-uPAR antibody and inhibitors of Rho kinase, JNK and NF-κB on the LPA-induced cell invasion, the AGS cells were preincubated with the neutralizing antibody to uPAR, Y-27632, SP600125, and BAY 11-7082 for 1 h and added to 5 μM LPA for 24 h.

RESULTS

Induction of uPAR by LPA in Human Gastric Cancer AGS Cells

The AGS cells were incubated with LPA for various times, and the level of uPAR mRNA in the cells was determined. As shown in Figure 1A, the level of uPAR mRNA increased 1 h after incubating the cells with LPA, with the

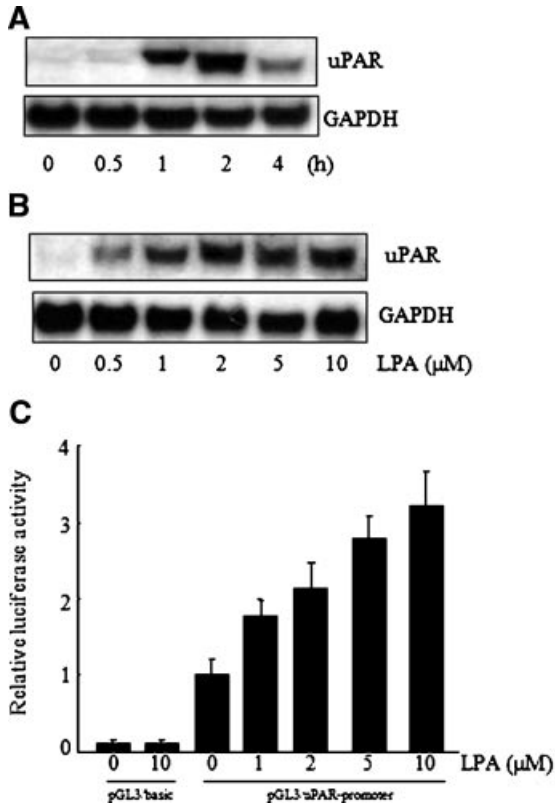

Fig. 1. Induction of uPAR by LPA in human gastric cancer AGS cells. The AGS cells were incubated with 5 μM LPA for 0–4 h (**A**) and 0–10 μM LPA for 2 h (**B**). After incubation, Northern blot analysis was performed. **C**: AGS cells transiently transfected with pGL3-uPAR reporter construct. The transfected cells were incubated with 0–10 μM LPA for 2 h and the luciferase activity was determined using a luminometer. The data represents the mean ± SD from triplicate measurements.

highest level being observed at 2 h. It was also found that LPA induced the uPAR mRNA expression in a dose-dependent manner at 0–10 μM (Fig. 1B). Next, the effect of LPA on transcriptional regulation of the uPAR gene was examined. To this end, the AGS cells were transiently transfected with the promoter-reporter construct (pGL3-uPAR) of the human uPAR gene and the luciferase gene. The AGS cells transfected with pGL3-uPAR showed an increase in promoter activity in a dose-dependent manner between 0 and 10 μM (Fig. 1C).

Involvement of LPA Receptors in LPA-Induced uPAR Expression

In order to examine the involvement of LPA receptors in uPAR induction by LPA, the total RNA was extracted from the AGS cells, and mRNA levels of LPA1, LPA2, and LPA3 were analyzed by RT-PCR. Among the three LPA receptors, LPA2 and LPA3 were expressed in AGS cells, whereas LPA1 mRNA was not detected (Fig. 2A). Specific siRNA oligonucleotides were used to determine which of the LPA receptors are involved in the LPA-induced expression of uPAR. The specific siRNA directed toward LPA2 and LPA3 reduced the basal LPA2 and LPA3 mRNA level (Fig. 2B,C). The induction of LPA1 mRNA by LPA was also reduced by the specific LPA1 siRNA (Fig. 2D). Each siRNA against LPA1, LPA2 and LPA3 partially inhibited the LPA-induced expression and promoter activity of uPAR mRNA (Fig. 2E,F).

Involvement of Rho-Family GTPases in LPA-Induced uPAR Expression

The Rho-family GTPases have been implicated in tumor invasion [Price and Collard, 2001] and are activated by LPA [Radeff-Huang et al., 2004]. Therefore, this study examined the involvement of the Rho-family GTPases in LPA-induced uPAR expression. As shown in Figure 3A, Y-27632, a Rho kinase inhibitor, blocked LPA-induced uPAR expression in a dose-dependent manner. In addition, the induction of uPAR promoter activity by LPA was significantly inhibited when the dominant negative Rho subfamily (RhoA, Rac1, and Cdc42) was co-transfected with pGL3-uPAR into the AGS cells (Fig. 3B).

Involvement of JNK MAPK in LPA-Induced uPAR Expression

The levels of phosphorylated Erk-1/2, JNK, and P38 MAPK in the AGS cells exposed to LPA for various periods were measured in order to determine the MAPK signaling pathways involved in the LPA-induced induction of uPAR. The LPA treatment led to remarkable increases in the level of Erk-1/2 and JNK phosphorylation. However, LPA did not induce the phosphorylation of P38 MAPK. The levels of the Erk-1/2, JNK, and P38 MAPK proteins were not altered significantly after the LPA treatment (Fig. 3C). In order to examine the specific roles of MAPKs in LPA-induced uPAR expression, the AGS cells were pretreated with PD98059 (a MEK inhibitor), SB203580 (a P38 MAPK inhibitor), and SP600125 (a JNK inhibitor) before the LPA treatment. As shown in Figure 3D, SP600125 partially blocked the LPA-induced expression of uPAR but PD98059 and SB203580 had no effect. PD98059 and SP600125 inhibited

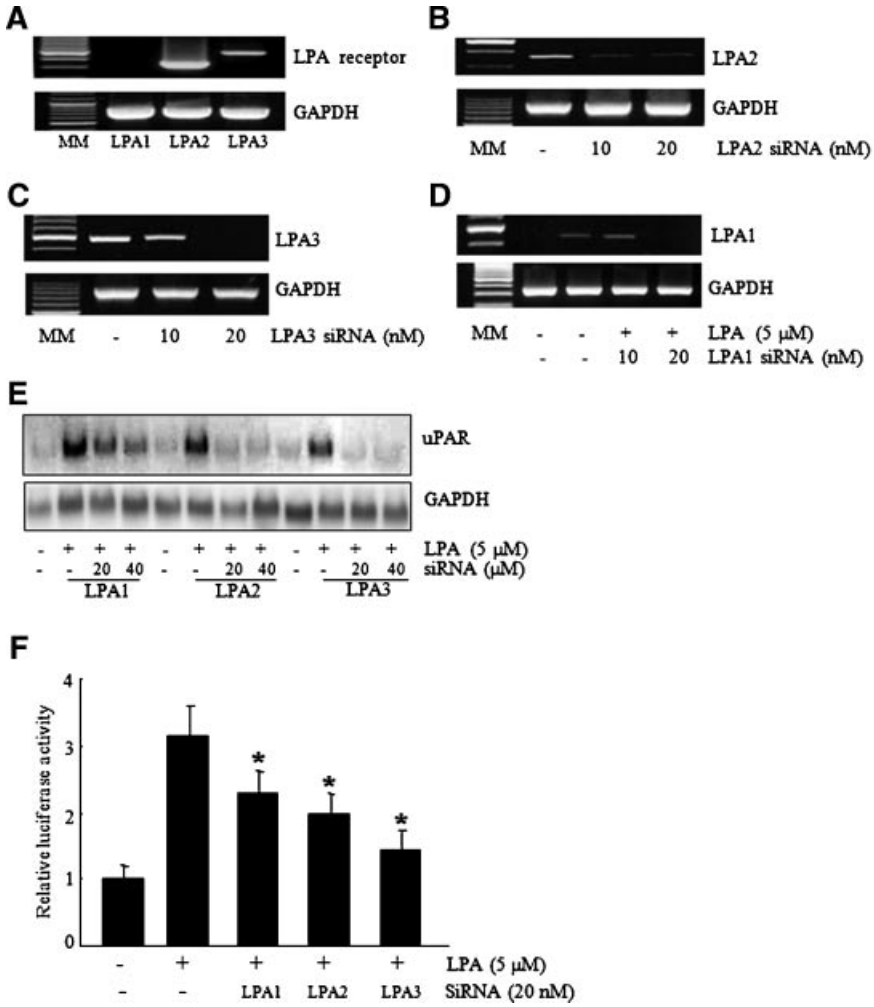

Fig. 2. Involvement of LPA receptors in LPA-induced uPAR expression in human gastric cancer AGS cells. **A**: The total RNA was extracted from the AGS cells and the mRNA levels of LPA1, LPA2, LPA3, and GAPDH was analyzed by RT-PCR. **B**,**C**: The AGS cells transiently transfected with the specific LPA2 and LPA3 siRNA oligonucleotides for 5 h. After stabilization for 48 h, the total RNA for LPA2, LPA3 and GAPDH mRNA were determined by RT-PCR. **D**: AGS cells transiently transfected with the specific LPA1 siRNA oligonucleotides for 5 h. The AGS cells, after stabilization for 48 h, were incubated with 5 μM LPA for 2 h. After incubation, RT-PCR was performed. **E**: AGS cells transiently transfected with the specific LPA1 and LPA2 and LPA3 siRNA oligonucleotides for 5 h. The AGS cells, after stabilization for 48 h, were incubated with 5 μM LPA for 2 h. After incubation, Northern blot analysis was performed. **F**: AGS cells were being transfected with specific LPA1 and LPA2 and LPA3 siRNA oligonucleotides, and transiently transfected with the pGL3-uPAR reporter construct. The transfected cells were incubated with 5 μM LPA for 2 h and the luciferase activities were determined using a luminometer. The data represents the mean ± SD from triplicate measurements. $^*P < 0.05$ versus LPA.

the LPA-induced activation of Erk-1/2 and JNK-1/2 in a dose-dependent manner, respectively (Fig. 3E).

To examine the role of JNK on transcriptional regulation of the uPAR gene, the AGS cells transfected with the pGL3-uPAR were pre-treated with PD98059, SB203580, and SP600125 before the LPA treatment. Consistent with Figure 3D, only SP600125 inhibited the LPA-induced uPAR promoter activity (Fig. 3F). In addition, the LPA-induced uPAR promoter activity was inhibited in a dose-dependent manner when the expression vector encoding a mutated c-jun (TAM67) were co-transfected with pGL3-uPAR into the AGS cells (Fig. 3G).

Effect of LPA on the Activation of Transcriptional Factors NF-κB and AP-1

Earlier studies suggested that the transcription factors, NF-κB and AP-1, are essentially involved in uPAR expression in the AGS cells [Kim et al., 2005] and that the transcription

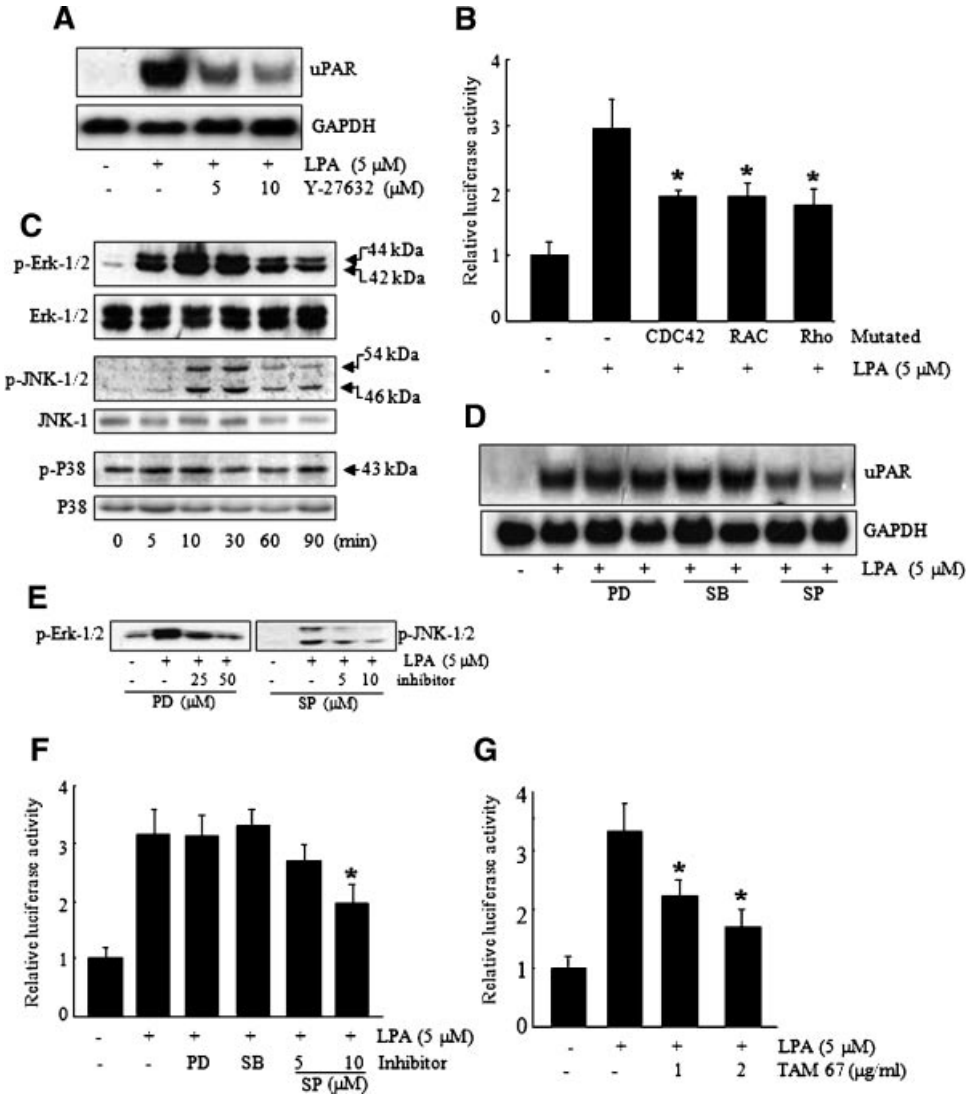

Fig. 3. Involvement of Rho-family GTPases and JNK MAPK in LPA-induced uPAR expression in human gastric cancer AGS cells. **A**: AGS cells, after being pretreated with 0–10 μM Y-27632 for 1 h, were incubated with 5 μM LPA for 2 h. After incubation, the cell lysates were determined for the uPAR mRNA by Northern blot analysis. **B**: Dominant negative mutants of Rho, Rac, and CDC42 were cotransfected with pGL3-uPAR into AGS cells. After incubation with 5 μM LPA for 2 h, the luciferase activities were determined using a luminometer. **C**: The AGS cells were incubated with 5 μM LPA for various periods and the levels of phosphorylated Erk-1/2, JNK, and P38 MAPK in the cell lysates were determined by Western blot analysis. **D**: AGS cells, after being pretreated with PD98059 (PD, 25 and 50 μM), SB203580 (SB, 5 and 10 μM) and SP600125 (SP, 5 and 10 μM) for 1 h, were incubated with 5 μM LPA for 2 h. After incubation, the uPAR mRNA in the cell lysates were determined by Northern blot analysis. **E**: AGS cells, after being pretreated with PD98059 (PD) and SP600125 (SP) for 1 h, were incubated with 5 μM LPA for 2 h. After incubation, the levels of phosphorylated Erk-1/2 and JNK in the cell lysates were determined by Western blot analysis. **F**: The transfected cells transiently transfected with pGL3-uPAR, after being pretreated with PD98059 (PD, 50 μM), SB203580 (SB, 10 μM) and SP600125 (SP), were incubated with 5 μM LPA for 2 h. After incubation, the cells were lysed, and the luciferase activity was measured using a luminometer. **G**: An expression vector encoding a mutated c-jun (TAM67) were cotransfected with pGL3-uPAR into AGS cells. After incubation with 5 μM LPA for 2 h, the luciferase activities were determined using a luminometer. The data represents the mean ± SD from triplicate measurements. $^*P < 0.05$ versus LPA.

factors are activated by LPA in the prostate cancer and bronchial epithelial cells [Hwang et al., 2006; Saatian et al., 2006]. Therefore, this study examined the effect of LPA on the activation of NF-κB and AP-1 in AGS cells. As shown in Figure 4A,B, the LPA treatment caused remarkable increases in the amount of NF-κB that could form a complex with the radiolabeled oligonucleotide probe in EMSA. The involvement of NF-κB in the induction of uPAR by LPA was examined by pretreating the AGS cells with 10 μM BAY11-7082 (a specific

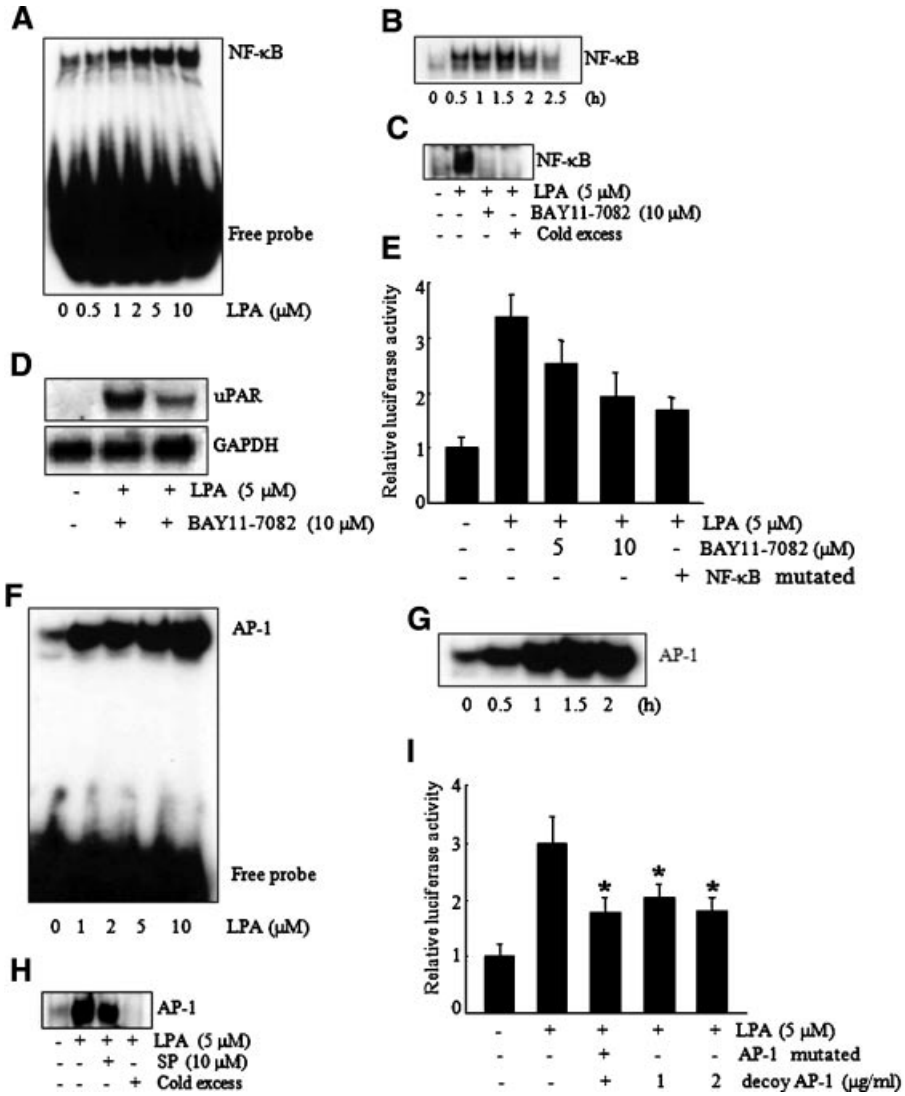

Fig. 4. Activation of transcription factor NF-κB and AP-1 during uPAR expression by LPA. AGS cells were treated with 0–10 μM LPA for 2 h (**A**) and 5 μM LPA for 0–2.5 h (**B**). After incubation, the nuclear extracts from the cells were analyzed by EMSA for the activated NF-κB using a radiolabeled oligonucleotide probe. **C**: AGS cells, after being pretreated with 10 μM BAY11-7082 for 1 h, were incubated with 5 μM LPA for 2 h, and then determined for the activated NF-κB by EMSA. **D**: AGS cells, after being pretreated with 10 μM BAY11-7082 for 1 h, were incubated with 5 μM LPA for 2 h. After incubation, the cell lysates were determined for the uPAR mRNA by Northern blot analysis. **E**: The AGS cells transiently transfected with pGL3-uPAR, after being pretreated with BAY11-7082 (5 and 10 μM), were incubated with 5 μM LPA for 2 h. Where indicated, the mutated pGL3-uPAR (converted the NF-κB site GGGAGGAGT to GGATCCAGT) was transfected into the cells. After incubation with 5 μM LPA for 2 h, the luciferase activities were determined using a luminometer. AGS cells were treated with 0–10 μM LPA for 2 h (**F**) and 5 μM LPA for 0–2 h (**G**). After incubation, the nuclear extracts from the cells were analyzed by EMSA to determine the level of activated AP-1 using a radiolabeled oligonucleotide probe. **H**: AGS cells, after being pretreated with 10 μM SP600125 for 1 h, were incubated with 5 μM LPA for 2 h, and then determined for the activated AP-1 by EMSA. **I**: AP-1 decoy oligonucleotide (1, 2 μg/ml) was cotransfected with pGL3-uPAR into the AGS cells. Where indicated, mutated pGL3-uPAR (converted the AP-1site TCCATGAGTCA to TCGGAATTCCA) was transfected into the cells. After incubation with 5 μM LPA for 2 h, the luciferase activities were determined using a luminometer. The data represents the mean ± SD from triplicate measurements. $^*P < 0.05$ versus LPA.

inhibitor of NF-κB) before the LPA treatment. BAY11-7082 inhibited the LPA-induced NF-κB-probe complex formation (Fig. 4C). BAY11-7082 also blocked the uPAR mRNA expression and promoter activity (Fig. 4D,E). The AGS cells transfected with pGL3-uPAR mutated in NF-κB binding site resulted in a decrease in uPAR promoter activity by LPA (Fig. 4E). In addition, the amount of AP-1 that could form a complex with the radiolabeled oligonucleotide probe was

increased in the AGS cells treated with LPA in a dose- and time-dependent manner (Fig. 4F,G). SP600125 (a JNK inhibitor) partially inhibited the LPA-induced AP-1-probe complex formation, suggesting the JNK pathway is involved in the AP-1 activation by LPA (Fig. 4H). Moreover, when the AGS cells were transfected with the uPAR promoter-reporter construct, in which the AP-1 binding site had been mutated or co-transfected, an AP-1 decoy oligonucleotide with pGL3-uPAR decreased LPA-induced uPAR promoter activity (Fig. 4I).

Effect of LPA on AGS Cell Invasiveness

It was suggested that the expression of uPAR is essential for the invasive phenotype of cancer cells. The role of LPA-induced uPAR in the AGS cell invasion was evaluated by incubating the cells with the specific antibodies to uPAR in a modified Boyden invasion chamber. As shown in Figure 5A, the cell invasiveness was increased by incubation with LPA. However, the LPA-treated cells partially lost their Matrigel invasiveness after incubation with the uPAR neutralizing antibodies, whereas they did not lose their matrigel invasiveness after incubation with the non-specific IgG. These results suggest that the uPAR induced by LPA plays an important role in the invasiveness of gastric cancer cells. In order to confirm that the Rho-family GTPases, JNK and NF-κB are involved in the LPA-induced invasiveness, the AGS cells were treated with Y-27632, SP600125, and BAY11-7082 before the LPA treatment. As shown in Figure 5B, all the inhibitors (Y-27632, SP600125, BAY11-7082) blocked the Matrigel invasiveness induced by LPA. However, the inhibitor alone was not significantly changed the cell invasiveness. This suggests that the Rho-family GTPases, JNK, and NF-κB signals activated by LPA upregulate uPAR, leading to an increase in the gastric cancer cell invasiveness (Fig. 6).

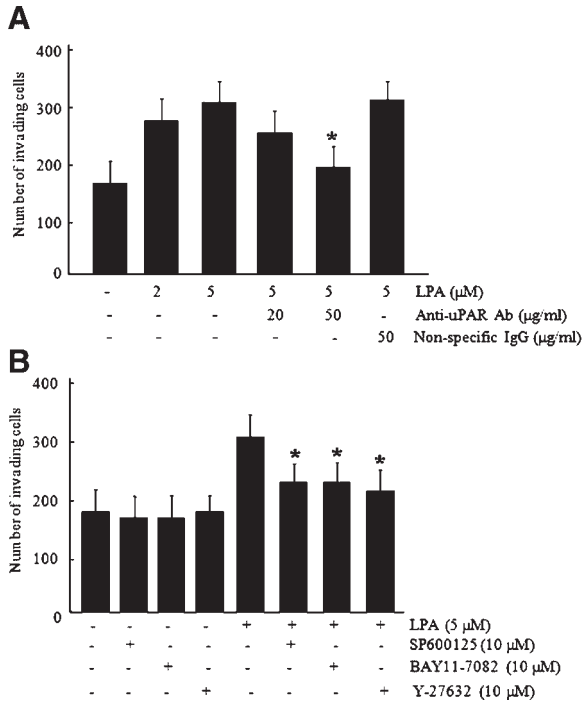

Fig. 5. Effects of LPA and inhibitors on AGS cell invasiveness. AGS cells (10^5) were incubated with 0–5 μM LPA in a Boyden Matrigel for 24 h in the presence or absence of anti-uPAR antibodies (20–50 μg/ml) (**A**), 10 μM Y-27632, 10 μM SP600125, and 10 μM BAY 11-7082 (**B**). After incubation, the cells invading the undersurface of the chambers were counted using a phase contrast light microscope after staining with a Diff-Quick stain kit. The data represents the mean ± SD from triplicate measurements. $^*P < 0.05$ versus LPA.

DISCUSSION

Lysophosphatidic acid (LPA) is bioactive phospholipid that is present in serum and is induced by various genes related to cancer angiogenesis and metastasis. LPA is produced through the action of lysophospholipids from inflammatory cells and platelets [Aoki et al., 2002]. Therefore, LPA has the potential to induce cancer invasion and metastasis. Several studies have provided the following evidence for the association between LPA and cancer progression: (i) Cancer cells produce more LPA than normal cells [Radeff-Huang et al., 2004]; (ii) LPA can induce the expression of proteases

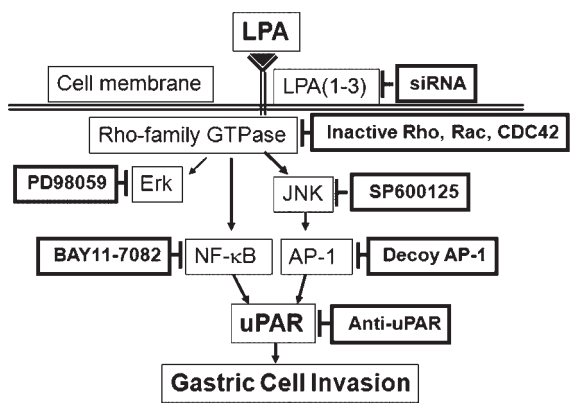

Fig. 6. Possible intracellular signaling pathways for the LPA-induced expression of uPAR and cell invasion in human gastric AGS cells.

including urokinase plasminogen activator [Li et al., 2005] and MMPs [Wu et al., 2005]; (iii) LPA can promote aggressive behavior in cancer cells by inducing the expression of cyclooxygenase-2 [Symowicz et al., 2005]; and (iv) LPA can induce expression of angiogenic factors such as IL-8 [Zhao et al., 2005], monocyte chemoattractant protein-1 [Kaneyuki et al., 2007], and vascular endothelial growth factor [Hu et al., 2001].

The present study found that LPA can induce the expression of uPAR and stimulate the cell invasiveness in human gastric cancer AGS cells, suggesting that the overexpression of uPAR by LPA is involved in the increased cell invasiveness.

LPA elicits its cellular responses through at least three different receptors, LPA1/Edg-2, LPA2/Edg-4, and LPA3/Edg-7 [Moolenaar, 1999]. The three LPA receptors are distributed quite differently in human tissues. Furthermore, it was suggested that a malignant transformation results in the aberrant new appearance or changes in the levels of LPA receptor expression. Guo et al. [2006] suggested that LPA1 gene expression is significantly higher in cancers from human prostate specimens than in benign tissues. In gastric cancer, Yamashita et al. [2006] suggested that the LPA receptor is differentially expressed in a various types of gastric cancer cell lines. In this study, among the three LPA receptors, the AGS cells continuously expressed LPA2 and LPA3 mRNA, and LPA1 mRNA expression was induced by LPA. Inhibition of the LPA receptors by siRNA reduced the LPA-induced expression and transcriptional activity of uPAR mRNA.

Cancer cell migration and invasion are central to the process of metastasis, and regulation of this process will be a target for cancer therapy. In particular, reorganization of the actin cytoskeleton is the primary mechanism for most types of cell migration. Rho-family GTPases such as Rho, Rac and Cdc42 regulates actin reorganization and gives cancer cells the ability to metastasize [Etienne-Manneville and Hall, 2002]. These results showed that the dominant negative Rho, Rac and Cdc42 mutants as well as the Rho-associated kinase (a downstream of Rho) inhibitor, Y-27632, were capable of down-regulating the uPAR promoter activity, which suggests that Rho-family GTPases are involved in LPA-induced uPAR expression. However, the Rho-family GTPases involved in uPAR expression have not identified. Price and Collard [2001] implicated the Rho-family GTPases in tumor invasion and activation by LPA.

MAPKs have been shown to regulate the expression and invasion of uPAR in a number of cell types, including gastric cancer [Kim et al., 2005]. However, the role of Erk-1/2, P38 and JNK MAPK in uPAR regulation appears to be cell type and stimuli specific. Previous studies suggested that Erk-1/2 and JNK MAPKs activation are involved in uPAR expression in gastric cancer [Kim et al., 2005]. In this study, this activation of Erk-1/2 and JNK preceded the induction of uPAR expression. However, the upregulation of uPAR was only attenuated by the selective JNK inhibitor (SP600125). In addition, transfection of the expression vector encoding a mutated c-jun (TAM67) decreased the uPAR promoter activity. These results suggest that the JNK signaling pathway plays some role in upregulating the uPAR gene by LPA. Phosphorylatation of c-jun results in the acquisition of enhanced transcription activity of complexes containing AP-1. In the absence of serine phosphorylation, c-jun is degraded by a ubiquitin-dependent proteolytic pathway [Manning and Davis, 2003].

Wang et al. [2000] reported that several transcription factors, including AP-1 and NF-κB, are present in the proximal 5′ region of the uPAR gene. In addition, previous studies suggested that AP-1 and NF-κB are involved in the signaling pathway for uPAR induction [Kim et al., 2005]. These results suggest that NF-κB and AP-1 are also involved in the signaling pathway for uPAR induction by LPA in AGS cells: (i) LPA treatment increased the amount of NF-κB and AP-1, which can form a complex with the radiolabeled oligonucleotide prove in EMSA; (ii) a LPA treatment also increases the NF-κB and AP-1-dependent transcriptional activity, as revealed by the Luc reporter construct assay; and (iii) Site-directed mutagenesis studies on the uPAR promoter revealed that NF-κB and AP-1 are essential for uPAR induction by LPA.

This study demonstrated that LPA enhances the expression of uPAR and cell invasiveness in human gastric cancer cells. As shown in Figure 6, LPA-induced uPAR expression is mediated through LPA receptors, Rho-GTPases, JNK, NF-κB and AP-1 signaling pathways, which in turn, stimulates the cell invasiveness.

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