Activation of Hypoxia-Inducible Factor-1α Is Necessary for Lysophosphatidic Acid – Induced Vascular Endothelial Growth Factor Expression

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Abstract

Purpose: Lysophosphatidic acid (LPA) plays an important role in mediating cell proliferation, survival, and tumor invasion and angiogenesis. This bioactive phospholipid at the concentration in ascitic fluid stimulates the growth of malignant ovarian tumors by increasing the expression of vascular endothelial growth factor (VEGF). In the present study, we investigated whether LPA activates hypoxia inducible factor-1 (HIF-1), a key transcriptional complex in tumor progression and metastasis, thereby increasing the expression of VEGF.

Experimental Design: Immunoblotting, reverse transcription-PCR, ELISA, immunofluorescence, and chromatin immunoprecipitation assay were used to examine the expression of VEGF and HIF-1α in various cancer cells. Specific HIF-1α small interfering RNA was transfected to various cancer cells to determine the role of HIF-1α in LPA-induced VEGF expression.

Results: LPA induced expressions of VEGF and HIF-1α in OVCAR-3, CAOV-3, PC-3, and SK-Hep1 cells but not in SKOV-3 and Hep-3B cells. In OVCAR-3 and PC-3 cells, the phosphoinositide 3-kinase/Akt/mammalian target of rapamycin/p70S6K and p42/p44 mitogen-activated protein kinase pathways were required for LPA-induced HIF-1α and VEGF expressions, whereas only the phosphoinositide 3-kinase/mammalian target of rapamycin/p70S6K pathway was important in SK-Hep1 cells. Immunofluorescence microscopy assay showed translocation of HIF-1α to nucleus by LPA, and chromatin immunoprecipitation assay revealed the binding of HIF-1α to the promoter of VEGF by LPA. Importantly, we found that small interfering RNA – induced reduction of HIF-1α expression significantly attenuated VEGF expression by LPA.

Conclusions: Our results show for the first time that LPA induces VEGF via HIF-1α activation and reveal a critical role of HIF-1α in LPA-induced cancer cell proliferation and angiogenesis.

Lysophosphatidic acid (1-acyl-LPA or 2-acyl-LPA) is a lipid mediator that is involved in multiple cellular events of almost every mammalian cell type (1). The estimated concentrations of active, albumin-bound LPA in serum are in the range of 1 to 5 μmol/L (2). Upon binding to G protein – coupled receptors Edg2/LPA1 (3), Edg4/LPA2 (4), and Edg7/LPA3 (5), LPA exerts diverse biological effects, including cell proliferation/survival, induction of neurite retraction, inhibition of gap junctional communication, and cell motility (6, 7). In ovary cancer, LPA contributes to the development, progression, and metastasis and is increased in both plasma and ascites of ovary cancer patients, reaching 80 μmol/L concentration (8 – 10). Ovary cancer cells also produce LPA, thereby maintaining an LPA-rich microenvironment (8, 11). Elevated LPA levels have been detected in 98% of ovary cancer patients, including 90% of patients with stage I disease, suggesting that LPA promotes early events in ovary carcinoma dissemination.

The hypoxia-inducible factor-1 (HIF-1) plays a central role in tumor progression and angiogenesis. This protein is a heterodimeric transcription factor, which regulates hypoxia-activated genes, and consist of a constitutively expressed HIF-1β subunit and a highly regulated HIF-1α (12 – 14). Upon binding to the hypoxia-responsive element within target gene, HIF-1 activates transcription of various hypoxia-inducible genes like angiogenic factors [vascular endothelial growth factor (*VEGF*)] as well as proliferation/survival factors [insulin-like growth factor-2 (*IGF2*), *NOS2*, WAF-1, and transforming growth factor-α] and extracellular matrix metabolism (urokinase-type plasminogen activator receptor and matrix metalloproteinase 2; ref. 13). The amount of HIF-1α is mainly regulated by either

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oxygen-dependent or oxygen-independent ways. In the presence of oxygen, HIF-1α is rapidly degraded by von Hippel-Lindau (VHL) – mediated ubiquitination and subsequent degradation by the proteasome (15). However, in hypoxia, HIF-1α is stabilized by binding with HIF-1β in the nucleus. Hypoxia-independent regulation of HIF-1α has also been shown to occur by a variety of growth factors and cytokines, including insulin, IGF-1, IGF-2, transforming growth factor, platelet-derived growth factor, epidermal growth factor, and interleukin-1 (16 – 22). In addition, oncogenic activation (such as Ha-*ras*, *myc*, or *src*) or loss of tumor suppressor function (such as p53, PTEN, or VHL) is also associated with HIF-1-mediated tumor progression (23).

VEGF is a dimeric glycoprotein that is critically important in both normal and tumor-associated angiogenesis (24).

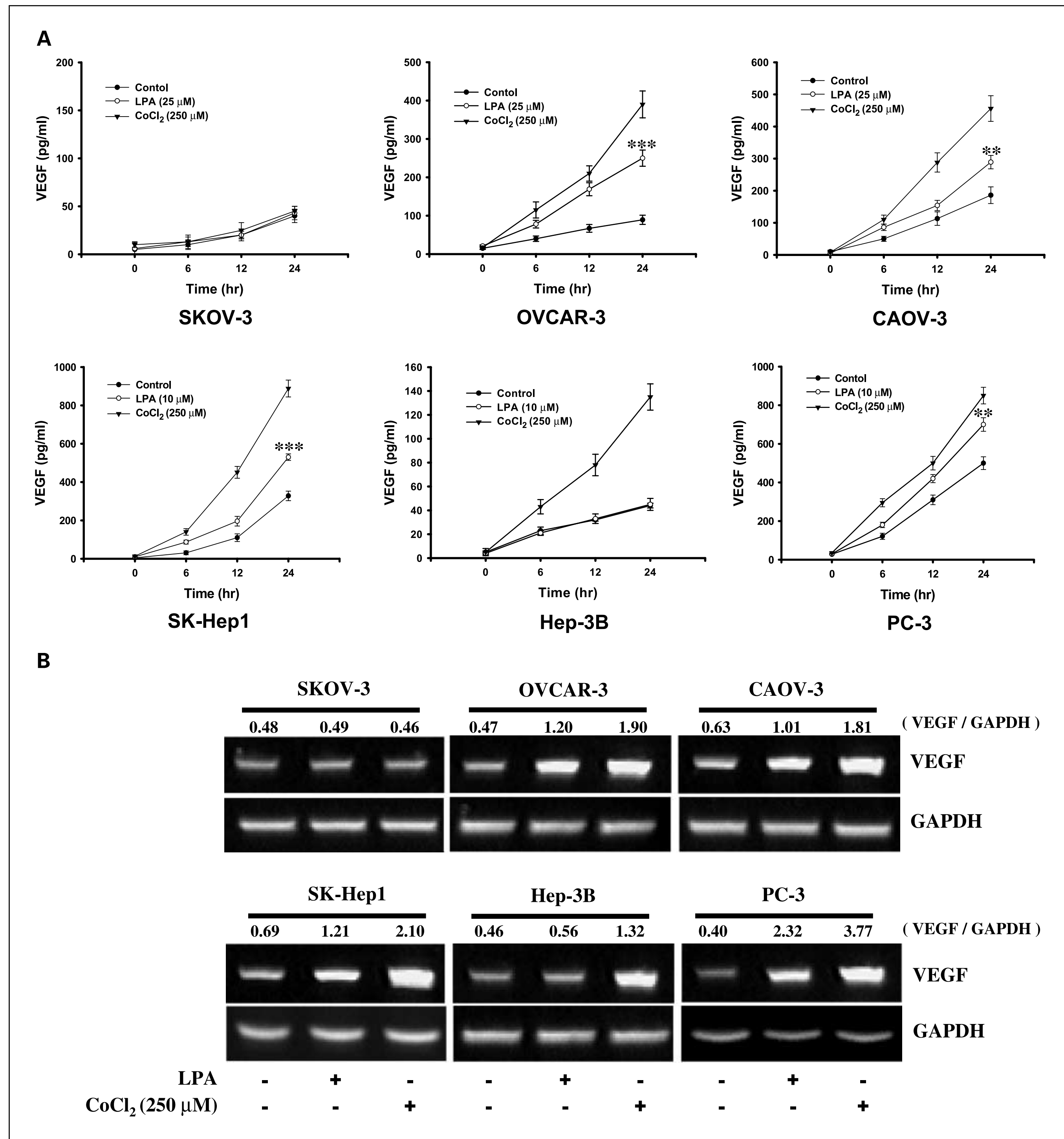

Fig. 1. LPA induces VEGF and HIF-1α expressions. *A,* the cells (5×10^5) were serum starved and incubated with LPA or $CoCl_2$ for the indicated time periods. VEGF protein level in the conditioned medium of tested cells was determined by ELISA. *B,* effects of LPA on VEGF mRNA. The cells were serum starved and exposed to LPA or $CoCl_2$ (250 μmol/L) for 24 hours. Total RNA was isolated, and RT-PCR was done with specific VEGF primers.

Overexpression of VEGF has been associated with tumor progression and poor prognosis in several tumors, including ovarian carcinoma (25) and hepatoma (26). The VEGF promoter has two transcriptional regulatory sites (SP1 and HRE), and they are located at −90 to −50 and −985 to −939 upstream of the transcription start site of VEGF, respectively (27). Each transcriptional regulatory site is important for the expression of VEGF in nonhypoxic cells. Although HER-2 and IGF-1 induce transcriptional activation of the *VEGF* gene by HIF-1 (28, 29), HGF increases VEGF expression through an SP1 transcription factor binding site (30).

LPA has been reported to induce the expression of VEGF in ovary (31, 32) and colon cancer (33). However, the detailed intracellular mechanisms of LPA-induced VEGF expression in cancer cells are not known. In the present study, we provide the evidence that HIF-1α is the key regulator for LPA-induced VEGF expression in cancer cells.

Materials and Methods

Reagents and antibodies. LPA was purchased from Avanti Polar Lipids (Alabaster, AL). LY294002, PD98059, and rapamycin were from Calbiochem (San Diego, CA). HIF-1α polyclonal antibody (for chromatin immunoprecipitation assay) was obtained from Chemicon (Temecula, CA); β-actin monoclonal antibody was from Sigma-Aldrich (St. Louis, MO), and HIF-1α monoclonal antibody (for Western blotting) was from BD (San Diego, CA). Phospho-antibodies [AKT, extracellular signal-regulated kinase, p70S6K, and mammalian target of rapamycin (mTOR)] were obtained from Cell Signaling (Beverly, CA), and enhanced chemiluminescence reagents and Sepharose 4B were from Amersham-Pharmacia Biotech (Piscataway, NJ). LipofectAMINE 2000 was obtained from Invitrogen (Carlsbad, CA); chromatin immunoprecipitation assay kit was from Upstate (Waltham, MA); and cycloheximide was purchased from Sigma-Aldrich. All other reagents used were of the purest grade available.

Cell culture. All cancer cells lines (SKOV-3, CAOV-3, OVCAR-3, PC-3, SK-Hep1, and Hep-3B) were purchased from the American Type Culture Collection (Manassas, VA). Cells were maintained in DMEM or RPMI 1640, supplemented with 10 % fetal bovine serum in a humidified atmosphere containing 5 % CO_2 at 37°C. For experimental purposes, the cells (5×10^5) were plated in six-well culture dishes. After 1 day, the cells were serum starved overnight before use.

ELISA assay. VEGF production in culture mediums was examined by using a human VEGF-specific ELISA kit (Pierce Biotechnology, Rockford, IL) according to the manufacturer's instructions.

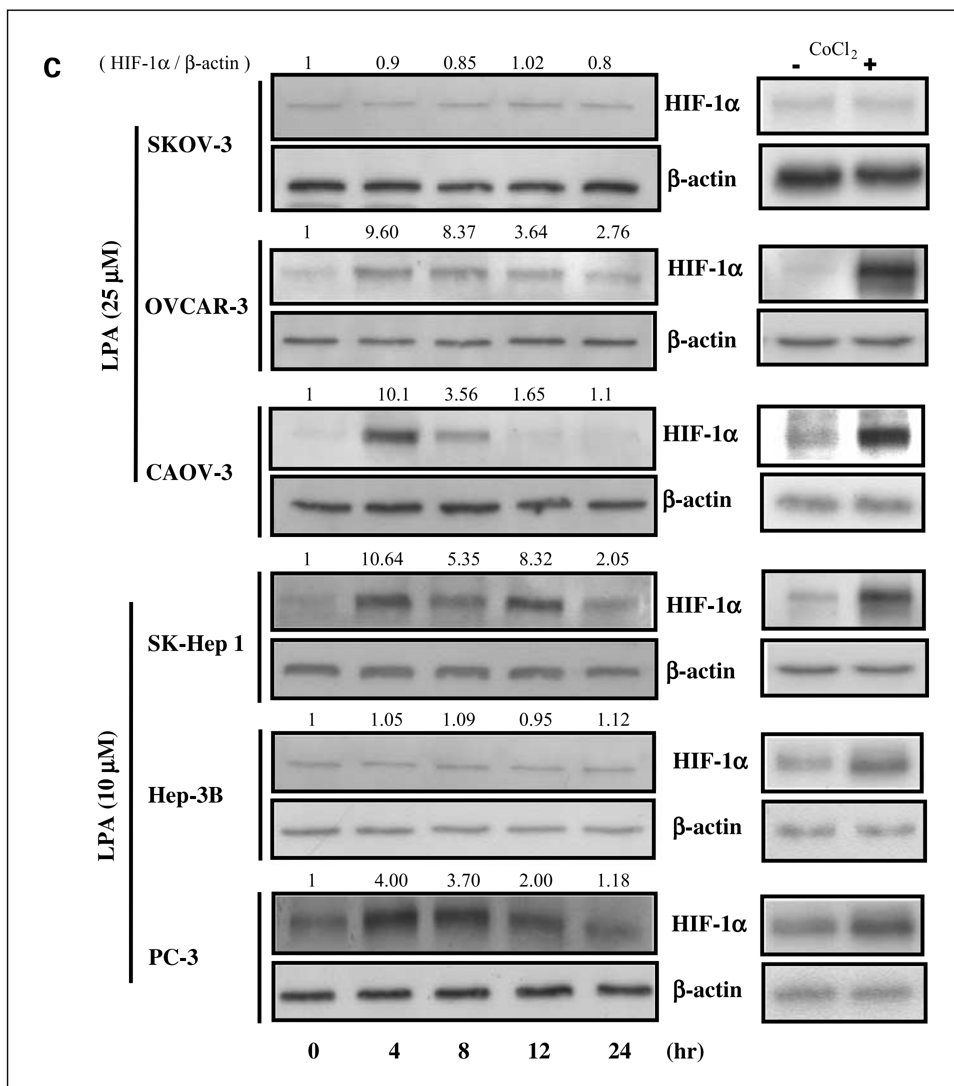

Fig. 1 *Continued. C,* the serum-starved cells were incubated with or without LPA or $CoCl_2$ for the indicated time periods. HIF-1α protein level of the cells tested was determined by immunoblotting with anti-HIF-1α antibody. The concentrations of LPA used were 10 μmol/L for SK-Hep1, Hep-3B, and PC-3 cells and 25 μmol/L for OVCAR-3, SKOV-3, and CAOV-3 cells. Representative of three experiments with similar results. Points, mean; bars, SE. **, $P < 0.025$; ***, $P < 0.001$, significantly different from the LPA-unstimulated cells.

Reverse transcription-PCR. Total cellular RNA (1 μg) isolated from cultured cell lines was used for reverse transcription. The cDNA was then subjected to PCR amplification with primer sets for HIF-1α, VEGF, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH): HIF-1α forward, 5′-TCCATGTGACCATGAGGAAA-3′; HIF-1α reverse, 5′-TATCCAGGCTGTGTCGACTG-3′; VEGF forward, 5′-CTACCTCCACCATGCCAAGT-3′; VEGF reverse, 5′-ATCTGCATGGTGATGTTGGA-3′; GAPDH forward, 5′-CATCTTCCAGGAGCGAGA-3′; GAPDH reverse, 5′-CTGCTTCACCACCTTCTTGAT-3′. Initial denaturation at 94°C for 3 minutes was done only for the first cycle, and the sequence of PCR amplification was followed by denaturation at 94°C for 30 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 30 seconds (32 cycle for HIF-1α, 28 cycle for GAPDH). The samples were resolved on a 1.2 % agarose gel and stained with ethidium bromide, and their intensity was compared using EagleSight Software v. 3.2 (Stratagene, La Jolla, CA).

Immunoblot assay. Quiescent cells were treated with LPA (indicated concentrations) for the period indicated. The stimulation was terminated by aspirating the medium and solubilizing the cells in 500 μL of ice-cold radioimmunoprecipitation assay buffer (34). Lysates were resolved by 10% SDS-PAGE and transferred to polyvinylidene difluoride membrane. Polyvinylidene difluoride membranes with proteins were blocked with 5% nonfat dried milk in PBS (pH 7.2) and incubated overnight at 4°C with the antibodies indicated. The membranes were washed twice with washing buffer (0.1% Tween 20 in PBS) and incubated with secondary antibodies (horseradish peroxidase–conjugated antibodies) for 1 hour at room temperature. After washing twice with washing buffer, the bands were visualized using enhanced chemiluminescence.

Immunofluorescence microscopy assay. Immunofluorescence was done as previously described (34). HIF-1α antibody was diluted 1:200, and all secondary antibodies were diluted 1:1,000. Fluorescence was visualized using a Leica DMR immunofluorescence microscope with DFC480 digital camera.

Chromatin immunoprecipitation assay. Chromatin immunoprecipitation was done using a kit purchased from Upstate Biotechnology (Charlottesville, VA) according to the protocol recommended by the manufacturer. The region of −1386 to −1036 of the *VEGF* promoter gene was amplified by PCR from the immunoprecipitated chromatin using the following primers: sense, 5′-CAGGTCAGAAACCAGCCAG-3′; antisense, 5′-CGTGATGATTCAAACCTACC-3′. The 350-bp PCR product was resolved on a 1.2% agarose gel and visualized by ethidium bromide staining and UV illumination.

Short interfering RNA. Small interfering RNA (siRNA) corresponding to the *HIF-1α* gene was designed and synthesized by BLOCK-iT RNA

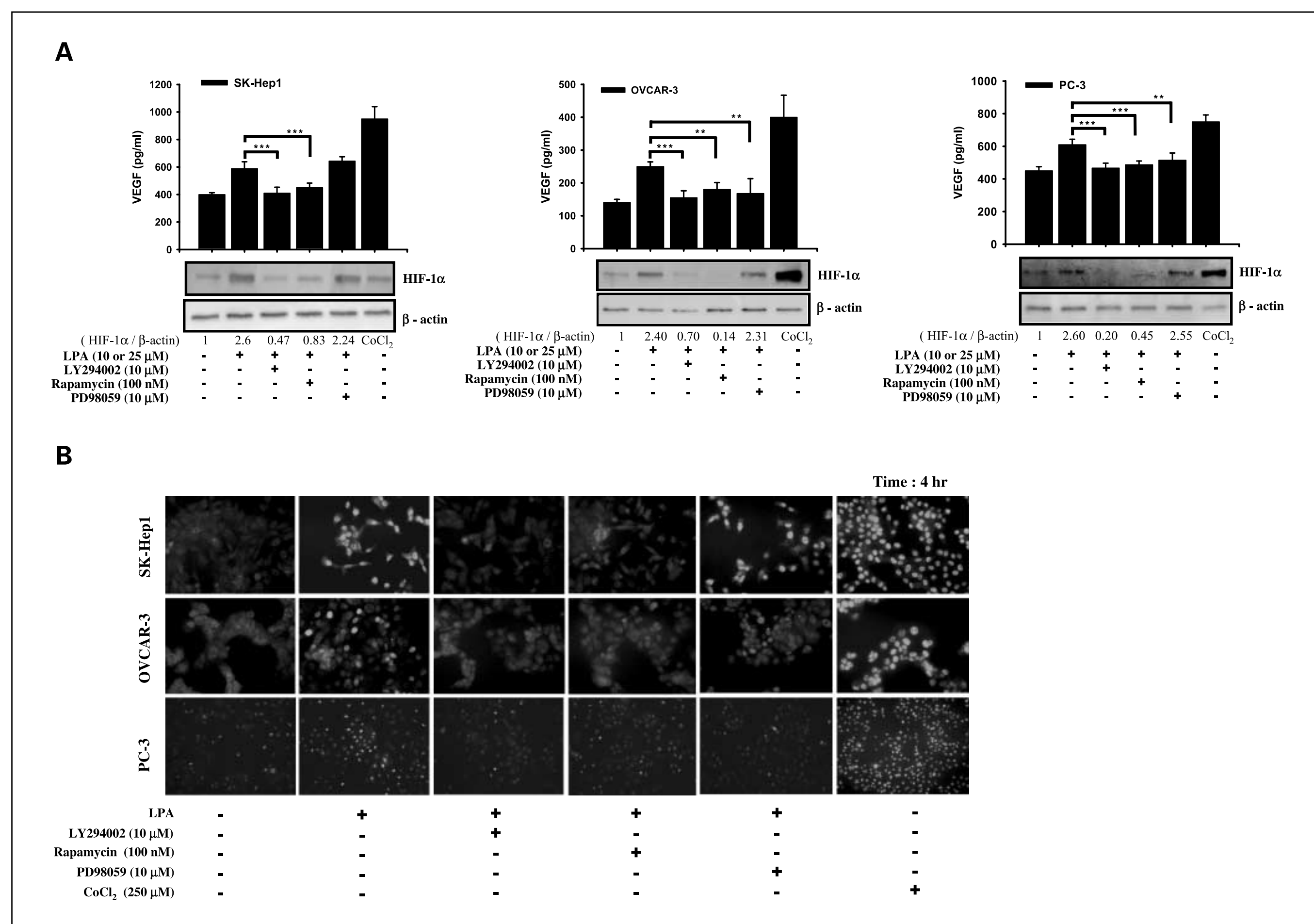

Fig. 2. *A,* PI3K/Akt/mTOR/p70S6K and p42/p44 MAP kinase pathways are involved in LPA-induced VEGF and HIF-1α expressions. Effects of pharmacologic inhibitors on LPA-induced HIF-1α (*bottom channel*) and VEGF expressions (*top channel*). Serum-starved cells were pretreated for 2 hours with vehicle or inhibitors and then exposed to LPA for 4 hours (for HIF-1α) or 24 hours (for VEGF). HIF-1α protein level of tested cells was determined by immunoblotting with anti-HIF-1α antibody. *B,* nuclear translocation of HIF-1α by LPA. The cells seeded in four-chamber slide were serum starved for 16 hours and treated for 2 hours with vehicle or inhibitor. The cells were then activated with LPA or $CoCl_2$ for 4 hours and immunostained with a mouse monoclonal anti-HIF-1α antibody. The concentrations of LPA used were 10 μmol/L for SK-Hep1 and PC-3 cells and 25 μmol/L for OVCAR-3 cells. The concentration of $CoCl_2$ used was 250 μmol/L as a positive control. The inhibitors used in these reactions were 10 μmol/L LY294002, 100 nmol/L rapamycin, or 10 μmol/L PD98059. Representative of three experiments with similar results. Columns, mean; bars, SE. **, $P < 0.025$; ***, $P < 0.001$, significantly different from the LPA-stimulated cells.

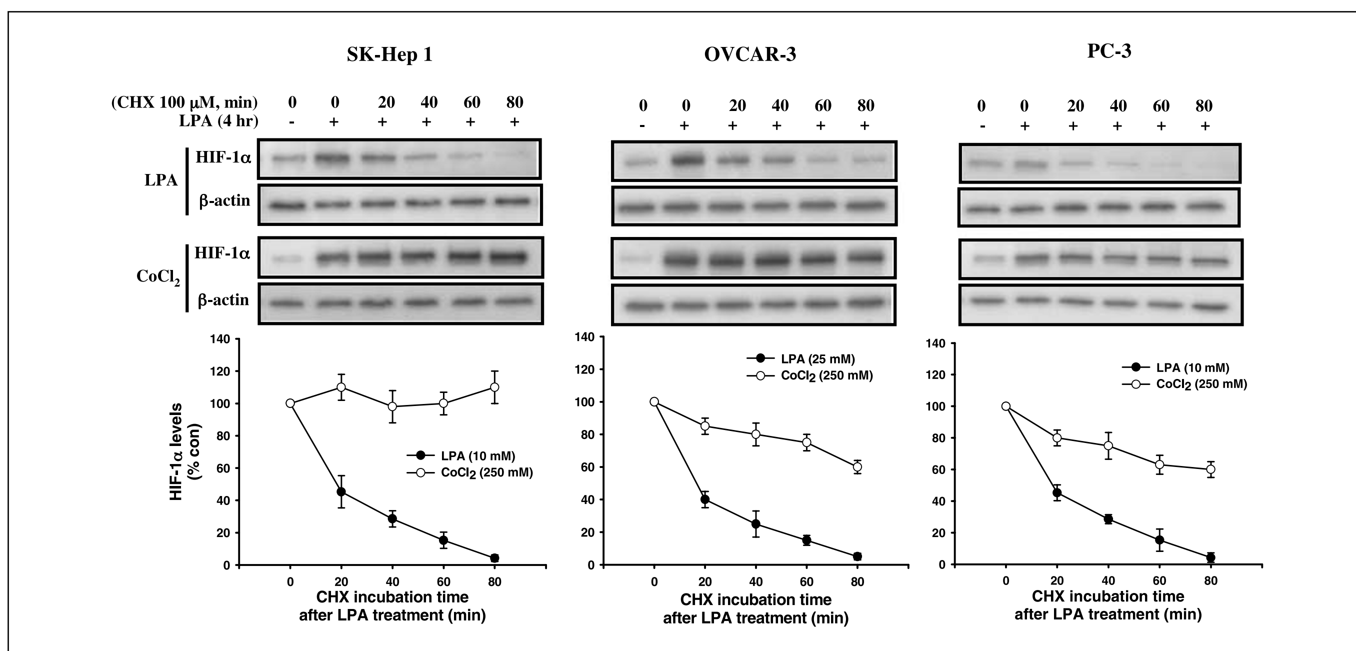

Fig. 3. Effects of LPA on HIF-1α protein stability. Serum-starved cells were pretreated with LPA or $CoCl_2$ (250 μmol/L) for 4 hours, and then cycloheximide (*CHX*) was added to a final concentration of 100 μmol/L. Cells were harvested at indicated times, and whole-cell lysates were subject to immunoblot assay to assess HIF-1α and β-actin (as loading control). Densitometry was used to measure the autoradiographic HIF-1α signal. Bottom, results. The concentrations of LPA used were 10 μmol/L for SK-Hep1 and PC-3 cells and 25 μmol/L for OVCAR-3 cells. One representative result from three experiments.

interference Express (Invitrogen). The following sequences were used: (positive sequence) forward, 5′-AGTTAGTTCAAACTGAGTTAATCCC-3′; reverse, 5′-GGGATTAACTCAGTTTGAACTAACT-3′; (negative control) forward, 5′-GGGAATCTCTGAGTTCAAATATACT-3′; reverse, 5′-AGTATATTTGAACTCAGAGATTCCC-3′. siRNA duplexes were used for transfection at a final concentration of 40 pmol/L. Cells were plated onto 12-well tissue culture dishes a day before the transfection, and the cells were transiently transfected with the LipofectAMINE 2000 (Invitrogen) according to the manufacture's instruction.

Statistical analysis. Results are expressed as mean ± SD, and an analysis was done by Student's t test. Ps < 0.05 were considered statistically significant.

Results

LPA induces VEGF and HIF-1α expressions in cancer cells. To test whether LPA modulates the levels of VEGF in our system, ovarian cancer (SKOV-3, OVCAR-3, and CAOV-3), prostate cancer (PC-3), and hepatoma (SK-Hep1 and Hep-3B) cells were incubated with LPA or 250 μmol/L $CoCl_2$ for indicated period of time, and then the conditioned media of each cancer cells were tested for the VEGF production through ELISA. As shown in Fig. 1A, there was no change by LPA, if any, in the VEGF production of SKOV-3 and Hep-3B cells. However, the expression of VEGF in OVCAR-3, CAOV-3, PC-3, and SK-Hep1 cells was increased by LPA in a time-dependent manner. As a positive control of hypoxia, the cells were incubated with hypoxia-mimicking agent $CoCl_2$. Interestingly, $CoCl_2$ increased the expression of VEGF in all cells except SKOV-3 cells. To examine whether LPA-induced VEGF expression occurred transcriptionally, the cells were incubated with or without LPA for 24 hours. Consistent to the data of VEGF protein, LPA increased the level of VEGF mRNA in OVCAR-3, CAOV-3, PC-3, and SK-Hep1 cells but not in SKOV-3 and Hep-3B cells (Fig. 1B). To test the involvement of HIF-1α in LPA-induced VEGF expression, the cancer cells were treated with LPA for indicated time period, and the expression of HIF-1α was monitored by immunoblotting with anti-HIF-1α antibody. LPA increased the expression of HIF-1α in OVCAR-3, CAOV-3, PC-3, and SK-Hep 1 cells, reaching a maximum level at 4 hours

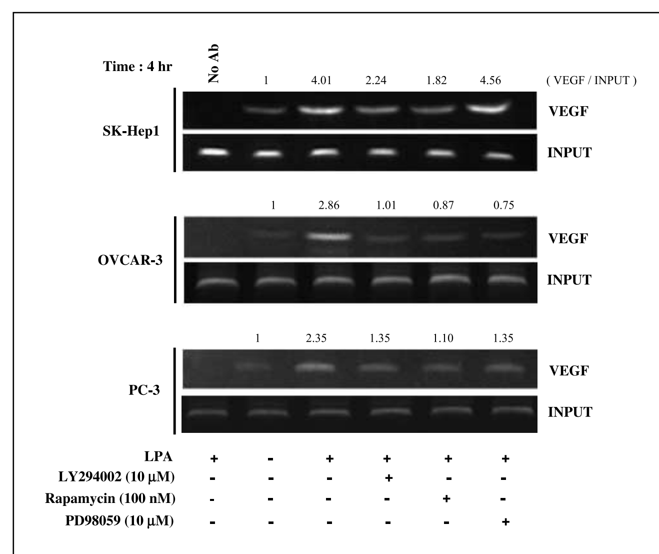

Fig. 4. LPA induces the binding of HIF-1α to the VEGF promoter. Chromatin immunoprecipitation assay with HIF-1α antibody was done as described in Materials and Methods. The serum-starved cells were pretreated for 2 hours with vehicle or inhibitors and then exposed to LPA for 4 hours. The immunoprecipitated DNA was purified, and the region from −1386 to −1036 bp of the human VEGF promoter was amplified by PCR (32 cycles). The concentrations of LPA used were 10 μmol/L for SK-Hep1 and PC-3 cells and 25 μmol/L for OVCAR-3 cells. The inhibitors used in these reactions were 10 μmol/L LY294002, 100 nmol/L rapamycin, or 10 μmol/L PD98059. Representative of three experiments with similar results.

of incubation (Fig. 1C). However, the expression of HIF-1α in SKOV-3 and Hep-3B cells was not increased by LPA. Furthermore, $CoCl_2$ did not give any influence to the expression of HIF-1α in SKOV-3 cells. These results are consistent with the data of LPA-induced VEGF expression (Fig. 1A), suggesting that there is a close relationship between the expressions of VEGF and HIF-1α by LPA in cancer cells tested. To determine the effect of LPA on transcriptional expression of HIF-1α, we analyzed the mRNA levels of HIF-1α by reverse transcription-PCR in cancer cells treated with or without LPA. LPA treatment did not, if any, change the amount of HIF-1α mRNA of cancer cells (Supplementary Fig. S1).

Phosphoinositide 3-kinase/Akt/mTOR/p70S6K and p42/p44 mitogen-activated protein kinase pathways are involved in LPA-induced VEGF and HIF-1α expressions. Because the significance of the phosphoinositide 3-kinase (PI3K)/Akt/mTOR/p70S6K (35, 36) and p42/p44 mitogen-activated protein kinase (MAPK; ref. 37) pathways has been proposed for VEGF expression, we analyzed the effects of LPA on the activities of Akt, mTOR, p70S6K, and p42/p44 MAPK. LPA increased phosphorylations of Akt, mTOR, p70S6K, and p42/p44 MAPK in OVCAR-3, PC-3, and SK-Hep1 cells (Supplementary Fig. S2). Next, to identify the signaling factors involved in LPA-induced VEGF and HIF-1α expressions, we used pharmacologic inhibitors of PI3K (LY294002), mTOR (rapamycin), and p42/p44 MAPK (PD98059). LY294002 and rapamycin markedly inhibited the LPA-induced HIF-1α and VEGF (Fig. 2A) expressions in all cells tested. However, the LPA-induced HIF-1α and VEGF expressions in SK-Hep1 were not inhibited by PD98059.

Because our data showed that LPA significantly increased level of the HIF-1α protein (Fig. 1C), we examined by immunofluorescence analysis whether this bioactive phospholipid molecule promotes the nuclear translocation of HIF-1α protein independent of hypoxia. As shown in *lane 2* of Fig. 2B, LPA increased the amount of HIF-1α in the nucleus of OVCAR-3 and PC-3 cells that was abrogated by pretreatment with LY294002, rapamycin, and PD98059. On the other hand, however, pretreatment of SK-Hep1 cells with PD98059 did not show any dramatic inhibition of HIF-1α translocalization induced by LPA.

LPA does not stabilize HIF-1α protein. We studied the effect of cycloheximide to investigate whether the increased amount of HIF-1α by LPA occurred in the process of protein stabilization. Thus, cancer cells were incubated with LPA or $CoCl_2$ for 4 hours followed by cycloheximide treatment for an indicated time period. As shown in Fig. 3, the amount of HIF-1α was only slightly reduced when the cells were treated with $CoCl_2$ in the presence of cycloheximide for 80 minutes. However, the expression of HIF-1α in the LPA-activated cells profoundly decreased to the basal level by cycloheximide incubation for 80 minutes, suggesting that LPA does not stabilize HIF-1 α protein in tested cells.

LPA induces binding of HIF-1α to VEGF promoter. To elucidate the interaction of HIF-1α at native hypoxia response elements of the VEGF promoter by LPA, chromatin immunoprecipitation assay system was used. Fractionated chromatins from control and LPA-stimulated cells were immunoprecipitated with anti-HIF-1α antibody followed by PCR analysis of native HIF-1 binding sites from VEGF genes. As shown in Fig. 4, HIF- 1α bound to the VEGF promoter by LPA was precipitated with anti-HIF-1α antibody. Controls either lacking the antibody (*lane 1*) or using the anti-HIF-1α antibody with lysate from untreated cells (*lane 2*) were included to confirm the specificity of the assay. Pretreatment of the cells (OVCAR-3 and PC-3) with LY294002, rapamycin, and PD98059 profoundly inhibited LPA-induced binding of HIF-1α to the VEGF promoter. Consistent to our earlier data (Fig. 2A), PD98059 did not inhibit the binding of HIF-1α to the VEGF promoter in SK-Hep1 cells.

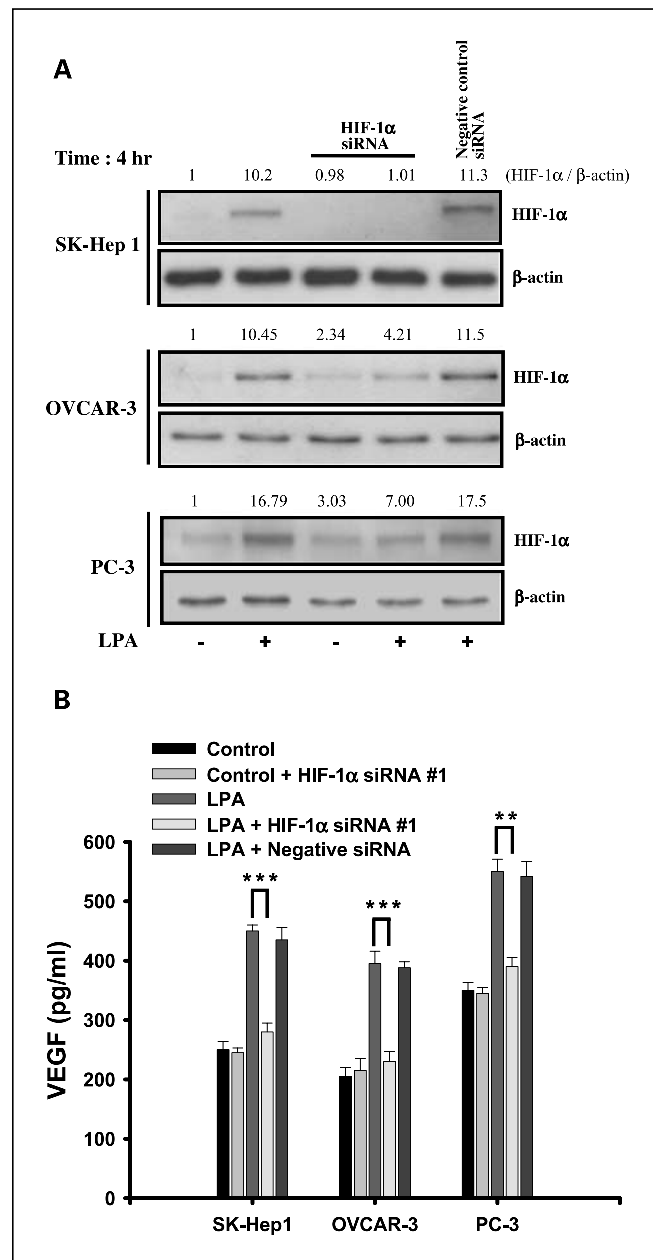

Fig. 5. Roles of HIF-1α in LPA-induced VEGF expression. The cells were transfected with 40 nmol/L HIF-1α-specific siRNA for 24 hours and then serum starved for 24 hours. HIF-1α was detected by immunoblotting with HIF-1α antibody in whole-cell extract after 4 hours of LPA. *A,* HIF-1α siRNA reduced LPA-inducible HIF-1α expression. *B,* inhibition of LPA-induced VEGF expression by HIF-1α siRNA. The concentrations of LPA used were 10 μmol/L for SK-Hep1 and PC-3 cells and 25 μmol/L for OVCAR-3 cells. Representative of three experiments with similar results. Columns, mean; bars, SE. **, $P < 0.025$; ***, $P < 0.001$, significantly different from the LPA-stimulated cells.

HIF-1α siRNA inhibits LPA-induced VEGF expression. To gain further insight into the functional significance of HIF-1α in LPA-induced VEGF expression, we determined whether reduced expression of HIF-1α affected LPA-induced VEGF expression. Cancer cells were transfected with HIF-1α siRNA for 24 hours and then stimulated with or without LPA. Treatment of cancer cells with HIF-1α siRNA significantly reduced LPA-induced HIF-1α expression, whereas control siRNA had no detectable effect (Fig. 5A). It is noted that HIF-1α siRNA was specific for targeting HIF-1α because the expression of β-actin was not changed. Decreasing HIF-1α expression by its siRNA significantly inhibited LPA-induced VEGF expression in cancer cells (Fig. 5B).

Discussion

In this study, we show the first evidence that LPA induces VEGF expression by activation of HIF-1α in various cancer cells. LPA-induced HIF-1α activation seems to be regulated by translational regulation rather than protein stabilization, which are governed by different signaling pathway depending on cancer cell lines. Both PI3K/Akt/mTOR/p70S6K and p42/p44 MAPK pathways are required for LPA-induced HIF-1α activation in ovarian cancer and prostate cancer, whereas only PI3K/mTOR/p70S6K pathway is required in hepatoma.

The expression of VEGF can be modulated by either HIF-1α-dependent or HIF-1α-independent mechanisms. Although HER-2 and IGF-1 induce transcriptional activation of the *VEGF* gene by HIF-1 (28, 29), HGF increases VEGF expression through an SP1 transcription factor binding site (30). Furthermore, Hu et al. (31) suggested the involvement of Fos-Jun and Jun-Sp1 interaction with the VEGF promoter. However, our data clearly show that LPA induced VEGF expression was mediated by HIF-1α. This conclusion is based on four pieces of evidence. First, the induction pattern of VEGF and HIF-1α in the tested cancer cells by LPA was identical. Although the expression of HIF-1α was concurrently increased by LPA with VEGF, it was not induced in SKOV-3 and Hep-3B cells that did not induce VEGF by LPA. Second, LPA-induced VEGF expression was inhibited by the same pharmacologic inhibitors for HIF-1α expression. Third, LPA induced the binding of HIF-1α to HRE of VEGF. Finally, RNA interference directed against HIF-1α abolished the induction of VEGF protein by LPA.

A number of different signaling pathways, including PI3K/Akt/mTOR/p70S6K or p42/p44 MAPK pathway, are involved in VEGF induction by way of activation of HIF-1α. Tacchini et al. (38) claim that HGF activates the DNA binding of HIF-1 through PI3K pathway in HepG2 cell line, and HER-2 and insulin induce the expressions of HIF-1 and VEGF through PI3K and AKT signaling pathways in nonhypoxic cells (28). However, p42/p44 MAPK pathway is also important for IGF-1-induced HIF-1α activation and VEGF (29). Our results show that signaling pathways of LPA-induced expressions of HIF-1α and VEGF are cancer cell type specific. Activation of both the PI3K/Akt/mTOR/p70S6K and p42/p44 MAPK pathways is required for the induction of HIF-1α and VEGF expression by LPA in ovarian cancer cells; however, p42/p44 MAP kinase pathway seems not to be involved in hepatoma cells because PD98059 could not block LPA-induced HIF-1α expression (Fig. 2A). Immunofluorescence data clearly showed that, unlike in ovarian and prostate cancer cells, PD98059 treatment could not completely block LPA-induced HIF-1α translocation into the nucleus by LPA in hepatoma cells (Fig. 2B). We also found in the present study that there was no direct cross-talk between PI3K/Akt/mTOR/p70S6K and p42/p44 MAPK pathways in response to stimulation with LPA because the blockage of either pathway did not affect the activities of signaling factors in the other pathway (Supplementary Fig. S2). Because translational regulatory proteins are substrates of the PI3K/Akt/mTOR/p70S6K cascade, it is likely that LPA increases HIF-1α translation via this pathway. However, the mechanism of how the p42/p44 MAPK activates HIF-1α still remains to be determined. Because HIF-1α transactivation is a complex process involving multiple proteins, it is likely that these p42/p44 MAPKs act on HIF-1α interacting proteins rather than HIF-1α itself to stimulate transactivation.

Under normoxic conditions, HIF-1α is hydroxylated at proline residues 402 and 564 by proline hydroxylases, recognized by pVHL, polyubiquitinated, and degraded by the proteasomes (39). Therefore, we initially focused our attention on the question of whether LPA treatment enables HIF-1α to escape from proteasomal degradation. However, our present data clearly show that HIF-1α protein stability was not affected by LPA stimulation. When cancer cells were treated with cycloheximide, HIF-1α protein was degraded in the presence of LPA (Fig. 3). Translation of HIF-1α mRNA seems to be significantly up-regulated by LPA and overcomes the rate of normoxic degradation. Moreover, LPA did not significantly induce HIF-1α mRNA (Supplementary Fig. S1).

In conclusion, our data clearly show that LPA stimulates the expression of HIF-1α to induce VEGF expression, which is the first evidence of direct linkage of LPA to HIF-1α activation and VEGF expression. Our results will help to understand the molecular mechanism for LPA-induced angiogenesis and cell motility and useful approach for inhibition of angiogenesis in tumor progression.

References

1. van Leeuwen FN, Giepmans BN, van Meeteren LA, Moolenaar WH. Lysophosphatidic acid: mitogen and motility factor. Biochem Soc Trans 2003;31:1209–12.
2. Eichholtz T, Jalink K, Fahrenfort I, Moolenaar WH. The bioactive phospholipid lysophosphatidic acid is released from activated platelets. Biochem J 1993;291:677–80.
3. Hecht JH, Weiner J, Post SR, Chun J. Ventricular zone gene-1 (vzg-1) encodes a lysophosphatidic acid receptor expressed in neurogenic regions of the developing cerebral cortex. J Cell Biol 1996;135:7906–10.
4. An S, Bleu T, Hallmark OG, Goetzl EJ. Characterization of a novel subtype of human G protein-coupled receptor for lysophosphatidic acid. J Biol Chem 1998;273:7906–10.
5. Bandoh K, Aoki J, Hosono H, et al. Molecular cloning and characterization of a novel human G-protein-coupled receptor, EDG7, for lysophosphatidic acid. J Biol Chem 1999;274:27776–85.
6. Tokumura A. A family of phospholipid autacoids: occurrence, metabolism and bioactions. Prog Lipid Res 1995;34:151–84.
7. Mukai M, Imamura F, Ayaki M, et al. Inhibition of tumor invasion and metastasis by a novel lysophosphatidic acid (cyclic LPA). Int J Cancer 1999;81:918–22.
8. Fang X, Gaudette D, Furui T, et al. Lysophospholipid growth factors in the initiation, progression, metastases, and management of ovarian cancer. Ann N Y Acad Sci 2000;905:188–208.
9. Xu Y, Gaudette DC, Boynton JD, et al. Characterization of an ovarian cancer activating factor in ascites

from ovarian cancer patients. Clin Cancer Res 1995;1:1223–32.

10. Westermann AM, Havik E, Postma FR, et al. Malignant effusions contain lysophosphatidic acid (LPA)-like activity. Ann Oncol 1998;9:437–42.

11. Fang X, Schummer M, Mao M, et al. Lysophosphatidic acid is a bioactive mediator in ovarian cancer. Biochim Biophys Acta 2002;1582:257–64.

12. Semenza GL. Regulation of mammalian O_2 homeostasis by hypoxia-inducible factor 1. Annu Rev Cell Dev Biol 1999;15:551–78.

13. Semenza GL. Targeting HIF-1 for cancer therapy. Nat Rev Cancer 2003;3:721–32.

14. Semenza GL. Oxygen-regulated transcription factors and their role in pulmonary disease. Respir Res 2000;1:159–62.

15. Huang LE, Arany Z, Livingston DM, Bunn HF. Activation of hypoxia-inducible transcription factor depends primarily upon redox-sensitive stabilization of its α subunit. J Biol Chem 1996;271:32253–9.

16. Zelzer E, Levy Y, Kahana C, Shilo BZ, Rubinstein M, Cohen B. Insulin induces transcription of target genes through the hypoxia-inducible factor HIF-1α/ARNT. EMBO J 1998;17:5085–94.

17. Feldser D, Agani F, Iyer NV, Pak B, Ferreira G, Semenza GL. Reciprocal positive regulation of hypoxia-inducible factor 1α and insulin-like growth factor 2. Cancer Res 1999;59:3915–8.

18. Hellwig-Burgel T, Rutkowski K, Metzen E, Fandrey J, Jelkmann W. Interleukin-1β and tumor necrosis factor-α stimulate DNA binding of hypoxia-inducible factor-1. Blood 1999;94:1561–7.

19. Richard DE, Berra E, Pouyssegur J. Nonhypoxic pathway mediates the induction of hypoxia-inducible factor 1α in vascular smooth muscle cells. J Biol Chem 2000;275:26765–71.

20. Gorlach A, Diebold I, Schini-Kerth VB, et al. Thrombin activates the hypoxia-inducible factor-1 signaling pathway in vascular smooth muscle cells: role of the p22(phox)-containing NADPH oxidase. Circ Res 2001;89:47–54.

21. Haddad JJ. Land SC. A non-hypoxic, ROS-sensitive pathway mediates TNF-α-dependent regulation of HIF-1α. FEBS Lett 2001;505:269–74.

22. Stiehl DP, Jelkmann W, Wenger RH, Hellwig-Burgel T. Normoxic induction of the hypoxia-inducible factor 1α by insulin and interleukin-1β involves the phosphatidylinositol 3-kinase pathway. FEBS Lett 2002;512:157–62.

23. Bardos JI, Ashcroft M. Hypoxia-inducible factor-1 and oncogenic signalling. Bioessays 2004;26:262–9.

24. Dvorak HF, Orenstein NS, Carvalho AC, et al. Induction of a fibrin-gel investment: an early event in line 10 hepatocarcinoma growth mediated by tumor-secreted products. J Immunol 1979;122:166–74.

25. Wong C, Wellman TL, Lounsbury KM. VEGF and HIF-1α expression are increased in advanced stages of epithelial ovarian cancer. Gynecol Oncol 2003;91:513–7.

26. Yoshiji H, Kuriyama S, Yoshii J, et al. Vascular endothelial growth factor tightly regulates *in vivo* development of murine hepatocellular carcinoma cells. Hepatology 1998;28:1489–96.

27. Loureiro RM, D'Amore PA. Transcriptional regulation of vascular endothelial growth factor in cancer. Cytokine Growth Factor Rev 2005;16:77–89.

28. Laughner E, Taghavi P, Chiles K, Mahon PC, Semenza GL. HER2 (neu) signaling increases the rate of hypoxia-inducible factor 1α (HIF-1α) synthesis: novel mechanism for HIF-1-mediated vascular endothelial growth factor expression. Mol Cell Biol 2001;21:3995–4004.

29. Miele C, Rochford JJ, Filippa N, Giorgetti-Peraldi S, Van Obberghen E. Insulin and insulin-like growth factor-I induce vascular endothelial growth factor mRNA expression via different signaling pathways. J Biol Chem 2000;275:21695–702.

30. Reisinger K, Kaufmann R, Gille J. Increased Sp1 phosphorylation as a mechanism of hepatocyte growth factor (HGF/SF)-induced vascular endothelial growth factor (VEGF/VPF) transcription. J Cell Sci 2003;116:225–38.

31. Hu YL, Tee MK, Goetzl EJ, et al. Lysophosphatidic acid induction of vascular endothelial growth factor expression in human ovarian cancer cells. J Natl Cancer Inst 2001;93:762–8.

32. Huang MC, Lee HY, Yeh CC, Kong Y, Zaloudek CJ, Goetzl EJ. Induction of protein growth factor systems in the ovaries of transgenic mice overexpressing human type 2 lysophosphatidic acid G protein-coupled receptor (LPA2). Oncogene 2004;23:122–9.

33. Shida D, Kitayama J, Yamaguchi H, et al. Lysophosphatidic acid (LPA) enhances the metastatic potential of human colon carcinoma DLD1 cells through LPA1. Cancer Res 2003;63:1706–11.

34. Lee J, Jung ID, Chang WK, et al. p85 β-PIX is required for cell motility through phosphorylations of focal adhesion kinase and p38 MAP kinase. Exp Cell Res 2005;307:315–28.

35. Fukuda R, Hirota K, Fan F, Jung YD, Ellis LM, Semenza GL. Insulin-like growth factor 1 induces hypoxia-inducible factor 1-mediated vascular endothelial growth factor expression, which is dependent on MAP kinase and phosphatidylinositol 3-kinase signaling in colon cancer cells. J Biol Chem 2002;277:38205–11.

36. Segrelles C, Ruiz S, Santos M, Martinez-Palacio J, Lara MF, Paramio JM. Akt mediates an angiogenic switch in transformed keratinocytes. Carcinogenesis 2004;25:1137–47.

37. Fukuda R, Kelly B, Semenza GL. Vascular endothelial growth factor gene expression in colon cancer cells exposed to prostaglandin E2 is mediated by hypoxia-inducible factor 1. Cancer Res 2003;63:2330–4.

38. Tacchini L, Dansi P, Matteucci E, Desiderio MA. Hepatocyte growth factor signalling stimulates hypoxia inducible factor-1 (HIF-1) activity in HepG2 hepatoma cells. Carcinogenesis 2001;22:1363–71.

39. Epstein AC, Gleadle JM, McNeill LA, et al. C. elegans EGL-9 and mammalian homologs define a family of dioxygenases that regulate HIF by prolyl hydroxylation. Cell 2001;107:43–54.