Loss of phospholipase D2 impairs VEGF-induced angiogenesis

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Vascular endothelial growth factor (VEGF) is a key mediator of angiogenesis and critical for normal embryonic development and repair of pathophysiological conditions in adults. Although phospholipase D (PLD) activity has been implicated in angiogenic processes, its role in VEGF signaling during angiogenesis in mammals is unclear. Here, we found that silencing of PLD2 by siRNA blocked VEGF-mediated signaling in immortalized human umbilical vein endothelial cells (iHUVECs). Also, VEGF-induced endothelial cell survival, proliferation, migration, and tube formation were inhibited by PLD2 silencing. Furthermore, while PLD2 knockout mice exhibited normal development, loss of PLD2 inhibited VEGF-mediated ex vivo angiogenesis. These findings suggest that PLD2 functions as a key mediator in the VEGF-mediated angiogenic functions of endothelial cells. [BMB Reports 2016; 49(3): 191-196]

INTRODUCTION

VEGF signaling mediates an essential rate-limiting step of angiogenesis in endothelial cells (1, 2). From embryo to adult, angiogenesis plays key roles in development and basic life phenomena to maintain homeostasis of such vital functions as oxygen/nutrient supply and waste elimination (3, 4). Furthermore, an excess or shortage of angiogenesis affects various diseases, such as cancer, inflammatory disorders, obesity, asthma, diabetes, multiple sclerosis, ischemic heart disease and pre-eclampsia (3-5). While VEGF is crucial for both physiological and pathological angiogenesis, it is dramatically upregulated during pathological angiogenesis, such as tumor and diabetic retinopathy (6, 7). Therefore, discovery and characterization of the key factor that mediates VEGF signaling in endothelial cells is essential for understanding the roles of angiogenesis in pathophysiological conditions.

Phospholipase D (PLD) is a phosphatidylycholine (PC)-hydrolyzing enzyme that generates phosphatidic acid (PA) and choline (8-11). In mammals, two PLD isozymes, PLD1 and PLD2, have been identified and have ∼50% sequence homology (8-11). PLD1 and PLD2 can be activated by a variety of upstream signals, such as growth factors, hormones, neurotransmitters, and phospholipids (8-11). PLD functions as a key amplifier/modulator between upstream signals and downstream mediators by producing the second messenger, PA (8-11). In mammals, two PLD isozymes, PLD1 and PLD2, have been identified and have similar functions in cellular and ex vivo tissue levels. Therefore, this study provides new information regarding the regulation of VEGF-induced angiogenesis.

RESULTS

PLD isotype-specific signaling pathway in iHUVECs

PLD1 and PLD2 mediate not only similar functions, but also PLD isoform-specific functions (10, 11, 14-16). First, we investigated whether each PLD mediates isotype-specific angiogenic signaling in iHUVECs. After silencing of each PLD isotype, we assessed the activation of key mediators of angiogenesis in endothelial cells. We found that both PLD1 and PLD2 silencing blocked phosphorylation of S6-kinase and ERK in iHUVECs (Fig. 1A). However, silencing of PLD2, but not PLD1, reduced Src phosphorylation. In addition, when we investigated the expression levels of genes related to angiogenesis in iHUVECs after silencing of PLD1 or PLD2 (Fig. 1B and 1C), silencing of PLD2, but not PLD1, significantly decreased the ANGPT1 mRNA level (Angiopoietin 1) (Fig. 1D). Furthermore, mRNA levels of KDR2 (VEGF receptor-2) and MMP2 (matrix metal-
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VEGF as a key mediator of angiogenesis activates multiple intracellular signaling pathways—including the phosphoinositide 3-kinase (PI 3-kinase), AKT, ERK, and Src pathways—in endothelial cells (1, 2). First, we investigated the roles of PLD isoforms in VEGF-mediated signaling after siRNA silencing of PLD1 or PLD2 in iHUVECs. We verified that VEGF increased the phosphorylation of S-6-kinase, ERK, and Src in a time-dependent manner (Fig. 2A), and a low VEGF concentration (2 ng/ml) induced the phosphorylation of S-6-kinase, ERK, and Src in iHUVECs (Fig. 2B). PLD1 or PLD2 silencing blocked the VEGF-induced phosphorylation of ERK, S-6-kinase, and Src in iHUVECs (Fig. 2C). In particular, p-ERK and p-S-6-kinase levels were inhibited more significantly by PLD2 silencing than PLD1 silencing. In the case of Src, silencing of PLD2, but not PLD1, inhibited Src phosphorylation (Fig. 2C). These results suggest that PLD2 is essential for VEGF signaling in iHUVECs and that the VEGF-PLD2 pathway differs from the VEGF-PLD1 pathway.

PLD2 deficiency leads to defects in VEGF-induced cell survival, proliferation, migration, and tube formation in iHUVECs
VEGF signaling mediates various endothelial cell functions—such as migration, proliferation, and survival—via multiple intracellular signaling pathways, including the Src, phosphoinositide 3-kinase (PI 3-kinase), AKT, and ERK pathways (1, 2, 4). Thus, we investigated the effect of PLD2 on the role of VEGF in the survival, proliferation, migration, and tube formation in iHUVECs. As shown in Fig. 3A and 3B, VEGF enhanced the survival and proliferation of iHUVECs. However, silencing of PLD2 blocked VEGF-induced cell survival and proliferation (Fig. 3A and 3B). Moreover, VEGF-induced migration and tube formation (in vitro angiogenesis) were also inhibited by silencing of PLD2 (Fig. 3C and 3D). Tube formation assay of in vitro angiogenesis has been reported to represent cell adhesion, migration, protease activation, and tubule formation in endothe-
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Fig. 3. PLD2 plays key roles in VEGF-induced iHUVEC survival, proliferation, migration and tube formation. (A and B) Following siRNA silencing of PLD2, iHUVECs were starved for 24 h and then treated with VEGF (20 ng/ml) for 48 h. Cell viability was measured by MTT assay. Cells were enumerated using a hemocytometer. (C) Following siRNA silencing of PLD2, iHUVECs were starved for 24 h and then detached. The detached cells were replated on the upper well of a chemotaxis chamber. Chemotactic migration was measured in the absence or presence of VEGF (10 ng/ml). Scale bar, 100 μm. (D) Following siRNA silencing of PLD2, iHUVECs were starved for 24 h and then detached. Cells were replated on matrigel-coated dishes in the absence or presence of VEGF (40 ng/ml). The white arrows indicate intact branching points of network. The yellow arrows indicate points where the branching network failed to occur. Scale bar, 250 μm. Data are representative of at least two independent experiments. Error bars indicate S.D. *P < 0.05.

Fig. 4. Aortas of Pld2 knockout mice show reduced VEGF-induced sprouting. (A) Mouse embryos and pups were genotyped at days E15.5 and P0, respectively. (B) Aortas were isolated from control and Pld2 knockout mice and implanted on matrigel. Aortas were treated with the indicated VEGF dose for the indicated time. Scale bar, 100 μm. (C) Aortas were treated with VEGF (20 ng/ml) in the absence or presence of 1-butanol or t-butanol. Scale bar, 100 μm. (D) Aortas from control and Pld2 knockout mice were treated with VEGF (40 ng/ml) for 5 days. Scale bar, 500 μm. Data are representative of at least two independent experiments.

PLD2 plays a role in VEGF-induced sprouting from aorta

Our in vitro data suggest that PLD2 might also mediate VEGF angiogenesis in vivo. We generated Pld2 knockout mice to reveal the roles of PLD2 in angiogenesis (18). First, we investigated the effect of PLD2 deficiency on tissue and organ development. As shown in Fig. 4A, at day E15.5, when fetal organogenesis was completed (19), we found that the genotypes of fetuses exhibited a Mendelian ratio, as expected.

Furthermore, mouse pups at P0 were born with a Mendelian ratio and showed normal development. Next, we investigated the effect of PLD2 deficiency on VEGF-induced angiogenesis. VEGF induces sprouting from mouse aorta in an ex vivo aortic ring assay, which is a more physiologically relevant model of angiogenesis and preserves certain key features of in vivo angiogenesis, which is mediated by various cell types (endothelial cells, macrophages, pericytes, smooth muscle cells and fibroblasts) (20, 21). VEGF-induced endothelial cell (EC) sprouting from the aorta occurred in a dose- and time-dependent manner (Fig. 4B). 1-Butanol, a blocker of PA generation from PLD, inhibited VEGF-induced EC sprouting from the aorta. However, t-butanol, as a control treatment, did not inhibit VEGF-induced EC sprouting (Fig. 4C). These results indicate that PLD activity is important for VEGF-mediated EC sprouting. When we investigated the involvement of PLD2 in ex vivo angiogenesis (aortic ring assay) using Pld2 knockout mice, we also found that PLD2 deficiency inhibited VEGF-induced aortic sprouting (Fig. 4D). These data suggest that PLD2 is involved.
in VEGF-mediated angiogenesis, although the cell-type specific roles of PLD2 in the aortic ring, which comprises multiple cell types, are unclear.

**DISCUSSION**

Here, we elucidated that PLD2 functioned as a key modulator of VEGF signaling pathways (ERK, S-6-kinase, and Src) in iHUVECs. This function of PLD2 was essential for survival, proliferation, migration, and tube formation of endothelial cells, which mediate new vessel formation or angiogenesis from existing vessels. Also, using an ex vivo Pld2-knockout mouse model, we revealed PLD2 to be an essential mediator of VEGF-mediated angiogenesis. Therefore, this work enhances our understanding of the intracellular mediators of VEGF-induced angiogenesis.

VEGF binds to and activates VEGF receptor-2 (VEGFR-2) on endothelial cells. VEGF binding induces the tyrosine phosphorylation of VEGFR-2 at multiple sites (Y951, Y1054, Y1059, Y1175, and Y1214) (2, 22-24). These tyrosine-phosphorylated sites recruit several downstream molecules—such as TSh, Shb, Sck, and PLC— which mediate multiple functions—such as migration (Src or FAK pathway), survival (PI3 kinase-AKT-S-6-kinase pathway), proliferation (ERK pathway) and vascular permeability (eNOS pathway)—of endothelial cells (2, 22-33). Our results indicate that PLD2 silencing inhibits VEGF-mediated phosphorylation of S-6-kinase, ERK, and Src in iHUVECs (Fig. 2C). Thus PLD2 may play a role as a key regulator of these key mediators of angiogenesis (ERK, S-6-kinase, and Src).

Recently, it has been reported that Src functions upstream of PLD1 in VEGF-mediated signaling of human retinal microvascular endothelial cells (HRMVECs) (12). However, we found that PLD1 functioned upstream of Src in VEGF signaling and PLD1 silencing resulted in a slight increase in VEGF-induced phosphorylation of Src compared to control silencing, while PLD2 silencing resulted in a reduction in VEGF-mediated Src phosphorylation in iHUVECs (Fig. 2C). This result is in agreement with a previous report that PLD1 silencing inhibits VEGF-induced phosphorylation of Src and Pyk2, levels, but PLD2 silencing had no significant effect (a slight reduction) on Src and Pyk2 phosphorylation in HeLa cells (14). Furthermore, mRNA levels of angiogenic related-genes were differently regulated by silencing of each PLD isotype in iHUVECs (Fig. 1D-F). Although whether these results are cell-type or signal specific is unclear, they suggest that PLD2 mediates VEGF signaling in a manner different from PLD1.

VEGF-neutralizing antibodies and VEGFR inhibitors have been used to target pathological angiogenesis, such as tumor angiogenesis (2, 34). However, as shown in knockout-mouse studies of VEGF and VEGFR, deficiency of these molecules caused embryonic lethality and defects in organ development due to failure of angiogenesis (2, 34-39). These data indicate that molecules that target VEGF and VEGFR may affect physiological angiogenesis. Here, we found that Pld2-knockout mice were born normally (a Mendelian frequency) (Fig. 4A). Therefore, PLD2 did not critically affect normal development and physiological angiogenesis. However, PLD2 deficiency inhibited VEGF (40 ng/ml)-mediated ex vivo angiogenesis (Fig. 4D). In mouse and human, the physiological level of VEGF is ∼0.065 ng/ml and 0.137 ng/ml, respectively (40, 41). In pathological tissue such as tumors, the VEGF level is 10-200 fold higher than that in normal tissue (42). Taken together, these reports and our findings suggest that PLD2 plays a key role in a high local concentration of VEGF, such as in pathological conditions. Therefore, we suggest that angiogenic targeting of PLD2, which is a key downstream molecule of VEGF signaling, may facilitate development of a drug targeted specifically at pathological conditions, while not negatively affecting the overall physiological condition.

**MATERIALS AND METHODS**

**Animal**

All mice were kept in an animal facility at the Pohang University of Science and Technology (POSTECH). Animal experiments were performed according to the institutional guidelines of POSTECH. To generate whole-body Pld2-knockout (KO) mice, Pld2fl/fl mice were crossed with Protamine-Cre (Jackson Laboratory) and Pld2 KO mice then maintained without the Cre allele. Pld2 KO mice were backcrossed with C57BL/6 for at least 14 generations.

**Cell culture and transfection**

Immortalized iHUVECs were maintained at 37°C in 5% CO2 in M199 medium supplemented with 10% FBS (Lonza), 5 mM L-glutamine (Life Technologies), endothelial cell growth supplement (BD Biosciences), penicillin (100 units/ml) and streptomycin (0.1 mg/ml). For PLD silencing, iHUVECs were transfected with control siRNA (luciferase GL2 duplex; Dharmaco Research) or PLD1 and/or PLD2 siRNA (20 nM duplex) using Lipofectamine (Invitrogen) according to the manufacturer’s instruction and then cultured for 48 h.

**Western blotting**

Samples were lysed in lysis buffer (20 mM Tris/pH 8.0, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 1.0% Triton X-100, 1.0% sodium deoxycholate, and protease inhibitor cocktail (Sigma)). After centrifugation at 10,000 × g for 10 min at 4°C, the protein concentration of supernatants was determined by Bradford assay. Laemmli sample buffer (5X) was added to the samples, which were then separated by SDS-PAGE and transferred onto nitrocellulose membranes (0.2 μm pore size). The membranes were blocked in blocking solution (5% non-fat milk in TTBS), then incubated with primary antibodies for 4 h or overnight at room temperature, followed by incubation with secondary antibodies (horseradish peroxidase (HRP)-conjugated) for 1 h. Blots were detected using an enhanced chemiluminescence kit.
Quantitative PCR (qPCR)
Total RNA from iHUVECs was isolated using TRIzol reagent, and cDNAs were generated from 1 μg of total RNA. qPCR was performed with HotStart-IT SYBR Green and a Bio-Rad iCycler iQ instrument using specific primers (PLD1: F: TGCGTCTACA TCCAACATATAA, R: AGGTCAATCCCTCCCAAAA, PLD2: F: GCTCCCCCTCACCTCCAG, R: GCAGGTAGGCATTAGGTA TG, ANGPT1: F: GACAGCAGGAAAAACAGACACAG, R: CACA AGCATTAAACACCACATC, KDR2: F: CAGAGTTGCTGGAAACA TTTGG, R: CAGGAACAGGGTACGGTAGCC, MMP2: F: TACC CCAAGCAGTGAACCA, R: AGCACCCGATCCAGTTAT). The relative mRNA quantities were calculated by the comparative Ct method after normalization to GAPDH.

MTT assay
MTT assay were performed as previously described, with some modifications (43). Cells were plated in 12-well plates and transfected with control or PLD2 siRNA using Lipofectamine (Invitrogen). After culturing for a further 2 days, cells were starved for 24 h and then treated with VEGF (20 ng/ml) for 48 h. Tetrazolium dye, MTT (0.5 mg/ml) was added to each well and the plates were incubated for 4 h at 37°C. Remaining MTT was removed and DMSO was added to each well to dissolve formazan crystals. Absorbance at 540 nm was measured using a spectrophotometer.

Proliferation
Cells were plated in six-well plates and transfected with control or PLD2 siRNA using Lipofectamine (Invitrogen). After being cultured for a further 2 days, cells were starved for 24 h and then treated with VEGF (20 ng/ml) for 48 h. For enumeration, cells were detached with trypsin and then counted using a hemocytometer.

Migration
Cell migration was measured using a modified Boyden chemotaxis chamber (NeuroProbe Inc.) as previously described, with some modifications (44). Polycarbonate membranes (8 μm pore size) were coated with collagen (20 μg, Sigma). Culture medium containing VEGF (10 ng/ml) was added to the bottom wells of the chamber. After starvation for 24 h, cells were detached and then resuspended in serum-free medium. The cells in the upper chamber were plated at a density of 1 × 10^5 cells. The chambers were incubated at 37°C for 3 h. Non-migrating cells in the upper chamber were removed from the membrane, and migrated cells were stained with Hoechst dye (Sigma).

Tube formation
After starvation for 24 h, iHUVECs (1 × 10^5 per well) were plated into 48-well culture plates coated with growth factor-reduced matrigel (BD Biosciences) and then incubated for 9 h at 37°C in M199 medium containing VEGF (40 ng/ml). Images were obtained using a Zeiss Axiosvert 135 microscope.

Aortic ring assay
After euthanasia, mice were perfused with cold PBS (10 ml) containing 1% FBS. The aortic ring was excised from each mouse and cut into 1 mm lengths. The aortic rings were implanted in growth factor reduced Matrigel (BD Biosciences) and incubated with EBM-2 medium supplemented with VEGF (20 or 40 ng/ml) for 5 days.

Statistical analysis
The data are presented as means ± SD. Statistical analyses were performed using Student’s t-test. * P < 0.05.

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