Localization of VEGFR-2 and PLD$_2$ in endothelial caveolae is involved in VEGF-induced phosphorylation of MEK and ERK

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2004; 10.1152/ajpheart.00786.2003.—To clarify the role of caveolae
in VEGF/VEGF receptor-2 (VEGFR-2)-mediated signaling cascades,
primary cultured human umbilical vein endothelial cells (HUVECs)
were fractionated to isolate caveolae-enriched cell membranes. Inter-
estingly, VEGFR-2, phospholipase D$_2$ (PLD$_2$), and Ras were enriched
in caveolae-enriched fractions. Moreover, VEGF increased PLD ac-
tivity in a time- and dose-dependent manner in HUVECs, whereas a
ligand specific for VEGFR-1 placental growth factor did not change
PLD activity. A PLD inhibitor, 1-butanol, almost completely sup-
pressed VEGF-induced ERK phosphorylation and cellular prolifera-
tion, whereas the negative control for 1-butanol, 3-butanol, did not
produce significant changes. Addition of phosphatidic acid negated
the 1-butanol-induced suppression. Pharmacological analyses using
several inhibitors indicated that PKC-δ regulates the VEGF-induced
activation of PLD/ERK. Thus PLD$_2$ could be involved in MEK/ERK
signaling cascades that are induced by the VEGF/VEGFR-2/PKC-δ
pathway in endothelial cells. Pretreatment with the cholesterol deple-
tion agent methyl-β-cyclodextrin (MβCD) almost completely disas-
sembled caveolar structures, whereas the addition of cholesterol to
MβCD-treated cells restored caveolar structures. Pretreatment with
MβCD largely abolished phosphorylation of MEK/ERK by VEGF,
whereas the addition of cholesterol restored VEGF-induced MEK/
ERK phosphorylations. These results indicate that intact caveolae are
required for the VEGF/VEGFR-2-mediated MEK/ERK signaling cas-
cade.

caveolin-1; protein kinase C-δ; signaling; vascular endothelial growth
factor; phospholipase D

THERE ARE six known members of the VEGF family: VEGF,
placental growth factor (PlGF), VEGF-B, VEGF-C, VEGF-D,
and orf virus VEGF (also called VEGF-E) (8, 23, 40). The
VEGF family proteins are secreted as dimeric glycoproteins.
They display differential interactions with three related recep-
tor tyrosine kinases: VEGFR-1/Flt-1, VEGFR-2/KDR/Flk-1,
and VEGFR-3/Flt-4 (8, 23, 40). VEGF exerts its action by
binding to VEGFR-1 and VEGFR-2 (8, 23, 40). VEGFR-1 and
VEGFR-2 are expressed predominantly in vascular endothelial
cells, but a few additional types of cells express one or both of
these receptors (23). Of these, VEGFR-2 is expressed in
hematopoietic stem cells, megakaryocytes, retinal progenitor

cells, and transformed tumorigenic cell types (23). On activa-
tion of VEGFR-2 in endothelial cells, three major second
messenger pathways elicit cell proliferation, migration, and
survival, which are essential steps for angiogenesis (10, 14, 18,
21, 22, 28, 38, 39, 41, 42). These pathways are the following:
mitogen-activated protein/ERK kinase (MEK)/ERK cascade,
phosphatidylinositol (PI) 3-kinase/serine-threonine protein ki-
nase/Akt cascade, phospholipase C-γ/intracellular Ca^{2+}/PKC
cascades (10, 14, 18, 21, 22, 28, 38, 39, 41, 42).

Caveolae, which are flask-shaped membrane invaginations,
were first detected on the surface of endothelial cells (24).
There is growing evidence that caveolae may act as structurally
and biochemically distinct plasma membrane compartments
that localize and regulate transmembrane signaling events (1,
27, 34). This fact is based on the finding that caveolae are
enriched for receptors and components of signaling cascades
(9, 32, 35). Caveolae are formed from lipid rafts by polymer-
ization of caveolins, which are hairpin-like palmitoylated in-
tegral membrane proteins that tightly bind cholesterol (1, 9, 26,
27, 35, 36). Interestingly, VEGFR-2 is colocalized with caveo-
lin-1 in the caveolae of endothelial cells (6, 7, 19). This finding
suggests that the VEGFR-2 signaling machinery may also be
localized in the endothelial caveolae. However, it is not known
how the VEGFR-2 signaling machinery is activated in the
endothelial caveolae on VEGF binding.

In this study, we examined the distributions of caveolin-1,
VEGFR-2, and the key signaling molecules of VEGFR-2 in
primary cultured human umbilical vein endothelial cells
(HUVECs). Interestingly, VEGFR-2 is localized in the endo-
thelial caveolae, together with phospholipase D$_2$ (PLD$_2$) and
Ras. Furthermore, this study provides evidence that PKC and
PLD, in response to activation by VEGF/VEGFR-2, participate
in the activation of MEK and ERK, which control several
cellular processes in vascular endothelial cells.

EXPERIMENTAL PROCEDURES

Materials and preparation of endothelial cells. Recombinant hu-
man VEGF$_{165}$ was purchased from R&D Systems (Minneapolis,
MN). Antibodies for caveolin-1 and immunoglobulin heavy chain
binding protein (BiP) were purchased from BD Transduction Labo-
ratory (San Diego, CA). Antibody for Ras was purchased from
Upstate Biotechnology (Waltham, MA). Antibodies for phospho-
MEK (Ser217/221), MEK, phospho-ERK1/2 (Thr202/Tyr204) and
ERK1/2 were purchased from Cell Signaling Technology (Beverly,
MA). Antibodies for VEGFR-2 and Raf-1 were purchased from Santa
Cruz Biotechnology (Santa Cruz, CA). Antibody for PLD$_{1/2}$ (15) was

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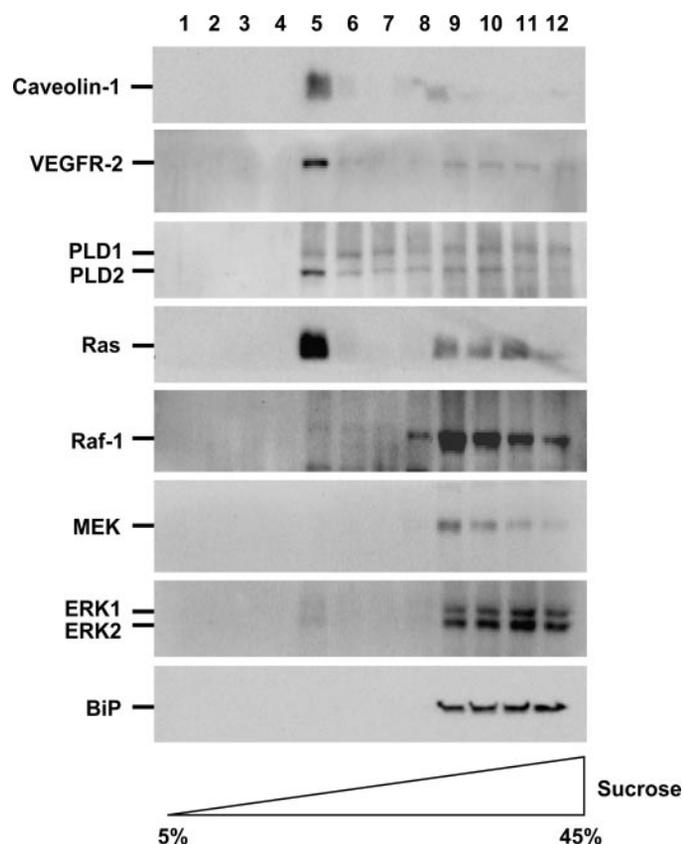@postech.ac.kr).

Fig. 1. Distribution of VEGF receptor-2 (VEGFR-2) signaling related molecules in sucrose density gradient fractionation of endothelial cells. Human umbilical vein endothelial cell (HUVEC) lysates were fractionated by a sucrose density gradient (5–45%). Each fraction was separated by SDS-PAGE and subjected to immunoblotting with the indicated antibody. Caveolin-1, VEGFR-2, phospholipase D_2 (PLD$_2$), and Ras were mainly found in *fraction 5*, whereas Raf-1, mitogen-activated ERK kinase (MEK), ERK1, ERK2, and heavy chain binding protein for endoplasmic reticulum (BiP) were mainly found in *lanes 8–12*, which are noncaveolar membrane fractions. Results were similar in 3 independent experiments.

a kind gift of Dr. Pann-Gill Suh (POSTECH, Pohang, Korea). Staurosporine, GF109203X, BAPTA-AM, and wortmannin were purchased from Sigma-Aldrich (St. Louis, MO). SU5614, brefeldin-A, Go6976, rottlerin, RO31–8220, phosphatidic acid (PA), and methyl-β-cyclodextrin (MβCD) were purchased from Calbiochem (San Diego, CA). Media, FBS, and most other biochemical reagents were purchased from Sigma-Aldrich unless otherwise specified. HUVECs were prepared from human umbilical cords by collagenase digestion, were grown in 20% FBS-containing medium 199 (13), and were between *passages 2* and *3* when used for the experiments in this study. The HUVECs were incubated in 1% FBS-containing medium for 16 h before performing each experiment.

Cell fractionation. Separation of light and heavy membrane fractions from HUVECs was performed according to Song et al. (37). Briefly, the HUVECs were suspended to 2 ml of 0.5 M sodium carbonate buffer (pH 11.0) containing phosphatase inhibitors and protease inhibitors. The cell suspension was homogenized, adjusted to 45% sucrose by the addition of 80% sucrose prepared in MBS buffer, and placed into ultracentrifugation tubes. A 5–45% discontinuous sucrose gradient was then formed on top. This sample was centrifuged at 39,000 rpm for 6 h in a SW-41 rotor (Beckman Instruments; Palo Alto, CA). The fractions were collected from the top in 1-ml amounts except for *fractions 1–4*, which were collected as 0.9-ml fractions. After the addition of a sample loading buffer, the fractions were

boiled, separated by SDS-PAGE, and transferred to nitrocellulose membranes. The membranes were immunoblotted with the desired antibodies. All signals were detected by chemiluminescent detection according to the manufacturer's protocol (Amersham; Buckinghamshire, UK).

Assay of PLD activity. PLD activity was assayed by measuring the formation of phosphatidyl-butanol, the product of PLD-mediated transphosphatidylation, in the presence of 1-butanol, as previously described, with a slight modification (25). The HUVECs were preincubated with 3 μCi/ml of [³H]myristic acid (DuPont-NEN; Boston, MA) for 4 h. Transphosphatidylation was catalyzed in the presence of 0.4% 1-butanol, and cells were exposed to the indicated concentration of VEGF for the indicated times in the absence or presence of inhibitors. The reaction was terminated with ice-cold methanol and cells were scraped from the plates. The lipid phase was extracted and developed by thin layer chromatography on silica gel-60 plates (Merck; Darmstadt, Germany) with the use of chloroform-methanol-acetic acid (9:1:1) as the solvent. The formation of [³H]phosphatidyl-butanol was expressed as a percentage of the total ³H-labeled lipid to account for differences in cell labeling efficiency.

Western blot analysis. The treated HUVECs were harvested with lysis buffer (50 mM Tris·HCl, 100 mM NaCl, 0.1% SDS, 1% Nonidet P-40, 50 mM NaF, 1 mM Na_3VO_4, 1 μg/ml aprotinin, 1 μg/ml pepstatin, and 1 μg/ml leupeptin). After the addition of the sample
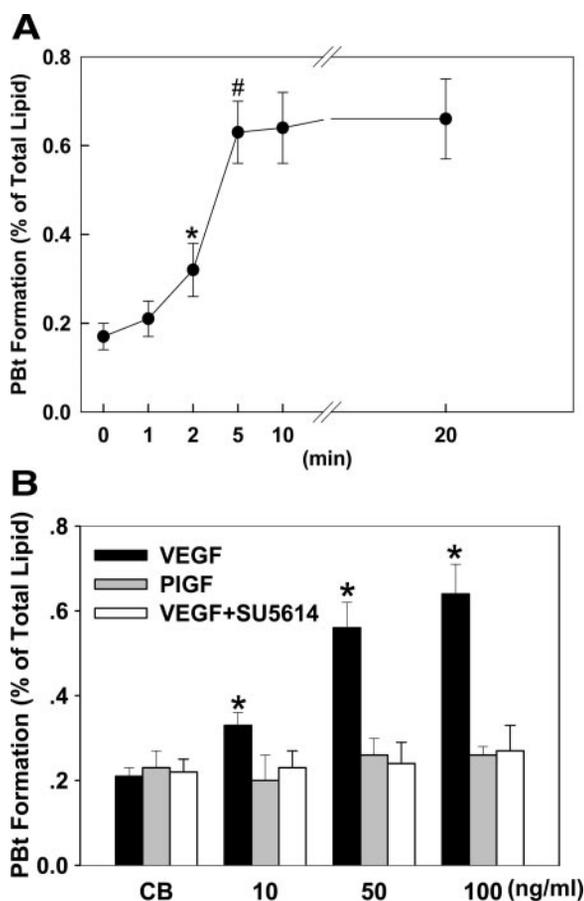

Fig. 2. VEGF increases PLD activity. HUVECs were labeled with ³H-labeled myristic acid for 4 h, and the cells were incubated with VEGF (50 ng/ml) for the indicated times (*A*) or the cells were treated with the indicated amounts of VEGF, placental growth factor (PlGF), or VEGF plus SU5614 (10 μM) for 5 min (*B*). PLD activity was then measured and expressed as formation of phosphatidyl butanol (PBt). Bars and solid circles represent means ± SD of 5 independent experiments. *$P < 0.05$ vs. control buffer (CB) or *time 0*; #$P < 0.05$ vs. time 2 min.
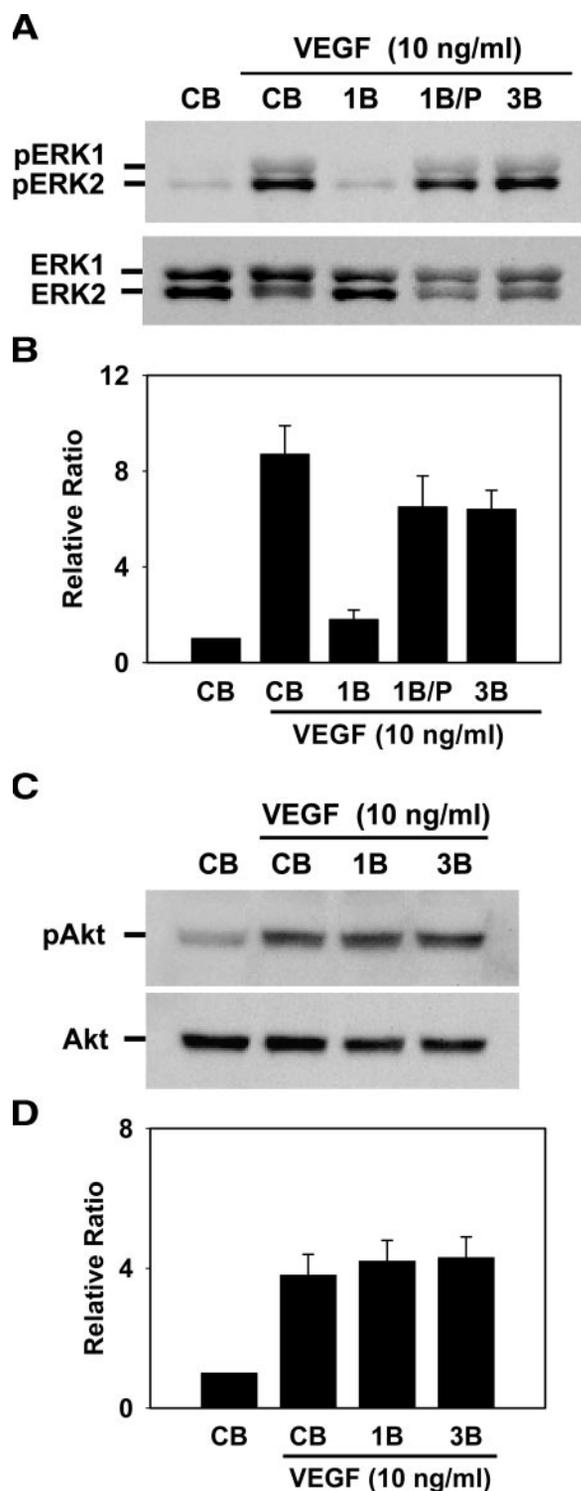
Fig. 3. Effect of PLD inhibition on VEGF-induced ERK1/2 and Akt activations. HUVECs were pretreated with 0.4% 1-butanol (1B) or 0.4% 3-butanol (3B) for 5 min; the cells were then treated with VEGF (10 ng/ml) for 10 min. For cells that received phosphatidic acid (PA) treatment, PA (200 μM) and VEGF (10 ng/ml) were added to cells treated with 1B (0.4% for 5 min) (1B/P). *A* and *C*: cell lysates (50 μg of total protein) were boiled in reducing buffer and separated by SDS-PAGE. Blots were probed with anti-phospho-ERK antibody or anti-phospho-Akt antibody (*top*). The membrane was stripped and reprobed with anti-ERK1/2 or anti-Akt antibody (*bottom*) to verify equal loading of protein in each lane. *B* and *D*: densitometric analyses are presented as the relative ratio of phospho-ERK2 to ERK2 or phospho-Akt to Akt. The ratio relative to CB is arbitrarily presented as 1. Bars represent means ± SD from 3 experiments.

loading buffer, lysates were boiled, separated by SDS-PAGE, and transferred to a nitrocellulose membranes. The membranes were immunoblotted with the appropriate primary antibody, washed, and incubated with horseradish peroxidase-conjugated secondary antibody. Signals were visualized by chemiluminescent detection according to the manufacturer's protocol (Amersham).

Proliferation assay. For the proliferation assay, DNA synthetic activity was measured as previously described (14). In brief, HUVECs were plated in 24-well plates at a density of 2×10^4 cells/cm^2 in medium 199 containing 2% FBS. After 12 h, plates were changed to fresh medium with the indicated agents. After 24 h, fresh medium and agents were added, and the cells were incubated for another 24 h. The cells were then washed with PBS, and the DNA amount was measured with PicoGreen fluorescent reagent (Molecular Probes; Eugene, OR) using a fluorescence spectrophotometer equipped with a microplate reader (Molecular Devices; Sunnyvale, CA).

Transmission electron microscopy. The HUVECs were treated with MβCD (5 mM) for 30 min or 1 h plus cholesterol (0.2 mM) for 30 min. Control and treated HUVECs were rinsed two times with PBS and fixed with 2% glutaraldehyde in sodium cacodylate buffer (0.1 M cacodylate and 0.1 M sucrose) at pH 7.4 for 12 h. Cells were subsequently postfixed with 1% osmium tetroxide in 0.1 M sodium cacodylate at pH 7.4 for 1 h. Samples were further dehydrated in graded alcohol solutions and embedded in epon. After the samples hardened, the cells were lifted with embedding medium. Transverse sections of 50 nm were cut with a diamond knife, stained first with uranyl acetate and subsequently with lead citrate, and examined by transmission electron microscopy at 80 kV (ZEOL1020 EX2, Japan).

Data analyses. Data are expressed as means ± SD. Statistical significance was tested using one-way ANOVA, followed by the Student-Newman-Keuls test. Statistical significance was set at $P < 0.05$.

RESULTS

Distribution of VEGFR-2 and its signaling molecules. To isolate caveolin-enriched endothelial cell membranes, HUVEC homogenate was fractionated using sucrose density centrifugation. Twelve fractions were recovered and subjected to Western blot analysis. Caveolin-1, the structural protein of caveolae,
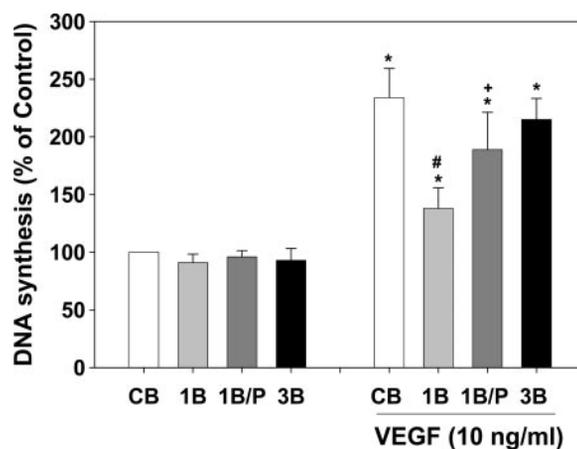

Fig. 4. Effect of PLD inhibition on VEGF-induced DNA synthetic activity. HUVECs were plated in medium 199 supplemented with 2% FBS. After a 12-h incubation period, the cells were treated with CB, 1B (0.04%), or 3B (0.04%) in the absence or presence of VEGF (10 ng/ml). For cells that received PA treatment, PA (20 μM) was added to cells treated with 1B (0.04%, 1B/P) in the absence or presence of VEGF (10 ng/ml). After 48 h, the DNA amount was measured with PicoGreen fluorescent reagent. Bars represent means ± SD from 5 experiments. *$P < 0.05$ vs. CB; #$P < 0.05$ vs. CB plus VEGF; +$P < 0.05$ vs. 1B plus VEGF.

was dominantly enriched in *fraction 5*, which represented the light membrane fraction (Fig. 1). BiP, a marker for endoplasmic reticulum, was enriched in noncaveolar *fractions 9–12*. These data indicate that the fractionation method was able to separate the caveolae from the heavier membrane fractions. Consistent with previous reports (6, 7, 19), VEGFR-2 was dominantly enriched in *fraction 5* (Fig. 1). To screen for components involved in VEGFR-2 signaling, other signaling molecules were tested. PLD_2 and Ras were enriched in *fraction 5*, whereas Raf-1, MEK, ERK1, and ERK2 were enriched in *fractions 8–12* (Fig. 1). These results suggest that VEGFR-2 may interact with PLD_2 and Ras in endothelial caveolae.

VEGF increases PLD activity. Because our data suggested that PLD might be involved in VEGFR-2 signaling in endothelial cells, we next examined whether VEGF-induced VEGFR-2 activation could regulate PLD activity in HUVECs. The addition of VEGF (50 ng/ml) increased PLD activity in a time-dependent manner (Fig. 2*A*). VEGF increased PLD activity as early as 1 min, produced maximal activity during 1–5 min, and produced maximal accumulation of product by 5 min (Fig. 2*A*). The maximum accumulation of PLD product was 3.1-fold. Moreover, VEGF (10, 50, and 100 ng/ml) increased

PLD activity in a dose-dependent manner, whereas the VEGFR-1-specific ligand PlGF (10, 50, and 100 ng/ml) did not change PLD activity (Fig. 2*B*). Furthermore, pretreatment with a specific VEGFR-2 antagonist (SU5614, 10 μM) completely inhibited the VEGF-induced PLD activity (Fig. 2*B*). Thus VEGF-induced PLD activity is mainly dependent on VEGFR-2 activation in endothelial cells.

VEGF-induced PLD activity is involved in ERK activation and proliferation. Because PLD is known to activate MEK/ERK signaling through mobilization of Raf-1 (29), we next examined whether VEGF-induced MEK/ERK activation may be partly attributed to VEGF-induced PLD activity. A PLD inhibitor, 1-butanol (0.4%), almost suppressed VEGF-induced ERK1/2 phosphorylation (Fig. 3, *A* and *B*). The addition of PA (200 μM) restored the suppressed effect of 1-butanol (0.4%) on VEGF-induced ERK1/2 phosphorylation. The negative control for 1-butanol, 3-butanol (0.4%), did not produce a dramatic change in VEGF-induced ERK1/2 phosphorylation. To address the specificity of 1-butanol-mediated inhibition of PLD on VEGF-induced ERK1/2 phosphorylation, we measured the effect of 1-butanol on another known VEGFR-2 signaling event, the VEGF-induced phosphorylation of Akt. Neither

A

Fig. 5. Effect of PKC inhibition on VEGF-induced ERK1/2 activation and PLD activity. *A*: HUVECs were labeled with [³H]myristic acid for 4 h, and the cells were pretreated with 100 nM staurosporine (ST), 1 μM GF109203X (GF), 1 μM BAPTA-AM (BA), 50 μg/ml brefeldin-A (BF), 30 nM wortmannin (WT), 100 nM Go6976 (G6), 100 nM rottlerin (RT), or 100 nM RO31–8220 (RO) for 30 min and then incubated with VEGF (50 ng/ml) for 5 min. PLD activity was then measured and expressed as formation of PBt. Bars represent means ± SD of 5 experiments. *$P < 0.05$ vs. CB; #$P < 0.05$ vs. VEGF (50 ng/ml) only. *B*: cells were pretreated with the same inhibitors for 30 min, then VEGF (10 ng/ml) was added and incubated for 10 min. The cell lysates were boiled in reducing buffer and separated by SDS-PAGE. Each lane contains 50 μg of total protein from the cell lysates. Blots were probed with anti-phospho-ERK antibody (*top*). The membrane was stripped and reprobed with anti-ERK1/2 (*bottom*) to verify equal loading of protein in each lane. *C*: densitometric analyses are presented as the relative ratio of phospho-ERK2 to ERK2. The ratio relative to CB is arbitrarily presented as 1. Bars represent means ± SD from 3 experiments.
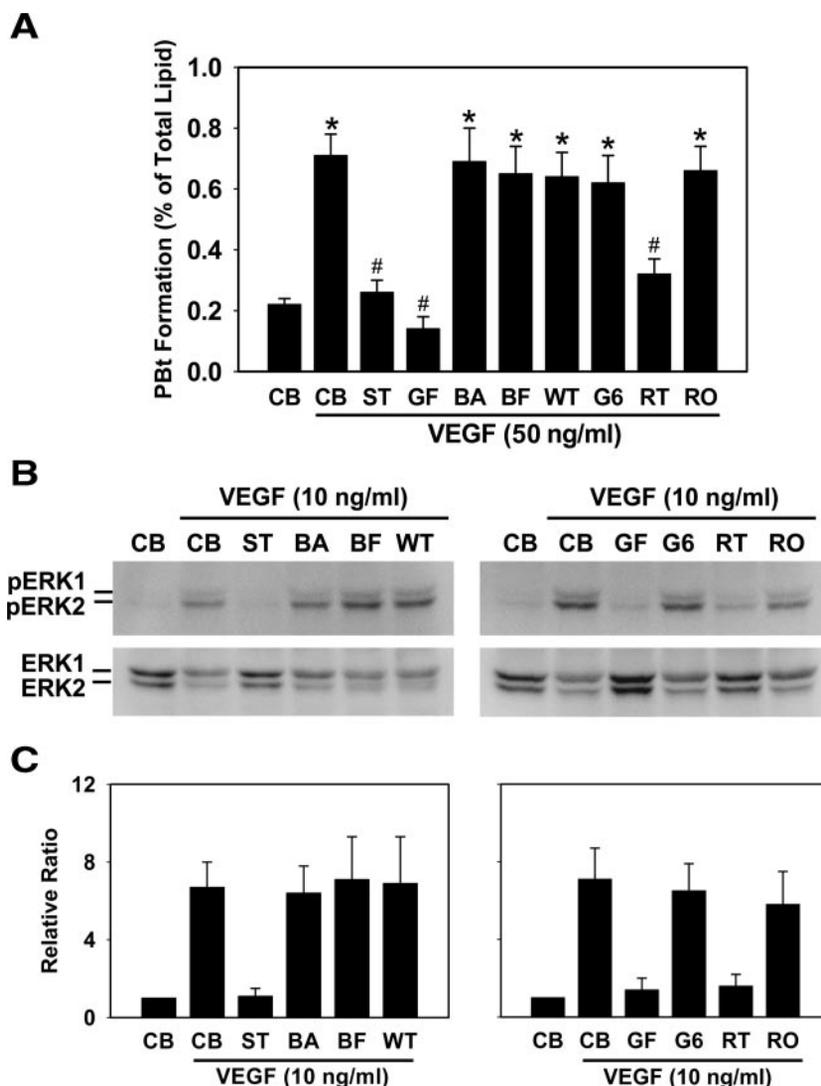
1-butanol nor 3-butanol produced any effect on VEGF-induced Akt phosphorylation (Fig. 3, *C* and *D*). Thus VEGF-induced PLD activity is preferentially involved in ERK1/2 activation, but not in Akt activation, in endothelial cells. Because VEGF-induced ERK activation is closely involved in the proliferation of endothelial cells (21, 28, 38), we next examined the effect of PLD inhibition on VEGF-induced proliferation of endothelial cells by measuring DNA synthetic activity. 1-Butanol (0.04%) significantly suppressed VEGF-induced DNA synthetic activity, whereas the addition of PA (20 μM) restored the suppressed effect of 1-butanol (0.04%) on VEGF-induced DNA synthetic activity (Fig. 4). The negative control for 1-butanol, 3-butanol (0.04%), did not produce a significant change in VEGF-induced DNA synthetic activity. Thus VEGF-induced PLD activity is involved in the proliferation of endothelial cells.

PKC-δ regulates VEGF-induced PLD/ERK activation. Because PLD is regulated by ADP ribosylation factor (ARF) and PKC (4, 5, 11, 12, 16, 17, 20, 31), we determined how these transducers are involved in VEGF-induced activation of PLD and ERK. As expected, the universal PKC inhibitors staurosporine (100 nM) and GF109203X (1 μM) abolished VEGF-induced PLD activity (Fig. 5*A*). In contrast, intracellular Ca^{2+} inhibitor BAPTA-AM (1 μM), ARF inhibitor brefeldin-A (50 μg/ml), and PI3-kinase inhibitor wortmannin (30 nM) did not significantly change VEGF-induced PLD activity (Fig. 5*A*). Notably, PKC-δ inhibitor rottlerin (100 nM) significantly suppressed VEGF-induced PLD activity, whereas PKC-α and -β inhibitor Go69761 (100 nM) and PKC-α, -β, and -γ inhibitor RO31–8220 did not significantly change VEGF-induced PLD activity. Accordingly, pretreatment of universal PKC inhibitors staurosporine and GF109203X and PKC-δ inhibitor rottlerin dramatically abolished VEGF-induced ERK1/2 phosphorylation, whereas other inhibitors did not produce a dramatic change (Fig. 5, *B* and *C*). Thus our results suggest that PKC-δ mainly regulates VEGF-induced activation of PLD and ERK.
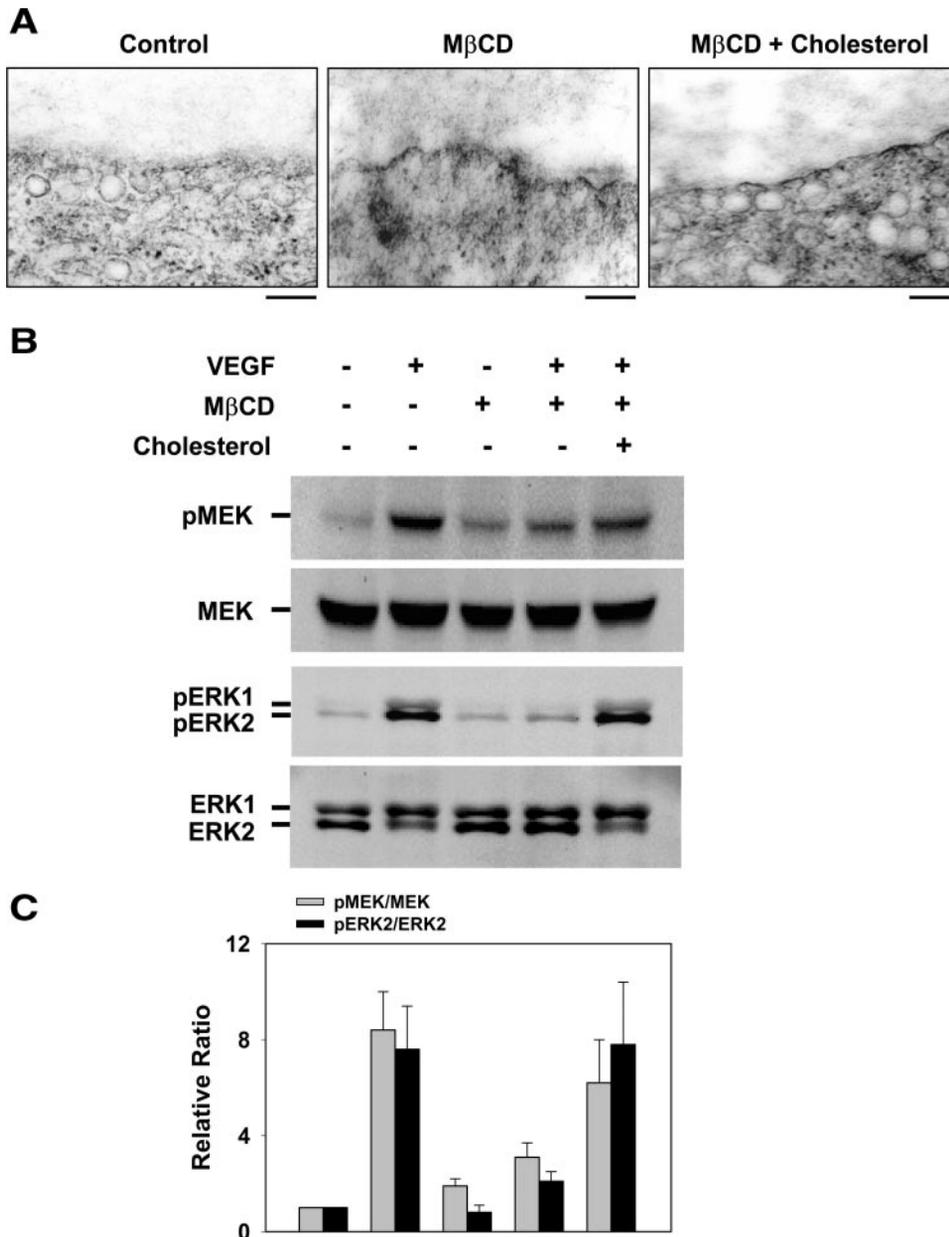

Fig. 6. Effect of depletion and repletion of cholesterol on caveolar structure and VEGF-induced MEK and ERK1/2 activations. *A*: transmission electron micrograph of HUVECs treated with control buffer (30 min), methyl-β-cyclodextrin (MβCD; 5 mM for 30 min), or MβCD (5 mM for 1 h) plus cholesterol (0.2 mM) for 30 min. Discrete round invaginations, ~50–100 nm in diameter, are observed beneath the plasma membrane of control cells. MβCD completely abolished these caveolar structures. However, repletion of cholesterol apparently restored the caveolar structures. Bar = 200 nm. *B*: after depletion or repletion of cholesterol, the HUVECs were treated with VEGF (10 ng/ml) for 10 min. The cell lysates were boiled in reducing buffer and separated by SDS-PAGE. Each lane contains 50 μg of total protein from the cell lysates. Blots were probed with anti-phospho-MEK antibody or anti-phospho-ERK antibody (*top*). The membrane was stripped and reprobed with anti-MEK or anti-ERK1/2 (*bottom*) to verify equal loading of protein in each lane. *C*: densitometric analyses are presented as the relative ratio of phospho-MEK to MEK or phospho-ERK2 to ERK2. The ratio relative to CB is arbitrarily presented as 1. Bars represent means ± SD from 3 experiments.

VEGF-induced activation of MEK/ERK requires intact caveolae. We next investigated whether intact endothelial caveolae are essential for localization of VEGFR-2, PLD, and Ras. Cholesterol is an essential component for normal assembly of caveolae (1, 3). Depletion of cholesterol using MβCD has been shown to disassemble caveolae and thus represents a useful tool to study caveolae-dependent processes (30). Transmission electron microscopy analyses revealed several vesicular invaginations (caveolae) in primary cultured endothelial cell membranes (Fig. 6A). Pretreatment with MβCD (5 mM for 30 min) almost completely disassembled these structures (Fig. 6A). Under these conditions, caveolin-1, VEGFR-2, and Ras were redistributed to *fractions 8–12*, and the characteristic localization of PLD_2 disappeared (data not shown). These results suggest that the integrity of caveolar structures is essential for the localization of VEGFR-2 and its close signaling molecules. The addition of cholesterol (0.2 mM for 1 h) to the MβCD-treated cells restored the caveolar structures (Fig. 6A). This result confirms that cholesterol is an essential substance for maintenance and generation of caveolae. We next examined the effect of the caveolar structure on VEGF-induced activation of MEK/ERK. Pretreatment with MβCD (5 mM for 30 min) largely abolished VEGF-induced MEK and ERK1/2 phosphorylations (Fig. 6, *B* and *C*). Notably, repletion of cholesterol (0.2 mM for 30 min) restored VEGF-induced phosphorylation of MEK and ERK1/2. These results indicate that an intact caveolar structure is preferentially required for the VEGF/VEGFR-2-mediated MEK/ERK signaling cascade.

DISCUSSION

Caveolae, which are flask-shaped membrane invaginations, were first detected on the surface of endothelial cells around 50 years ago (24). In fact, vascular endothelial cells are one of the most abundant sources of caveolae. In a series of electron microscopy studies, Palade and co-workers (2, 33) provided compelling evidence for caveolae as transendothelial carriers by showing the sequential movements of probes from one side of the endothelial layer to the other. Since then, many molecular and cellular studies have brought new insights into caveolar structure and function, and these findings shed new light on many areas of cellular physiology, including signal transduction. Caveolae are enriched for components of signaling cascades, such as heterotrimeric G proteins, Src family kinases, platelet-derived growth factor receptors, epidermal growth factor receptors, and PKC (9, 26, 32, 35). Thus caveolae could be the main platform for signal transduction in endothelial cells.

The fractionation method was able to effectively separate the heavier membrane fractions containing caveolae (Fig. 1). Notably, a relatively larger subfraction of PLD_2 is also enriched in caveolin-1-containing caveolae, although only a trace amount of PLD_2 is present in noncaveolar fractions. In comparison, our data indicate that PLD_1 is evenly distributed in noncaveolar fractions. Interestingly, Seymour et al. (31) suggested that VEGF-induced PLD activity in endothelial cells, although they did not define which type of VEGFR or PLD is involved. Therefore, we hypothesized that VEGFR-2 might regulate PLD activity through activation of caveolae-enriched PLD_2. Indeed, VEGF-induced VEGFR-2 activation rapidly increased PLD activity in a dose-dependent manner, whereas PlGF-induced VEGFR-1 activation did not produce any change in

PLD activity. Moreover, inhibition of VEGFR-2 activation with SU5614 completely abolished VEGF-induced PLD activity. Unfortunately, there are currently no reliable methods for discrimination between PLD_1 and PLD_2 activity. However, considering that PLD_2 is localized in the endothelial caveolae, it is likely involved in VEGFR-2-induced activation of total PLD activity (Fig. 7).

PLD catalyzes the hydrolysis of phosphatidylcholine to produce PA and choline. PA itself can then be converted to diacylglycerol or lysophosphatidic acid, second messengers that activate various downstream signaling events (5, 11, 20). PLD is known to be involved in one of the MAPK pathways, specifically the pathway by which Raf-1 acts on MEK and ERK (29). To test this possible pathway in endothelial cells, the effect of a PLD inhibitor, 1-butanol, on VEGF-induced ERK phosphorylation was examined. The addition of 1-butanol almost totally suppressed VEGF-induced ERK phosphorylation, whereas it did not produce any significant change in VEGF-induced Akt. Moreover, the negative control for 1-butanol, 3-butanol, did not produce a significant change in VEGF-induced MEK and ERK phosphorylations. Although we extensively tried to ablate PLD_1 or PLD_2 expression specifically using anti-sense and interference RNA technologies, these experiments did not work well. Therefore, we cannot exclude the possibility that 1-butanol has a nonspecific effect on VEGF-induced MEK and ERK phosphorylations. However, given that addition of PA restored the 1-butanol-induced suppressive effect, VEGF-induced PA formation through activation of PLD activity may be involved in VEGF-induced ERK phosphorylation in endothelial cells (Fig. 7). Furthermore, our
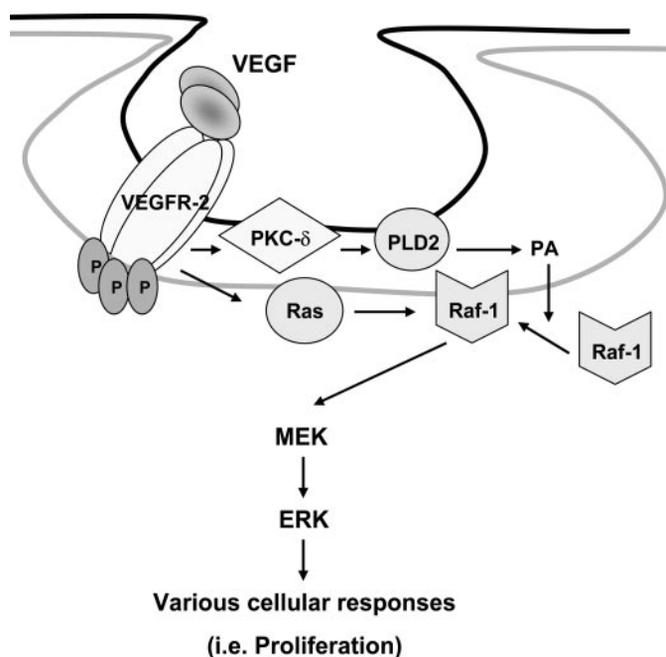

Fig. 7. Schematic diagram showing how VEGF induces ERK activation through VEGFR-2 localized on caveolae. VEGF binds to VEGFR-2 and activates PKC-δ, PLD_2, and Ras. All these components are located in the caveolae of vascular endothelial cells. PA is released on PLD_2 activation. The increased level of PA translocates Raf-1 from the cytoplasm to the membrane and subsequently activates Raf-1. In turn, activated Raf-1 activates MEK and ERK. Activated MEK and ERK evoke several cellular processes in endothelial cells.

results indicated that PLD inhibition with 1-butanol suppressed both VEGF-induced ERK activation and VEGF-induced proliferation in endothelial cells. These two effects were temporally correlated. Given that VEGF-induced ERK activation is closely involved in the proliferation of endothelial cells (21, 28, 38), VEGF-induced PLD/ERK activation could be important for proliferation of endothelial cells. Considering that VEGF-induced proliferation of endothelial cells is one of essential steps in VEGF-induced angiogenesis, PLD in endothelial cells could play an important role in angiogenesis.

Two phosphatidylcholine-specific mammalian isoforms of PLD (PLD_1 and PLD_2) have been isolated and characterized (5, 20). Currently, specific PLD_1 and PLD_2 inhibitors are not available. PLD_1 and PLD_2 are activated differently by ARF, Rho, and PKC in different cell types (4, 5, 11, 12, 16, 17, 20, 31). Our data demonstrate that pretreatment with PKC inhibitor totally suppressed VEGF-induced PLD activity and ERK phosphorylation, whereas pretreatment with ARF inhibitor did not change VEGF-induced PLD activity or ERK phosphorylation. Thus, similar to previous finding (31), VEGF-induced PLD activity and ERK phosphorylation are totally dependent on PKC activation. Therefore, we further examined which PKC isoenzyme is mainly responsible for VEGF-induced PLD/ERK2 activations. With the use of several inhibitors, our analyses indicate that the PKC-δ isoenzyme mainly regulates these activations. We previously demonstrated that PKC-δ mediates the direct phosphorylation-dependent activation of PLD_2 in PC12 cells (12). Likewise, PLD_2 may be involved in VEGF/VEGFR2/PKC-δ-induced MEK/ERK signaling cascades in endothelial cells (Fig. 7).

We next investigated whether intact endothelial caveolae are essential for localization and signaling of VEGFR-2. When the caveolae were disassembled, the localization of VEGFR-2 was shifted to noncaveolar fractions. Furthermore, under conditions that disassemble caveolae, VEGF-induced MEK/ERK activation was largely abolished. This observation is similar to that in a recent study (19). In addition, under conditions that reassemble caveolae, VEGF-induced MEK/ERK activation almost completely recovered. This result indicates that intact caveolae are essential and critical as a platform for VEGFR-2-induced MEK/ERK signaling cascades.

Taken together, two important findings regarding VEGFR-2 signaling in vascular endothelial cells were found in this study. First, PKC-δ/PLD_2 could be partly involved in VEGF/VEGFR-2-induced MEK/ERK activations and cellular proliferation. Second, normal caveolar structure is essential and critical for VEGF-induced MEK/ERK activations.

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