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Biochemical and Biophysical Research Communications 308 (2003) 101–105
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Localization of Tie2 and phospholipase D in endothelial caveolae is involved in angiopoietin-1-induced MEK/ERK phosphorylation and migration in endothelial cells

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Received 19 June 2003

Abstract

Angiopoietin-1 (Ang1) and its receptor, Tie2, play critical roles in blood vessel formation. Ang1 triggers a variety of signaling events in endothelial cells leading to vasculogenic and angiogenic processes. However, the underlying mechanism for Ang1/Tie2 signaling is not fully understood. Here, we show that Tie2 and phospholipase D (PLD) are localized in the caveolae, specialized subdomains of the endothelial cell plasma membrane enriched with signaling molecules. Interestingly, Ang1 increased PLD activities in a dose- and time-dependent manner. Ang1-induced MEK/ERK activation was abrogated when PLD was inhibited, suggesting that PLD mediates Ang1-induced MEK/ERK activation. Moreover, PLD inhibitor, 1-butanol, inhibited Ang1-induced endothelial cell migration. Our results indicate that: (1) caveolae may be the platform for Tie2/PLD association in endothelial cells; (2) PLD is a new mediator of Ang1/Tie2-induced signaling pathway, and it participates in MAPK activation and endothelial cell migration.

Caveolae, which are flask-shaped membrane invaginations, were first detected on the surface of endothelial and epithelial cells about 40 years ago [1]. There is growing evidence that caveolae may act as structurally and biochemically distinct plasma membrane compartments that localize and regulate transmembrane signaling events [2]. This hypothesis is based on the finding that caveolae are enriched for components of signaling cascades, such as heterotrimeric G proteins, Src family kinases, platelet-derived growth factor receptors, and EGF receptors [3].

Interestingly, VEGFR-2 is co-localized with caveolin-1 in the caveolae of endothelial cells [4,5]. This finding suggests that the VEGFR-2 signaling machinery may also be localized in the endothelial caveolae. Besides VEGF, another endothelial cell specific growth factor, angiopoietin-1 (Ang1) and its specific receptor, Tie2, have been identified as key molecules in vasculogenesis and angiogenesis [6]. The Ang1/Tie2 system enhances migration, sprouting, and survival of endothelial cells [7–11]. These processes are mediated through pathways that include phosphatidylinositol 3-kinase, Akt, focal adhesion kinase, Raf/Ras/mitogen-activated protein zkinase (MAPK), and Dok-R/Dok-2/Nck/Pak [8–12]. However, it is not known whether the Tie2 signaling machinery is localized in the caveolae of endothelial cells, as is the case for VEGFR-2.

In this report, we examined the distribution of Tie2 and its related key molecules in human umbilical vein endothelial cells (HUVECs). Interestingly, we found that Tie2 is localized in the caveolae of HUVECs, together with phospholipase D (PLD). PLD was recently found to be an important effector enzyme in signaling pathways through its product, phosphatidic acid (PA) and its secondary metabolites, lyso-phosphatidic acid (LPA) and diacylglycerol (DAG) [13]. Furthermore, we provide evidence that PLD participates in the Ang1/Tie2-induced MAPK pathway, resulting in endothelial cell migration.

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doi:10.1016/S0006-291X(03)01341-X

Materials and methods

Materials. Production of recombinant angiopoietin-1 (Ang1) and its biochemical assay with Tie2 phosphorylation were performed as previously described [14]. Media and serum were obtained from Life technology (Gaithersburg, MD). Mitogen-activated protein/extracellular signal-regulated kinase (ERK) (MEK) 1/2 inhibitor PD 98059 was obtained form Calbiochem (San Diego, CA). Antibodies against phospho-MEK1/2 (Ser217/221), phospho-ERK1/2 (Thr202/Tyr204), MEK, and ERK were purchased from Cell Signaling Technology (Beverly, MA). Polyclonal antibody for PLD1/2 was generated as previously described [15]. Most other biochemical reagents were purchased from Sigma–Aldrich unless otherwise specified. HUVECs were prepared from human umbilical cords by collagenase digestion and maintained as previously described [14]. The primary cultured cells used in this study were between passage 2 and 3.

Fractionation of caveolin enriched membrane. Separation of light and heavy membrane fraction was according to Song et al. [16]. Confluent 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 with 10 strokes of a Dounce homogenizer, three 20-s bursts of a Polytron homogenizer, and five 20-s bursts of a sonicator. The homogenate was then adjusted to 45% sucrose by addition of 80% sucrose prepared in MES buffer (25 mM MES, pH 6.5, 150 mM NaCl, 1 mM EGTA, 1 mM Na_3VO_4, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 μg/ml pepstatin), and placed into ultracentrifugation tubes. A 5–35% discontinuous sucrose gradient (both in MES buffer) was then formed on top. This sample was centrifuged at 39,000 rpm for 6 h in a SW-40 rotor (Beckman instruments, Palo Alto, CA). Fractions were collected from the top in 1-ml amounts, except for fractions 1–4, which were collected as 0.8-ml fractions.

Immunostaining of HUVECs. Approximately 30–50% confluent HUVECs were washed with phosphate-buffered saline (PBS) and fixed for 10 min at −20 °C with methanol. Fixed cells were rinsed with PBS and were permeabilized with 0.1% Triton X-100 in PBS for 10 min. The cells were washed with PBS and incubated with blocking solution (10% FBS in PBS) for 1 h at room temperature. The cells were then incubated overnight at 4 °C with rhodamine conjugated anti-caveolin-1 antibody (Santa Cruz biotechnology, Santa Cruz, CA) and then washed with PBS. Then, the cells were incubated again with anti-Tie2 antibody (R&D system, Minneapolis, MN) or anti-pan PLD antibody [15] diluted 1/150 with blocking solution. After washing with PBS, the cells were incubated with FITC-conjugated anti-goat or anti-rabbit antibodies (Santa Cruz biotechnology, Santa Cruz, CA). The slides were mounted and examined by confocal microscope, and images were captured with LSM 510 (Zeiss, Gottingen, Germany).

PLD assay. PLD activity was assayed by measuring the formation of PtdButOH, the product of PLD-mediated transphosphatidylation, in the presence of 1-butanol as previously described with a slight modification [17]. HUVECs were grown to confluence in a 6-well plate in M-199 medium containing 20% serum, then, the cells were incubated in serum free medium for 16 h. Starved cells were preincubated for 4 h with 3 μCi/ml of [^{3}H]myristic acid (Dupont NEN, Boston, MA). Transphosphatidylation was catalyzed in the presence of 0.4% 1-ButOH and cells were stimulated with the indicated concentration of Ang1 for the indicated times. The reaction was terminated with ice-cold methanol and cells were scraped. The lipid-phase was extracted and developed by TLC on silica gel-60 plates (Merck, Darmstadt, Germany) using chloroform:methanol:acetic acid (9:1:1) as the solvent. The formation of [^{3}H]phosphatidylbutanol was expressed as a percentage of the total [^{3}H]lipid to account for cell labeling efficiency differences.

Western blotting. The treated HUVECs were washed two times with PBS, dissolved in sample buffer (50 mM Tris–HCl, 100 mM NaCl, 0.1% SDS, 1% NP-40, 50 mM NaF, 1 mM Na_3VO_4, 1 μg/ml aprotinin, 1 μg/ml pepstatin, and 1 μg/ml leupeptin), boiled, separated by SDS–PAGE, and transferred to nitrocellulose membrane. The membranes were treated and the phosphorylation levels of MEK1/2 (Ser217/221) or ERK1/2 were analyzed according to the manufacturer's protocol (Cell Signaling Technology), and the membrane was reprobed with anti-MEK1/2 or anti-ERK1/2 antibody to verify equal loading of protein in each lane. All signals were detected by chemiluminescent detection according to the manufacturer's protocol (Amersham, Buckinghamshire, UK).

Cell migration assay. The migration assay in HUVECs was performed using a modified 48-well Boyden chamber (Neuroprobe, Cabin John, MD) as previously described [11]. Indicated reagents in M199 containing 1% bovine serum albumin were placed in the bottom or upper wells of the chamber. Polycarbonate filters with 8 μm pores (Poretics, Livermore, CA) were coated with 50 μg/ml fibronectin and 0.2% gelatin and placed between the test substances and the upper chambers. Cells were trypsinized, washed twice in M199, and resuspended in M199 containing 1% bovine serum albumin. We placed 5×10^4 cells into each well in the upper chamber, then incubated for 12 h at 37 °C in a humidified chamber with 5% CO_2. After incubation, the nonmigrated cells were removed from the upper side of the filters with a cotton ball. The filters were fixed with methanol, mounted onto microscope slides, and stained with Diff-Quik solution. The migrated cells were counted at 100× magnification using a microscope.

Data analyses. Data are expressed as means ± standard deviation (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 and discussion

To isolate caveolin-enriched endothelial cell membranes, HUVECs homogenate was fractionated using sucrose density centrifugation. Twelve fractions were recovered and subjected to Western blot analysis. Caveolin-1, the structural protein of caveolae, was dominantly enriched in fraction 5, which represented the light membrane fraction containing caveolae (Fig. 1A). Bip, a marker for endoplasmic reticulum, was enriched in noncaveolar fractions 9–12. These data indicate that our fractionation method was able to separate the caveolae from the heavier membrane fractions. Very interestingly, we found that Tie2 was mainly enriched in fraction 5 and rarely detected in fractions 9–12 (Fig. 1A). Immunofluorescence staining showed that Tie2 and caveolin-1 were mainly located on the plasma membrane of endothelial cells (Fig. 1B). In addition, both proteins were co-localized in discrete patches. These data indicate that Tie2 is mainly localized to endothelial caveolae. Importantly, this characteristic localization of Tie2 in caveolae could act as an efficient platform for Ang1 binding. The Ang1 structure consists of a carboxy-terminal fibrinogen-like domain that is responsible for receptor binding, a central coiled-coil domain that oligomerizes these fibrinogen-like domains, and a short amino-terminal domain that superclusters oligomers into variably sized multimers [18,19]. Thus, Ang1 is a multimer held together by its disulfide crosslinks and coiled-coil structures [19]. To achieve Tie2 receptor multimerization and activation,

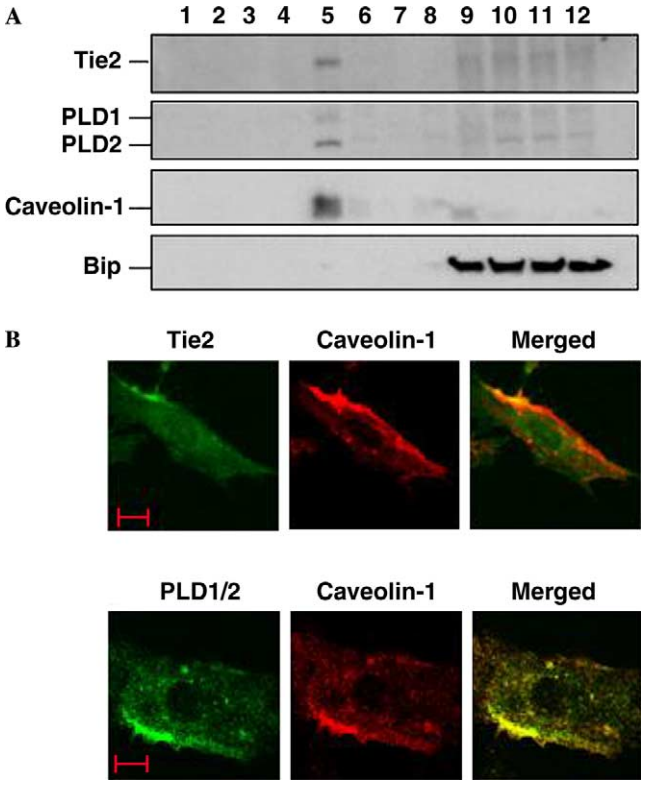

Fig. 1. Tie2 and PLD2 are localized in the caveolin-1 enriched low-density membrane fraction in endothelial cells. (A) HUVECs were fractionated and subjected to Western blot analysis with corresponding antibodies. Tie2 and PLD2 are localized in fraction 5, which is the same fraction enriched for caveolin-1. Bip, a marker for endoplasmic reticulum, is localized in fractions 9–12, which represent noncaveolae membrane fractions. (B) Confocal microscopy of immunofluorescence staining in HUVECs shows co-localization of Tie2, PLD, and caveolin-1. HUVECs were fixed and stained with rhodamine-conjugated anti-caveolin-1 antibody, then the immunostained samples were double immunostained with anti-Tie2 antibody or anti-PLD antibody and visualized with FITC-conjugated corresponding secondary antibodies. The yellow regions of the merged images indicate the co-localization of caveolin-1 and Tie2 (upper panels) or caveolin-1 and PLD (lower panels). Bar = 10 μm.

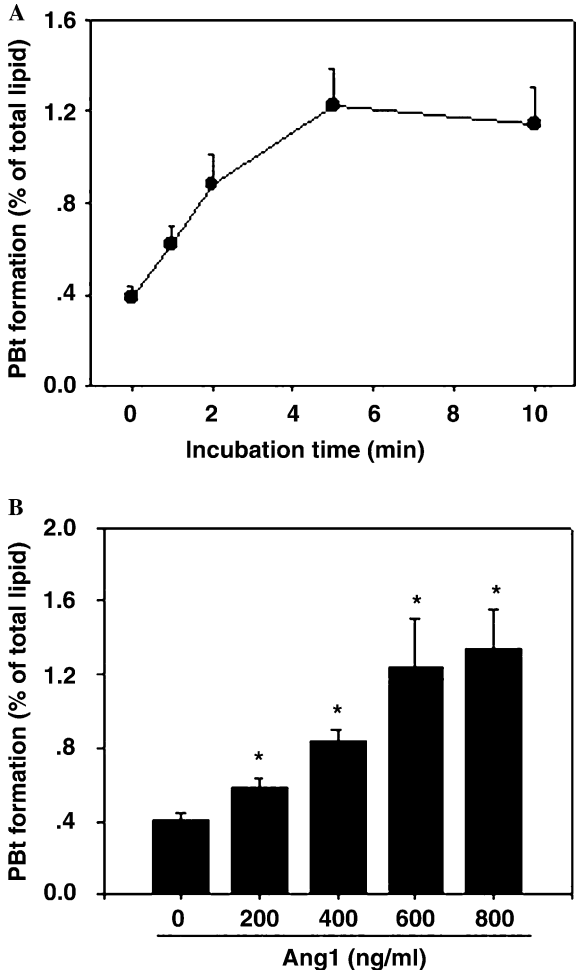

Fig. 2. Ang1 increases PLD activity in time- and dose-dependent manners in HUVECs. (A) HUVECs were labeled with [^{3}H]myristic acid and incubated with 400 ng/ml of Ang1 for the indicated times. (B) Labeled HUVECs were incubated for 5 min with the indicated amounts of Ang1. PLD activity was measured as formation of [^{3}H]PtdButOH/total lipid. Dots and bars represent means ± SD of 3 experiments. *$p < 0.05$ versus time 0 or control buffer.

Ang1 uses a modular and oligomeric/multimeric structure unlike that of any other known growth factor [18,19]. Therefore, enriched Tie2 localization in caveolae could be meaningful for efficient transmembrane signaling events to multimeric Ang1.

During screening for signal molecules in membrane fractions of endothelial cells, we also found that both PLD1 and PLD2 were mainly enriched in fraction 5, while trace amounts were present in fractions 9–12 (Fig. 1A). Immunofluorescence staining showed that PLD1/2 and caveolin-1 were mainly co-localized on the plasma membrane of endothelial cells in discrete patches (Fig. 1B). These data suggested that PLD might be involved in Tie2 signaling in endothelial cells.

Because Ang1 is an agonistic ligand of Tie2 [6], we examined whether Ang1-induced Tie2 activation could regulate PLD activity in HUVECs. PLD activity was measured using formation of phosphatidylbutanol (PtdButOH). Addition of Ang1 (400 ng/ml) increased PLD activity in a time-dependent manner (Fig. 2A). Ang1 increased PLD activity as early as 1 min and produced a maximal accumulation at 5 min. The maximum accumulation in PLD activity was 3.3-fold. Moreover, Ang1 (0–800 ng/ml) increased PLD activity in a dose-dependent manner (Fig. 2B). Thus, Ang1-induced Tie2 activation is closely involved in regulation of PLD activity in endothelial cells.

PLD catalyzes the hydrolysis of phosphatidylcholine to produce PA and choline. PA itself can then be converted to DAG or LPA, second messengers that activate various downstream signaling events [13,20]. PLD is known to be involved in one of MAPK pathways, specifically the pathway by which Raf and Ras act on mitogen-activated protein kinase (MEK) and extracellular

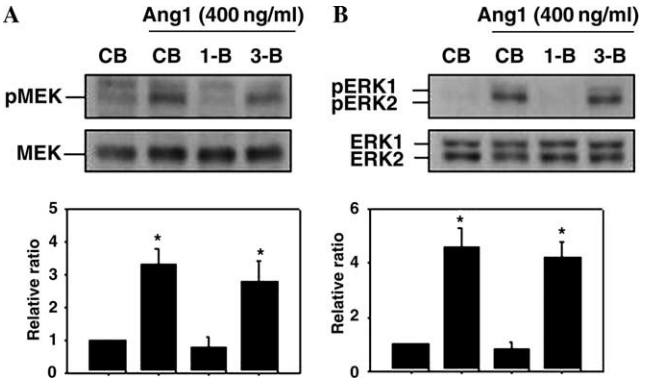

Fig. 3. PLD is involved in Ang1-induced MEK/ERK phosphorylation and endothelial cell migration. HUVECs were incubated for 16 h in 1% serum-containing M-199 medium. The cells were incubated with control buffer (CB), 1-butanol (0.4%, 1-B), or 3-butanol (0.4%, 3-B) for 5 min and then incubated with Ang1 (400 ng/ml) for 10 min. After treatment, cell lysates were harvested. Each lane contains 50 μg of total protein from the cell lysates. Blots were probed with anti-phospho-MEK (Ser217/221) antibody or anti-phospho-ERK1/2 antibody (upper panels). The membrane was stripped and reprobed with anti-MEK antibody or anti-ERK antibody (middle panels) to verify equal loading of protein in each lane. Densitometric analyses are presented as the relative ratio of phospho-MEK to MEK or phospho-ERK2 to ERK (lower panels). The relative ratio to CB is arbitrarily presented as 1. Numbers represent means ± SD from 3 experiments. *$p < 0.05$ versus CB.

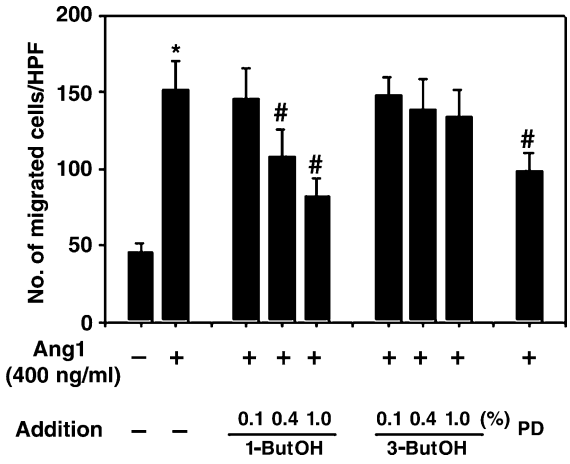

Fig. 4. PLD is involved in Ang1-induced MEK/ERK phosphorylation and endothelial cell migration. HUVECs were incubated for 16 h in 1% serum-containing M-199 medium. Ang1 (400 ng/ml) or control buffer in M199 containing 1% BSA was placed in the lower wells of the chamber, while the indicated concentration of 1-butanol, 3-butanol, or PD98059 in M199 containing 1% bovine serum albumin was placed in the upper wells of the chamber. HUVECs (5.0×10^4) were placed in the upper chamber, then incubated for 12 h at 37 °C. The migrated cells were stained with Diff-Quik solution and counted at 100× magnification using a microscope. Bars represent means ± SD from 3 experiments. *$p < 0.05$ versus control buffer #$p < 0.05$ versus Ang1 (400 ng/ml) only.

signal-regulated kinase (ERK) [21]. We recently reported that Ang1 activated the Raf/Ras/MEK/ERK pathway for endothelial cell migration [12]. Therefore, we hypothesized that PLD could be involved in this cell migration pathway. To test this hypothesis, we first examined the effect of a PLD inhibitor, 1-butanol, on Ang1-induced MEK/ERK phosphorylation in HUVECs. 1-Butanol (0.4%) almost totally suppressed Ang1-induced MEK/ERK phosphorylation (Fig. 3). The negative control for 1-butanol (0.4% 3-butanol) did not produce any changes in Ang1-induced MEK/ERK phosphorylation. A previous study indicated that PLD-induced PA could be a positive regulator in mobilization of cytosolic Raf to membrane-bound Raf for activating Raf/Ras/MEK/ERK signaling cascades [22]. Thus, co-localization of Tie2 and PLD in caveolae is meaningful for efficient and prompt coupling to Raf/Ras/MEK/ERK upon Ang1 binding to Tie2.

Raf/Ras/MEK/ERK activation in endothelial cells is involved in proliferation, survival, and migration [12]. However, Ang1 does not affect the proliferation of endothelial cells [6]. Ang1 does affect cell survival, but Ang1-induced cell survival is mainly dependent on the PI 3′-kinase/Akt signaling pathway [10]. Therefore, we used endothelial cell migration to assay for the effect of Ang1 on PLD activity. Ang1 (400 ng/ml) increased migration approximately 3.3-fold after 12-h treatment (Fig. 3B). 1-Butanol (0.1%, 0.4%, and 1.0%) inhibited Ang1-induced endothelial cell migration in a dose-dependent manner, while 3-butanol (0.1%, 0.4%, and 1.0%) did not produce any inhibition (Fig. 4). Notably, 1-butanol (0.4%) inhibited approximately 40–45% of Ang1-induced endothelial cell migration. This effect is comparable to MEK1/2 inhibitor PD98059 (50 nM), which induced inhibition of approximately 45–50% of Ang1-induced endothelial cell migration. These data indicate that PLD is closely involved in the Ang1/Tie2-Raf/Ras/MEK/ERK pathway for endothelial cell migration.

From this study, we found two novel findings regarding the caveolae of endothelial cells. First, the specific endothelial cell receptor, Tie2, is localized in caveolae, as other receptors are. Second, PLD is also localized in caveolae of endothelial cells. We found that Ang1 can increase PLD activity, and furthermore, Ang1-induced PLD can be positive regulator for Raf/Ras/MEK/ERK signaling cascades and endothelial cell migration through generation of PA.

Acknowledgments

This work was supported by the Creative Research Initiatives of the Korean Ministry of Science and Technology, and by the Brain Korea 21 Project.

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