LPP3 localizes LPA$_6$ signalling to non-contact site in endothelial cells

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Abstract
Lysophosphatidic acid (LPA) is an emerging angiogenic factor, as knockdown of its producing enzyme, autotaxin and receptors caused severe developmental vascular defects in both mice and fish. In addition, overexpression of autotaxin in mice caused similar vascular defects, indicating that the extracellular amount of LPA must be tightly regulated. Here, we focused on an LPA-degrading enzyme, lipid phosphate phosphatase 3 (LPP3), and showed that LPP3 was localized in specific cell-cell contact sites of endothelial cells and suppresses LPA signalling via LPA₆ receptor. In HEK293 cells, overexpression of LPP3 dramatically suppressed activation of LPA₆. In human umbilical vein endothelial cells (HUVECs), LPA induced actin stress fiber formation via LPA₆, which was prominently up-regulated by LPP3 knockdown. LPP3 was localized to cell-cell contact sites and was missing in non-contact sites to which LPA-induced actin stress fiber formation mediated by LPA₆ was restricted. Interestingly, the expression of LPP3 in HUVECs was dramatically increased after forskolin-treatment, in which Notch signalling was involved. These results indicate that LPP3 regulates and localizes LPA signalling in endothelial cells thereby stabilizing vessels via Notch signalling for proper vasculature.
Introduction

LPA regulates a wide variety of cellular processes in vertebrates including the migration, adhesion, proliferation, differentiation and cell death, and thereby influences multiple *in vivo* events ranging from organogenesis to development of cancer (Ishii et al., 2004; Lin et al., 2010). LPA is mainly produced by autotaxin (ATX), an extracellular enzyme that converts lysophospholipids to LPA, and exerts its action through at least six G protein-coupled receptors (LPA<sub>1-6</sub>) specific to LPA (Chun et al., 2010). A major question in this field is how extracellularly-produced LPA is controlled. Lipid phosphate phosphatases (LPPs) are key factors controlling LPA metabolism. LPPs are integral membrane enzymes that dephosphorylate lipid phosphates such as sphingosine-1-phosphate (S1P) and LPA (Brindley and Pilquil, 2009; Pyne et al., 2005).

In vertebrates, at least three LPP genes (*LPP1-3*) have been identified (Kai et al., 1997; Roberts et al., 1998). LPA under the control of these LPA-related genes was recently shown to be a key regulator of the development of embryonic vasculature in both mice and zebrafish (Escalante-Alcalde et al., 2003; Panchatcharam et al., 2014; Tanaka et al., 2006; Yukiura et al., 2011).

ATX knockout mice died around E9.5 due to the defects of blood vessel formation in the yolk sac, allantois and embryos (Tanaka et al., 2006). Similar vascular defects were observed when ATX expression was suppressed in zebrafish embryos (Yukiura et al., 2011). In the zebrafish embryos, ATX knockdown caused retarded elongation (or sprouting) of intersegmental vessels (ISVs). Knockdown of multiple LPA receptors (LPA<sub>1</sub>, LPA<sub>4</sub> and LPA<sub>6</sub>) resulted in similar defects in ISV formation (Yukiura et al.,
2011). LPP3 knockout mice failed to form a chorio-allantoic placenta and yolk sac vasculature around E9.5 (Escalante-Alcalde et al., 2003). This phenotype was also reproduced in endothelial cell-specific LPP3 knockout mice (Panchatcharam et al., 2014). Very recently, we established transgenic mice overexpressing ATX and found that overexpression of ATX in embryo caused similar vascular defects, as well as vascular defects in retina when ATX was overexpressed conditionally in neonate. Because endothelial cells but not mural cells are involved in vascular formation in the embryonic stages, it is assumed that the level of LPA, balanced by ATX and LPP3, affects endothelial cell functions via LPA receptors in a highly coordinated fashion, thereby regulating blood vessel formation. To understand regulation of LPA signalling, we examined the roles of LPA and LPP3 using human umbilical vein endothelial cells (HUVECs).
Results and Discussion

To examine the role of LPP3 in endothelial cells more precisely, we first examined the effects of LPA on HUVEC functions. We found that LPA dramatically induced the formation of actin stress fibers and intracellular gaps (Fig. 1A) and increased the cells' permeability (Fig. 1B). We also quantified the LPA effect by counting the number of intercellular gaps (Supplementary Fig. 1). The most strongly expressed LPA receptor in HUVECs was LPA₆ (Fig. 1C). The LPA-induced actin stress fiber formation was suppressed by silencing LPA₆ and restored by reintroducing LPA₆ in HUVECs (Fig. 1D and E). The LPA-induced increase in the permeability of HUVECs was also suppressed by silencing LPA₆ (Fig. 1F). LPA-induced actin stress fiber formation and intracellular gaps were suppressed by either treatment with siRNA for Gα₁₃, siRNA for RhoA, or Y27632, a ROCK inhibitor (Fig. 1G). Consistent with this, we confirmed that activation of RhoA (Supplementary Fig. 2A) but not Rac1 (data not shown) in LPA-stimulated HUVECs as judged by measuring the level of activated form of RhoA (GTP-bound RhoA).

We next characterized LPP3 as an LPA-degrading enzyme. We confirmed that mammal LPP3s (mouse and human) degraded LPA both at protein (Fig. 2A) and cellular levels (Fig. 2B). When overexpressed in HEK293 cells, LPP3 prominently suppressed the activation of LPA₆ but not the activation of the receptor for platelet-activating factor (PAF) in HEK293 cells (Fig. 2C and D) as judged by a transforming growth factor-α (TGFα) shedding assay (Inoue et al., 2012). These results indicated that LPP3 negatively regulated LPA₆ signalling at the cellular level.
We next examined the effect of knockdown of LPP3, which was found to be highly expressed in HUVECs (Fig. 3A). In control HUVECs, treatment with 0.1 µM LPA but not 1 µM LPA failed to induce the rearrangement of the cytoskeleton and the disruption of cell-cell adhesion (Fig. 3D). Knockdown of LPP3 by siRNA treatment resulted in lower adhesive activity of HUVECs to cell culture plate (data not shown) and lower LPA degrading activity in HUVECs (Fig. 3B and C). In the HUVECs treated with siRNA for LPP3, the lower LPA concentration (0.1 µM) did induce actin stress fiber formation and disrupted cell-cell adhesion (Fig. 3D). The effect was reversed by re-expression of mouse LPP3 (mLPP3) but not by catalytically inactive mLPP3, in which catalytic serine 198 was replaced with threonine (Fig. 3D). The effect of LPP3 knockdown was attributed to an enhanced LPA₆ signalling, since the effect of the siRNA for LPP3 was cancelled by simultaneous knockdown of LPA₆ in the cells (Fig. 3D). These results were confirmed by quantification of the number of LPA-induced intercellular gaps (Supplementary Fig. 1B). In addition, we confirmed that knockdown of LPP3 by siRNA treatment resulted in enhanced RhoA activation induced by LPA (Supplementary Fig. 2B).

During our experiments using HUVECs, we noticed that the effect of LPA was highly affected by cell density, especially by the presence of cell-cell contact. Sparse HUVECs were more susceptible to LPA than confluent HUVECs as shown by the formation of actin stress fibers (Fig. 4A) This might be due to lack of cell-cell
adhesion, since HUVECs treated with forskolin (FK), which enhanced cell-cell adhesion (Fukuhara et al., 2005; Stelzner et al., 1989), showed marked resistance to LPA stimulation (Fig. 4A). Interestingly, the effect of FK was canceled by LPP3 knockdown (Fig. 4A), indicating that LPP3 is involved in the resistance to LPA in FK-treated HUVECs. This was confirmed by quantification of the number of cells with actin stress fibers (Supplementary Fig. 1C), since evaluation of LPA effect based on intercellular gaps could not be applied to sub-confluent cells. We also found that in FK-treated cells LPA-induced RhoA activation was significantly attenuated (supplementary Fig. 2C). Furthermore, the LPP3 expression level was ~1.5 fold higher in confluent HUVECs than in nonconfluent HUVECs and was dramatically increased in FK-treated HUVECs (Fig. 4B). The FK-induced up-regulation of LPP3 was prominently suppressed by PKA inhibitor (H89) but not by EPAC inhibitor (ESI-09) (Supplementary Fig. 3). We also found that in FK-treated HUVECs, LPP3 was predominantly localized to cell-cell contact sites (Fig. 4C). Co-staining with VE-cadherin antibody confirmed the result (supplementary Fig. 4A). Such sub-cellular localization of LPP3 was not observed in either confluent or nonconfluent cultures (Fig. 4C). In HUVEC monolayers scratched with tips, LPP3 was not localized to the site where neighboring HUVECs were absent (non-contact site, Fig. 4D and supplementary Fig. 4B). Interestingly, leader cells, i.e., cells located at the edge of the HUVEC sheets, showed strong actin stress fiber formation after LPA stimulation (Fig. 4E), indicating that these cells dramatically responded to LPA. These results indicate that the site of LPA action was restricted to the non-contact site of HUVECs. This effect was canceled
by treating the cells with either siRNAs for LPA₆, Gα₁₃ or RhoA, or Y27632 (Fig. 4E). The localized LPA₆ signalling to the non-contact sites appeared to be specific for LPA, because it was not induced by a PAR ligand, thrombin (Fig. 4F). We also applied a phosphatase-resistant LPA₆ agonist, OMPT (Jiang et al., 2013), and found that OMPT induced formation of actin stress fibers in all the HUVECs (Fig. 4F). These results indicate that the localized activation of LPA₆ at non-contact sites in the leader cells was due to the absence of LPP3 in these sites.

In endothelial cells, contact-dependent gene expression is partly regulated by Notch signalling. The enhanced expression of LPP3 in FK-treated HUVECs was suppressed by Notch inhibitor, DAPT (Fig. 4G). Furthermore, the expression of LPP3 was up-regulated when HUVECs were cultured on a plate coated with Dll4 (Fig. 4H) but not Jagged1 (data not shown). We also found that on Dll4-coated plate LPA-induced RhoA activation was significantly attenuated (supplementary Fig. 2D). Thus, in endothelial cells, LPA signaling appears to be regulated by Notch signaling through modulation of LPP3 expression.

Recently, LPA was found to enhance vascular permeability upon inflammation (Panchatcharam et al., 2014). Thus, LPA seems to be a vascular arture destabilizing factor in pathological conditions. In this study, we found that LPP3 enhanced the cell-cell interaction by downregulating LPA₆ signalling. Previous reports also indicated that LPP3 enhanced cell-cell interaction through its RGD domain (or RGE domain in mice LPP3) and integrins (Humtsoe et al., 2005; Humtsoe et al., 2003;
Wary and Humtsoe, 2005). Thus, LPP3 appeared to stabilize blood vessels by multiple ways.

LPA is considered to be ubiquitously distributed because its synthetic enzyme, ATX, and its precursor, lysophosphatidylcholine (LPC), are also ubiquitously distributed (Aoki et al., 2008). In addition, many types of endothelial cells express high levels of LPA6 (Ren et al., 2013). These findings appear to indicate that LPA is always active and induces massive vascular permeability. However, as we showed in this study, this is not the case because LPP3 is highly expressed in endothelial cells and protect them from the LPA effect. We demonstrate that LPP3 was expressed locally and restricted LPA signalling at the subcellular level. LPA signalling was profoundly suppressed at cell-cell contact sites, where LPP3 was accumulated, and was fully active in non-contact sites, where LPP3 was absent (Fig. 4). Jia et al. reported that a dityrosine motif present in the second cytoplasmic portion of LPP3 serves as basolateral targeting signal in MDCK cells (Jia et al., 2003). Probably, HUVECs use a similar mechanism to locate LPP3 to the cell-cell contact site (lateral site). Interestingly, another LPP isoform, LPP1, distributes specifically to the apical surface of MDCK cells. Thus, it is possible that each LPP isoform regulates the lipid phosphate signalings in specific domains of the cells.

We also showed that LPP3 expression was regulated transcriptionally by Notch signalling (Fig. 4G and H). Notch signalling is known to contribute to the stability of newly formed vessels by inducing gene expression and establishing firm adherens junctions (Phng and Gerhardt, 2009). Thus, it is reasonable to assume that
Notch signalling stabilizes newly formed vessels by inducing LPP3 and down-regulating LPA signalling.
Materials and Methods

Reagents and antibodies

1-Oleoyl LPA was purchased from Avanti Polar Lipids Inc. Stealth siRNAs against human LPP3, LPA$_6$, G$\alpha_{13}$ and RhoA were purchased from Invitrogen. DAPT, thrombin, forskolin and anti-Flag M2 antibody were purchased from Sigma. Alexa Fluor 594 phalloidin was purchased from Molecular Probes. Recombinant human Dll4 were from R&D Systems.

Cell culture and transfection

HUVECs were purchased from Kurabo and were maintained in HuMedia-EG2 with a growth additive set. HEK293T cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM; Nissui, Tokyo, Japan) containing 10% fetal calf serum and antibiotics. HUVECs and HEK293 cells were transfected by using NEON electroporation system (Invitrogen) and Lipofectamine 2000 (Invitrogen), respectively.

Permeability assay

Permeability assay was performed as described previously (Fukuhara et al., 2005) using FITC-labeled dextran (molecular weight, 42,000) as an indicator of permeability.

Evaluation of GPCR activation

Activation of LPA$_6$ receptor and PAF receptor were evaluated by a TGF-$$\alpha$$ shedding assay as described previously (Inoue et al., 2012) using cDNAs for human LPA$_6$ and human PAFR, except that cDNA for human LPP3 was co-transfected.

LPA degradation assay

Cells (HUVECs or HEK293 cells transfected with cDNA for LPP3) or membrane fraction, which had been transiently transfected with LPP3 plasmid constructs, were incubated in M199 containing 1% BSA and 10 $\mu$M LPA. The amount of LPA remained in the media was determined by LC-MS/MS as previously described (Inoue et al., 2011).
Immunofluorescence staining.
For immunofluorescence cell staining, HUVECs cells were cultured on collagen-I-coated glass-bottom dishes, fixed in 4% PFA-PBS, permeabilized in 0.1% Triton X-100 in PBS for 10 min, incubated with 3% BSA-PBS containing 10% goat serum, incubated with Alexa Fluor-594-conjugated phalloidin at room temperature. Images were captured and line scan plot analysis was performed with a Zeiss LSM 700 confocal laser scanning microscope. Quantification of the data was performed by counting the number of intercellular gaps or the number of cells with or without actin stress fiber.

Quantitative RT-PCR analysis
Total RNA was reverse-transcribed using High-Capacity cDNA RT Kits (Applied Biosystems) according to the manufacturer’s instructions. PCR reactions were performed with SYBR Premix Ex Taq (Takara Bio) and were monitored by ABI Prism 7300 (Applied Biosystems). Standard plasmids ranging from $10^2$ to $10^6$ copies per well were used to quantify the absolute number of transcripts of cDNA samples. The numbers of transcripts were normalized to the number of a house-keeping gene, GAPDH in the same sample.

Primers used in human gene expressions are listed below:
LPP1, 5’-CTGGAGCGATGTGTTGACTG-3’ and 5’-GTTGGTGTTCATGCAGAGTTG-3’;
LPP2, 5’-CTACCGTCCAGATACCATCACC-3’ and 5’-GTTGAAGTCCGAGCGAGAATAG-3’;
LPP3, 5’-TGGCAGGATTGTGCTCAAGG-3’ and 5’-CAATAATGTCCACAGGGTAAAGG-3’;
HEY1, 5’-GAGAAGCAGGGATCTGCTAA-3’ and 5’-CCCAAACGATAGTCCCAT-3’;

Note that primer sets including LPA1-6 and GAPDH were same as previously described.(Inoue et al., 2011)

Evaluation of activation of RhoA and Rac1
Activation of RhoA and Rac1 was examined using G-LISA system (Cytoskeleton, Inc.)
according to the manufacture’s instruction. In this system, level of activated (GTP-bound) RhoA and Rac1 was determined using antibodies specific to the activated form of RhoA and Rac1.

**Statistical analyses.**
All statistical analyses were carried out using Prism software (GraphPad). A P-value less than 0.05 was considered to be significant.
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References


Fig 1. LPA induced actin stress fiber formation through LPA$_6$-G$\alpha_{13}$-RhoA-ROCK pathway
(A) Immunofluorescence analysis of the distribution of F-actin in HUVECs treated with
1 µM LPA by Alexa594-phalloidin staining. The arrows indicate intercellular gaps. (B) Vascular permeability induced by LPA stimulation as judged by leak of FITC-labeled dextran across the monolayer-cultured HUVECs. Error bars indicate s.d. (n=4). (C) qRT-PCR analysis of LPA receptors. Total RNA from HUVECs in confluent culture was reverse-transcribed and the resulting cDNAs were subjected to qRT-PCR. The number of the transcripts was normalized to GAPDH in the same sample. (D) Gene silencing efficacy of siRNAs for LPA_6 in HUVECs. 48 hrs after siRNAs treatment, HUVECs were harvested, and LPA_6 mRNA were quantitated by qRT-PCR. (E) HUVECs were pretreated with siRNA for LPA_6 or siRNA for LPA_6 and siRNA-resistant hLPA_6 cDNA. 48 hrs after the transfection, cells were immunostained for F-actin as in (A). (F) HUVECs were treated with siRNA for LPA_6 and LPA-induced vascular permeability was evaluated as in (B). Error bars indicate s.d. (n=4). P-value was estimated by one-way ANOVA with Bonferroni’s posttest analyses, **P < 0.01, ***P < 0.001. (G) HUVECs were treated with siRNAs for Go_{13} and RhoA or Y27632 (ROCK inhibitor). 48 hrs after the transfection, cells were immunostained for F-actin as in (A). Scale bar in (E) and (G), 20 µm.
Fig. 2. LPP3 attenuates LPA₆ activation in HEK293 cells

LPA degrading activity of LPP3 was determined at proteins (A) and cellular levels (B). Membrane fractions of HEK 293 cells transfected with cDNAs for LPP3 or catalytically inactive LPP3 (A) or the cells themselves (B) were incubated with LPA and the LPP activity was determined by measuring LPA level remained by LC-MS/MS. Error bars indicate s.d. (n=3). (C and D) Inhibition of LPA and PAF signalling by LPP3. HEK293 cells that had been transfected with cDNAs for hLPA₆, hPAFR, hLPP3 and inactive form of hLPP3 in combination as indicated were stimulated with LPA (C) or PAF (D) at indicated concentration and the activation of receptors was evaluated using TGFα shedding assay. Error bars indicate s.d. (n=3). P-value was estimated by one-way ANOVA with Bonferroni’s posttest analyses, ***P < 0.001.
Fig. 3. LPP3 negatively regulates LPA signalling in endothelial cells

(A) Expression of LPPs in HUVECs. qRT-PCR was performed as in Fig. 1C. (B) Gene silencing efficacy of siRNAs for LPP3 in HUVECs as judged by qRT-PCR. (C) LPA degrading activity in HUVECs. LPA degrading activity of HUVECs transfected with siRNAs for LPP3 and/or mLPP3 cDNA in combination was evaluated by measuring LPA level remained in the culture media by LC-MS/MS. (D) Staining of F-actin in HUVECs. HUVECs were transfected in combination with siRNAs for LPP3 and LPA6 and mouse LPP3 (mLPP3, both active and inactive form) cDNA as indicated. 48 hrs after transfection, HUVECs were stimulated with LPA at indicated concentration. The arrows indicate intercellular gaps. Scale bar, 20 µm.

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Fig. 4. LPP3 determines the sub-cellular localization of LPA signalling in endothelial cells.
(A) Sub-confluent, confluent and forskolin (FK)-pretreated (for 30 min) HUVECs were
stimulated with LPA at indicated concentration for 30 min. F-actin was stained with
Alexa594-phalloidin (red). Unlike sub-confluent and confluent HUVECs, cells with
strong adhesion did not respond to LPA, which was reversed by treatment of the cells
with siRNA for LPP3. (B) Cell density-dependent expression of LPP3 in HUVECs.
Expression of LPP3 in sub-confluent, confluent and FK-treated (2 hrs) HUVECs was
determined by qRT-PCR. Error bars indicate s.d. (n=4). (C and D) Sub-cellular
localization of LPP3. HUVECs transfected with cDNA for flag-tagged LPP3. (C)
HUVECs (sub-confluent, confluent or FK-treated) were stained with anti-flag antibody
(green) and Alexa594-phalloidin (red). (D) FK-treated HUVECs were scratched using
tip, and after 30 min cells were stained as in (C). sc; scratched area. (E and F) Leader
cells were highly susceptible to LPA stimulation. (E) Confluent HUVECs were
scratched, and after 30 min cells were stimulated with 1 µM LPA and stained with
Alexa594-phalloidin. HUVECs were also pre-treated with siRNAs for LPA6, Gα13 or
RhoA or Y27632. (F) The scratched HUVECs were also stimulated with OMPT (a
phosphatase-resistant LPA analog, 1 µM) or thrombin (1 U/mL). (G and H) Notch-induced expression of LPP3 in HUVECs. (G) FK-induced LPP3 expression in
HUVECs was suppressed by Notch inhibitor, DAPT (10 µM for 2 hrs). (H) The Notch
ligand, rDll4 (1 µg/ml), enhanced the expression of LPP3 in HUVECs, which were
suppressed by DAPT. Other Notch-target gene, HEY1, showed a similar expression
pattern. 24 hr after rDll4 stimulation, expression of LPP3 and HEY1 was determined by
qRT-PCR. Error bars indicate s.d. (n=4). P-values were estimated by one-way ANOVA
with Bonferroni’s posttest analyses in B and H and by student’s t-test in D, *P < 0.05,
***P < 0.001.