SHORT REPORT

LPP3 localizes LPA6 signalling to non-contact sites in endothelial cells

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ABSTRACT

Lysophosphatidic acid (LPA) is emerging as an angiogenic factor, because knockdown of the enzyme that produces it (autotaxin, also known as ENPP2) and its receptors cause severe developmental vascular defects in both mice and fish. In addition, overexpression of autotaxin in mice causes 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, also known as PPAP2B), and showed that LPP3 was localized in specific cell–cell contact sites of endothelial cells and suppresses LPA signalling through the LPA6 receptor (also known as LPAR6). In HEK293 cells, overexpression of LPP3 dramatically suppressed activation of LPA6. In human umbilical vein endothelial cells (HUVECs), LPA induced actin stress fibre formation through LPA6, which was substantially upregulated by LPP3 knockdown. LPP3 was localized to cell–cell contact sites and was missing in non-contact sites to which LPA-induced actin stress fibre formation mediated by LPA6 was restricted. Interestingly, the expression of LPP3 in HUVECs was dramatically increased after forskolin treatment in a process involving Notch signalling. These results indicate that LPP3 regulates and localizes LPA signalling in endothelial cells, thereby stabilizing vessels through Notch signalling for proper vasculature.

KEY WORDS: LPA receptor, LPP3, Endothelial cell, Lysophosphatidic acid

INTRODUCTION

Lysophosphatidic acid (LPA) regulates a wide variety of cellular processes in vertebrates, including 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, also known as ENPP2), an extracellular enzyme that converts lysophospholipids to LPA, and exerts its action through at least six G-protein-coupled receptors (LPA1–LPA6, also known as LPAR1–LPAR6) 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 (SIP) and LPA (Brindley and Pilquil, 2009; Pyne et al., 2005). In vertebrates, at least three LPP genes (LPP1, LPP2 and LPP3; also known as PPAP2A, PPAP2C and PPAP2B, respectively) have been identified (Kai et al., 1997; Roberts et al., 1998). LPA, under the control of these LPPs, has recently been 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 die at around embryonic day (E)9.5 owing to the defects in blood vessel formation in the yolk sac, allantois and embryos (Tanaka et al., 2006). Similar vascular defects are observed when ATX expression is suppressed in zebrafish embryos (Yukiura et al., 2011). In the zebrafish embryos, ATX knockdown causes retarded elongation (or sprouting) of intersegmental vessels (ISVs). Knockdown of several of the LPA receptors (LPA1, LPA4 and LPA6) also results 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 is 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 the embryo causes similar vascular defects, as well as vascular defects in retina when ATX is overexpressed conditionally in neonates (Yukiura et al., 2015). 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 by signalling through 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 fibres and intracellular gaps (Fig. 1A), and increased the permeability of the cells (Fig. 1B). We also quantified the LPA effect by counting the number of intercellular gaps (Fig. S1). The most strongly expressed LPA receptor in HUVECs was LPA6 (Fig. 1C). The LPA-induced actin stress fibre formation was suppressed by silencing LPA6 with small interfering RNA (siLPA6) and restored by reintroducing LPA6 in HUVECs (Fig. 1D,E). The LPA-induced increase in the permeability of HUVECs was also suppressed by silencing LPA6 (Fig. 1F). LPA-induced actin stress fibre formation and intracellular gaps were suppressed by either treatment with siRNA for Gt13 (siGt13), siRNA for RhoA (siRhoA), or Y27632, a ROCK inhibitor (Fig. 1G). Consistent with this, we confirmed that activation of RhoA (Fig. S2A) but not Rac1 (data not shown) in
Fig. 1. See next page for legend.
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 that mammalian 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,D) as judged by a transforming growth factor-α (TGFα) shedding assay (Inoue et al., 2012). These results indicate that LPP3 negatively regulates 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 (siLPP3) resulted in lower adhesion of HUVECs to cell culture plate (data not shown) and lower LPA-degrading activity in HUVECs (Fig. 3B,C). In the HUVECs treated with siRNA for LPP3, the lower LPA concentration (0.1 μM) did induce actin stress fibre 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 the catalytic serine 198 is replaced with threonine (Fig. 3D). The effect of LPP3 knockdown could be attributed to an enhanced LPA6 signalling, because the effect of siRNA for LPP3 was negated by simultaneous knockdown of LPA6 in the cells (Fig. 3D). These results were confirmed by quantification of the number of LPA-induced intercellular gaps (Fig. S1B). In addition, we confirmed that knockdown of LPP3 by siRNA treatment resulted in enhanced RhoA activation induced by LPA (Fig. S2B).

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 fibres (Fig. 4A). This might be due to a lack of cell–cell adhesion, because HUVECs treated with forskolin, which enhances cell–cell adhesion (Fukuhara et al., 2005; Stelzner et al., 1989), showed marked resistance to LPA stimulation (Fig. 4A). Interestingly, the effect of forskolin was negated by LPP3 knockdown (Fig. 4A), indicating that LPP3 is involved in the resistance to LPA in forskolin-treated HUVECs. This was confirmed by quantification of the number of cells with actin stress fibres (Fig. S1C), given that evaluation of LPA effect based on intercellular gaps cannot be applied to sub-confluent cells. We also found that, in forskolin-treated cells, LPA-induced RhoA activation was significantly attenuated (Fig. S2C). Furthermore, the LPP3 expression level was ~1.5 fold higher in confluent HUVECs than in nonconfluent HUVECs, and was dramatically increased in forskolin-treated HUVECs (Fig. 4B). The forskolin-induced upregulation of LPP3 was prominently suppressed by a protein kinase A (PKA) inhibitor (H89) but not by a cAMP-dependent...
exchange protein (EPAC) inhibitor (ESI-09) (Fig. S3). We also found that in forskolin-treated HUVECs, LPP3 was predominantly localized to cell–cell contact sites (Fig. 4C). Co-staining with anti-VE-cadherin antibody confirmed the result (Fig. S4A). Such subcellular 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 neighbouring HUVECs were absent (non-contact site, Fig. 4D; Fig. S4B). Interestingly, leader cells, that is, cells located at the edge of the HUVEC sheets, showed strong actin stress fibre formation after LPA stimulation (Fig. 4E), indicating that these cells dramatically responded to LPA. These results indicate that the site of LPA action is restricted to the non-contact site of HUVECs. This effect was negated by treating the cells with siRNAs for LPA6, Gα13 or RhoA, and upon Y27632 treatment (Fig. 4E). The localized LPA6 signalling to the non-contact sites appeared to be specific for LPA, because it was not induced by the protease-activated receptor (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 fibres in all 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 forskolin-treated HUVECs was suppressed by the Notch inhibitor DAPT (Fig. 4G). Furthermore, the expression of LPP3 was upregulated when HUVECs were cultured on a plate coated with Dll4 (Fig. 4H) but not jagged-1 (data not shown). We also found that on a Dll4-coated plate LPA-induced RhoA activation was significantly attenuated (Fig. S2D). Thus, in endothelial cells, LPA signalling appears to be regulated by Notch signalling through modulation of LPP3 expression.
Recently, LPA has been found to enhance vascular permeability upon inflammation (Panchatcharam et al., 2014). Thus, LPA seems to be a vasculature-destabilizing factor in pathological conditions. In this study, we found that LPP3 enhanced the cell–cell interactions by downregulating LPA6 signalling. Previous reports have also indicated that LPP3 enhances cell–cell interaction through its RGD domain (or RGE domain in mice LPP3) and integrins (Humtsoe et al., 2003, 2005; Wary and Humtsoe, 2005). Thus, LPP3 appears to stabilize blood vessels through several mechanisms.

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

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**Fig. 4.** See next page for legend.
Fig. 4. LPP3 determines the subcellular localization of LPA signalling in endothelial cells. (A) Sub-confluent, confluent and forskolin (FK)-pretreated (for 30 min) HUVECs were stimulated with LPA at the indicated concentration for 30 min. F-actin was stained with Alexa-Fluor-594–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 forskolin-treated (2 h) HUVECs was determined by quantitative (q)RT-PCR. Error bars indicate s.d. (n=4). (C) Subcellular localization of LPP3. HUVECs were transfected with cDNA for Flag-tagged LPP3. (C) HUVECs (sub-confluent, confluent or forskolin-treated) were stained with anti-Flag antibody (green) and Alexa-Fluor-594–phalloidin (red). (D) Forskolin-treated HUVECs were scratched using a tip, and after 30 min cells were stained as in C, sc, scratched area. (E,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 Alexa-Fluor-594–phalloidin. HUVECs were also pre-treated with siRNAs for LPA6, Gα13 or RhôA, or with 5 µM Y27632. (F) The scratched HUVECs were also stimulated with OMPT (a phosphatase-resistant LPA analog, 1 µM) or thrombin (1 U/ml). (G,H) Notch-induced expression of LPP3 in HUVECs. (G) Forskolin-induced LPP3 expression in HUVECs was suppressed by Notch inhibitor, DAPT (10 µM for 2 h). (H) The Notch ligand, recombinant (r)Dll4 (1 µg/ml), enhanced the expression of LPP3 in HUVECs, which were suppressed by DAPT. Another Notch-target gene, HEY1, showed a similar expression pattern. At 24 h after rDll4 stimulation, expression of LPP3 and HEY1 was determined by qRT-PCR. Error bars indicate s.d. (n=4). *P<0.05, ***P<0.001 (one-way ANOVA with Bonferroni’s post-test analyses in B and H, and Student’s t-test in D). Scale bars: 20 µm.

always active and induces massive vascular permeability. However, as we have shown in this study, this is not the case because LPP3 is highly expressed in endothelial cells, which protects 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. have reported that a diryosine motif present in the second cytoplasmic portion of LPP3 serves as a basolateral targeting signal in MDCK cells (Jia et al., 2003). It is likely that 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 lipid phosphate signalling in specific domains of the cells.

We also found that LPP3 expression is regulated transcriptionally by Notch signalling (Fig. 4G,H). Notch signalling is known to contribute to the stability of newly formed vessels by inducing LPP3 and downregulating LPA signalling.

MATERIALS AND METHODS
Reagents and antibodies
1-Oleoyl LPA was purchased from Avanti Polar Lipids Inc. Stealth siRNAs against human LPP3, LPA6, Gα13 and RhoA were purchased from Invitrogen. DAPT, thrombin, forskolin, Y27632 and anti-Flag M2 antibody were purchased from Sigma. Alexa-Fluor-594–phalloidin was purchased from Molecular Probes. Recombinant human DI4 was from R&D Systems.

Cell culture and transfection
HUVECs were purchased from Kurabo and were maintained in HumaMedia-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 the NEON electroporation system (Invitrogen) and Lipofectamine 2000 (Invitrogen), respectively.

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

Evaluation of G-protein-coupled receptor activation
Activation of LPAα receptor and PAF receptor were evaluated by a TGF-α shedding assay as described previously (Inoue et al., 2012) using cDNAs for human LPAα 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 a membrane fraction from cells that had been transiently transfected with LPP3 plasmid constructs, were incubated in M199 containing 1% BSA and 10 µM LPA. The amount of LPA remaining in the medium was determined by liquid chromatography tandem mass spectrometry (LC-MS/MS) as previously described (Inoue et al., 2011).

Immunofluorescence staining
For immunofluorescence cell staining, HUVECs were cultured on collagen-I-coated glass-bottomed dishes, fixed with 4% PFA in PBS, permeabilized in 0.1% Triton X-100 in PBS for 10 min, incubated with 3% BSA in PBS containing 10% goat serum, and 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 fibre.

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 plasmds 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: LPP1, 5'-CTGGAGCGGA-TGTTGACTG-3' and 5'-GGTTGTTTCATGCAGATTG-3'; LPP2, 5'-CATCCGTCCAGATACCATCACC-3' and 5'-GGTTGAAGCGCAGGAGAATAG-3'; and HEY1, 5'-GCAGAGGAGCAGGACTGCTA-3' and 5'-CCAAAAAAAACTCCGATATCAT-3'. Note that primer sets for LPA1–LPA6 and GAPDH were the same as previously described (Inoue et al., 2011).

Evaluation of activation of RhoA and Rac1
Activation of RhoA and Rac1 was examined using a G-LISA system (Cytoskeleton) according to the manufacturer’s instruction. In this system, the 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). P<0.05 was considered to be significant.

Competing interests
The authors declare no competing or financial interests.

Author contributions
H.Y. designed the research plans, performed most of the experiments and wrote the draft of manuscript. K.I., R.K. and A.I. performed LC-MS/MS experiments, experiments involving HUVEC cells and the TGF-α shedding assay, respectively. J.A. designed most of the research plans and wrote the manuscript.

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Supplementary information
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