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Role of lipid phosphate phosphatase 3 (LPP3) in human aortic endothelial cell function.

1Z. Touat-Hamici, 1H. Weidmann, 2Y. Blum, 1C. Proust, 1H. Durand, 1F. Iannacci, 1V. Codoni
4,5P. Gaignard, 4,5P Thérond, 2M. Civelek, 3A.S. Karabina, 2A.J. Lusis, 1F. Cambien, 1*E. Ninio

1Sorbonne Universités, UPMC, INSERM UMR_S 1166, ICAN, Genomics and Pathophysiology of Cardiovascular Diseases Team, F-75013, Paris, France
2University of California, Los Angeles, David Geffen School of Medicine, Los Angeles, CA 90095-1679, USA
3Sorbonne Universités, UPMC, INSERM UMR_S 933, Hôpital Armand-Trousseau, Paris, France
4APHP, Hôpital de Bicêtre, Service de Biochimie, 94275 Le Kremlin Bicêtre, France
5Université Paris Sud, UR Lip(Sys)², UFR de Pharmacie, Châtenay-Malabry, 92296, France

Correspondence:
Z. Touat-Hamici (zahia.touat@upmc.fr) and E. Ninio (ewa.ninio@upmc.fr)
Sorbonne Universités, UPMC, INSERM UMR_S 1166, ICAN, Genomics and Pathophysiology of Cardiovascular Diseases Team, 91 Bd de l'Hôpital, F-75013, Paris, France

Abstract

Aims:
Lipid phosphate phosphatase 3 (LPP3; PPAP2B) is a transmembrane protein dephosphorylating and thereby terminating signalling of lipid substrates including lysophosphatidic acid (LPA) and sphingosine-1-phosphate (S1P). Human LPP3 possesses a cell adhesion motif that allows interaction with integrins. A polymorphism (rs17114036) in PPAP2B is associated with coronary artery disease, which prompted us to investigate the possible role of LPP3 in human endothelial dysfunction, a condition promoting atherosclerosis.

Methods and results:
To study the role of LPP3 in endothelial cells we used human primary aortic endothelial cells (HAECs) in which LPP3 was silenced or overexpressed using either wild type or mutated cDNA constructs. LPP3 silencing in HAECs enhanced secretion of inflammatory cytokines, leukocyte adhesion, cell survival and migration and impaired angiogenesis, whereas wild-type LPP3 overexpression reversed these effects and induced apoptosis. We also demonstrated that LPP3 expression was negatively correlated with VEGF expression. Mutations in either the catalytic or the RGD domains impaired endothelial cell function and pharmacological inhibition of S1P or LPA restored it. LPA was not secreted in HAECs under silencing or overexpressing
LPP3. However the intra- and extracellular levels of S1P tended to be correlated with LPP3 expression, indicating that S1P is probably degraded by LPP3.

Conclusions:
We demonstrated that LPP3 is a negative regulator of inflammatory cytokines, leukocyte adhesion, cell survival and migration in HAECs, suggesting a protective role of LPP3 against endothelial dysfunction in humans. Both the catalytic and the RGD functional domains were involved and S1P, but not LPA, might be the endogenous substrate of LPP3.

Abbreviations
LPP3, Lipid Phosphate Phosphatase 3; hLPP3, human LPP3, HAECs, Human Aortic Endothelial Cells; PPAP2B, Type 2 Phosphatidic Acid Phosphatase β; LPA, Lysophosphatidic Acid; S1P, Sphingosine-1-Phosphate; C1P, Ceramide-1-Phosphate; PA, Phosphatidic Acid; VEGF, Vascular Endothelial Growth Factor; RGD, Arginine-Glycine-Aspartate; RAD, Arginine-Alanine-Aspartate; PLA2G4A, Phospholipase A2, Group IVA; PTGS2, Prostaglandin-Endoperoxide Synthase 2 (Cyclooxygenase 2); IL1b, IL6 And IL8, Interleukin-1β, -6, -8; MCP1, Monocyte Chemoattractant Protein 1 (CCL2 Chemokine (C-C Motif) Ligand 2); SELE, Selectin E; VCAM1, Vascular Cell Adhesion Molecule 1; ICAM1, Intercellular Adhesion Molecule 1; DMSO, Dimethylsulfoxide; KDR, Kinase Insert Domain Receptor; LEF1, Lymphoid Enhancer-Binding Factor 1; MAPK, Mitogen Activated Protein Kinase; EDG1, Endothelial Differentiation Gene 1, SPR1, Sphingosine-1-Phosphate Receptor 1, ANGPT2; Angiopoietin 2.

1. Introduction
Endothelial cells have a key role in vascular homeostasis as under quiescent conditions they maintain an anti-inflammatory, anti-thrombotic and anti-proliferative environment. However, several risk factors activate endothelial cells inducing what is known as endothelial dysfunction. This leads to increased vascular permeability, leukocyte adhesion and inflammation, all of which underlie atherosclerosis, the major cause of cardiovascular disease.

LPP3 is an integral membrane protein that degrades various lipid phosphate mediators. LPP3, is encoded by the PPAP2B gene which is located in a locus associated with the risk of coronary artery disease (CAD) in recent Genome-Wide Association Studies (GWAS). The major allele A (f=0.91) of the rs17114036 variant located in the 5th intron of PPAP2B was associated with an increased risk of CAD (odds ratio, 1.17; \(P=3.81 \times 10^{-19}\)). The major allele of rs6588635, a proxy SNP of rs17114036, was associated with lower PPAP2B mRNA levels in human aortic endothelial cells (HAECs) a finding later confirmed directly using the
rs17114036 probe in microarray expression studies using the same HAECs by co-authors of this study. Interestingly, it was shown recently that in macrophages, the CAD risk variant is rs72664324 rather than rs17114036 (both in high LD) which operates to alter the response of macrophages to oxLDL by altering binding of C/EBP beta to an enhancer site regulating PPAP2B expression. Finally, an upregulation of Ppap2b expression in aortic endothelial cells and in atherosclerotic lesions isolated from Apoe−/− mice was reported.

The in vitro and in vivo studies performed by Panchatcharam et al. in mice are consistent with a role of LPP3 in atherosclerosis. First, targeted inactivation of Ppap2b in smooth muscle cells (SMC) enhanced intimal hyperplasia and promoted SMC dedifferentiation. After vascular injury LPP3 expression limited cellular response to lysophosphatidic acid (LPA) and impaired SMC phenotype modulation. Second, targeted Ppap2b inactivation in endothelial cells enhanced inflammatory responses and vascular permeability.

LPP3 belongs to the phosphatidic acid-phosphatase (PAP) enzymes family. In humans, three PAP genes are known: PPAP2A, PPAP2C and PPAP2B, which encode LPP1, LPP2 and LPP3, respectively. LPP3 is an integral membrane protein, with six transmembrane domains that is found in both plasma and intracellular membranes. The third hydrophilic loop contains the catalytic domain that localizes to the extracellular side of the plasma membrane or luminal side of internal membranes. Ppap2b inactivation in mice results in early embryonic lethality due to impaired vasculogenesis, indicating that LPP3 is essential for normal vascular development [for review]. LPPs dephosphorylate and thereby terminate the signalling of a broad range of lipid substrates including phosphatidic acid (PA), lysophosphatidic acid (LPA), ceramide-1-phosphate (C1P), sphingosine-1-phosphate (S1P), and diacylglycerol pyrophosphate. LPA and S1P are implicated in several signalling pathways involved in atherosclerosis. S1P contributes to vascular development and endothelial barrier functions through the regulation of cellular proliferation, differentiation, migration and angiogenesis and acts as an intra- and extra-cellular mediator. LPA is involved in cell migration, proliferation and differentiation, contributing to neovascularization and to the induction and the release of proteases leading to cell invasiveness. Human LPP3 possesses a cell adhesion motif of arginine-glycine-aspartate (RGD) that allows interactions with αvβ3 and α5β1 integrins, promoting cell adhesion and intracellular signalling.

Our study was focused on human primary endothelial cells, as they constitute the first barrier of the vessel wall and their dysfunction is crucial in atherosclerosis. The rs17114036 in the last intron of PPAP2B constitutes an expression quantitative trait locus (eQTL), associated with a decreased PPAP2B expression in homozygotes bearing the major A allele. On the other
hand, when using the public eQTL database (GTEx portal), we found that the rs17114036 in the PPAP2B locus was not associated with the expression of PPAP2B in various other tissues, thus pointing out the specific role of LPP3 in human primary endothelial cells.

The absence of functional studies on the role of LPP3 in primary HAECs, with the exception of a paper published during the revision of our manuscript, prompted us to determine the downstream mechanisms implicating LPP3 in endothelial dysfunction by combining LPP3 knockdown and overexpression, as well as targeted mutations of the RGD motif. We found that in HAECs, PPAP2B/LPP3 downregulates the expression and production of the inflammatory cytokines IL6, IL8 and the expression of the chemokine MCP1, as well as diminishing cell viability through induction of apoptosis and S1P-mediated migration. LPP3 also prevents leukocyte adhesion to HAEC monolayers. Together, these data strongly support a protective role of LPP3 against endothelial dysfunction and atherosclerosis in humans.

2. Materials and Methods (detailed Materials and Methods are on line in the Supplementary Materials)

2.1 Cell culture
For the functional studies, human aortic endothelial cells (HAECs) were purchased (PromoCell, Germany; all males of the rs17114036 AA genotype) and used at passages 3 to 7. To study the correlations according to the genotype of HAECs between LPP3 expression and those of selected genes, we used the database established with HAECs isolated from the aortic explants of 147 heart transplant donors of anonymous origin (108 males as deduced from DNA analysis) through the UCLA transplant program approved by UCLA Institutional Review Board.

2.2 ELISA assays for cytokines and MCP1
ELISA assays were performed in cell supernatants to detect soluble forms of IL1β, IL6 and IL8 and MCP1 using the respective human DuoSet ELISA kits (R&D systems).

2.3 Human peripheral blood mononuclear cell adhesion assay
Human peripheral blood mononuclear cells (hPBMC) were obtained from the Etablissement Français du Sang, Rungis, France and were isolated from 6 donors of Buffy coats using a Ficoll density gradient. hPBMC were labelled with calcein-AM, then incubated with HAEC monolayers and the number of fluorescent adherent hPBMC was assessed with an inverted
epi-fluorescence microscope NIKON ECLIPSE Ti/Intensilight C-HGFIIE using an FITC filter and 4 x 10 magnification. Adherent hPBMC were quantitated in each well by automated counting using ImageJ software (NIH). In controls (siCtrl, Ctrl) 1000 to 1800 cells/well were counted.

2.4 Cell viability assay
Cell viability was performed using the tetrazolium salt WST-1 assay. Cells were treated with VEGF-165 (VEGF) or LPP3 substrates during the last 24h prior to addition of WST-1 solution for 3h, the absorbance was measured at 450 nm.

2.5 Cell proliferation assay
Cell proliferation was assayed using the addition of 5-bromo-2'-deoxyuridine (BrdU) to the plates for 6h and the absorbance was quantified at 450 nm.

2.6 Cell migration
Transfected HAECs were cultured to reach confluence. Cells were treated with VEGF or LPP3 substrates during the last 24h. Linear scratches were performed in the cell monolayers using a 1000 µl pipette tip and then the cells were incubated for further 24h. Images of the gap were obtained at 0 and 16h with fully motorized inverted microscope (Nikon Eclipse TiS) using bright field at 4 x 10 magnification. The wounded area was analysed using ImageJ software (NIH) by quantification of the surface of wounded area at 16h as compared to 0h.

2.7 Angiogenesis tube formation in Matrigel
Angiogenesis assays were performed using 15-well μ-angiogenesis slides (Ibidi, Germany). The slides were coated with Matrigel and HAECs were seeded at 10,000 cells/well and incubated for 24h. Images of newly structured tubes were captured and tube formation was quantified by counting the number of tubular and branching point structures.

2.8 Genome-Wide Expression analysis and pre-processing of expression data
Transcriptomic analysis of total RNA was performed using the Illumina HT-12 v4 BeadChip. Briefly, RNA was extracted from HAECs and 250 ng of total RNA was reverse transcribed, amplified and biotinylated using the Illumina TotalPrep RNA Amplification Kit. Each biotinylated cRNA was hybridized to a single BeadChip and scanned using the Illumina Hiscan array.
2.9 Immunohistochemistry (IHC) staining

IHC was performed using standard protocols. Briefly, IHC was performed on 5-μm sections of Tissue MicroArray (TMA) blocks. Deparaffinized and rehydrated sections were incubated with primary antibodies, and then with a kit UltraVision LP Detection System (Thermo Fisher Scientific, UK). Slides were counterstained with aqueous hematoxylin and mounted with Immunomount (Shandon, Cergy-Pontoise, France).

2.10 Statistical analysis

All data points were obtained from the mean of the experimental points from 2 or 3 replicates. Comparisons between 2 groups were performed using the Wilcoxon paired test. Comparisons between more than 2 groups were performed using the Kruskall-Wallis test followed by Dunn post-hoc test (XLStat 2013, Addinsoft, New York, USA). A significance threshold at p<0.05 was used for all tests.

3. Results

3.1 LPP3 is expressed in human vascular wall

Immunostaining of human atherosclerotic aortas (tissues microarrays) showed that LPP3 is expressed in endothelial cells, intimal macrophages and in the smooth muscle cells of the media (Figure S1). The LPP3 transcript was also present in human atherosclerotic carotid tissue as assessed by RT-qPCR (data not shown).

3.2 Transcriptional analysis of HAECs silenced for LPP3

To investigate the function of human LPP3 we proceeded by knockdown or overexpression strategies in HAECs from different donors. Two LPP3 siRNAs (siLPP3(1) and siLPP3(2)) or LPP3 over-expression plasmids were transiently transfected into HAECs. mRNA knockdown or overexpression was confirmed by RT-qPCR, and its impact on LPP3 protein level was evaluated by immunoblotting. siLPP3(1), suppressed the expression of LPP3 mRNA by 80% to 90% (Figure 1A) and the corresponding protein level strongly diminished compared to the control siRNA (Figure 1B). siLPP3(2) was also effective as it suppressed the expression of LPP3 mRNA by about 80% (Figure S2) compared to the control siRNA, finally we preferentially used siLPP3(1) for the functional analysis. When LPP3 was overexpressed in
HAECs, a mean 2-fold increase in mRNA (Figure 1A) and a substantial increase in protein levels was observed (Figure 1B).

We explored the consequences of siRNA-mediated LPP3 silencing in HAECs from 5 donors by global gene expression profiling using the HumanHT-12 v4 Expression BeadChip which targets more than 47,000 probes. Using an adjusted p-value threshold of 0.05, we found 1941 genes that were differentially expressed between siLPP3 transfected HAECs and the control siRNA (scrambled) (Table S1). Among the genes differentially expressed, 342 had a fold change higher than 1.5. Statistical enrichment of specific biological processes and molecular functions annotated in Gene Ontology (GO http://www.geneontology.org/) was assessed by Gene Set Enrichment Analysis (GSEA) using the Genetrail software. Significant pathways were identified (FDR-corrected p-values <0.05). Among them, cell cycle, cell adhesion, cell migration, angiogenesis, blood vessel development, leukocyte activation and differentiation and inflammatory response were found (Table S2). All these signalling pathways are related to either endothelial cell dysfunction or neovessel formation.

3.3 LPP3 downregulates VEGF expression

VEGF and several proinflammatory cytokines induce LPP3 expression in mice25. We showed that both LPP3 mRNA and protein levels (n=2) were upregulated, in HAECs after 24h treatment with VEGF165 (Figure 1A and 1B). Next, we evaluated the expression of VEGF mRNA in HAECs silenced for LPP3 or overexpressing it. We observed a 2-fold increase in VEGF expression in cells silenced for LPP3 and a small significant decrease of VEGF expression in LPP3 overexpressing cells (Figure 2). In addition LPP3 knockdown increased the expression level of the VEGFA receptor (KDR) 1.3-fold in our microarray data (adj. P.value=0.0005) (Table S1; line 562). These observations indicate a possible retrocontrol mechanism between VEGF and LPP3 in HAECs.

3.4 siLPP3 upregulates the expression of pro-inflammatory cytokines

To determine the effects of LPP3 levels on inflammatory cytokines in HAECs, we assessed the mRNA levels of major proinflammatory mediators: IL6, IL8, IL1β and MCP1 (or CCL2) following knockdown of LPP3. We found that siLPP3 significantly increased the mRNA levels of these mediators, reaching nearly 4-fold for IL6 and IL8 and 2-fold for MCP1, as compared to control siRNA (Figure 3A). IL1β mRNA level was upregulated up to 4-fold in LPP3 silenced HAECs (Figure S3A). LPP3 overexpression, on the other hand, did not dramatically change the IL6, IL8, and MCP1 mRNA expression (Figure S4A), except for IL1β a decrease of 20% was observed (Figure S3B); suggesting that a basal level of LPP3 mRNA is sufficient to
maintain a low level of inflammatory molecules. Next, in order to assess whether the upregulation of cytokine- and MCP1-specific mRNAs in LPP3 silenced cells lead to an increased secretion of these compounds, we measured their presence in culture media using ELISA. As shown in Figures 3B, the level of IL8 was increased in the media of LPP3 silenced HAECs as compared to control siRNA and the level of IL6 (p=0.055) and MCP1 were only slightly increased without reaching significance. In agreement with no change in mRNA levels observed in HAECs overexpressing LPP3, no change in the cytokine levels was observed in these cells (Figure S4B). IL1β level was not affected by siLPP3 (Figure S3C) which may suggest that LPP3 silencing is independent of inflammasome activation, or that an additional signal could be required for the IL1β secretion.

Among the genes affected by the LPP3 silencing, PTGS2 exhibited among the highest fold change of expression (Table S1). PTGS2 (or COX2) in concert with PLA2G4A are implicated in atherosclerosis and cardiovascular disease. We tested the impact of LPP3 on PLA2G4A and PTGS2 expression in HAECs, and observed that siRNA-mediated LPP3 silencing increased mRNA levels of both genes (Figure S5A), on the other hand, LPP3 overexpression decreased PTGS2 level, but not that of PLA2G4A (Figure S5B).

We tested whether or not the expression of the above genes could be affected by the rs17114036 in PPAP2B, in a collection of HAECs from 147 donors (119 homozygotes for the major allele A and 28 heterozygotes AG). We compared the correlations of LPP3 expression with these genes, according to their genotype. Indeed, we found a strong correlation between LPP3 expression and IL6, IL8, PLA2G4A, PTGS2, VCAM1 and SELE only in HAECs bearing the protective G allele. The AA homozygotes showed no correlation between PPAP2B and these genes. Correlation values of PPAP2B with selected genes are represented in (Figure 4), although in statistical analysis, only the correlation between PPAP2B and IL8 and PTGS2 remained significant. These data support the hypothesis of the protective role of LPP3 in inflammatory response, although such significant correlations do not necessarily imply a causal relationship.

3.5 Role of LPP3 in leukocyte adhesion

As a consequence of vascular inflammation, leukocytes are recruited to endothelial surface and transmigrate into the intimal space of the arteries. Induction of adhesion molecules and chemokine expression in response to inflammatory stimuli plays a critical role in leukocyte adhesion to the endothelium\(^1\). To investigate whether or not LPP3 expression impacts leukocyte adhesion, we first evaluated adhesion molecules and chemokine expression in HAECs. Using RT-qPCR, we measured the expression of 3 major adhesion molecules, SELE,
ICAM1 and VCAM1 and showed that LPP3 silencing as compared with the control siRNA treatment induced a 11-, 3-, and 1.5-fold increase of their respective mRNA levels, (Figure 5A) accompanied by the increase in their protein levels (Figure 5C). LPP3 overexpression significantly decreased ICAM1 mRNA as compared to the control plasmid, whereas VCAM1 and SELE mRNAs were not affected by LPP3 overexpression (Figure 5B).

In western blots SELE and ICAM1 proteins were decreased whereas VCAM1 protein level remained unchanged compared to LPP3 silenced cells (Figure 5C). We further investigated whether LPP3 expression affects the recruitment of leukocytes, after transfection of HAECs with either siLPP3 or LPP3 plasmids. HAECs were incubated for 1h with fluorescent calcein AM-labelled human blood leukocytes, and as shown in Figure 5D and E, leukocyte attachment was markedly enhanced when the cells were transfected with siLPP3 as compared to control siRNA. Conversely, overexpression of LPP3 significantly decreased leukocyte adhesion to the HAECs monolayer, as compared to control plasmid (Figure 5D and E). These observations are concordant with the increased protein expression of adhesion molecules in siLPP3 silenced cells and with the decreased expression in LPP3 overexpressing cells (Figure 5B).

3.6 LPP3 downregulates cell survival and proliferation

In order to assess the role of LPP3 in survival and proliferation of HAECs, we performed the mitochondrial activity WST1 assay to determine cell viability and a BrDU incorporation assay to measure cell proliferation rate. LPP3 silencing significantly increased cell viability by 30% (Figure 6A). VEGF treatment only slightly decreased cell survival in control cells (p=0.072) but did not modify it in siLPP3 silenced cells (Figure 6A) suggesting that LPP3 itself is crucial to control cell viability. Interestingly, neither LPP3 silencing nor VEGF treatment modified HAECs' proliferation rate as compared to control; however, when siLPP3 HAECs were stimulated with VEGF we observed a small increase of cell proliferation as compared to controls stimulated with VEGF (Figure S6A). Overexpression of LPP3 decreased both cell survival (-30%; Figure 6B) and proliferation rate measured by BRDU incorporation (-40%; Figure S6B) as compared to the control plasmid.

To investigate which LPP3 substrate is involved in the survival of HAECs, we performed the WST1 assay on HAECs (n=3) under silencing or overexpression of LPP3. siLPP3 transfected cells were treated with 1µM of either PF8380 (an inhibitor of LPA synthesis) or FTY720 (a S1P receptor inhibitor), while LPP3 overexpressing cells were treated with LPP3 substrates (1µM) either (2S)-OMPT (LPA mimetic resistant to dephosphorylation) or S1P (S1P receptor
agonist). siLPP3 increased WST1 labelling but treatment of the silenced cells with either PF8380 or FTY720 prevented the siLPP3-induced increase of cell survival (Figure 6C). Conversely, when HAECs were transfected with an LPP3 plasmid, neither (2S)-OMPT nor S1P compensated the decrease of survival induced by LPP3 overexpression (data not shown).

Next, HAECs were transfected with plasmids carrying PPAP2B cDNA mutations obtained by site-directed mutagenesis in 2 major functional domains. Two mutations H249P or H251P were introduced in the catalytic domain, and one mutation was introduced into the ligand domain by transforming RGD to RAD. Both H249P and H251P as the RGD – to - RAD mutation reduced cell survival to some extent as compared to the wild-type (Figure 6D). however, only RGD-to-RAD mutation induced a small decrease of cell proliferation rate as measured by BrDU assay (Figure S6C). These results indicate that both the catalytic and RGD domains are probably involved in the regulation of cell survival.

We conclude that LPP3 silencing substantially increases the viability of HAECs and supplementation with the specific drugs that impede LPA or S1P signalling results in an inhibition of cell viability. In contrast, overexpression of LPP3 significantly decreased cell survival and this effect could not be compensated by adding (2S)-OMPT (LPA mimetic) or S1P, suggesting that the RGD motif of LPP3 may play a role in the regulation of cell viability in HAECs.

In order to examine whether or not LPP3 level impacts apoptosis we performed several assays. In the first assay, annexin V/7-AAD binding was performed in LPP3 overexpressing cells using 2 different plasmid concentrations and showed that there was an increased fraction of both annexin V+/7-AAD− expressing HAECs showing early apoptosis (data not shown) and annexin V+/7-AAD+ expressing HAECs showing late apoptosis (Figure S7A and B). However, LPP3 knockdown only slightly decreased annexin V/7-AAD labeling. In the second assay, LPP3 knockdown induced a marginal (p=0.054) decrease of caspase-3/-7 activity, but when LPP3 was overexpressed the caspase-3/-7 activity increased approximately 2-fold as compared to the control plasmid (Figure 6E).

Finally, to further understand the mechanism underlying the LPP3 involvement in cell viability, we assessed whether or not LPP3 levels affect ERK1/2 phosphorylation. LPP3 knockdown
increased ERK1/2 phosphorylation as compared to control siRNA, whereas, LPP3 overexpression did not affect ERK1/2 phosphorylation (Figure S7C). Therefore, LPP3 regulates cell viability through the induction of apoptosis via the activation of caspase-3 and -7 and ERK1/2 phosphorylation.

3.7 LPP3 downregulates cell migration

Migration of endothelial cells is a key initiating event in the formation of new blood vessels. To investigate whether LPP3 is involved in HAECs migration, we performed wound-healing assays. To compare the differences in migratory behaviour, the surface of the wounded area was determined after 16 h of various treatments. Cells were treated or not with VEGF which is an activator of endothelial cell migration. When HAECs were silenced for LPP3 the migration distance in the dish increased 1.6-fold as compared to the siRNA control (Figure 7A). HAECs silenced for LPP3 and treated by VEGF showed slightly increased migration as compared siCtrl (Figure S8). LPP3 overexpression decreased the wounded area up to 50% as compared to the control plasmid (Figure 7A). To determine if the catalytic or the ligand domain of LPP3 is involved in cell migration, we used the same approach as described above to study cell survival. When LPP3 was silenced, treatment of HAECs with PF8380 did not modify the migration of HAECs, indicating that inhibition of LPA is not sufficient to restore the LPP3 silencing effect (Figure 7B). When siLPP3 cells were treated with FTY720 the wounded area was reduced by 30%, as compared to siLPP3, pointing to an important role for S1P in cell migration in this model (Figure 7A). When (2S)–OMPT was added to LPP3 over-expressing cells, no change in the migration was observed, as compared to the control plasmid (Figure 7C). This observation is in agreement with our results obtained with PF8380, suggesting that LPA production is not required for LPP3-regulated endothelial cell migration. In contrast, addition of S1P to HAECs overexpressing LPP3 tended to increase (p = 0.075) migration 3.6-fold as compared to the control plasmid (Figure 7C). Together, these results suggest that LPP3 may downregulate HAEC migration through S1P degradation.

3.8 LPP3 regulates angiogenesis

To investigate whether or not LPP3 participates in the formation of tubes, a hallmark of angiogenesis, we performed a Matrigel tube formation assay in HAECs under- or overexpressing LPP3. At early times (3-6h), LPP3 silenced cells started to organize into tube structures more effectively than the siRNA control cells and this process was delayed when
LPP3 was overexpressed (not shown), consistent with our results obtained both on cell viability (Figure 6) and migration (Figure 7).

After 24 hours LPP3 silencing strongly decreased tube formation (~63%) as compared to the control siRNA (Figure 8A), suggesting that the angiogenic structures formed at 6h (data not shown) became disorganized. On the contrary, overexpression of LPP3 increased the formation of tubes by 50% as compared to the control plasmid (Figure 8B). Branching point structure number was regulated in the same proportion as the number of tubes (data not shown). In order to determine which LPP3 substrate is involved in the regulation of angiogenesis, we treated the LPP3 silenced cells with the PF8380 and showed that inhibiting LPA supply did not affect the number of tube structures as compared to the control (DMSO), whereas FTY720 treatment nearly restored the number of tubes in LPP3 siRNA transfected cells towards the level of control siRNA (Figure 8C). Treatment of LPP3 overexpressing cells with either S1P or (2S)-OMPT decreased LPP3-induced angiogenesis (Figure 8D). Next, we investigated which of the LPP3 functions is responsible for this regulation using either catalytic or RAD LPP3 mutants. We observed that both catalytic mutants (H249P or H251P) as well as RAD mutant decreased the number of tubes as compared to the plasmid carrying the wild-type LPP3 (Figure 8E). Our results suggest that both RGD ligand and the enzymatic domains of LPP3 are involved in LPP3-regulated angiogenesis. Among LPP3 substrates, S1P is likely the one implicated in LPP3-mediated regulation of angiogenesis.

3.9 LPP3 degrades both intra- and extracellular S1P

We showed that S1P, but not LPA, was the major substrate of LPP3 involved in cell migration and angiogenesis. To determine whether S1P is degraded by LPP3 under our experimental conditions, and to assess if LPP3 degrades preferentially extra- or intracellular pools of S1P, we measured its levels using reverse phase HPLC, in both culture media and cellular extracts from LPP3-silenced or overexpressing HAECs. The S1P content in intact HAECs was low, ranging from 3.1 to 4.2 pmol/10^6 cells (intracellular) and 1.1nM (extracellular). However, in LPP3 silenced HAECs (n=3) both intra- (Figure S9A) and extracellular (Figure S9B) S1P levels increased, whereas in LPP3 overexpressing HAECs the intracellular levels of S1P decreased (Figure S9A) and became undetectable in supernatants (Figure S9B). As the number of donors was too low to apply the statistical analysis, these results must be confirmed in the future on a larger number of donors. In parallel, we measured the level of LPA in the same HAEC supernatants by mass spectrometry, using 2 different protocols and appropriate internal standards, and in neither of the experimental conditions (+/- VEGF), could we detect LPA.
Thus in our experiments, LPP3 might control both intra- and extracellular pools of S1P in HAECs.

4. Discussion

The novelty of our study is the use of a new integrated approach, combining transcriptomics and functional cellular studies to demonstrate that LPP3 constitutes an important component of human aortic endothelial cell physiology. Indeed, silencing LPP3 in HAECs readily enhanced inflammation, cell survival and migration, and impaired angiogenesis, whereas, LPP3 overexpression had the opposite effects. In various human cells LPP3 expression is induced by growth factors and inflammatory cytokines, including VEGF. In this study, we demonstrated for the first time that modulation of LPP3 expression by either knockdown or overexpression negatively correlated with VEGF expression suggesting a negative feedback control loop between VEGF and LPP3 in HAECs. This is in agreement with former studies showing that VEGF increases LPP3 protein expression in HUVECs. Additionally, LPA, one of the LPP3 substrates, was shown to induce VEGF expression in ovarian cancer cells, however LPP3 expression was not studied in this context.

4.1 Anti-inflammatory role of LPP3 in HAECs

We showed here that LPP3 silencing in HAECs, promoted their pro-inflammatory response by increasing IL1β mRNA and MCP1 expression and both expression and secretion of IL6 and IL8. Basal LPP3 is sufficient to maintain a low level of inflammatory cytokines because LPP3 overexpression did not modify cytokine expression. In addition, we showed that LPP3 silencing significantly increased the expression of adhesion molecules, which correlated with an increased human blood leukocytes adhesion to HAEC monolayers. In the future work we plan to use the specific blocking antibodies directed against adhesion molecules to ascertain that LPP3 knockdown is indeed involved in leukocyte adhesion via these molecules. Interestingly, it was shown previously in HUVECs that a high level of S1P induces the expression of SELE and VCAM1 via NFκB. Moreover, in epithelial cells, LPP3 downregulates the inflammatory response by degrading S1P and LPA and thus inhibiting MAPK and NFκB activation, and the production of IL6, IL8 and IL1β. Of note, several genes involved in the IκB kinase/NF-κB pathway were upregulated in LPP3-silenced HAECs (Table S2), which is in accordance with a LPP3 protective role in inflammation, although in some settings in vivo NFκB readily contributed to the resolution of inflammation. Recently, it was reported that targeted inactivation of Ppap2b in mice increases inflammation and vascular...
permeability induced by LPS mainly due to the accumulation of LPA. Altogether, these results confirm that LPP3 has a key role in inflammation and endothelial adhesiveness for leukocytes.

4.2 Differential co-expression of PPAP2B with proinflammatory genes stratified by PPAP2B genotype in HAECs

In our study, a comparative analysis, in a collection of HAECs from 147 donors (119 AA homozygotes and 28 AG heterozygotes) of the two genotypes of rs17114036 showed a strong correlation of the expression level of PPAP2B with the inflammatory genes (IL6, IL8, PLA2G4A and PTGS2) when the protective minor allele G was present. However in AA homozygotes, there was no correlation. During endothelial dysfunction, there is an increase of VEGF expression concomitant with the increase of these pro-inflammatory mediators and adhesion molecules. Therefore an increased LPP3 expression could be a defensive mechanism tending to reduce the expression of proinflammatory genes. The lack of such correlation in the AA homozygotes might therefore be explained by the fact that the expression of LPP3 is too low in these cells to counteract the inflammatory response.

4.3 LPP3 is involved in HAECs' survival

In the present study, LPP3 overexpression strongly decreased HAECs' survival by inducing apoptosis and decreased their proliferation rate. Several studies have shown that degradation of LPA and S1P by LPP3 alters both activation of ERK1/2 and caspases 3/7, indeed, in the present work we showed that in HAECs overexpressing LPP3, the caspases 3/7 were activated and accompanied by an increased annexin V binding to apoptotic cells, thus pointing to the LPP3 pro-apoptotic potential. In parallel, LPP3 knockdown increased ERK 1/2 phosphorylation favouring cell survival. This is consistent with the previous study showing that S1P inhibits the activation of caspases 3/7-dependent apoptotic pathway downstream of ceramide, a pathway called the "sphingolipid rheostat" that governs the cell fate leading either to survival or to apoptosis.

Here, we demonstrated that the loss of either catalytic or adhesion function of LPP3 decreased to the similar extent the number of viable cells. Furthermore, both LPA supply and S1P receptor inhibitors blocked cell survival induced by LPP3 silencing, consistent with the previous studies showing that LPP3 inhibits survival via degradation of its lipid substrates. However, when LPP3 was overexpressed, neither LPA mimic nor S1P was able to reverse the inhibition of cell survival, indicating that the RGD domain was involved. Although, the precise
mechanism remains to be defined, we suggest that LPP3 may regulate cell survival via both catalytic and RGD domains.

4.4 S1P plays a major role in HAECs' migration and angiogenesis

We showed that LPP3 downregulates cell migration as measured by wound healing assay. Since the S1P receptor inhibitor blocked siLPP3-induced migration and conversely, the addition of the S1P nearly reversed the inhibition of migration mediated by LPP3 overexpression, we conclude that the anti-migratory effect of LPP3 in HAECs depends solely on the degradation of S1P. Therefore the role of S1P in HAECs is consistent with the former studies which demonstrated that S1P stimulates migration through its receptor S1PR1 (previously EDG1) in human umbilical endothelial and smooth muscle cells\textsuperscript{39,40}. Indeed, S1PR1 was upregulated under LPP3 knockdown conditions in our transcriptomic data (Table S1; line 313). In contrast, Panchatcharam et al.\textsuperscript{5} showed that LPP3 is involved in smooth muscle cell migration in mice through degradation of LPA; this discrepancy could be due to different cells and different species.

LPP3 is a key element in cell-cell interactions due to its RGD motif which interacts with integrins\textsuperscript{26}, and it allows initiation of angiogenesis either by bFGF or VEGF\textsuperscript{25}. Our work demonstrated that LPP3 tightly regulates the number of tubes and branching point structures, consistent with its pro-angiogenic role. We showed that both the catalytic and RGD domains were necessary to sustain angiogenesis. When LPP3 was silenced, inhibiting S1P receptor, but not LPA formation, tended to restore the angiogenesis. These results suggest that LPP3 regulates angiogenesis possibly by controlling the availability of S1P.

We showed that although the LPP3 silencing in HAECs increased the expression of the pro-angiogenic factor VEGF, their angiogenic response was strongly decreased, possibly because of the excessive accumulation of S1P and the upregulation of S1PR1 expression, as shown in our transcriptomic data (Table S1). However, the loss of \textit{S1pr1} gene in mice is lethal \textit{in utero} due to a defect in vessel maturation\textsuperscript{41}, indicating that S1P is indispensable for vessel stabilization termination of angiogenesis\textsuperscript{42}. Indeed, there is a gradual expression of S1PR1 from the mature sprouting region to the growing vascular front during blood vessel formation\textsuperscript{43}. In this process S1P activates S1PR1 which in turn inhibits VEGF signalling and suppresses sprouting, promotes junction formation and recruitment of mural cells to prevents excessive sprouting \textsuperscript{43}. Our results on angiogenesis in HAECs support these studies, as LPP3 silencing prevented angiogenesis, because the S1P level was likely too high to initiate sprouting. In contrast, when LPP3 was overexpressed, sprouting was favoured.
4.5 Summary and limitations of our study

To summarize (Figure S10), our transcriptomic and functional studies using primary HAECs from 3 to 13 different donors show that LPP3 downregulates both inflammation and leukocyte adhesion by reducing expression of cytokines and adhesion molecules in these cells. LPP3 also downregulates cell growth by degrading both S1P and LPA. LPP3 inhibits cell migration by controlling the levels of S1P and the expression of its receptor S1PR1. We finally showed that LPP3 may degrade intra- and extracellular S1P in HAECs, the latter result due to the limited number of samples (n=3) should be confirmed in the future on a larger number of HAECs donors. In contrast to S1P, we were unable to detect LPA in the supernatants of HAECs, however we cannot exclude an intracellular role for LPA. Indeed, in HAECs silenced for LPP3 addition of an inhibitor of LPA decreased cell survival to the similar extent as the antagonist of S1P receptors.

The main limitation of our study was the access to a large collection of cultivable HAECs with different genotypes in respect to the leading SNP rs17114036\(^3\) permitting the functional studies which could reveal the difference between those genotypes. In the future, such an approach will shed a light on the transcriptional control of LPP3 and open the way to novel pharmacological interventions to combat endothelial dysfunction.

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Conflict of interest

None

References


Figure 1. Modulation of Lipid phosphate phosphatase 3 (LPP3) expression in HAECs. HAECs were transfected for 48h with either siRNA (siCtrl or siLPP3) or with the expression plasmids (Ctrl or hLPP3); siCtrl transfected cells were treated or not with 100 nM VEGF for additional 24h (A). The level of LPP3 mRNA was determined by RT-qPCR. The results (mean of duplicate measurements) are shown as relative LPP3 mRNA levels as compared to individual respective controls set at 1, each dot within vertical scatter plots represents a single donor (n=8); the mean +/- standard error of mean (SEM) is depicted. *: p<0.05, **: p<0.001.

Lysates from HAECs transfected either with siLPP3 or siCtrl (treated or not with VEGF) and either hLPP3 expression plasmids or Ctrl plasmids were analyzed by immunoblotting using a rabbit polyclonal anti-LPP3 antibody and re-probed with anti-tubulin antibody to ensure equal loading and transfer. Immunoblot from one representative experiment out of 2 is shown (B).

Figure 2. LPP3 downregulates VEGF expression in HAECs. HAECs were transfected with either siRNA (siCtrl or siLPP3) or with hLPP3 expression plasmids (Ctrl or LPP3) for 48 h. The level of VEGF mRNA was determined by RT-qPCR. The results (mean of duplicate measurements) are shown as relative VEGF mRNA levels as compared to individual respective controls set at 1, each dot within vertical scatter plots represents a single donor (n=8); the mean +/- standard error of mean (SEM) is depicted. *: p<0.05, **: p<0.0001.

Figure 3. siLPP3 upregulates the expression of pro-inflammatory mediators in HAECs. HAECs were transfected with siRNA (siCtrl or siLPP3) for 48 h. mRNA relative levels of IL6, IL8 and MCP1 were determined by RT-qPCR (A). The results (mean of duplicates) are shown as relative mRNA levels as compared to individual respective controls set at 1, each dot within vertical scatter plots represents a single donor (n=8); the mean +/- SEM is depicted. The IL6, IL8 and MCP1 concentrations were determined in supernatants of HAECs cultured for 24 hours in serum-free media using ELISA (B). The results (mean of triplicates) are shown, each dot within vertical scatter plots represents a single donor (n=5); the mean +/- SEM is depicted. *: p<0.05,**: p<0.001, ***: p<0.0001. In panel B we also give the p-value of 0.055 for IL6 to show the trend.

Figure 4. Correlation between PPAP2B/LPP3 expression and other genes for the AA and AG genotypes. (A) Circle plots of the correlations. Edges are colored in red/blue for postive/negative correlations respectively and are weighted according to the correlation value. (B) Statistical comparison of the correlation coefficients between the two genotypes (red; positive
and green; negative correlation). Values of the correlations are reported for each genotype, with the p-value into brackets. The p-value related to the comparison tests is given in the last column.

**Figure 5. LPP3 down-regulates leukocyte recruitment to HAEC monolayers.** HAECs were transfected with siRNA (siCtrl or siLPP3) or with hLPP3 expression plasmids (Ctrl or LPP3) for 48 h. mRNA relative levels of adhesion molecules SELE, VCAM-1 and ICAM-1 were determined by RT-qPCR (A, B). The results (mean of duplicates) are shown as relative mRNA levels as compared to individual respective controls set at 1, each dot within vertical scatter plots represents a single donor (n=7-8); the mean +/- SEM is depicted. Lysates were analyzed by immunoblotting with specific antibodies and re-probed with an anti-tubulin antibody (the SELE membrane) to ensure equal loading and transfer (C). Leukocyte adhesion was assessed using a hPBMC adhesion assay as described in Material and Methods. HAECs were transfected with either siRNA (siCtrl or siLPP3) or with hLPP3 expression plasmids (Ctrl or LPP3) for 48 h, and then incubated with calcein-AM labelled hPBMC. Images of fluorescent cells were captured with an epi-fluorescence microscope (D) and the adherent cells were quantified by automated counting using ImageJ and represented as a relative change over the control (E). The results (mean of duplicates) are shown, each dot within vertical scatter plots represents a single leukocyte donor (n=5-6) tested on 3 HAECs donors; the mean +/- SEM is depicted.

* p<0.05, **: p<0.001. Bar scale in panel D indicates 500µm.

**Figure 6. LPP3 affects viability of HAECs.** HAECs were transfected with either siRNA (siCtrl or siLPP3) (A) or with hLPP3 expression plasmids (Ctrl or LPP3) (B) for 48 h. Cell viability was measured using the WST-1 assay and the results (% of respective controls; mean of duplicates) are shown, each dot within vertical scatter plots represents a single donor (n=7); the mean +/- SEM is depicted. To show the variability of controls each point was calculated as % of the mean of controls set as 100%. HAECs transfected with either siLPP3 or siCtrl were treated with inhibitors of either LPA formation (PF8380) or S1P receptor (FTY720) (C). HAECs transfected with Ctrl- or LPP3-containing plasmids were treated with LPA mimic ((2S)-OMPT) or S1P (D). HAECs were transfected with either control plasmids, WT LPP3 or with mutants of the catalytic domain (H249P or H251P) or of the adhesion motif (RGD->RAD), subsequently the WST-1 assays were performed. The results (mean of triplicates) in panels C, D and E (% of respective controls) are shown, each dot within vertical scatter plots represents a single donor (n=3); the mean +/- SEM is depicted. Caspase-3 and -7 activity was assessed using Apo-ONE® Homogeneous Caspase-3/7 Assay (F) and the relative fluorescence was
measured using a microplate reader. The results (mean of duplicates) are shown as % of respective controls set at 100%, each dot within vertical scatter plots represents a single donor (n=8); the mean +/- SEM is depicted.

*: p<0.05, **: p<0.001. When more than 3 groups were compared a global p-value is: panel A: p=0.006; panel C: p=0.08; panel D: p=0.04. In panel A we also give the p-value of 0.072 for siCtrl vs siCtrl+VEGF and in panel E the p=0.054 for siLPP3 to show the trend.

**Figure 7. LPP3 downregulates HAECs migration.** HAECs were transfected with either siRNA (siCtrl or siLPP3) or with hLPP3 expression plasmids (Ctrl or LPP3) for 48 h. Cell monolayers were wounded with 1000µl pipette tips (right panel) and incubated for 16h to assess cell migration. Wounded areas were imaged at 0h and 16h. Results expressed in % of the respective controls (mean of duplicates) are shown, each dot within vertical scatter plots represents a single donor (n=7); the mean +/- SEM is depicted (A). Bar scale in micrographs in panel A indicates 500µm. siLPP3 and siCtrl transfected HAECs were treated with inhibitors of either LPA formation (PF8380) or S1P receptor (FTY720) (B). LPP3-containing plasmid or Ctrl plasmid transfected cells were treated with LPA mimics ((2S)-OMPT) or S1P (C). The results (mean of duplicates) in panel B and C are shown, each dot within vertical scatter plots represents a single donor (n=3); the mean +/- SEM is depicted. To show the variability of controls each point was calculated as % of the mean of individual controls set as 100%.

*: p<0.05, ***: p<0.0001. In panel C we give the p-value of 0.075 to show the trend. When more than 3 groups were compared a global p-value is: panel B: p=0.02, panel C: p=0.03

**Figure 8. LPP3 is a positive regulator of angiogenesis.** HAECs were transfected with either siRNA (siCtrl or siLPP3) (A) or with hLPP3 expression plasmids (Ctrl or LPP3) (B) for 48 h. Cells were harvested and seeded on Matrigel and incubated for an additional 48h. Microphotographs of cultures were taken at 24h and angiogenesis was quantified by counting the tube-like and branching point structures, using an automated ImageJ program. Bar scale in panel A and B indicates 500µm. siLPP3 and siCtrl transfected HAECs were treated with inhibitors of either LPA formation (PF8380) or S1P receptor (FTY720) (C). HAECs transfected with either hLPP3 expression plasmids or Ctrl plasmids were treated with LPA mimics ((2S)-OMPT) or S1P (D). HAECs were transfected with control plasmid, wild-type LPP3 or mutants of the catalytic domain (H249P or H251P) or the adhesion motif RGD->RAD (E). The results expressed as % of respective controls (mean of duplicates) are shown, each dot within vertical scatter plots represents a single donor (n=4-6); the mean +/- SEM is depicted. To show the variability of controls each point was calculated as % of the mean of individual controls set as 100%.
*: p<0.05, ***: p<0.0001. When more than 3 groups were compared a global p-value is: panel C: p=0.0002; panel D: p=0.06 and panel E: p=0.0004.