Supplementary Material

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

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Materials and Methods

Oligonucleotides and Chemicals

Recombinant human vascular endothelial growth factor-165 (VEGF-165) was purchased from R&D Systems (VEGF 293-VE/CF). Lipid substrates were purchased from Cayman Chemical: (2S)-OMPT (10005707), PF8380 (12018), FTY720 (10006292) and S1P (62570). Human IL1β/IL-1F2, IL6, MCP1 and CXCL8/IL8 DuoSet ELISA (enzyme-linked immunosorbent assay) kits were purchased from R&D Systems.

siRNA oligonucleotides were purchased from Ambion/Applied Biosystems: Silencer Select Negative Control siRNA (4390843), PPAP2B (4390824;siRNA(1): ID s16386, siRNA(2): ID s16385). Plasmids were purchased from Origen: hLPP3: *PPAP2B* cDNA (RC203480) and control: pCMV-Entry vector (PS100001). *PPAP2B* mutant constructs were made in the laboratory using *PPAP2B* cDNA (Origen).

Mutant primers were: catalytic site (H249->P):

Forward. 5'TCTCGCGTATCAGACCAAAAGCACCATCCCAGTGAT

Reverse. 5'ATCACTGGGA- TGGTGCTTTTGGTCTGATACGCGAGA ;

catalytic site (H251->P):
Forward. 5'TCTCGCGTATCAGACCACAAGCAACATCCCAGTGAT
Reverse. 5'ATCACTGGGATGTTGCTTGTGGTCTGATACGCGAGA ;
adhesion motif (RGD-> RAD):
Forward.5'CTACAGATGCAGAGCTGATGACAGCAAAG
Reverse. 5'CTTTGCTGTCATCAGCTCTGCATCTGTAG.

Cell culture and transfections

Human aortic endothelial cells (HAECs) were either purchased (PromoCell, Germany; all males bearing the rs17114036 AA genotype) or 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(1). Cells were cultured in Endothelial Cell Growth Media (Lonza) supplemented with growth factors (EGM-2 bullet kit: 2% FBS, 0.4% hFGF-2, 0.1% VEGF, 0.1% R3-IGF1, 0.1% hEGF, 0.1% ascorbic acid, 0.1% heparin, 0.1% GA-100) in a humidified 5% CO2 atmosphere at 37°C. All assays were performed using HAECs from at least 3 different donors (passages 4-6). Prior to assays, HAECs were split in 6-wells plates and grown to 70-80% of confluence and then transfected for 48h either with 20nM of siRNA using Lipofectamine RNAiMax (Life Technologies), or with 1µg of plasmids (and 2µg for the annexin V/7-AAD assay) using Turbofect (Thermo Scientific). 24 hours post-transfection, the media were replaced with fresh complete media containing either 100ng/ml VEGF-165 or various concentrations of LPA or S1P agonists ((2S)-OMPT, FTY720, PF8380 or S1P). HAECs were maintained in cultures for further 24 hours before assays.
All protocols involving humans (Human aortic endothelial cells; HAECs) were approved by UCLA Institutional Review Board and NIH, were conform to the Helsinki Declaration of 1975 as revised in 1983 and a written consent was given for the use of the human material.

ELISA assays for cytokines

HAECs were cultured in 6-well plates and transfected with either siRNA or plasmids containing the *PPAP2B* constructs. After transfection cells were cultured 48h in serum-free medium, and cell supernatants were collected and centrifuged at 300 g for 5 minutes to remove cellular debris. ELISA assays were performed in cell supernatants to detect soluble forms of IL1β, IL6, IL8 and MCP1 (CCL2) using respective human DuoSet ELISA kits (R&D Systems) following manufacturer's instructions. The experiments were performed in duplicate in supernatants from 5 donors.

Human peripheral blood mononuclear cells adhesion assay

Prior to adhesion assay endothelial cells were seeded into 12-well culture plates, transfected with either siRNA or plasmids for 48 hours and treated by adequate stimuli during 24 hours. Human peripheral blood mononuclear cells (hPBMC) were isolated from a single-donor Buffy coats (Etablissement Français du Sang, Rungis, France) using Ficoll density gradient centrifugation for 20 min at 1200 rpm, next the cells were resuspended in RPMI medium at $3x10^6$ cells/ml(2). Cells were labelled with 10 µM calcein-AM (Life Technologies) for 30 min at 37°C in the dark, and then were sedimented and washed three times with PBS to remove excess dye and resuspended in phenol red-free RPMI (with 10% FBS) at a density of $3x10^6$ cells/ml. HAECs monolayers were washed after various treatments with PBS and incubated with calcein-AM labeled hPBMCs ($3x10^6$ cells/well) at 37°C for 1h. HAECs monolayers were washed at least 3 times with PBS to remove the non-adherent calcein-AM labelled cells and the media was replaced with 1 ml PBS. Images of fluorescent cells were captured with epi-fluorescence microscope NIKON ECLIPSE Ti with Nikon Intensilight C-HGFIE using FITC filter at 4X10 magnification, and the adherent cells were quantified by automated counting using ImageJ software (NIH). The results (mean of duplicates) are shown, each dot within vertical scatter plots represents a single leukocyte donor (n=6) tested on 3 HAECs donors.

Cell viability assay

Cell viability assessment was performed according to a colorimetric assay using the tetrazolium salt WST-1 (4-[3-(4-iodophenyl)-2H-5-tetrazolio]-1-3-benzene disulfonate), based on the conversion of WST-1 into formazine by mitochondrial dehydrogenase enzyme in viable cells. 10,000 cells/well were seeded in 96-well plates in 100 µl medium and transfected for 48 hours. Cells were treated with the adequate treatments during the last 24 hours. Then 10 µl of WST-1 solution were added to each well, followed by 3 hours incubation. Absorbance was measured at 450 nm in a Flexstation 3 microtiter plate reader (Molecular devices, CA. USA). The results (mean of triplicates) are shown, each dot within vertical scatter plots represents a single donor (n=7: Figure 5A and B), (n=3 : Figure 5B; C, D and E) or (n=8 : Figure 5F).

Cell proliferation assay

Cell proliferation test was carried out using the ELISA kit (Calbiochem) containing the thymidine analogue the 5-bromo-2'-deoxyuridine (BrdU), which is incorporated into the DNA of proliferating cells. Briefly, one day prior to the ELISA test, HAECs were seeded in 96-well plates at 10.000 cells/well in 100µl medium, treated with various agents and then cultured at 37°C for 24h to allow adherence. BrdU (10 µM) was added for 6 hours to the plates, next the cells were fixed and incubated with anti-BrdU antibody conjugated with peroxidase which reacts with its substrate to yield a product quantified at 450 nm using a Flexstation 3

microtiter plate reader (Molecular devices, CA. USA). The results (mean of triplicates) are shown, each dot within vertical scatter plots represents a single donor (n=7 : Figure S5A) or (n=3 : Figure S5B).

Apo-ONE® Homogeneous Caspase-3/7 Assay

Apoptosis was measured with the Apo-ONE® Homogeneous Caspase-3/7 Assay (Promega, Madison, WI, USA) using rhodamine 110, bis-(N-CBZ-L-aspartyl-L-glutamyl-L-valyl-L-aspartic acid amide; Z-DEVD-R110) as a substrate for both Caspase-3 and -7. Briefly, HAECs from 8 donors were seeded in 96-well plates and transfected for 48h with either siLPP3 or LPP3 plasmid or their respective controls. The combined activity of Caspase-3 and -7 was measured after 6h of incubation with the cells as an increase of rhodamine 110 fluorescence after cleavage of the substrate Z-DEVD-rhodamine. Cells were labeled with Hoechst 33342 at the end of incubation, in order to determine the exact cell amount in each well.

Fluorescence was read using a FLEXSTATION microplate reader at excitation 499 nm and emission 521 nm. Rhodamine fluorescence intensities were normalized to those of Hoechst. The results (mean of duplicates) are shown, each dot within vertical scatter plots represents a single donor (n=8).

ANNEXIN V-FITC / 7-AAD Assay

Apoptosis of HAECs was analyzed by annexin V-FITC/ 7-AAD binding assay based on the binding properties of annexin V to phosphatidylserine (PS) and on the specificity of 7-amino-actinomycin D (7-AAD) to bind to double-stranded DNA with a high-affinity between GC base pairs. 7-AAD was used for the distinction between the early- and the late apoptotic or the necrotic cells. Both floating and adherent cells were harvested and washed by PBS, then stained according to the manufacture's instruction. 5×10^5 of collected cells were incubated in the dark for 15 minutes with 100 µL of 1×Binding Buffer containing 10 µL of annexin V-FITC and 20 µL of 7-ADD Viability Dye (Beckman-Coulter, Inc. Marseille, France) on ice. Samples were diluted with 400 µL of 1×Binding Buffer and immediately analyzed by a flow cytometer (LSRFortessa - Becton-Dickinson). Data are presented as percentage of annexin V+/7AAD cells for early apoptosis and annexin V+/7AAD+ cells for late apoptosis. Statistical analyses were generated from HAECs obtained from 4 donors.

Cell migration

Transfected HAECs were cultured for 48h to reach 90% confluence and then treated with mitomycin (Sigma-Aldrich) for 2h. Two linear scratches per well were performed in the cell monolayer using a 1000µl pipette tip. HAECs were treated with adequate substrates and

were incubated at 37°C and 5% CO2 for the next 16h. Images of the gap were obtained at 0 and 16h with a 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. The results (mean of duplicates) are shown, each dot within vertical scatter plots represents a single donor (n=7 : Figure 6A and B) or (n=3 : Figure 6C and D).

Angiogenesis tube formation in Matrigel

In vitro angiogenesis assays were performed using 15-well µ-angiogenesis slides (Ibidi, Germany). The slides were coated with 10µl of Matrigel (VWR) per well and then left to polymerize for 30 min at 37 °C. Transfected HAECs were seeded at 10.000 cells/well in triplicate, were treated with various agents and were incubated at 37°C for additional 24h. The images of newly structured tubes were captured at 6 and 24h using 4 x 10 magnification (Nikon). Tube formation was quantified manually by counting the number of tubular and branching point structures. The results (mean of duplicates) are shown, each dot within vertical scatter plots represents a single donor (n=6: Figures 8A and B) or (n=4 : Figures 8C, D and E).

RNA extraction and real-time PCR

Total RNA was extracted 48h post-transfection using the mirVANA-miRNA isolation kit (Life Technologies) and RNA quality was assessed by the 2100 Bioanalyser (Agilent technologies, Canada). RNA integrity (RIN) was between 8 to 10. Synthesis of cDNA was carried out using the Super Script II Reverse Transcriptase (Life technologies). Real-time PCR was performed using Mx3005P QPCR System SYBR Green (Thermo Scientific). The amplification program was: 95 °C for 15 min, 40 cycles of 95 °C for 30 s and one cycle of 95°C for 1 min, 60°C for 30 s and 95°C for 30 s. Data were analyzed using MxPro® software using the ΔΔCT method and normalized to the ubiquitin and GAPDH control genes for each donor. Controls were set to 1. Experiments were performed in duplicate using 3 to 8 donors.

Protein extraction and Western blot analysis

Cells were lysed by the Mammalian Whole Cell Protein Extraction Kit supplemented with the protease inhibitor cocktail (Promocell, Germany) and the protein concentration was measured using Quick Start Bradford reagent (BioRad). Protein extracts (50 µg) were separated in 4-12% NuPAGE Novex gels (Life Technologies) and transferred onto PVDF membranes (BioRad). The membrane was probed with adequate antibodies. the rabbit anti-LPP3, kindly provided by Dr Susan S. Smyth, Division of Cardiovascular Medicine, The Gill Heart Institute, Lexington, KY(3) and anti-tubulin mouse monoclonal antibody (Invitrogene). HRP-conjugated anti-rabbit or anti-mouse (DAKO) corresponding secondary antibodies were

used for detection. The immunoblot corresponds to one representative experiment out of 2. The chemiluminescence signal was detected using Pierce ECL Plus Western blotting substrate (Thermo Scientific) using ImageQuant LAS 4000 (GE Healthcare). Scanned bands were normalized with tubulin for all proteins and additional normalization with total ERK1/2 for phosphor-ERK1/2 antibody. Normalization was performed by quantification of the density of the bands using ImageJ software (NIH).

Antibodies used were: the rabbit anti-LPP3, kindly provided by Dr Susan S. Smyth, Division of Cardiovascular Medicine, The Gill Heart Institute, Lexington, KY(3). VCAM-1(H-276) (#sc-8304), E-selectin (H-300) (#sc-14011), ICAM-1(H-108) (#sc-7891) from Santa Cruz (TX – USA). Phospho-p44/42 MAPK (Erk1/2)(Thr202/Tyr204) (#4376) and p44/42 MAPK (Erk1/2) (#9102) from Cell signalling (MA – USA). Alpha-tubulin (B-5-1-2) (#32-2500) from Invitrogene (USA).

Plasmid constructs

Site-directed mutagenesis (Agilent QuickChange II XL Site-Directed Mutagenesis kit; Agilent Technologies) was performed using *PPAP2B* wild type cDNA (4390824) (Origen). Mutated primers from Sigma-Genosys (Sigma-Aldrich) were used to generate expression plasmids carrying different mutations. After PCR and Dpn I digestion, XL10-Gold Ultracompetent cells provided with the kit were transformed with the different mutant plasmids. All constructs were sequenced.

Genome-Wide Expression analysis and pre-processing of expression data

Total RNA was extracted from 5 HAECs donors using mirVANA-miRNA kit (Life Technologies) as described above. Transcriptomic analysis of total RNA was performed using the *Illumina* HT-12 v4 BeadChip (http://www.Illumina.com). 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 (Ambion/Applied Biosystems). Each biotinylated cRNA (750 ng) was hybridized to a single BeadChip at 58°C for 16–18 hours. BeadChips were scanned using the *Illumina Hiscan array*.

The summary probe-level data delivered by the *Illumina* scanner (mean and SD computed over all beads for a particular probe) was loaded in *Genome Studio*. The pre-processing was done by the *Illumina* software, at the level of the scanner and by *Genome Studio* included: correction for local background effects, removal of outlier beads, computation of average bead signal and SD for each probe and gene, calculation of detection *P*-values using negative controls present on the array, quantile normalization across arrays, check of outlier samples using a clustering algorithm, check of positive controls. Analyses were carried out on the mean level for all probes in each gene. To stabilise variance across expression levels, *arcsinh* transformation was applied to the expression data. Compared to a log

transformation, this transformation has the advantage not to discard negative expression values which can occur in Illumina data.

The *Illumina HT-12 v4 BeadChip* targets more than 47,000 probes derived from the National Center for Biotechnology Information Reference Sequence (NCBI) RefSeq Release 38 (November 7, 2009) and other sources. A gene was declared significantly expressed in the dataset, i.e. expressed above background (as measured by the negative controls present on each array), when the detection P-value calculated by Genome Studio software was <0.05 in more that 5% of the samples.

Immunohistochemistry (IHC) staining

IHC was performed using standard protocols with the following primary monoclonal antibodies directed against: CD68 monoclonal antibody (mab) (Dako, Trappes, France; 1/500), alpha-actin mab (Dako; 1/4000), CD34 mab (Sigma, 1/200), CD3 mab (Dako, 1/100) and the polyclonal rabbit anti-LPP3 (Sigma Aldrich; 1/50). Briefly, IHC was performed on 5-µm sections of Tissue MicroArray (TMA) blocks. Deparaffinized and rehydrated sections were incubated for 30 min at room temperature with primary antibodies, washed, and incubated for 30 minutes with a kit UltraVision LP Detection System (Thermo Fisher Scientific, UK). After washing, the HRP Polymer DAB Plus Chromogen substrate was added (Thermo Fisher Scientific). Slides were counterstained with aqueous hematoxylin and mounted with Immunomount (Shandon, Cergy-Pontoise, France).

Determination of S1P levels

Methanol (1ml) containing 2.5 µl concentrated HCl was added to 500 µL of HAECs suspension or supernatants. C17-S1P (27 pmoL) was added as internal standard and lipids were extracted by addition of 1 ml chloroform, 200 µl NaCl (4 M) and 100 µl NaOH (3 M). The alkaline aqueous S1P-containing phase devoid of other sphingoid bases and of the majority of hydrophobic PL was transferred to a clean tube. The alkaline aqueous phase was acidified with 100 µl concentrated HCl and extracted twice with 1.5 ml chloroform. The organic phases were evaporated and the dried lipids were dissolved in 100 µl of methanol. A derivatization mixture of 5 mg *o*-phthaldialdehyde, 100 µl ethanol, 5 µl 2-mercaptoethanol and 5 ml boric acid (3% v/w) was prepared and adjusted to pH 10.5 with 10 N KOH. Nine µl of the derivatization mixture was added to the lipids and the solution incubated for 15 minutes at 37°C. The derivatives were analyzed with a Shimadzu HPLC system using an RP 18 Kromasil column (2.1 mm i.d. x 150 mm) maintained at 45°C. Separation was performed with the isocratic eluent containing methanol: K_2HPO_4 (0.07 M) (78:22 v/v) at a flow rate of 0.25 mL/min. The derivatives were detected selectively using a Shimadzu spectrofluorometer with an excitation wavelength of 334 nm and an emission wavelength of 440 nm. S1P was

quantified by comparison of its fluorescent signal with that of the derivative of the internal standard (coefficients of variation <5%). The results (mean of triplicates) are shown, each dot within vertical scatter plots represents a single donor (n=3).

Determination of LPA

LPA Extraction

All LPA standards were of highest purity and purchased from Avanti Polar Lipids (Alabaster, Alabama). 300 µl of cell supernatant were spiked with LPA C17:1 as internal standard and extracted by Bligh and Dyer(4), substituting chloroform with dichloromethane. The extracts were dried under vacuum and the residues were re-dissolved in buffer A:buffer B (50:50, v/v). Buffer A consisted of water/acetonitrile (60:40, v/v) containing 10 mM ammonium acetate and buffer B consisted of isopropanol/dichloromethane/methanol (60:20:20, v/v) containing 10 mM ammonium acetate).

Reverse-phase liquid chromatography and mass spectrometry

The molecular species LPA C16:0, 18:0, 18:1, 18:2, 20:4 were analysed using a modified LC-MS/MS method(5). The LPA molecular species were separated by reverse phase chromatography on a 1.7uM 2.1x100 mm CSH C18 column (Waters, Milford, MA) using a stepwise gradient starting with buffer A:B=50:50 to buffer A:B=0:100 over 1 min. Mass spectral data were recorded on a SCIEX 6500 QTrap mass spectrometer equipped with an IonDrive Turbo V source (SCIEX, Framingham, MA). LPA molecular species were measured using MRM pairs with the instrument operating in the positive ion mode. Individual LPA molecular species were identified by matching their MRM signal and chromatographic retention time with those of pure identical standards.

Quantification of LPA molecular species

Individual LPA species were normalized to the internal standard LPA C17:1 and the ratios of peak areas were compared with those of authentic standards used for standard curves. Using this method, the lower detection limit for most LPA species was about 20 fg on the column.

Statistical analyses

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

Statistical analysis of microarray data was performed in the R environment (version 3.0.1) using the *lumi* package of Bioconductor(4). The variance stabilizing transformation(5) was applied to raw data. A variance-based filtering of gene expressions was further performed using the *nsFilter* function from the *genefilter* package with default parameters (interquartile range and variance cutoff of 0.5). Differential expression analysis was performed using the *limma* package of Bioconductor. To account for the pairing of each siRNA sample with its control, the difference between gene expressions of both samples was considered as the variable to be tested to 0. Analysis was further adjusted for beadchip. The Benjamini-Hochberg procedure was applied to adjust for multiple testing.

Table S1. HAECs (n=5) were transfected with LPP3 siRNA(1) or control siRNA. Total RNA was prepared and whole-genome expression profiling was performed using Illumina HumanHT-12 v4 Expression BeadChip which targets 47,000 probes. The table show the list of statistically significant differentially expressed genes (FDR-adjusted p-value (BH) <0.05) in siLPP3 silenced as compared to siCtrl cells.

Table S2. GO biological processes enrichment. HAECs (n=5) were transfected with LPP3 siRNA(1) or control siRNA. Total RNA was prepared and global gene expression analysis was performed. cDNAs were labelled and hybridized to the Illumina HumanHT-12 v4 Expression BeadChip which targets 47,000 probes. Statistical enrichment of specific biological processes and molecular functions annotated in Gene Ontology (GO http://www.geneontology.org/) was assessed by GSEA using Genetrail software. Significant GO biological processes were selected (FDR<0.05).

Mechanism	Biological processes	category	Genes	FDR	Enrichment
Inflammation	Inflammatory response	GO:0006954	161	0.000888283	Up
	Cytokine activity	GO:0005125	76	0.00291964	Up
	Cytokine production	GO:0001816	124	0.00511788	Up
	Chemokine activity	GO:0008009	19	0.0078495	Up
	Chemotaxis	GO:0006935	87	0.00845029	Up
	Cell adhesion	GO:0007155	382	9.36178e-06	Up
	Cell adhesion molecule binding	GO:0050839	25	0.00324616	Up
	Leukocyte activation	GO:0045321	176	0.00951024	Up
	Regulation of leukocyte migration	GO:0002685	22	0.015871	Up
Cell survival	Cell cycle	GO:0007049	706	0	Down
	Regulation of cell cycle	GO:0051726	324	0.00117872	Down
	Positive regulation of cell proliferation	GO:0008284	231	0.00614088	Up
	Caspase inhibitor activity	GO:0043027	10	0.0131256	Up
	I-kappaB kinase/NF-kappaB cascade	GO:0007249	127	0.0210446	Up

Cell migration	Response to wounding	GO:0009611	289	1.51154e-05	Up
	Wound healing	GO:0042060	112	0.00891874	Up
	Cell migration	GO:0016477	260	0.000168581	Up
	Regulation of cell migration	GO:0030334	127	0.00149412	Up
Angiogenesis	Angiogenesis	GO:0001525	151	0.000576977	Up
	Blood vessel development	GO:0001568	220	0.00153458	Up
	Blood vessel morphogenesis	GO:0048514	184	0.00286085	Up
MAPK signaling	MAPK signaling pathway	04010	148	0.00108394	Up
NOS	positive regulation of nitric oxide biosynthetic process	GO:0045429	15	0.00149412	up
NOS	regulation of nitric oxide biosynthetic process	GO:0045428	19	0.00607399	up

Table S3: Primer sequences

Human genes	Primer sequences
GAPDH	For. 5' -TGCACCACCAACTGCTTAGC Rev. 5' -CAGTCTTCTGGGTGGCAGTGA
Ubiquitin	For. 5' -TTTTTGGGAATGCAACAACTT Rev. 5' -CACTTGGTCCTGCGCTTGA
PPAP2B (LPP3)	For. 5’ -TTTACTATGTGGCCTTCCAAAAT Rev. 5’ -ACTAGAACATGAACACTTAAACC
VEGF A	For. 5' -TCTACCTCCACCATGCCAAGT Rev. 5' -GATGATTCTGCCCTCCTCCTT
IL1β	For. 5’ -GTGTAGATCCCAAAAATTACCCA Rev. 5 -CCAGGAAGACGGGCATGTT
IL6	For. 5' -AACCTGAACCTTCCAAAGATG Rev. 5' -TCTGGCTTGTTCCTCACTACT
IL8	For. 5' -TCTGGACCCCAAGGAAAACT Rev. 5' -TCTTGGATACCACAGAGAATGAATTT
SELE	For. 5' -GATGAGAGGTGCAGCAAGAAG Rev. 5' -CTCACACTTGAGTCCACTGAAG
ICAM1	For. 5’ -CAGTGACCATCTACAGCTTTCCGG Rev. 5' -GCTGCTACCACAGTGATGATGATGACAA

VCAM1	For. 5' -TGAGTTTTGAGAACGAACACTCTTATCT Rev. 5' -GCTTACAGTGACAGAGCTCCCATT
MCP1/CCL2	For. 5' - TCTCGCCTCCAGCATGAAAGT Rev. 5' -GCATTGATTGCATCTGGCTGA
PLA2G4A	For. 5' -CGACGTCCTTCTCTGGTATTGAAT Rev. 5' -GATGCTGGAAGTGACTATCAAAGTGATA
COX2/PTGS2	For. 5' -ATATGTTCTCCTGCCTACTGGAA Rev. 5' -GCCCTTCACGTTATTGCAGCTG
ANGPT2	For. 5' -CAGTTCTGAGGATGTTAATCGTC Rev. 5' -AGACATTCACAGAACTTAACACC

Legends to the supplementary Figures

Figure S1. LPP3 expression in vascular cells. IHC was performed on 5-µm sections of TMA blocks. Deparaffinized and rehydrated sections were incubated with primary antibodies monoclonal antibodies against CD68 (macrophages), alpha-actin (smooth muscle cells), CD34 (endothelial cells), CD3 (lymphocytes; served as negative control for monoclonal antibodies) and the polyclonal rabbit anti-LPP3 and rabbit serum. IHC were revealed using the HRP Polymer DAB Plus Chromogen substrate. Slides were counterstained with aqueous hematoxylin and mounted with Immunomount. Original magnification 40 x 10.

Figure S2. Silencing of LPP3 with siLPP3(2). HAECs were transfected for 48h with siLPP3 (siRNA(2): ID s16385) or siCtrl. The level of LPP3 mRNA was determined by RT-qPCR and the results (mean of duplicates) are expressed as a relative LPP3 mRNA level over the control, each dot within vertical scatter plots represents a single donor (n=5) **: $p<0.001$.

Figure S3. LPP3 downregulates IL1β mRNA level but not IL1 β secretion. HAECs were transfected with siRNAs (siCtrl or siLPP3) (A) or hLPP3 expression plasmids (Ctrl or LPP3) (B) for 48h. mRNA relative level of IL1β was determined by RT-qPCR. The results (mean of duplicates) are shown, each dot within vertical scatter plots represents a single donor (n=8). The cytokine concentrations were determined using ELISA in supernatants of HAECs cultured for 24 hours in serum free media. The results (mean of duplicates) are shown, each dot within vertical scatter plots represents a single donor (n=5) (C, D). *: $p<0.05$, **: $p<0.001$.

Figure S4 Overexpression of LPP3 does not modify proinflammatory cytokines formation in HAECs. HAECs were transfected with hLPP3 expression plasmids (Ctrl or LPP3) for 48 h. mRNA relative levels of IL6 IL8 and MCP1 were determined by RT-qPCR (A). The results were expressed as mRNA fold change of Ctrl set as 1 (mean of duplicates) are shown, each dot within vertical scatter plots represents a single donor (n=4); 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. To show the variability of controls each point was calculated as % of the mean of individual controls set as 100%.

Figure S5. LPP3 downregulates PLA2G4A and PTGS2 mRNA levels. HAECs were transfected for 48h with either siLPP3 or siCtrl (A) and either hLPP3 expression plasmids or Ctrl plasmids (B). mRNA relative levels of PLA2G4A and PTGS2 (COX2) were determined by RT-qPCR. The results were expressed as mRNA fold change of Ctrl set as 1 (mean of duplicates) are shown, each dot within vertical scatter plots represents a single donor (n=4) *: $p<0.05$.

Figure S6. Effect of LPP3 on cell proliferation. HAECs were transfected with siRNAs (siCtrl or siLPP3) and either treated or not with VEGF for 48h (A). HAECs were transfected with hLPP3 expression plasmids (Ctrl or LPP3) for 48h (B). HAECs were seeded in 96-well plates and transfected for 48h with either control plasmid, WT LPP3 or mutants of the catalytic domain (H249P or H251P) or the adhesion motif (RGD->RAD) (C). Cell proliferation was measured using the BrDU assay. The results (mean of duplicates) are shown, each dot within vertical scatter plots represents a single donor (n=5: A, B) or (n=3: D); 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%. *: $p<0.05$, **: $p<0.001$. When more than 3 groups were compared a global p-value is: panel A: $p=0.074$ and panel C: $p=0.01$.

Figure S7. LPP3 increases apoptosis in HAECs. HAECs were transfected with siRNAs (siCtrl or siLPP3) or hLPP3 expression plasmids (Ctrl or LPP3) for 48h. Apoptosis was measured using the annexin V/7AAD labeling by flow cytometry. Scatter-plots represent data from 4 donors, 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%. Global p-value is $p=0.0001$ for the right panel; **: $p<0.001$ (A). Cell labeling gates from one representative donor are presented (B). Data are presented as percentage of annexin V+/7AAD+ positive cells. Lysates from HAECs transfected either with siLPP3 or siCtrl and either hLPP3

expression plasmids or Ctrl plasmids were analyzed by immunoblotting using anti-phospho-ERK1/2 antibody and re-probed with an anti-ERK1/2 antibody to assess total ERK1/2 labelling. An anti-tubulin antibody was used to ensure equal loading and transfer (C). Immunoblot from 1 experiment is shown out of 2.

Figure S8. LPP3 downregulates HAECs migration. HAECs were transfected with siRNA (siCtrl or siLPP3) and either treated or not with VEGF for 48h. 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 (mean of duplicates) are expressed in % of the respective controls 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 individual controls set as 100%. Global p-value is p=0.0001; *: p<0.05, **: p<0.001.

Figure S9. S1P levels in HAECs' extracts and supernatants. HAECs were transfected with either siRNA (siCtrl or siLPP3) or with hLPP3 expression plasmids (Ctrl or LPP3) for 48h. Cell extracts (A) and supernatants (B) were collected. Lipids were extracted and derivative mixtures were analyzed by HPLC. The results (mean of duplicates) are shown, each dot within vertical scatter plots represents a single donor (n=3); the mean +/- SEM is depicted.
nd, not detected (limit <0.5 nM).

Figure S10. Graphical summary of principal results explaining the function of LPP3 in HAECs.

A. Endothelial dysfunction: LPP3 knockdown in HAECs leads to an increased VEGF expression and upregulation of S1P levels. In addition, leukocyte adhesion cell survival and migration, inflammatory signaling pathways involved in endothelial dysfunction were induced. Overexpression of LPP3 diminished VEGF expression and S1P levels restoring normal endothelial function.

B. Angiogenesis: LPP3 knockdown in HAECs leads to increased VEGF expression and to sustained S1P formation. This resulted in tube formation but subsequently inhibited the resolution phase of angiogenesis involved in cellular junction and branching point formation. Overexpression of LPP3 permitted normal angiogenesis.

Abbreviations: sphingosine kinase (SK); sphingosine (Sph)

References

1. Romanoski CE, Lee S, Kim MJ, Ingram-Drake L, Plaisier CL, Yordanova R, Tilford C, Guan B, He A, Gargalovic PS, Kirchgessner TG, Berliner JA, Lusis AJ. Systems genetics analysis of gene-by-environment interactions in human cells. *Am J Hum Genet*. 2010;**86**:399–410.

2. Pégorier S, Stengel D, Durand H, Croset M, Ninio E. Oxidized phospholipid: POVPC binds to platelet-activating-factor receptor on human macrophages. Implications in atherosclerosis. *Atherosclerosis*. 2006;**188**:433–443.

3. Sciorra VA, Morris AJ. Sequential actions of phospholipase D and phosphatidic acid phosphohydrolase 2b generate diglyceride in mammalian cells. *Mol Biol Cell*. 1999;**10**:3863–3876.

4. Du P, Kibbe WA, Lin SM. lumi: a pipeline for processing Illumina microarray. *Bioinforma Oxf Engl*. 2008;**24**:1547–1548.

5. Lin SM, Du P, Huber W, Kibbe WA. Model-based variance-stabilizing transformation for Illumina microarray data. *Nucleic Acids Res*. 2008;**36**:e11.