Mechanosensitive PPAP2B Regulates Endothelial Responses to Atherorelevant Hemodynamic Forces

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Rationale: PhosPhatic Acid Phosphatase type 2B (PPAP2B), an integral membrane protein known as lipid phosphate phosphatase (LPP3) that inactivates lysophosphatidic acid, was implicated in coronary artery disease (CAD) by genome-wide association studies. However, it is unclear whether genome-wide association studies–identified coronary artery disease genes, including PPAP2B, participate in mechanotransduction mechanisms by which vascular endothelia respond to local atherorelevant hemodynamics that contribute to the regional nature of atherosclerosis.

Objective: To establish the critical role of PPAP2B in endothelial responses to hemodynamics.

Methods and Results: Reduced PPAP2B was detected in vivo in mouse and swine aortic arch (AA) endothelia exposed to chronic disturbed flow, and in mouse carotid artery endothelia subjected to surgically induced acute disturbed flow. In humans, PPAP2B was reduced in the downstream part of carotid plaques where low shear stress prevails. In culture, reduced PPAP2B was measured in human endothelial cells under atherosusceptible waveform mimicking flow in human carotid sinus. Flow-sensitive microRNA-92a and transcription factor KLF2 were identified as upstream inhibitor and activator of endothelial PPAP2B, respectively. PPAP2B suppression abrogated atheroprotection of unidirectional flow; inhibition of lysophosphatidic acid receptor 1 restored the flow-dependent, anti-inflammatory phenotype in PPAP2B-deficient cells. PPAP2B inhibition resulted in myosin light-chain phosphorylation and intercellular gaps, which were abolished by lysophosphatidic acid receptor 1/2 inhibition. Expression quantitative trait locus mapping demonstrated PPAP2B coronary artery disease risk allele is not linked to PPAP2B expression in various human tissues but significantly associated with reduced PPAP2B in human aortic endothelial cells.

Conclusions: Atherorelevant flows dynamically modulate endothelial PPAP2B expression through miR-92a and KLF2. Mechanosensitive PPAP2B plays a critical role in promoting anti-inflammatory phenotype and maintaining vascular integrity of endothelial monolayer under atheroprotective flow.

Key Words: atherosclerosis ■ endothelial cells ■ genome wide association study ■ hemodynamics ■ microRNAs ■ permeability

Atherosclerosis, a regional inflammatory disorder of arteries, originates and develops preferentially at sites of curvature, branching, and bifurcation in elastic and muscular arteries where endothelia are activated by local disturbed flow.1–4 In these arterial regions of atypical vascular geometry, repeated cardiac cycles impose complicated patterns of multidirectional hemodynamics at variable frequencies leading to fluid disturbance featuring oscillation, flow reversal, and low average shear stress. In vivo and in vitro investigations have established the causative role of disturbed flow in provoking atherosusceptible endothelia characterized by cobblestone morphology, low-grade inflammation, and compromised vascular integrity.5–7 Conversely, in arterial regions resistant to atherogenesis, unidirectional high-shear stress promotes atheroprotective endothelia that are anti-inflammatory, antipermeable, elongated, and aligned with the direction of flow. Multiple molecular actions have been mechanistically linked to the hemodynamics-mediated endothelial phenotypes, such as activation of nuclear factor-κB,8,9 protein kinase Cζ,9,10 bone morphogenic protein
Nonstandard Abbreviations and Acronyms

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<td>AA</td>
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<td>eQTL</td>
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<td>HAEC</td>
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<td>KLF2</td>
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4,11 angiopoietin-2,12 and unfolded protein response13 by disturbed flow or upregulation of vascular protective molecules, such as endothelial nitric oxide synthase, KLF2,14-15 KLF4,16,17 and Sirtuin-119 by unidirectional shear stress. Bioactive lipids are critical regulators of cellular growth, death, senescence, adhesion, migration, inflammation, and intracellular trafficking20; however, it remains largely unknown whether mechanical stimuli actively modulate bioactive lipid-mediated signaling in cells constantly exposed to physical cues, such as vascular endothelia subjected to continuous hemodynamic forces.

Extracellular lysophosphatidic acid (LPA) is an endogenous lipid messenger abundant in the circulation which acts on its cognate G-protein–coupled receptors known as lysophosphatidic acid receptors (LPARs) to trigger intracellular signaling necessary for neural and vascular development, embryo implantation, and innate defense.21 LPA binds to a repertoire of membrane receptors coupled with diverse intracellular pathways to achieve distinct cellular actions. Abnormal activation of LPA signaling is implicated in various human diseases, such as cancer, fibrotic disorders, metabolic syndrome, and cardiovascular diseases.22-24 LPA accumulates in human atherosclerotic plaques25 and plasma LPA is elevated in patients with acute coronary syndrome.26 In ApoE-deficient mice, systemic inhibition of LPA receptors using pharmacological antagonists notably reduced the atherosclerotic burden.27 LPA-activated cellular pathways are negatively regulated by lipid phosphate phosphatases, a cohort of integral membrane proteins that hydrolyze extracellular LPA and therefore limit access of lipid phosphates to their active sites. Although elevated LPA has been associated with vascular dysfunction,25,28 it is unclear whether endothelial responses to LPA stimulation are mediated by atherorelevant hemodynamics.

Herein, we tested the hypothesis that activation of LPA signaling is an unrecognized molecular mechanism that contributes to the atherosusceptible endothelia associated with disturbed flow. Specifically, we postulated that atheroprotective hemodynamics elevates endothelial PhosPhatidic-Acid-Phosphatase-type-2B (PPAP2B), also known as lipid phosphate phosphatases 3 that hydrolyses LPA and therefore suppresses LPA receptor–mediated cellular signaling.29 PPAP2B has been implicated in cardiovascular diseases by genome-wide association studies (GWAS) showing that risk allele at single-nucleotide polymorphism (SNP) rs17114036 predicts coronary artery disease independent of traditional risk factors, such as cholesterol and diabetes mellitus.30 A recent expression quantitative trait locus (eQTL) study further linked the risk allele at SNP rs6588635, proxy to SNP rs17114036 (r^2=0.831), to lower PPAP2B expression in human aortic endothelial cells (HAEC).31 Moreover, Panchatcharam et al32 recently reported that inducible inactivation of PPAP2B in endothelial and hematopoietic cells leads to vascular activation mediated by LPA receptor–dependent signaling. However, mechanoregulation and noncoding RNA modulation of PPAP2B are poorly understood. In this study, we demonstrate that endothelial PPAP2B, significantly suppressed in arterial regions exposed to disturbed flow, mediates the anti-inflammatory and antipermeable endothelial phenotype associated with atheroprotective flow. Moreover, disturbed flow–induced microRNA-92a (miR-92a) was identified as a novel negative regulator governing the mechanosensitivity of PPAP2B. The investigation provides new molecular insights of bioactive lipid-mediated signaling in determining the atherorelevant endothelial phenotypes in relation to spatial hemodynamics. Our data further elucidate the critical roles of mechanical stimuli and noncoding RNAs in modulating LPA signaling whose dysregulation is associated with a variety of human diseases.

**Methods**

A detailed Methods is available in the Online Data Supplement.

**Results**

**Reduced Endothelial PPAP2B Is Associated With Disturbed Flow In Vivo and In Vitro**

Mechanoregulation of endothelial PPAP2B by atherorelevant hemodynamics was investigated using in vivo animal models and complementary in vitro flow device that accurately reproduced arterial flow waveforms measured in humans. First, endothelia were harvested from the lesser curvature of swine AA that is atherosusceptible because of chronic disturbed flow and from the nearby descending thoracic aorta that is relatively atheroresistant owing to unidirectional shear stress.33 Endothelial purity was confirmed by showing high expression of endothelial marker vascular endothelial (VE)-cadherin and undetectable levels of smooth muscle cell marker SM22 alpha (Figure 1A), consistent with previous studies.34 Western blotting detected a 55±8% lower expression of endothelial PPAP2B in AA compared with nearby descending thoracic aorta, establishing an association between reduced endothelial PPAP2B and chronic disturbed flow in vivo (Figure 1A). Reduced endothelial PPAP2B associated with disturbed flow in vivo was further demonstrated in the mouse aorta. Endothelium-enriched RNA was collected from the intima of lesser curvature of mouse aorta exposed to chronic disturbed flow and from the greater curvature subjected to unidirectional flow as previously described.35 Figure 1B showed a 57±8% lower expression of intimal PPAP2B in the atherosusceptible lesser curvature of mouse aorta. In contrast, PPAP2B expression was not modulated in the...
media/adventitia in the lesser curvature of the mouse aorta when compared with the greater curvature (Online Figure IA).

To test whether atherorelevant shear stresses causatively modulate endothelial PPAP2B, we adapted the dynamic flow system, an in vitro flow apparatus developed by Dai et al36 to apply well-defined flow waveforms corresponding to arterial geometries and flow profiles in humans. HAEC were subjected to atherosusceptible flow mimicking wall shear stress in human carotid sinus or to atheroprotective flow representing wall shear stress in human distal internal carotid artery.36 Real-time quantitative polymerase chain reaction (qPCR) detected significantly higher (2.4-fold) PPAP2B transcripts in HAEC subjected to 24-hour atheroprotective flow compared with atherosusceptible shear stress (Figure 1C). Prolonged exposure (72 hours) further augmented PPAP2B mRNA (5.24-fold) and protein (7.21-fold) in HAEC exposed to atheroprotective flow (Figure 1C).

The aforementioned swine and mouse models provided in vivo evidence linking endothelial PPAP2B expression to chronic atherorelevant flows. To further elucidate endothelial PPAP2B response to acute disturbed flow in vivo, we performed surgery to ligate 3 of the 4 caudal branches of mouse left common carotid artery (LCA; Figure 1D). The partial ligation introduced acute oscillatory wall shear stress in LCA but had no significant effect on the blood flow in the right common carotid artery.37 We isolated carotid intimal RNA samples with high endothelial purity; qRT-PCR detected high expression of endothelial marker platelet endothelial cell adhesion molecule 1 (PECAM1) (average threshold cycle: 20.9±0.4), whereas expression of smooth muscle α-actin is negligible (average threshold cycle: 41.7±1.1). In contrast, high smooth muscle α-actin expression was detected in media/adventitia (total 14 samples) isolated from abovementioned arterial regions (average threshold cycle:...
18.1±0.7), whereas PECAM1 expression is insignificant (average threshold cycle: 37.2±0.4). A 55±4% reduction of endothelial PPAP2B mRNA was detected in ligated LCA compared with nonsurgical right common carotid artery 48 hours after the partial ligation (Figure 1D). In contrast, no significant regulation of PPAP2B was detected in the media/adventitia isolations from the ligated LCA compared with nonsurgical right common carotid artery (Online Figure IB).

To address the relevance of these findings to human disease, PPAP2B mRNA abundance was measured in both upstream and downstream sections of human carotid plaques that are exposed to distinct hemodynamics leading to modulation of mechanosensitive genes. Consistent with results from swine and mouse, PPAP2B expression was significantly reduced in the downstream sections of human carotid plaques where low shear stress prevails when compared with the upstream sections (Figure 1E).

miR-92a Suppresses Mechanosensitive PPAP2B Through 3′-Untranslated Region Binding

The regulatory mechanisms contributing to the mechanosensitivity of PPAP2B are not clearly defined. We hypothesized that flow-sensitive miRNAs could be unrecognized post-transcriptional regulators of endothelial PPAP2B. Bioinformatics tool TargetScan predicted an evolutionarily conserved putative binding site for miR-92a in the 3′-untranslated region (UTR) of human PPAP2B. Notably, previous studies have demonstrated higher endothelial miR-92a levels in the lesser curvature of swine AA in vivo and in cells subjected to disturbed flow in vitro. As shown in Figure 2A, miR-92a mimics significantly abolished the elevation of PPAP2B in HAEC subjected to 72-hour atheroprotective flow. Similarly, miR-92a mimics suppressed PPAP2B, whereas miR-92a inhibitors elevated PPAP2B in HAEC under static conditions at the transcriptional and translational levels (Figure 2B and 2C, respectively). Modulation of miR-92a in HAEC also led to regulation of integrin subunit α5 (Online Figure II), consistent with the previous studies. Furthermore, luciferase reporter assays demonstrated a direct functional interaction between miR-92a and human PPAP2B 3′UTR. Full-length 3′UTR of human PPAP2B was inserted downstream of a secreted Gaussia luciferase in a mammalian vector that also expresses secreted alkaline phosphatase as internal control for transfection-normalization. Intracellular delivery of miR-92a mimics significantly repressed the Gaussia luciferase in HAEC (Figure 2D) and in HEK-293 cells (Online Figure III) expressing the above-mentioned luciferase vectors; knockdown of endogenous miR-92a in HAEC using specific inhibitors enhanced the luciferase activity (Figure 2E). Mutations that disrupt the base-paired complement between human PPAP2B 3′UTR and miR-92a seed region (Figure 2F) completely eliminated the miR-92a-mediated suppression and enhancement of luciferase activity (Figure 2D and 2E, respectively), establishing that miR-92a directly inhibited human PPAP2B through 3′UTR recognition at the predicted binding site. Moreover, consistent with miR-92a suppression of PPAP2B, real-time qPCR demonstrated elevated endothelial miR-92a expression in the LCA of mice subjected to the partial ligation (Online Figure IVA) and in HAEC exposed to atherosusceptible flow (Online Figure IVB) where PPAP2B is reduced by disturbed flow. In agreement with these

![Figure 2](http://circres.ahajournals.org/)

**Figure 2.** miR-92a suppresses Phosphatidic Acid Phosphatase type 2B (PPAP2B) expression by targeting its 3′-untranslated region (UTR). A, miR-92a mimic diminished elevated PPAP2B expression induced by atheroprotective waveform in human aortic endothelial cells (HAEC). B, miR-92a mimic suppressed PPAP2B mRNA and protein levels in HAEC under static conditions. C, miR-92a inhibitor elevated PPAP2B mRNA and protein levels in HAEC under static conditions. D, miR-92a mimic decreased the activity of luciferase reporter containing the human PPAP2B 3′UTR in HAEC. E, miR-92a inhibitor increased the activity of luciferase reporter containing human PPAP2B 3′UTR in HAEC. F, Site-directed mutagenesis of miR-92a–binding site in the PPAP2B 3′UTR diminished the effect of miR-92a on the luciferase reporter activity in HAEC (D and E). All data are represented as means±SEM. n=3 to 5. *P<0.05, t test. n.s. indicates no significant difference.
findings, increased miR-92a was reported in the lesser curvature of mouse aorta (compared with the greater curvature) and downstream sections of human carotid plaques (compared with the upstream sections)38 where disturbed flow was prevalent and PPAP2B was reduced (Figure 1B and E).

**KLF2 Activates Endothelial PPAP2B Expression**

We then investigated the regulation of PPAP2B expression by KLF2, a flow-sensitive transcription factor14-16 and a direct downstream target of miR-92a.38,39 Adenovirus-mediated KLF2 overexpression (17.2±2.6-fold mRNA) led to a significant upregulation of PPAP2B in HAEC (Figure 3A). Dual-luciferase reporter assay was used to define the human PPAP2B promoter and more importantly to examine its response to transcription factor KLF2. The putative human PPAP2B promoter (1.1 kb) was cloned into the upstream of a Firefly luciferase, which significantly increased the luciferase activity by 25-fold when compared with the promoter-less controls (Figure 3B). KLF2 overexpression notably enhanced the activity of luciferase containing the putative human PPAP2B promoter but not promoter-less luciferase (Figure 3B). Real-time qPCR showed reduced endothelial KLF2 in the mouse LCA subjected to partial ligation (Figure 3C) and in HAEC exposed to atherosusceptible flow (Figure 3D), consistent with the KLF2 activation of vascular PPAP2B.

**Mechanosensitive PPAP2B Mediates Endothelial Cell Alignment Under Flow**

It is well documented that vascular endothelial cells elongate and align with each other in the direction of unidirectional flow in vitro and in vivo. To probe the putative role of PPAP2B in flow-mediated endothelial functions, we performed cell alignment analyses in HAEC exposed to 72-hour atheroprotective shear stress using dynamic flow system, identifying a uniformly aligned monolayer of elongated HAEC along with the flow direction, whereas cells under static conditions maintained a cobblestone morphology (Figure 4). Cell alignment was quantified by measurement of stress fiber angles to the direction of shear force, showing a 13° angle that is comparable with those reported in human umbilical vein endothelial cells under steady flow of 12 dynes/cm². Knockdown of PPAP2B using specific small interfering RNA (siRNA) noticeably impaired the alignment of HAEC monolayer under atheroprotective flow (Figure 4), implicating PPAP2B in flow-mediated endothelial mechanotransduction. The critical role of PPAP2B in endothelial alignment was consistently supported by quantification of the angle between the primary axis and the flow direction (Online Figure V).

**Atheroprotective Flow Attenuates Endothelial LPA Signaling and Reduces Major Species of Bioactive LPA**

Mechanosensitivity of endothelial PPAP2B that degrades LPA indicates atheroprotective flow is an unrecognized negative regulator of LPAR(s)-mediated cell signaling that has been linked to endothelial inflammation.27,28 Online Figure VI demonstrated that 10 μmol/L of LPA (4 hours) significantly stimulated proadhesive vascular cell adhesion protein 1 (VCAM1) expression in HAEC that were preconditioned by atherosusceptible waveform for 72 hours. VCAM1 expression was notably reduced in cells preconditioned by atheroprotective flow for 72 hours followed by LPA treatment. High-performance liquid chromatography electrospray ionization tandem mass spectrometry42 was used to determine the direct link between atherorelevant flows and the concentrations of bioactive LPA. Several LPA species detected in basal endothelial culture media (EGM-2, Lonza) were significantly lower when collected from HAEC subjected to atheroprotective flow (72 hours) when compared with atherosusceptible flow (Online Figure VII). Moreover, siRNA-mediated PPAP2B knockdown led to an increase of most LPA species in the media to the level similar to those collected from cells exposed to atherosusceptible flow. Consistently, PPAP2B overexpression in HAEC resulted in reduced LPA species in the media collected from cells exposed to atherosusceptible waveform (Online Figure VIII).

**Flow-Sensitive PPAP2B Contributes to the Anti-Inflammatory/Adhesive Endothelial Phenotype by Inactivating LPAR1-Mediated Signaling**

To investigate the causative role of PPAP2B and LPA signaling in directing the anti-inflammatory endothelial phenotype associated with atheroprotective shear stress, PPAP2B-targeting siRNAs and a LPA receptor antagonist were used in HAEC subjected to 72-hour atherorelevant flows. Increased PPAP2B expression under atheroprotective shear stress (Figure 5A) was associated with elevated KLF2 levels (Figure 5B) and a significant suppression of proinflammatory genes monocyte chemotactant protein-1 (MCP1), VCAM1, and E-selectin (SELE) when compared with cells exposed to atherosusceptible flow.
Figure 4. Phosphatidic Acid Phosphatase type 2B (PPAP2B) knockdown abolishes endothelial alignment induced by atheroprotective waveform. A. Human aortic endothelial cell monolayer displayed uniformly elongated morphology parallel to the direction of atheroprotective waveform (72 hours), instead of random polygonal structure under static conditions, as indicated by cell borders stained with VE-cadherin (green), stress fiber (red) marked by phalloidin, and nuclei demonstrated by 4′,6-diamidino-2-phenylindole. PPAP2B knockdown by small interfering RNAs (siRNAs) significantly abrogated unidirectional flow-induced cell polarity. B. Cell alignment is quantified as the angle of stress fibers (red) from the flow direction. Three stress fibers were measured per cell, with 30 to 50 cells measured per condition. All data are represented as means±SEM. n=5. *P<0.05, 2-way ANOVA. siCtrl indicates control siRNA; and siPPAP2B, PPAP2B siRNA.

(Online Figure X–XIVD). siRNA-mediated knockdown of PPAP2B during atheroprotective flow (Figure 5A) had no effect on the KLF2 expression (Figure 5B) but resulted in an increased expression of these inflammatory genes to levels similar or higher than in cells exposed to atherosusceptible flow (Figure 5C–5E). KLF2 expression was not affected by PPAP2B knockdown in HAEC under static condition (Online Figure IX). LPA receptor 1/3 (LPAR1/3) antagonist, Ki16425, restored the anti-inflammatory endothelial phenotype (reduced expression of proinflammatory genes) in siPPAP2B-treated cells under atheroprotective flow (Figure 5F). Increased MCP1, VCAM1, and SELE expression by PPAP2B knockdown in cells exposed to atheroprotective flow were validated by a second set of siRNA that targets human PPAP2B (Online Figure X). Conversely, overexpression of human PPAP2B notably reduced MCP1, VCAM1, and SELE expression in HAEC under static conditions (Online Figure XI). PPAP2B overexpression suppressed the proinflammatory endothelial phenotype in cells exposed to atherosusceptible flow, as demonstrated by reduced expression of MCP1, VCAM1, and SELE (Online Figure XIB). Consistent with the contributory role of LPA signaling in promoting inflammation in PPAP2B-deficient cells, knockdown of endogenous PPAP2B had no effect on tumor necrosis factor-α stimulated expression of MCP1, VCAM1, and SELE (Online Figure XII) but significantly increased the expression of proinflammatory/adhesive genes in HAEC treated with LPA (Online Figure XIII). Together, these data suggest that endothelial inflammation associated with disturbed flow is significantly regulated by signaling via LPA through LPAR1/3 receptors. PPAP2B, which reduces LPA concentration, underlies the anti-inflammatory endothelia associated with the atheroprotective hemodynamics by inactivating LPAR1/3-mediated signaling.

Similarly, Ki16425 significantly reduced expression of MCP1, VCAM1, and SELE in HAEC under static conditions (Online Figure XIV). Functional consequences of PPAP2B knockdown in HAEC were further determined by leukocyte–endothelial interactions, using human acute monocytic leukaemia cell line THP-1. PPAP2B deficiency greatly increased the numbers of THP-1 cells adherent to the endothelial monolayer (Figure 5G). Moreover, LPAR antagonist Ki16425 partially restored the antiadhesive phenotype in PPAP2B-deficient HAEC, consistent with the anti-inflammatory role of PPAP2B via inhibition of the LPA signaling. Given the undetectable levels of LPAR3 transcripts in HAEC as assessed by real-time qPCR (data not shown), it is likely that the effect of Ki16425 (LPAR1/3 antagonist) is largely attributable to inhibition of LPAR1 in these cells. Accordingly, siRNA-mediated knockdown of endogenous LPAR1 resulted in reduced expression of MCP1, VCAM1, and SELE in HAEC (Online Figure XIVB), suggesting that LPAR1 activation accounts for the increased inflammation/adhesion in PPAP2B-deficient cells. Expression of MCP1, VCAM1, and SELE was not changed in cells with knockdown of endogenous LPAR2 (Online Figure XIVC), consistent with similar leukocyte adhesion in PPAP2B-deficient cells treated with or without LPAR2 antagonist H2L5186303 (Online Figure XIVD).

Flow-Sensitive PPAP2B Maintains the Endothelial Monolayer Integrity by Suppressing LPAR1- and 2-Mediated Signaling

In addition to chronic inflammation, increased vascular permeability is the hallmark of the atherosusceptible endothelial phenotype, whereas the underlying molecular mechanisms are poorly understood. We tested the hypothesis that lower PPAP2B contributes to the compromised vascular integrity associated with disturbed hemodynamics. As demonstrated in Figure 6A, exposure to atheroprotective flow notably reduced the formation of intercellular gaps (0% gap area) in HAEC monolayers compared with control cells under disturbed atherosusceptible flow (0.87% gap area). Knockdown of PPAP2B under atheroprotective flow significantly increased intercellular gap area (from 0% to 0.68%). Compromised endothelial monolayer integrity has been mechanistically linked to increased cell contractility resulting from myosin light chain (MLC) phosphorylation. Western blotting with phospho-MLC antibodies revealed a suppressed MLC phosphorylation in HAEC exposed to atheroprotective flow, which was restored on PPAP2B knockdown (Figure 6B). Similarly, PPAP2B knockdown in HAEC under static conditions resulted in an
increased intercellular gap formation (Figure 6C). Consistent with the role PPAP2B in hydrolyzing LPA, worsened monolayer integrity was detected in PPAP2B-deficient cells treated with LPA (Figure 6D). Electric Cell-substrate Impedance Sensing detected reduced monolayer resistance (Figure 6E), and Western blot showed elevated MLC phosphorylation in HAEC monolayer with PPAP2B knockdown (Figure 7A).

Phosphorylation of MLC is controlled at least in part by Rho-family small GTPase RhoA and its effector Rho-dependent protein kinase (ROCK) through ROCK-dependent phosphorylation and inhibition of MLC phosphatase (MLCP), resulting in increased actomyosin-based contractility and consequent elevated permeability of endothelial cells. An inhibitor of ROCKs, Y-27632, completely abrogated the induced MLC phosphorylation in PPAP2B-deficient cells (Figure 7B). Finally, MLC phosphorylation in PPAP2B-deficient cells was abrogated by siRNAs targeting LPAR1 or LPAR2 (Figure 7C). Together, our data suggest the following model for the induction of atherosusceptible phenotype of endothelia by disturbed flow. Atherosusceptible flow promotes downregulation of PPAP2B through an increased expression of PPAP2B-targeting miR-92a. A decrease in PPAP2B levels results in accumulation of LPA, which drives inflammatory/adhesive endothelial phenotype through LPAR1 signaling and increased endothelial permeability through LPAR1/2 contractile signaling (Figure 7D).

**CAD Risk Allele at rs17114036 Is Associated With Lower PPAP2B Expression in HAEC**

PPAP2B was implicated in CAD by GWAS but the association of the risk SNP rs17114036 in the locus, and the expression of PPAP2B in human tissues is poorly defined. Our
data demonstrated the atheroprotective role of PPAP2B by inhibiting endothelial inflammation and permeability, implicating lower vascular PPAP2B expression is associated with the CAD risk allele at rs17114036 and increased susceptibility to CAD. eQTL mapping provides a functional reference to examine the effect of disease-associated SNPs on the expression of genes in various tissues. Recently, eQTL mapping of gene expression in HAEC isolated from 147 human donors identified the risk allele at SNP rs6588635, proxy to SNP rs17114036 (r²=0.831), is associated with lower endothelial PPAP2B expression. To better understand the regulation of PPAP2B by the CAD risk locus, we imputed the genotypes of 147 donors based on the 1000 genomes reference panel and calculated the association of PPAP2B expression and the imputed SNPs in the region. There was a perfect overlap between the association of SNPs in the 1p32.2 locus with CAD susceptibility (Figure 8A) and the PPAP2B expression in HAEC (Figure 8B). These results suggested that CAD risk SNPs increase the disease susceptibility by regulating PPAP2B expression in endothelial cells. Minor allele frequency of the most significantly associated risk SNP (rs17114036) is 9.575% in our data set with 28 individuals of heterozygous carriers (AG); the rest were risk allele (AA) carriers. Stratification of individuals based on their genotypes of the SNP rs17114036 indicated that the risk allele (AA) was significantly associated with lower expression of PPAP2B (P=6.6×10⁻¹³; Figure 8C), consistent with the atheroprotective role of PPAP2B suggested in this study. These data collectively demonstrate that risk allele A at rs17114036 in humans is associated with increased CAD susceptibility and lower expression of PPAP2B in HAEC. Furthermore, we interrogated publically available eQTL data sets and determined that the SNPs in the risk locus were not associated with PPAP2B expression in whole blood, monocytes and macrophages, lymphoblastoid cell lines, skin fibroblasts and adipose tissue, brain, and liver, suggesting that while this gene may be expressed in the interrogated tissues, the CAD risk locus regulates the expression of PPAP2B in an endothelium-specific manner.
Discussion

Disturbed arterial flow has been linked to atherosusceptible endothelia that contribute to the limited distribution of atherosclerosis at predictable vascular sites, despite exposure of the entire arterial tree to systemic risk factors. Herein, we proposed that reduced PPAP2B resulting from disturbed flow sensitizes the endothelia to local LPA that favors the inflammatory and permeable vascular functions associated with atherogenesis. In this study, we highlight the importance of this system in responding to mechanical flow patterns that augment or inhibit atherogenesis through several strategies to modulate LPA/LPAR signaling in endothelial cells (LPA treatment of endothelial cells; downregulation of the expression of enzyme PPAP2B using either siRNA or miR-92a mimic and the use of LPAR antagonists). Recent GWAS has linked PPAP2B to CAD susceptibility but mechanoregulation of PPAP2B and its upstream regulators are poorly understood. We used in vivo swine and murine along with a complementary in vitro flow device to establish that atherorelevant hemodynamic forces causatively modulate endothelial PPAP2B expression which is suppressed by disturbed flow-induced miR-92a. In addition, PPAP2B expression is significantly reduced in the downstream sections of the human carotid plaques where disturbed flow is prevalent and miR-92a is upregulated. Moreover, mechanosensitive PPAP2B expression is critical for mediating cell alignment, promoting anti-inflammatory phenotype, and maintaining vascular integrity in endothelial monolayer subjected to unidirectional blood flow. The atheroprotective role of endothelial PPAP2B is further addressed by eQTL mapping demonstrating significant association between PPAP2B CAD risk allele (rs17114036) and reduced PPAP2B expression in HAEC, which was not detected in other major human tissues. Collectively, these studies elucidate novel atherorelevant mechanotransduction mechanisms mediated by GWAS-identified CAD gene PPAP2B in vascular endothelial cells.

Although mechanoregulation of LPA signaling and arterial site–dependent response to LPA stimulation have not been suggested, LPA-activated cellular signaling was previously implicated in vascular dysfunction and atherogenesis. First, LPA is a potent lipid messenger that acts on LPA receptors to stimulate endothelial inflammation, promote stress fiber formation, and increase monolayer permeability. Second, atherosclerosis in Apoe−/− mice is notably reduced by systemic inhibition of LPA receptors using the pharmacological antagonist Ki16425. Consistent with the enzymatic function of PPAP2B in degrading LPA, enhanced leukocyte adhesion and increased vascular leakage were reported in Tie2-mediated genetic deletion of PPAP2B in C57BL/6 mice. Our data provide the first line of evidence suggesting vascular site–specific responses to LPA activation as the result of regional endothelial PPAP2B expression associated with spatial hemodynamics. LPA signaling is proposed to act locally, consistent with our investigation addressing focal LPA sensitization as an unrecognized molecular signature contributing to the atherosusceptible endothelia activated by disturbed flow in limited vascular regions.
It is proposed that flow-mediated, site-specific endothelial functional differences do not result in significant vascular injury unless exposed to additional systemic risk factors. Our results indicated that endothelia exposed to disturbed flow are primed to LPA stimulation which might be further augmented by additional atherogenic factors because LPA is produced during mild oxidation of low-density lipoprotein and accumulates in human atherosclerotic plaques. Recently, plasma long-chain unsaturated LPA was reported to be elevated in patients with acute coronary syndrome. Indeed, our data demonstrate a sensitized inflammation in PPAP2B-deficient cells treated with LPA but not tumor necrosis factor-α.

PPAP2B has been shown to hydrolyze bioactive lipids, such as LPA and sphingosine-1-phosphate. Atheroprotective flow decreased LPA species and sphingosine-1-phosphate in media that is dependent on the elevated endothelial PPAP2B, because PPAP2B knockdown increased major LPA species (Online Figure VII) and sphingosine-1-phosphate (Online Figure XV) under atheroprotective flow. Our study addressed the contribution of LPA receptors mediating the elevated inflammation and enhanced permeability in PPAP2B-deficient cells (Figure 6D) using small molecules and siRNAs specifically targeting LPA signaling. Disturbed flow–induced inflammation is attributable to elevated LPA as a result of lower PPAP2B expression and consequent LPAR1 activation. The interruption of LPAR 1/3 signaling by receptor antagonist Ki16425 restored the unidirectional flow-mediated, antiadhesive endothelial monolayer in PPAP2B-deficient HAEC. Because LPAR3 is not expressed in HAEC, this implicates LPAR1 as the cognate receptor. This result accords with the reported regulatory role of LPAR1 in activating nuclear factor-κB, a critical transcription factor which promotes the proinflammatory endothelial phenotype associated with disturbed hemodynamics. Our data are consistent with the ability of Ki16425 to reduce LPA-induced inflammation in mouse lungs stimulated by lipopolysaccharide and in arterial vascu-
tures of Apoe−/− mice. Conversely, activation of LPAR1 and LPAR2 leads to ROCK-mediated MLC phosphorylation and compromised vascular integrity in PPAP2B-deficient endothelia, as inhibition of LPAR1, LPAR2, and RhoA/ROCK significantly attenuated MLC phosphorylation and intercellular gap formation promoted by PPAP2B knockdown. Activation of RhoA by LPAR1 and LPAR2 was reported, and the causative role of RhoA in stimulating MLC phosphorylation and monolayer permeability was demonstrated in pulmonary endothelia. LPA is a potent stimulator of endothelial stress fiber and intercellular gap formation. Here, we demonstrated that chronic atheroprotective flow can indirectly inactivate RhoA and MLC phosphorylation by promoting PPAP2B expression. Our data demonstrate that PPAP2B is decreased in HAEC under atherosusceptible flow within 5 hours (Online Figure XVIA) and is accompanied by the increased LPA in the media (Online Figure XVIB). We propose that rapid mechanotransduction responses contribute to the decrease of endothelial PPAP2B associated with acute or chronic disturbed flow in vivo. Indeed, miR-92a and KLF2, the upstream regulators of PPAP2B demonstrated here, are modulated by atherorelevant flow within a few hours and the regulation remains with the hemodynamic application. The rapid response of PPAP2B
to atherorelevant hemodynamic indicates the putative role of PPAP2B in rapid endothelial response to flow, such as radical formation. Indeed, LPA signaling has been linked to reactive oxygen species production in ovarian cancer cells.54

Recent GWAS investigations have identified 46 loci associated with CAD, including a risk allele at rs17114036 located in human PPAP2B gene (Chromosome 1p32.2) that predicts increased CAD susceptibility (odds ratio=1.17; P=3.81×10⁻¹⁹).30 GWAS studies have led to discovery of novel genes influencing pathogenesis of CAD, particularly new biology in lipid metabolism.55 Significant association of the risk SNP with the expression level of PPAP2B in endothelial cells but not in other tissues supports the involvement of PPAP2B as a probable causal gene related to the CAD 1p32.2 locus in the atheroprotective role of PPAP2B in mediating endothelial health. The molecular mechanism leading to reduced endothelial PPAP2B expression associated with the CAD risk allele is unclear. This will be the subject of a future study.

Endothelium-enriched miR-92a has recently emerged as a major small noncoding RNA that mediates atherosusceptible endothelia by inhibiting transcription factors KLF2 and KLF4.34,39 Systemic delivery of miR-92a inhibitors reduced atherosclerosis burden in mice.38,56 In addition, miR-92a inhibition significantly improved neoangiogenesis in animal models of ischemia-induced injury.50,57 In this study, in silico prediction and experimental validation have led to identification of miR-92a as a novel post-transcriptional suppressor of mechanosensitive PPAP2B that inhibits vascular inflammation and permeability. Our data demonstrate that PPAP2B is significantly lower in vivo in swine AA, lesser curvatures of mouse AA, and in partial-ligated mouse LCA where disturbed flow is prevalent and endothelial miR-92a expression is increased. This is in agreement with the reduced PPAP2B levels in the downstream sections of human carotid plaques where low shear stress prevails and miR-92a is elevated.39 Moreover, KLF2 was identified here as a positive transcription activator of PPAP2B, supporting the coordinated action of miR-92a in inhibiting PPAP2B. Conversely, PPAP2B inhibition has no effect on KLF2 expression and its sensitivity to atheroprotective flow, indicating that the anti-inflammatory role of PPAP2B is not because of its modulation of KLF2, and that GWAS-implicated CAD gene PPAP2B can contribute to the atheroprotective function of KLF2 by inhibiting the LPA signaling in vascular endothelia. Our data further support the miR-92a-mediated suppression of PPAP2B because miR-92a has been demonstrated as a negative regulator of endothelial KLF2.34,59 Interestingly, PPAP2B knockdown led to the reduction of nitric oxide synthase 3 transcripts (Online Figure XVII) suggesting that flow-sensitive PPAP2B can promote vascular health by increasing endothelial nitric oxide synthase expression. Conversely, nitric oxide synthase inhibitor L-N-Nitroarginine methyl ester and endothelial nitric oxide synthase–targeting siRNAs have no significant effect on the endothelial PPAP2B expression (Online Figure XVIII).

Regional targeting of dysregulated endothelial miR-92a in disturbed flow-prevalent arterial sites is predicted to restore vascular health, therefore providing an attractive strategy for future developments of arterial wall–based atherosclerosis therapy complementary to current pharmacological treatments that aim to reduce systemic risk factors. We have recently engineered lesion-targeting polyelectrolyte complex micelles as an innovative delivery system for therapeutic nucleotides and tested their effectiveness in inhibiting atherogenic miR-92a in inflammatory endothelia in vitro,58 establishing the proof of concept for modulating regional expression of endothelial PPAP2B in treating atherosclerotic diseases.

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We thank Drs Catherine Reardon, Godfrey Getz, James Liao, and Irena Levitan for critical discussions of the article, and Dr Matthew Churpek for suggestions of statistical analyses.

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Disclosures

None.

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11. Chang K, Weiss D, Suo J, Vega JD, Giddens D, Taylor WR, Jo H. Bone morphogenetic protein antagonists are coexpressed with bone morphogenetic protein 4 in endothelial cells exposed to unstable flow in vitro in mouse aortas and in human coronary arteries: role of bone morphogenetic...


What Is Known?

• Phosphatidic Acid Phosphatase type 2B (PPAP2B), also known as lipid phosphate phosphatase 3, is implicated in coronary artery disease (CAD) by genome-wide association studies.

• Atherosclerosis occurs preferentially at arterial sites where endothelia are activated by local disturbed flow.

• Lysophosphatidic acid, a bioactive lipid hydrolyzed by PPAP2B and elevated in CAD patients, contributes to vascular dysfunction and atherogenesis.

What New Information Does This Article Contribute?

• Reduced PPAP2B was detected in atherosusceptible arterial sites exposed to disturbed flow in mice, swine, and humans, resulting from elevated miR-92a and decreased KLF2.

• PPAP2B mediates the anti-inflammatory/permeable phenotype in endothelia under unidirectional flow by inhibiting lysophosphatidic acid signaling.

• In humans, CAD risk allele at rs17114036 is associated with reduced PPAP2B in aortic endothelia.

Genome-wide association studies have identified ≈46 genetic loci associated with CAD. We hypothesize that genome-wide association studies-identified CAD gene(s) participate in mechanotransduction mechanisms by which vascular endothelia are activated by local disturbed flow leading to focal origin of atherosclerosis. We report that PPAP2B is significantly reduced in atherosusceptible sites associated with disturbed flow–induced miR-92a, consistent with decreased endothelial PPAP2B linked to CAD risk allele. PPAP2B inhibition abolishes the atheroprotection of unidirectional flow. Modulation of miR92a–PPAP2B signaling axis in atherosusceptible endothelia may alleviate atherosclerotic burdens.
Mechanosensitive PPAP2B Regulates Endothelial Responses to Atherorelevant Hemodynamic Forces

Congqing Wu, Ru-Ting Huang, Cheng-Hsiang Kuo, Sandeep Kumar, Chan Woo Kim, Yen-Chen Lin, Yen-Ju Chen, Anna Birukova, Konstantin G. Birukov, Nickolai O. Dulin, Mete Civelek, Aldons J. Lusis, Xavier Loyer, Alain Tedgui, Guohao Dai, Hanjoong Jo and Yun Fang

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Online Methods

Porcine endothelial cell isolation
Endothelial cells from porcine aorta were isolated as described previously\(^1\). Aortic tissues were freshly collected from adult pigs (5-mo-old; ~200 lb). Endothelial cells were harvested from aortic regions illustrated in Figure 1A by gentle scraping. Cells were transferred directly to lysis buffer for protein extraction. Representative cell scrapes were periodically transferred to glass microscope slides to monitor cell purity by immunostaining for EC marker PECAM1, smooth muscle cell marker SM22-alpha as well as leukocyte marker CD45, as previously described\(^1\). Endothelial purity was detected at greater than 95%, consistent with previous studies\(^1\). Moreover, swine protein lysates were immunoblotted for EC marker VE-cadherin and smooth muscle cell marker SM22-alpha to verify the endothelial purity, as shown in Figure 1A. Samples with detectable levels of SM22-alpha were discarded. All procedures were in accordance with National Institutes of Health guidelines, and approved by the Institutional Animal Care and Use Committee.

Regional isolation of endothelial RNA from mouse aortic arch
Mouse aortic arch was harvested and carefully dissected under stereo microscope. After removal of periadventitial fat, aortic arch was opened en face. The lesser curvature (LC) and greater curvature (GC) regions, as illustrated in Figure 1B, were separated and placed on a nitrocellulose membrane soaked in isopropanol for 5 min. Then the media/adventitia were peeled away while the endothelia adherent to the membrane. Total RNA was extracted using Qiagen miReasy kit. LC and GC regions were pooled from 2 to 3 mice, as described previously\(^5\).

Application of athero-relevant flows in vitro
Athero-protective or athero-susceptible waveform from human carotid arteries\(^6\) was replicated using a flow device consisting of a computerized stepper motor UMD-17 (Arcus Technology) and a 1° tapered stainless steel cone, which was modified based on the flow system developed by Dai et al\(^6\). The flow devices were placed in 37°C incubator with 5% CO\(_2\). HAEC at 100% confluence, maintained in EGM2 medium containing 4% dextran in 6-well plates, were subjected to athero-protective or athero-susceptible flow waveform for 24 or 72 hours.

Mouse acute disturbed flow model (partial carotid ligation)
Acute disturbed flow was created by partial ligation of wild type C57BL/6 mouse left common carotid artery (LCA), as described previously\(^7\). In brief, wild type C57BL/6 mice (Jackson Laboratories) were anesthetized under isoflurane. A small incision in the neck was made to expose LCA. Among the four caudal branches of LCA, only superior thyroid artery (STA) was left intact while external carotid artery (ECA), internal carotid artery (ICA), and occipital artery (OA) were ligated with 6-0 silk suture, as shown in Figure 1D. Reduced and oscillatory flow in the LCA after ligation was predicted by computational fluid dynamics and detected by high-resolution ultrasound measurements while blood flow in right carotid artery (RCA) did not change significantly compared to that of pre-ligation\(^7\). Two days after the LCA partial ligation, mice were sacrificed. Intimal RNA as well as medial/adventitial RNA was isolated from both RCA and ligated LCA for real-time quantitative PCR (qPCR) analyses. All mice were housed in a specific pathogen-free facility and used in accordance with National Institute of Health guidelines and protocols approved by the Institutional Animal Care and Use Committee.

Intimal and medial/adventitial RNA isolation form carotid arteries
Mouse were killed with CO\(_2\) inhalation and perfused with saline containing heparin via the left ventricle. LCA and RCA were isolated and cleaned of periadventitial fat. The lumen was then
quickly flushed with 150 ul of QIAzol (Qiagen) using a 29-gauge syringe. The eluate was collected for intimal RNA isolation using RNeasy mini kit (Qiagen) according to the manufacturer’s instructions. The remaining artery after QIAzol flushing was used for medial and adventitia RNA isolation. To test endothelial enrichment of the intimal RNA, real-time qPCR was performed on endothelial marker PECAM1, smooth muscle cell marker α-SMA, and leukocyte marker CD45. Intimal RNA is nearly free of medial/adventitial RNA contamination.\(^7\)

**PPAP2B expression in human atherosclerotic lesions**

The study was approved by the local ethical committee and all participants gave written informed consent, as described previously.\(^8\) PPAP2B mRNA abundance was assessed in human atherosclerotic plaques obtained from patients undergoing carotid endarterectomy. Carotid samples were cut into upstream and downstream parts of atherosclerotic lesions. Total RNAs were prepared using Trizol. PPAP2B expression was quantified and normalized to GAPDH.

**Expression quantitative trait locus (eQTL) mapping in Humans**

Gene expression profiling of PPAP2B and neighboring loci and genotyping of PPAP2B risk alleles were performed on endothelial cells (HAEC) isolated from healthy aortas of 147 donors under control and proatherogenic conditions. Association analysis was described previously.\(^9\)

**Cells**

HAEC was obtained from Lonza and maintained in EGM2 medium (Lonza). HEK was maintained in DMEM with 10% FBS. THP-1 was maintained in RPMI-1640 medium supplemented with 10% FBS and 0.05 mM 2-mercaptoethanol.

**Reagents and antibodies**

LPAR1/3 antagonist Ki16425 (working concentration: 10 µM) was purchased from Cayman Chemical (Cat# 10012569). LPAR2 antagonist H2L5186303 (working concentration: 1 µM) was purchased from Tocris (Cat# 4878). ROCK inhibitor Y-27632 (working concentration: 10 µM) was purchased from BD Biosciences (Cat# 562822). LPA was obtained from Santa Cruz Biotechnology (Cat# 22556-62-3). High molecular weight dextran was obtained from Sigma (Cat# 31392-50G). L-NAME was purchased from Sigma (Cat# N5751). Antibodies are listed in Online Table I.

**mRNA and miR quantitative real-time PCR**

Total RNA was isolated using mirVana miRNA isolation kit (Life Technologies) or Direct-zol RNA MiniPrep (Zymo Research), unless stated otherwise. mRNA was reverse-transcribed into cDNA using SuperScript III (Life Technologies) or High Capacity cDNA Reverse Transcription kit (Life Technologies). miRNA was reverse-transcribed using TaqMan MicroRNA reverse transcription kit (Life Technologies). cDNA quantification was performed on LightCycler 480 II (Roche) using LightCycler 480 Master Hydrolysis Probes for miR or SYBR Green I Master for mRNA. Absolute quantification of each gene of interest was normalized to the geometric mean of beta-actin, ubiquitin, and GAPDH. The expression level of miR-92a was normalized to sno-U6 RNA. PCR primers are listed in Online Table II.

**Western Blot**

Whole cell extracts were separated by NuPAGE 4-12% Bis-Tris Protein Gels (Life Technologies) and transferred to PVDF membrane using iBlot Gel Transfer System (Life Technologies). After blocking with 5% non-fat milk in TBST, the membrane was incubated with primary antibody against proteins of interest, followed by incubation with HRP-conjugated
secondary antibody. Blots were developed with the ECL system (Pierce). Luminescence was detected by ChemiDoc MP Imager (Bio-rad).

**Transfection of miR mimics and inhibitors**
Endogenous expression of miR-92a was inhibited by transfection with 50 nM miR-92a hairpin inhibitors (Dharmacon). Over-expression of miR-92a was performed with 1 nM miR-92a mimic (Dharmacon). The transfection was performed using Lipofectamine RNAiMAX according to manufacturer’s instructions.

**Luciferase assay of PPAP2B 3’UTR**
Wild-type or mutated PPAP2B 3’UTR was inserted downstream of Gaussia luciferase (GLuc) gene in pEZX-MT05 vector (GeneCopoeia). Secreted Alkaline Phosphatase (SEAP) driven by CMV promoter in the same vector served as control reporter. HAECs or HEK cells were transfected with given vectors by using Lipofectamine LTX (Invitrogen) 24 hrs after introduction of hsa-miR-92a mimics or inhibitors. Secreted Gluc and SEAP activity was measured by Secrete-Pair™ Dual Luminescence Assay Kit (GeneCopoeia) on Cytation3 (BioTek).

**Mutagenesis of miR-92a target site in PPAP2B 3’UTR**
HmiT055492-MT05 plasmid containing PPAP2B 3’UTR was purchased from GeneCopoeia. As shown in Figure 2F, mutagenesis of the putative miR-92a target site within PPAP2B 3’UTR was performed using QuickChange Lightning kit (Agilent Technologies) according to the manufacturer's protocol. Mutation was confirmed by DNA sequencing.

**KLF2 overexpression in HAEC via adenovirus**
HAECs were infected with adenovirus expressing KLF2 or GFP (Vector Biosystems Inc) at 200 m.o.i. (multiplicity of infection) using GeneJammer Transfection Reagent (Agilent Technologies) according to manufacturer’s instructions. Virus was removed after 24 hours.

**PPAP2B overexpression in HAEC via plasmid**
HAECs were seeded at 4x10⁵ cells/well in 6-well plate or 8x10⁴ cells/well in 24-well plate and transfected with 5 ug (6-well plate) or 1 ug (24-well plate) of PPAP2B expression or null plasmid (GeneCopoeia) using Lipofectamine LTX (Invitrogen). After 1 hour, transfection mixture was replaced with fresh growth medium EGM2. Any subsequent experiment on transfected cells was conducted 24 hours after transfection.

**siRNA and transfection**
Control siRNAs and siRNAs targeting PPAP2B, LPAR1, LPAR2, and NOS3 (eNOS) were purchased from Qiagen. PPAP2B siRNA (S103043761, Qiagen) was used for most of the experiments for PPAP2B knockdown. A second set of PPAP2B siRNA (S103081995, Qiagen) was used to validate siRNA specificity, as showed in Online Figure IX. The transfection, using 50 nM of siRNA for cells in 24-well plate or 100 nM of siRNA for cells in 6-well plate, was performed using Lipofectamine RNAiMAX(Life Technologies) according to manufacturer’s instructions.

**Immunofluorescence staining**
Cells in 6-well plates were fixed in PBS containing 3.7% formaldehyde, permeabilized with 0.25% Triton X-100 in PBS containing 0.1% Tween 20, and blocked with 2% BSA in PBS containing 0.1% Tween 20. To stain F actin, cells were incubated with PromoFluor-546 conjugated phalloidin. To stain VE-Cadherin, cells were incubated with rabbit polyclonal antibody against VE-Cadherin, followed by Alexa Flour 488 Goat Anti-Rabbit IgG. Images were acquired at 20X on Nikon Elipse TE300.
Endothelial cell alignment
Cells were cultured and stained on 6-well plate. Images were captured at 1 cm from the center of the well at 20X on Nikon Elipse TE300, meanwhile the flow direction were marked for each image. Then cell alignment was quantified using two different methods on the same images. In Figure 4, we measured the angle of stress fibers to flow direction, as described previously\(^\text{10}\). Three stress fibers were randomly selected and measured per cell. In Online Figure V, we measured the angle of longest axis to flow direction\(^\text{11}\). Total 30-50 cells were used to quantify the cell alignment for each given condition at every experiment. Five independent experiments were analyzed; the average values were shown in Figure 4B and Online Figure V. Due to the fine details and space constraint, we are limited to show cropped representative images.

Endothelial cell monolayer gap quantification
Cells were cultured and stained on 6-well plate. To acquire consistent images, a cross was drawn from the center of the well with 2 cm in length on the back of 6-well plate. One image was captured at each of the 4 endpoints of the cross at 20X on Nikon Elipse TE300. Intracellular gaps were quantified as the percent area of monolayer not covered by cells in Image J using the 4 images for each condition in each independent experiment. Three independent experiments were performed and total 12 images were examined in each given experimental condition. Due to the fine details, we are limited to show cropped representative images.

Measurement of transendothelial electrical resistance
Cell permeability was evaluated by measuring transendothelial electrical resistance (TER) across HAEC monolayers on 8 well electrode arrays 8W10E+ (Applied Biophysics) by an electrical cell-substrate impedance sensing system Model 1600R (Applied Biophysics) as described previously\(^\text{11}\).

Leukocyte-Endothelial Cell Adhesion Assay
THP-1 cells were labeled with 5 µM of calcein-AM (Life Technologies) for 30 minutes. Then, fluorescence-labeled THP-1 cells were incubated with PPAP2B or control siRNA-transfected HAECs for 1 hour. After washing with pre-warmed PBS, the adherent THP-1 was visualized with Elipse TE300 and the fluorescence intensity was measured with Cytation 3 (BioTek).

Expression quantitative locus (eQTL) mapping
Gene expression profiling as well as genotyping and association analysis of human aortic endothelial cells (HAEC) isolated from 147 donors was described previously\(^\text{9}\).

LPA and S1P measurement
LPA was extracted from culture media by a modified Bligh-Dyer method followed by the measurements employing high-performance liquid chromatography electrospray ionization tandem mass spectrometry (HPLC ESI-MS/MS) technology, as described previously\(^\text{12}\). The measurements of LPA and S1P were performed by the Lipidomics Core Facility at Virginia Commonwealth University and Wayne State University.

Statistics
Data are expressed as mean ± SEM. Differences between two groups were analyzed by 2-tailed unpaired Student’s t test. One-way and two-way ANOVA were performed on multiple group comparisons for single and two variables, respectively. \(P < 0.05\) was considered significant.
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References


Online Figure I. PPAP2B expression was not different in the media/adventitia between the lesser curvature (LC) and the greater curvature (GC) of mouse aorta (A) or between mouse ligated LCA and non-surgical RCA (B). All data are represented as mean ± SEM. n = 5-6. t-test.
Online Figure II. miR-92a overexpression decreased ITGA5 (A), while inhibition of miR-92a increased ITGA5 expression (B) in HAEC. All data are represented as mean ± SEM. n = 5. * p < 0.05, t-test.
Online Figure III. miR-92a mimics (1 µmol/L) significantly repress the activity of luciferase containing human PPAP2B 3’UTR in HEK-293 cells. All data are represented as mean ± SEM. n = 3. * P < 0.05, t-test.
Online Figure IV. Elevation of endothelial miR-92a by disturbed flow in vivo and in vitro. (A) Endothelial miR-92a was significantly increased in mouse left carotid artery (LCA) that was subjected to partial ligation (48 hrs) to create acute disturbed flow when compared with non-surgical right carotid artery (RCA). (B) miR-92a abundance in HAEC was significantly increased by athero-susceptible waveform in vitro. All data are represented as mean ± SEM. n = 3. * p < 0.05, t-test.
Online Figure V. Cell alignment is quantified as the angle of longest axis from the flow direction. siCtrl: control siRNA; siPPAP2B, PPAP2B siRNA. All data are represented as mean ± SEM. n = 5. * p < 0.05, two-way ANOVA.
**Online Figure VI.** Precondition with athero-protective waveform (72 hrs) ameliorates LPA (10 µmol/L, 4 hrs)-induced VCAM1 expression in HAEC. All data are represented as mean ± SEM. n = 3. * p < 0.05, t-test.
Online Figure VII. Lower concentrations of LPA species in medium collected from HAEC subjected to athero-protective waveform for 72 hours when compared with athero-susceptible waveform, especially unsaturated LPA species. PPAP2B knockdown by siRNA elevated LPA in medium even under athero-protective waveform. All data are represented as mean ± SEM. n = 3. * p < 0.05, t-test.
Online Figure VIII. Lower concentrations of LPA species in medium collected from HAEC subjected to PPAP2B overexpression via adenovirus under athero-protective waveform for 72 hours. All data are represented as mean ± SEM. n = 3. * p < 0.05, t-test.
Online Figure IX. PPAP2B knockdown by siRNA did not affect KLF2 expression in HAEC. All data are represented as mean ± SEM. n = 8. t-test.
**Online Figure X.** A second set of siRNA targeting PPAP2B showed similar efficiency in PPAP2B inhibition (A) and increased MCP1 (B), VCAM1 (C) and SELE (D) expression under athero-protective waveform. All data are represented as mean ± SEM. n = 3. * p < 0.05, t-test.
Online Figure XI. PPAP2B overexpression via plasmid transfection decreased MCP1, VCAM1, and SELE expression under static conditions (A) as well as athero-susceptible waveform (B). All data are represented as mean ± SEM. n = 3. * p < 0.05, t-test.
Online Figure XII. TNFα (5 ng/mL, 4 hrs)-stimulated expression of MCP1(A), VCAM1(B) and SELE(C) was not affected by PPAP2B siRNA. All data are represented as mean ± SEM. n = 6. * p < 0.05, t-test.
Online Figure XIII. LPA (10 µmol/L, 4 hrs)-stimulated expression of MCP1(A), VCAM1(B) and SELE(C) was increased by PPAP2B siRNA. All data are represented as mean ± SEM. n = 8. * p < 0.05, t-test.
Online Figure XIV. LPA receptor 1 inhibition, either by Ki16425 (A) or siRNA knockdown (B), decreases transcriptional expression of MCP1, VCAM1, and SELE in HAEC under static conditions. LPA receptor 2 inhibition by siRNA knockdown has no effect on MCP1, VCAM1, and SELE expression (C). (D) LPA receptor 2 inhibition by H2L5186303 did not affect fluorescence labeled THP-1 cell adhesion to endothelial monolayer induced by knockdown of PPAP2B by siRNA. All data are represented as mean ± SEM. n = 3-6. * p < 0.05, t-test.
Online Figure XV. Lower concentrations of S1P in medium collected from HAEC subjected to athero-protective waveform for 72 hours when compared with athero-susceptible waveform. PPAP2B knockdown by siRNA elevated S1P in medium even under athero-protective waveform. All data are represented as mean ± SEM. n = 3. * p < 0.05, t-test.
Online Figure XVI. Short-term athero-susceptible waveform (5 hr) decreased PPAP2B expression (A), accompanied by higher LPA concentrations in medium (B) compared with athero-protective waveform. All data are represented as mean ± SEM. n = 3. * p < 0.05, t-test.
Online Figure XVII. PPAP2B knockdown by siRNA decreased eNOS (NOS3) in HAEC. All data are represented as mean ± SEM. n = 8. * p < 0.05, t-test.
Online Figure XVIII. Nitric Oxide (NO) inhibition had no effect on PPAP2B expression in HAEC. (A) Inhibition of NO production by L-NAME (100 μM) did not affect PPAP2B expression under static conditions or athero-protective flow. (B) Knockdown of eNOS (NOS3) by siRNA did not affect PPAP2B expression under static conditions. All data are represented as mean ± SEM. n = 3-6, t-test.