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Mechano-Sensitive PPAP2B Regulates Endothelial Responses to Athero-Relevant Hemodynamic Forces

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Abstract

Rationale—PhosPhatidic-Acid-Phosphatase-type-2B (PPAP2B), an integral membrane protein that inactivates lysophosphatidic acid, was implicated in coronary artery disease (CAD) by genome-wide-association-studies (GWAS). However, it is unclear whether GWAS-identified CAD genes including PPAP2B participate in mechanotransduction mechanisms by which vascular endothelia respond to local athero-relevant 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 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 aortic endothelial cells (HAEC) under athero-susceptible 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 athero-protection of unidirectional flow; Inhibition of lysophosphatidic acid receptor 1 (LPAR1) restored the flow-dependent, anti-inflammatory phenotype in PPAP2B-deficient cells. PPAP2B inhibition resulted in myosin-light-chain phosphorylation and intercellular gaps, which

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DISCLOSURES

None.

were abolished by LPAR1/2 inhibition. Expression-quantitative-trait-locus-mapping demonstrated PPAP2B CAD risk allele is not linked to PPAP2B expression in various human tissues but significantly associated with reduced PPAP2B in HAEC.

Conclusions—Athero-relevant flows dynamically modulate endothelial PPAP2B expression through miR-92a and KLF2. Mechano-sensitive PPAP2B plays a critical role in promoting anti-inflammatory phenotype and maintaining vascular integrity of endothelial monolayer under athero-protective flow.

Keywords

Hemodynamics; endothelial cell; microRNA; Genome Wide Association Studies; atherosclerosis; vascular permeability

INTRODUCTION

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 “athero-susceptible” 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 “athero-protective” endothelia that are anti-inflammatory, anti-permeable, 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[5, 8], protein kinase Cζ[9, 10], bone morphogenic protein 4[11], angiopoietin-2[12] and unfolded protein response[13] by disturbed flow or up-regulation of vascular protective molecules such as eNOS, KLF2[14-16], KLF4[17, 18] and Sirtuin-1[19] by unidirectional shear stress. Bioactive lipids are critical regulators of cellular growth, death, senescence, adhesion, migration, inflammation, and intracellular trafficking[20]; 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 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 plaques[25] and plasma LPA is elevated in patients with acute coronary syndrome[26]. In ApoE-deficient mice, systemic inhibition of LPA receptors employing pharmacological antagonists notably reduced the

atherosclerotic burden[27]. LPA-activated cellular pathways are negatively regulated by lipid phosphate phosphatases (LPP), 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 athero-relevant hemodynamics.

Herein we tested the hypothesis that activation of LPA signaling is an unrecognized molecular mechanism that contributes to the athero-susceptible endothelia associated with disturbed flow. Specifically, we postulated that athero-protective hemodynamics elevates endothelial PhosPhatidic Acid Phosphatase type 2B (PPAP2B), also known as lipid phosphate phosphatases 3 (LPP3) 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[31]. Moreover, Smyth and colleagues recently reported that inducible inactivation of PPAP2B in endothelial and hematopoietic cells leads to vascular activation mediated by LPA receptor-dependent signaling[32]. However, mechano-regulation and non-coding 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 anti-permeable endothelial phenotype associated with athero-protective flow. Moreover, disturbed flow-induced microRNA-92a (miR-92a) was identified as a novel negative regulator governing the mechano-sensitivity of PPAP2B. The investigation provides new molecular insights of bioactive lipid-mediated signaling in determining the athero-relevant endothelial phenotypes in relation to spatial hemodynamics. Our data further elucidates the critical roles of mechanical stimuli and non-coding RNAs in modulating LPA signaling whose dys-regulation is associated with a variety of human diseases.

METHODS

A detailed methods section is available in the Online Data Supplement.

RESULTS

Reduced endothelial PPAP2B is associated with disturbed flow in vivo and in vitro

Mechano-regulation of endothelial PPAP2B by athero-relevant 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 aortic arch that is athero-susceptible due to chronic disturbed flow and from the nearby descending thoracic aorta that is relatively athero-resistant owing to unidirectional shear stress[33]. Endothelial purity was confirmed by showing high expression of endothelial marker VE-cadherin and undetectable levels of

smooth muscle cell marker SM22 alpha (Figure 1A), consistent with previous studies[6, 34]. Western blotting detected a 55 ± 8% lower expression of endothelial PPAP2B in aortic arch (AA) compared to nearby descending thoracic aorta (DT), 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 athero-susceptible 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 to the greater curvature (Online Figure I-A).

To test whether athero-relevant shear stresses causatively modulate endothelial PPAP2B, we adapted the “dynamic flow system”, an *in vitro* flow apparatus developed by Gimbrone and colleagues[36] to apply well-defined flow waveforms corresponding to arterial geometries and flow profiles in humans. HAEC were subjected to “athero-susceptible” flow mimicking wall shear stress in human carotid sinus, or to “athero-protective” flow representing wall shear stress in human distal internal carotid artery[36]. Real-time qPCR detected significantly higher (2.4-fold) PPAP2B transcripts in HAEC subjected to 24-hr athero-protective flow compared to athero-susceptible shear stress (Figure 1C). Prolonged exposure (72 hrs) further augmented PPAP2B mRNA (5.24-fold) and protein (7.21-fold) in HAEC exposed to athero-protective flow (Figure 1C).

The aforementioned swine and mouse models provided in vivo evidence linking endothelial PPAP2B expression to “chronic” athero-relevant flows. To further elucidate endothelial PPAP2B response to “acute” disturbed flow in vivo, we performed surgery to ligate three of the four caudal branches of mouse left common carotid (LCA) artery (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 (RCA)[37]. We isolated carotid intimal RNA samples with high endothelial purity; real-time qPCR detected high expression of endothelial marker PECAM1 (average threshold cycle: 20.9±0.4) while 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) while 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 non-surgical RCA 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 to non-surgical RCA (Online Figure I-B).

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 mechano-sensitive genes[38]. Consistent with results from swine and mouse, PPAP2B expression was significantly

reduced in the downstream sections of human carotid plagues where low shear stress prevails as compared to the upstream sections (Figure 1E).

miR-92a suppresses mechano-sensitive PPAP2B through 3'-untranslated region (UTR) binding

The regulatory mechanisms contributing to the mechano-sensitivity of PPAP2B are not clearly defined. We hypothesized that flow-sensitive miRNAs could be unrecognized posttranscriptional regulators of endothelial PPAP2B. Bioinformatics tool TargetScan predicted an evolutionarily-conserved putative binding site for miR-92a in the 3'-UTR of human PPAP2B. Notably, previous studies have demonstrated higher endothelial miR-92a levels in the lesser curvature of swine aortic arch in vivo[6, 34] and in cells subjected to disturbed flow *in vitro*[38, 39]. As shown in Figure 2A, miR-92a mimics significantly abolished the elevation of PPAP2B in HAEC subjected to 72-hr athero-protective flow. Similarly, miR-92a mimics suppressed PPAP2B whereas miR-92a inhibitors elevated PPAP2B in HAEC under static conditions at the transcriptional and translational levels (Figures 2B and 2C, respectively). Modulation of miR-92a in HAEC also led to regulation of Integrin subunit alpha 5 (Online Figure II), consistent with the previous studies[40]. 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 abovementioned luciferase vectors; knockdown of endogenous miR-92a in HAEC employing 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 (Figures 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 IV-A) and in HAEC exposed to athero-susceptible flow (Online Figure IV-B) where PPAP2B is reduced by disturbed flow. In agreement with these findings, increased miR-92a was reported in the lesser curvature of mouse aorta (compared with the greater curvature) and downstream sections of human carotid plagues (compared with the upstream sections)[38] where disturbed flow was prevalent and PPAP2B was reduced (Figures 1B and E).

KLF2 activates endothelial PPAP2B expression

We then investigated the regulation of PPAP2B expression by KLF2, a flow-sensitive transcription factor[14-16] and a direct downstream target of miR-92a[34, 39]. Adenovirus-mediated KLF2 over-expression (17.2 ± 2.6 fold mRNA) led to a significant up-regulation of PPAP2B in HAEC (Figure 3A). Dual-luciferase reporter assay was employed 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 over-expression 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 athero-susceptible flow (Figure 3D), consistent with the KLF2 activation of vascular PPAP2B.

Mechano-sensitive 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-hr athero-protective shear stress employing 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 to those[41] reported in human umbilical vein endothelial cells under steady flow of 12 dynes/cm^2. Knockdown of PPAP2B employing specific siRNA noticeably impaired the alignment of HAEC monolayer under athero-protective 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).

Athero-protective flow attenuates endothelial LPA signaling and reduces major species of bioactive LPA

Mechano-sensitivity of endothelial PPAP2B that degrades LPA indicates athero-protective flow is an unrecognized negative regulator of LPA receptor(s)-mediated cell signaling that has been linked to endothelial inflammation[27, 28]. Online Figure VI demonstrated that 10 μmol/L of LPA (4 hrs) significantly stimulated pro-adhesive Vascular Cell Adhesion Protein 1 (VCAM1) expression in HAEC that were preconditioned by athero-susceptible waveform for 72 hrs. VCAM1 expression was notably reduced in cells pre-conditioned by athero-protective flow for 72 hrs followed by LPA treatment. High-performance liquid chromatography electrospray ionization tandem mass spectrometry (HPLC ESI-MS/MS)[42] was employed to determine the direct link between athero-relevant 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 athero-protective flow (72 hrs) as compared to athero-susceptible 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 athero-susceptible flow. Consistently, PPAP2B over-expression in HAEC resulted in reduced LPA species in the media collected from cells exposed to athero-susceptible waveform (Online Figure VIII).

Flow-sensitive PPAP2B contributes to the anti-inflammatory/adhesive endothelial phenotype by inactivating LPA Receptor 1-mediated signaling

To investigate the causative role of PPAP2B and LPA signaling in directing the anti-inflammatory endothelial phenotype associated with athero-protective shear stress,

PPAP2B-targeting siRNAs and a LPA receptor antagonist was utilized in HAEC subjected to 72-hr athero-relevant flows. Increased PPAP2B expression under athero-protective shear stress (Figure 5A) was associated with elevated KLF2 levels (Figure 5B) and a significant suppression of pro-inflammatory genes Monocyte Chemoattractant Protein-1 (MCP1), Vascular Cell Adhesion Protein 1 (VCAM1) and E-selectin (SELE) as compared to cells exposed to athero-susceptible flow (Figures 5C-5E). siRNA-mediated knockdown of PPAP2B during athero-protective 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 athero-susceptible flow (Figures 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 pro-inflammatory genes) in siPPAP2B-treated cells under athero-protective flow (Figures 5F). Increased MCP1, VCAM1 and SELE expression by PPAP2B knockdown in cells exposed to athero-protective flow were validated by a second set of siRNA that targets human PPAP2B (Online Figure X). Conversely, over-expression of human PPAP2B notably reduced MCP1, VCAM1 and SELE expression in HAEC under static conditions (Online Figure XI-A). PPAP2B over-expression suppressed the pro-inflammatory endothelial phenotype in cells exposed to athero-susceptible flow, as demonstrated by reduced expression of MCP1, VCAM1 and SELE (Online Figure XI-B). Consistent with the contributory role of LPA signaling in promoting inflammation in PPAP2B-deficient cells, knockdown of endogenous PPAP2B had no effect on TNFα stimulated expression of MCP1, VCAM1 and SELE (Online Figure XII) but significantly increased the expression of pro-inflammatory/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 athero-protective 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-A). Functional consequences of PPAP2B knockdown in HAEC were further determined by leukocyte-endothelial interactions, using monocytic 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 anti-adhesive phenotype in PPAP2B-deficent 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 XIV-B), 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 XIV-C), consistent with similar leukocyte adhesion in PPAP2B-deficient cells treated with or without LPAR2 antagonist H2L5186303 (Online Figure XIV-D).

Flow-sensitive PPAP2B maintains the endothelial monolayer integrity by suppressing LPA Receptor 1 and 2-mediated signaling

In addition to chronic inflammation, increased vascular permeability is the hallmark of the athero-susceptible endothelial phenotype[7] while 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 athero-protective flow notably reduced the formation of intercellular gaps (0% gap area) in HAEC monolayers compared with control cells under disturbed athero-susceptible flow (0.87% gap area). Knockdown of PPAP2B under athero-protective flow significantly increased intercellular gap area (from 0% to 0.068%). Compromised endothelial monolayer integrity has been mechanistically linked to increased cell contractility resulting from myosin light chain (MLC) phosphorylation[43]. Western blotting with phospho-MLC antibodies revealed a suppressed MLC phosphorylation in HAEC exposed to athero-protective flow, which was restored upon 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 (ECIS) 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-kinase (ROCK) through ROCK-dependent phosphorylation and inhibition of MLC phosphatase (MLCP), resulting in an increased actomyosin-based contractility and consequent elevated permeability of endothelial cells[43, 44]. An inhibitor of ROCKs, Y-27632, completely abrogated the induced MLC phosphorylation in PPAP2B-deficient cells (Figure 7A). Furthermore, both LPAR1/3 antagonist Ki16425 and LPAR2 antagonist H2L5186303 decreased intercellular gap formation 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 athero-susceptible phenotype of endothelia by disturbed flow. Athero-susceptible 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[30] 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 athero-protective 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. Expression quantitative trait locus (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 ($r^2 = 0.831$), is associated with lower endothelial PPAP2B expression[31]. In order to better understand the regulation of PPAP2B by the CAD risk locus, we imputed the genotypes of 147 donors based on the 1,000 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 dataset 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 ($P=6.6\times10^{-13}$, Figure 8C), consistent with the athero-protective role of endothelial 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 datasets and determined that the SNPs in the risk locus were not associated with PPAP2B expression in whole blood[45], monocytes and macrophages[46], lymphoblastoid cell lines, skin fibroblasts and adipose tissue[47], brain[48] and liver[49], 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 athero-susceptible 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 lysophosphatidic acid (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 a number of strategies to modulate LPA/LPAR signaling in endothelial cells (LPA treatment of endothelial cells; down-regulation 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[30] but mechano-regulation of PPAP2B and its upstream regulators are poorly understood. We employed in vivo swine and murine along with a complementary *in vitro* flow device to establish that athero-relevant 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 up-regulated[38]. Moreover, mechano-sensitive 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 athero-protective 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 athero-relevant mechanotransduction mechanisms mediated by GWAS-identified CAD gene PPAP2B in vascular endothelial cells.

Although mechano-regulation 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[25, 28]. Second, atherosclerosis in *Apoe*-/- mice is notably reduced by systemic inhibition of LPA receptors employing the pharmacological antagonist Ki16425[27]. 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[32]. 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[23], consistent with our investigation addressing focal LPA sensitization as an unrecognized molecular signature contributing to the athero-susceptible 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[1]. Our results indicated that endothelia exposed to disturbed flow are primed to LPA stimulation which might be further augmented by additional atherogenic factors since LPA is produced during mild oxidation of LDL and accumulates in human atherosclerotic plaques[25]. Recently, plasma long-chain unsaturated LPA was reported to be elevated in patients with acute coronary syndrome[26]. Indeed, our data demonstrate a sensitized inflammation in PPAP2B-deficient cells treated with LPA but not TNFα.

PPAP2B has been shown to hydrolyze bioactive lipids such as lysophosphatidic acid and sphingosine-1-phosphate (S1P). Athero-protective flow decreased LPA species and S1P in media that is dependent on the elevated endothelial PPAP2B, because PPAP2B knockdown increased major LPA species (Online Figure VII) and S1P (Online Figure XV) under athero-protective flow. Our study addressed the contribution of LPA receptors mediating the elevated inflammation and enhanced permeability in PPAP2B-deficient endothelial cells (Figure 6D) employing 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, anti-adhesive endothelial monolayer in PPAP2B-deficient HAEC. Since 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 NF-kB[50], a critical transcription factor which promotes the pro-inflammatory endothelial phenotype associated with disturbed hemodynamics[5]. Our data are consistent with the ability of Ki16425 to reduce LPA-induced inflammation in mouse lungs stimulated by lipopolysaccharide[51] and in arterial vasculatures of *Apoe*-/- mice[27]. 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[21] and the causative role of RhoA in stimulating MLC phosphorylation and monolayer permeability was demonstrated in pulmonary endothelia[52]. LPA is a potent stimulator of endothelial stress fiber and intercellular gap formation[25]. Our investigation suggested that disturbed blood flow probably elevates vascular permeability in athero-susceptible regions by inhibiting PPAP2B and promoting LPA-induced cell contraction locally. Acute fluid shear stress was shown to modulate Rho-mediated signaling in endothelial cells[44, 53]. Here we demonstrated that chronic athero-protective flow can indirectly inactivate RhoA and MLC phosphorylation by promoting PPAP2B expression.

Our data demonstrate that PPAP2B is decreased in HAEC under athero-susceptible flow within five hours (Online Figure XVI-A) and is accompanied by the increased LPA in the media (Online Figure XVI-B). 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 athero-relevant flow within a few hours and the regulation remains with the hemodynamic application[14-16, 39]. The rapid response of PPAP2B to athero-relevant 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 and to the athero-protective 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 non-coding RNA that mediates athero-susceptible 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 neovascularization in animal models of ischemia-induced injury[40, 57]. In this study, *in silico* prediction and experimental validation have led to identification of miR-92a as a novel post-transcriptional suppressor of mechano-sensitive PPAP2B that inhibits vascular inflammation and permeability. Our data demonstrate that PPAP2B is significantly lower in vivo in swine aortic arch, lesser curvatures of mouse aortic arch 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[38]. 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 athero-protective flow, indicating that the anti-inflammatory role of PPAP2B is not due to its modulation of KLF2, and that GWAS-implicated CAD gene PPAP2B can contribute to the athero-protective function of KLF2 by inhibiting the LPA signaling in vascular endothelia. Our data further support the miR-92a-mediated suppression of PPAP2B since miR-92a has been demonstrated as a negative regulator of endothelial KLF2[34, 39]. Interestingly, PPAP2B knockdown led to the reduction of nitric oxide synthase 3 (eNOS) transcripts (Online Figure XVII) suggesting that flow-sensitive PPAP2B can promote vascular health by increasing eNOS expression. Conversely, nitric oxide sythase inhibitor L-NAME and eNOS-targeting siRNAs have no significant effect on the endothelial PPAP2B expression (Online Figure XVIII).

Regional targeting of dys-regulated 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

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Nonstandard Abbreviations and Acronyms

PPAP2B	PhosPhatidic-Acid-Phosphatase-type-2B
HAEC	human aortic endothelial cell
LPA	lysophosphatidic acid
LPAR1	lysophosphatidic acid receptor 1
LPAR2	lysophosphatidic acid receptor 2
Eqtl	expression quantitative trait locus

MLC	myosin-light-chain
ROCK	Rho-dependent protein kinase
SNP	single nucleotide polymorphism
L-NAME	L-N-Nitroarginine methyl ester

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Novelty and Significance

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 (GWAS).
- Atherosclerosis occurs preferentially at arterial sites where endothelia are activated by local disturbed flow.
- Lysophosphatidic acid (LPA), 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 athero-susceptible 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 LPA signaling.
- In humans, CAD risk allele at rs17114036 is associated with reduced PPAP2B in aortic endothelia.

GWAS have identified ~46 genetic loci associated with CAD. We hypothesize that GWAS-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 athero-susceptible sites associated with disturbed flow-induced miR-92a, consistent with decreased endothelial PPAP2B linked to CAD risk allele. PPAP2B inhibition abolishes the athero-protection of unidirectional flow. Modulation of miR92a-PPAP2B signaling axis in athero-susceptible endothelia may alleviate atherosclerotic burdens.

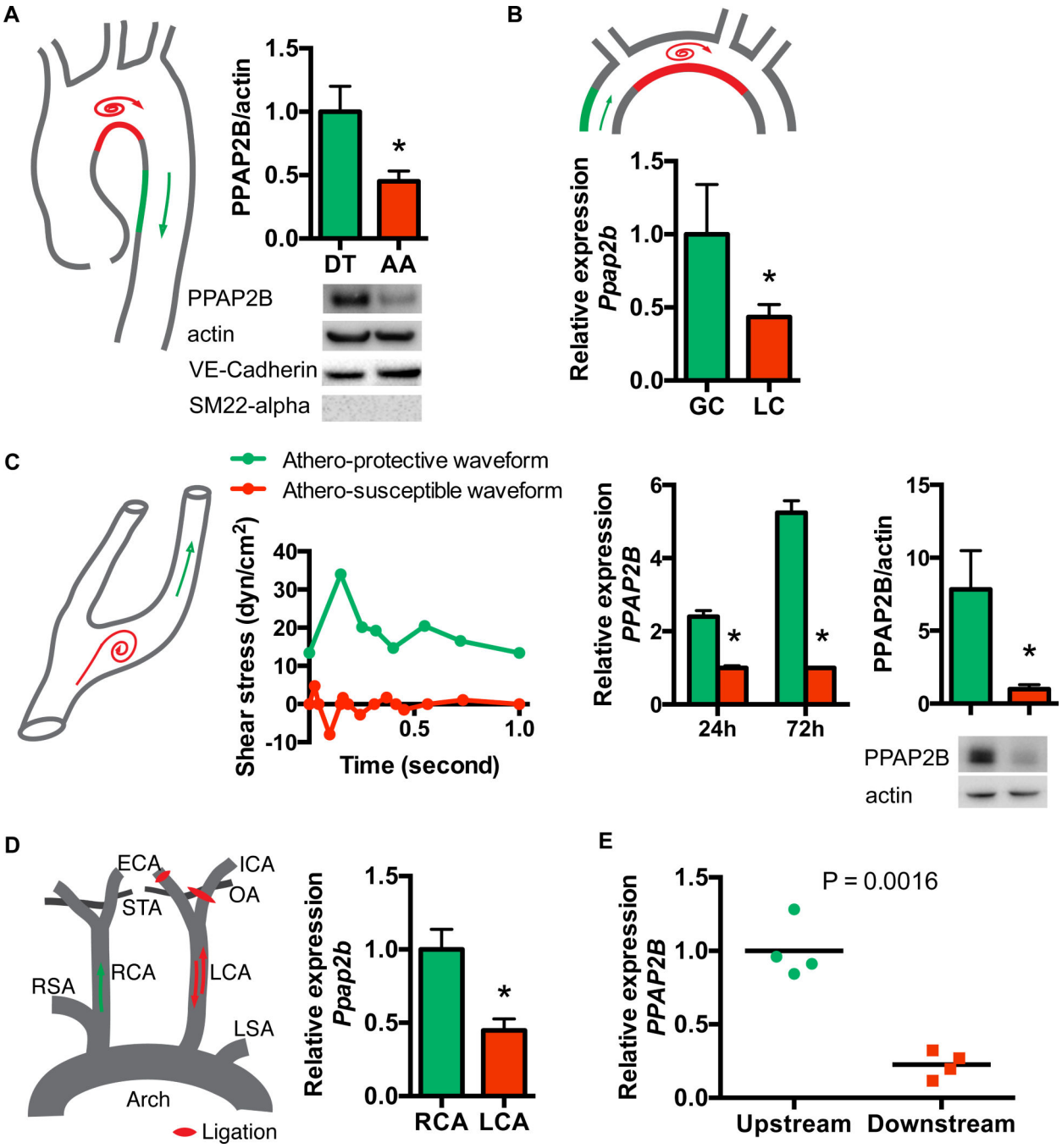

Figure 1. Low endothelial PPAP2B expression associated with disturbed flow *in vitro* and in vivo

(A) Diagram of porcine aorta illustrates unidirectional flow in descending thoracic aorta (DT) and disturbed flow near the lesser curvature of aortic arch (AA). PPAP2B protein abundance was significantly lower in endothelial cells (EC) isolated from AA. **(B)** Diagram of mouse aortic arch illustrates unidirectional flow in greater curvature (GC) and disturbed flow near the lesser curvature (LC). PPAP2B mRNA abundance was significantly lower in EC isolated from LC. **(C)** Diagram shows two distinct flows in human carotid bifurcation. Athero-protective and athero-susceptible waveforms represent hemodynamics in human

distal internal carotid artery and carotid sinus, respectively. PPAP2B mRNA abundance was elevated in human aortic endothelial cells (HAEC) under athero-protective waveform for 24 hours and 72 hours. PPAP2B protein was increased in HAEC under athero-protective waveform for 72 hours. **(D)** Diagram illustrates partial ligation in mice to generate acute disturbed flow in the left carotid artery (LCA). PPAP2B mRNA abundance was decreased in EC from LCA by acute disturbed flow. **(E)** PPAP2B mRNA abundance in human atherosclerosis lesions. Upstream and downstream sections of the atherosclerotic plaque were obtained from human carotid endarterectomy samples (n=4). All data are represented as mean ± SEM. n = 3-5. * $P < 0.05$, t-test.

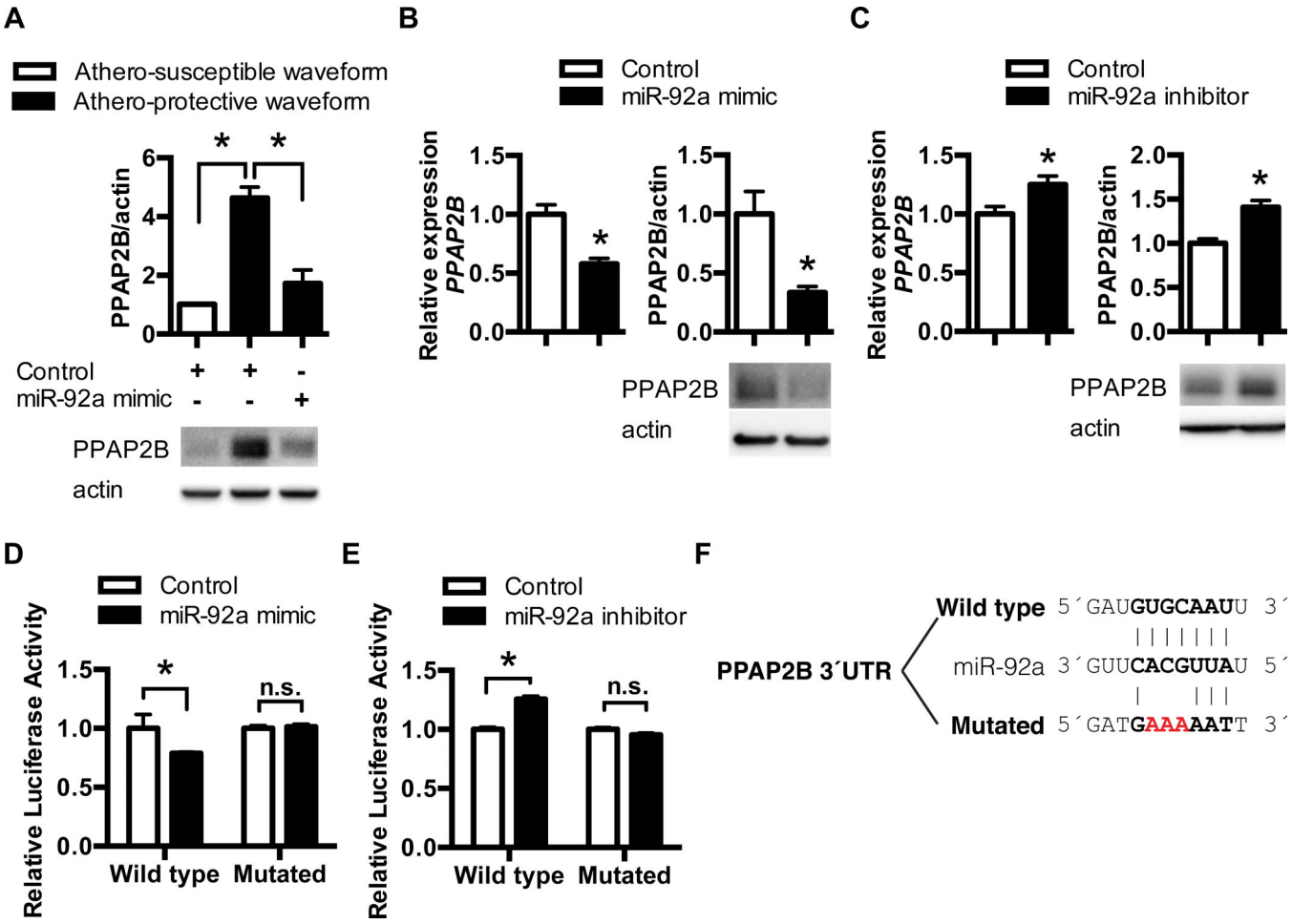

Figure 2. miR-92a suppresses PPAP2B expression by targeting its 3′UTR

(A) miR-92a mimic diminished elevated PPAP2B expression induced by athero-protective 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,E)**. All data are represented as mean ± SEM. n = 3-5. * $P < 0.05$, t-test.

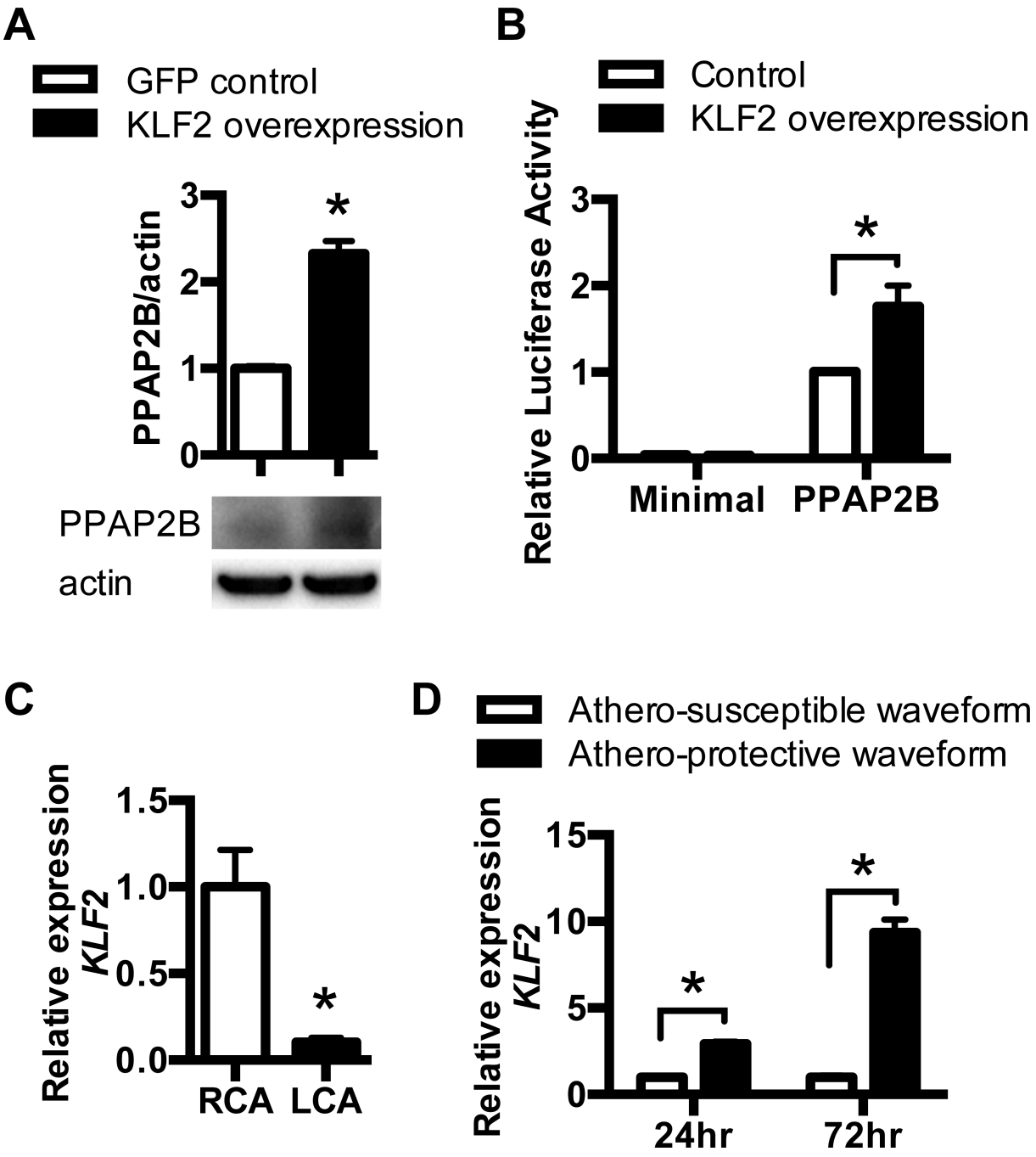

Figure 3. KLF2 promotes PPAP2B expression by activating its promoter activity

(A) KLF2 overexpression via adenoviral transduction increased PPAP2B protein in HAEC. **(B)** PPAP2B promoter drove Firefly luciferase reporter activity, compared with minimal promoter, which was further increased by KLF2 overexpression via plasmid transfection. **(C-D)** KLF2 expression was decreased in endothelial cells isolated from ligated mouse LCA **(C)** and in HAEC exposed to athero-susceptible waveform **(D)**. All data are represented as mean ± SEM. n = 3-5. * $P < 0.05$, t-test.

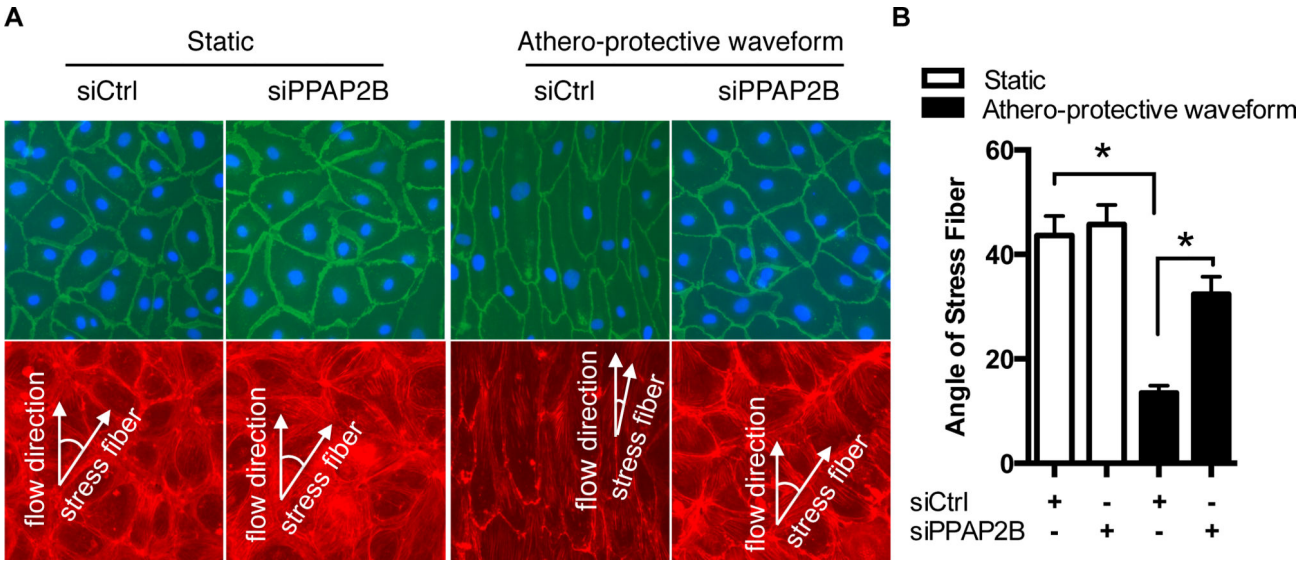

Figure 4. PPAP2B knockdown abolishes endothelial alignment induced by athero-protective waveform

(A) Human aortic endothelial cell (HAEC) monolayer displayed uniformly elongated morphology parallel to the direction of athero-protective waveform (72 hrs), instead of random polygonal structure under static conditions, as indicated by cell borders stained by VE-Cadherin (green), stress fiber (red) marked by phalloidin, and nuclei demonstrated by DAPI. PPAP2B knockdown by 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 30-50 cells measured per condition. siCtrl: control siRNA; siPPAP2B, PPAP2B siRNA. All data are represented as mean ± SEM. n = 5. * $P < 0.05$, two-way ANOVA.

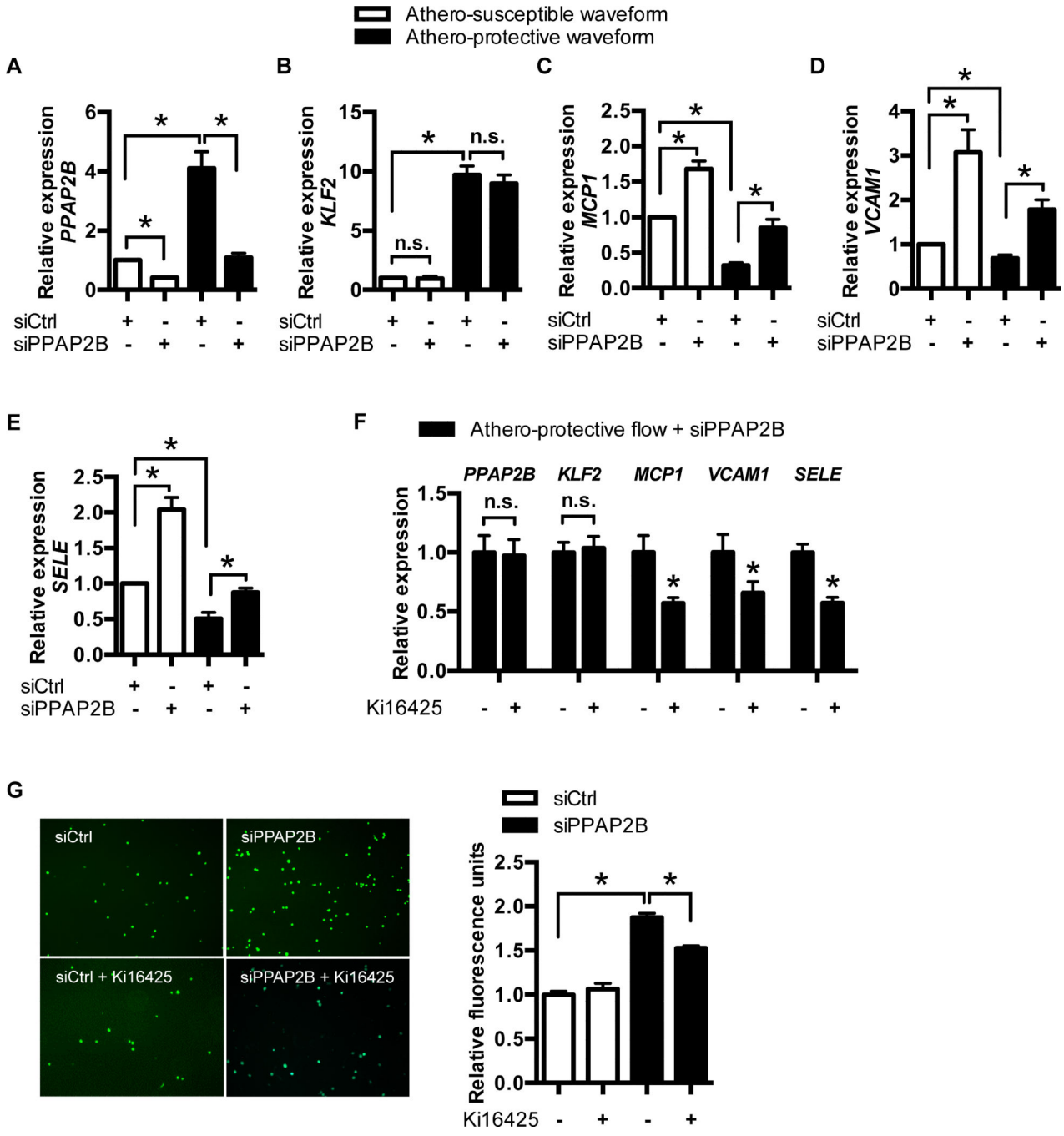

Figure 5. Flow-sensitive PPAP2B attenuates endothelial inflammation by inhibiting LPA signaling

(A) PPAP2B-targeting siRNAs abrogated induced PPAP2B expression (mRNA) in HAEC subjected to athero-protective waveform (72 hrs). **(B)** KLF2 expression (mRNA) was elevated in athero-protective waveform and not affected by PPAP2B siRNA treatment. **(C-E)** Athero-protective waveform (72 hrs) significantly reduced expression (mRNA) of MCP1, VCAM1, and E-Selectin (SELE) in HAEC monolayer compared with their expressions under athero-susceptible waveform. siRNA-mediated inhibition of PPAP2B

stimulated the pro-inflammatory/adhesive endothelial phenotype in HAEC under athero-protective waveform. **(F)** LPA receptor 1/3 antagonist Ki16425, did not affect PPAP2B expression (mRNA), but restored the anti-inflammatory property by inhibiting MCP1, VCAM1, and SELE (mRNA) in PPAP2B-deficent HAEC under athero-protective waveform. **(G)** Knockdown of endogenous PPAP2B in HAEC increased THP-1 cell adhesion (green) to endothelial monolayer, whereas Ki16425 compounds reduced the leukocyte-endothelial interaction in PPAP2B-deficient HAEC. Representative images are shown and cell adhesion was measured by fluorescence intensity of labeled THP-1 cells. siCtrl: control siRNA; siPPAP2B, PPAP2B siRNA. All data are represented as mean ± SEM. n = 4-6. * $P < 0.05$, twoway ANOVA for (A) - (E) and (G), t-test for (F).

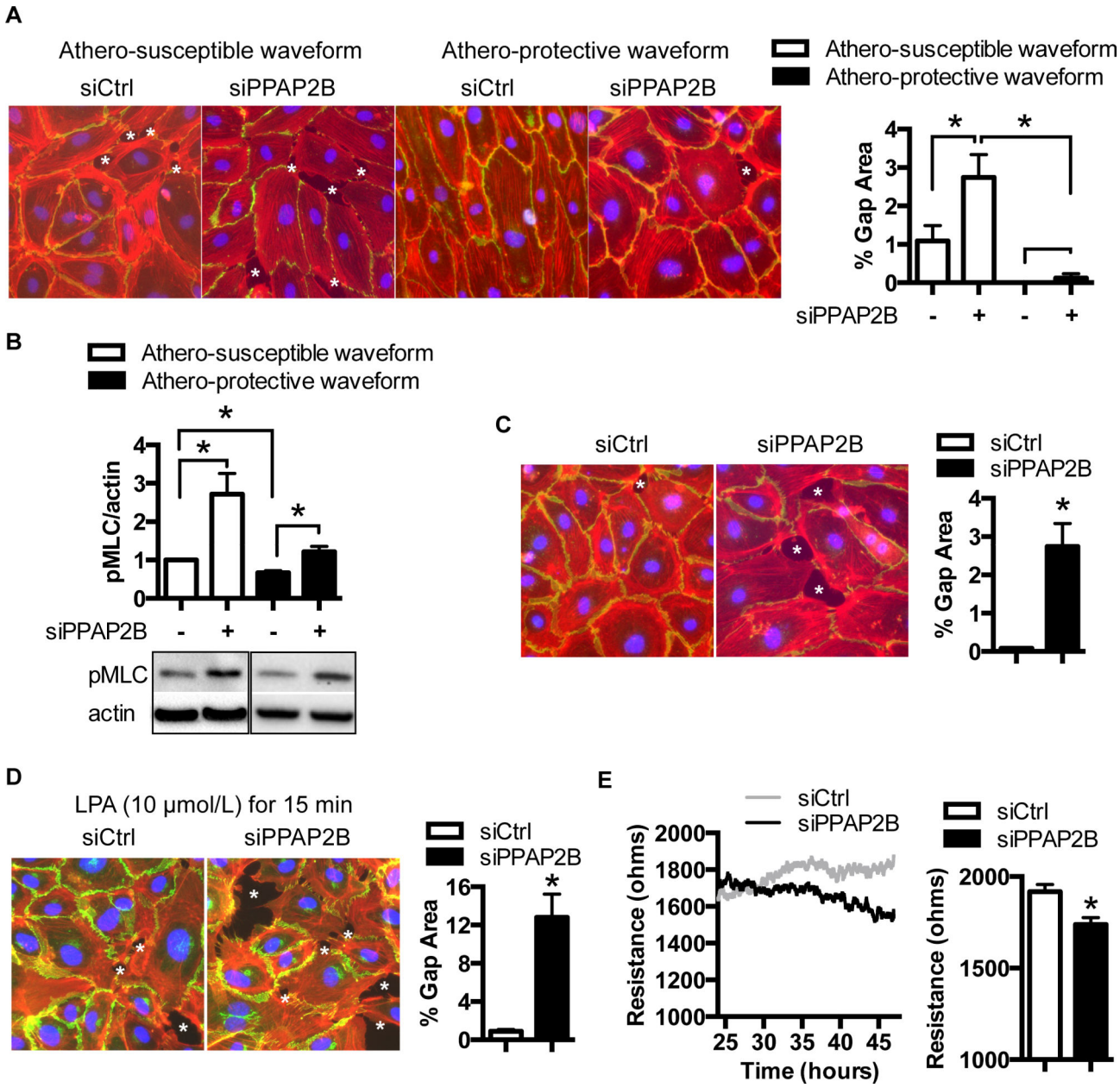

Figure 6. Inhibition of flow-sensitive PPAP2B results in intercellular gap formation and increased permeability of HAEC monolayer

(A) HAEC monolayer, under either athero-susceptible or protective waveform (72 hrs), was stained for VE-Cadherin (Green) and stress fiber (Red) to show intercellular gaps (*), which was quantified by the percentage of area covered by intercellular gaps. Reduced intercellular gap formation was detected in HAEC exposed to athero-protective waveform compared with HAEC subjected to athero-susceptible waveform. PPAP2B siRNAs resulted in compromised monolayer integrity of HAEC exposed to athero-protective waveform, shown by increased gap formation. **(B)** Phosphorylated myosin light chain (pMLC) in HAEC monolayer was decreased by athero-protective waveform (72 hrs). PPAP2B knockdown significantly reversed the suppression of pMLC by athero-protective waveform. **(C)**

PPAP2B inhibition by siRNAs led to compromised HAEC monolayer integrity under static conditions, which was further decreased by LPA treatment **(D),** as shown by increased intercellular gap formation. **(E)** PPAP2B knockdown resulted in weakened HAEC barrier function, shown by real-time resistance measurements conducted by Electric Cell-substrate Impedance Sensing. Time indicated hours after PPAP2B knockdown. siCtrl: control siRNA; siPPAP2B, PPAP2B siRNA. All data are represented as mean ± SEM. n = 3-5. * $P < 0.05$, two-way ANOVA for (A) and (B), t-test for (C) - (E).

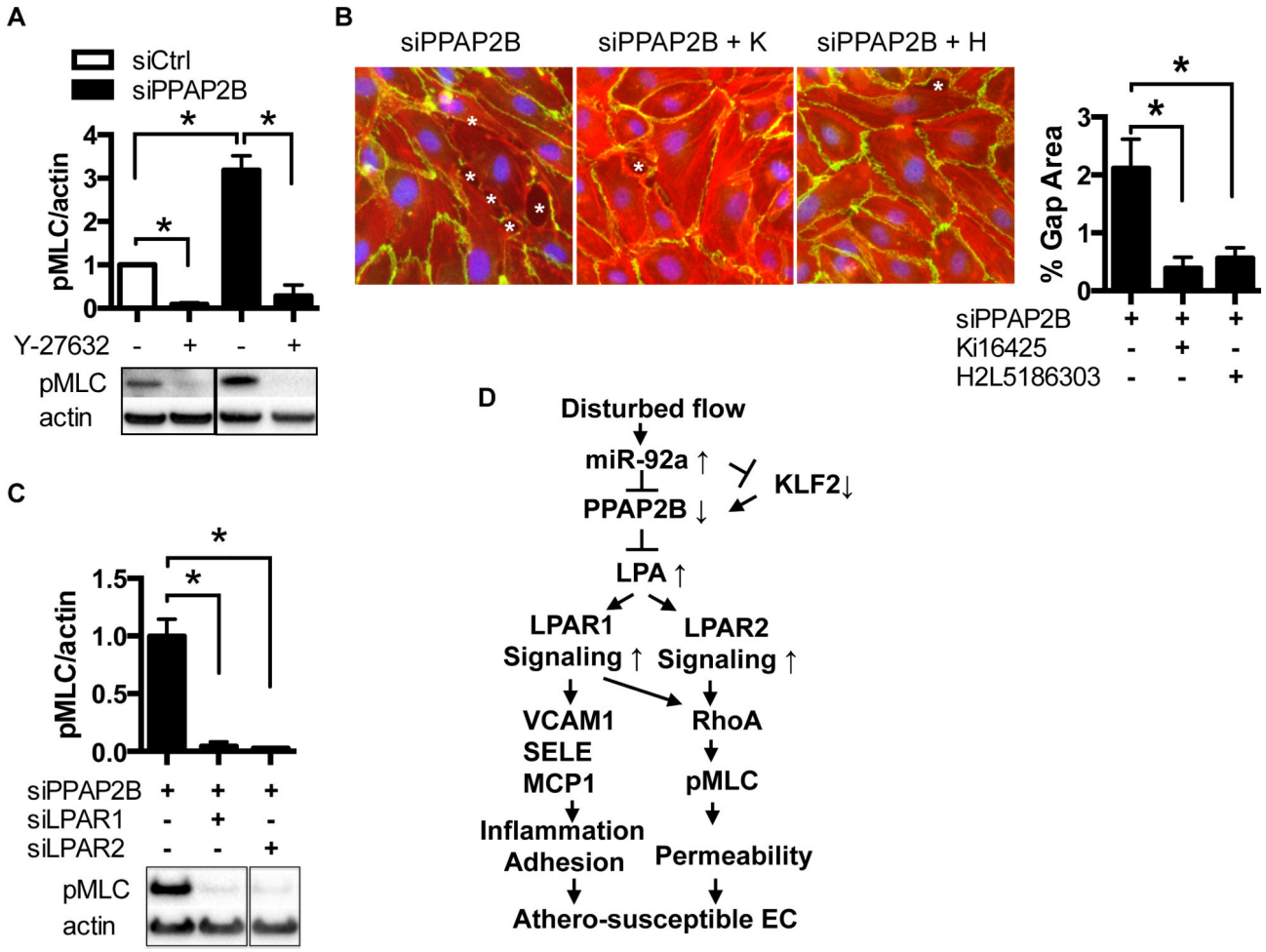

Figure 7. LPAR1 and LPAR2 inhibitions block the effects of PPAP2B inhibition on intercellular gap formation and MLC phosphorylation in HAEC

(A) Rock inhibitor Y-27632 blocked the elevation of pMLC induced by PPAP2B inhibition. **(B)** PPAP2B inhibition by siRNAs led to compromised HAEC monolayer integrity under static condition, which was rescued by LPAR1 or LPAR2 inhibition by Ki16425 and H2L5186303 respectively, as shown by intercellular gaps. DMSO was added to all treatments, as both LPAR1 and LPAR2 inhibitors were resolved in DMSO. **(C)** PPAP2B inhibition by siRNA led to elevated pMLC under static condition, which was blocked by LPAR1 or LPAR2 inhibition using siRNAs. **(D)** Graphic representation of the effects of flow-sensitive PPAP2B on atherosusceptibility in HAEC. siCtrl: control siRNA; siPPAP2B, PPAP2B siRNA. All data are represented as mean ± SEM. n = 3-5. * $P < 0.05$, two-way ANOVA for (A), one-way ANOVA for (B) and (C).

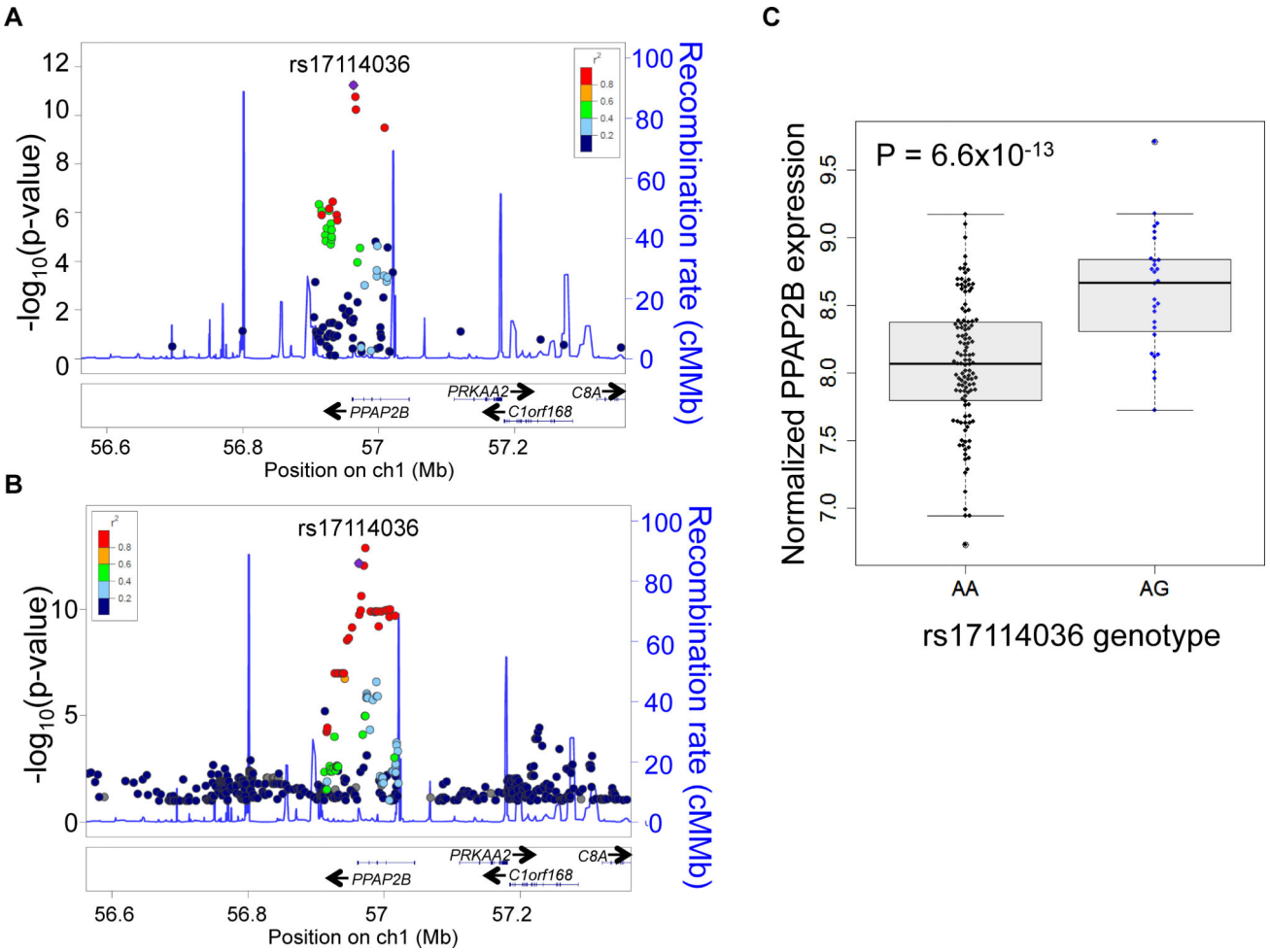

Figure 8. Regional association plot highlighting the PPAP2B genomic locus. (A) CAD risk association based on the CARDIoGRAMplusC4D consortium data

(B) Association of PPAP2B expression in HAEC. The purple diamond figure shows the most significantly associated SNP (rs17114036) in the region. The plots were generated using LocusZoom. Recombination rates are based on European populations from the 1000 Genomes Project. **(C)** Expression level of PPAP2B stratified based on the peak association SNP. P-value of association was calculated using a mixed model approach to account for population structure.