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Lipid Phosphate Phosphatase 3 Maintains NO-Mediated Flow-Mediated Dilation in Human Adipose Resistance Arterioles

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Abstract

Microvascular dysfunction predicts adverse cardiovascular events despite absence of large vessel disease. A shift in the mediator of flow-mediated dilation (FMD) from nitric oxide (NO) to mitochondrial-derived hydrogen peroxide (H_2O_2) occurs in arterioles from patients with coronary artery disease (CAD). The underlying mechanisms governing this shift are not completely defined. Lipid phosphate phosphatase 3 (LPP3) is a transmembrane protein that dephosphorylates lysophosphatidic acid, a bioactive lipid causing a receptor-mediated increase in reactive oxygen species. A single nucleotide loss-of-function polymorphism in the gene coding for LPP3 (rs17114036) is associated with elevated risk for CAD, independent of traditional risk factors. LPP3 is suppressed by miR-92a, which is elevated in the circulation of patients with CAD. Repression of LPP3 increases vascular inflammation and atherosclerosis in animal models. We investigated the role of LPP3 and miR-92a as a mechanism for microvascular dysfunction in CAD. We hypothesized that modulation of LPP3 is critically involved in the disease-associated shift in mediator of FMD. LPP3 protein expression was reduced in left ventricle tissue from CAD relative to non-CAD patients ($p=0.004$), with mRNA expression unchanged ($p=0.96$). Reducing LPP3 expression (non-CAD) caused a shift from NO to H_2O_2 (% maximal dilation: Control 78.1±11.4% vs Peg-Cat 30.0±11.2%; $p<0.0001$). miR-92a is elevated in CAD arterioles (Fold change: 1.9±0.0.1 $p=0.04$), while inhibition of miR-92a restored NO-mediated FMD (CAD) and enhancing miR-92a expression (non-CAD) elicited H_2O_2-mediated dilation ($p<0.0001$). Our data suggests LPP3 is crucial in the disease-associated switch in the mediator of FMD.

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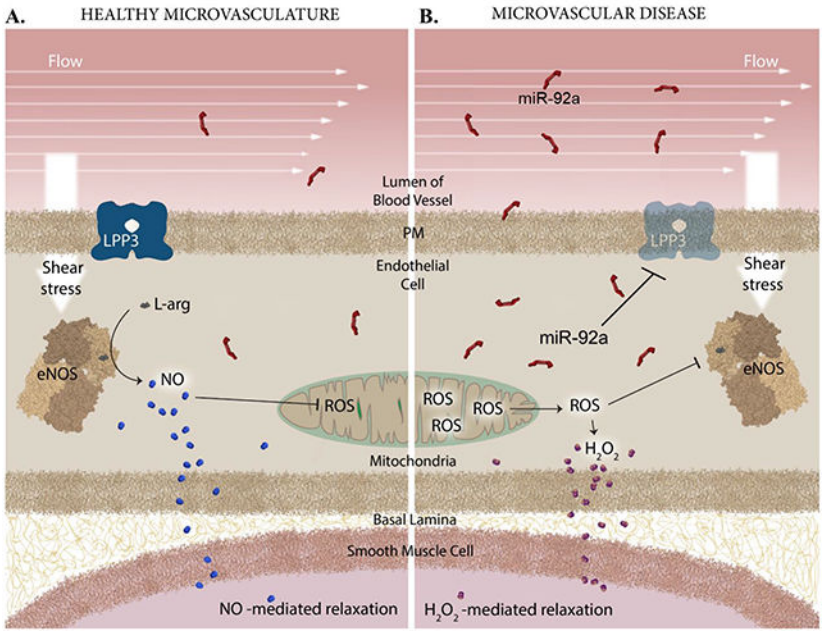
Graphical Abstract.

Maintenance of Lipid Phosphate Phosphatase 3 (LPP3) Maintains Microvascular Function in Health and Disease.



A. In healthy microvasculature, LPP3 helps tranduce the production of shear-induced nitric oxide (NO) formation to induce smooth muscle relaxation (flow-mediated dilation).

B. In microvascular disease, such as coronary artery disease, miR-92a inhibits expression of LPP3, and increases production of mitochondrial-derived reactive oxygen species (ROS) to cause a switch in the mechanism of microvascular flow-mediated dialtion from NO to H_2O_2

Keywords

FMD; LPP3; miR-92a; CAD

Introduction

Shear stress is one of the most physiologically relevant regulators of endothelial-dependent vasomotor tone, stimulating the release of nitric oxide (NO) from the endothelium, acting in a paracrine fashion on the underlying smooth muscle cells to elicit dilation of conduit and resistance-size vessels (flow-mediated dilation, FMD) (Moncada *et al.*, 1988; Palmer *et al.*, 1988). Presence of NO promotes vascular quiescence by maintaining an anti-inflammatory, anti-thrombotic and anti-proliferative environment (Kubes *et al.*, 1991; Ahluwalia *et al.*, 2004). Alterations in endothelial function in the form of reduced NO bioavailability and elevation in reactive oxygen species (ROS) are some of the earliest manifestations of vascular disease (e.g., endothelial dysfunction)(Stauss *et al.*, 1999; Ponnuswamy *et al.*, 2012).

In resistance arterioles (50 – 250 μm) from patients with prior diagnosed CAD, the mediator of FMD shifts away from NO to hydrogen peroxide (H_2O_2) (Liu *et al.*, 2003; Miura *et al.*, 2003; Liu *et al.*, 2011). Although H_2O_2 is sufficient to preserve vasodilator capacity to shear stress, the loss of NO corresponds with a simultaneous reduction in vascular quiescence. These changes in vascular milieu have significant clinical implications, especially at the level of microcirculation where they are highly predictive of the development of clinical cardiovascular disease (Pepine *et al.*, 2010; van de Hoef *et al.*, 2014). The basis of this shift in vasodilator mechanism with CAD is poorly understood, thus uncovering the mechanisms leading to the shift could provide insights into the earliest manifestations of atherosclerosis and further our understanding of vascular pathophysiology.

Advances in bioactive lipid signaling have identified novel factors that play important roles in maintaining normal vascular function (Freed *et al.*, 2014). Lipid phosphate phosphatase (LPP3), an integral plasma membrane protein, dephosphorylates and terminates outside-in signaling of lysophosphatidic acid (LPA), a glycerol-based lipid. Elevated levels of LPA are implicated in cardiovascular disease and are associated with vascular dysfunction (Smyth *et al.*, 2008; Morris & Smyth, 2013; Panchatcharam *et al.*, 2014; Smyth *et al.*, 2014; Chabowski *et al.*, 2018). An increase in LPA levels has been observed with LPP3 knockdown/knockout mouse models (Busnelli *et al.*, 2017; Chandra *et al.*, 2018), while overexpression of LPP3 significantly reduces LPA levels (Wu *et al.*, 2015). Genome-wide association studies identified risk alleles in the gene coding for LPP3, the presence of which might cause a decrease in expression of LPP3, and an increase in CAD susceptibility (Schunkert *et al.*, 2011). Additionally, miR-92a, a negative regulator of LPP3 (Wu *et al.*, 2015), is elevated in vascular tissue and the circulation of patients with CAD (de Winther & Lutgens, 2014; Rong *et al.*, 2017; Zhang *et al.*, 2017), while inhibition of miR-92a improves vascular function in a murine model of atherosclerosis, and improves wound healing (Loyer *et al.*, 2014; Gallant-Behm *et al.*, 2018). Inhibition of miR-92a is an attractive therapeutic target and is the subject of clinical trials on wound healing (NCT03603431, NCT03494712) (Abplanalp *et al.*, 2020). Lower expression of LPP3 drive by miR-92a may cause LPA to accumulate and enhance signaling through LPA receptors 1 and 3, which have been implicated in microvascular dysfunction (Chabowski *et al.*, 2018) and atherosclerosis (Smyth *et al.*, 2014). Together, these findings demonstrate that LPP3 may play an integral role in maintaining normal vascular function by regulating LPA signaling.

Despite strong implications that LPP3 might play a mechanistic role in vascular function, little evidence exists corroborating these findings in human microcirculation. In this study, we compared expression of LPP3 in human heart and resistance arterioles from patients with and without CAD. We also examined whether increasing expression of LPP3 via targeted suppression of miR-92a function would restore NO-mediated vasodilation in resistance arterioles from patients with CAD. We hypothesized that modulation of LPP3 in resistance arterioles would elicit shifts in the mediator of FMD in arterioles from non-CAD and CAD patients: A decrease in expression of LPP3 in arterioles from non-CAD patients cause a loss of NO-mediated FMD with a compensatory shift to H_2O_2 as the mediator of FMD, while elevated levels of LPP3 in arterioles from CAD patients restore NO-mediated FMD.

Methods

All data is available upon reasonable request to Senior Authors.

Ethical Approval and Tissue acquisition

Protocols for de-identified, surgical discard tissue acquisition and processing were approved by the Institutional Review Board (IRB) of the Medical College of Wisconsin (PRO00000114). All tissue used within this study was deemed discarded surgical tissue, collected through a de-identified process and was deemed exempt by MCW IRB from requiring informed consent. The study conformed to the standards set by the Declaration of Helsinki, except for registration in a database. Human adipose tissue (subcutaneous and visceral) was obtained as discarded tissue at the time of surgery and immediately placed in 4°C HEPES buffer [(in mM) NaCl 275, KCl 7.99, $MgSO_4$ 4.9, $CaCl_2 \cdot 2H_2O$ 3.2, KH_2PO_4 2.35, EDTA 0.07, glucose 11, HEPES acid 20)]. Cardiac tissue was obtained through the Wisconsin Donor Network. Inclusion and exclusion criteria pertain to all subject groups. De-identified patient demographics are collected and stored using an assigned non-PHI containing ID number. Tissue from patients without clinically diagnosed CAD and no more than one risk factor (Table 1, n = 8 independent samples) were categorized as the non-CAD group. Risk factors used for classification were derived from a patient information survey completed by medical personnel in a de-identified manner and include cigarette smoking, diabetes mellitus, hypertension, hyperlipidemia, and family history of cardiovascular disease. Conditions such as obesity, hyperuricemia or rheumatoid arthritis are also occasionally present. Tissue was defined as CAD were obtained from tissue from subjects with clinically diagnosed CAD (Table 1, n = 10 independent samples). Tissues from subjects with multiple cardiovascular risk factors but no CAD were excluded.

Cannulated Arteriole Preparation and Flow-Mediated Dilation

Human resistance arterioles (101±31 μm, n = 33 non-CAD, 22 CAD) were isolated from adipose tissue and excess adipose/connective tissue was removed. The arterioles were cannulated onto glass micropipettes of matched impedance in an organ chamber filled with Krebs Solution ([in mM] pH 7.4, 37°C NaCl 123, KCl 4.7, $MgSO_4$ 1.2, $CaCl_2 \cdot 2H_2O$ 2.5, $NaHCO_3$ 16, EDTA 0.03, glucose 11, KH_2PO_4 1.2), as previously published (Kuo *et al.*, 1991; Miura *et al.*, 2003). Arterioles were pressurized, and pre-constricted (30–50% of max. diameter) with endothelin-1 (ET-1) prior to initiation of flow. On average, 0.36±0.01 nM of ET-1 was needed to achieve desired stable constriction, and the dose used was not influence by vessel treatment. Flow through the cannulated arterioles was achieved by changing the height of each reservoir in equal amounts in opposite directions, thus minimizing any changes in intraluminal pressure. In response to flow, internal diameters were measured using on-screen calibrated calipers after 5 minutes at a given pressure gradient. Two flow-response curves were generated; first with vehicle, then a second with inhibitor(s), including L-NAME or PEG-cat (see Pharmacological Intervention). Time controls were generated with no intervention between the two flow-response curves. At the end of each experiment, papaverine (100 μM) was added to assess vasodilator capacity/smooth muscle function(Kadlec *et al.*, 2017; Chabowski *et al.*, 2018). Data are reported as a percent

of the maximal diameter obtained by exposure to the endothelial-independent vasodilator papaverine.

Pharmacological Intervention

The pharmacological agents added to the organ bath constituted ≤1 % of the total circulating bath volume. To determine whether the mediator of FMD was NO or H_2O_2, the nitric oxide synthase (NOS) inhibitor [N^{ω}-nitro-L-arginine methyl ester (L-NAME), 100 μM] or the H_2O_2 scavenger polyethylene glycol-catalase (Peg-Cat) 500 U/mL, respectively, were added to the organ chamber for 30 min prior to pre-constriction. Two consecutive pressure gradient (flow) studies were performed on the majority of the vessels, where the first flow study was the control, while the second flow study was with either L-NAME or Peg-Cat. Prior studies have shown these doses to effectively block NO and H_2O_2 components of FMD, respectively (Miura *et al.*, 2003; Phillips *et al.*, 2007).

Molecular Interventions

To assess the mechanism of vasodilation under various molecular interventions, isolated arterioles and human umbilical vein cells (HUVEC; ATCC) were transfected with control or targeting siRNAs (detailed below) as our group has previously described. (Kadlec *et al.*, 2017; Chabowski *et al.*, 2018). Briefly, one end of isolated arterioles were cannulated and siRNA (control or targeting siRNA) were infused into the lumen of the arterioles. The free end of the arteriole was then tied closed, followed by slipping the cannulated end off of the pipette, cinching it closed as well. In this way, the endothelial cells are directly exposed to the siRNA. For functional studies, the tied ends were clipped off, and the arterioles were cannulated as described above. Depending on the experimental condition, the following transfection agents were used: silencer negative control (Ambion, AM4611, final concentration 50 nM, 24 hour) or LPP3 (Ambion, AM16708; final concentration 50 nM, 24 hour) targeting siRNA, silencer negative control anti-miR (scramble, miR inhibitor, Qiagen, YI00199006-DDA, final concentration 50 nM, 48 hours), anti-miR-92a (miR-92a inhibitor, ThermoFisher, Assay ID PM12524), final concentration 50 nM, 48 hours) or miR-92a mimic (ThermoFisher, Assay ID MC12524, final concentration 50 nM, 48 hours). All the transfections were carried out using Opti-MEM™ media (ThermoFisher, 31985062) supplemented with Lipofectamine RNAiMax (ThermoFisher, 13778030) to improve transfection efficiency. After intraluminal incubation, the vessels were removed from the media and placed in fresh cold HEPES buffer, the suture ties were cut off and the vessels were either re-cannulated for functional assessment or snap frozen in liquid nitrogen and stored at −80°C for molecular studies.

Western Blot

Human hearts from deceased subjects were obtained through the organ procurement protocols at Versiti/Blood center of Wisconsin and Wisconsin Donor Network. Adipose resistance arterioles were collected from surgical discard tissue, and HUVEC lysates were both collected following transfection. Proteins from all tissues (arterioles, left ventricle [LV]) were prepared via standard protocols (Beyer *et al.*, 2016; Ait-Aissa *et al.*, 2019). The following primary antibodies were used to detect target proteins: LPP3 (Thermo Scientific, 39-1000, 1:500) and GAPDH (Abcam, ab8245, 1:10000). After incubation with the primary

antibodies, the membranes were incubated for 1-hour in room temperature with secondary anti-mouse antibodies conjugated to horseradish peroxidase [Millipore, AP308P, 1:5,000] followed by the chemiluminescent substrate (Bio-Rad) for 5 min. Once the primary target (LPP3) was detected, the membranes were stripped using commercial stripping buffer (Restore PLUS Western Blot Stripping Buffer, Invitrogen #46430) for 2 hours in room temperature. The membranes were then incubated again in 5% milk blocking solution for an hour in room temperature, washed and incubated with primary antibody against GAPDH, followed by the secondary peroxidase-conjugated anti-mouse antibody (Millipore, AP308P, 1: 10,000) and chemiluminescent substrate as described above. Expression of proteins was quantified using the digitized images (TIFF extension) and Image J (www.imagej.net) software using the densitometry analysis. The values obtained for LPP3 expression were normalized to values obtained for corresponding GAPDH expression and reported in the results section as relative Units.

RNA Isolation from Left Ventricle and Resistance Arterioles

Freshly frozen LV samples of tissue were thawed and homogenized using the tissue rupturer (Fisher Scientific, PowerGen 125) in TRIzol™ (Thermo Fisher, 15596026) as previously (Ait-Aissa *et al.*, 2019). Isolated and cleaned human adipose resistance arterioles were snap frozen in liquid nitrogen and stored in −80°C until further processing. Total RNA was extracted from samples using RNAqueous™-Micro Total RNA Isolation Kit (Ambion, AM1931). 25 ng of total RNA was used to synthesize cDNA using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, 4368814). RNA was quantified using NanoDrop™ 2000/C spectrophotometer (Thermo Fisher). Samples were stored in −80°C until further use.

Quantitative Real-Time Polymerase Chain Reaction

Quantification of the target gene expression was performed using PrimerPCR™ SYBR® Green Assay for LPP3 (BioRad, PPAP2B, Human Catalog #10025636), and Qiagen QuantiTect Primer Assay for eNOS (Hs_NOS3_1_SG, QT00089033) and normalized to 18s (Hs_RRN18S_1_SG, QT100199367). miRNA levels were assessed in similar ways. Previously frozen arterioles were used for total RNA extraction via Monarch® Total RNA Miniprep Kit (New England BioLabs, T2010S) according to manufacturer recommendations. RNA quantified with NanoDrop™ (Thermo Scientific, 13-400-518) and a total of 10 ng of RNA per sample was used for the Reverse Transcriptase reactions (Qiagen, miScript® II RT Kit, 218160) and quantitative PCR analysis (Qiagen, miScript SYBR® Green PCR Kit, 218073). Detection of miR-92a was achieved using hsa-miR-92a-3p primer (Qiagen, YP00204258) and was normalized to RNU6 (Qiagen, MS00033740). Expression analysis was performed using BioRad CFX96 Touch Real-Time PCR Detection System.

Statistical analysis

All data tested by parametric tests are expressed as mean ± SD. For vascular studies, the data are expressed as a percent of maximal diameter, where 100% represents full relaxation to the maximal diameter noted throughout the experiment. Generally this was the diameter following papaverine. To compare dilator responses relationship, a 2-way ANOVA RM was used with pressure gradient (flow stimulus intensity) and treatment as parameters,

with multiple comparisons made using Tukey post hoc tests. Non-Gaussian distribution was assumed for all the expression studies (protein, mRNA); thus, non-parametric tests (Mann Whitney U-test) were used to test for significance. Where samples were paired, the Wilcoxon test was used. Appropriate statistical tests are reported in Figure Legends. All analyses were performed using GraphPad Prism 7.04 (GraphPad Software, San Diego, CA) with statistical significance defined at $P<0.05$.

Results

Discarded hearts were collected from a total of 18 patients, 10 with CAD and 8 non-CAD. Adipose tissue (subcutaneous, visceral) was collected from a total of 55 patients, where 33 were categorized as non-CAD while 22 had clinically diagnosed CAD. Patient demographics are detailed in Table 1 for heart tissue and Table 2 for adipose tissue.

LPP3 Expression is Reduced in Human LV and Adipose Arterioles from Patients with CAD

To investigate whether the expression of LPP3 correlates with presence of CAD, we compared the protein expression and mRNA transcript in the human LV tissue and adipose resistance arterioles from patients with and without CAD. In LV samples from patients with CAD LPP3 protein expression was significantly reduced compared to patients classified as non-CAD (Mann Whitney non-parametric U-test p = 0.004; Figure 1A). Conversely, there were no differences in LPP3 mRNA levels between patients with and without CAD in LV tissue or adipose resistance arterioles (Figure 1B and 1C, Mann Whitney non-parametric U-test, p = 0.96 and 0.14, respectively).

Knockdown of LPP3 in Non-CAD Arterioles Induces CAD-like Dilator Phenotype

LPP3 siRNA tended to reduce expression of LPP3 by approximately 50% in HUVECs (Figure 2A; Mann Whitney U-test p = 0.05), with directionally similar trends using adipose arterioles from non-CAD patients (Figure 2B; Wilcoxon t-test, p = 0.13). Non-CAD adipose arterioles transfected with control siRNA-maintained NO-mediated FMD (inhibited by L-NAME) (% Max. Diameter at 100 cm H_2O: Control 77.5±7.1, n=4 vs L-NAME 12.2±8.7; 2-way RM ANOVA, p < 0.0001 vs. control), while Peg-Cat, had no effect on the dilation (Figure 2C. 2-way RM ANOVA p = 0.49). When non-CAD arterioles were treated with LPP3-targeting siRNA, Peg-Cat but not L-NAME significantly reduced dilation to flow (% Max. Diameter at 100 cm H_2O: Control 78.1±11.4, n=9 vs. Peg-Cat 30.0±11.2; 2-way RM ANOVA, p < 0.0001, Figure 2D) indicating that suppression of physiological LPP3 via siRNA causes a pathological switch in the mechanism of dilation.

Blocking miR-92a, an Inhibitor of LPP3 Expression, Restores NO-Mediated FMD in Human Arterioles from Subjects with CAD

Expression of miR-92a was increased in adipose resistance arterioles from patients with CAD relative to non-CAD (Mann Whitney U-test, p = 0.04, n = 15 each group; Figure 3A). Transfection (48-hours) of adipose arterioles from patients with CAD using anti-miR92a abolished mRNA expression of miR-92a (Wilcoxon Signed Rank test, p = 0.03, n = 6 each group; Figure 3B) and induced a switch in the mechanism of vasodilation from H_2O_2 to NO, (% maximal dilation: Control 71.3±7.7, n=7 vs L-NAME 1.4±26.1, 2-way RM ANOVA p

< 0.0001, n = 5 – 7; Figure 3D) with no effect of the control siRNA on the mechanism of dilation (% maximal dilation: Control 76.4±8.1, vs Peg. Cat 6.4±12.7, 2-way RM ANOVA p < 0.0001; n = 3 – 5; Figure 3C). Interestingly, anti-miR92a transfection did not change LPP3 (n = 13 each) or eNOS mRNA expression (n = 13 each) in adipose arterioles from patients with CAD (Figure 4 A & B, Wilcoxon Signed Rank test p = 0.15 and 0.54 respectively)

Next, we determined whether decreasing LPP3 expression in arterioles from non-CAD patients would elicit a switch in the mechanism of dilation from NO to H_2O_2. Arterioles from non-CAD patients were transfected with a miR92a mimic or control. Paradoxically, miR-92a mimic increased LPP3 (Figure 5A; Wilcoxon Signed Rank test p = 0.03, n = 6 each) did not alter eNOS mRNA expression (Figure 5B; Wilcoxon Signed Rank test p = 0.34, n = 6 each), but did induce a switch in the mechanism of FMD from NO to H_2O_2 (Figure 5C; % maximal dilation: Control 73.8±16.6, vs. Peg-Cat 23.5±30.5, 2-way RM ANOVA p < 0.0001; n = 5 – 6)

Discussion

This is the first study to demonstrate that LPP3 contributes to the regulation of the mediator of FMD in the human microcirculation. There are three major novel findings. First, we demonstrate that LPP3 protein expression is reduced in the LV from patients with CAD relative to non-CAD, while there are no differences in mRNA expression in either the LV or adipose resistance arterioles. Second, and unexpectedly, reducing the expression of LPP3 in adipose resistance arterioles from non-CAD patients via siRNA elicits a pathological switch in the mechanism of vasodilation from NO to H_2O_2. Third, we demonstrate that expression of miR-92a, the negative regulator of LPP3, is increased in adipose resistance arterioles from CAD patients relative to non-CAD, and inhibition of miR-92a in adipose resistance arterioles from CAD subjects induces a switch in the mediator of FMD from H_2O_2 to NO. Conversely, miRNA-92a mimic results in a pathological switch in the mechanism of FMD in arterioles from non-CAD patients, despite paradoxical increases in LPP3 expression. Collectively, these findings suggest that LPP3 may be required for maintaining normal endothelial function (NO-mediated FMD). Changes in the expression/function of this protein may have consequences at the microvascular level, potentially driving the functional vascular phenotype typically observed in patients with CAD.

LPP3 Expression within the Human Microcirculation

Several large genomic studies examining SNPs associated with cardiovascular (dys)function identified important polymorphisms in gene sequences coding for proteins with key functions in endothelial and smooth muscle cells (Coronary Artery Disease Genetics, 2011; Erbilgin *et al.*, 2013). These new CAD risk loci revived the interest in vascular wall-specific mechanisms that contribute to vascular pathologies. A genetic variant, rs17114036, located at 1p32.2 in a noncoding region of the sequence for LPP3 is a risk allele that is highly predictive of increased susceptibility for CAD independent of traditional risk factors such as hypertension, obesity, or type 2 diabetes mellitus (Schunkert *et al.*, 2011). Because the loci containing major risk-associated LPP3 SNP is associated with lower LPP3 protein levels (Erbilgin *et al.*, 2013), only a few studies have evaluated the expression of LPP3 specifically

in the vasculature from either animal models or humans, (Wu *et al.*, 2015), but none in the microcirculation. Considering the prognostic importance of microvascular dysfunction, we evaluated the expression of LPP3 in human tissues relevant to the cardiovascular function. Samples taken from human LV from subjects with clinically diagnosed CAD confirmed a reduction in protein expression, however transcription, (mRNA) was not different in either the LV or adipose resistance arterioles between CAD and non-CAD, which would be in line with a posttranscriptional effect of miRNA-92a to decrease LPP3 protein levels. However, with present data, it is unclear whether the observed reduction in protein expression is a result of, or a contributor to cardiovascular disease, and several groups have evaluated the role of LPP3 in cardiovascular function through *in vitro* and *in vivo* pharmacological and genetic inhibition of LPP3 function/expression. For example, genetic knockout of LPP3 is embryonically lethal due to an underdeveloped vasculature (Escalante-Alcalde *et al.*, 2003; Ren *et al.*, 2013), while inducible knockout results in vascular cell proliferation and increased inflammation (Panchatcharam *et al.*, 2013; Panchatcharam *et al.*, 2014). Tissue-specific inactivation of LPP3 also results in the development of atherosclerotic lesions, and elevation in circulating lipids, such as LPA, the main substrate of LPP3, is known to play a role in atherosclerosis (Busnelli *et al.*, 2017). Decreased expression of LPP3 is associated with SMC proliferation around regions of vascular injury (Panchatcharam *et al.*, 2013), along with upregulation of inflammatory markers (Wu *et al.*, 2015). These effects are likely the result of increased LPA signaling as blocking either LPA receptors or autotaxin (the enzyme responsible for generating majority of the circulating LPA) significantly reduced these responses (Panchatcharam *et al.*, 2014; Wu *et al.*, 2015).

Role of LPP3 in Maintaining Microvascular Function

While the majority of studies focus on the large, conduit arteries, little is known about the role LPP3 plays in the microcirculation. Small arterioles do not develop atherosclerosis, but like large vessels, experience activated endothelium and hyperplasia of the tunica media. However, more important might be the loss of endothelial function in the form of reduced bioavailability of NO and increased ROS as these compounds are the two major components of microvascular endothelial dysfunction (Crea *et al.*, 2014; Gutterman *et al.*, 2016). Previous data from our lab demonstrates the effects of microvascular dysfunction in CAD (switch in mechanism from NO to H_2O_2) (Phillips *et al.*, 2007; Beyer *et al.*, 2016). To evaluate whether LPP3 is involved in maintaining NO-mediated FMD in non-CAD arterioles, the mediator of FMD was assessed in non-CAD adipose arterioles transfected with siRNA targeting LPP3. While not reaching statistical significance, there was a trend toward a 50% reduction in LPP3 expression (Figure 2) with a shift from NO-mediated FMD to H_2O_2, recapitulating the functional phenotype observed in the presence of CAD. This observation suggests that reduced expression and/or function of LPP3 may contribute to the CAD phenotype characterized by FMD being mediated by H_2O_2.

Considering that 1) the function of LPP3 is to deactivate localized LPA signaling by removing the phosphate group (Morris & Smyth, 2014), 2) LPA levels are elevated in subjects with risk factors for or CAD itself (Dohi *et al.*, 2012), and 3) studies from our laboratory show that increasing LPA redirects the mediator of FMD from NO to H_2O_2 in non-CAD arterioles (Chabowski *et al.*, 2018), it is intriguing to speculate that in CAD, the

H_2O_2-mediated FMD is a result of elevated LPA signaling caused by decreased expression of LPP3. Whether a direct causative relationship exists between reduced expression of LPP3, elevated LPA signaling and loss of NO-mediated FMD requires further investigation.

miR-92a and Microvascular Function

Several studies identified miR-92a as an important regulator of vascular function with implications in cardiovascular pathophysiology (Bonauer *et al.*, 2009; de Winther & Lutgens, 2014; Huang *et al.*, 2017; Wang *et al.*, 2022). Additionally, miR-92a has been identified as a shear-sensitive, direct negative regulator of LPP3 expression in the HAECs (Wu *et al.*, 2015). Recent evidence demonstrates that miR-92a regulates arterial stiffness in mice by regulating vascular smooth muscle phenotype (Wang *et al.*, 2022). Collectively, these findings provide evidence that manipulation of miR-92a is a viable cardiovascular therapeutic. Indeed, current clinical trials (NCT03603431, NCT03494712) (Abplanalp *et al.*, 2020), are investigating the effects of repressing miR-92a on cardiovascular parameters. The present study is the first to report that miR-92a transcript expression is elevated in human adipose arterioles from CAD patients vs. non-CAD (Figure 3A). The reciprocal changes in expression of LPP3 (decreased in CAD) and miR-92a (elevated in CAD) are consistent with the observation that the mediator of FMD was restored to NO after treatment with anti-miR-92a (Figure 3D). Collectively, miR-92a represents a new target for improving microvascular endothelial dysfunction in CAD.

No difference in LPP3 expression was observed in CAD arterioles treated with anti-miR-92a oligo and control anti-miR oligo. (Figure 4A). This was unexpected and suggests that a different mechanism might be responsible for the functional phenotype. It is possible that the decrease in miR-92a was sufficient to restore NO-mediated dilation, but not sufficient to be detected by molecular means. A more likely explanation is that miR-92a has targets other than LPP3 that could influence the mediator of dilation. In fact, miR-92a has been shown to regulate Kruppel-like Factor 2 and 4, which are transcription factors capable of regulating eNOS expression under certain conditions (Wu *et al.*, 2011; Fang & Davies, 2012; Loyer *et al.*, 2014). However, when LPP3 and eNOS expression were evaluated under anti-miR-92a treatment, transcript levels were unchanged (Figure 4A and 4B).

Experimental Considerations

LPP3 protein was not detected (Western blot or IHC) in human adipose arterioles from subjects with or without CAD despite using similar protocols. Three different commercially available antibodies: PPAP2B (Novusbio, Cat. Number: NBP1-82825), VCIP (ZV002, ThermoFisher, Cat. Number: 39-1000) and PPAP2B (Sigma, Prestige Antibodies, Cat. Number: HPA028892) were tested with lysates for Western blot prepared from human arterioles (data not shown). Other studies use human-sourced cultured cell preparations, or overexpression of tagged/flagged LPP3 for detection of the tag rather than the LPP3 or animal LPP3 knockout/overexpression models. It is possible that LPP3 is not expressed in human adipose arterioles. This is unlikely considering LPP3 has been detected in in the endothelium of large vessels (Wu *et al.*, 2015; Aldi *et al.*, 2018), cultured endothelial cells from large human vessels (Wu *et al.*, 2015; Touat-Hamici *et al.*, 2016), mouse endothelium (Panchatcharam *et al.*, 2014), and in microvascular endothelial cells (Chatterjee *et al.*, 2016).

LPP3 has also been detected in SMC of animal and human large vessels (Panchatcharam *et al.*, 2013; Van Hoose *et al.*, 2022) but with limited data on microvasculature. It is possible that the protein levels in human arterioles are below the detection limit using the immunological methods and other, more sensitive methods need to be employed, such as mass spectrometry. At present, we cannot differentiate whether the shift from NO to H_2O_2-mediated FMD is due to reduced expression of LPP3 or the increase in LPA. Furthermore, we did not investigate the source of H_2O_2 generation within these experiments, however previous evidence from our lab has demonstrated that mitochondria are the primary source of H_2O_2 in CAD (Liu *et al.*, 2003). It is also possible that the absence/reduction of expression of LPP3 interferes with the canonical function of LPP3 (de-phosphorylation of LPA and other lipids), causing the dysfunction. Finally, it is possible that endothelial cells are much more sensitive and require less LPP3. Even a minimal reduction in LPP3 in endothelial cells could induce a phenotypical change. Furthermore, while the knockdown of LPP3 initiated intraluminally would affect endothelial cells first, the vascular smooth muscle would not be spared from the molecular modulation. However, it is possible that these small expression changes in the tunica intima and media thus reductions in LPP3 in endothelial cells, but not vascular smooth muscle would not be observed using Western blot techniques.

Studies involving transfecting vessels with different agents (siRNA or miR) are limited by the length of time the vessels are viable post-dissection. The upper limit for most human arterioles is 48 hours. It is possible that a functional effect is observed before a significant knock-down is achieved. Transfection times beyond the 48-h mark were not attempted due to the high degree of failed quality control at 72 or 96 hours (over 50%, unpublished data). However, higher concentrations need to be selected with caution as they may induce reagent-dependent vascular toxicity, masking the true effect of the transfection.

Previous work by our group has demonstrated that the switch in dilator mechanism in the microvasculature is a phenomenon associated with the presence of CAD and aging per se does not result in this switch (Beyer *et al.*, 2017). It should be noted that while this study was not designed nor powered to investigate sex differences, it should be pointed out that there is a disparate number of females that were included in the non-CAD group, and a larger number of male patients in the CAD group. Previous investigations by our group have not found sex differences in this switch in dilator mechanism(Beyer *et al.*, 2017).

Conclusion

Collectively, observations made in this study support the concept that LPP3 plays a critical role in maintaining normal microvascular endothelial function, and decreased expression is seen with CAD. Our study extends these findings into the human microcirculation, where loss of LPP3 recapitulates the phenotype observed in arterioles from subjects with CAD. miR-92a, an inhibitor of LPP3 expression is elevated in arterioles from subjects with CAD. Inhibition of this miR restores NO-mediated FMD in CAD arterioles. Whether this is through an increase in eNOS rather than LPP3 is an area for future investigation. LPP3 resides in the cell membrane where it degrades LPA. Whether the observed changes in the mechanism of FMD under reduced expression of LPP3 are a result of increased LPA signaling is an important focus of future work.

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Data Availability Statement:

All data is available upon request.

Non-Standard Abbreviations and Acronyms

CAD	Coronary artery disease
ET-1	Endothelin-1
FMD	Flow-mediated dilation
H_2O_2	Hydrogen peroxide
HUVEC	Human umbilical vein cells
L-NAME	L-N^G-Nitro arginine methyl ester
LPA	Lysophosphatidic Acid
LPP3	Lipid phosphate phosphatase 3
LV	Left ventricle
miR	Micro RNA
NO	Nitric oxide
NOS	Nitric oxide synthase
Peg-Cat	Polyethylene glycol catalase
ROS	Reactive oxygen species
siRNA	Silent interfering RNA

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Key Points

- LPP3 expression is reduced in patients with coronary artery disease (CAD).
- Loss of LPP3 in CAD is associated with an increase in the LPP3 inhibitor, miR-92a.
- Inhibition of LPP3 in the microvascular of healthy patients mimics the CAD FMD phenotype.
- Inhibition of miR-92a restores NO-mediated FMD in the microvasculature of CAD patients.

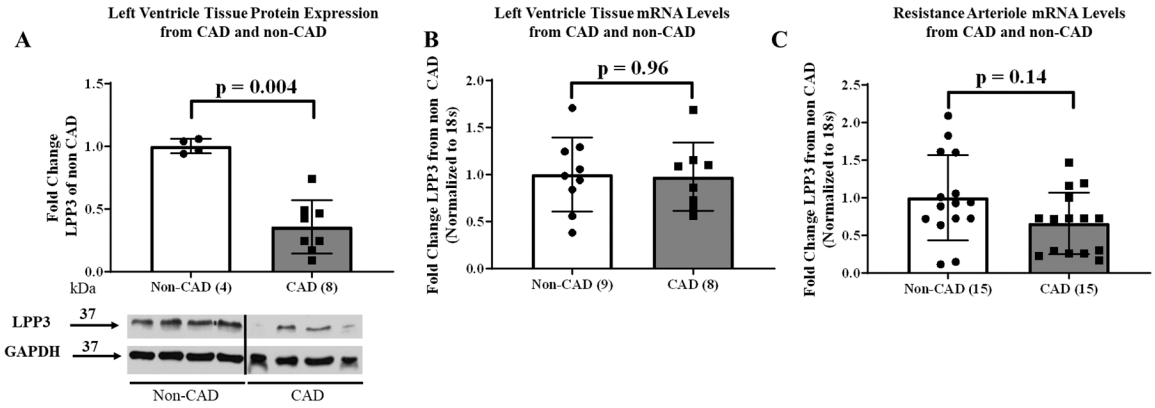

Figure 1.

Expression of LPP3 in human heart and adipose arterioles. **A**) LPP3 protein expression in human heart left ventricle from non-CAD (open bar, black circle) and CAD patients (grey bar, black square) (Mann Whitney U-test p = 0.004, n = 4 non-CAD, 8 CAD). Protein levels of LPP3 were significantly reduced in the presence of CAD. Blots represent 3 CAD and 3 non-CAD patient samples. **B**) LPP3 mRNA expression in human heart left ventricle from non-CAD and CAD patients. Presence of clinically diagnosed CAD had no effect on mRNA levels (Mann Whitney U-test, p = 0.96, n = 9 independent non-CAD samples, 8 independent CAD samples). **C**) LPP3 mRNA expression in human adipose arterioles from non-CAD and CAD subjects. LPP3 mRNA levels are not significantly reduced in arterioles from subjects with CAD (Mann Whitney U-test p = 0.14 n = 15 each group). Statistical analysis for protein and mRNA were performed using Mann Whitney non-parametric tests and are expressed as mean ± SD. (n) indicates the number of subjects.

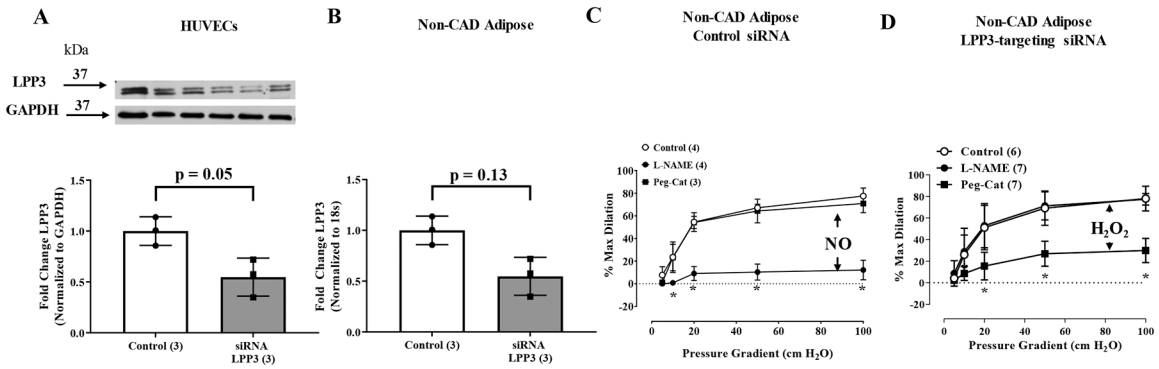

Figure 2.

Effects of LPP3 downregulation on flow-induced dilation (FMD) in non-CAD vessels. Confirmation of siRNA-driven knock-down of **A**) LPP3 Protein in HUVECs (Mann-Whitney U-test p = 0.05, n = 3 for both groups) and **B**) mRNA with RT-qPCR in non-CAD human vessels (Wilcoxon signed-rank test p = 0.13, n = 3 for both groups). **C**) The magnitude of FMD is preserved in human arterioles treated for 24 hours with scramble siRNA. L-NAME inhibits the dilation, while Peg-Cat has no effect. (2-way RM ANOVA, *p<0.0001 vs. control curve, n = 3 – 4) **D**) The magnitude of FMD is preserved in non-CAD human arterioles treated for 24-hours with LPP3-tergetting siRNA. Peg-Catalase significantly reduces FMD, whereas L-NAME has no effect (2-way RM ANOVA, p < 0.0001 vs. control curve, n = 6 – 7). Statistical analysis for protein and mRNA were performed using Mann Whitney and Wilcoxon non-parametric tests, respectively, while FMD responses were performed using a 2-way RM ANOVA. Results are expressed as mean ± SD. (n) indicates the number of subjects.

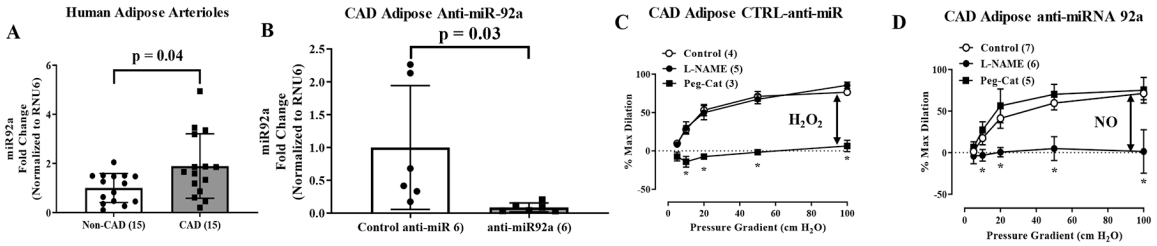

Figure 3.

Role of miR-92a in regulation the mechanism of FMD in CAD vessels. **A**) Levels of miR-92a is increased in vessels from subjects with CAD as compared to non-CAD. Expression normalized to RNU6 and expressed as fold change from non-CAD. (Mann Whitney U-test, p = 0.04 vs. non-CAD, n = 15 each group). **B**) Expression of miRNA-92a is decreased after treatment for 48 hours with anti-miR-92a in CAD arterioles in comparison to scramble anti-miR (Wilcoxon signed-rank test, p = 0.03 vs. control anti-miR, n = 6 matched pairs). **C**) The magnitude of FMD is preserved in human arterioles treated for 48 hours with control anti-miR. Peg-Catalase inhibits the dilation, while L-NAME has no effect (2-way RM ANOVA, *p < 0.0001 vs control curve, n = 3 – 5). **D**) The magnitude of FMD is preserved in CAD human arterioles treated for 48 hours with anti-miR-92a. L-NAME significantly reduces FMD, whereas Peg-Cat has no effect (2-way RM ANOVA, *p < 0.0001 vs control curve, n = 5 – 7). Statistical analysis for mRNA were performed using Mann Whitney and Wilcoxon non-parametric tests, respectively, while FMD responses were performed using a 2-way RM ANOVA. Results are expressed as mean ± SD. (n) indicates the number of subjects

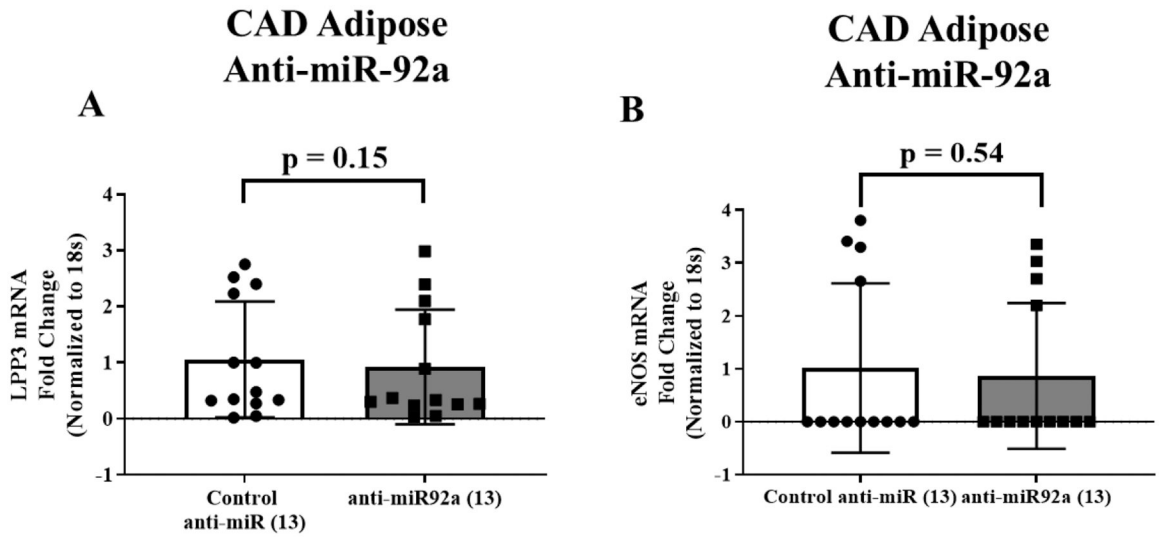

Figure 4.

Effects of miR-92a downregulation on expression of LPP3 and eNOS. **A**) Transcript expression of LPP3 are not different from control in response to 48-hours treatment with anti-miR-92a in adipose arterioles from patients with CAD. Expression normalized to 18s (Wilcoxon test, p = 0.15, n = 13 matched pairs). **B**) Transcript levels of eNOS are unchanged in response to 48-hours treatment of anti-miR-92a in adipose arterioles from patients with CAD. Expression normalized to 18s. (Wilcoxon test, p = 0.54, n = 13 matched pairs). Statistical analysis for mRNA were performed using Wilcoxon non-parametric comparisons. Results are expressed as mean ± SD (n) indicates the number of subjects

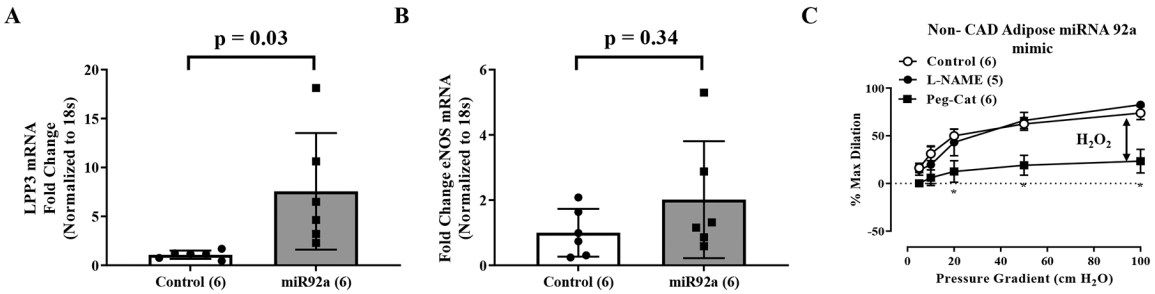

Figure 5.

miR-92a mimic does not decrease expression of LPP3 or eNOS in resistance arterioles from non-CAD patients. **A**) Transcript levels of LPP3 are significantly elevated in response to 48-hours of treatment with miR-92a mimic (Wilcoxon signed-rank test, p = 0.03 vs control, n = 6 matched pairs). Expression normalized to 18s. **B**) Transcript levels of eNOS are not increased in response to 48-hours treatment with miR-92a mimic. Expression normalized to 18s (Wilcoxon signed-rank test, p = 0.34, n = 6 matched pairs). **C**) Treatment (48 hours) with miR-92a mimic induces a switch from NO to H_2O_2 in resistance arterioles from non-CAD patients. Peg-Cat significantly reduces FMD, whereas L-NAME has no effect. (2-way RM ANOVA, *p < 0.0001 vs control curve, n = 5 – 6). Statistical analysis for mRNA were performed using Wilcoxon signed-rank test, while FMD responses were performed using a 2-way RM ANOVA. Results are expressed as mean ± SD. (n) indicates the number of subjects.

Table 1.

Patient Demographics for Heart Samples

	Non-CAD (n=8)	CAD (n=10)
Sex, Male/Female	2/6	4/6
Age, years (mean±SD)	45.4±15.7	55.8±5.5 *
Body mass index (mean±SD)	29.6±8.3	35.8±6.3
Underlying Conditions		
Coronary artery disease	0	10
Diabetes mellitus	0	5
Hypertension	0	7
Hyperlipidemia	0	4
Myocardial infarction	0	4
Congestive heart failure	0	1
None of the above	8	0

* $p<0.0001$ Non-CAD vs CAD.

Table 2:

Patient Demographics for Arterioles

	Non-CAD (n=33)	CAD (n=22)
Sex, Male/Female	8/25	18/4
Age, years (mean±SD)	49±16	71±10 *
Body mass index (mean±SD)	29.3±5.2	29.3±7.2
Underlying Conditions		
Coronary artery disease	0	22
Diabetes mellitus	1	7
Hypertension	3	15
Hyperlipidemia	2	15
Myocardial infarction	0	4
Congestive heart failure	1	3
A-Fib	0	5
None of the above	26	0

* $p<0.0001$ Non-CAD vs CAD.