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sphingosine-1-Phosphate-Induced Vasodilation in Human Resistance Arterioles During Health and Disease

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Abstract

Background: Preclinical studies suggest that sphingosine-1-phosphate (S1P) influences blood pressure regulation primarily through nitric-oxide (NO)-induced vasodilation. Since microvascular tone significantly contributes to mean arterial pressure, the mechanism of S1P on human resistance arterioles was investigated. We hypothesized that S1P induces NO-mediated vasodilation in human arterioles from adults without coronary artery disease (nonCAD) through activation of two receptors, S1PR₁ and S1PR₃. Further, we tested whether this mechanism is altered in vessels from patients diagnosed with coronary artery disease (CAD).

Methods: Human arterioles (50-200 μm in luminal diameter) were dissected from otherwise discarded surgical adipose tissue, cannulated, and pressurized. Following equilibration, resistance vessels were pre-constricted with endothelin-1 (ET-1) and changes in internal diameter to increasing concentrations of S1P (10⁻¹² to 10⁻⁷ M) in the presence or absence of various inhibitors were measured.

Results: S1P resulted in significant dilation that was abolished in vessels treated with S1PR₁ and S1PR₃ inhibitors and in vessels with reduced expression of each receptor. Dilation to S1P was significantly reduced in the presence of the NOS inhibitor L-NAME and the NO scavenger cPTIO. Interestingly, dilation was also significantly impaired in the presence of PEG-catalase, apocynin, and specific inhibitors of NADPH oxidases (NOX) 2 and 4. Dilation in vessels from patients diagnosed with coronary artery disease (CAD) was dependent on H₂O₂ alone which was only dependent on S1PR₃ activation.

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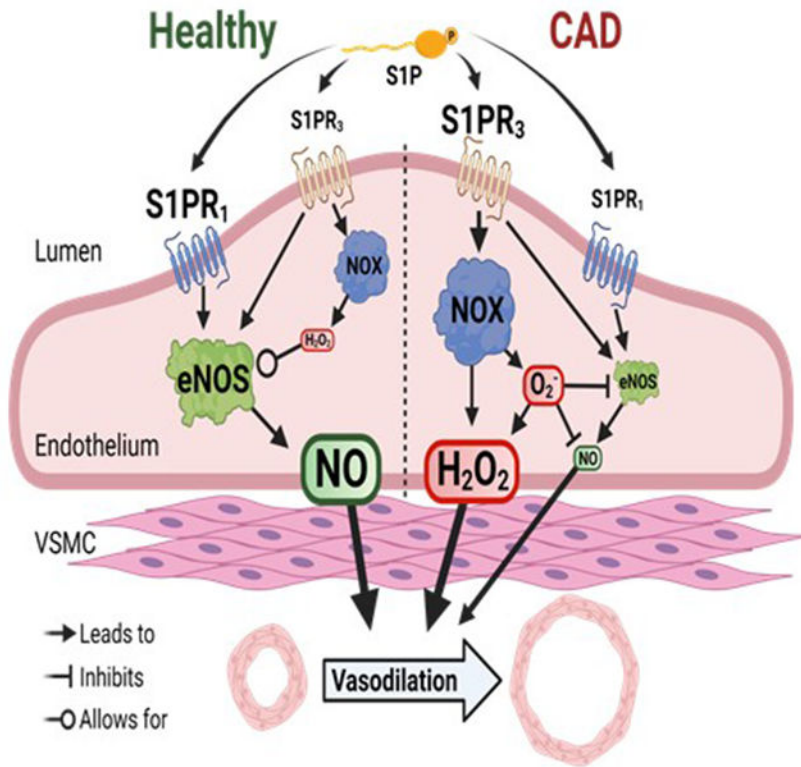
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Disclosures

The authors have no financial conflicts of interest to disclose.

Conclusions: These translational studies highlight the inter-species variation observed in vascular signaling and provide insight into the mechanism by which S1P regulates microvascular resistance and ultimately blood pressure in humans.

Graphical Abstract



Keywords

Endothelium; Microvascular; Vasodilation; Nitric Oxide; sphingosine-1-phosphate (S1P); microvascular tone; CAD; translational; human

INTRODUCTION

Hypertension remains the dominating risk factor for the development of cardiovascular disease, the number one cause of mortality both in the United States and globally. Maintaining normal blood pressure requires tight control of arterial tone predominantly in the resistance arterioles, a subtype of blood vessels that regulate peripheral vascular resistance. Resistance within arterioles, or microvascular tone, is modulated by various stimuli including shear-induced vasoactive mediators such as nitric oxide (NO)¹, hydrogen sulfide (H₂S)², and reactive oxygen species (ROS) such as H₂O₂³, to name a few. While many vasoactive compounds exist that freely diffuse out of the endothelium to relax surrounding smooth muscle, NO production via endothelial nitric oxide synthase (eNOS or NOS3) is preferred due to its anti-inflammatory properties on surrounding tissues as opposed to the damaging effects of H₂O₂⁴.

Circulating sphingolipids such as sphingosine-1-phosphate (S1P) have been shown to activate eNOS/NOS3 to cause NO-mediated dilation and reduce blood pressure in animals⁵. Within endothelial cells, S1P is formed by the phosphorylation of sphingosine by one of two sphingosine kinase isoforms (SpK 1 and 2). S1P then exits the cell exclusively through transporter Spinster-2^{6–8} and participates in “inside-out signaling” where it can activate its receptors within the endothelial cell membrane. The importance of S1P and its role in blood pressure homeostasis is highlighted by rodent *in vivo* studies showing that inhibition of S1P formation results in endothelial dysfunction and elevated mean arterial pressure⁹. S1P-induced activation of eNOS/NOS3 has been shown to be via endothelial membrane-bound G-protein coupled receptors, namely S1P receptors 1 and 3 (S1PR_{1&3}). S1PR₁ is the dominant receptor in the endothelium¹⁰, whereas S1PR₃ is present in both the endothelial and smooth muscle layer of arteries¹¹. Activation of S1PR₃ along with S1PR₂ in vascular smooth muscle cells has been shown to induce constriction¹² which is in sharp contrast to the NO-mediated vasodilatory effects observed by S1PR₃ in the endothelium. While the majority of studies suggest a strong link between S1PR₁ and NO formation, the role of S1PR₃ is more elusive with prior work demonstrating that it is capable of increasing ROS¹³.

Although the beneficial effects of S1P in rodents is clear, the diversity in mechanisms of vasodilation between species is dramatic¹⁴ and no studies to-date have examined S1P signaling in the human vasculature. The effects of S1P on microvascular tone during the disease state (e.g. cardiovascular disease) are also not well established. Here, we investigated the effect of S1P on human microvascular tone and sought to elucidate the mechanism(s) by which S1P elicits dilation in peripheral arterioles from subjects without coronary artery disease (nonCAD) as well as those diagnosed with coronary artery disease (CAD).

METHODS

The authors declare that all supporting data are available within the article (and its Supplemental Material).

Human Specimen Acquisition

Surgical discard human adipose tissue (pericardial, subcutaneous, visceral) was collected for the isolation of arterioles. Patients were included if they were under the age of 80 and had 0-1 risk factor(s) for CAD (e.g., hypertension, hyperlipidemia, diabetes mellitus type 1 or 2, congestive heart failure), or had a confirmed formal diagnosis of CAD via cardiac catheterization. For more detailed information regarding the collection of human specimens for this study please refer to the Supplemental Material. The research protocols for tissue acquisition and processing were approved by the Institutional Review Board of the Medical College of Wisconsin.

Microvascular Reactivity Studies

Human resistance arterioles with luminal diameters of 50-200 μ m ($113 \pm 17\mu$ m, mean \pm SD) were dissected from adipose tissue within 36 hrs of specimen arrival. Microvessels were cannulated on glass micropipettes of matched resistance in an organ chamber and

pressurized at 30 mmHg and 60 mmHg for 30 min each in warm Krebs buffer while bubbled with a 74%/21%/5% mixture of N₂, O₂, and CO₂, respectively as previously reported^{15, 16}. Following equilibration, arterioles were pre-constricted with endothelin-1 (ET-1) to 30-70% of their passive diameters. Vessel wall diameter changes in response to agents were then measured by visualizing the internal wall and using a digital caliper videomicroscopy system. Measurements were made both pre- and post-S1P administration at 1 min intervals.

During equilibration at 60 mmHg, vessels were exposed for 30 min to one of several different compounds: nitric oxide synthase (NOS) inhibitor N ω -nitro-L-arginine methyl ester (L-NAME) (100 μ M), the NO scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazole-1-oxyl-3-oxide (cPTIO) (100 μ M), polyethylene glycol-catalase (PEG-Catalase) 500 U/mL, S1PR₁ inhibitor W146 (10 μ M), S1PR₃ inhibitor CAY10444 (10 μ M), NADPH-oxidase (NOX) inhibitor apocynin (300 μ M), NOX4 inhibitor GKT137831 (1 μ M) or NOX2 inhibitor GSK2795039 (1 μ M). All interventions were administered into the organ bath and constituted 1% of the total bath volume and all concentrations represent the final concentrations in the organ bath. Increasing concentrations of S1P were administered (10⁻¹² M to 10⁻⁶ M) at 1-minute intervals. For endothelial viability assessment, acetylcholine was administered in increasing concentrations (10⁻⁹ M to 10⁻⁴ M) at 2-minute intervals. Up to two studies were performed in each vessel, separated by a wash (organ chamber bath replaced x3). At the end of the study, arterioles were exposed to the endothelial-independent dilator papaverine (100 μ M) to determine the vessel's maximal diameter. Microvessels that did not achieve a stable constriction with ET-1 for 10 min or dilated less than 75% of maximal resting diameter to papaverine were not included in the data analysis.

siRNA in Human Arterioles

Decreased expression of S1PR₁ and S1PR₃ was achieved as previously described¹⁷. Arterioles were cannulated on glass micropipettes at one end and transfused with either the negative control siRNA or target protein siRNA (50nM), both with Lipofectamine RNAiMAX (Invitrogen). Both ends of the arterioles were tied off and vessels were incubated in fresh EBM-2 medium overnight (16-20 hrs). Both ends of the vessels were cut following intraluminal exposure then cannulated for functional studies. Confirmation of decreased expression in arterioles was performed using immunohistochemistry.

Detection of NO in Microvessels

Fluorescent detection of NO in cannulated vessels was achieved using the Enzo Life Sciences NO detection Kit (Enzo Life Sciences, Enzo Biochem, Farmingdale, New York). Cannulated vessels were intraluminally exposed to the NO fluorescent probe (1:400 in HEPES buffer) for 2 hrs then pre-constricted with ET-1 to 50% of its passive diameter. Images were taken using an Olympus IX73 microscope following exposure to S1P (10⁻⁷) and maximal flow (P 100 cmH₂O) every min up to 5 min. The NOS inhibitor L-NAME was used to confirm probe specificity. Relative intensity was measured at excitation and emission wavelengths of 650/670 using ImageJ software. The total corrected fluorescence (TCF) = integrated density – (vessel area x mean background intensity) was calculated for

each vessel. Data was normalized and reported as fold change from the pre-S1P or pre-flow baseline value.

Immunohistochemistry

Confirmation of S1PR₁ and S1PR₃ expression in human arterioles was accomplished using immunohistochemistry. Microvessels collected from nonCAD adult patients (0-1 risk factors for CAD) or those diagnosed with CAD were fixed in 10% zinc formalin buffer for 24-72 hrs. Samples were then embedded in paraffin and cut into 4µm slices using a HMS355 microtome. S1P receptor expression was assessed by immunolabeling with a rabbit polyclonal IgG antibody against human S1PR₁ (1:200, 1 hr incubation) and rabbit polyclonal IgG against human S1PR₃ (1:100, overnight incubation). Sections were then incubated with secondary antibody or normal serum IgG (negative control). Immunohistochemical staining proceeded with biotinylated goat anti-rabbit IgG followed by alkaline phosphatase streptavidin and alkaline phosphatase chromogen substrate. A Leica Bond MAX immunostainer was used. Following staining, slides underwent heat-induced epitope retrieval (HIER) for 10 min. Slides were scanned with a NanoZoomer HT slide scanner (Hamamatsu, Japan).

Cell Culture

Human umbilical vein endothelial cells (HUVEC; ATCC, Manassas, VA, USA) were cultured in endothelial cell growth medium (EGM-2; Lonza, Basel, Switzerland), supplemented with 5% fetal bovine serum, growth factors, heparin, hydrocortisone, and ascorbic acid (EGM-2 SingleQuot; Lonza, Basel, Switzerland). Cells were used at passage 4-5 at 70-90% confluency. Treatments included 1% FBS with S1P vehicle (methanol), NOX4 inhibitor, NOX4 inhibitor vehicle (ethanol), and PEG-Catalase for 30 min. Following the 30 min incubation, cells were exposed to S1P (10^{-7}) for 10 min.

Western Blot

Treated HUVECs were scraped and submerged in cold lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% deoxycholic acid, 0.1% SDS, 0.5% NP40), which was previously mixed with a protease and phosphatase inhibitor cocktail (Roche, Basel, Switzerland). The cell lysates were sonicated and kept at -80°C until further use. Protein concentration was determined using the BCA protein assay (Thermo Scientific, Waltham, MA, USA). Samples were then subjected to electrophoresis using 4-20% SDS-PAGE gel and transferred to nitrocellulose membranes. Primary antibodies for proteins of interest were then mixed in 1% BSA and added for 1 hr (eNOS, 1:1000, rabbit, monoclonal, Cell Signaling, and phosphorylated-eNOS Ser1177, 1:1000, rabbit, monoclonal, Cell Signaling). Expression of GAPDH (1:1000, rabbit, monoclonal, Cell signaling, #D16H11) was used as a loading control. Peroxidase conjugated secondary antibodies and chemiluminescent substrate (Bio-Rad, Hercules, CA, USA) were used for detection.

Materials

1% bovine serum albumin (BSA) solution was used to dissolve endothelin-1 (Millipore Sigma, St. Louis, MO, USA). Methanol (MeOH) and 0.3M NaOH solution were used to

reconstitute S1P (Millipore Sigma, St. Louis, MO, USA and Cayman Chemical, Ann Arbor, MI, USA) according to the manufacturer's recommendations. W146 (Cayman Chemical & Tocris Bioscience, Minneapolis, MN, USA) was reconstituted in MeOH and 0.03M NaOH. CAY10444 and c-PTIO (Cayman Chemical) were dissolved in dimethylformamide and water, respectively. GKT137831 (Cayman Chemical) and GSK2795039 (Millipore Sigma) were both dissolved in ethanol. Apocynin and PEG-Catalase (Millipore Sigma) were dissolved in MeOH and water, respectively. Acetylcholine (Millipore Sigma) was diluted in water. S1P receptor antibodies were purchased from Invitrogen by Thermo Fisher (Thermo Fisher Scientific, Waltham, MA, USA). ROS-ID® NO Detection kit (Enzo Life Sciences, Enzo Biochem, Farmingdale, New York) was dissolved in HEPES buffer at the time of the experiment.

Statistical Analysis

For functional vascular studies, data is presented as mean \pm SEM. Dilation is expressed as a percentage of maximal dilation with 100% dilation representing the maximal dilation achieved with the endothelium-independent dilator papaverine. % maximal dilation = (vessel diameter – stable diameter after ET-1 constriction) / (maximal diameter – stable diameter after ET-1 constriction). To compare the dose-response relationship, a two-way ANOVA was used with dose-response to S1P and treatment as parameters with a Geisser-Greenhouse correction. When a significant difference was observed between curves ($p < 0.05$), results were compared using a Dunnett's multiple comparisons test. For NO detection studies, all data was normalized and reported as fold-change from baseline. A student's t-test was performed between intervention (S1P dose or time at maximal flow) and intervention + L-NAME. Western blot data were plotted as ratios of interventions compared against S1P analyzed using a ratio t-test; interventions were compared against S1P. All analyses were performed using GraphPad Prism 9.1.0 (GraphPad Software, San Diego, CA, USA) with statistical significance defined at $p < 0.05$.

RESULTS

Demographic data for patients is included in Table 1. nonCAD individuals are defined as those having 0-1 risk factor(s) for CAD while the CAD group consisted of patients with a confirmed clinical diagnosis of significant (obstructive) coronary atherosclerosis. Risk factors for CAD included hypertension, hyperlipidemia, active smoker, diabetes mellitus (type 1 and 2), and congestive heart failure. A total of 69 surgical discard specimens were collected from adults aged 22-79. 47 of the 69 patients had 0 risk factors for CAD, while others had only one risk factor. 15 patients had a diagnosis of CAD and could have more than 1 risk factor for the disease. The average age was 42.6 ± 10.8 yrs (mean \pm SD) and 67.5 ± 10.2 for the nonCAD and CAD groups, respectively. BMI was similar between the nonCAD and CAD groups. The initial diameters at cannulation and after administration of papaverine (mean \pm SD), were $113 \pm 17 \mu\text{m}$ and $164 \pm 22 \mu\text{m}$, respectively.

S1PR₁ and S1PR₃ contribute to S1P-induced dilation in arterioles from nonCAD adults

To confirm that both S1PR₁ and S1PR₃ are present in human resistance arterioles, immunohistochemistry was performed. As shown in Figure 1A, both S1PR₁ and S1PR₃

are present in the endothelium of microvessels from nonCAD adults as indicated by the brown staining which is absent in the negative controls (Fig S1A).

To determine whether exogenous S1P is capable of eliciting dilation in human arterioles, increasing doses of exogenous S1P (10^{-12} M to 10^{-7} M) were administered to pressurized microvessels pre-constricted with ET-1. A linear, dilatory dose response curve was observed (Figure 1B) with maximal dilation occurring at 10^{-7} (% maximal dilation [MD] mean \pm SEM, 55 ± 4.1 , $n=12$) which was significantly increased compared to vehicle control (%MD 1.9 ± 1.0 , $n=5$ ‡ $p<0.01$, * $p<0.05$) (Figure S1B). Interestingly, administration of 10^{-6} S1P elicited constriction and reduced the diameters on average to 22.7 ± 5.8 , $n=12$ (Figure S3A), suggesting higher doses of S1P cause constriction, a phenomenon previously reported in animal studies¹⁸. To determine whether the response to S1P requires activation of S1PR₁ and/or S1PR₃, a dose response curve (DRC) to S1P was performed both in the presence of the S1PR₁ antagonist W146 and the S1PR₃ antagonist CAY10444. As shown in Figure 1B, maximal dilation to S1P was significantly reduced with inhibition of either S1PR₁ or S1PR₃ compared to the administration of S1P alone (%MD 1.7 ± 4 , $n=5$ and 19.7 ± 8.7 , $n=6$, respectively, versus 55 ± 4.1 for S1P alone, ‡ $p<0.01$, * $p<0.05$), suggesting that both receptors are critical for S1P-induced dilation in humans. To demonstrate the viability of the endothelium in the presence of these inhibitors, dose response curves to the endothelial-dependent vasodilator acetylcholine (Ach) were performed following incubation with either W146 or CAY10444. Despite the presence of these inhibitors, vessels maintained the ability to dilate to Ach ($80\% \pm 7.6$, $n=4$ and $89.1\% \pm 2.7$, $n=4$, for W146 and CAY10444, Figure S2B).

To confirm the role of S1PR₁ and S1PR₃ in this mechanism of dilation, siRNA against both receptors was utilized to decrease expression in nonCAD human arterioles. As shown in Figure 1C, a decreased amount of brown staining is observed in the endothelium (denoted by red box) in microvessels intraluminally exposed to siRNA for S1PR₁ and S1PR₃. Dilation to S1P was significantly decreased in vessels treated with siRNA against S1PR₁ (%MD 11.2 ± 4.9 , $n=5$) compared to S1P alone (%MD 55 ± 4.1 , $n=12$, ‡ $p<0.01$). Likewise, a significant reduction in S1P-induced maximal dilation was seen in arterioles treated with siRNA against S1PR₃ (%MD 36.8 ± 4.1 , $n=5$) compared to untreated vessels (‡ $p<0.01$) (Figure 1D). S1P was capable of eliciting dilation in arterioles intraluminally treated with negative control siRNA (Figure S1C).

S1P-induced dilation in human microvessels requires formation of NO

To determine the role of NO in dilation, we performed S1P DRCs in the presence of the NOS inhibitor L-NAME and cPTIO, a potassium salt capable of scavenging NO. As shown in Figure 2, nonCAD vessels exposed to either L-NAME or cPTIO exhibited significantly reduced dilation to S1P (%MD 15.8 ± 6.5 , $n=5$, and 9.5 ± 3.1 , $n=5$ for L-NAME and cPTIO, respectively) compared to S1P alone (%MD 55.0 ± 4.1 , $n=12$, ‡ $p<0.01$).

To confirm that S1P induces formation of NO in microvessels, NO was detected with a fluorescent probe and measured using microscopy. After intraluminal incubation with the probe, vessels were exposed to increasing concentrations of S1P (10^{-12} M to 10^{-7} M). Figure 2C illustrates that administration resulted in a slight increase in signal intensity which

was quenched by the addition of L-NAME at 10^{-11} M of S1P ($n=3$, $*p<0.05$). Since shear stress is a robust stimulus for NO production, changes in fluorescence intensity were also measured in vessels exposed to maximal flow (pressure gradient of 100cm H₂O) to serve as a comparison. NO formation rose dramatically within 30 s of being exposed to maximal flow (Figure 2D) as indicated by an increase in fluorescence intensity which peaked at 300 s (5 min) of flow ($n=8$). The signal was significantly reduced in the presence of L-NAME at 30, 60, and 120 s of flow ($n=3$, $*p<0.05$). Figures 2E and 2F are representative images for NO detection from S1P and flow, respectively.

H₂O₂ is necessary for NO-mediated S1P-induced dilation

It has been shown that H₂O₂ sensitizes S1P receptors in endothelial cells and results in a higher degree of eNOS activation compared to S1P alone¹⁹. To understand the role of H₂O₂ in S1P-mediated vasodilation, S1P DRCs were performed in nonCAD human microvessels during exposure to PEG-Catalase which resulted in impaired dilation compared to S1P (%MD 8.4 ± 3.1 , $n=5$, $\ddagger p<0.01$, Figure 3A). Since sphingolipids are known to acutely activate NADPH oxidases (NOXs), we then tested whether inhibition of NOX would cause a similar impairment in dilation. Microvessels treated with the non-specific NOX inhibitor apocynin exhibited a significant decrease in S1P-induced dilation (%MD 10.1 ± 2.9 , $n=5$) compared to S1P alone (%MD 55.0 ± 4.1 , $n=12$, $\ddagger p<0.01$, Figure 3B). To determine which specific NOX is required for dilation, inhibitors for the two major NOX isoforms in endothelial cells, NOX2 and NOX4, were administered during exposure to S1P. The use of either inhibitor significantly decreased S1P-induced dilation (%MD 22.9 ± 6.6 , $n=5$ and 11.2 ± 8.3 , $n=5$, for NOX2 and NOX4, respectively) compared to S1P ($\ddagger p<0.01$, Figure 3C–D). Finally, to add mechanistic insight into the role of NOX in S1P vasodilation, western blots were performed in human umbilical vascular endothelial cells (HUVECs) to determine if NOX4, an enzyme that has been shown to be necessary for eNOS signaling²⁰, is required for S1P-induced increases in phosphorylated eNOS²¹. Figure 4 shows that the Ser1177 phosphorylated:total eNOS ratio is decreased in endothelial cells treated with both S1P and the NOX4 inhibitor compared to S1P alone ($n=4$ for all groups, $*p<0.05$). Of note, constriction to S1P was observed at the higher dose (10^{-6}) in nonCAD arterioles treated with the various inhibitors as well as siRNA (Figure S3).

Dilation to S1P during disease relies primarily on activation of S1PR₃ and formation of H₂O₂

Similar to arterioles from nonCAD individuals, both S1PR₁ and S1PR₃ were identified in microvessels collected from patients with CAD (Figure 5A). Although S1P elicited dilation in vessels from individuals with disease (%MD 56.3 ± 12.6 , $n=5$), this effect was not significantly reduced during inhibition of S1PR₁ (%MD 30.8 ± 13.4 , $n=5$, Figure 5B) but rather was only impaired during exposure to the S1PR₃ antagonist (%MD 8.1 ± 8.4 , $n=4$, $*p<0.05$) as opposed to S1P alone (%MD 56.3 ± 12.6 , $n=5$) (Figure 5C). To establish the mediator of S1P-induced dilation in human vessels during disease, we exposed the vessels to L-NAME and PEG-Catalase. Exposure of the vessels to L-NAME did not significantly impact the dilation caused by S1P (%MD 27 ± 6 , $n=5$, Figure 5D), however was significantly impaired with PEG-Catalase (%MD 12.4 ± 8.8 , $n=5$, $*p<0.05$) compared to S1P alone (%MD 56.3 ± 12.6 , $n=5$, Figure 5E). Ach-induced dilation did not differ in

arterioles from patients with CAD versus nonCAD (Figure S2A). A relative constriction was also observed following administration of a higher dose of S1P (10^{-6}) in presence of all inhibitors (Figure S4).

DISCUSSION

To our knowledge this is the first study to examine the mechanism by which S1P elicits vasodilation in humans during both health and disease. There are several major findings here, the first of which is that S1P promotes dilation in the human microvasculature in physiological concentrations, suggesting a possible major role in blood pressure homeostasis. We hypothesized and confirmed that S1P receptors 1 and 3 are present in the endothelium of resistance arterioles. Both pharmacological inhibition and genetic knock-down of S1PR₁ and S1PR₃ supported our hypothesis that both receptors are critical for S1P-induced dilation in microvessels from nonCAD individuals. Our fourth major and unexpected finding was that the S1P pathway requires both NO and H₂O₂ to elicit dilation, and activation of NADPH oxidases (NOX 2 and 4) is a key contributor to this mechanism. However, unlike the robust formation of NO during shear, exogenous exposure to S1P results in a modest increase in NO. Finally, although both receptors are present in the microvascular endothelium from patients with disease (CAD), dilation from S1P relies primarily on activation of S1PR₃ as opposed to S1PR₁. These translational findings suggest that S1P signaling in the human endothelium regulates vascular tone however the mechanism differs between nonCAD adult individuals versus those with cardiovascular disease.

S1P signals through three G-protein coupled receptors (GPCRs), S1PR₁₋₃. Expression of these receptors in vascular tissue is heterogenous, with S1PR₁ being the dominant receptor in the endothelium¹⁰, S1PR₂ being more highly expressed in the medial layer, and S1PR₃ being expressed in both the endothelium and smooth muscle cells¹¹. The downstream signaling of each receptor has also proven unique as S1PR₁ signals exclusively through the G_{α_i} subunit²², S1PR₃ signals through G_i, G_q, and G_{12/13}, and S1PR₂ relies primarily on G_{12/13}^{10, 23-27}. Several studies done in human cell lines²⁸⁻³⁰ and rodents³¹ have shown that increases in NO upon administration of S1P are dependent on activation of S1PR₁³² and S1PR₃^{33, 34}, which lead to eNOS activation through PI3K and Akt that is initiated by the shared G_i subunit. Our data suggests that inhibition or decreased expression of either receptor results in decreased dilation suggesting a necessary communication between the two downstream signaling pathways. With the exception of pharmacological inhibition of S1PR₁, residual dilatory capacity to S1P was observed in vessels with reduced activity or expression of either receptor. The residual dilation may be the result of incomplete inhibition of the targeted receptor, activity of the non-targeted receptor, or dilation through an unknown mechanism.

Generation of mouse models in which S1PR₁ is deleted in endothelial cells by tamoxifen-activated Cre recombinase (S1PR₁^{flox/flox} or S1PR₁ ECKO) have been crucial to understand the role of S1P in blood pressure regulation in animals. Using this mouse model, Cantalupo et al. elegantly demonstrated that S1PR₁, as opposed to S1PR₃, is primarily responsible for blood pressure homeostasis³⁵. Others have provided compelling evidence that S1PR₃ is

equally important in controlling arterial pressure. Nofer et al. have shown that high-density lipoprotein (HDL) exerts its dilatory effects through bound S1P and activation of S1PR₃³³. Forrest et al. also proposed that S1PR₃ is essential for blood pressure homeostasis as arterial pressure was significantly increased in S1PR₃^{-/-} mice³⁶. It is important to note that these preclinical studies were not only done in animals, but also in varying sized vessels from different vascular beds. Although these studies have added insight to the importance of S1PR signaling, the abundance of S1PR₁ versus S1PR₃ in large (conduit vessels) versus small (arterioles) is unknown as well as how receptor expression changes from health to disease. It is clear from our data and others that both receptors have an important role in vascular tone and blood pressure regulation. Perhaps the more interesting question is whether expression of one receptor over the other is more beneficial for long-term vascular health and if receptor expression or signaling is altered in the early stages of hypertension.

While the majority of studies have focused on how S1P reduces vascular tone through formation of NO, only a few have suggested an important role for H₂O₂. Dantas and colleagues have reported that dilation from S1P relies exclusively on eNOS-derived NO, as opposed to other endothelium-derived hyperpolarizing factors (EDHFs)³¹. Our results suggest that NO formed during exposure to S1P is modest compared to NO generated during increased flow, although still critical as S1P-induced dilation is inhibitable by both L-NAME and cPTIO. One possible explanation for this finding could be that the level of NO produced from S1P is below the threshold of detection for the fluorescent probe. Another possibility is the fact that S1P also promotes ROS formation, therefore NOX-produced superoxide could potentially quench S1P-induced NO thus falling below the threshold for detection. Finally, the majority of S1P in human plasma is bound to HDL³⁷, however for this study, as common in other studies^{30, 31, 38}, S1P was administered directly, unbound to lipoproteins. It is possible that S1P triggers a higher level of NO formation if bound to HDL.

Based on our findings, the human vasodilatory response to S1P requires production of both NO and H₂O₂, presumably through nitric oxide synthase (NOS) and NOX, respectively. Although the deleterious effects of H₂O₂ on the cardiovascular system are well-documented in the literature³⁹, formation of this ROS during health has also proven critical. Generation of H₂O₂ has been shown to be a necessary component in the activation of eNOS during laminar shear stress⁴⁰. Some speculate the source of H₂O₂ is the key factor in the determination of whether its downstream result is beneficial or detrimental to the vascular wall. For instance, emerging evidence suggests that NOX4-derived H₂O₂ is vasculoprotective⁴¹⁻⁴³. Furthermore, Tian et al. have demonstrated that H₂O₂ promotes eNOS localization to the plasma membrane to enhance NO production during shear⁴⁴. More specifically, studies performed in BAECs have shown that activation of NOX4 with subsequent activation of eNOS is critical in NO-formation during flow⁴⁵. Our data add to this growing body of evidence as we found that NOX 2 and 4 are needed for dilation from S1P. Further, we provide data suggesting that S1P-induced increases in phosphorylated eNOS rely on activation of NOX4. While studies have suggested that NO is the primary mediator responsible for S1P dilation, our study underscores the importance of ROS in the mechanism by which S1P may regulate blood pressure in humans. The H₂O₂ formed from S1P may itself trigger dilation through various proposed mechanisms including activation of

transient receptor potential vanilloid 4 (TRPV4) channels⁴⁶, or act as a component of eNOS activation⁴⁷.

Although animal studies have been instrumental in deciphering the S1P signaling pathway, the use of discarded surgical tissue allowed for interrogation of S1P signaling specific to humans and also shed light on how this mechanism changes during times of disease, a model which is difficult to replicate in animals. Interestingly, although both S1PR₁ and S1PR₃ were both identified and found to be functional in arterioles from patients with CAD, dilation to S1P was observed to be predominantly due to activation of S1PR₃. Of note, since collection of tissue from diseased patients is more scarce, only pharmacological studies were conducted. A key hallmark of cardiovascular disease is decreased NO bioavailability due to increased cellular oxidative stress⁴⁸. Our data here suggests that dilation due to acute S1P exposure relies primarily on activation of S1PR₃ and formation of reactive oxygen species (H₂O₂). Although S1P may regulate tone by promoting vasorelaxation, the mechanism by which this is accomplished may cause further oxidative damage. We have previously shown that chronic exposure (16-20 hrs) to S1P is capable of restoring flow-induced NO-mediated dilation in vessels from CAD patients⁴⁹. This, combined with the fact that plasma S1P levels are reduced in patients with CAD, suggests that during times of low S1P exposure or a high oxidative stress environment, the S1P signaling pathway is pro-oxidant but can be manipulated over time to reestablish a healthy endothelium.

Study limitations

As common in other studies^{30, 31, 38} including this study, S1P was administered freely dissolved in methanol. This may not accurately represent the physiological effects of S1P in human blood, as most of the lipid is bound to either albumin or HDL³⁷. Another issue is that to enhance clarity during video microscopy, arterioles were stripped of perivascular adipose tissue (PVAT) prior to cannulation. As such, possible interactions between S1P and PVAT remain unknown, and it is possible that S1P also elicits effects on PVAT which ultimately influence tone on the vessel it surrounds. Finally, a major limitation of this study is the amount of heterogeneity that exists among the patients from which tissue was collected. Please refer to the Supplemental Material for a complete description of limitations associated with the use of human tissue for translational research.

Perspectives

Preclinical studies have revealed an important role for S1P in the regulation of blood pressure. Although S1P is effective at reducing microvascular tone, the mechanism of dilation in humans is more complex and favors formation of oxidative stress in individuals with cardiovascular disease. The question remains whether targeting of S1PR₁ is a safe and effective strategy to reduce blood pressure in this patient population.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations and Acronyms

BAEC	bovine aortic endothelial cells
CAD	coronary artery disease
cPTIO	2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazole-1-oxyl-3-oxide
EDHF	endothelial-derived hyperpolarizing factor
eNOS	endothelial nitric oxide synthase
FID	flow-induced dilation
H₂O₂	hydrogen peroxide
HUVEC	human umbilical vein endothelial cells
L-NAME	N ω -nitro-L-arginine methyl ester
MD	maximal dilation
NADPH	nicotinamide adenine dinucleotide phosphate
NO	nitric oxide
NOX	nicotinamide adenine dinucleotide phosphate oxidase
PEG-Catalase	polyethylene glycol - catalase
ROS	reactive oxygen species
S1P	sphingosine-1-phosphate
S1PR	sphingosine-1-phosphate receptor

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Novelty and Relevance

What is New?

Physiologic levels of S1P elicit dilation in human resistance arterioles through activation of S1PR₁ and S1PR₃.

Both S1PR₁ and S1PR₃ are expressed in microvessels from nonCAD adult patients as well as individuals diagnosed with CAD.

S1P signaling in arterioles from nonCAD adults requires formation of both NO and H₂O₂, as opposed to predominantly H₂O₂ in vessels from subjects with CAD.

What is Relevant?

S1P signaling in nonCAD humans requires formation of both NO and reactive oxygen species.

The mechanism of S1P-induced dilation is altered during disease and favors formation of H₂O₂ via activation of S1PR₃.

Clinical/Pathological Implications

S1P signaling has emerged as a potential therapeutic target in the regulation of blood pressure. The mechanism by which S1P causes dilation in human resistance arterioles is altered during disease and may amplify production of intracellular oxidative stress.

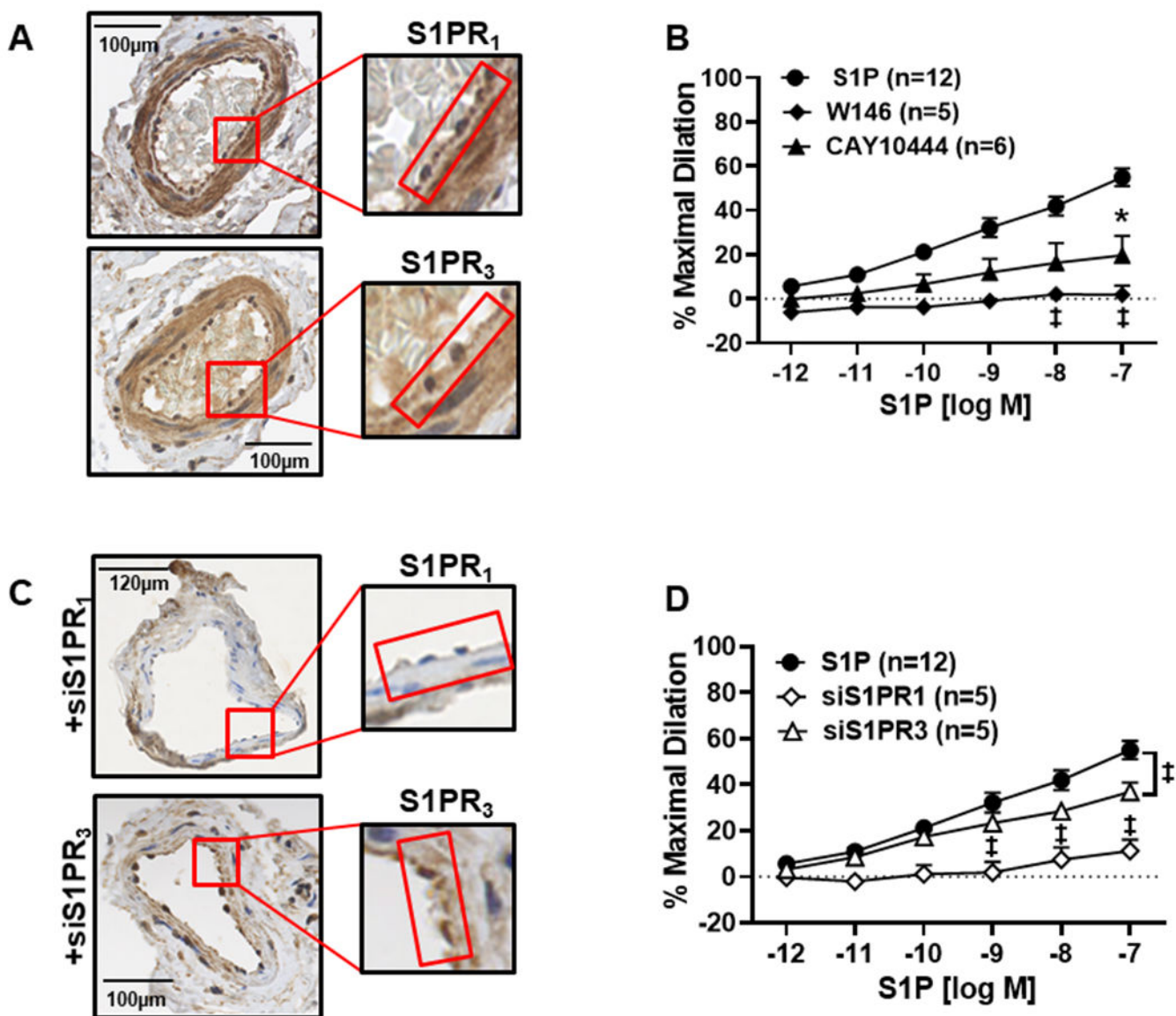


Figure 1. S1P elicits dilation in human arterioles from nonCAD patients via activation of S1PR₁ or S1PR₃.

A) Immunohistochemistry (IHC) staining (representative images of n=5) shows that S1P receptors 1 and 3 are expressed in the human microvasculature from nonCAD adults (left). The endothelium is highlighted by the red box. **B)** S1P elicits a dose-dependent dilation (10^{-12} to 10^{-7} , n=12) which is inhibited by the S1PR₁ inhibitor W146 (10 μ M, n=5) and CAY10444 (10 μ M), an S1PR₃ inhibitor (n=5) compared to S1P alone (n=12, ‡p<0.01, *p<0.05) **C)** Confirmation of siRNA-mediated knock-down of S1PR₁ and S1PR₃ in human arterioles with endothelium outlined in red (representative images from n=3). **D)** Dilation to S1P is significantly decreased in microvessels treated with either siS1PR₁ (n=5) or siS1PR₃ (n=5, ‡p<0.01 and *p<0.05) siRNA treatment versus S1P. Data are expressed as mean % maximal dilation \pm SEM.

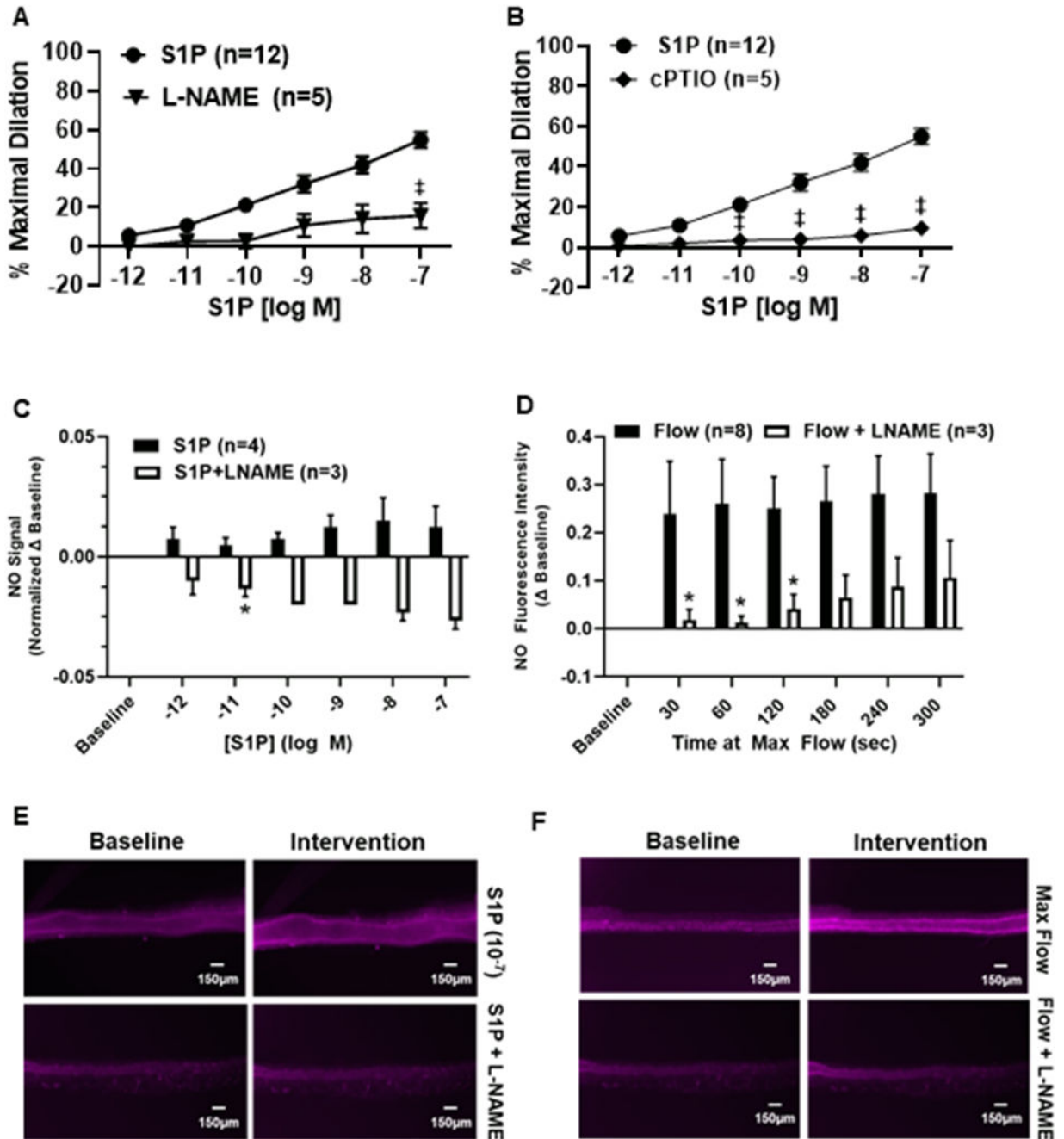


Figure 2. Role of NO in S1P-induced dilation of the human microvasculature.

A) S1P-induced dilation in arterioles from nonCAD patients is reduced in the presence of the nitric oxide synthase (NOS) inhibitor L-NAME (100 μ M, n=5, ‡p<0.01) L-NAME versus S1P alone, as well as during exposure to the NO scavenger cPTIO (100 μ M, n=5, ‡p<0.01) **(B).** **C)** NO increases from baseline following exposure to increasing doses of S1P (n=4) which is quenched in the presence of L-NAME (100 μ M, n=3). **D)** NO formation in response to maximal flow (20 μ L/min, n=8) which is significantly reduced with L-NAME (100 μ M, n=3, *p<0.05). **E)** Representative images of NO production in vessels exposed to S1P +/-

L-NAME (100 μ M). **F**) Representative images of NO formation in arterioles exposed to maximal flow (20 μ L/min) +/- L-NAME (100 μ M). Data are expressed as the mean \pm SEM.

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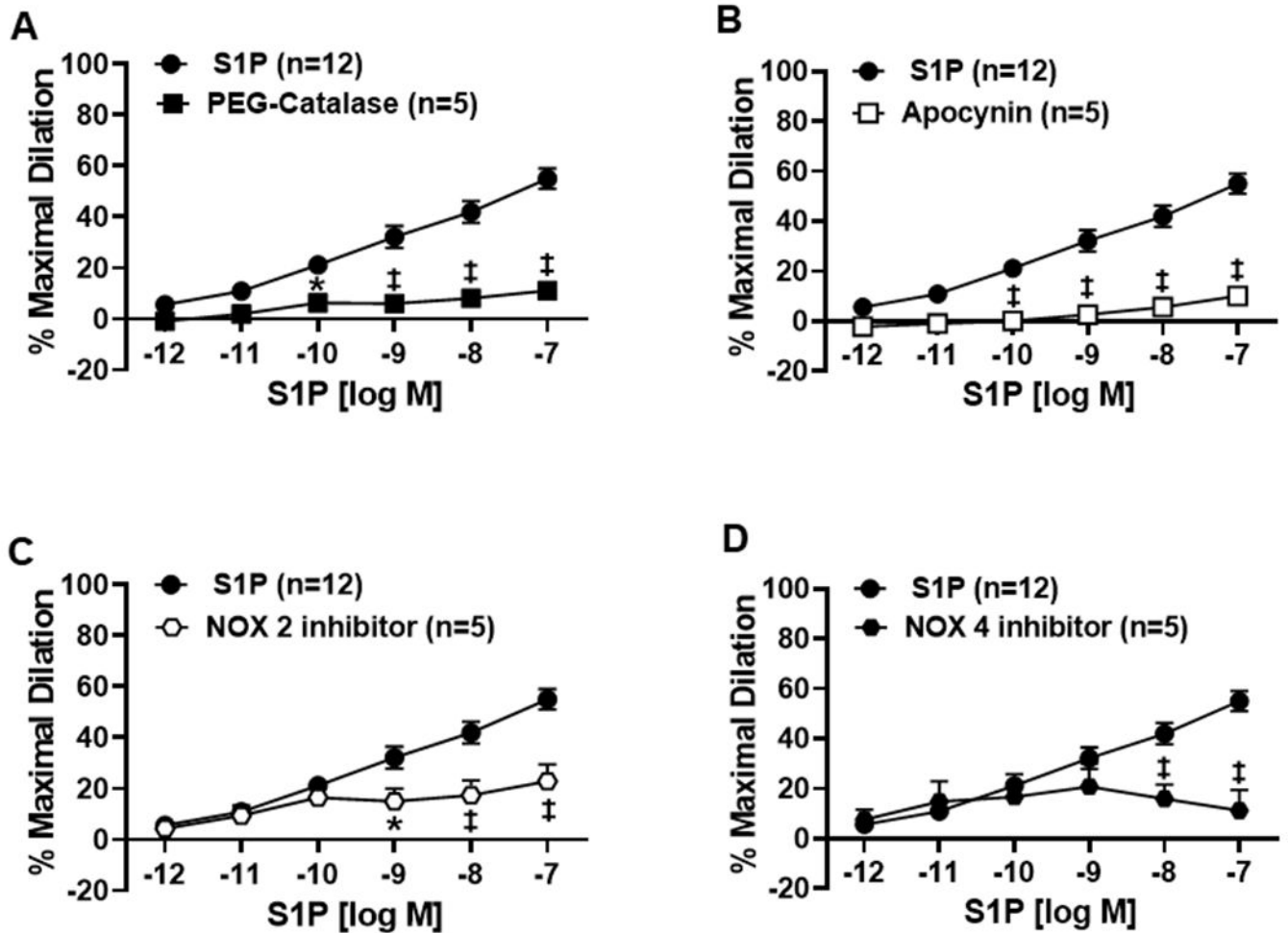


Figure 3. H₂O₂ production via NOX is necessary for S1P-induced dilation in microvessels from nonCAD subjects.

A) Dilation elicited by S1P is significantly reduced in the presence of PEG-Catalase (500U, n=5), the non-specific NOX inhibitor apocynin (300μM, n=5) (B), the specific NOX2 inhibitor GSK2795039 (1μM, n=5) (C), and the NOX4 inhibitor GKT137831 (1μM, n=5) compared to S1P alone (n=12, ‡p<0.01, *p<0.05) (D).

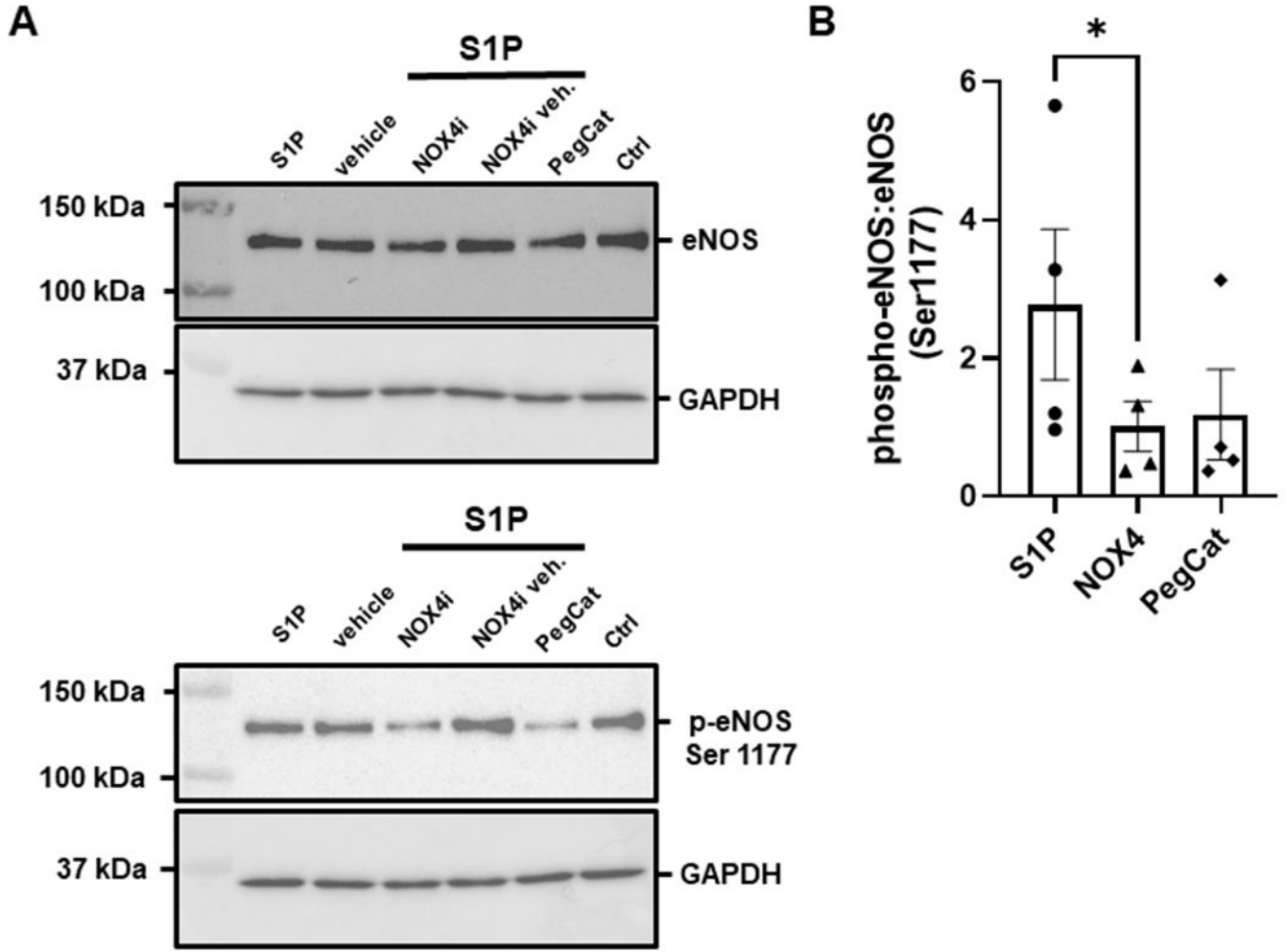


Figure 4. Role of H₂O₂ in phosphorylated eNOS from S1P.

S1P-induced Ser1177 phosphorylation of eNOS in HUVECs (n=4) is impaired in the presence of PEG-Catalase (500U, n=4) and significantly reduced during inhibition of NOX4 with GKT137831 (1μM, n=4). Representative images of western blots on the left (A) and phospho-eNOS:eNOS ratio comparison on the right (B). Data are expressed as the mean ratio of phosphorylated eNOS to total eNOS±SEM. *p<0.05 compared to S1P alone.

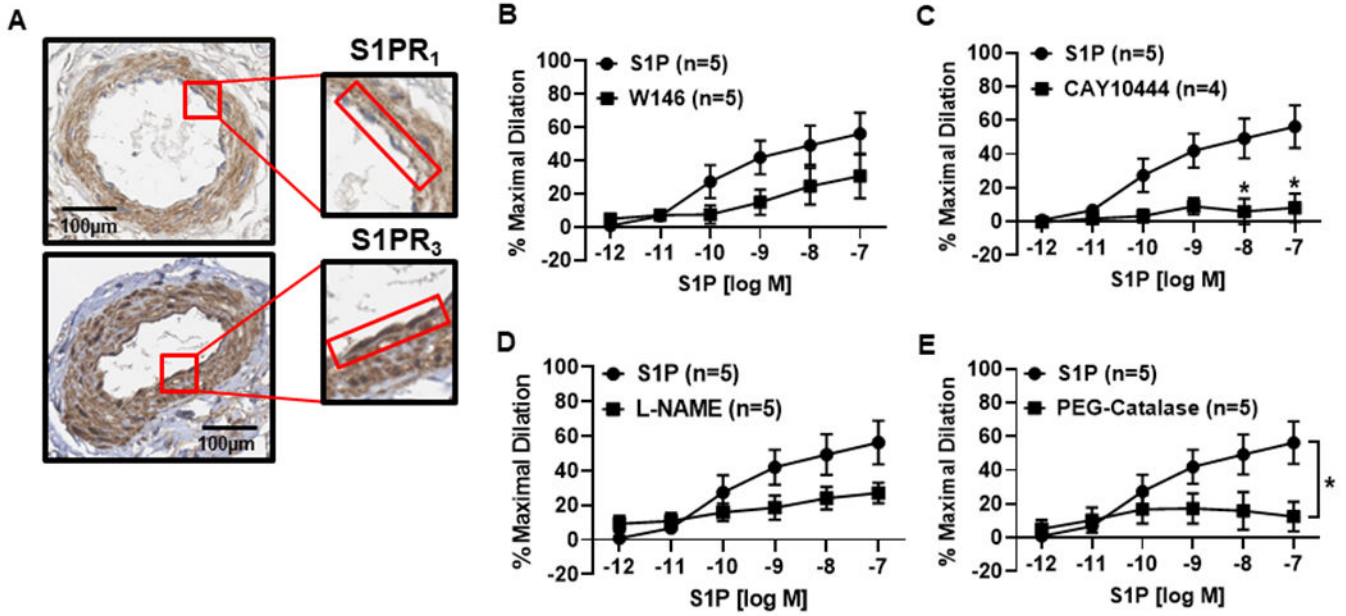


Figure 5. The S1P signaling pathway during disease (CAD).

A) Representative immunohistochemistry (IHC) images showing staining (representative images of n=5) of S1P receptors 1 and 3 in the microvascular endothelium from patients with CAD. The endothelium is highlighted by the red box. **B)** S1P induced a dose-dependent dilation (10^{-12} to 10^{-7}) was not significantly reduced in the presence of the S1PR₁ inhibitor W146 (10 μ M, n=5) in arterioles collected from patients diagnosed with CAD. **(C)** Dilation to S1P was significantly impaired during exposure to the S1PR₃ inhibitor CAY10444 (10 μ M, n=5) compared to S1P alone (n=5). **D)** In vessels from patients with CAD, dilation was not significantly impaired with the addition of L-NAME (100 μ M, n=5), but was significantly reduced in the presence of PEG-Catalase (500U, n=5) **(E)**. Data are expressed as mean % maximal dilation \pm SEM., *p<0.05 compared to S1P treatment alone.

Table 1.**Sample demographics.**

A total of 69 discarded human samples (54 nonCAD and 15 CAD) were utilized for this study. A subject was categorized as nonCAD if they carried no more than one risk factor for CAD (e.g. hypertension, hyperlipidemia, diabetes mellitus, or congestive heart failure). Subjects categorized as CAD had to have angiographic evidence of obstructive CAD and could have more than 1 risk factor for disease.

Patient Demographics: Total Samples (n = 69)	nonCAD (n=54)	CAD (n=15)
<u>Characteristics</u>		
Sex, M/F/unknown	2/51/1	10/5/0
Age, years (average±SD)	42.6±10.8	67.5±10.2
BMI (average±SD)	29.2±6.8	30.0±6.6
<u>Underlying disease/risk factor(s)</u>		
Hypertension	4	8
Hyperlipidemia	2	11
Diabetes Mellitus	1	3
Congestive Heart Failure	0	3
None of the Above	47	0

M = male, F = female, nonCAD = non-coronary artery disease, CAD = coronary artery disease, BMI = body mass index