ORIGINAL RESEARCH

Manipulation of the Sphingolipid Rheostat Influences the Mediator of Flow-Induced Dilation in the Human Microvasculature

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***Background*—**Elevated levels of ceramide, a sphingolipid known to cause a transition from nitric oxide (NO)– to hydrogen peroxide–dependent flow-induced dilation (FID) in human arterioles, correlate with adverse cardiac events. However, elevations of ceramide are associated with changed concentrations of other sphingolipid metabolites. The effects of sphingolipid metabolites generated through manipulation of this lipid pathway on microvascular function are unknown. We examined the hypothesis that inhibition or activation of the ceramide pathway would determine the mediator of FID.

***Methods and Results*—**Using videomicroscopy, internal diameter changes were measured in human arterioles collected from discarded adipose tissue during surgery. Inhibition of neutral ceramidase, an enzyme responsible for the hydrolysis of ceramide, favored hydrogen peroxide–dependent FID in arterioles from healthy patients. Using adenoviral technology, overexpression of neutral ceramidase in microvessels from diseased patients resulted in restoration of NO-dependent FID. Exogenous sphingosine-1-phosphate, a sphingolipid with opposing effects of ceramide, also restored NO as the mediator of FID in diseased arterioles. Likewise, exogenous adiponectin, a known activator of neutral ceramidase, or, activation of adiponectin receptors, favored NO-dependent dilation in arterioles collected from patients with coronary artery disease.

***Conclusions*—**Sphingolipid metabolites play a critical role in determining the mediator of FID in human resistance arterioles. Manipulating the sphingolipid balance towards ceramide versus sphingosine-1-phosphate favors microvascular dysfunction versus restoration of NO-mediated FID, respectively. Multiple targets exist within this biolipid pathway to treat microvascular dysfunction and potentially improve patient outcomes. **(*J Am Heart Assoc*. 2019;8:e013153. DOI: 10.1161/JAHA.119.013153.)**

Key Words: adiponectin • flow-induced dilation • microvascular dysfunction • sphingosine-1-phosphate

The endothelium is largely responsible for maintaining a nonproliferative, atheroprotective environment in the vasculature.[1] A significant portion of this protection is attributable to the formation and release of nitric oxide (NO) during flow-induced dilation (FID), the physiological mechanism by which arterioles dilate in response to an increase in shear stress. NO is the predominant mediator of FID in healthy adult individuals; however, NO bioavailability decreases with the onset of disease and is replaced with an inflammatory compensatory mediator, hydrogen peroxide (H_2O_2).[2] This transition from NO-dependent to H_2O_2-mediated FID, a form of microvascular dysfunction, is a known predictor of future cardiovascular events and favors the formation of atherosclerosis.[1] We have previously shown that ceramide, a bioactive sphingolipid known to be increased in the plasma of patients with coronary artery disease (CAD), and an independent predictor of major adverse cardiovascular events in otherwise healthy individuals,[3] is capable of initiating this switch in mechanism by increasing mitochondrial reactive oxygen species.[4] Further, inhibition of ceramide formation in diseased vessels can restore NO-dependent FID.

Interestingly, sphingolipid metabolites can exert opposing effects on the endothelium. While ceramide is known to increase mitochondrial-derived reactive oxygen species and promote inflammation, through a series of steps it can be converted to sphingosine-1-phosphate (S1P), a sphingolipid that has been shown to increase intracellular NO levels and maintain mitochondrial integrity.[5] For instance, ceramide can be converted to sphingosine via neutral ceramidase (NCdase), an enzyme located in the plasma membrane conveniently close to the ceramide-generating enzyme, neutral sphingomyelinase. The close proximity of these enzymes in the plasma membrane suggests that tight regulation of the

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Clinical Perspective

What Is New?

- To our knowledge, this is the first study to demonstrate that altering the sphingolipid balance through pathway manipulation influences the mediator formed during flow-induced dilation.
- In arterioles from healthy patients, manipulating the pathway towards ceramide formation promotes microvascular dysfunction, whereas interventions that increase sphingosine-1-phosphate reverses dysfunction associated with coronary artery disease.

What Are the Clinical Implications?

- Microvascular dysfunction is a precursor to many chronic diseases including coronary artery disease.
- Sphingolipids are now recognized as critical signaling molecules in cardiovascular disease as well as cancer, therefore understanding how they alter microvascular function may provide future potential areas of prevention or treatment of primary as well as cancer-induced cardiovascular disease.

formation and breakdown of these lipids is critical. Sphingosine can then undergo phosphorylation by sphingosine kinase (SpK) to form S1P. The balance of these lipids, often referred to as the "sphingolipid rheostat," can have a profound effect on cellular environment with the potential to influence the mediator of FID. For these reasons, knowledge of how this pathway can be manipulated may lead to novel therapies for treatment and prevention of cardiovascular disease (CVD).

Since NCdase can regulate ceramide levels by metabolizing ceramide to sphingosine, this enzyme may serve as a potential target that allows for adjustment of the rheostat towards S1P and NO-dependent FID. Although small molecule activators of NCdase currently do not exist, adiponectin, an adipocyte-derived cytokine, has recently been shown to activate NCdase and decrease intracellular ceramide levels.[6] Likewise, ceramidase activity is decreased, leading to increased ceramide concentration in cells lacking adiponectin receptors. Further, Wang and colleagues[7] demonstrated that the anti-inflammatory, antioxidative effects of adiponectin are largely caused by activation of endothelial NCdase through binding of adiponectin receptor (AdipoR) 1. Based on these findings, we hypothesized that shifting the sphingolipid rheostat favoring ceramide or S1P formation would result in an H_2O_2- or NO-mediated response to increased flow, respectively. This question was addressed by investigating: (1) whether inhibition of NCdase could initiate the switch in mechanism of dilation from NO to H_2O_2 in healthy arterioles; (2) whether increased expression of NCdase or activation via adiponectin could restore NO-dependent FID in arterioles from patients with disease; (3) whether exogenous S1P or inhibition S1P formation would restore NO-mediated FID in diseased arterioles and produce the diseased phenotype, respectively; and (4) whether adiponectin restores an NO-dependent mechanism in diseased arterioles via activation of NCdase. While many studies have concentrated on the effects of exogenous ceramide or S1P, one of the unique features of this study is the more relevant manipulation of enzymes that govern the rheostat leading to alteration of the FID phenotype.

Materials and Methods

All data and supporting materials have been provided with the published article.

Tissue Acquisition

Freshly discarded human adipose tissue (subcutaneous, peritoneal, visceral) was collected from patients undergoing surgical procedures. Tissue samples were immediately placed in ice-cold HEPES buffer and transported on ice to the laboratory. Patient demographics (age, sex, and comorbidities) were obtained through the Medical College of Wisconsin's REDCap database in a deidentified manner. All protocols were approved by the local institutional review board at the Medical College of Wisconsin.

Measurement of FID by Videomicroscopy

Human adipose arterioles were dissected and placed in a 35-mm × 10-mm dish to be cleaned of all perivascular adipose tissue. Approximately 10 to 20 arterioles were dissected per tissue/patient to be distributed to various treatments. They were then cannulated on glass micropipettes of matched impedance and placed in an organ chamber. Vessels were bathed in warm Kreb's buffer and bubbled with a 74%/21%/5% mixture of nitrogen, oxygen, and carbon dioxide, respectively. Arterioles were pressurized using open reservoirs and allowed to equilibrate for 30 minutes at 30 mm Hg followed by 30 minutes at 60 mm Hg. Once equilibrated, endothelin-1 was used to preconstrict the vessel to 30% to 50% of its passive diameter. To ensure that the various treatments did not interfere with endothelin-1–induced constriction, diameters were measured after 10 minutes of endothelin-1 administration to confirm integrity of the arteriole. Internal vessel wall diameters were then measured before and during increasing flow rates with constant intraluminal pressure. Pressure gradients of 5 to 100 cmH_2O were used to initiate increases in flow. Healthy human non-CAD arterioles were

incubated for 16 hours with the NCdase inhibitor Ceranib-1 (10^{-5} mol/L) or the SpK inhibitor (5×10^{-6} mol/L). Arterioles from patients with CAD were pretreated with S1P (10^{-6} mol/L), adiponectin (2 μg/mL), the adiponectin receptor agonist AdipoRon (5×10^{-6} mol/L), or Ceranib-1 and adiponectin (10^{-5} mol/L and 2 μg/mL, respectively). To determine the primary mediator of FID, the NO synthase inhibitor, N^{ω}-nitro-L-arginine (L-NAME; 10^{-4} mol/L), the H_2O_2 scavenger polyethylene glycol-catalase (PEG-catalase; 500 U/mL), or both were added to the organ bath 30 minutes before initiating flow. No more than 2 studies were performed on each vessel and each study was separated by a wash (organ chamber bath replaced 3 times) and 30 minutes of equilibration at 60 mm Hg. Following completion of the second study, the direction of maximal flow (100 cmH_2O) was reversed to confirm matched impedance between the glass pipettes. To determine the arteriole's maximal diameter, an endothelium-independent vasodilator (papaverine, 10^{-4} mol/L) was then administered to the organ bath at the end of each experiment.

Adenoviral Transfection and Expression

Adenoviral vectors containing a single promoter for GFP alone (Ad-GFP (green fluorescent protein), 50 μL, 4.69×10^{9} TU/mL) or a vector with dual promoters for both NCdase and GFP (Ad-NCdase-GFP, 50 μL, 2.1×10^{10} TU/mL) were generated at the Viral Vector Core at the Blood Center of Wisconsin. The Ad-GFP or Ad-NCdase-GFP virus was injected into the microvessel lumen using the Nanoliter Injector with SMART-Touch Controller (World Precision Instruments). Following adenoviral administration, both ends of the arteriole were tied allowing for containment of the virus in the vessel lumen. Microvessels were then incubated at 37°C with 5% carbon dioxide for 16 to 20 hours in EBM media (Lonza Bioscience). Following incubation, the ends of the vessel were cut and the vessel cannulated for videomicroscopy. Virus incorporation into the endothelium was confirmed using an Olympus CKX53 with fluorescence (Olympus America Inc).

Immunohistochemistry

Immunohistochemistry was used to assess NCdase expression in human arterioles collected from healthy patients without CAD and those with CAD, as previously described.[8] Briefly, microvessels dissected from human adipose tissue (≈100 μm in diameter) were fixed in 10% zinc formalin buffer for 24 to 72 hours. The samples were then embedded in paraffin and cut into 4-μm sections using an HMS355 microtome. NCdase expression was assessed by immunolabeling with a mouse monoclonal antibody IgG_{2b} against NCdase (1:200). Sections were then incubated with secondary antibody or normal serum IgG (negative control). Immunohistochemical staining is detected with biotinylated goat anti-rabbit IgG followed by alkaline phosphatase streptavidin and alkaline phosphatase chromogen substrate. A BOND-MAX immunostainer (Leica Biosystems Inc) was used for immunostaining. Slides then underwent heat-induced epitope retrieval for 10 minutes. Slides were visualized and scanned with a NanoZoomer HT slide scanner (Hamamatsu).

Materials

Dimethyl sulfoxide was used to reconstitute SpK inhibitor (Abcam), AdipoRon hydrochloride (Tocris Bioscience), and Ceranib-1 (Tocris Bioscience). A 1% BSA solution was used to dissolve endothelin-1 (Sigma-Aldrich). NCdase primary antibody was purchased by Santa Cruz (sc-374634). Adiponectin (Recombinant Human gAcrp30/Adipolean, PeproTech US) was reconstituted in 0.1% BSA. S1P was dissolved in methanol (Sigma-Aldrich). To show that dimethyl sulfoxide, methanol, or BSA had no effect on microvessel studies, vehicle control curves were run in parallel to experimental conditions.

Statistical Analyses

A Fischer exact test was used to compare baseline demographic data for healthy patients without CAD versus those with CAD. Data points are expressed as means±SEMs. FID is expressed as a percentage of maximal relaxation from endothelin-1 constriction, with 100% representing full relaxation to the maximal diameter obtained by the addition of papaverine. To compare flow-response relationships, a 2-way ANOVA was used with flow gradient and treatment as parameters. When a significant difference was observed between curves ($P<0.05$), responses at individual concentrations were compared using a Holm-Sidak multiple comparison test. All analyses were performed using GraphPad Prism, version 7.04. Statistical significance was defined as $P<0.05$.

Results

A total of 80 discarded human tissue samples were collected from surgical procedures. Experiments were performed using microvessels dissected from human adipose of 32 heathy patients without CAD and 48 patients with CAD. Patients were classified as healthy if they had ≤1 risk factor (hypertension, hyperlipidemia, diabetes mellitus, congestive heart failure) for CAD. Patients had to have the formal diagnosis of CAD to be considered a diseased patient. The initial diameters before and after administration of papaverine (mean±SD) were 141±31 μm and 170±46 μm, respectively, for non-CAD arterioles, and 110±43 μm and 140±58 μm,

Table. Patient Demographics

Characteristics	Healthy (n=32)	CAD (n=48)
Men/women	9/23	36/12
Age, average±SD, y	50±13	66±8
Underlying diseases/risk factors		
CAD*	0	48
Hypertension*	3	33
Hyperlipidemia*	3	28
Diabetes mellitus*	2	19
Congestive heart failure	3	5
None of the above	29	0

*$P<0.01$ for patients without coronary artery disease (CAD) versus patients with CAD.

respectively, for adipose microvessels from patients with CAD. Demographic data for these patients are included in Table.

Inhibition of NCdase Promotes H_2O_2-Dependent FID

To determine whether inhibition of ceramide hydrolysis via inhibition of NCdase would favor H_2O_2-dependent FID, non-CAD human adipose arterioles were incubated for 16 hours with the NCdase inhibitor Ceranib-1. As illustrated in Figure 1A, incubation with Ceranib-1 alone did not affect the vessel's ability to vasodilate in response to flow, as compared with the vehicle-treated control (%maximal dilation [MD] 83.4±6.4 [n=6]; Ceranib-1 versus 69.9±8.5 [n=7]; vehicle). Previously, we have shown that FID in arterioles collected from human patients without CAD is dependent on the formation of NO, which is confirmed again in Figure 1A. Exposure to the NO synthase inhibitor, L-NAME, significantly reduced the vasodilatory capacity due to flow (%MD 32.8±18.6 [n=7]) compared with vehicle-treated control (%MD 69.9±8.5 [n=7]). However, L-NAME had no effect on FID in non-CAD vessels first incubated with Ceranib-1 (Figure 1B, %MD 83.6±4.1 [n=7]), whereas PEG-catalase eliminated FID (%MD 1.9±4.9 [n=7]), suggesting that pharmacological inhibition of NCdase, or preventing the breakdown of ceramide, can shift the mechanism of FID in non-CAD arterioles from NO to H_2O_2.

NCdase Expression in Human Microvessels

Using immunohistochemistry, NCdase expression was assessed in non-CAD arterioles, arterioles collected from patients with CAD, and vessels from diseased patients that were intraluminally incubated with an NCdase adenovirus. As shown in Figure 2A, enhanced punctate staining is observed in the endothelium (red arrow) in the non-CAD arteriole compared with the CAD arteriole (Figure 2B). Following incubation with the Ad-NCdase, a CAD arteriole has a similar punctate staining pattern (Figure 2C) as the non-CAD arteriole. Antibody specificity was confirmed using a negative control (Figure 2D).

Overexpression of NCdase Restores NO-Dependent FID

To assess whether overexpression of NCdase could affect the mediator of FID, Ad-NCdase-GFP was injected into the lumen of microvessels from patients with CAD and incubated overnight for 16 hours. Figure 3A illustrates that the overall magnitude of FID is unaffected in vessels collected from patients with CAD that have been intraluminally exposed to Ad-GFP or Ad-GFP-NCdase compared with vehicle-treated control (%MD 71.0±6.2 [n=7] versus 76.7±3.5 [n=8] versus 69.4±7.7 [n=6], respectively, for Ad-GFP, Ad-GFP-NCdase, and vehicle control).

Our prior work has shown that FID in vessels collected from patients with CAD is H_2O_2-mediated. As shown in Figure 3B, CAD arterioles incubated with Ad-GFP continued to vasodilate to H_2O_2 in response to flow as L-NAME had no effect compared with Ad-GFP alone, (%MD 73.9±7.1 [n=7] versus 71.0±6.2 [n=7], respectively); however, dilation was significantly impaired following administration of PEG-catalase (%MD 42.7±13.0 [n=10]), suggesting that the virus alone had no effect on the mediator of FID. However, overnight intraluminal exposure to Ad-NCdase-GFP restored NO-dependent FID in arterioles from diseased patients as PEG-catalase had no effect on FID compared with Ad-NCdase-GFP alone (%MD 78.9±4.1 [n=7] versus 76.7±3.5 [n=8], respectively), whereas administration of L-NAME resulted in significant impairment of flow-mediated vasodilation (%MD 48.3±12.8% [n=7]) (Figure 3C). Representative fluorescent images of vessels treated with Ad-GFP and Ad-GFP-NCdase are compared with nontreated arterioles in Figure 3D.

Inhibition of SpK Promotes H_2O_2-Dependent FID, Whereas Exogenous Administration of S1P Favors Dilation Via NO

To determine whether inhibition of S1P formation through blockade of SpK can affect the mediator of FID, non-CAD arterioles were incubated with the SpK inhibitor. Figure 4A shows that incubation with the SpK inhibitor alone does not impair the ability of the vessel to dilate compared with vehicle-treated control (%MD 80.0±4.2 [n=7] versus 69.9±8.5 [n=6], respectively). Inhibition of S1P formation in non-CAD vessels results in a significant decrease in FID

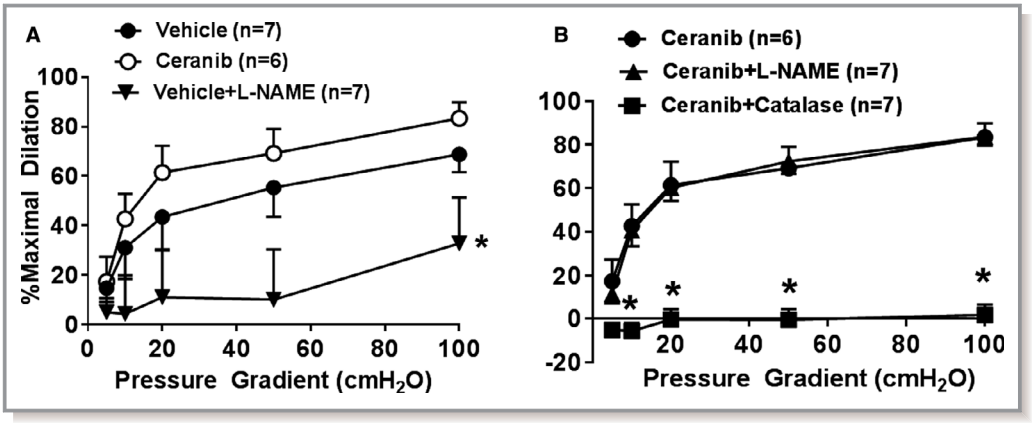

Figure 1. Inhibition of neutral ceramidase (NCdase) drives dilation towards hydrogen peroxide in non–coronary artery disease (non-CAD) arterioles. **A**, Flow-induced dilation is impaired in non-CAD arterioles exposed to N^{ω}-nitro-L-arginine (L-NAME) (n=7), whereas incubation with the NCdase inhibitor Ceranib-1 (10^{-5} mol/L, 16–20 hours) did not affect the magnitude of dilation compared with vehicle (n=6 and n=7, respectively). **B**, The response to flow was impaired in non-CAD vessels incubated with Ceranib-1 in the presence of polyethylene glycol-catalase (PEG-catalase) (500 U) (n=7), whereas L-NAME had no effect (n=7). **P*<0.05 vs vehicle for curve mean (**A**) or at specific pressure gradients (**B**).

when exposed to PEG-catalase (%MD 11.2±4.7 [n=7]) compared with SpK inhibitor alone (%MD 80.0±4.2 [n=7]); however, it is unaffected by L-NAME (%MD 82.5±3.9) (Figure 4B). In arterioles from patients with CAD, exogenous administration of S1P restores NO-dependent FID. Figure 4C shows that S1P alone does not impair FID compared with vehicle control (%MD 80.6±3.8 [n=8] versus 74.6±5.9 [n=6], respectively) and vessels collected from patients with CAD primarily rely on H_2O_2, as dilation is significantly decreased when exposed to PEG-catalase (%MD −3.9±2.2 [n=6]). However, in CAD arterioles treated with S1P, PEG-catalase had no effect (%MD 82.4±5.4 [n=7]), whereas FID was significantly reduced in the presence of L-NAME compared with S1P alone (%MD 0.3±2.0 [n=8] versus 80.6±3.8 [n=8]) (Figure 4D).

Exogenous Adiponectin or Activation of the Adiponectin Receptor Restores NO-Dependent FID in Diseased Arterioles

Since previous studies have shown that adiponectin increases activity of NCdase and thus decreases intracellular ceramide, we next assessed whether adiponectin alone was capable of

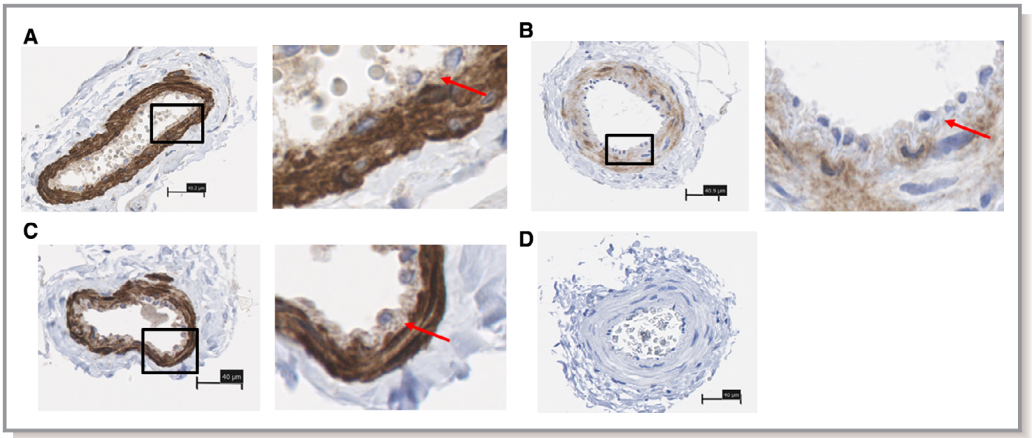

Figure 2. Neutral ceramidase (NCdase) expression in human arterioles. Representative images from 3 patients (3 non–coronary artery disease [non-CAD], 3 coronary artery disease [CAD], and 3 CAD+adenovirus with promoters for GFP and NCdase [Ad-GFP-NCdase]). Areas of the endothelium (box) are magnified to show staining specifically within the endothelial layer (red arrow). The area of staining is increased in non-CAD (**A**) compared with CAD arterioles (**B**). Following overnight administration of Ad-GFP-NCdase, the total amount of staining is increased (**C**) compared with a CAD control vessel (**B**). Primary antibody was removed to determine specificity (**D**). Bar=40 μm.

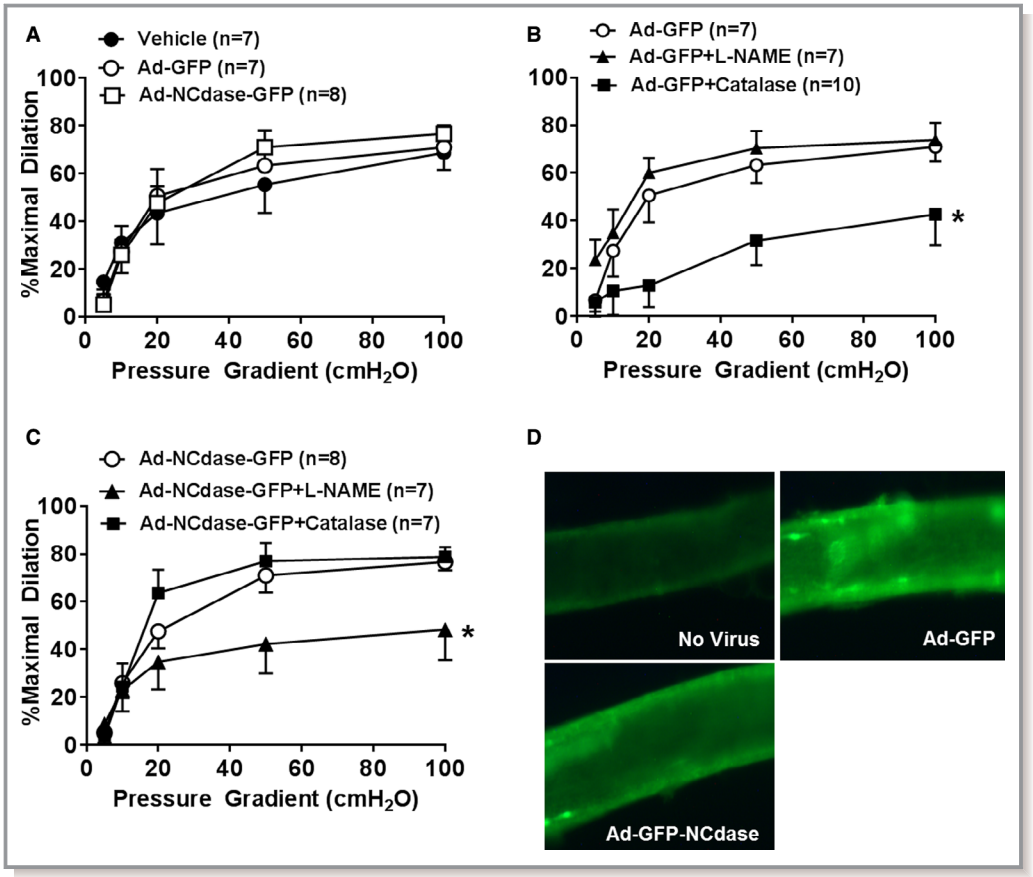

Figure 3. Overexpression of neutral ceramidase (NCdase) restores nitric oxide–dependent flow-induced dilation (FID) in diseased arterioles. **A**, Treatment with either adenovirus with promoters for GFP (Ad-GFP) (n=7), or Ad-NCdase-GFP (n=8), has no effect on dilation in response to flow compared with vehicle (n=7). **B**, Exposure to N^{ω}-nitro-L-arginine (L-NAME) has no effect on FID in arterioles collected from patients with coronary artery disease (CAD) that have been treated with Ad-GFP alone (n=7) compared with vehicle (n=7), whereas the response to flow is impaired following exposure to polyethylene glycol-catalase (PEG-catalase) (n=10). **C**, FID is impaired in CAD microvessels treated with Ad-NCdase-GFP following incubation with L-NAME (n=7), whereas PEG-catalase has no effect (n=7) compared with Ad-NCdase-GFP alone (n=8). *P<0.05 for response curve averages.

initiating a switch in FID mechanism from one dependent on the formation of H_2O_2 to NO. Adipose microvessels collected from patients with CAD were incubated overnight (16 hours) with exogenous globular adiponectin. As shown in Figure 5A, FID was significantly reduced in CAD arterioles treated with PEG-catalase compared with vehicle control (%MD −3.9±2.2 [n=6] versus 74.6±5.9 [n=6], respectively); however, adiponectin alone had no effect on the vasodilatory capacity in response to flow (%MD 68.3±3.9 [n=9]). PEG-catalase had no effect in arterioles from diseased patients following chronic overnight exposure to adiponectin (%MD 61.0±9.1 [n=7]), whereas maximal dilation was significantly decreased in the presence of L-NAME (%MD 38.3±11.7 [n=6]) compared with adiponectin alone (%MD 61.0±9.1 [n=7]) (Figure 5B).

To determine whether a similar effect could be observed by activating adiponectin receptors, AdipoRON, a nonselective adiponectin receptor agonist was also applied overnight to CAD arterioles. Treatment with AdipoRON alone did not affect FID compared with vehicle-treated control (%MD 63.9±8.3 [n=7] versus 69.4±7.7 [n=6]) (Figure 5C); however, FID was significantly impaired following exposure to both AdipoRON and L-NAME in vessels from patients with CAD compared with AdipoRON alone (%MD 33.0±11.1 [n=6] versus 63.9±8.3 [n=7]). PEG-catalase had no effect on flow-mediated dilation in CAD vessels treated with AdipoRON, as shown in Figure 5D (%MD 61.5±7.8 [n=6]).

Plasticity in FID Mechanism is Observed Following Administration of Adiponectin During NCdase Inhibition

Following the observations that inhibition of NCdase via treatment with Ceranib-1 promotes H_2O_2-dependent FID in arterioles from patients without CAD, and that exposure to

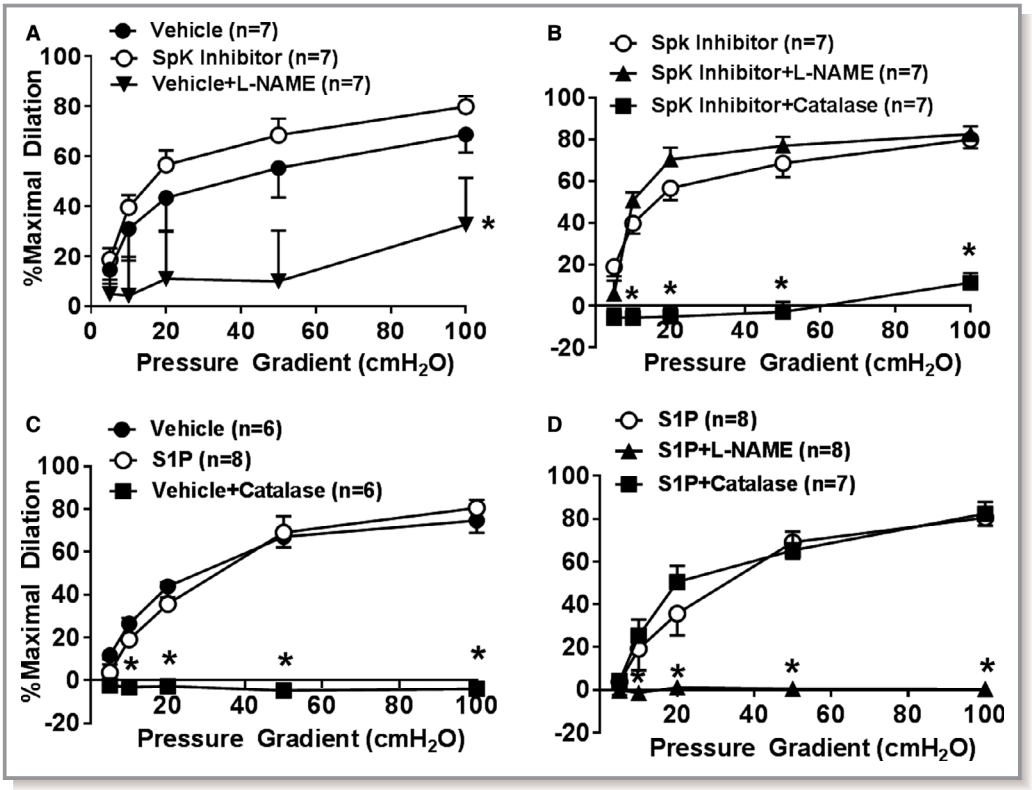

Figure 4. Effect of sphingosine-1-phosphate (S1P) on the mediator of flow-induced dilation (FID). **A**, Non–coronary artery disease (non-CAD) arterioles incubated with the sphingosine kinase (SpK) inhibitor (5×10^{-6} mol/L, 16–20 hours) reached normal dilation capacity compared with vehicle (n=7 and n=7, respectively). **B**, The response to flow was inhibited in non-CAD arterioles incubated with SpK inhibitor overnight in the presence of polyethylene glycol-catalase (PEG-catalase) (500 U) (n=7) compared with SpK inhibitor alone (n=7), whereas N^{ω}-nitro-L-arginine (L-NAME) had no effect (n=7). **C**, Dilation was abolished in arterioles from diseased patients following exposure to PEG-catalase (n=6) compared with vehicle (n=6). CAD arterioles incubated with S1P (10^{-6} mol/L, 16–20 hours) had no effect on FID compared with vehicle (n=6 and n=8, respectively). **D**, CAD arterioles incubated with S1P in the presence of L-NAME had impaired dilation, whereas PEG-catalase had no effect (n=8 and n=7, respectively) compared with S1P alone (n=8). *P<0.05 for curve averages (**A**) or at specific pressure gradients (**B** through **D**).

adiponectin, an adipokine known to activate NCdase, restores NO-dependent dilation in diseased arterioles, we next hypothesized that adiponectin-induced restoration of NO-dependent FID would not occur in the presence of NCdase inhibition with Ceranib-1. To test this, microvessels from patients with CAD were first incubated with Ceranib-1 (4 hours) to allow for inhibition of NCdase, followed by addition of adiponectin for 16 hours. As shown in Figure 6A, PEG-catalase blocked FID in CAD arterioles (%MD −3.9±2.2 [n=6]); however, vessels treated with Ceranib-1 and adiponectin remained unaffected compared with vehicle control (70.6±4.1 [n=6] versus 69.4±7.7 [n=6], respectively). Interestingly, FID was unaffected by both L-NAME (%MD 63.3±7.0 [n=8]) and PEG-catalase (%MD 69.5±8.5 [n=6]) in CAD arterioles treated with both Ceranib-1 and adiponectin, yet it was significantly reduced when L-NAME and PEG-catalase were combined (Figure 6B) (%MD 32.9±9.9 [n=6] compared with Ceranib-1 and adiponectin alone %MD 70.6±4.1 [n=6]), suggesting that both NO and H_2O_2 contribute interdependently to this mechanism of vasodilation.

Discussion

A large amount of evidence now indicates that sphingolipids, more importantly the balance of these bioactive lipids, can have a significant impact on cellular homeostasis and cardiac risk.[9] This is the first study to show that targeted manipulation of the sphingolipid pathway can alter the mechanism of FID, which can potentially promote, prevent, or reverse microvascular dysfunction, a condition that precedes the formation of atherosclerosis. There are several major findings in this study. First, manipulation of NCdase alone is sufficient to alter the mechanism of FID where inhibition of NCdase causes microvascular dysfunction in an otherwise normal non-CAD arteriole, and overexpression of NCdase in microvessels from patients with

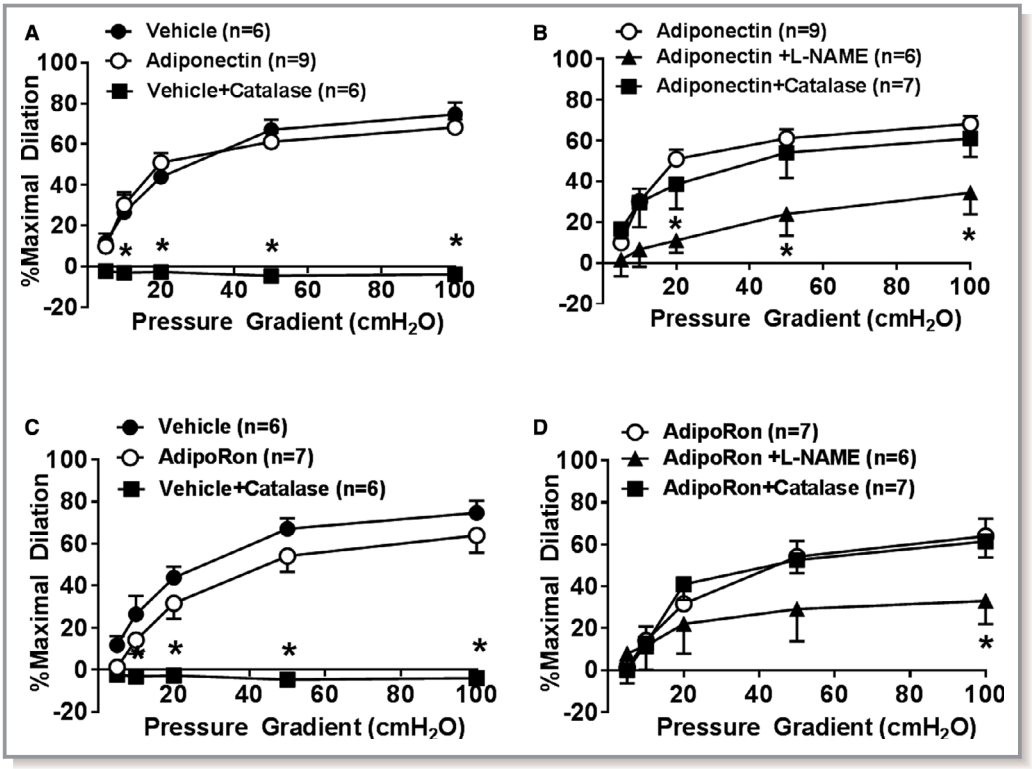

Figure 5. Exogenous adiponectin or activation of adiponectin receptors restores nitric oxide–dependent flow-induced dilation in microvessels from diseased patients. **A**, Coronary artery disease (CAD) arterioles treated with adiponectin (2 μg/mL, 16–20 hours) did not alter the dilation capacity of the vessel in response to flow compared with vehicle (n=9 and n=6, respectively). **B**, Dilation to flow was inhibited in CAD vessels incubated with adiponectin in the presence of N^{ω}-nitro-L-arginine (L-NAME) (n=6) compared with adiponectin alone (n=6), whereas polyethylene glycol-catalase (PEG-catalase) had no effect. **C**, Vessels from patients with CAD treated with AdipoRon (5×10^{-6} mol/L, 16–20 hours) dilated normally in response to flow compared with vehicle (n=7 and n=6, respectively). **D**, Dilation to flow was impaired in CAD vessels first treated with AdipoRon in the presence of L-NAME, whereas PEG-catalase had no effect (n=6 and n=7, respectively) compared with AdipoRon alone (n=7). *P<0.05 for specific pressure gradients.

disease allows for restoration of NO-dependent FID. Second, preventing phosphorylation of sphingosine to form S1P is also detrimental to non-CAD arterioles and promotes H_2O_2-dependent FID. Third, exogenous administration of S1P can restore an NO-mediated mechanism of FID in diseased microvessels. Fourth, exogenous adiponectin or activation of the adiponectin receptor is also capable of restoring NO as the mediator in vessels from patients with disease. Finally, administration of adiponectin during NCdase inhibition results in dual mediators of FID, suggesting a mechanism of plasticity. Together, these results shed light on how manipulation of this lipid pathway affect what vasoactive compound is produced and released in response to flow and ultimately influences the microvasculature to either prevent or promote disease. A schematic illustrating this concept is presented in Figure 7.

Sphingolipid Balance and FID

The physiological and pathophysiological roles of sphingolipids continue to emerge at a rapid pace providing convincing evidence that these lipid messengers are involved in most, if not all, critical cellular processes.[10] Sphingolipid metabolites, most notably ceramide and S1P, are now considered vital signaling molecules that modulate vascular homeostasis and inflammation.[11] Interestingly, ceramide and S1P have *opposing* effects on the endothelium. In addition to inducing endothelial dysfunction, more recent data have shown that ceramides, specifically long-chain ceramides, Cer(d18:1/16:0), Cer(d18:1/18:0), Cer (d18:1/24:0), and Cer(d18:1/24:1), are associated with major adverse cardiovascular events in individuals without CAD.[3] On the contrary, S1P has been demonstrated to exert beneficial effects on the vasculature by increasing intracellular NO levels and maintaining the integrity of the endothelium.[5]

Our current study indicates that manipulation of NCdase, a type II transmembrane protein responsible for hydrolysis of ceramide to sphingosine, can have profound effects on the mediator of FID. Exposure to Ceranib-1, a small molecule shown to effectively inhibit NCdase, causing accumulation of

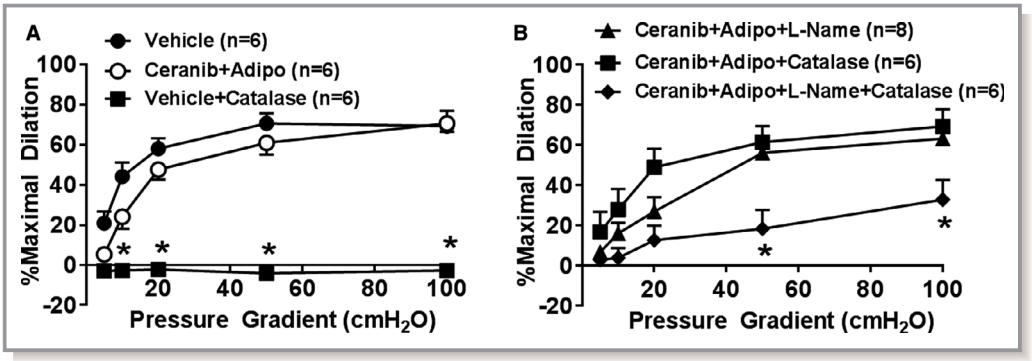

Figure 6. Effect of adiponectin administration with concurrent neutral ceramidase (NCdase) inhibition on the mediator of flow-induced dilation (FID). **A**, Microvessels from patients with coronary artery disease (CAD) maintained dilator capacity following exposure to Ceranib-1 for 4 hours followed by administration of adiponectin (n=6) compared with vehicle (n=6). **B**, Neither N^{ω}-nitro-L-arginine (L-NAME) (n=8) nor polyethylene glycol-catalase (PEG-catalase) (n=6) impaired FID in CAD arterioles treated with both Ceranib-1 and adiponectin; however, dilation was significantly decreased in the presence of both drugs (n=6) compared with vehicle. *P<0.05 at specific pressure gradients.

ceramide along with a reduction in sphingosine and S1P,[12] was sufficient to initiate the transition to H_2O_2-dependent FID in non-CAD vessels. While ceramidases are not currently a target in the treatment of CVD, they have evolved as desirable targets in the treatment of various forms of cancer. This is primarily because of evidence of increased ceramidase expression and activity in tumor cells.[13] Although inhibition of ceramidase may prove to be beneficial as a cancer therapy, it will likely have damaging effects on the microvasculature and possibly accelerate CVD. On the other hand, activation of ceramidases, specifically NCdase, may be an attractive strategy to reduce intracellular ceramide levels and decrease CVD risk in patients with known elevated levels of plasma ceramide. Presently, no commercially available activators of ceramidase exist; however, in the current study, increasing expression of NCdase using adenoviral technology restored the healthy FID phenotype in diseased vessels, suggesting that targeting NCdase may be a feasible approach to preventing the harmful vascular effects of ceramide.

Adiponectin as a Regulator of the Sphingolipid Rheostat

Adiponectin, an adipocyte-derived cytokine, has been shown to reduce oxidative stress, inhibit leukocyte-endothelial cell interactions, decrease smooth muscle proliferation, increase cytosolic NO levels, and promote NO-dependent vasodilation.[14,15] Epidemiological data have shown a close correlation between decreased plasma levels of adiponectin and endothelial dysfunction in humans.[16] Likewise, on an atherogenic diet, adiponectin-knockout mice demonstrate impaired endothelium-dependent vasodilation.[17] Traditionally, the beneficial effects of adiponectin were considered to be through AdipoR1 and AdipoR2 activation of AMP-activated protein kinase resulting in phosphorylation of endothelial NO synthase and production of NO.[18] However, more recent data have shown that adiponectin decreases cellular ceramide levels independent of AMP-activated protein kinase. Further, ceramidase activity is decreased leading to increased ceramide concentration in cells lacking adiponectin receptors.[6] More recent data have suggested that both adiponectin receptors have intrinsic ceramidase activity, therefore stimulation of either AdipoR1 or AdipoR2 can effectively decrease ceramide levels independently of NCdase activation. Vasiliauskaite-Brooks and colleagues[19] were able to demonstrate that both receptors have basal ceramidase activity that is accelerated 25-fold upon activation of the receptors either by adiponectin or via the AdipoR1/R2 agonist AdipoRON.

The results from the current study are the first to show that exogenous administration of adiponectin can reverse endothelial dysfunction in human arterioles. Restoration of NO-dependent FID was also observed in microvessels from diseased patients that were exposed to AdipoRON, a nonselective agonist of both AdipoR1 and AdipoR2. To determine whether the adiponectin-induced switch to NO-dependent FID in arterioles from patients with CAD was caused by activation of NCdase, arterioles were first incubated with the NCdase inhibitor Ceranib-1 before adiponectin exposure. In an interesting twist, it was observed that both NO and H_2O_2 contribute to FID under these conditions as dilation was decreased only in the presence of both NO synthase inhibition with L-NAME and H_2O_2 scavenging with PEG-catalase. While this condition of adiponectin exposure during ceramide hydrolysis inhibition significantly decreased FID, ≈25% of dilation caused by flow remained. This suggests that adiponectin may promote plasticity with regard to FID mediators, which may be another potential benefit from this adipokine. Previous data from our

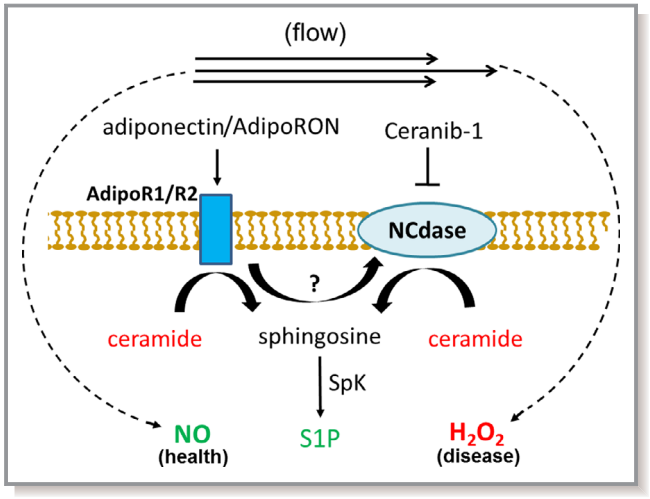

Figure 7. Schematic illustration highlighting the effect of the sphingolipid pathway on the mediator of flow-induced dilation (FID). Shear stress from increased blood flow results in the formation and release of nitric oxide (NO) or hydrogen peroxide (H_2O_2) in adults without coronary artery disease (CAD) versus those with CAD, respectively. Inhibition of neutral ceramidase (NCdase) favors ceramide accumulation favoring FID mediated by H_2O_2. Likewise, preventing formation of intracellular sphingosine-1-phosphate (S1P) through inhibition of sphingosine kinase (SpK) also results in H_2O_2-dependent dilation in vessels from patients without CAD. Overexpression of NCdase, exogenous S1P, or adiponectin, or activation of adiponectin receptors, restores NO-dependent dilation in arterioles from patients with CAD. Adiponectin, or the nonselective adiponectin receptors 1 and 2 (AdipoR1/R2) agonist AdipoRon, is capable of either activating NCdase or has intrinsic ceramidase activity allowing for the hydrolysis of ceramide.

laboratory have indicated that this same effect is observed in CAD arterioles that overexpress peroxisome proliferator-activated receptor γ coactivator 1-α (PGC-1α). Adiponectin is known to activate NAD-dependent deacetylase sirtuin-1 (SIRT1) via the AdipoR1, which then subsequently de-acetylates and activates PGC-1α. It has also been suggested that the AMP-activated protein kinase pathway is responsible for adiponectin-initiated activation of PGC-1α.[20] It is plausible that adiponectin influences the mediator of FID via 2 distinct pathways, one that involves AMP-activated protein kinase–induced activation of SIRT1, and the other via activation of NCdase to manipulate intracellular ceramide levels. While the significance of using 2 vasodilators with opposing effects, anti-inflammatory and proinflammatory for NO and H_2O_2, respectively, currently remains unclear, future studies will focus on this phenomenon.

Just as with adiponectin, in the current study exposure to S1P alone restored NO as the mediator of FID in diseased arterioles. S1P can exert its action on either extracellular receptors or on intracellular targets. Most of the beneficial effects of S1P (eg, cellular growth, angiogenesis, vasodilation) are thought to be caused by activation of S1P receptors found on the endothelial cell surface.[21] However, S1P, generated by SpK, can also exert its affects directly within the cell. Although the exact relationship between S1P and SIRT1 is not completely understood, Gao et al[22] have shown that S1P, like adiponectin, is also capable of increasing expression of SIRT1 in endothelial cells. The current study also demonstrated that simply inhibiting intracellular formation of S1P via inhibition of SpK in non-CAD arterioles forces the switch in FID mechanism from NO to H_2O_2. Of note, S1P generated intracellularly can also participate in "inside-out signaling," where S1P moves outside of the cell and then is capable of activating the S1P receptors that reside on the plasma membrane.[23] Whether the change in mediator of FID is caused by lack of direct intracellular effects of S1P versus activation of S1P receptors is a future area of investigation. Likewise, whether SIRT1 and/or PGC-1α is the final common pathway of both adiponectin and S1P remains an intriguing question.

Clinical Implications of Sphingolipid Pathway Manipulation

The role bioactive lipids have in the prevention or promotion of CVD continues to grow.[24] Since elevated plasma ceramide levels now coincide with adverse cardiac events, this measurement may become a standard of care and join the more traditional lipid profile of total cholesterol, low-density lipoprotein, and high-density lipoprotein.[25] The fact that ceramide causes microvascular dysfunction, which precedes large vessel disease, suggests that strategies to decrease ceramide levels, and mitigate its effect on the vasculature, could prove beneficial by avoiding the progression of disease. This may be achieved through activation of NCdase, or by tipping the sphingolipid balance towards S1P either via the use of S1P agonists or by activating downstream targets of S1P such as SIRT1 or PGC-1α.

Sphingolipids have also gained an extraordinary amount of attention in the realm of cancer research. It is now understood that most, if not all, chemotherapies increase ceramide plasma levels, which likely contributes to their effectiveness in decreasing tumor burden.[26] Further, patients with increased activity or expression of NCdase appear to be chemoresistant.[26,27] For these reasons, chemotherapies, which directly increase ceramide and/or inhibit NCdase, have emerged as potential novel treatments. Elevated plasma S1P levels have been shown to be associated with tumor metastasis.[28] For this reason, suppressing the actions of S1P with a neutralizing antibody has materialized into a cancer therapy.[29] As cancer rates continue to climb,[30] the use of these treatments will escalate as well, potentially increasing the prevalence of CVD. To circumvent this, efforts need to be targeted toward understanding how to effectively treat the cancer while limiting the harmful effects on the systemic microvasculature.

Study Limitations

One of the main limitations of the current study is the inability to effectively measure changes in sphingolipid concentrations following manipulation of the pathway. The current "gold standard" for measuring sphingolipids is liquid chromatography tandem-mass spectrometry, which would require collection of a large pool of microvessels to achieve an adequate amount of starting material. The measurement would also reflect the whole vessel (endothelium, smooth muscle layer, and adventitia) as opposed to the endothelial layer alone. Since FID occurs at the level of the endothelium and we have previously shown that the smooth muscle layer acts as a depot for ceramide, use of the whole vessel to quantify these lipids may not reflect the functional observations. Because of the same limitations, it is equally challenging to measure NCdase activity in human arterioles. Current assays utilize C12-NBD-Cer (NBD-C12:0, d18:1), a fluorescent ceramide analog that releases NBD-dodecanoic acid upon activation by NCdase in a neutral pH environment. These compounds are then separated using thin layer chromatography and quantified using a chromatoscanner.[31] This again would require use of multiple intact microvessels to achieve an adequate amount of starting material and would fail to accurately reflect NCdase activity specifically within the endothelium.

Although the use of discarded tissue is a highly translational approach to study human microvascular function, there are some limitations as well. Certain variables cannot be controlled, such as other comorbidities or use of prescribed medications. To limit the effect of medications, microvessels were thoroughly washed before reactivity studies. It is also possible that although patients do not have the formal diagnosis of CAD, they indeed have atherosclerotic plaque at a subclinical level. In an attempt to mitigate these potential effects, patients were classified as having "non-CAD" only if they had no more than 1 risk factor for CAD. Despite these limitations, the study of human arterioles provides mechanistic insight that unfortunately cannot be determined using animal models.

Conclusions

Overall, this study suggests that the metabolites formed within the sphingolipid pathway can exert a dramatic effect on the vasodilator produced during FID. Inhibition of the pathway favoring ceramide accumulation or prevention of S1P formation favors an H_2O_2-dependent mechanism, whereas shunting the pathway towards S1P promotes a more normal phenotype where the mechanism of FID relies on formation of NO. Microvascular dysfunction precedes the formation of atherosclerosis and elevated ceramide levels are correlated with major adverse cardiac events. Pharmacological interventions to decrease ceramide and increase S1P levels may prove to be an effective strategy to prevent or treat the complications of large artery disease. Likewise, manipulating this pathway may aid in preventing accelerated CVD observed in patients who have received chemotherapy.

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Disclosures

None.

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