Ceramide Changes the Mediator of Flow-Induced Vasodilation From Nitric Oxide to Hydrogen Peroxide in the Human Microcirculation

Julie K. Freed, Andreas M. Beyer, John A. LoGiudice, Joseph C. Hockenberry, David D. Gutterman

Rationale: Mitochondrial-derived hydrogen peroxide (H$_2$O$_2$) regulates flow-induced dilation (FID) in microvessels from patients with coronary artery disease. The relationship between ceramide, an independent risk factor for coronary artery disease and a known inducer of mitochondrial reactive oxygen species, and FID is unknown.

Objective: We examined the hypothesis that exogenous ceramide induces a switch in the mediator of FID from nitric oxide to H$_2$O$_2$.

Methods and Results: Internal diameter changes of resistance arterioles from human adipose and atrial tissue were measured by video microscopy. Mitochondrial H$_2$O$_2$ production was assayed in arterioles using mito peroxyl yellow 1. Polyethylene glycol–catalase, rotenone, and Mito-TEMPO impaired FID in healthy adipose arterioles pretreated with ceramide, whereas $N^\omega$-nitro-l-arginine methyl ester had no effect. Mitochondrial H$_2$O$_2$ production was induced in response to flow in healthy adipose vessels pretreated with ceramide, and this was abolished in the presence of polyethylene glycol–catalase. Immunohistochemistry demonstrated ceramide accumulation in arterioles from both healthy patients and patients with coronary artery disease. $N^\omega$-nitro-l-arginine methyl ester reduced vasodilation to flow in adipose as well as atrial vessels from patients with coronary artery disease incubated with GW4869, a neutral sphingomyelinase inhibitor, whereas polyethylene glycol–catalase had no effect.

Conclusions: Our data indicate that ceramide has an integral role in the transition of the mediator of FID from nitric oxide to mitochondrial-derived H$_2$O$_2$, and that inhibition of ceramide production can revert the mechanism of dilation back to nitric oxide. Ceramide may be an important target for preventing and treating vascular dysfunction associated with atherosclerosis. (Circ Res. 2014;115:525-532.)

Key Words: ceramides ■ mitochondria ■ nitric oxide ■ reactive oxygen species ■ sphingomyelin phosphodiesterase

vasodilation to shear stress (flow-induced dilation [FID]) is an endothelium-dependent process important for maintaining vascular homeostasis. Impaired FID is a powerful predictor of future cardiovascular events. Increased shear during flow stimulates endothelial release of vasoactive substances including nitric oxide (NO), prostacyclin, and endothelial-derived hyperpolarizing factors. We have previously shown that risk factors for, or the presence of coronary artery disease (CAD), evoke a transition in the endothelial mediator of FID from NO to mitochondrial-derived hydrogen peroxide (H$_2$O$_2$). Although both factors elicit smooth muscle relaxation, the nonvasomotor effects of each are generally opposite with NO-stimulating anti-inflammatory, antithrombotic, and antiproliferative pathways, compared with the proinflammatory, prothrombotic, and proatherogenic nature of H$_2$O$_2$. The mechanism by which this transition occurs with the onset of disease represents a major gap in our understanding of microvascular control.

Accumulating evidence reveals that a class of bioactive metabolites, known as sphingolipids, play an essential role in cardiovascular pathophysiology. Their function is diverse and ranges from modulating cell proliferation and angiogenesis to atherosclerosis and cell death. Ceramide, a prototypical sphingolipid product of sphingomyelinase, is produced in endothelial cells, found in human plasma, and is a risk factor for atherosclerosis. Furthermore, ceramide is known to stimulate mitochondrial reactive oxygen species (ROS) production. Therefore, we tested whether ceramide might play a role in the switch from NO to H$_2$O$_2$ in the mechanism of FID in the human microcirculation. We addressed this question by examining (1) whether exogenous ceramide can convert the mediator of FID from NO to H$_2$O$_2$ in healthy non-CAD arterioles, (2) whether ceramide induces mitochondrial production of H$_2$O$_2$, and (3) whether...
inhibition of ceramide production restores NO as the mediator of dilation in arterioles from patients with chronic CAD.

Methods

Tissue Acquisition
Fresh human adipose tissue (visceral, subcutaneous, peritoneal) and right atrial appendages from patients undergoing surgical procedures were obtained as discarded surgical specimens. Tissues were placed in ice-cold HEPES buffer or cardioplegia solution for adipose and atrial tissue, respectively. Deidentified patient demographic data were collected using the Generic Clinical Research Database at the Medical College of Wisconsin. All protocols were approved by the local Institutional Review Board at the Medical College of Wisconsin and the Zablocki VA Medical Center.

Measurement of FID by Video Microscopy
Briefly, isolated arterioles were cannulated on glass micropipettes and secured in an organ chamber with circulating Krebs buffer. After equilibration, endothelin-1 was added to assess viability and to preconstrict the vessels by 30% to 50% of their passive diameter. Internal diameters were measured at steady state before and during intraluminal flow at pressure gradients of 5 to 100 cm H2O. The following inhibitors were added to the bath 30 minutes before initiation of flow; NO synthase inhibitor Nω-nitro-ʟ-arginine methyl ester (10−4 mol/L), H2O2 scavenger polyethylene glycol–catalase (PEG–catalase; 500 U/mL), mitochondrial antioxidant Mito-TEMPO (10−4 mol/L), or electron transport chain (ETC) complex I inhibitor rotenone (10−5 mol/L). C-2 ceramide (10−5 mol/L) or the neutral sphingomyelinase (NSmase) inhibitor GW4869 (4×10−6 mol/L) was incubated with arterioles from healthy patients and patients with CAD, respectively, for 16 to 20 hours before the experiment. Papaverine, (10−4 mol/L) an endothelium-independent vasodilator, was added at the end of each experiment to determine the vessel’s maximal diameter, and then the direction of maximal flow was reversed in the presence of maximal dilation (MD) to confirm matched impedance between pipettes.

Fluorescence Detection of Mitochondrial H2O2
Mito perox yellow 1 (MitoPY1) was used to evaluate mitochondrial-derived H2O2 in vessels during flow. After cannulation in a warmed chamber (37°C) containing HEPES buffer (pH, 7.4), arterioles were perfused intraluminally with MitoPY1 (10−5 mol/L for 1 hour). Vessels pretreated with vehicle or ceramide (10−3 mol/L, 16–20 hours) were then exposed to either no flow (remained pressurized at 80 cm H2O, 0 gradient) or flow at a pressure gradient of 100 cm H2O in the presence or absence of PEG–catalase (500 U/mL). Changes in mitochondrial H2O2 fluorescence probed by MitoPY1 were examined under fluorescence microscopy equipped with a krypton/argon laser fluorescence microscope (model TE200 Nikon Eclipse) using an excitation wavelength of 488 nm and measured emission between 530 and 590 nm. Baseline measurements of fluorescence were obtained in the absence of flow and every minute during 5 minutes of intraluminal flow. Images were then analyzed for fluorescence intensity in arbitrary units using Metamorph (Universal Imaging Corp) subtracting background fluorescence. Relative average fluorescence intensity was normalized for surface area and presented as percent change from baseline (before initiation of flow). All vessels used for comparison on a given day were obtained from the same patient and studied using the same imaging parameters. See the Supplemental Methods for complete protocol.

Immunohistochemistry
Immunohistochemistry was performed to visualize ceramide expression in human arterioles from discarded adipose tissue as previously described. Briefly, isolated arterioles (≈200 μm in diameter) were fixed in zinc formalin buffer for 24 to 72 hours and processed for paraffin embedding. Samples were sectioned on an HMS355 microscope at 4 μm. Immunolabeling was performed using a mouse anti-human monoclonal antibody against ceramide. Immunostaining was performed using a Leica Bond MAX Immunostainer. Slides were deparaffinized and subject to heat-induced epitope retrieval for 10 minutes at pH 6.0. The primary antibody was optimal at 1:200 using the Bond Refine-HRP detection system. Slides were scanned with a NanoZoomer HT slide scanner (Hamamatsu, Japan). Staining was quantified within the vessel wall using Metamorph (Universal Imaging Corp) and reported as average area percent or the total stained area divided by the total wall area (square micrometers).

Statistical Analyses
Data are expressed as means±SEM. 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. Fisher exact test was used to compare baseline characteristics for patients without and with CAD. All analyses were performed using SigmaStat, version 3.5. Statistical significance was defined as P<0.05.

Results
Discarded human tissue was collected from a total of 56 patients. Results were tabulated using adipose arterioles from 42 patients without CAD and 14 patients with CAD. In those with CAD, 10 vessels were from adipose and 4 from atrial appendages. The diameters of arterioles before and after (passive diameter) administration of papaverine are as follows (mean±SD): 171±56 and 174±62, respectively, for adipose vessels from healthy patients; 176±62 and 185±72, respectively, for adipose vessels from patients with CAD; and 81±33 and 91±30, respectively, for coronary arterioles from patients with CAD. Patient demographic information is summarized in the Table.

Ceramide Induces a Transition in the Mediator of FID
As shown in Figure 1, after incubating human adipose arterioles overnight (16–20 hours) with C-2 ceramide, FID was maintained compared with vehicle-treated control (%MD,
Inhibition of NO synthase with \(\text{N}^\omega\)-nitro-\(\text{l}\)-arginine methyl ester inhibited FID in the vehicle group (%MD, 13.8±7.7; \(n=7\)) but had no effect on FID in the ceramide-treated group (%MD, 84.1±7.4; \(n=5\)). PEG–catalase abolished dilation in the ceramide-incubated vessels (%MD, 21.4±8.2; \(n=5\)) but had no effect on vehicle-treated vessels (%MD, 86.4±7.7; \(n=5\)). When arterioles were treated acutely (30 minutes) with ceramide, FID remained NO dependent (%MD, 16.3±3.7; \(n=5\), data not shown). Overnight treatment with ceramide did not affect dilation to sodium nitroprusside, indicating endothelial specificity to the effects of ceramide.

Ceramide Increases Mitochondrial H\(_2\)O\(_2\) in Response to Flow

To directly assess whether ceramide induces mitochondrial-derived H\(_2\)O\(_2\) in response to increases in shear, semiquantitative analysis of MitoPY1, a fluorescent probe used for imaging H\(_2\)O\(_2\) specifically in the mitochondria,\(^{14}\) was performed. As shown in Figure 2, an increase in mitochondrial-derived H\(_2\)O\(_2\) was observed at both 1 minute (% change from baseline [%Δ], 88±34; \(n=5\)), ceramide versus −14±9.2; \(n=5\)), vehicle) and 5 minutes of maximal flow (100 cm H\(_2\)O; %Δ, 130±52.6; \(n=5\)), ceramide versus −16±14; \(n=5\), vehicle) in healthy human adipose arterioles preincubated with ceramide compared with vehicle. To confirm that the increase in MitoPY1 fluorescence was attributable to H\(_2\)O\(_2\) as opposed to other peroxide species, additional studies were performed in the presence of PEG–catalase. PEG–catalase completely blocked the increase in MitoPY1 fluorescence in ceramide-treated arterioles exposed to maximum flow at 1 minute (%Δ, −16.3±15.3; \(n=4\)) and 5 minutes (%Δ, −16.7±17.4) compared with ceramide alone (%Δ, 88±34; \(n=5\)).

Mitochondria Is the Source of FID-Induced H\(_2\)O\(_2\) Release in Ceramide-Treated Vessels

To determine whether the source of H\(_2\)O\(_2\) is mitochondrial, dilation was measured in the presence of the ETC complex I inhibitor rotenone (1 \(\mu\)mol/L). Figure 3A shows that rotenone alone does not affect FID (%MD, 74.7±9.6; \(n=5\)), whereas vasodilation is significantly reduced in ceramide-treated arterioles in the presence of rotenone (%MD, 38.3±14.1; \(n=5\)) compared with ceramide alone (%MD, 78.6±5.8; \(n=10\)). Treatment with the mitochondrial-targeted

### Table. Patient Demographics

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Healthy ((n=42))</th>
<th>CAD ((n=14))</th>
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<td>10/4</td>
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<td>Diabetes mellitus(^*)</td>
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<tr>
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</tr>
<tr>
<td>None of the above</td>
<td>37</td>
<td>0</td>
</tr>
</tbody>
</table>

CAD indicates coronary artery disease; F, female; M, male; and \(n\), number of patients.

\(^*\)P<0.05 for healthy patients vs patients with CAD.

78.6±5.8 \([n=10]\), ceramide versus 76.8±4.9 \([n=10]\), vehicle).

![Figure 1. Effect of exogenous ceramide on flow-induced dilation (FID).](image-url)

A. The magnitude of dilation is not affected with overnight incubation of ceramide alone compared with vehicle-treated control \((n=10\) and 10, respectively). B. FID in healthy adipose arterioles is reduced in the presence of \(\text{N}^\omega\)-nitro-\(\text{l}\)-arginine methyl ester \((\text{l-NAME})\), however is maintained if first preincubated with ceramide \((n=7\) and 5, respectively). C. Polyethylene glycol–catalase has minimal effect on FID in healthy arterioles, however impairs FID in ceramide-treated arterioles \((n=5\) and 5, respectively). D. Sodium nitroprusside \((\text{SNP})\)-induced dilation is not reduced by ceramide compared with vehicle-treated control \((n=4\) and 6, respectively). \(^*\)P<0.05 vs vehicle at specific pressure gradients. \(n\) indicates number of patients.
antioxidant, Mito-TEMPO, resulted in a similar reduction in FID (Figure 3B). Mito-TEMPO decreased FID in arterioles incubated overnight with ceramide (%MD, 20.6±10.1 [n=5], Mito-TEMPO versus 78.6±5.8 [n=10], ceramide alone). Mito-TEMPO alone did not affect FID (%MD, 87.8±1.5; n=5).

Ceramide Presence in Human Arterioles
Immunohistochemistry was used to evaluate the presence of ceramide in arterioles from healthy patients (non-CAD) and those with CAD. Figure 4 is representative of 3 experiments. Interestingly, ceramide appears to be predominantly located in the smooth muscle layer. The average area percent of staining did not differ between the 2 groups (non-CAD [mean±SD], 27.6±8.7% and CAD, 25.3±6.2%), nor was there a difference in staining density between the 2 groups (data not shown). However, the total stained area was greater in the CAD arterioles (5503±1802 μm²) versus non-CAD (1994±619 μm²). Likewise, the total vascular cross-sectional area was larger in CAD versus non-CAD vessels (2.2×10⁴±0.4×10⁴ versus 0.7×10⁴±0.3×10⁴ μm², respectively). The secondary antibody was specific (Figure 4C).

Inhibition of NSmase Reverts Mechanism of FID From H₂O₂ to NO in Arterioles From Patients With CAD
Adipose arterioles from patients with CAD were incubated overnight with the specific noncompetitive NSmase inhibitor GW4869 (4×10⁻⁶ mol/L, 16–20 hours). In these vessels, dilation to flow was attenuated by Nω-nitro-ʟ-arginine methyl ester (Figure 5; %MD, 29.5±4.9; n=5) but not by PEG–catalase (%MD, 64.1±5.6; n=5). Incubation with GW4869 did not alter the magnitude of FID (%MD, 73.9±4.9 [n=9] versus 81.5±4.6 [n=8], vehicle). Thus, inhibiting ceramide production reverts the mechanism of dilation from H₂O₂ to NO in vessels from subjects with CAD.

To determine whether a similar switch in mechanism occurs in the coronary circulation, arterioles isolated from right atrial appendages from patients with CAD were treated in a similar fashion. As shown in Figure 6A, incubation with GW4869 alone had no effect on dilation (%MD, 75.4±6.4 [n=4], GW4869 versus 85.6±3.4 [n=4], vehicle). Inhibition of NO synthase with Nω-nitro-ʟ-arginine methyl ester reduced FID in CAD atrial vessels treated with GW4869, whereas PEG–catalase had no effect. (Figure 6B; %MD, 12.6±2.2 [n=4] versus 73.1±9.7 [n=4], respectively).

Discussion
This study is the first to demonstrate that ceramide plays a pivotal role in switching the primary mediator of FID in human arterioles.

Figure 3. Role of mitochondria in flow-induced dilation (FID) in ceramide exposed vessels. A, Vasodilation in response to flow is impaired in arterioles pretreated with ceramide and rotenone compared with ceramide alone. Rotenone alone had no effect on FID (n=5, 10, and 5, respectively). B, Mito-TEMPO significantly decreased the response to flow in ceramide-treated vessels compared with ceramide alone. Mito-TEMPO alone had no effect (n=5, 10, and 5, respectively). †P<0.05 vs ceramide curve. *P<0.05 vs ceramide at specific pressure gradients.

Figure 4. Immunohistochemistry was used to evaluate the presence of ceramide in arterioles from healthy patients (non-CAD) and those with CAD. Figure 4 is representative of 3 experiments. Interestingly, ceramide appears to be predominantly located in the smooth muscle layer. The average area percent of staining did not differ between the 2 groups (non-CAD [mean±SD], 27.6±8.7% and CAD, 25.3±6.2%), nor was there a difference in staining density between the 2 groups (data not shown). However, the total stained area was greater in the CAD arterioles (5503±1802 μm²) versus non-CAD (1994±619 μm²). Likewise, the total vascular cross-sectional area was larger in CAD versus non-CAD vessels (2.2×10⁴±0.4×10⁴ versus 0.7×10⁴±0.3×10⁴ μm², respectively). The secondary antibody was specific (Figure 4C).

Figure 2. Effect of ceramide on production of mitochondrial hydrogen peroxide (H₂O₂) in response to flow. A, Representative images of cannulated vessels incubated in vehicle or ceramide overnight±polyethylene glycol–catalase, after intraluminal incubation with mito peroxy yellow 1 (MitoPY1) at baseline, 1 minute, and 5 minutes of maximum intraluminal flow. B, Quantification of mitochondrial H₂O₂ expressed as percent change in MitoPY1 fluorescence from baseline at 1 minute and 5 minutes of maximum flow. *P<0.05 vs vehicle. †P<0.05 vs ceramide+catalase.
arterioles with the onset of CAD. The major novel findings are 3-fold. First, overnight exposure to exogenous ceramide can evoke a transition from NO to H$_2$O$_2$ as the mediator of FID. Second, ceramide-induced increases in H$_2$O$_2$ require an intact mitochondrial ETC. Third, inhibition of the ceramide-producing enzyme NSmase can restore NO as the mediator of FID in patients with chronic CAD. These findings show that ceramide is a critical mechanistic component of the transition that takes place from the NO-mediated pathway of microvascular dilation observed in healthy individuals to an H$_2$O$_2$-driven signaling pathway of FID seen in patients with atherosclerotic disease.

Vasodilation to shear stress is not only important to maintain tissue perfusion, but is highly predictive of future cardiovascular events. Endothelial-dependent dilation is mediated by hyperpolarization or by release of vasoactive factors which include NO, prostacyclin, and other endothelium-derived hyperpolarizing factors (eg, epoxyeicosatrienoic acid or H$_2$O$_2$). Although each factor is capable of eliciting dilation, each has a different effect on vascular biology. For instance, in vessels from subjects with CAD, endothelial release of H$_2$O$_2$ creates an environment that promotes inflammation, thrombosis, and atherosclerosis.

**Ceramide and Mitochondrial H$_2$O$_2$-Dependent Dilation**

Prior evidence suggests that the endothelial mitochondria are the source of H$_2$O$_2$ responsible for FID in patients with CAD. Elevated levels of plasma sphingomyelin, the precursor to ceramide, are an independent risk factor for CAD and a known inducer of mitochondrial dysfunction. This is the first study to our knowledge demonstrating a link between ceramide and mitochondrial-derived H$_2$O$_2$ in microvessels from patients with CAD.

Ceramide consists of a family of bioactive sphingolipids formed via multiple pathways. The most rapid generation of ceramide occurs through sphingomyelin hydrolysis by acid or neutral

![Figure 4](image_url)

**Figure 4.** Ceramide accumulation in arterioles from healthy patients vs patients with coronary artery disease (CAD). Representative images from 3 patients (3 healthy, 3 CAD). The total area of staining is decreased in arterioles from healthy patients (A) vs patients with CAD (B); however, area stained/total area did not differ between groups. Specificity of the antibody was examined by removal of the primary antibody (C). Bar=40 μm.

![Figure 5](image_url)

**Figure 5.** Inhibition of neutral sphingomyelinase (NSmase) reverts the mediator of flow-induced dilation (FID) back to nitric oxide in vessels from adipose tissue from patients with coronary artery disease (CAD). A. Incubation with the specific NSmase inhibitor GW4869 (4 μmol/L, 16–20 hours) did not affect the overall magnitude of dilation to flow compared with vehicle-treated control (n=9 and 8, respectively). B. The response to flow was inhibited in CAD vessels first incubated with GW4869 in the presence of Nω-nitro-ʟ-arginine methyl ester (l-NAME; 100 μmol/L) compared with GW4869 alone, whereas polyethylene glycol–catalase (500 U) had no effect (n=5, 9, and 5, respectively). *P<0.05 vs GW4869 at specific pressure gradients.
sphingomyelinas. Ceramide can also be formed by reacetylation of sphingosine, known as the salvage pathway, as well as de novo by the condensation of palmitate and serine. Ceramidase enzymes found within the cytosol are responsible for the catabolism of ceramide back to sphingosine. This complexity of ceramide formation and removal implies that cellular levels of ceramide are tightly regulated as is true with many key signaling molecules. Overall, the de novo synthesis of ceramide contributes little to the overall amount of ceramide within the cell, with most of its generation coming from the sphingomyelinases found in the cell membrane (NSmase), or within lysosomes (ASmase). The current study examined specifically the role of NSmase because this is the only ceramide-producing enzyme found within the sphingomyelin-rich caveolae, allowing close contact to luminal flow and intimate connection with a primary signaling location in the cell membrane. Czarny et al demonstrated that NSmase activity dramatically increases within the first 2 minutes of increased flow in the membrane fraction of the rat pulmonary artery, whereas there is no change in ASmase activity. Previous studies have shown that ceramide can increase ROS levels through activation of nicotinamide adenine dinucleotide phosphate-oxidase (NADPH) oxidase, xanthine oxidase, and the mitochondrial ETC, specifically at the Q site of complex III. Zhang et al previously showed that administration of exogenous ceramide increases NADPH oxidase activity, resulting in decreased vasodilation to bradykinin in small bovine arterioles. However, prior evidence in human tissue suggests that the primary source of H$_2$O$_2$ that mediates FID in microvessels from patients with CAD is the mitochondrial ETC. The current study supports the mitochondria as a predominant source of ceramide-induced ROS formation, but it is recognized that cross-talk exists between intracellular sites of ROS production in the form of ROS-induced ROS release. It is possible that ceramide exerts its effect on multiple cellular sites of ROS formation. However, the mitochondria seem to play an obligatory role in the ROS generation during shear based on the effectiveness of the ETC complex I inhibitor rotenone and the mitochondrial-specific antioxidant Mito-TEMPO in abolishing FID in arterioles from healthy subjects who were pretreated with ceramide.

NO-dependent FID was not affected by acute exposure (30 minutes) to ceramide, whereas vessels exposed to ceramide chronically (16–20 hours) transitioned from an NO- to an H$_2$O$_2$-dependent mechanism of dilation. This highlights the complexity involved in this transition. For instance, it is known that there exists a constant flux between formation of the cell-damaging ceramides and the more benign sphingosines such as sphingosine-1-phosphate, known as the sphingolipid rheostat. It is feasible that expression of ceramidase, the enzyme that converts ceramide to sphingosine, may decrease during chronic exposure. Likewise, longer exposure may be necessary to elicit changes in NO bioavailability. A study by Zhang et al showed that ceramide accumulation, in vivo and in vitro, increases the association of protein phosphatase 2A with endothelial NO synthase, subsequently decreasing phosphorylation at Ser1177 and Ser617, thus decreasing the amount of available NO. Although ceramide is known to increase mitochondrial-derived

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**Figure 6.** Inhibition of neutral sphingomyelinase (NSmase) reverts the mediator of flow-induced dilation (FID) back to nitric oxide (NO) in atrial vessels from patients with coronary artery disease (CAD). **A.** Incubation with the specific NSmase inhibitor GW4869 (4 μmol/L, 16–20 hours) had no effect on FID compared with vehicle (n=4). **B.** The response to flow was inhibited in CAD vessels first incubated with GW4869 in the presence of Nω-nitro-L-arginine methyl ester (L-NAME; 100 μmol/L), whereas polyethylene glycol–catalase (500 U) had no effect (n=4). *P<0.05 vs GW4869 at specific pressure gradients.

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**Figure 7.** A schematic diagram illustrating the involved pathways. Under normal conditions in healthy adults, exposure of the endothelial layer to shear stress activates endothelial nitric oxide synthase (eNOS) causing elevation of nitric oxide (NO) which primarily serves as the mediator of smooth muscle dilation. In the disease state, increased levels of ceramide formed via neutral sphingomyelinase (NSmase) trigger mitochondrial reactive oxygen species formation, which both decrease the bioavailability of NO and ultimately change the mediator of vasodilation to hydrogen peroxide (H$_2$O$_2$). Inhibition of NSmase with the specific noncompetitive inhibitor GW4869 can revert the mechanism of dilation back to NO.
ROS,\textsuperscript{27} therefore ceramide-induced decreases in NO likely contribute to this alteration in dilation as well. Future studies are needed to understand how ceramide metabolism and regulation of NO contribute to this transition in mechanism because this could provide novel avenues for drug discovery.

Interestingly, our study suggests that ceramide concentrates predominantly in the smooth muscle layer of arterioles from patients with CAD. This observation is in agreement with a study by Augé et al\textsuperscript{28} which demonstrated that activation of NSmase and subsequent ceramide accumulation are associated with smooth muscle cell proliferation. Although ceramide may be responsible for hyperplasia associated with disease, the medial layer might also serve a reservoir for lipid actions in the endothelium. This finding is also supported by the fact that vascular endothelial cells tend to not accumulate cholesterol or lipid as compared with other various cell types such as smooth muscle cells or macrophages. Studies have shown that after exposure to elevated levels of lipid, endothelial cells upregulate specific transporters such as ATP-binding cassette G1.\textsuperscript{29} Although the average percent area of ceramide staining did not differ between the 2 groups, and the average vascular wall area was larger in the CAD arterioles, the total stained area was greater in the CAD vessels suggesting that the overall amount of ceramide is elevated in arterioles from patients with CAD.

The use of an inert form of ceramide would be beneficial in determining the specificity of ceramide in modifying vascular function. Often the precursor to ceramide, dihydroceramide, which lacks the \textit{trans}-4,5 double bond, is used as a negative control; however, the data have been conflicting because dihydroceramide-4,5 double bond, is used as a negative control; trans

Conclusions and Clinical Implications

The present study confirms that exogenous ceramide can cause a shift in vasoactive mediators from NO to mitochondrial-derived \textit{H$_2$O$_2$}, and that inhibition of NSmase, a key enzyme in ceramide production, can revert the mechanism of dilation back to NO. Although the majority of clinically significant lesions involve the coronary conduit arteries, microvascular dysfunction is a key risk factor for cardiovascular events, even in the absence of large artery disease.\textsuperscript{31} Accumulating evidence suggests a correlation between elevated ceramide levels and type 2 diabetes mellitus\textsuperscript{32} as well as smoking,\textsuperscript{33} both potent instigators of endothelial microvascular dysfunction. The ability to decrease overall ceramide levels through inhibition of NSmase or via activation of ceramidase can have a profound impact on multiple clinical scenarios that are attributable to oxidative stress including vascular inflammation and thrombosis. Therefore, thorough understanding of ceramide signaling and regulatory mechanisms within the vasculature may allow for the development of new therapeutics and a means to improve microcirculation in disease.

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Disclosures

None.

References


Potential Study Limitations

An important limitation to studies in isolated arterioles is the inability to quantify vascular cell target molecules. Small sample volumes prevented us from quantifying ceramide levels in the tissue. The diacylglycerol kinase assay has been used to measure ceramide levels between 25 pmol and 2 nmol; however, the specificity of this assay has been questioned.\textsuperscript{30} The amount of starting sample required for other methods such as thin-layer chromatography, high-performance liquid chromatography, and mass spectrometry surpasses the total amount that can be isolated from a patient sample, making accurate measurement of ceramide from human microvessels a challenge.

By necessity, our healthy samples of tissue were collected from subjects with a variety of diseases and who may be taking ≥1 medications. There may also be patients who have subclinical atherosclerotic plaque and are misclassified as normal. To minimize this risk, only subjects with no more than 1 risk factor for CAD and no evidence of CAD (see Table) were classified as not having CAD. We argue that the benefit of directly examining responses in human vessels outweighs the methodological limitations described. However, we further try to minimize these restrictions by washing vessels with 60 mL of buffer before experimentation to eliminate most pharmacological agents and by searching for confounding effects of related diagnoses, age, and sex.

Fluorescent probes, such as dichlorodihydrofluorescein, dihydroethidium, and mitochondrial superoxide indicator (mitoSOX), have been used extensively to measure production of \textit{H$_2$O$_2$}, superoxide (O$_2$\textsuperscript{−}), and mitochondrial-derived O$_2$\textsuperscript{−}, respectively.\textsuperscript{11} Although each has the ability to detect changes in intracellular ROS, they have limitations and shortcomings as well.\textsuperscript{12} The present study used a newer fluorescent probe, MitoPY1, to detect levels of mitochondrial-derived \textit{H$_2$O$_2$}. This boronate-based probe contains a triphenylphosphonium group similar to mitoSOX that targets the fluorophore to the mitochondria, allowing it to react with \textit{H$_2$O$_2$}. It is possible that MitoPY1 may react with other forms of ROS; however, in the present study, PEG–catalase, which is specific for \textit{H$_2$O$_2$}, abolished the increase in fluorescence indicating that the majority of ROS being detected was \textit{H$_2$O$_2$}.31


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Supplemental Material

Supplemental Methods

Measurement of FID by Videomicroscopy:

Measurements of internal diameter were made on isolated, pressurized human resistance arterioles from human visceral adipose tissue or human coronary arterioles (HCA) using videomicroscopy as previously described. Briefly, isolated arterioles were cannulated on glass micropipettes and secured in an organ chamber containing Kreb’s buffer consisting of (in mmol/L) 123 NaCl, 4.7 KCl, 2.5 CaCl₂, 1.2 MgSO₄, 16 NaHCO₃, 0.026 EDTA, 1.2 KH₂PO₄, and 11 glucose. The preparation was then placed on the stage of an inverted microscope (Olympus CK2, magnification 200X) coupled to a CCD video camera (WV-BL200, Panasonic), video monitor (Panasonic), and calibrated videomicrometer (VIA-100K, Boeckeler Instruments Inc., resolution = 0.4µm). The chamber bath was bubbled continuously with 21% O₂, 5% CO₂, and 74% N₂ to maintain a pH of 7.40 ± 0.5 and a PO₂ of 140 ± 10mmHg while temperature was maintained at 37°C. Vessels were slowly pressurized over an hour from an intraluminal pressure of 40cmH₂O to a final pressure of 80cmH₂O. Following equilibration, endothelin-1 was added to assess viability and to pre-constrict the vessel to 30-50% of its diameter after pressurization. Internal diameters were measured at steady-state before and during intraluminal flow at pressure gradients of 5 to 100cmH₂O. The following inhibitors were added to the bath 30 minutes prior to initiation of flow; nitric oxide synthase (NOS) inhibitor Nω-nitro-L-arginine (L-NAME; 10⁻⁴ mol/L), H₂O₂ scavenger polyethylene glycol-catalase (PEG-Catalase; 500U/mL), mitochondrial antioxidant Mito-TEMPO (10⁻⁶ mol/L), or electron transport chain (ETC) complex I inhibitor rotenone (10⁻⁶ mol/L). C-2 Ceramide (10⁻⁵ mol/L) or the neutral sphingomyelinase inhibitor GW4869 (4x10⁻⁶ mol/L) was incubated with arterioles from healthy patients and patients with CAD, respectively, for 16-20hrs prior to the experiment. Papaverine, (10⁻⁴ mol/L) an endothelium-independent vasodilator, was added at the end of each experiment to determine the vessel’s maximal diameter, then the direction of maximal flow was reversed in the presence of maximal dilation, to confirm matched impedance between pipettes.

Supplemental References