Peroxynitrite Disrupts Endothelial Caveolae Leading to eNOS Uncoupling and Diminished
Flow-Mediated Dilation in Coronary Arterioles of Diabetic Patients

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Abstract

Peroxynitrite (ONOO\textsuperscript{−}) contributes to coronary microvascular dysfunction in diabetes mellitus (DM). We hypothesized that in DM ONOO\textsuperscript{−} interferes with the function of coronary endothelial caveolae, which plays an important role in nitric-oxide (NO)-dependent vasomotor regulation. Flow-mediated dilation (FMD) of coronary arterioles was investigated in DM (n=41) and nonDM (n=37) patients undergoing heart surgery. NO-mediated coronary FMD was significantly reduced in DM patients, which was restored by ONOO\textsuperscript{−} scavenger, iron-(III)-tetrakis(N-methyl-4'pyridyl)porphyrin-pentachloride or uric-acid, whereas exogenous ONOO\textsuperscript{−} reduced FMD in nonDM subjects. Immuno-electronmicroscopy demonstrated an increased 3-nitrotyrosine formation (ONOO\textsuperscript{−} specific protein nitration) in endothelial plasma membrane in DM, which colocalized with caveolin-1 (Cav-1), the key structural protein of caveolae. The membrane-localized Cav-1 was significantly reduced in DM and also in high glucose-exposed coronary endothelial cells. We also found that DM patients exhibited a decreased number of endothelial caveolae, whereas exogenous ONOO\textsuperscript{−} reduced caveolae number. Correspondingly, pharmacological (methyl-β-cyclodextrin) or genetic disruption of caveolae (Cav-1-knockout mice) abolished coronary FMD, which was rescued by sepiapterin, the stable precursor of NO synthase cofactor, tetrahydrobiopterin. Sepiapterin also restored coronary FMD in DM patients. Thus, we propose that ONOO\textsuperscript{−} selectively targets and disrupts endothelial caveolae, which contributes to NO synthase uncoupling, hence reduced NO-mediated coronary vasodilation in DM patients.

Key words: Coronary Microvascular Disease, Caveolae, Peroxynitrite, NO synthase uncoupling
Diabetes mellitus (DM) is associated with impaired function of coronary resistance arteries. The consequence is a mismatch between myocardial blood supply and demand leading to ischemic episodes in the diabetic heart (1). Reduced myocardial perfusion may develop even in the absence of occlusive coronary artery disease. For instance, Nitenberg et al have demonstrated that in DM patients with angiographically normal coronary arteries cold pressor test-induced dilation was reduced, as estimated by coronary surface area during quantitative angiography (2). Intracoronary injection of acetylcholine (ACh), which normally dilates arteries, caused vasospasm preferentially in diabetic patients (3). In small coronary arteries dissected from the heart of diabetic patients, Miura et al (4) and recently our group (5) demonstrated ACh-induced constriction. The underlying mechanism(s) responsible for coronary microvascular dysfunction remained poorly understood in human DM.

Flow-mediated dilation (FMD), the intrinsic regulatory mechanism of resistance arteries in response to increases in wall shear stress (WSS) (6), is one of the key determinants of myocardial perfusion (7). The endothelium-dependent FMD is responsible for normalizing WSS and provides a feed-forward mechanism for increases in myocardial blood flow and metabolite-induced vasodilation (6). The problem is that this regulatory mechanism often fails in the diseased heart. Early studies have shown that brachial artery FMD is reduced in patients with type-1 (8) and type-2 DM (9). Previously we demonstrated that in a mouse model of type-2 DM (db/db mice) WSS-induced dilation is diminished in coronary arterioles, which is due to the reduced availability of NO (10; 11). In DM, among other key pathological mechanisms responsible for reduced NO synthase activation (12; 13), the direct interaction of NO with superoxide anion (14) has received substantial attention. It is of particular importance that the NO-superoxide interaction not only reduces NO bioavailability but also generates the reactive peroxynitrite (ONOO-). ONOO- has numerous detrimental effects in the cardiovascular system.
and plays a crucial role in the development of DM-associated vascular pathology (15). Emerging evidence indicates that ONOO- preferentially targets vascular endothelium and specific subcellular compartments within (15). The footprint of enhanced ONOO- production is 3-nitrotyrosine (3-NT) formation. 3-NT-modified proteins were detected even in the normal rat aorta endothelium, with a distinct localization to mitochondria (16). Some evidence indicates that in aging rat, 3-NT formation is enhanced and more widely distributed within subcellular compartments of the endothelium (17). Yet, it is unknown whether ONOO- preferentially targets endothelial plasma membrane microdomains, such as caveolae, a specific type of cholesterol rich membrane lipid raft, which plays a key role in WSS-induced, NO-dependent regulation of arterial diameter (18).

Accordingly, in this study we set out to investigate alterations in coronary FMD in DM patients and to elucidate the impact of ONOO-, which we hypothesized, preferentially targets endothelial caveolae. In this study we demonstrate that NO-mediated coronary FMD is markedly reduced in patients with DM. We show that ONOO- targets and disrupts endothelial membrane caveolae, which subsequently predisposes NO synthase for uncoupling in DM.
Research Design and Methods

Patients

Protocols were approved by the Institutional Review Board at our Institution. Consecutive patients undergoing heart surgery were enrolled in this study. Patients were divided into two groups with or without documented DM. Patients were included irrespective of DM duration.

Assessment of FMD in human coronary arterioles

Videomicroscopy of isolated human coronary arterioles were performed as previously described (19). Briefly, coronary arterioles (diameter:~100µm) were dissected from the right atrial appendages obtained from patients at the time of heart surgery. Arterioles were cannulated and pressurized (70 mmHg) and changes in diameter were measured with a videocaliper (Colorado).

Intraluminal flow was induced by changing perfusion pressure with equal degree but in opposite direction, in three steps (ΔP: 25-50-90 cmH₂O). Figure 1a shows that ΔP elicited increases in flow (~8 to ~35 µL/min), which resulted in increases in WSS (from ~5 to ~15 dyne/cm²). To assess the role of NO, ONOO- and NOS cofactor, tetrahydrobiopterin (BH₄) in affecting coronary FMD, coronary arterioles were incubated with NOS inhibitor, Nω-nitro-L-arginine methyl ester (L-NAME, 2x10⁻⁴ M, for 30-min), selective ONOO- sequesters, iron(III)tetrakis(N-methyl-4'pyridyl)porphyrin-pentachloride (FeTMPyP, 10⁻⁴ M) and uric acid (10⁻⁴ M, for 30-min) or stable BH₄ precursor, sepiapterin (10⁻⁵ M, for 30-min), respectively; and coronary FMD was reassessed. In other protocols following incubation with FeTMPyP or sepiapterin, vessels were exposed to additional L-NAME and coronary FMD was obtained again. In separate protocols arterioles were exposed to exogenous ONOO- (10⁻⁵ M) and FMD was
assessed. To investigate the role of caveolae, arterioles were pre-incubated with methyl-β-cyclodextrin (mβCD, 2x10^{-3} M, for 60-min), which is known to disrupt caveolae (20).

**Detection of superoxide anion production**

Superoxide production in coronary arteries was measured by using the dihydroethidium (DHE) method, as described previously (10; 11). Briefly, cryosections (8 µm thick) from snap-frozen human atrium were incubated with DHE (10 µM, for 30-min) with or without pretreatment with superoxide scavenger, Tiron (5 mM, 30-min). Slides were washed in PBS, and sections were covered with DAPI-Vectashield. Fluorescence intensity measurements (excitation: BP545/25, emission: BP605/70) of oxidized, nuclei-trapped DHE were performed in nuclei in the vessel wall and normalized to nuclei area.

**Immunohistochemistry and proximity ligation assay for co-localization**

Atrial appendages from nonDM and DM patients were fixed in 4% paraformaldehyde and paraffin embedded. Consecutive sections (8 µm thick) were blocked with normal donkey serum (1-hour) and immune-labeled with monoclonal anti-caveolin 1 (Abcam, 1:100, overnight 4°C) and polyclonal anti-3-NT (Sigma, 1:1000, overnight 4°C) antibodies. Immuno-fluorescent labeling was performed with corresponding Cy3 and Cy5 secondary antibodies (Jackson Immunoresearch). DAPI was used for nuclear staining. For non-specific binding the primary antibody was omitted. Images were collected with fluorescent microscopy. The co-localization between caveolin-1 and 3-NT was calculated with linear regression and Pearson’s correlation coefficient (r), as described by Zinchuk et al (21), in DM and nonDM patients by an investigator unaware of samples’ identity.
In additional experiments *in situ* Proximity Ligation Assay (PLA, Duolink, Sigma) was performed to detect co-localization of Cav-1 and 3-NT according to manufacturer instructions. Briefly, PLA reactions were performed using same primary antibodies (anti-Cav-1, 1:100 and anti-3-NT, 1:1,000), followed by a pair of oligonucleotide-labeled secondary antibodies (Orange PLA probes). In this assay PLA probes create a positive signal only when the epitopes of the target proteins are in close proximity (<40 nm). The signal from each detected pair of PLA probes was then counted using fluorescence microscopy (excitation: BP545/25, emission: BP605/70). For negative controls primary antibodies were omitted.

**Immmuno-electron microscopy**

*Immunogold labeling for 3-NT.* The right atrial appendages were fixed in 4% formaldehyde 0.2% glutaraldehyde in 0.1 M NaCacodylate buffer (pH 7.4). Sections were cut with Leica EM UC6 ultramicrotome (Leica Microsystems, Inc, Bannockburn, IL). Sections were blocked in normal goat serum (1-hour) and incubated with primary rabbit anti-3-NT polyclonal antibody (1:10, overnight 4°C, Sigma) or anti-Cav-1 polyclonal antibody (1:100, overnight 4°C, Cell Signalling) and with additional gold-labeled (10 nm) secondary antibody. Sections were observed in a JEM 1230 transmission electron microscope (JEOL USA Inc). Counting of anti-3-NT-immunogold and anti-Cav-1-immunogold particles within coronary endothelial cells was evaluated by an investigator unaware of samples’ identity.

**Assessment of caveolae number in coronary endothelial cells**

Electron microscopy of atrial samples was performed as described above. Images selected for analysis contained at least one red blood cell to ensure accuracy in identifying the endothelial cell layer. The number of caveolae per µm² of cell membrane was counted, as described
previously (22) and compared in DM and nonDM patients by an investigator unaware of samples’ identity. In separate protocols, atrial samples were exposed to increasing concentrations of exogenous ONOO- (1, 10 and 100 µM), and the number of endothelial caveolae was calculated.

**Western immunoblot**

Western immunoblot analysis was carried out as described previously (5). Briefly, coronary arterioles were homogenized in radio-immunoprecipitation assay buffer and protein concentration was measured by Bradford assay. Equal amount of proteins were loaded for gel electrophoresis. After blotting, a polyclonal anti-3-NT antibody (dilution, 1:1,000) was used for the detection of 3-NT. Membranes were re-probed with anti-β-actin IgG (dilution, 1:5,000) to normalize for loading variations. Corresponding horseradish peroxidase-labeled secondary antibody was used, and chemiluminescence was visualized autoradiographically.

**Membrane fractionation of cultured human coronary artery endothelial cells (HCAEC)**

HCAEC (Lonza, Walkersville, CA, USA) were grown in EBM-2 medium. Cells were maintained in normal (5.5 mM) or high glucose medium (25 mM, HG, for 24-hour). HCAEC were then harvested for cell fractionation using ultracentrifugation. In brief, cells were washed in PBS and lysed in MSE extraction buffer (10mM Tris–HCl, 220mM D-mannitol, 70.1mM sucrose, 1mM EGTA, 0.025% BSA). Protein was measured (Bio-Rad) and equal amount of protein was centrifuged at 100,000×g for 20 min. Supernatant was collected as cytosolic fraction, and pellet contained the membrane fraction. Equal amounts of total lysate, cytosolic, and membrane fractions were loaded for Western blot analysis.
Assessment of endothelium-dependent dilation in caveolin-1 knockout mice

Protocols involving mice were approved by the Institutional Animal Care and Use Committee. Videomicroscopy and wire myography to assess coronary FMD (10; 11) and relaxation of aorta was performed as described previously (23). Briefly, the heart and thoracic aorta was excised from caveolin-1 knockout (Cavtm1Mls/J, Jackson Laboratory) and wild-type mice. Mouse coronary arteries (diameter:~100µm) were isolated and pressurized (70 mmHg). Arteries were pre-constricted with thromboxane analogue, U46619 (10⁻⁹ M). Diameter changes of arterioles to increases in WSS and ACh (10⁻⁹ -10⁻⁶ M) were measured with videomicroscopy. Aorta sections (2 mm in length) were mounted on wire myograph to measure isometric force generation. Aorta were pre-constricted with phenylephrine (10⁻⁶ M) and relaxation was assessed in response to ACh (10⁻⁹ -10⁻⁶ M). Coronary arteries and aortic rings were incubated with sepiapterin (10⁻⁵ M, 30-min) or sepiapterin plus L-NAME (2x10⁻⁴ M, 30-min) and WSS- and ACh-induced responses were reassessed.

Data analysis

Statistical analyses were performed using IBM SPSS Statistics 19. Agonist-induced arteriole responses were expressed as changes in diameter as a percentage of the maximal dilation, defined as passive diameter of the vessel at 70 mmHg in calcium-free PSS. Aortic relaxations in mice were presented as percent changes in force following agonist administration. Statistical analysis was performed by repeated-measures ANOVA, followed by Tukey’s post hoc test. \( P < 0.05 \) was considered statistically significant. Data are expressed as mean ± standard error of the mean.
Results

Patients. Patient demographics and clinical data are presented in Table 1. The age, gender, clinical parameters, underlying diseases and medications were similar between the two populations, except the documented DM, glucose levels and the use of insulin or oral antidiabetics. Patients with DM were more likely to undergo coronary artery bypass graft (CABG) surgery than nonDM. Thus, the potential influence of anti-diabetic medications and the type of the surgery on the measured endpoints cannot be entirely excluded in this study.

NO-dependent FMD is diminished in coronary arterioles of DM patients. In isolated coronary arterioles, a spontaneous tone developed in response to 70 mmHg intraluminal pressure. There were no significant differences between the active (nonDM: 105±7 µm; DM: 94±7 µm) and passive (nonDM: 143±8 µm; DM: 123±7 µm) diameters in DM and nonDM patients. Incubation with sepiapterin, ONOO- sequesters, FeTMPyP, uric acid and/or L-NAME had no effect on the spontaneously developed tone (data not shown).

In coronary arterioles of nonDM patients, increases in intraluminal flow, via increasing WSS (Figure 1a), elicited dilations, whereas coronary arterioles from DM patients exhibited a diminished FMD (Figure 1b and 1c). The NOS inhibitor, L-NAME reduced coronary FMD in nonDM, but not in DM patients (Figure 1d). Dilations to the NO-donor, sodium-nitroprusside were similar in DM and nonDM patients (Figure 1e).

ONOO- contributes to the diminished coronary arteriolar FMD. We found a significantly increased DHE-detected, Tiron-inhibited superoxide production in coronary arteries of DM patients, when compared to nonDM (Figure 2a and 2b). Moreover, the increased level of 3-NT in coronary arterioles indicated an augmented ONOO- production in DM patients (Figure 2c).
Incubation of coronary arterioles with the selective ONOO- scavengers, FeTMPyP (Figures 2d) or uric acid (Figures 2e), significantly enhanced coronary FMD in DM, whereas exogenous application of ONOO- reduced coronary dilation in nonDM patients (Figure 2f).

**ONOO- targets endothelial caveolae in DM.** The subcellular localization of 3-NT was investigated in coronary arterioles using immune-electronmicroscopy and immunohistochemistry approaches. While 3-NT immunogold labeling was less prominent within the endothelium of nonDM patients, in DM the number of 3-NT immunogold particles were increased and more widely distributed, with a significantly increased presence in the luminal plasma membrane of endothelium (Figure 3a and Table 2).

To test whether ONOO- targets endothelial membrane caveolae co-immunostaining of 3-NT and caveolin-1 (Cav-1) was performed. In coronary endothelial cells a greater degree of co-localization between 3-NT and Cav-1 was found in DM patients, as indicated by Pearson’s correlations of fluorescence intensities (Figure 3b and 3c). Co-localization of 3-NT and Cav-1 was confirmed by in situ proximity ligation assay in further independent experiments (Figure 3d and 3e).

**Membrane-localized Cav-1 is reduced in coronary endothelium of DM patients.** We found no significant change in total Cav-1 expression between coronary arteries of DM and nonDM patients (Figure 4a). When distribution of Cav-1 immuno-gold particles was investigated by EM we found that membrane-localized Cav-1 was significantly reduced in DM endothelium (Figure 4 and Table 2). To confirm these changes in the membrane Cav-1 content we also employed primary cultured HCAECs that were exposed to high glucose (25 mM) concentrations. We found
that exposure of HCAECs to high glucose significantly reduced membrane Cav-1 content, whereas it did not alter total and cytosolic level of Cav-1 (Figure 4c and 4d).

**ONO0- reduces the number of endothelial caveolae.** Electron microscopy was employed to evaluate the number of endothelial caveolae in DM and nonDM patients (Figure 5a). We found a significantly reduced number of endothelial caveolae in DM patients, when compared to nonDM (Figure 5b). Moreover, coronary arteries were exposed to exogenous ONOO-. ONOO-, in a dose dependent manner reduced the number of endothelial caveolae (Figure 5c and 5d). ONOO-, at the highest concentration, disrupted the integrity of endothelial membrane, which was lacking any caveolae, and also showed signs of membrane blebbing.

**Lack of caveolae contributes to endothelial NOS uncoupling in DM.** We found that administration of sepiapterin, a stable precursor of BH₄ significantly enhanced coronary FMD in DM patients, in an NO-dependent manner, while it did not affect dilation in non-DM patients (Figure 6a). Pharmacologic disruption of caveolae with mβCD entirely abolished FMD in coronary arterioles in nonDM patients, which was partially restored by additional administration of sepiapterin (Figure 6b).

To provide additional evidence for the lack of caveolae contributing to eNOS uncoupling, Cav-1 knockout mice (CavKO) were employed. A phenotypic characteristic of CavKO mice is the complete lack of endothelial caveolae (24), what we also observed in this study (data not shown). We found that increases in WSS elicited dilations in wild-type but caused constrictions in coronary arteries of CavKO mice, which was converted to dilation by sepiapterin, in NO dependent manner (Figure 6c and 6d). There was no difference in ACh-induced coronary dilation in wild type and CavKO mice either in the absence or presence of sepiapterin, and the responses were only partially inhibited by L-NAME (Figure 6e). In the aorta of CavKO mice
administration of sepiapterin significantly enhanced relaxation to ACh; and the response was abolished by additional L-NAME, suggesting a greater contribution of NO to ACh response in the mouse aorta ((Figure 6c and 6d).
**Discussion**

This study demonstrates that in coronary resistance arteries of diabetic patients increased production of ONOO- targets and disrupts endothelial caveolae, which in turn contributes to uncoupling of eNOS and results in a diminished flow-mediated dilation (FMD). This conclusion is supported by key findings that 1) in coronary arterioles of DM patients ONOO- sequester restored, whereas in nonDM patients exogenous ONOO- reduced FMD. 2) 3-NT staining was localized at the endothelial plasma membrane in a close proximity to caveolin-1. 3) Coronary arterioles of DM patients exhibited reduced level of membrane-localized caveolin-1 and also a reduced number of endothelial caveolae. 4) The BH₄ precursor sepiapterin enhanced coronary FMD after pharmacological or genetic (caveolin-1 knockout mice) disruption of caveolae, and finally 5) sepiapterin restored coronary FMD in DM patients.

Solid line of evidence indicates that increased production of ONOO- plays an important role in the development of cardiovascular complications in DM (15). ONOO- is a powerful oxidizing agent that causes rapid depletion of sulfhydryl groups, causes DNA damage, protein nitration of aromatic amino acid residues in proteins, such as 3-NT (15). Cellular mechanisms to prevent the deleterious effect of ONOO- are not clearly defined (25). Previous studies have shown a significantly enhanced formation of 3-NT in the coronary endothelium in sepsis (26), in the coronary artery wall in human transplant coronary artery disease (27), and also in coronary vessels in animal model of DM (28). In this study we found an elevated superoxide anion and also increased 3-NT formation in coronary microvessels of DM patients. Given that the two patient populations exhibited similar co-morbidities it is likely that among various confounding factors DM and most likely high blood glucose concentration is one of the major contributors for enhanced ONOO- formation in patients undergoing heart surgery. Importantly, in this study we
found that ONOO-, in a reversible manner, diminishes NO-dependent FMD in coronary arterioles of DM patients.

Next, we aimed to elucidate ONOO- targeted subcellular mechanisms that are responsible for diminished NO availability in coronary arterioles of DM patients. Under physiological conditions ONOO- production, as detected by 3-NT, is restricted to the mitochondria of the vascular endothelium (16). WSS-dependent activation of eNOS, on the other hand, is primarily coupled to regulatory mechanisms at the endothelial plasma membrane, although some evidence indicates involvement of mitochondria in this process (29). Among others platelet endothelial cell adhesion molecule-1 (30; 31) and endothelial caveolae (18) located at the plasma membrane have been shown to mediate wall shear stress-dependent vasodilation in rodents. Given that ONOO- causes lipid peroxidation (15) we wondered whether ONOO- preferentially targets and interferes with vasoregulatory mechanisms intrinsic to the endothelial plasma membrane. In this study we found that 3-NT immunogold particles were more abundant in the plasma membrane of coronary endothelium in DM patients. We also demonstrated that 3-NT staining was in close proximity (less than 40 nm) to caveolin-1, the main scaffolding protein of caveolae. There are four tyrosine groups within caveolin-1 that can be potentially nitrated. Whether 3-nitration of caveolin-1 has any functional consequence is entirely unknown. To investigate possible effects of ONOO- and caveolin-1 interaction in DM, we measured caveolin-1 expression in coronary arteries and found no significant changes between DM and nonDM patients. Interestingly, immune-electronmicroscopy has revealed that the membrane-localized caveolin-1 is significantly reduced in the coronary endothelium of DM patients. The reduced level of membrane caveolin-1 was also confirmed in cultured human coronary endothelial cell that were exposed to high glucose concentrations for 24 hours. Based on these results we raised the possibility that alterations in the membrane-localized caveolin-1 content may influence the formation and/or
stability of endothelial caveolae in DM. Indeed, in this study we found that DM patients exhibited a significantly reduced number of coronary endothelial caveolae. We also demonstrated that short-term administration of exogenous ONOO- elicited a dose-dependent, significant caveolae loss. In line with this observation, an earlier study has shown that even a short-term myocardial ischemia, due to a temporary occlusion of coronary artery in anesthetized dogs, elicited a significant loss of caveolae in coronary vessels (32). A later study by Peterson et al showed that bovine aortic endothelial cells acutely exposed to a superoxide-generating napthoquinolinedione resulted in loss of endothelial cell caveolae (33). Collectively, we propose that a reduced membrane-localized caveolin-1 may lead to destabilization and/or disruption of endothelial caveolae in coronary arterioles of DM patients. We suggest that a direct interaction between ONOO- and cavelin-1 proteins is responsible for this pathology. Whether ONOO-induced, 3-nitration of caveolin-1 contributes to this process or only represents a ‘footprint’ of caveolin-1 targeting by ONOO- has yet to be elucidated.

How disruption of endothelial caveolae by ONOO- contributes to an impaired NO synthesis is not well understood. A study by Peterson et al demonstrated that caveolin-1, eNOS, and GTPCH-I, the rate-limiting enzyme for BH4 biosynthesis, all are localized in the endothelial caveolea (34). This and other studies indicated a crucial role for caveolae in compartmentalization of enzymes that are required for NO synthesis (35). It is also known that the interaction between caveolin-1 with eNOS is inhibitory (36-38). Loss of caveolae, through exposing endothelial cells to oxidized LDL, cyclosporine or mβCD has been shown to displace eNOS from plasma membrane, which is, however, associated with a diminished NO production (39-41). A study by Zhang et al showed that in endothelial cells the plasma membrane targeted eNOS, although was more efficient to produce NO when compared to the Golgi targeted eNOS, was more vulnerable for caveolae disruption by mβCD (42). Moreover, a recent elegant study
demonstrated that a noninhibitory mutant of the caveolin-1 scaffolding domain, without interfering with other functions of endogenous caveolin-1, such as to form caveolae, significantly enhanced eNOS-derived NO synthesis (43). These studies indicated that under normal condition the interaction between caveolin-1 and eNOS directs eNOS to the caveolae, and that this compartmentalization seems important for eNOS activation and NO production. Given that loss of caveolae structure/function may ultimately lead to an impaired NO synthesis. To furnish functional evidence for this scenario, in this study human coronary arterioles were acutely treated with mβCD to disrupt vascular caveolae. We found that mβCD completely abolished NO-dependent coronary FMD in nonDM patients. Correspondingly, we found that increases in flow induced coronary arterial constrictions in caveolin-1 knockout mice. Our data are the first, to our knowledge, to provide evidence for the crucial role for caveolae in mediating wall shear stress-induced dilation in human coronary arterioles.

The impaired flow-induced coronary dilation after caveolae disruption can be mediated by several mechanisms. Due to the observed close proximity of eNOS to GTPCH-I (34) we raised the possibility that the availability for eNOS cofactor, BH₄ is limited after caveolae disruption, which ultimately leads to eNOS uncoupling in DM patients (44; 45). Interestingly, in the caveolin-1 knockout mice the ratio of BH₄ to BH₂ is reduced in the myocardium, which leads to eNOS uncoupling and may contribute to the cardiopulmonary phenotype of these mice (46). In this study we demonstrated that in mβCD treated vessels the stable BH₄ precursor, sepiapterin partially restored dilation to flow in human coronary arterioles. Moreover, in mice lacking caveolae, we found that following sepiapterin administration flow-induced coronary dilation was restored to the control levels, whereas sepiapterin enhanced aortic relaxation. Importantly, the diminished FMD in coronary arterioles of DM patients was also augmented by sepiapterin administration. Collectively, these results are the first to demonstrate, that impairment in flow-
induced, NO-dependent coronary dilation in DM patients is due to eNOS uncoupling, which is likely to be mediated by ONOO- dependent disruption of endothelial caveolae. It should be noted that ONOO-’s influence on the function of eNOS might not be limited to disruption of coronary endothelial caveolae. It has been shown that ONOO- directly interacts and reduces the level of BH$_4$, as it has greater affinity for BH$_4$ than that of ascorbic acid and glutathione (47). Chen et al demonstrated that exposure of human eNOS to ONOO- resulted in a dose-dependent loss of activity with a marked destabilization of the eNOS dimer (48). They found that both free and eNOS-bound BH$_4$ were oxidized by ONOO-, however, full oxidation of eNOS protein-bound BH$_4$ required significantly higher ONOO- concentrations (48). Based on the presented data we also support the direct interaction between peroxynitrite and BH$_4$, which explains why peroxynitrite scavenger and sepiapterin fully restore coronary dilation in DM patients, in spite of having a reduced number of caveolae. Based on the results of this study we propose a novel ‘upstream’ mechanism by which ONOO- targets and disrupts coronary endothelial caveolae and that a disrupted endothelial caveolae predisposes BH$_4$ to ONOO- dependent oxidation in DM (Figure 6g). Restoring endothelial caveolae via ONOO- sequestration and/or treatment with a stable BH$_4$ precursor may facilitate NO production in the diseased coronary artery, a therapeutic strategy, which may prove beneficial to improve cardiovascular morbidity and mortality in DM.
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Table 1

Patient demographics, diseases, and medications

|                         | nonDM | DM    | P    |
|-------------------------|-------|-------|------|
| n                       | 37    | 41    |      |
| Male                    | 26    | 29    | 1.000|
| Age (y)                 | 65 ± 15 | 69 ± 10 | 0.180|
| Body weight (Kg)        | 83 ± 25 | 89 ± 18 | 0.282|
| BMI (Kg/m²)             | 28.8 ± 7.4 | 31.1 ± 6.1 | 0.135|
| Systolic Blood Pressure (mmHg) | 135 ± 25 | 136 ± 26 | 0.944|
| Diastolic Blood Pressure (mmHg) | 73 ± 12 | 71 ± 12 | 0.492|
| Serum glucose (mg/dL)   | 115 ± 35 | 157 ± 71 | 0.004*|

Underlying disease, n

|                         | nonDM | DM    | P    |
|-------------------------|-------|-------|------|
| Type 1 diabetes         | 0     | 2     | 0.494|
| Type 2 diabetes         | 0     | 39    | <0.0001*|
| Hypertension            | 26    | 35    | 0.189|
| Hyperlipidemia          | 15    | 21    | 0.498|
| Coronary artery disease | 23    | 30    | 0.474|
| Peripheral vascular disease | 2     | 5     | 0.438|
| Congestive Heart failure | 9     | 5     | 0.237|

Medications, n

|                         | nonDM | DM    | P    |
|-------------------------|-------|-------|------|
| Aspirin                 | 25    | 31    | 0.807|
| Lipid lowering          | 20    | 25    | 0.655|
| Insulin                 | 0     | 14    | <0.0001*|
| Drug Type                  | NonDM | DM | p-value |
|---------------------------|-------|----|---------|
| Oral antidiabetic         | 0     | 27 | <0.0001*|
| Beta blocker              | 23    | 29 | 0.636   |
| ACE inhibitor             | 11    | 15 | 0.636   |
| Diuretic                  | 10    | 16 | 0.344   |
| Anticoagulant             | 8     | 14 | 0.317   |
| Calcium channel blocker   | 8     | 10 | 1.000   |

| Surgical procedure, %    | NonDM | DM | p-value |
|--------------------------|-------|----|---------|
| CABG                     | 29    | 40 | 0.039*  |
| Valve replacement        | 8     | 4  | 0.209   |

n, number of nonDM and DM patients studied; For continues variables mean ± SD are shown. * indicate statistical difference. All categorical risk factors were examined by Fisher’s exact tests, while continuous variables were assess by Student’s t-test between the two patient groups. ACE, angiotensin-converting enzyme.
Table 2

Quantification of 3-NT and Cav-1 immunogold distribution

|                  | nonDM | DM    | P     |
|------------------|-------|-------|-------|
| **3-NT nanogold** |       |       |       |
| Total in endothelium/µm² | 11.0±1.4 | 42.4±9.4 | 0.077 |
| Luminal membrane/µm | 1.2±0.5  | 7.9±1.4 | 0.029* |
| Abluminal membrane/µm | 1.2±0.5  | 3.6±1.3 | 0.189 |
| Cytosol/µm² | 8.6±1.4  | 18.0±5.5 | 0.223 |
| Luminal/total (%) | 12±3   | 44±6   | 0.016* |
| Cytosol/total (%) | 77±6   | 39±5   | 0.007* |
| **Cav-1 nanogold** |       |       |       |
| Total in endothelium/µm² | 19.7±2.9 | 20.9±4.9 | 0.852 |
| Luminal membrane/µm | 4.0±0.4  | 2.0±0.5 | 0.042* |
| Abluminal membrane/µm | 1.6±0.5  | 2.7±0.6 | 0.217 |
| Cytosol/µm² | 9.3±1.8  | 13.4±3.2 | 0.330 |
| Luminal/total (%) | 38±4   | 15±1   | 0.023* |
| Cytosol/total (%) | 47±2   | 64±4   | 0.033* |

Data are number of immunogold particles/endothelial surface area (µm²) or number of particles on membrane/length (µm). In the case of 3-NT immunogold labeling a total of 117.5 µm (10 cells from 4 nonDM patients) and 127.5 µm (9 cells from 4 DM patients) membrane was analyzed. In the case of Cav-1 immunogold labeling a total of 214.1 µm (11 cells from 4 nonDM patients) and 184.7 µm (12 cells from 4 DM patients) membrane was analyzed. Data are mean ± SEM. * indicate statistical difference P<0.05 (Student’s t-test).
Figure Legends

**Figure 1.** (a) Step increases in inflow and outflow pressure (ΔP, 25, 50 and 90 cmH$_2$O) elicited increases in intraluminal flow (white circles) and resulted in increases in wall shear stress (WSS) (black circles). (b) Representative recordings and (c) summary data of changes in diameter of coronary arterioles isolated from patients without (nonDM, white circles, n=13) and with DM (black circles, n=16) in response to increases in WSS. (d) WSS-induced changes in diameter of coronary arterioles of DM (black circles, n=4) and nonDM patients (white circles, n=5) in the absence and presence of the nitric oxide synthase inhibitor NG-nitro-L-arginine methyl ester (L-NAME, white and black diamonds). (e) Changes in diameter of coronary arterioles isolated from nonDM (white circles, n=7) and DM (black circles, n=7) subjects in response to NO donor, sodium nitroprusside (SNP). Data are means ± SEM. * DM vs. nonDM patients. # nonDM vs. nonDM+L-NAME. P<0.05.

**Figure 2.** (a) Representative fluorescent images of DHE staining and (b) summary data of fluorescent intensities/nuclei area (arbitrary unit, AU) in coronary arteries of nonDM (n=4) and DM patients (n=3). DAPI: blue, DHE fluorescence: red, auto-fluorescence: green (at 488 nm). Purple color indicates overlap of blue nuclei and strong DHE red fluorescence. Scale bar: 50 µm. * DM vs. nonDM patients. # DM vs. DM+Tiron. P<0.05. (c) Representative Western immunoblot shows 3-NT formation in coronary arterioles of nonDM and DM patients. St: molecular weight standard. 3-NT-BSA was used for positive control and anti-β-actin was used for loading variations. (d-e) WSS-induced changes in diameter of coronary arterioles from patients with DM (black circles, n=5) in the presence of ONOO- scavengers, (d) iron-(III)-tetrakis(N-methyl-4'pyridyl)porphyrin-pentachloride (FeTMPyP, black triangles, n=5) or (e) uric
acid (black triangles, n=5), and also in the simultaneous presence of FeTMPyP or uric acid and L-NAME (back diamonds, n=4). (f) WSS-induced changes in diameter of coronary arterioles from nonDM patients (white circles, n=5) after bolus exposure to exogenous ONOO- (10 µM, white triangles, n=4). Data are means ± SEM. * indicate significant effects of various treatments. P<0.05.

Figure 3. (a) Electron micrograph illustration shows subcellular distribution of anti-3-NT immunogold particles (10 nm) in coronary arteriolar endothelium in nonDM (left) and DM patient (right). For detailed quantitative assessment of 3-NT nanogold distribution see Table 2. Black arrowheads indicate 3-NT localization in the close proximity to the luminal or abluminal plasma membrane, whereas white arrows point cytosolic localization. EC: endothelium, L: vessel lumen, R: red blood cell. (b) Representative immunohistochemistry images for co-localization of Cav-1 and 3-NT in coronary arterioles of nonDM and DM patients. Blue: DAPI (nuclei), red: Cav-1, green: 3-NT. Yellow color illustrates the overlap of red and green signal, white: auto-fluorescence (at 488 nm). Scale bar = 20 µm. (c) Summary data of Pearson’s correlation analysis for determining the degree of co-localization of Cav-1 and 3-NT. Multiple region-of-interests (data are from 26-26 region of interest from 3 nonDM and 3 DM patients) along endothelial cells were selected manually and a scatter plot of Cav-1 and 3-NT fluorescent intensities (normalized to the background adjacent to the region of interest) was generated. ImageJ (NIH) was used to determine pixel signal intensities for each region of interest. (d) Representative immunohistochemistry images of proximity ligation assay (PLA) for co-localization of Cav-1 and 3-NT in coronary endothelium of nonDM (upper panels) and DM patients (lower panels). (e) Summary data represents PLA positive signals obtained in 5 nonDM and 7 DM patients. 3 coronary vessels from each patients were analyzed for the number of PLA positive interactions.
(shown as red blobs) around the endothelial (ENDO) nuclei (DAPI shown in blue). Green: auto-fluorescence (at 488 nm). White arrowheads point PLA positive signals. Scale bar = 15 µm. Data are means ± SEM. * nonDM vs. DM patients. P<0.05.

**Figure 4.** (a) Representative Western immunoblot and summary data of densitometry analysis show Cav-1 expression in coronary arterioles of nonDM and DM patients (N=6-6). Anti-β-actin was used for normalizing loading variations. (b) Electron micrograph illustration shows subcellular distribution of anti-Cav-1 immunogold particles (10 nm) in coronary arteriolar endothelium in nonDM (upper) and DM patient (lower). For detailed quantitative assessment of Cav-1 nanogold distribution see Table 2. EC: endothelium, L: vessel lumen, RBC: red blood cell. (c-d) Representative Western immunoblot and summary data of densitometry analysis of 3 independent experiments show Cav-1 expression in total lysate (c) or membrane and cytosole fractions (d) of human coronary artery endothelial cells in the presence of normal glucose (NG, 5.5 mM) or after high glucose (HG, 25 mM) exposure. Anti-β-actin was used for normalizing loading variations. * NG vs. HG. P<0.05.

**Figure 5.** Electron microscopy for quantitative analysis of caveolae. (a) Scheme for analysis of the electron microscopy images: Endothelial cells (EC) were identified as those regions adjacent to red blood cells (RBC). VSMC: vascular smooth muscle cell (left). A photo editor was used to establish a threshold gate to assist in identifying caveolae in multiple regions of interest (ROI) (middle). Caveolae (black arrowhead) within endothelial cells were defined as apical or basal invaginations open to the surface, and membranes for analysis had to contain at least one caveolae (right). (b) Representative electron micrograph illustration of the coronary endothelium...
in nonDM (upper, white arrowheads point caveolea) and DM patients (lower, black arrowheads point caveolea). (c) Summary data show comparison of the number of endothelial membrane caveolae per µm² in nonDM (white circles, data are from 53 membrane regions from 3 patients) and DM patients (black circles, data from 46 membrane regions from 3 patients). (d) Electron micrograph illustration and (e) summary data demonstrates the effects of increasing concentrations (1 - 100 µM) of exogenous ONOO- on the number of coronary endothelial caveolae. Scale bar: 0.5 µm. Black arrows point caveolae. Data are means ± SEM. * nonDM vs. DM patients. # indicate significant effect of ONOO- treatment. P<0.05.

Figure 6. (a) WSS-induced changes in diameter of coronary arterioles of nonDM patients (white circles and dashed lines, n=5) and DM (black circles, and dashed lines, n=7) before and after incubation with sepiapterin (Sep, white and black up triangles in nonDM and DM, respectively), in the absence and presence of L-NAME (white and down triangles, in nonDM and DM, respectively). * nonDM vs. DM patients, # DM vs. Sep, $ nonDM or DM vs. Sep+NAME. P<0.05. (b) WSS-induced changes in diameter of coronary arterioles of nonDM patients (white circles and dashed lines, n=6) in the presence of mβCD (white diamonds, n=4) and in the simultaneous presence of mβCD and sepiapterin (Sep, white triangles, n=4). * nonDM vs. mβCD, # mβCD vs. mβCD+Sep. P<0.05. (c) Representative recordings and (d) summary data of changes in diameter of coronary arterioles isolated from wild type (WT, while circles, N=4) or Cav-1 knockout mice (CavKO, black circles, n=4) in response to increases in flow or (e) increasing concentrations of acetylcholine (ACh, 10⁻⁹ – 10⁻⁶ M) in the absence and presence of sepiapterin (Sep, white and black up triangles in WT and CavKO, respectively) or in the simultaneous presence of Sep plus L-NAME (white and black down triangles in WT and CavKO, respectively). * WT vs. CavKO, # CavKO vs. Sep, $ WT or CavKO vs. Sep+NAME.
P<0.05. (f) Percent changes in aortic relaxation in response to increasing concentration of acetylcholine (ACh, $10^{-9} - 10^{-6}$ M) in wild type (WT, white circles, n=8) and caveolin-1 knockout mice (CavKO, black circles, n=8), in the absence and presence of sepiapterin (Sep, white and black up triangles, in WT and CavKO, respectively) or in the simultaneous presence of Sep plus L-NAME (white and black down triangles in WT and CavKO, respectively). * CavKO vs. Sep, # WT or CavKO vs. Sep+NAME. Data are means ± SEM. (g) Schematic drawing illustrates the proposed novel mechanism by which ONOO- targets and disrupts coronary endothelial caveolae and that a disrupted caveolae predisposes eNOS cofactor, BH$_4$ to ONOO-dependent oxidation, resulting in a diminished WSS-induced, NO-mediated coronary dilation in DM.
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5
Figure 6