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Mitochondrial Regulation of Diabetic Vascular Disease: An Emerging Opportunity

Michael E. Widlansky[1] and **R. Blake Hill, PhD**[2]

[1]Department of Medicine, Division of Cardiovascular Medicine and Department of Pharmacology, Medical College of Wisconsin, Milwaukee, WI, USA

[2]Department of Biochemisty, Medical College of Wisconsin, Milwaukee, WI, USA

Abstract

Diabetes-related vascular complication rates remain unacceptably high despite guideline-based medical therapies that are significantly more effective in individuals without diabetes. This critical gap represents an opportunity for researchers and clinicians to collaborate on targeting mechanisms and pathways that specifically contribute to vascular pathology in patients with diabetes mellitus. Dysfunctional mitochondria producing excessive mitochondrial reactive oxygen species (mtROS) play a proximal cell-signaling role in the development of vascular endothelial dysfunction in the setting of diabetes. Targeting the mechanisms of production of mtROS or mtROS themselves represents an attractive method to reduce the prevalence and severity of diabetic vascular disease. This review focuses on the role of mitochondria in the development of diabetic vascular disease and current developments in methods to improve mitochondrial health to improve vascular outcomes in patients with DM.

Keywords

Diabetes; Endothelium/Vascular Type/Nitric Oxide; Coronary Artery Disease; Mitochondria

Introduction:

Public health interventions to reduce smoking and tradition anti-hypertensive and cholesterol-lowering pharmacological therapies have been successful in reducing the prevalence of atherosclerotic disease in the general population.[1] Unfortunately, these therapies have met with significantly less success in individuals with diabetes mellitus

Correspondence to: Michael E. Widlansky; R. Blake Hill.

Corresponding Author: Michael E. Widlansky, MD, MPH, FACC, FAHA, Associate Chief of Academic Affairs, Division of Cardiovascular Medicine, Associate Professor, Medicine, and Pharmacology, Medical College of Wisconsin, 8701 W Watertown Plank Road, Milwaukee, WI 53226, Phone: (414) 955-6737, Fax: (414) 955-6203, mwidlans@mcw.edu.

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(DM). Despite aggressive therapies, the risk of myocardial infarction or stroke in patients with diabetes is seven to ten time that in individuals without diabetes.[2] Also, diabetes-related microvascular diseases, including retinopathy, nephropathy, neuropathy, and limb ischemia, occur at a rate of twenty-five times that seen in non-diabetic individuals.[2] DM vascular disease remains a leading cause of CV disease, renal failure, amputations, and blindness in the US.[3–6] The results of intensive glycemic control trials have been overall disappointing concerning their impact on both macrovascular and end-organ microvascular diseases. Therefore, there remains a critical gap in our ability to prevent and ameliorate diabetic vascular disease, likely related to our limited understanding of unique mechanisms driving vascular disease in DM.

While the full range of mechanisms remains unclear, the development of clinically relevant vascular disease in patients both with and without DM begins with the development of phenotypical vascular endothelial dysfunction. The persistence and severity of endothelial dysfunction play a critical role in the development of initial manifestations of disease as well repeat events in those with established disease.[7,8] Abnormal systemic glucose levels critically contributes to impaired endothelial function in patients with both type 1 and type 2 DM.[9–11] However, the our understanding of the proximal regulators of vascular endothelial function in DM remains limited. In part due to the unique local environment in endothelial cells, endothelial mitochondria play a critical role in regulating vascular endothelial function at both local and systemic levels. In particular, in patients with DM, emerging evidence suggests a unique role for perturbed mitochondrial function in the early pathogenesis and eventual clinical manifestations of diabetic vascular disease.[12–15]

This review will focus on the emerging field connecting the form and function of endothelial cell mitochondria to the development of vascular endothelial dysfunction in DM. Also, this article will address potential methods for targeting mitochondria health to inhibit, reverse, and mitigate vascular disease in patients with both type 1 and type 2 DM.

Importance of the Vascular Endothelium

Wilhelm His Sr., a Swiss anatomist, first coined the term “endothelium” to identify the layer of cells lining the luminal surface of blood vessels.[16] Early theories of this layer of cells as an inert, smooth layer designed to maximize laminar blood flow have long since given way to ample data supporting a critical role for the vascular endothelium in regulating vascular health.

The functioning of the endothelium reflects overall vascular health. In conditions of health, the vascular endothelium expresses few to no inflammatory markers, balances prothrombotic and pro-fibrinolytic factors, and tends toward more robust vasodilation when challenged with autocrine, paracrine, and endocrine stimulators of vasodilation.[7]

While multiple factors produced by the endothelium regulate vasodilation, angiogenesis, and inflammation, Nobel-prize winning work established nitric oxide (NO) as a central regulator of vascular health. NO induces vasodilation by diffusing from the endothelium to adjacent smooth muscle cells in conduit and resistance arteries. NO subsequently activates soluble guanyl cyclase and directly acts on calcium-activated potassium channels.[7] NO also limits

the expression of endothelial cell adhesion molecules critical for the recruitment of mononuclear cells important in the early development of and progression of atherosclerosis. NO inhibits platelet-platelet interactions and the expression of PAI-1, preserving the balance of thrombotic and fibrinolytic forces. Reduced NO bioavailability is a central feature of impaired vascular endothelial function.[17]

In humans, investigators can readily measure endothelial function using *in vivo* methods that indirectly measure NO bioavailability by quantifying endothelium-dependent vasodilation.[7] A large body of studies with tens of thousands of human subjects strongly supports the validity of these measurements as both measurements of NO bioavailability and predictors of future cardiovascular events in humans both with and without prevalent atherosclerotic disease.[18] Endothelial dysfunction occurs before the development of atheromatous disease and therefore is an attractive target for therapies to prevent both primary and secondary cardiovascular events. Interventions known to reduce cardiovascular risk also improve endothelial function in humans, fueling this attractive concept.[7]

Summary:

- The vascular endothelium is a critical regulator of overall vascular health
- While multiple autocrine, paracrine, and endocrine factors regulate vascular health, NO produced by the endothelium is a critical regulator of vascular endothelial function.
- NO bioavailability is readily measureable and a loss of NO bioavailability is a central component in the development of vascular endothelial dysfunction, an early precursor of the development of atheromatous vascular disease.

Diabetic Vascular Disease and the Role of Endothelial Dysfunction:

DM vascular disease differs pathophysiologically and phenotypically from that seen in non-DM individuals. DM large and medium-sized arterial disease characterized by more aggressive and diffuse involvement of the arterial tree. The greater lipid and macrophage content of diabetic atherosclerotic plaques, combined with generally thinner fibrous caps and larger necrotic cores, increases their vulnerability to plaque rupture.[19–21] Diabetic coronary atherosclerosis is characterized by longer atherosclerotic lesions and more diffuse disease with greater distal vessel involvement.[21,22] From the microvascular perspective, both the range and severity of unique DM-related microvascular complications (nephropathy, retinopathy, neuropathy, small vessel peripheral arterial disease, and diabetic microvascular cardiomyopathy) are strongly tied to dysglycemia.[23,24]

The unique phenotype of DM atherosclerotic and microvascular disease strongly implicates the presence additional pro-atherosclerotic stimuli and mechanisms amplifying and accelerating vascular dysfunction that subsequently lead to clinically evident disease earlier in life than in individuals without DM. Seminal work in the early 1990's demonstrated the presence of impaired endothelium-dependent vasodilation in individuals with both type 1 and type 2 diabetes secondary to a loss of NO bioavailability.[9,10] Dysregulation of glucose levels in DM, embodied by episodic hyperglycemia, hypoglycemia (often occurring

secondary to therapies), and glycemic excursions, are critical to the development of acute, chronic, and acute-on-chronic endothelial dysfunction in DM.[9–11,13,25–29] Innovative work employing a localized forearm hyperglycemic clamp demonstrates that short-term exposure to hyperglycemia (six hours at approximately 300 mg/dL) impairs NO bioavailability and endothelium-dependent vasodilation. Both acute hyper- and hypoglycemic exposures rapidly impair human vascular endothelial function through increased oxidative stress, inflammation, and a loss of NO bioavailability in both healthy non-DM subjects and patients with DM. [25–30] Chronic exposures to abnormal glucose concentrations, including high variability in systemic glucose levels in the setting of poor glycemic control, strongly correlate with greater impairment of endothelial function and accelerated adverse vascular remodeling.[31,32] Repeated bouts of hypoglycemia and hyperglycemic rebound may be relatively more toxic to the vascular endothelium than hyperglycemia alone.[31,32]

In addition to abnormal glucose levels, elevated circulating free fatty acid (FFA) levels in DM induce systemic and local vascular inflammation and reduce NO bioavailability.[33–35] Infusing FFAs into healthy individuals to levels seen in type 2 DM patients leads to higher circulating levels of endothelial cell adhesion molecules, myeloperoxidase levels, and tissue plasminogen activator inhibitor levels- all suggesting the inducement of a dysfunctional endothelial phenotype.[36] FFA infusion into type 2 DM patients leads to impaired endothelium-dependent vasodilation, reduced NO bioavailability, and elevated blood pressure.[37] Elevated FFAs in DM are also associated with microvascular disease and obesity-related insulin resistance.[38]

Summary:

- Diabetic vascular disease has a unique, aggressive phenotype throughout the vascular tree that is less responsive to current therapies.
- Abnormal glucose and free fatty acid levels synonymous with DM mechanistically contribute to the development of endothelial dysfunction in both type 1 and type 2 diabetes

Mitochondrial ROS and the Origins of Endothelial Dysfunction in DM

While the exact origins of vascular endothelial dysfunction in the setting of DM remain incompletely elucidated, strong evidence implicates mitochondrial dysfunction is an early event in the development of endothelial dysfunction in both type 1 and type 2 DM.[15] Typically, mitochondrial dysfunction manifests with impaired energy production and increased mtROS production. In endothelial cells, ROS are produced from multiple sources, including NADPH oxidase, xanthine oxidase, and uncoupled endothelium-derived NO synthase. Mitochondria within endothelial cells are also a major source of cellular ROS production. *In vitro* data suggest superoxide generated by mitochondria accounts for 0.2–2% of cellular oxygen consumption leading to organelle concentrations of superoxide of between 10–200 pM.[39,40] While monoamine oxidase, TCA cycle enzymes such as α-ketoglutarate and aconitase produce superoxide,[41,42] the mitochondrial inner membrane-based electron transport chain (ETC) is responsible for the majority of mtROS production.[43]

The protein complexes of the electron transport chain and their specific roles in ROS production have previously been extensively reviewed.[44] The primary sources of mtROS from the ETC are complexes I and III.[45,46] SHC-transformic protein $p66^{shc}$, located in the intermembrane space, also passes electrons into the ETC and ultimately induces the production of superoxide.[42] The relative amounts of superoxide produced are dependent on multiple factors, including availability and concentration of substrate (e.g., glucose or free fatty acids), pH, local oxygen tension, and overall level of oxidative stress in the environment from all cellular sources.[45]

In endothelial cells, due in larger part to the unique nature of endothelium's metabolic demand, mtROS play a prominent role as cell-signaling molecules.[44] Vascular endothelial cells overwhelmingly rely on glycolysis rather than the TCA cycle for cellular ATP needs under all but lowest and non-physiological glucose supply states.[47] This allows endothelial cells the freedom to leverage the products of the ETC for cell signaling purposes. Mitochondrial ROS production occurs in coordination with other key sources of cellular ROS, including xanthine oxidase and NADPH oxidase, suggesting mtROS play a critical role in regulating the overall endothelial cell redox state.[48,49] While superoxide itself makes a relatively poor cell signaling molecule due to its short half-life and poor diffusion capacity, superoxide is readily converted to hydrogen peroxide in both the mitochondrial matrix (by manganese superoxide dismutase, MnSOD) and intermembrane space (by copper-zinc superoxide dismutase, CuZn SOD). The half-life of hydrogen peroxide is nearly 100 times that of superoxide. Hydrogen peroxide is non-polar and therefore more readily diffuses across membranes. The existence of aquaporin 8 on the inner mitochondrial membrane also likely facilitates the transport of hydrogen peroxide out of the mitochondrial matrix and into the cytoplasm.[50,51] Nitric oxide, present at higher concentrations in endothelial cells than other cell populations, not only stimulates mitochondrial biogenesis but also inhibits complexes I and IV of the ETC.[52–55] Therefore, unique to endothelial cells, changes in NO bioavailability will be reflect by changes in mtROS production.[13,44] States of reduced NO bioavailability that characterize vascular endothelial dysfunction lead to increased mtROS production.[13]

Strong evidence supports the paradigm that mtROS regulate endothelial function under pathological conditions and exposures. In human coronary resistance vessels, hydrogen peroxide derived from mitochondrial superoxide produced from the ETC is critically involved in regulating flow-induced dilation.[56,57] Additionally, acute hypertension induced by weightlifting by untrained individuals changes the primary vasodilator molecule in human subcutaneous resistance vessels in young healthy individuals from NO to hydrogen peroxide derived from the mitochondria.[58]

Specific to DM, initial groundbreaking work demonstrated that exposing cultured bovine aortic endothelial cells to high glucose concentrations results in excessive levels of mtROS and activation of endothelial inflammatory pathways via NF-κB. This process is completely abrogated by blockade of the complexes of the electron transport chain or over-expression MnSOD.[59] Further work demonstrated that mtROS induced by high glucose concentrations inhibit glyceraldehyde 3-phosphate dehydrogenase.[60] This process lead to the inhibition of glycolysis and subsequent build-up of glyceraldehyde-3-phosphate, fructose-6-phosphate,

and glucose. The build-up of these glycolysis products activates multiple mechanisms known to contribute to diabetic vascular complications (e.g., protein kinase C activation, AGE production, and sorbitol).[15] Additionally, exposure of cultured bovine aortic endothelial cells to high glucose triggers enhanced methylation of histone H3K4 and reduced methylation of H3K9. These epigenetic changes lead to chronic NF-κB activation and subsequent chronic endothelial inflammation with reduced NO bioavailability.[61,62] Pharmacological and molecular interventions to reduce mtROS block these epigenetic modifications.[61,62] These data suggest mtROS signaling mechanisms may be in part responsible for the “metabolic memory” of the vasculature that accounts for the delay in vascular benefits seen in some glycemic control trials in type 1 DM.[63]

Inherited attributes of mitochondrial function may play a role in the elevated vascular risk in patients with type 2 diabetes. The oxidative phosphorylation capacity of mitochondria in insulin-resistant offspring of patients with type 2 DM is approximately 30% lower than that observed in non-insulin resistant controls.[64] Mice with an endothelial-specific inducible NF-κB knockout have delayed onset of insulin resistance and hypertension in the setting of a Western diet.[65] Taken in the context the central role mtROS play in regulating endothelial NF-κB expression, these two studies suggest that a portion of the heritable component of type 2 diabetes and its vascular complications may be attributed to inherited variations in mitochondrial function.

Taken together, these data strongly support the concept that under normal conditions, mtROS production is critical to maintaining a healthy endothelium and vasculature while excessive production of mtROS is deleterious to endothelial health. This concept, known as mitohormesis, suggests that the risk of adverse events secondary to free radical production is hormetic, rather than linear, and that low-levels of mtROS favor health and longevity.[66] Further support that mitohormesis occurs in the vascular endothelium by studies demonstrating chronic anti-oxidant therapy with polyphenols reduces MnSOD expression and results in impaired endothelium-dependent vasodilation in mouse renal arteries compared to shorter term polyphenol-based antioxidant therapy.[67] Exercise bouts acutely increase ROS production that is at least in part from mitochondria and treatment with anti-oxidants abrogates the positive effects of exercise on insulin sensitive in humans.[68] However, excessive mtROS production driven by the metabolic abnormalities in both type 1 and type 2 diabetes adversely impact the vasculature. 12,61–63,69

Summary:

- The primary source of mtROS is the oxidative phosphorylation process, including its interaction with p66shc.
- Due to their unique attributes, endothelial cells are able to use mtROS as cell signaling molecules. Physiological levels of mtROS appear important for maintain endothelial health, while excessive mtROS impair endothelial function.
- Abnormal glucose levels in DM contribute to both acute and chronic impairments in endothelial function mechanistically through mtROS signaling and interactions at protein, genomic, and epigenomic levels.

Targeting Mitochondrial ROS to Improve Vascular Health in DM

Overall, these data suggest excessive mtROS production in the DM endothelium activates signaling pathways resulting in both acute impairment of vascular endothelial function as well as chronic changes in the endothelial phenotype resulting in a long-term increase of vascular inflammation and impairment endothelium-dependent vasodilation (Figure 1). These data further suggest therapeutic strategies designed to reduce excessive mtROS production may reduce vascular risk in individuals with both type 1 and type 2 DM in a highly targeted manner.

Multiple different strategies may be employed to reduce the overall mtROS concentrations in the endothelium. One obvious strategy is the use of mitochondrial-targeted antioxidants to reduce overall mtROS concentrations. Animal studies using oral administration of mitochondria-targeted antioxidants, including MitoQ, show promise through improving endothelium-dependent vasodilation and reversing age- and hypertension- associated endothelial dysfunction in carotid artery and aorta .[70,71] In addition, *in vitro* studies of human skeletal muscle arteries show that *in vitro* exposure to MitoQ reverses age-associated impairments in endothelium-dependent vasodilation by increasing NO production from eNOS.[72] Data from our lab and others demonstrates that the mitochondrial-targeted anti-oxidants, including a small pilot study of oral mitochondrial anti-oxidant supplementation in older adults without DM,[73] can reverse impaired endothelium-dependent vasodilation and improve NO bioavailability in conduit (brachial) and subcutaneous resistance vessels in humans with T2DM.[12,13,73] However, in general, clinical trials employing antioxidants therapies to improve outcomes in cardiovascular disease have been mostly disappointing and antioxidant therapies are not recommended for primary or secondary prevention of atherosclerotic diseases.[74,75] While past failures with non-targeted anti-oxidants do not preclude the possibility that mitochondrial-targeted will succeed, the prior failures cast doubt on the likelihood of success with targeted-antioxidant approach necessitating consideration of alternative strategies. The reasons for the lack of clinical efficacy compared to experimental efficacy remain unclear, but may in part relate to the inability of antioxidants to adequately reduce their targets prior to oxidant damage occurring due to unfavorable pharmacokinetics.[76] In addition, indiscriminate mtROS quenching with antioxidants could negatively impact the favorable effects of physiological levels of mtROS as described previously.

A promising alternative strategy targets proximal regulators of mtROS production that are of particular importance in DM. Potential targets include mitochondrial protein acetylation, mitochondrial membrane integrity and fluidity, mitochondrial dynamics, and inner mitochondrial membrane potential (Figure 2). [44,77] The following sections will review the data regarding these potential targets and the evidence of their potential to influence vascular health in DM.

Summary:

- Targeting excessive mtROS production is an attractive pathway for pharmacological interventions to reverse endothelial function in DM.

- Data from animal studies and a small human pilot study in healthy older humans without DM suggest mitochondrial-targeted antioxidants might be effective.
- The targeted anti-oxidant strategy may be limited due to interfering with mitohormesis and pharmacokinetic considerations

Targeting Mitochondrial Protein Modifications:

At a cellular level in DM, excess free fatty acids and glucose concentrations stress endothelial cells and drive increasing concentrations of the products of the TCA cycle, including acetyl-CoA and NADH.[78,79] Increased acetyl-CoA groups and elevated NADH concentrations relative to NAD^+, through both enzymatic and non-enzymatic pathways, induce mitochondrial protein acetylation.[80,81] Deacetylation enzymes, such as those in the sirtuin family, mitigate this process. While high-glucose driven acetylation likely occurs on a wide range of mitochondrial proteins, acetylation of $p66^{shc}$ induces increased mtROS production from the ETC.[82] In addition, elevated glucose levels downregulate histone deacetylase enzyme sirtuin1 (SIRT1), leading to increased $p66^{shc}$ expression.[83] Elevated $p66^{shc}$ also reduces expression of MnSOD.[84,85] Importantly, increased mtROS production in endothelial cells triggered by persistent, hyperglycemia-induced activation of $p66^{shc}$ plays a critical role in the chronic, sustained impairment of vascular dysfunction in aortas in a type 1 DM mouse model and human aortic endothelial cells despite a return to normal glycemic conditions.[86]

These data suggest interventions targeting increasing expression of SIRT1 or reducing $p66^{shc}$ expression would reduce mtROS production and improve vascular endothelial function. Resveratrol (3,5,4′-trihydroxy-trans-stilbene), a natural phenol found in the skin of grapes, blueberries, and raspberries, is an activator of SIRT1. *Ex vivo* animal data using a type 2 diabetic mouse model demonstrate resveratrol improves endothelium-dependent vasorelaxation, and Sirt1 overexpression improves NO bioavailability and aortic endothelial function in a mouse ApoE knockout model of atherosclerosis and human umbilical vein endothelial cells (HUVECs).[87,88] *In vitro* studies on HUVECs show that resveratrol treatment increases NO bioavailability through increased production from eNOS and attenuates vascular inflammation through a reduction in oxidative stress.[89,90] Resveratrol's favorable impact on NO bioavailability is likely in part due to direct activation of eNOS through deacetylation of lysines 496 and 506 on eNOS as well as indirect activation via AMP Kinase and FOXO.[91,92] Each of these activities of SIRT1 increase NO bioavailability and subsequently suppress mitochondrial ETC activity.[13]

However, the efficacy of resveratrol in humans thus far has been disappointing. One small human study of 30 insulin-resistant adults demonstrated improved vascular function by fingertip plethysmography.[93] However, in skeletal muscle biopsies on these subjects, nextgeneration RNA sequencing of failed to demonstrate a reduction in SHC1 mRNA transcripts responsible for $p66^{shc}$ expression.[93] In addition, while resveratrol activates SIRT1 in animal and cell culture studies, only limited data directly demonstrate resveratrol reduces $p66^{shc}$ levels and none of these data are derived from endothelial cells or human arterial tissues.[94] These data suggest the observed ameliorative effects of resveratrol in animals, in cell culture, and in human studies may be secondary to favorable off-target effects of

resveratrol and SIRT1 activation rather than a direct impact on $p66^{shc}$ itself. These favorable off-target effects may include resveratrol's favorable effects on glucose homeostasis in patients with DM.[95]

As an alternative to resveratrol, interventions to increase the mitochondrial NAD^+/NADH ratio (it is lowered by excessive substrate in DM) have the potential to activate sirtuins, including SIRT1, and reduce mitochondrial protein acetylation.[96,97] Nicotinamide mononucleotide (NMN) and nicotinamide riboside (NR, also known as vitamin B3) are NAD^+ precursors that could be given to increase the NAD^+/NADH ratio. The available data on NMN and NR supplementation in animal models were recently thoroughly reviewed.[98] NMN favorably impacts insulin sensitivity and glucose metabolism in type 2 diabetic and aging mouse models.[99] NR supplementation protects against high fat-induced obesity by increasing oxidative metabolism and reducing liver inflammation in type 2 diabetic and healthy mouse models.[100,101] While both NMN and NR are available as dietary supplements, only one study to date has tested either of these as an intervention to improve vascular endothelial function. NMN reverses carotid artery endothelial dysfunction and reduces age-associated vascular stiffness in a mouse model of aging.[102] In this study, NMN supplementation at 300 mg/kg dose resulted in reduced vascular oxidative stress, increased NAD^+ content, and increased MnSOD expression. [102]

While increasing NAD^+ to stimulate sirtuin activity and to reduce mtROS production appears to be biologically plausible mechanism to exploit for human translational studies based on the previously cited data, the method by which NAD^+ concentrations are increased, the relative tissue expressions of intermediaries, the potential off-target effects of the intervention, and the overall cellular metabolic state may impact the efficacy and safety of this strategy.[98] Nicotinamide phophoribosyltransferase (NAMPTNAMPT), the rate-limiting enzyme in the NAD^+ salvage pathway, has additional paracrine and endocrine activity. Produced by visceral adipose (including perivascular fat) and endothelial cells, NAMPTNAMPTplasma levels are elevated in individuals with DM, and NAMPT induces vascular inflammation.[103–108] Elevated circulating NAMPT levels correlate with impaired brachial artery endothelial function and have been shown to be elevated in the macrophages in unstable human atheromatous plaques.[109–111] Whether NAMPT has deleterious effects or ameliorative effects may depend on whether NAMPTNAMPT is in a monomeric (pro-inflammatory) or a dimeric (anti-inflammatory) form.[112,113] MicroRNA-34a targets NAMPT mRNA and recently discovered small molecule P7C3 activates NAMPT, suggesting two potential pharmacological approaches to increase NAD^+ through increased NAMPT expression or activity.[114–116]

Summary:

- Mitochondrial protein acetylation driven by high glucose exposure mechanistically contributes to endothelial dysfunction in DM. Acetylation of p66shc, downregulation of sirtuin activity, and nutrient excess driving a low NAD+/NADH ratio all contribute to this process.
- Resveratrol, a SIRT1 agonist, has shown some promise in cell culture and animal studies while human studies have been limited and less convincing.

- Interventions to increase NAD+/NADH ratio as increase sirtuin activity. While a single animal study has look used this strategy to improve carotid artery endothelial function, no human data is yet available testing the efficacy of this strategy.

Targeting Inner Mitochondrial Membrane-Based Cardiolipin

A unique aspect of the structure of mitochondrial membranes is the high content of cardiolipin (~20%) in the inner membrane which houses the proteins of the ETC.[117,118] Cardiolipin is composed of a glycerol head group with two phosphotidylglyceride backbones with four bound fatty acid chains of different lengths and saturation. Cardiolipin is synthesized and remodeled directly in the mitochondrial inner membrane.[119] Cardiolipin plays a critical role in multiple mitochondrial functions including the transport of proteins into mitochondria; mitochondrial calcium handling; assembly and stabilization of ETC complexes and super-complexes for efficient electron transfer; proper cristae formation; and mitochondrial fusion and fission.[119,120]

Damage to or loss of cardiolipin strongly induces mitochondrial structural changes that include more rounded mitochondria with less well-organized cristae as well as increased mtROS production. Aging and diabetes have both been shown to be associated with reduced inner membrane cardiolipin content leading to reduced ETC efficiency and increased mtROS production.[121–123] Owing to its close association with ETC components, cardiolipin is highly susceptible to oxidative damage.[124] Our prior work demonstrates that in both mononuclear cells and in the endothelium of patients with type 2 DM, mitochondrial membranes contain less cardiolipin and produce increased mtROS relative to healthy control subjects.[12,125] Also, we found that greater cellular cardiolipin content correlates with greater endothelium-dependent vasodilation of the brachial artery while endothelium-dependent vasodilation of the brachial artery was inversely associated with mitochondrial superoxide production.[12]

These data strongly suggest that a pharmacological strategy toincrease cardiolipin content and to reduce cardiolipin damage could improve vascular function in humans with DM. One strategy employed to stabilize cardiolipin content in mitochondria involves small mitochondrial penetrating peptides. Szeto-Schiller (SS) tetra-peptides, with alternating aromatic and basic residues and a lack of membrane potential dependence for mitochondrial uptake, selectively target the mitochondrial inner membrane and selectively bind to cardiolipin accounting their concentration in the inner membrane.[126] Therefore, SS peptides make an ideal vehicle for targeting cardiolipin stabilization and reducing mtROS production from the ETC.[127] SS peptides favorably impact the interaction of cytochrome c and cardiolipin to facilitate proper transfer of electrons from complex III to complex IV and reduce mtROS production.[116,126,128]

Evidence from animal studies suggests SS peptides could potentially be useful in reducing the risk of vascular disease in diabetes. Studies in ApoE knockout mice demonstrate that 12 weeks of therapy with SS-31 results in reduced atherosclerotic plaque formation, reduced vascular oxidative stress, and reduced vascular inflammation.[129] In type 1 DM rat and mouse models of retinopathy, administration of SS-31 blocked the development of

microvascular retinal disease.[130,131] In a banded aortic model of pressure overload, SS-31 reduced expression of proteins associated with pressure-overload.[132] However, human studies have shown limited efficacy to date, and no studies have been performed exclusively in patients with DM. SS-31, when given in a single, four-hour infusion resulted in reduced left ventricular end-systolic and end-diastolic volumes and was well tolerated in patients with heart failure with systolic dysfunction.[133] Acute administration of SS-31 to patients following an ST-elevation myocardial infarction did not result in any decrease in infarct size.[134]

Summary:

- Cardiolipin is a diphosphatidylglycerol lipid that is largely unique to the mitochondrial inner membrane and critical to normal physiological mitochondrial function. Due to its structure, it is easily damaged by mtROS.
- Cardiolipin is damaged and reduced in the endothelial and mononuclear cells of patients with type 2 DM and cardiolipin content correlates positively with endothelial function in human resistance vessels.
- Szeto-Schiller (SS) tetra-peptides stabilize cardiolipin and reduce mtROS levels but to date human data demonstrate little efficacy.

Targeting Mitochondrial Dynamics Proteins

Phenotypically, endothelial cell mitochondrial dysfunction is preceded by changes in the dynamic network of these organelles, which undergo constant fission and fusion with their long axes aligned along cytoskeletal tracks leading to changes in size, shape, distribution, and number.[135–138] The proper balance of mitochondrial fission and fusion is critical for controlling mitochondrial function, allowing mitochondria to move to areas of energy demand, to repair damaged mitochondria, and to isolate irreversibly damaged mitochondria for autophagy.[139–142]

Regulation of the fission and fusion processes involves collaborative and coordinated activities of multiple proteins. In human diabetes and during acute, abnormal glucose exposures, mitochondria become highly fragmented.[69,143] This fragmentation indicates a regulatory imbalance of over-active mitochondrial fission activity relative to fusion.[125,144] Our prior work in endothelial cells suggests increased activity and expression of a critical cytosolic mitochondrial fission protein, the GTPase dynamin-related protein 1 (Drp1), and overexpression of one of its mitochondrial docking proteins, Fis1, play mechanistic roles in mitochondria-related endothelial dysfunction.[69,143] Drp1 is central to normal physiological mitochondrial fission.[136] Upon activation, Drp1 translocates from the cytosol to the mitochondria, binds one of the docking proteins on the mitochondrial outer membrane (Fis1, Mff, MiD49, MiD51), and assembles into multimers forming helical rings on the outer membrane to initiate fission.[136,145] Of the multiple mitochondrial outer membrane docking proteins, Drp1 may preferentially binds Fis1 in the setting of cellular stressors, including high glucose, in cell culture.[146–149]

Hyperglycemia also drives over-expression of Drp1 and Fis1 in the endothelium.[144] Drp1 and Fis1 interact to drive mitochondrial fission leading to excessive mtROS production.[144,150] Molecular suppression of Drp1 or Fis1 blocks hyperglycemia's adverse impact cultured human aortic endothelial cells.[144] Similarly, under clinically relevant low glucose conditions, molecular inhibition of Drp1 using siRNA blocks low-glucose induced impairments in endotheliumdependent vasodilation of human subcutaneous arterioles by preserving NO bioavailability in human resistance arteries.[69] Poor glycemic control in DM also increases fission of leukocytebased mitochondrial networks and promotes leukocyte-endothelial cell adhesion, furthering the vascular inflammatory process.[151] Putative Drp1 inhibitor Mitochondrial Division Inhibitor 1 (Mdivi-1) similarly reverses low-glucose induced endothelial dysfunction and impaired endothelium-dependent vasodilation of subcutaneous resistance arterioles from humans with type 2 diabetes.[69,152] Interestingly, both resveratrol and the common diabetes drug metformin also appear to inhibit Drp1-mediated mitochondrial fission amongst their multiple effects.[153]

These data suggest the attractive hypothesis that inhibiting the interaction of Drp1 and Fis1 could reverse vascular endothelial dysfunction in patients with either type1 or type 2 DM. The relative specificity of Drp1 for Fis1 under cellular stress adds to the attractiveness by suggesting inhibiting this interaction may have limited potential for adverse off-target effects under healthy conditions. Our prior data support this hypothesis by showing both Drp1 siRNA and Mdivi1 have no appreciable impact on the endothelium-dependent vasodilation of subcutaneous resistance vessels from healthy humans.[69] However, given Drp1's critical importance normal physiological fission processes, strategies that focus primarily on Drp1 inhibition may be too toxic for development. For example, a Drp1 missense mutation in mice impairs Drp1 activity and causes severe cardiomyopathy.[154] Mdivi1's effects may also not be specific to Drp1 inhibition. Recent data suggests Mdivi1 inhibits mitochondrial complex I and is a relatively poor Drp1 inhibitor casting doubt on whether Drp1 inhibition would play a significant role in Mdivi1's *in vivo* effects.[155] Recently, a small peptide, P110, was developed to inhibit the Drp1-Fis1 interaction.[156] Use of P110 in a rat myocardial infarct model resulted in protection from ischemia-reperfusion injury and improved ventricular function three weeks following acute myocardial infarction.[157] However, P110 may also interfere with physiologically important mitochondrial fission, such as that which occurs during exercise adaptation.[158] Dynasore is an additional inhibitor of GTPase activity of Drp1 that has shown an ability to inhibit endothelin-1 induced artery constriction which may be of enhanced importance in the diabetic vasculature.[159] Further fundamental work remains for the development of highly selective Drp1-Fis1 inhibitor for testing with respect to its potential efficacy in the treatment of diabetic vascular disease.

Targeting proteins that stimulate mitochondrial fusion may also be a promising method for reducing pathological mitochondrial fission and mtROS production in the DM endothelium. The fusion enhancing protein Optic Atrophy Protein 1 (OPA1) plays an important role in maintaining normal cristae structure and therefore assembly of the complexes of the respiratory chain and the fidelity of oxidative phosphorylation.[160] Coronary endothelial cells from a type 1 diabetes mouse model show decreased OPA1 expression (and Drp1 overexpression), possibility contributing excessive mitochondrial fission and mtROS production.[161] Mitofusins 1 and 2 (Mfn1/2) are GTPase enzymes which also catalyze

mitochondrial fusion.[162] In cultured rat aortic endothelial cells, exposure to high glucose and high palmitate concentrations leads to mitochondrial fragmentation and increased mtROS production that is in part related to downregulation of Mfn2 activitythat occurs through reduced ubiquitination.[163] While no specific agonist of OPA1 is available,fish oil has been reported to increase OPA1 and Mfn2 expression in the aortas of ApoE knockout mice in association with a reduction in atherosclerotic burden.[164] Small molecule 15-oxospiramilactone activates Mfn2 through inhibition of deubiquitination, but there are no data to date specifically testing this agent in DM models or humans[165]

While promising, multiple questions will need to be addressed when approaching reducing diabetic vascular complications through manipulation of mitochondria dynamics. The notion that greater fusion is always beneficial does not appear to stand up to our current understanding of mitochondrial dynamics.[166,167] Additionally, physiological mitochondrial fission in response to exercise in certain cells, including myocardium, may represent a normal adaptation to increased energy demands and necessary for chronic adaptations to exercise.[158] The exact dynamics proteins related to this response, the relevance of these findings to the vascular endothelium where mitochondrial energy demands significantly differ from cardiac myocytes, and how this response might be modulated in the setting of energy excess states like DM remain to be determined. These data further suggest specific targeting of a mitochondrial dynamics protein by a pharmacological approach may also need to include more selective targeting to the endothelium to avoid potential negative off-target effects.

Summary:

- Intact mitochondrial dynamics are critical for the maintenance of normal vascular endothelial function.
- Increased Drp1 and Fis1 levels and interaction appear to contribute to endothelial function in humans resistance vessels from patients with type 2 diabetes and in normal vessels exposure to abnormal glucose levels.
- Specific agonists for these and other dynamics proteins are not yet available for human use and may require endothelium-specific targeting.

Targeting Inner Mitochondrial Membrane Potential

Early data demonstrate that reducing mitochondrial membrane potential magnitude ($\Delta\psi_m$) through using pharmacological agents to partially dissipate membrane potential or overexpressing mitochondrial uncoupling protein 2 (UCP2) located on the inner mitochondrial member reduces mtROS production in endothelial cells driven by high glucose exposure.[60]

Also, our group demonstrated that reducing $\Delta\psi_m$ magnitude in human vessels through partial mitochondrial uncoupling with either FCCP or dinitrophenol reverses impaired endotheliumdependent vasodilation in resistance arteries from patients with type 2 DM in an eNOS and NOdependent manner.[12] These data suggest that reducing hyperpolarization of

the inner membrane of endothelium-based mitochondria in humans with DM could be a viable strategy to reduce diabetic vascular complications.

While agents like FCCP and dinitrophenol are not viable therapeutic options due to their toxicities, upregulation of UCP2 or opening of other channels on the mitochondrial inner membrane, including the mitochondrial permeability transport pore (mPTP), represent potential novel targets. Transgenic overexpression of UCP2 is protective against atherosclerosis formation while murine UCP2 KO leads to exaggerated atheromatous disease.[168] Capsaicin (8methyl-N-vanillyl-6-nonenamide) activates transient receptor potential vanilloid 1 channels leading to increased UCP2 expression and reverses hyperglycemia-induced endothelial dysfunction in aortic and mesenteric arteries in insulin-resistant db/db mice.[6] Further studies are necessary to translate the impact and mechanisms of effect of capsaicin on DM endothelial dysfunction. Targeting mPTP is complicated at this time by limited understanding of its molecular structure.[77]

Summary:

- Partial depolarization of the mitochondrial inner membrane reverses endothelial dysfunction in resistance vessels from patients with type 2 diabetes and vessels exposed to abnormal glucose levels.
- Methods for safely partially depolarizing the mitochondrial inner membrane in humans remain elusive. Possible targets including UCP2 and mPTP.

Targeting Mitophagy:

Mitophagy describes the signaling cascade mitochondria are degraded by autophagy. In this mitochondrial quality control process, unhealthy mitochondria damaged by excessive mtROS production move toward the perinuclear zone of the cell where they will ultimately be broken down in lysosomes, leaving the cell with a healthier mitochondrial population. [169] This process is initiated and propogated by signaling cascades that include outer mitochondrial membrane associated proteins PTEN-induced kinase 1 (PINK1), Parker, Mfn2, and Nix1.[163,170]

Intact mitophagy appears to protect the vascular endothelium against the development of endothelial dysfunction and cell death secondary to metabolic insults such as seen type 1 and type 2 diabetes. Palmitate exposure induces PINK1 and Parkin expression in human aortic endothelial cells and large arteries of mice with type 1 diabetes, leading to increased mitophagy and protecting endothelial cells against cell death.[171] Overall, recent data suggest impaired mitophagy contributes to the development of diabetes-related vascular dysfunction, atherosclerosis, and cardiomyopathy.[172]

Based on these data, interventions that strengthen mitophagy in diabetes may hold promise to improve vascular health. Trehalose, a disaccharide that promotes autophagy, has been shown to improve endothelium dependent vasodilation in the carotid arteries of older rats and forearm resistance vessels of older healthy humans.[173,174] However, in the human study trehalose did not impact muscular (brachial artery) endothelium-dependent vasodilation and the amount of intake necessary to see effect resulted in weight gain. In addition, trehalose

several off-target effects and, as a sugar substitute, might alter activity and composition of the gut microbiome in non-advantageous ways.[175] Significant work remains to develop and test the efficacy of mitophagy enhancement as a strategy to improve vascular function in diabetes.

Summary:

- Intact mitophagy importantly protects overall endothelial health and appears to be impaired in diabetes.
- More specific, targeted interventions are likely necessary to best test this strategy for improving endothelial function in humans with DM.

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Abbreviations:

Δψm	Mitochondrial Membrane Potential
CuZn SODq	Copper-Zinc Superoxide Dismutase
DM	Diabetes Mellitus
Drp1	Dynamin-related protein 1
ETC	Electron Transport Chain
FFA	Free Fatty Acids
HUVECs	Human Umbilical Vein Endothelial Cells
Mdivi-1	Mitochondrial Division Inhibitor 1
Mfn1/2	Mitofusin 1/2
MnSOD	Manganese Superoxide Dismutase
mPTP	Mitochondrial Permeability Transition Pore
mtROS	Mitochondrial Reactive Oxygen Species
NMN	Nicotinamide mononucleotide
NO	Nitric Oxide
NR	Nicotinamide Riboside
OPA-1	Optic Atrophy Protein 1
SIRT1	Sirtuin-1
SS	Szeto-Schiller

UCP2 Uncoupling Protein 2

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Summary:

The vascular complications of DM remain a significant source of morbidity and mortality despite gains made with a general risk reduction approach with current therapies.

Moretargeted, DM-specific approaches to reduce the morbidity and mortality of diabetic vascular disease remain a high priority to more effectively reduce the vascular risk burden in this growing patient population. Ample evidence suggests mitochondrial dysfunction characterized by excessive mtROS drives deleterious changes in DM endothelial function both acutely and chronically through signaling effects that directly impact proteins critical to vascular regulation. Multiple current approaches to reducing DM-associated excessive mtROS are under active investigation, each with strengths and potential drawbacks. While many findings from cell culture and animal studies appear promising, favorable findings in non-human studies must be validated in human studies before further development. A greater understanding of the structure, function, and interaction of mitochondrial-specific targets has the potential to yield new therapies to advance our ability to reduce the morbidity and mortality associated with vascular disease in patients with either type 1 and type 2 DM.

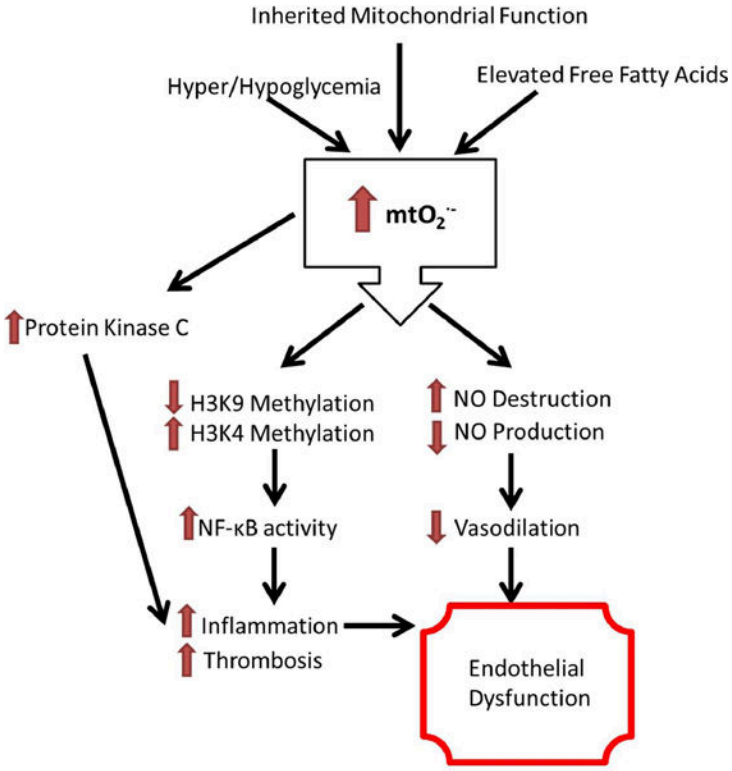

Figure 1: Induction of Vascular Endothelial Dysfunction in Diabetes Through Increased mitochondrial ROS production:

Abnormal glycemic levels, elevated free fatty acid levels, and genetic predisposition to suboptimal mitochondrial function drive excessive mitochondrial superoxide ($mtO_2^{\cdot-}$) production. Excessive $mtO_2^{\cdot-}$ directly reduces NO bioavailability both through direct reaction to make peroxynitrate as well as activating other ROS producing enzymes and uncoupling endothlelial NO synthase. In addition, $mtO_2^{\cdot-}$ activates protein kinase C and epigenetic changes leading to increased expression of the inflammatory transcription factor NF-κB. These changes lead to both acute and chronic impairments in vascular endothelial function in DM patients.

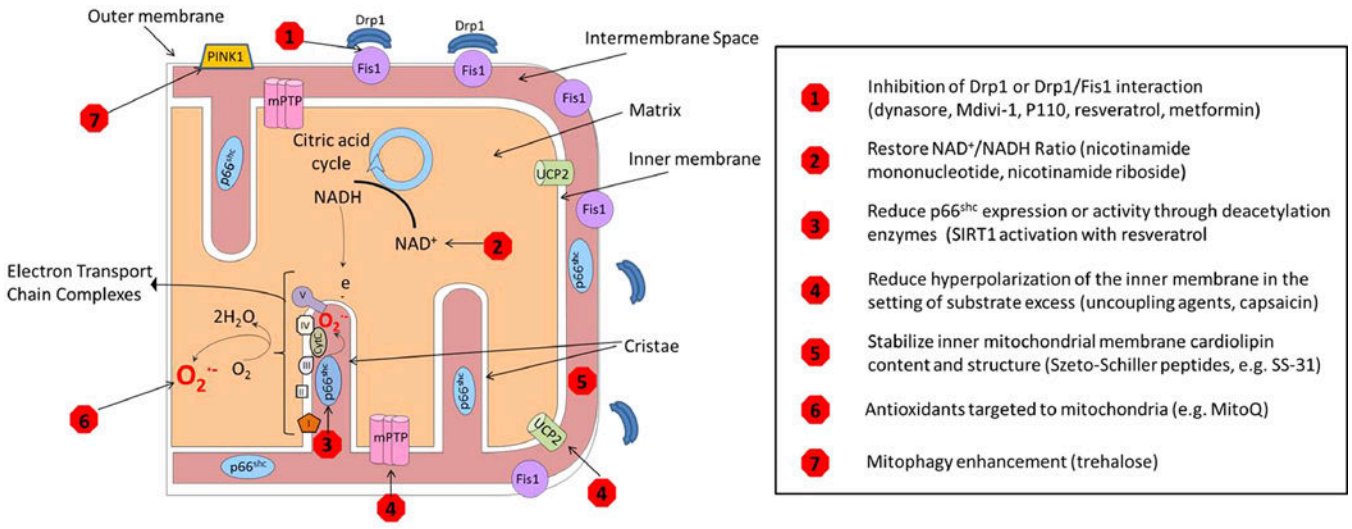

Figure 2: Potential Targets for Reduction of mitochondrial ROS production:

Reducing mtROS in patients with DM to improve vascular health could involve pharmacological interventions at multiple sites driving mtROS production in mitochondria. CytC- cytochrome c, Drp1- dynamin-related protein 1, mPTP- mitochondrial permeability transition pore, UCP2- mitochondrial uncoupling protein 2