Tackling endothelial dysfunction by modulating NOS uncoupling: new insights into its pathogenesis and therapeutic possibilities

Rinrada Kietadisorn, Rio P. Juni, and An L. Moens
Maastricht University Medical Centre, Cardiovascular Research Institute Maastricht, and Department of Cardiology, Maastricht, The Netherlands

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Kietadisorn R, Juni RP, Moens AL. Tackling endothelial dysfunction by modulating NOS uncoupling: new insights into its pathogenesis and therapeutic possibilities. Am J Physiol Endocrinol Metab 302: E481–E495, 2012. First published December 13, 2011; doi:10.1152/ajpendo.00540.2011.—Endothelial nitric oxide synthase (eNOS) serves as a critical enzyme in maintaining vascular pressure by producing nitric oxide (NO); hence, it has a crucial role in the regulation of endothelial function. The bioavailability of eNOS-derived NO is crucial for this function and might be affected at multiple levels. Uncoupling of eNOS, with subsequently less NO and more superoxide generation, is one of the major underlying causes of endothelial dysfunction found in atherosclerosis, diabetes, hypertension, cigarette smoking, hyperhomocysteinemia, and ischemia/reperfusion injury. Therefore, modulating eNOS uncoupling by stabilizing eNOS activity, enhancing its substrate, cofactors, and transcription, and reversing uncoupled eNOS are attractive therapeutic approaches to improve endothelial function. This review provides an extensive overview of the important role of eNOS uncoupling in the pathogenesis of endothelial dysfunction and the potential therapeutic interventions to modulate eNOS for tackling endothelial dysfunction.

endothelial dysfunction; nitric oxide synthase; nitric oxide synthase uncoupling; superoxide

THE ENDOTHELIAL LAYER OF BLOOD VESSELS is critical to vascular and myocardial health and plays an important role in the pathophysiology of hypertension and myocardial ischemia. It regulates the release of 1) endothelium-induced relaxing factors such as nitric oxide (NO) and endothelium-derived hyperpolarization factor (EDHF), 2) endothelium-derived contracting factors such as endothelin-1 and angiotensin, and 3) pro-inflammatory prothombolytic and 4) growth factors. Endothelial dysfunction is characterized by an impaired release of the aforementioned and has been defined as a blunting of the vasodilatory response to acetylcholine or hyperemia, both of which are known to produce NO-dependent vasodilatation. Disruption of the endothelium triggers a number of signaling cascades that converge on medial smooth muscle cells, which stimulates cell proliferation and migration and leads to pathological repair and development of neointimal hyperplasia (36). Endothelial dysfunction causes NO deficiency (80), which has been implicated in the underlying pathology of many cardiovascular diseases.

Endothelial nitric oxide synthase (eNOS) is the critical enzyme in the maintenance of vascular pressure by producing NO, a volatile gas that diffuses to the adjacent vascular smooth muscle. NO has a physiological role in the regulation of vascular tone, synaptic transmission, and cellular defense. In addition, it plays a major role in the relaxation of smooth muscle surrounding the arterioles (25) and maintaining vascular function by inhibition of vasoconstriction (220), platelet aggregation, leukocyte adhesion, and cell proliferation through the cGMP-dependent downstream signaling cascade. Moreover, NO limits oxidative phosphorylation in mitochondria (137). NO functions not only as a physiological regulator of cell respiration but also augments the generation of mitochondria-derived reactive oxygen species (ROS). The majority of ROS generation in the vasculature is derived from NADPH oxidases (NOX) and eNOS uncoupling. The latter takes place when oxidative stress oxidizes the fragile eNOS cofactor tetrahydrobiopterin (BH4).

This review gives an extensive overview of the role of eNOS uncoupling in the pathogenesis of endothelial dysfunction and how modulating eNOS uncoupling can tackle endothelial dysfunction.

Molecular Mechanism of eNOS Uncoupling

Evidence has shown that eNOS uncoupling is the underlying cause of endothelial dysfunction in animal experiments such as deoxycorticosterone acetate (DOCA)-salt hypertension (99), angiotensin II-induced hypertension (136), myocardial ischemia/reperfusion (I/R) injury (128), streptozotocin (STZ)-induced diabetes (73), as well as essential hypertension (71) and hypertension-induced heart failure (179). Importantly, uncoupling of eNOS in the pathogenesis of endothelial dysfunction in vascular disease states has been linked to the decrease of BH4 bioavailability due to enhanced oxidation of BH4. As an...
essential cofactor, BH4 is necessary for optimal eNOS activity (6). It facilitates NADPH-derived electron transferring from the eNOS reductase to the oxygenase domain to convert l-arginine to NO and l-citrulline. When BH4 levels are inadequate, eNOS becomes unstable and uncoupled, leading to subsequently less NO production and more superoxide generation. Moreover, the interaction between NO and superoxide leads to the formation of peroxynitrite, a potent oxidant, which further oxidizes BH4 (97).

BH4 is synthesized by the de novo or the salvage pathway. Through de novo pathway, BH4 is generated from guanosine-5′-triphosphate (GTP) via the rate-limiting enzyme GTP cyclohydrolase (GTPCH), 6-pyruvoyltetrahydropterin synthase, and sepiapterin reductase (164, 182). In the salvage pathway, BH4 is regenerated by its oxidized form 7,8-dihydrobiopterin (BH2) via dihydrofolate reductase (DHFR) or quinonoid dihydrobiopterin (qBH2) through dihydropteridine reductase (DHPR) (24, 182). Interestingly, BH2 can promote eNOS uncoupling because BH2, which has no eNOS cofactor property, can competitively replace eNOS-bound BH4 (33). In addition, the relative abundance of eNOS vs. BH4, together with the intracellular BH4:BH2 ratio, rather than absolute concentrations of BH4, is the key determinant of eNOS uncoupling (34). Recently, Crabtree et al. (32) demonstrated in BH4 deficiency hph-1 mice that DHFR plays a vital role in regulating the BH4:BH2 ratio and eNOS coupling in vivo, particularly when total biopterin availability is diminished.

The bioavailability of NO produced by eNOS might be affected at multiple levels, including 1) eNOS mRNA or protein expression, 2) availability of its substrate l-arginine, which might be competed by asymmetric dimethylarginine (ADMA), 3) availability of its cofactors, 4) protein-protein interaction such as caveolin-1 (cav-1) and heat shock protein 90 (Hsp90), 5) posttransitional modifications, and 6) reaction of NO with superoxide to yield peroxynitrite, which further reduce the bioavailability of BH4 (76). Although many studies have shown that eNOS overexpression generates vasoprotective effects by increasing endothelium-derived NO (86, 147), the expression of eNOS protein in endothelial dysfunction remains at normal or even, in some cases, increased levels (25). Indeed, elevated eNOS expression without further increase in BH4 levels, results in eNOS uncoupling because of the enzyme cofactor imbalance (13) (see Fig. 1).

The availability of the substrate l-arginine is required as the nitrogen donor for eNOS-derived NO. Although it is unlikely that plasma l-arginine levels would drop below the concentrations required for eNOS activity, decreased intracellular l-arginine caused by arginase may also lead to eNOS uncoupling. The expression of arginase in endothelial cells (ECs) can compete with eNOS for their common substrate (12) and regulating the BH4:BH2 ratio and eNOS coupling in vivo, particularly when total biopterin availability is diminished.

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Fig. 1. Central role of endothelial NO synthase (eNOS) uncoupling in the pathogenesis of endothelial dysfunction. eNOS is localized at the plasma membrane caveolae. In endothelial cells, eNOS is inactive when it is bonded with caveolin 1 (cav-1). When it becomes active, eNOS dissociates from cav-1 and binds with calmodulin (CAM) and heat shock protein 90 (Hsp90) and together with phosphorylation of serine sites (e.g., Ser1177). The functional eNOS protein is a dimer (so-called coupled eNOS). Tetrahydrobiopterin (BH4), an essential cofactor of eNOS, is necessary for optimal eNOS activity. BH4 facilitates NADPH-derived electron transferring from the eNOS reductase to the oxygenase domain to convert l-arginine to NO and l-citrulline. NO plays a major role in relaxation of smooth muscle surrounding arterioles and maintaining vascular function by inhibition of vasoconstriction, platelet aggregation, leukocyte adhesion, and cell proliferation through the cGMP-dependent downstream signaling cascade. Interaction between l-arginine and asymmetric dimethylarginine (ADMA; endogenous competitive inhibitor of NOS) is likely direct competition for eNOS. When availability of l-arginine or BH4 levels are inadequate, eNOS becomes unstable and uncoupled, leading to subsequently less NO production and more superoxide generation. Moreover, interaction between NO and superoxide leads to formation of peroxynitrite, a potent oxidant, which further oxidizes BH4, resulting in eNOS uncoupling as a vicious cycle, with subsequent endothelial dysfunction. Outside arrows indicate adjacent endothelial cells.
downregulate eNOS activity (217). Furthermore, studies show that oxidized low-density lipoprotein (OxLDL) is able to reduce endothelial L-arginine uptake, resulting in decreased local L-arginine and eNOS uncoupling (201). In addition, endogenous ADMA levels, a derivative of L-arginine, can act to competitively inhibit eNOS. The elevated levels of plasma ADMA are associated with oxidative stress within the vascular and development of endothelial dysfunction and cardiovascular diseases (1, 175).

Atherosclerosis-Induced eNOS Uncoupling

Endothelial dysfunction is considered to be an early marker for atherosclerosis, preceding angiographic or ultrasonic evidence of atherosclerotic plaque formation (117). Atherosclerosis starts with an innate immune response involving the recruitment and activation of monocytes/macrophages that respond to an excessive accumulation of modified lipids within the arterial wall, followed by an adaptive immune response (109). Wilcox et al. (213) demonstrated that atherosclerosis is associated with enhanced expression of all forms of NOS in the intima and adventitia. Furthermore, NOS-derived NO plays a dual role, as both anti- and proatherosclerotic effects are based on the course of disease progression. Endothelium-dependent vascular relaxation is impaired both in cholesterol-fed mice (92) and in isolated human coronary arteries (53), and this impairment is correlated with the degree of atherosclerosis (145). Administration of L-arginine (5, 18) or BH4 (65, 184) attenuates atherosclerotic lesion progression, whereas administration of NOS inhibitors blocks this protective effect (208), signifying a direct link between NO and atherosclerosis lesion formation. Recently, using an apoE<sup>-/-</sup> model of rapid atherosclerotic lesion formation after partial carotid ligation, Li et al. (108) demonstrated that NOS uncoupling and BH4 deficiency contribute to the vascular inflammation and abnormal cytokine milieu induced by disturbed flow without affecting systemic immune cells.

In apoE<sup>-/-</sup> mice, aortic superoxide production is diminished after administration of the NOS inhibitor L-NAME, suggesting that NOS is an important source of superoxide in this model (7, 214). In addition, BH4 deficiency is likely the major cause of eNOS uncoupling in atherosclerosis. apoE<sup>-/-</sup> mice overexpressing eNOS (apoE<sup>-/-</sup>/eNOS-Tg) showed eNOS dysfunction demonstrated by a decrease in NO production relative to eNOS expression, a marked reduction in vascular BH4, and an increase in superoxide formation, resulting in an acceleration of atherosclerotic lesion formation (146). In addition, oral BH4 administration (10 mg·kg<sup>-1·day</sup><sup>-1</sup>) to apoE<sup>-/-</sup>/eNOS-Tg mice reduced the formation of atherosclerotic lesion and vascular superoxide and increased eNOS-derived NO (146), indicating that the increase in eNOS expression alone, but not together with vascular BH4 levels, may result in eNOS uncoupling due to the stoichiometric relationships between endothelial BH4 and NOS activity (13). On the other hand, overexpression of GTPCH in apoE<sup>-/-</sup>/GTPCH-Tg mice is able to decrease endothelial superoxide production, increase aortic BH4 levels, and recouple eNOS. As a result, these double transgenic mice showed a significant decrease in aortic root atherosclerotic plaque compared with apoE<sup>-/-</sup> controls (7). Takaya et al. (178) demonstrated that, in the triple transgenic mice of eNOS- and GTPCH-overexpressing in apoE<sup>-/-</sup> (apoE<sup>-/-</sup>/eNOS-Tg/GTPCH-Tg), increases in vascular BH4 levels are associated with decreased eNOS-dependent superoxide production and results in reduced atherosclerotic plaque area. This study indicates that eNOS-derived superoxide plays a crucial role in atherosclerosis and that upregulation of GTPCH is able to restore eNOS function.

Elevated levels of C-reactive protein (CRP), a proinflammatory marker, have been also associated with atherosclerosis. Many data are evolving to suggest that CRP also promotes atherothrombosis (82, 149). Interestingly, studies in human endothelial cells showed that CRP, a proinflammatory marker, caused downregulation of eNOS activity and NO bioavailability (200, 205). Moreover, expression of human CRP in transgenic mice resulted in decreased eNOS activity (60, 126, 159). Singh et al. (167) demonstrated in human aortic endothelial cells (HAECs) that CRP inhibited GTPCH and stimulated NOX, leading to a decrease in BH4 and an increase in ROS levels. These resulted in uncoupling of eNOS and decreased eNOS activity, which was associated with decreased phosphorylation of Ser<sup>1777</sup> and decreased eNOS binding to Hsp90.

In healthy vasculatures, eNOS-derived NO prevents oxidative modification of low-density lipoprotein (LDL) (154). However, under oxidative stress, LDL is initially oxidized at the endothelial walls (38, 205a). OxLDL has been known to initiate the pathogenesis of atherosclerosis (169, 170). Moreover, OxLDL is also a potent inducer of superoxide; hence, it is a cause of oxidative stress (156). OxLDL increases synthesis of caveolin-1 (cav-1) (91) and inhibits the Akt survival pathways (26, 40, 56) attenuating eNOS activity with subsequent decreases in NO bioavailability.

During the inflammatory process in atherosclerosis, inducible NOS (iNOS) in endothelial cells and macrophages is upregulated and produces excessive amount of NO (20). Deletion of iNOS decreases plaque development in apoE<sup>-/-</sup> mice, indicating that iNOS does have proatherogenic effects (94). In addition, studies indicate that neuronal NOS (nNOS) also plays a role in protecting atherosclerotic plaque development in animal models (95, 190). This NOS isoform is upregulated in smooth muscle cells, macrophages, and endothelial cells in both early and advanced human atherosclerotic lesions (213). Recently, Seddon et al. (160) demonstrated in healthy humans that nNOS-derived NO is important for the control of blood pressure via regulation of basal vascular tone and blood flow, yet there are no data to indicate nNOS uncoupling is involved with atherosclerosis.

Diabetes Mellitus-Induced eNOS Uncoupling

Evidence indicates that endothelial dysfunction could play an initial and ultimately crucial role in the development of macrovascular and microvascular complications caused by diabetes mellitus (DM) in human and animal models of DM (197). Under hyperglycemic conditions, a hallmark of diabetes, eNOS-derived NO capacity is diminished (87). In vitro experiments have shown that protein kinase C (PKC)-mediated phosphorylation of eNOS protein may elicit the enzyme catalytic activity (74). Stimulation of endothelial cells with phorbol esters, direct activators of PKC (107), or glucose (30) elevates the expression of eNOS. Glucose also greatly enhances endothelial superoxide production (30), leading to increased vascu-
lar formation of the NO/superoxide reaction product peroxynitrite (93), which further promotes BH4 oxidation, with subsequent enhanced eNOS uncoupling (124).

Insulin is an essential hormone of metabolic homeostasis that has vasodilator action via PI3K/Akt pathway-dependent eNOS activation (138). In addition to the PI3K/Akt pathway, insulin can modulate eNOS activity by increasing BH4 synthesis via activation of GTPCH. Shinozaki et al. (165) have demonstrated that the GTPCH activity is significantly decreased in a rat model of insulin resistance, which leads to attenuation of endothelial BH4 levels and substantial increased BH2 levels, resulting in impairment of endothelium-dependent vasodilatation. Alteration of insulin-mediated vasodilatation has been associated with vascular insulin resistance, which is implicated in arterial hypertension and endothelial dysfunction. eNOS-derived NO regulates blood flow to insulin-sensitive tissues, including skeletal muscle (85), liver (161), and adipose tissue (85), and its activity is impaired in insulin-resistant individuals. An increasing body of evidence has shown the role of eNOS in regulating tissue sensitivity to insulin. An increase in systemic insulin resistance has been demonstrated in eNOS<sup>-/-</sup> mice that received a hyperinsulinemic euglycemic clamp (45, 162). Furthermore, a study in normotensive and nondiabetic individuals demonstrated that insulin resistance, measured by insulin-mediated glucose disposal, significantly correlated with increased plasma levels of ADMA, a potent endogenous NOS inhibitor (171). It was been demonstrated that NOS inhibition impaired microvascular recruitment and blunted muscle glucose uptake in response to insulin (206) indicating that eNOS plays a major role in the regulation of insulin sensitivity. Interestingly, an increase in eNOS expression is not always beneficial. It was demonstrated in Goto-Kakizaki rats, a model of insulin resistance, that elevated eNOS expression in aortic tissue was associated with increased superoxide production and decreased NO bioavailability. Incubation with l-NAME or deendothelialization of the aortic segments significantly decreased superoxide production.

Increased superoxide production in diabetes is not restricted to ECs and is demonstrably enhanced in the smooth muscle layer (116). Interestingly, adenosival transection of eNOS to diabetic vessels improved endothelial-dependent relaxation without altering superoxide production of vascular smooth muscle cells, an observation that may point to an important contribution of an impaired NOS function to endothelial dysfunction in diabetes.

Importantly, downregulation of DHFR has been observed in aortas of STZ-induced diabetic mice (143). As a key enzyme responsible for salvaging BH4, DHFR downregulation can result in BH4 deficiency and BH2 accumulation, both of which worsen eNOS uncoupling. Crabtree et al. (33) has demonstrated in hyperglycemic models of murine endothelial cells that eNOS uncoupling is due to the accumulation of BH2 in these cells. In addition, BH2 replaces BH4-eNOS binding and, hence, directly suppresses eNOS activity. This indicates that BH2-eNOS assembly does have a key role in the pathogenesis of diabetic vasculopathies. In STZ-diabetic rats, hyperglycemia-induced oxidative stress impairs the ability of dimethylarginine dimethylaminohydrolase (DDAH) to metabolize ADMA, leading to an elevation of ADMA and inhibition of endothelium-derived synthesis of NO (110).

Leo et al. (105) demonstrated in diabetic rats that hyperglycemia increased superoxide production, causing endothelial dysfunction, which was due to the impairment of both NO and EDHF-type relaxation. However, the degree of impairment in NO activity appears to be greater than the reduction in EDHF activity, as EDHF is able to elicit maximum relaxation when NO is inhibited, suggesting that NO is more susceptible to impairment by diabetes. Recent studies showed that attenuation of eNOS-derived NO in insulin-resistant C57BL/KsJ (diabetic, +db/+db) mice (98) and rat models of type 1 and type 2 DM (49) could be due to an impairment of eNOS activity through the augmented cav-1 expression, which further indicates that cav-1 plays an important role in cardiovascular complications in both types of diabetes.

Recently, Jo et al. (84) demonstrated in STZ-induced diabetic cardiomyopathy in eNOS<sup>-/-</sup>, iNOS<sup>-/-</sup>, nNOS<sup>-/-</sup>, and WT mice that iNOS uncoupling plays a major role in the pathophysiology of the diabetic heart. Although the oxidative/nitrosative stress markers, i.e., malondialdehyde (MDA), 4-hydroxynoneal (HNE), and nitrotyrosine (NT) are augmented in the diabetic heart, this increase in oxidative/nitrosative stress was significantly repressed in the iNOS<sup>-/-</sup> diabetic mouse heart. Importantly, oral administration of sepiapterin, a precursor of BH4 (10 mg·kg<sup>-1</sup>·day<sup>-1</sup> for 14 days) significantly increases myocardial BH4 in the control and diabetic hearts. However, a significant increase in the ratio BH4: BH2 is observed only in the diabetic heart and is associated with the inhibition of uncoupling NO, resulting in increasing iNOS-derived NO. Intriguingly, the absence of the increase in BH2 in the diabetic heart by administration of sepiapterin suggests that the salvage pathway of BH4 synthesis through DHFR is more activated in the diabetic heart than in the control heart. These findings may provide an important therapeutic implication in the treatment of NOS uncoupling-induced cardiovascular dysfunction by potentiating the salvage pathway of BH4 synthesis by sepiapterin administration. Furthermore, hyperglycemia significantly elicits the expression of iNOS both in in vitro (59, 218) and in vivo diabetic embryopathy mouse models associated with increased protein nitrosylation and enhanced NO and superoxide production, resulting in excess production of peroxynitrite, which stimulates the apoptosis pathway via c-Jun NH2-terminal kinase1/2 (JNK1/2) activation (218). Presently, no evidence demonstrates uncoupling of nNOS in DM.

**Hypertension-Induced eNOS Uncoupling**

It is broadly recognized that ROS contribute to the pathogenesis of hypertension. Increased levels of superoxide oxidize NO to peroxynitrite and subsequently to nitrite and nitrate. This results in a loss of “bioactive” NO-mediated vasodilatation, an increase in vasoconstriction, and subsequently an increase in systemic vascular resistance (64). Angiotensin II is known to play a major role in the initiation and progression of hypertension (122). It stimulates hypertension in part by ROS generation via eNOS uncoupling, leading to further increases in superoxide (24, 136). Furthermore, the reduction of NO caused by eNOS uncoupling promotes salt sensitivity and salt-induced hypertension (106). Landmesser et al. (99) demonstrated in DOCA salt hypertensive rats that uncoupled eNOS and NOX represent important sources of increased vascular ROS produc-
tion that ultimately oxidize vascular BH4. In addition, Takimoto et al. (179) and Moens et al. (134) revealed in a mouse model of transverse aortic constriction that eNOS uncoupling is a major source of superoxide in the pathogenesis of de novo and established pressure overload-induced heart failure. Compared with WT, eNOS−/− mice showed no cardiac hypertrophy, dilatation, and myocardial fibrosis because there was no detrimental eNOS-derived superoxide production.

Higashi et al. (71) demonstrated that BH4 augments endothelium-dependent vasodilatation in the forearm in normotensive as well as hypertensive individuals through the recoupling of eNOS and by increasing eNOS-derived NO production. Moreover, studies indicate that the use of NOS inhibitors with relative specificity for nNOS and iNOS (121, 155, 180) implicates reduced NO production by these two NOS isoforms as possible contributors to salt-induced hypertension. Indeed, nNOS-derived NO generation in autonomic efferent nerves participates in vasodilatation, blood flow increase, and hypotension (186, 187). SilToda et al. (166) demonstrated in a mild hypertensive mouse model (unilateral nephrectomy, with subcutaneous implantation of DOCA and saline feeding) increased cardiac oxidation, decreased NOS-derived NO generation and decreased cardiac BH4, resulting in NOS uncoupling. Interestingly, experiments of NOS inhibitors in this study revealed that nNOS is the largest contributor to cardiac superoxide production in diastolic dysfunction.

Smoking-Induced eNOS Uncoupling

Cigarette smoke (CS)-mediated oxidative stress downregulates eNOS levels, leading to reduced NO production and decreased endothelium-dependent vasodilatation in endothelial cells (48, 173). Edirisinghe et al. (48) revealed that CS-mediated downregulation of vascular endothelial growth factor receptor 2 expression and activation results in reduction of phosphorylated eNOS and total eNOS both in human lung microvascular endothelial cells and in mouse lungs. Decreased expression/activation levels of eNOS in response to CS have direct implications for endothelial functions such as cell migration, angiogenesis, and endothelium-dependent relaxation (47). Importantly, deficit of BH4 bioavailability caused by ROS scavengers in CS may lead to NOS uncoupling, at least in part, contributing to endothelial dysfunction in chronic smokers (69). In addition, CS has been shown to induce iNOS expression in pulmonary arteries (215). Furthermore, Lowe et al. (115) revealed that chemicals found in aqueous cigarette smoke extracts can directly affect eNOS by oxidizing BH4. Although both eNOS and nNOS are inhibited by cigarette smoke extracts, BH4 reactivates eNOS but not nNOS, whereas l-arginine protects nNOS but not eNOS. Thus, differential effects might be expected, and this may be the basis for isofrom-selective inhibition of nNOS over eNOS in penile tissue from rats treated with cigarette smoke (216).

Homocysteine-Induced eNOS Uncoupling

Increased plasma homocysteine (Hcy) has emerged as an independent risk factor for atherosclerosis and vascular disease (28). The pathophysiological roles of hyperhomocysteinemia (HHcy) in endothelial dysfunction are associated with increased thrombogenicity (46, 141), increased oxidative stress (191), overactivation of redox-sensitive inflammatory pathways (209), and atherogenesis (75). Uncoupling of eNOS is mainly responsible for Hcy-induced oxidative stress, because Hcy activates intracellular superoxide synthesis in human umbilical vein endothelial cells (HUVECs), and treatment with l-NAME markedly decreases superoxide generation, indicating that Hcy-induced oxidative stress is dependent mainly on NOS activity (188). In cultured porcine endothelial cells incubated with Hcy, it has been described that eNOS uncoupling contributes to Hcy-induced superoxide formation (42). The interaction between Hcy and eNOS uncoupling can be explained in several ways. First, Hcy elicits the cellular transport of l-arginine without altering eNOS activity, and eNOS generates superoxide rather than NO and forms peroxynitrite which later oxidizes BH4, leading to eNOS uncoupling (188). Third, studies with HUVECs have revealed that Hcy induces eNOS uncoupling through increasing superoxide and diminishing intracellular BH4 bioavailability (39, 188). Recently, He et al. (68) provided the first evidence that Hcy impairs coronary artery endothelial function. In addition, plasma levels of NO and BH4 are positively correlated and significantly decreased in patients with HHcy compared with controls. This suggests that the uncoupling of eNOS induced by HHCy in patients with chronic HHcy due to reduced BH4 levels may explain in part this adverse effect (88). Fourth, Hcy inhibits catabolic degradation activity of DDAH, causing ADMA accumulation; thus, it inhibits eNOS activity in vascular endothelium (172). Importantly, ADMA is associated with the increased eNOS uncoupling found in endothelium of patients with coronary artery disease (CAD) (10). Recently, Lemarie et al. (104) demonstrated in the endothelial progenitor cells (EPCs) of heterozygous methylenetetrahydrofolate reductase-deficient (Mthfr−/−) mice, which are mildly HHcy, and that ROS production is significantly increased and the eNOS dimer-to-monomer ratio significantly decreased compared with EPCs from wild-type mice, demonstrating eNOS uncoupling. This study indicates that eNOS uncoupling is a main cause of increased ROS formation in these mildly HHcy mice. Interestingly, the expression of sirtuin-1 (SIRT1), a NAD+-dependent protein deacetylase, is impaired at both mRNA and protein levels by Mthfr−/− mice. SIRT1 has been shown to exert protective effects against endothelial dysfunction by preventing stress-induced senescence (144) and, at vascular levels, to promote endothelium-dependent vasodilatation by deacetylating eNOS and increasing NO bioavailability (120). These results suggest that, in mildly HHcy mice, the uncoupling of eNOS increases ROS production, which leads to inhibition of SIRT1, then to premature senescence of EPCs, and thus eventually to endothelial dysfunction.

On the other hand, although there are no data showing that Hcy induces nNOS or iNOS uncoupling, Hcy has also been shown to upregulate iNOS, which may contribute to the inflammatory response that characterizes atherogenesis and may account for the adverse effects of Hcy (210). In addition, an increase in iNOS activity is a key contributor to a HHcy-mediated collagen/elastin switch and a resulting decline in aortic compliance (168).
I/R Injury-Induced eNOS Uncoupling

One of the events occurring in the early phase of I/R is vascular dysfunction associated with impaired endothelial function. ROS production has been implicated in endothelial damage, leading to endothelial dysfunction after I/R injury (176). ROS generation has been shown to be significantly increased during reperfusion. The largest increase in ROS level has been observed 15 min after reperfusion (15, 22, 89). This increase in reactive oxygen burst coincides with the development of endothelial dysfunction. Indeed, while 90 or 120 min of ischemia did not change vascular reactivity, vascular response to acetylcholine (Ach) was attenuated as early as 2.5 min after the onset of reperfusion (67, 189). A reduction in endothelial-dependent vasorelaxation after I/R injury has been associated with a decrease in NO bioavailability due to alteration of eNOS. Furthermore, endothelial-dependent vasorelaxation in response to serotonin was also impaired in pig carotid artery exposed to I/R, which was again improved with BH4 supplementation (185).

Several ROS generators during I/R injury have been determined. These include mitochondria (27), xanthine oxidase (3), NOX (51), neutrophils (212), and eNOS (131). eNOS uncoupling has been demonstrated to have a significant role in the occurrence of tissue damage after I/R injury. In the early phase of cardiac ischemia, there is an increase in Ca^{2+} uptake by cardiomyocytes and endothelial cells, leading to activation of eNOS with subsequent rapid and short-lived aggravation of NO concentration. This is followed not only by rapid consumption of L-arginine (the substrate of the enzymes) but also BH4 (the cofactor), triggering eNOS uncoupling and a decrease in NO bioavailability, which has been shown especially in the onset of reperfusion (61, 77).

It has been demonstrated that isolated rat hearts subjected to ischemia showed reduced BH4 levels with subsequent decreased eNOS activity and increased superoxide production (43). BH4 supplementation could restore impaired epicardial coronary epithelial function; moreover, it attenuated lipid peroxidation and improved cardiac functional recovery in rat models of global cardiac I/R (204). In accordance with that, impaired coronary flow during reperfusion phase could be improved by BH4 infusion, which was associated with increased eNOS activity and decreased NOS-dependent superoxide production (44). Moreover, a study in rat femoral artery showed that BH4 increased NO production and decreased H_{2}O_{2} release after I/R (152).

In addition to BH4, Hsp90, another posttranslational modulator of eNOS, has also been implicated in I/R injury. Hsp90 was proposed to prevent eNOS uncoupling and eNOS-derived superoxide production after chronic myocardial ischemia (163). It has been demonstrated that an Hsp90-transfected pig model of myocardial I/R showed reduction in infarct size and improved myocardial function. This effect was abrogated by administration of L-NAME and was associated with the ability of Hsp90 to act as an adaptor for Akt and phosphatase calcineurin, thereby promoting eNOS Ser^{177} phosphorylation and Thr^{495} dephosphorylation (96). All these findings enhance the essential role of eNOS in I/R injury and provide evidence for the potential of this protein as a therapeutic target.

Atrial Fibrillation-Induced eNOS Uncoupling

The association of eNOS and atrial fibrillation (AF) has been derived from the study of Minamino et al. (125), showing significantly reduced plasma levels of nitrite and nitrate and platelet cGMP in patients with AF compared with patients with sinus rhythm. Furthermore, Takahashi et al. (177) demonstrated that, compared with control patients with sinus rhythm, the increase of Ach-induced forearm blood flow was considerably smaller in patients with AF and was improved after cardioversion to sinus rhythm. These two studies proposed a correlation between AF and endothelial dysfunction with eNOS as a link. Altered hemodynamic condition has been implicated as an underlying mechanism that translates AF to endothelial dysfunction (23, 177). Cai et al. (23) suggested that decreased endocardial eNOS expression in a dog model of AF was due to turbulent blood flow occurring during AF. In an in vitro setting, it was demonstrated that HUVECs produce lower levels of NO when exposed to turbulent flow compared with when in laminar flow conditions. An increase in NO generation after laminar flow exposure appeared to be due to an upregulation of eNOS expression (142). Regulation of eNOS expression and NO generation by shear stress involves the opening of ion channels followed by eNOS protein phosphorylation and gene upregulation, leading to an increase in NO bioavailability (194). Although experimental animal studies have shown reduced eNOS expression in fibrillating atria (23, 58), a human study showed no difference in eNOS gene and protein expression in the atrium of AF and sinus rhythm (SR) patients (21).

In addition to eNOS, iNOS has been implicated in AF. Han et al. (63) reported an increased level of NO in the right atrium of AF patients, with subsequent generation of peroxynitrite, which was associated with an increase in iNOS expression. In addition, Nishijima et al. (140) reported increased expression of iNOS in a dog model of heart failure-induced AF. Despite some concerns about the methodology used (35, 62), the authors demonstrated that increased expression of iNOS in heart failure (HF) is accompanied by low NO and high superoxide production, which can be reversed by administration of BH4, suggesting the occurrence of iNOS uncoupling. Furthermore, 6-wk oral administration of 50 mg BH4 plus 3 g L-arginine twice daily reduced inducible AF and normalized heart failure-induced shortening of the left atrial myocyte action potential duration in this model (140).

Investigating the source of superoxide in the atrium of patients with AF, Kim et al. (90) demonstrated that gp91phox containing NOX in atrial myocytes was the main source of atrial superoxide production in SR and in AF patients. NOX was shown to contribute significantly to superoxide generation in fibrillating atria but not in patients with sinus rhythm, suggesting that increased levels of NOX-dependent oxidative stress induce eNOS uncoupling with a consequence of increased production of superoxide. Moreover, Reilly et al. (153) demonstrated that different atrial sources of ROS varied with the duration and substrate of AF. The authors reported that NOX was the main source of superoxide production in the left atrium of goats after 2 wk of AF and in patients who developed postoperative AF. Conversely, after 6 mo of AF, NOX showed no production of superoxide. Instead, NOS was the main source of oxidative stress in the presence of this long-standing AF. Interestingly, NOS-dependent superoxide production was

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associated with decreased BH4 level and BH4: BH2 ratio, suggesting that NOX-dependent superoxide generation during early periods of AF induces oxidation of BH4 as a cofactor of NOS leading to uncoupling of this enzyme. These results imply that AF treatments may be differentiated according to duration and substrate of AF. Whether agents that modulate eNOS will be able to alter AF in patients with atrial structural remodeling remain to be elucidated.

Endothelial Dysfunction and Endothelial Progenitor Cells

EPCs are circulating cells with the ability to differentiate into mature endothelium and have a role in endothelial repair and maintenance. Under certain pathophysiological conditions, for instance in CAD patients, this process seems to be blunted, resulting in reduced levels and migratory capacity of EPCs for neovascularization of ischemic tissue (198). In addition, both NO and oxidative stress, and in particular the balance between them, regulate the number and function of EPCs by direct and indirect mechanisms (52). Increasing evidence suggests that a damaged endothelial lining can be restored by circulating EPCs derived from bone marrow (41, 157, 207). A decrease in the number and function of EPCs has been associated with a large number of risk factors for atherosclerosis (112, 123). There is strong evidence that EPC-expressed eNOS is regulated under various physiological and pathophysiological conditions. Moreover, compounds or molecules that increase eNOS expression improve EPC function, whereas eNOS inhibitory substances have deleterious effects (183). By upregulating eNOS expression of EPCs, statins, estrogen, and erythropoietin (EPO) could enhance reendothelialization or augment neovascularization (37, 81, 100, 192). On the contrary, OxLDL or CRP attenuates EPCs survival, differentiation, and function by reducing eNOS expression of EPCs (118, 203).

It has been demonstrated that several substances affect the number and function of EPCs via an Akt/eNOS-related pathway. Indeed, the PI3K/Akt pathway is vital for regulating EPC recruitment, mobilization, and proliferation (139). Compounds that stimulate the PI3K/Akt protein kinase pathway can also activate eNOS (56). This association between eNOS and EPC count and activity appears to be crucial, because the expression of eNOS is essential for the mobilization of stem and progenitor cells (4), and perturbations in the PI3K/Akt/eNOS/NO signaling pathway or one of its members may result in EPC dysfunction (62). Recently, Cui et al. (36) demonstrated that transplantation of EPCs overexpressing eNOS can repair balloon-caused carotid artery injury in a rat model by inhibiting neointimal hyperplasia and restoring vascular function.

Diagnosis of Endothelial Dysfunction

The ability to detect endothelial dysfunction before overt cardiovascular disease manifests makes the diagnostic modalities attractive clinical tools for prevention and rehabilitation (103). In humans, endothelium-derived vasodilatation can be assessed by measuring increases in the diameter of large arteries (forearm or coronary) after 1) release of ischemia (e.g., arrested forearm circulation), 2) intra-arterial infusion of Ach, or 3) a sudden blood pressure elevation by placing the hands in ice water (cold pressure test). These measurements are based on the fact that conduit vessels can respond to alterations in blood flow by increasing vessel diameter via an endothelial-dependent mechanism (8, 150). These responses have been shown to reflect local bioactivity of endothelium-dependent NO generation and NO inactivation. These measurements are very sensitive to external conditions (room temperature, caffeine intake, etc.); hence, these measurements need to be performed under standard conditions (130) (see Fig. 2).

Fig. 2. Potential therapeutic options for treating endothelial dysfunction by modulating eNOS. The most straight-forward way to modulate eNOS is administration of its substrate L-arginine or its essential cofactor BH4 or BH4 analogs. Folic acid (FA) can modulate eNOS by improving BH4 bioavailability in the vasculature by preventing its oxidation, enhancing regeneration of BH4 from BH2, and chemically stabilizing BH4. Novel small molecules AVE9488 and AVE3085 are eNOS transcription enhancers. Statins can improve endothelial function by increasing eNOS stability and inhibiting NADPH oxidase upregulation. Also, statins can increase BH4 levels in vascular endothelial cells by potentiating GTPCH gene expression and BH4 synthesis.
Quantitative coronary angiography (QCA) measures the changes in the diameters of the coronary arteries in baseline conditions compared with vasodilatation induced by endothelial-dependent drugs. More recently, noninvasive QCA has been developed using computed tomography imaging (78) or magnetic resonance imaging (MRI) (181).

**Therapeutic Effects of eNOS Modulators**

**L-Arginine.** L-Arginine, a semiessential amino acid found in large quantities in fish, chicken, and beans, is the substrate for the production of NO. L-Arginine deficiency or the presence of its endogenous inhibitors, i.e., ADMA, may lead to eNOS uncoupling (174). L-Arginine activates oxygen uptake by eNOS by electron interaction with heme-bound oxygen (14). In addition, L-arginine may be able to restore the physiological status by normalizing the extracellular L-arginine:ADMA ratio (17). Although L-arginine improves both endothelium-dependent vasodilation and abnormal interactions of vascular cells, platelets, and monocytes, clinical studies with L-arginine have shown inconsistent effects on endothelial function. Acute and subacute L-arginine administration improves NO-dependent vasodilatation in a study of healthy elderly individuals (16) and patients with hypercholesterolemia (29) and coronary artery disease (2). On the other hand, chronic administration may not show the beneficial effect of L-arginine. The VINTAGE MI study demonstrated that 6 mo of oral L-arginine administration (3 g 3 times a day) does not improve clinical outcome and possibly increases risk of death in older patients with CAD (158). In addition, L-arginine supplementation also leads to an increase in Hcy production, which can result in worsening, not improving, endothelial function and atherosclerosis (114). Oral administration of L-arginine (9 g/day) to healthy postmenopausal women for 1 mo has no affect on major endocrine hormones or lipid profile. Although L-arginine could be the key to future treatment of cardiovascular disorders, it has not been possible to draw any general conclusion supporting the use of L-arginine for improving the clinical treatment in patients with endothelial dysfunction. In addition, it is not completely clear which types of endothelial dysfunction are related to L-arginine deficiency.

**BH4.** Since uncoupled eNOS can increase the production of ROS, promote BH4 oxidation, and self-limit its NO biosynthesis, modulating eNOS uncoupling is an attractive therapeutic approach in endothelial dysfunction. The most straightforward way to modulate eNOS is administration of its essential cofactor BH4 (133).

BH4 is an FDA-approved therapy for some forms of phenylketonuria (PKU), in which there is a deficiency in the hepatic enzyme phenylalanine hydroxylase. Supplementing with BH4 increases NO production by uncoupling eNOS in mice with hypertension-induced heart failure with subsequent reversal of cardiac hypertrophy and fibrosis (134). In vitro administration of BH4, or its physiological precursor sepiapterin, restored endothelium-dependent relaxation of resistance arteries in diabetic db/db mice (148), atherosclerotic vessels of humans and pigs (184), and patients with endothelial dysfunction (70, 72). Chronic oral administration of BH4 has been reported to improve endothelium function (31, 99), decrease vascular superoxide production, and increase vascular NO production. As a result, it blunts the increase in blood pressure in a hypertensive mice model (99). Recently, Li et al. (108) demonstrated in apoE−/− mice by using partial carotid ligation that oral BH4 supplementation in drinking water (10 mg·kg−1·day−1 for 1 wk) prevents NOS uncoupling and improves endothelial function associated with diminished monocyte adhesion and T-cell activation as well as blunted cytokine production from the vessel wall. In addition, short-term administration of BH4 (intra-arterial infusion 500 µg/min) improves endothelial-dependent vasodilatation to ACh in type 2 diabetes (70). Porkert et al. (151) demonstrated in subjects with poorly controlled hypertension that orally administering (400 mg or higher) BH4 daily has a significant and sustained antihypertensive effect. Importantly, this effect is associated with reversing the uncoupling of NOS and improving NO bioavailability (162). Although studies suggest that BH4 administration shows beneficial results for endothelial dysfunction, it may have limited long-term benefit in improving NO bioavailability (165).

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**Folic acid.** Folic acid (FA) is a chemically stable and inexpensive vitamin (Vit B9), which has direct and indirect superoxide scavenging effects. Although the best-known biological function of FA is to reduce plasma Hcy, the major effects of FA in the cardiovascular system are independent of Hcy lowering (135). Moat et al. (127) demonstrated in patients with CAD that FA dose-dependently improves endothelial function through a mechanism independent of Hcy lowering. Clinically, FA and 5-methyltetrahydrofolate (5-MTHF), the active form of FA, have been shown to restore endothelial function in patients with hypercholesterolemia (202), diabetes (195, 196), and atherosclerosis (11). FA increases the vascular bioavailability of BH4 and subsequently reduces eNOS-derived superoxide generation (11). FA restores the function of uncoupled eNOS by improving BH4 bioavailability in the vasculature by preventing its oxidation, enhancing the regen-
eration of BH4 from BH2, and chemically stabilizing BH4 (129, 134). Furthermore, Hyndman et al. (79) demonstrated that 5-MTHF is capable of binding the active site of NOS and mimicking the orientation of BH4. In addition, van Etten et al. (195) demonstrated that administration of 5-MTHF ameliorates endothelial dysfunction found in patients with type 2 diabetes by restoring impaired NO-mediated vasodilatation. Moreover, administration of FA improves EPC function by normalizing gene expression profiles in type 1 diabetes patients (196). However, recent clinical trials have failed to demonstrate a benefit of long-term use of FA in lowering the risk of recurrent cardiovascular disease or death after an acute myocardial infarction (19, 111), which can be explained by too low of a dosage (113). Therefore, an important distinction must be made between FA as a long-term, low-dose fortification or dietary supplement and as a short-term, high-dose treatment.

*eNOS transcription enhancers.* The novel small molecules AVE9488 and AVE3085 are eNOS transcription enhancers. AVE9488 has proven in vivo effects improving left ventricular remodeling in a rat model of myocardial infarction (54) and a mouse model of cardiac I/R injury (55). Wolffhart et al. (214) demonstrated in a model of experimental atherosclerosis that both AVE9488 and AVE3085 have vasoprotective properties, i.e., the increased endothelial NO generation associated with reduced cuff-induced neointima formation and reduced formation of atherosclerotic plaques in apoE−/− mice. This study indicates that both compounds showed the concomitant increase in eNOS transcription and eNOS protein levels; BH4 levels also increased, although GTPCH mRNA levels did not. In addition, the mechanisms for the increase in BH4 by these eNOS enhancers still remain unknown. Recently, Yang et al. (219) demonstrated that AVE3085 restored impaired endothelial function in a hypertensive model by upregulated expression of eNOS protein and mRNA, enhanced eNOS phosphorylation, and decreased formation of nitrotyrosine. While these preclinical data are very promising, no clinical studies are initiated at the moment.

*Statins.* Statins, hydroxymethylglutaryl-CoA reductase inhibitors, improve endothelial function by increasing eNOS stability (102) and bioavailability of NO (9), and in part by lowering LDL (119). In animal experiments, statins have been shown to reduce platelet activation and thrombus formation, which in part, are influenced by eNOS upregulation (101). Moreover, statins increase BH4 levels in vascular endothelial cells by potentiating GTPCH gene expression and BH4 synthesis, thereby preventing relative shortages of BH4 (66). Some statins, such as atorvastatin, decrease caveolin-1 expression in ECs, thereby allowing for the activation of eNOS by cofactors, resulting in promoting NO production (50). Wenzel et al. (211) demonstrated in STZ diabetic rats that chronic administration of atorvastatin (20 mg·kg−1·day−1 for 7 wk) improves endothelial function by normalizing endothelial BH4 level and GTPCH expression, reducing oxidative stress, preventing NOX upregulation, and preventing and reversing eNOS uncoupling.

Conclusions

In this review, we have discussed how eNOS uncoupling is one of the major underlying causes of endothelial dysfunction found in atherosclerosis, diabetes, hypertension, cigarette smoking, HCY, and I/R injury. Evidence suggests that modulating of eNOS by stabilizing eNOS function and suppressing eNOS-derived ROS is a promising therapeutic target for endothelial dysfunction. Therapeutics, such as l-arginine, BH4, FA, eNOS transcription enhancers (AVE9488 and AVE 3085), and statins have achieved vascular protection, improving endothelial function and ameliorating cardiovascular diseases in animal and/or human studies, yet this experimental evidence needs to be confirmed in clinical trials. Further understanding of the pathophysiology and the molecular biology of endothelial dysfunction is required to understand this discrepancy. Importantly, future efforts may be directed at identifying signaling pathways regulating NOS on oxidative/nitrosative stress and providing possible mechanisms that can recouple the uncoupled eNOS and other NOS isoforms. These new studies will provide us with a comprehensive understanding of the molecular basis of eNOS uncoupling, thereby identifying potential novel therapeutic approaches targeting the underlying signaling pathways for the prevention and treatment of progressive endothelial dysfunction in cardiovascular diseases.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

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