Modulating endothelial nitric oxide synthase: a new cardiovascular therapeutic strategy

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Zhang Y, Janssens SP, Wingler K, Schmidt HH, Moens AL. Modulating endothelial nitric oxide synthase: a new cardiovascular therapeutic strategy. Am J Physiol Heart Circ Physiol 301: H634–H646, 2011. First published May 27, 2011; doi:10.1152/ajpheart.01315.2010.—The pathogenesis of many cardiovascular diseases is associated with reduced nitric oxide (NO) bioavailability and/or increased endothelial NO synthase (eNOS)-dependent superoxide formation. These findings support that restoring and conserving adequate NO signaling in the heart and blood vessels is a promising therapeutic intervention. In particular, modulating eNOS, e.g., through increasing the bioavailability of its substrate and cofactors, enhancing its transcription, and interfering with other modulators of eNOS pathway, such as netrin-1, has a high potential for effective treatments of cardiovascular diseases. This review provides an overview of the possibilities for modulating eNOS and how this may be translated to the clinic in addition to describing the genetic models used to study eNOS modulation.

endothelial nitric oxide synthase uncoupling; modulators; superoxide; tetrahydrobiopterin; enhancers; nitric oxide donors

Uncoupling of eNOS has been described in 1) situations associated with endothelial dysfunction such as atherosclerosis (3, 70), diabetes mellitus (135), ischemia-reperfusion (I/R) injury (35, 138), hypertension (68), and chronic flow overload (78); 2) cardiac hypertrophy with ventricular remodeling (133); and 3) diastolic heart failure (149). eNOS also uncouples physiologically. For example, eNOS may uncouple postnatal in the lung, possibly leading to a developmental adaptation of the pulmonary vascular system to produce reactive oxygen species (ROS) (81). In addition, it has been demonstrated that purified eNOS is never fully coupled (46). eNOS can be modulated by directly or indirectly increasing the bioavailability of its cofactors and substrate, increasing its transcription and interfering with eNOS modulators, such as netrin-1, resulting in increased NO-production and/or less eNOS-dependent generation of free radicals.

In this review, we describe the different possibilities to modulate eNOS (see Fig. 1) and how the modulation of eNOS can be applied as a new therapeutic approach for cardiovascular diseases in which the uncoupling of eNOS has been described to be of pathogenetic importance.

The Role of NOS Substrate: l-Arginine

The exact mechanism that causes the uncoupling of NO synthase in physiological and pathophysiological conditions is unclear. Lack of both the substrate l-arginine (114, 156) and binding of BH4 (157) may be involved. l-Arginine stimulates oxygen uptake by coupled eNOS (46) and prevents superoxide...
formation within uncoupled eNOS by electronic interaction with the heme-bound oxygen (9). Steady-state L-arginine levels may be increased by either inhibiting L-arginine breakdown via arginase (54) or by supplying additional arginine. The latter approach has been tested in many clinical trials with contrasting results. Short- to medium-term administration of arginine improves NO-mediated vascular function in elderly humans as well as a variety of clinical conditions, including hypercholesterolemia, coronary artery disease, congestive heart failure, and peripheral artery disease (PAD) (13). However, in other trials L-arginine was not beneficial (11) or the benefits did not continue with chronic therapy. Importantly, one study has suggested possible harm in post-myocardial infarction patients treated with oral arginine (117), although the causal relationship between deaths and arginine administration has been questioned. Arginine-based nutritional adjunct to PAD therapy gained support from several short-term clinical studies. Nevertheless, in the Nitric Oxide in Peripheral Arterial Insufficiency Study (NO PAIN I), the largest randomized clinical trial of the longest duration in patients with PAD (151), the long-term administration of arginine (3 g/day) did not increase NO availability, walking distance, or vascular reactivity. Furthermore, the data indicated that long-term administration of L-arginine might even impair functional capacity. It was concluded that, as opposed to short-term administration, long-term administration may not be useful in patients with intermittent claudication due to PAD (151).

The exact reasons for the contrasting findings of clinical studies testing L-arginine supplementation are unknown, and it is not entirely clear if and why L-arginine oral administration may work at all. It has been suggested that prolonged L-arginine supplementation results in “arginine tolerance,” possibly involving the induction of arginases. Furthermore, oral arginine supplementation has low bioavailability: approximately 40% of dietary L-arginine is catabolized in the intestine, a further fraction is metabolized by liver arginase to urea, and only 1% of oral arginine becomes available as a substrate for NOS (14). Importantly, intracellular L-arginine levels may never decrease to the subsaturating levels for eNOS, even under nonpathophysiological conditions (28), and L-arginine is intracellularly generated (124). The 

\[ K_m \]

for L-arginine as a substrate for eNOS is 2–15 μM, and the cytoplasmic level of arginine is 600 to 900 μM. Thus arginine should not become limiting for NO formation at all. However, asymmetric dimethyl-L-arginine (ADMA), an endogenous competitive eNOS inhibitor that acts at the eNOS substrate binding site, is increased under pathophysiological conditions (20). It not only directly inhibits eNOS but also inhibits endothelial L-arginine uptake via cationic amino acid transporters (4). In vitro, ADMA uncouples eNOS and elevates ROS generation in the absence of BH4 but not in the presence of L-arginine (34). Moreover, basal and stimulated NO release appear to be affected differently by ADMA (2).

Irrespective of the underlying beneficial mechanism of L-arginine supplementation, L-arginine supplementation may sometimes be ineffective and even harmful. In rat hyperoxaluric kidneys and excessive crystal deposition, for example, L-arginine administration increases oxidative and nitrosative stress in an \( \text{NO}^\text{nitro-L-arginine methyl ester} (\text{L-NAME}) \text{ reversible manner} (54). Thus the supplementation of L-arginine is not a promising strategy to treat cardiovascular diseases.

The Role of NOS Cofactor BH4

BH4 has been recognized as an essential cofactor of eNOS since it was first described in 1989 (134). In its coupled state, BH4 is tightly bound to eNOS, and the amount of binding is correlated with eNOS activity (7). In the presence of its substrates (L-arginine and oxygen), eNOS catalyzes the formation of L-citrulline and NO. In the presence of subsaturating levels of BH4, the electron transfer in eNOS becomes uncoupled from L-arginine oxidation and NO formation. This uncoupling results in superoxide production by eNOS. When the bioavailability of BH4 or L-arginine declines, the eNOS dimer demonstrates a less tightly packed oxidase domain and a higher sensitivity to proteolysis (103). Multiple pleiotropic roles of BH4 in eNOS enzymatic function have been demonstrated: BH4 (1) facilitates the binding of L-arginine substrate to eNOS, (2) shifts the heme iron into a high spin state that increases enzyme activity, (3) is involved in electron transfer, and (4) stabilizes the NOS dimer (24, 63, 65, 108). Stoll et al. (128) demonstrated that eNOS also stabilizes BH4 by controlling its protonation state. BH4 itself has also direct intrinsic antioxidant effects (90). Furthermore, Landmesser et al. (68) provided evidence that eNOS uncoupling contributes to systemic hypertension in the DOCA-salt rat model of hypertension. In addition, the prevention of pressure overload-induced eNOS uncoupling by BH4 suggested a potential therapeutic option of this molecule for chronic pressure overload-induced ventricular remodeling (133). It has also been shown that an overexpression of eNOS cannot rescue atherosclerotic lesion in apolipoprotein E-knockout (ApoE−/−) mice, but a simultaneous supplementation of BH4 or an upregulation of the rate-limiting enzyme of BH4 synthesis, guanosine triphosphate cyclohydrolase-I (GTPCH-1), led to a recovery of eNOS bioactivity (i.e., eNOS recoupling), thereby improving endothelial function and reducing disease progression (102). Although eNOS expression was increased, NO release was not reversed without correspondingly increasing of BH4 in a spontaneously hypertensive rat model (27). The administration of L-arginine to bEnd.3 cells, without an administration of additional BH4, exacerbated ROS generation caused by indicating that eNOS uncoupling induced ROS generation because of relative BH4 deficiency (11). In vivo investigation of eNOS regulation clearly indicates a very tight stoichiometric relation between
eNOS and BH₄ to achieve an optimal eNOS coupling status (7). Once generated, superoxide reacts with NO to form the highly reactive intermediate peroxynitrite. BH₄ is unstable at pH 7.4 and is oxidized rapidly by low concentrations of peroxynitrite (87). An increased formation of peroxynitrite in turn oxidizes BH₄ to the BH₂ radical. In addition, the oxidized form BH₂ may compete with BH₄ for binding at eNOS with subsequently further exacerbated eNOS uncoupling (145).

However, the increased expression of GTPCH-1 can prevent the decrease in BH₄ levels in isolated cells and in atherosclerotic animal models (162). Aoki et al. (6) showed in human umbilical vein endothelial cells that fluvastatin, a 3-hydroxy-3-methyl-glutaryl-CoA reductase inhibitor, not only upregulated eNOS activity via enhancement of its phosphorylation at Ser¹⁷⁷ and Ser⁶³⁷ but also prevented eNOS uncoupling via elevating BH₄ levels through enhancing GTPCH expression.

Recently, it has been reported that thiol-specific reducing agents can restore impaired endothelium-dependent vasodilation caused by S-glutathionylation of eNOS as an alternative of BH₄ repletion for the recoupling of eNOS (22).

Because of the major pathogenic role of BH₄ deficiency in the onset of cardiovascular diseases, BH₄ supplementation has been explored as a new therapeutic strategy in many experimental models. It has been demonstrated that BH₄ improves I/R injury in isolated perfused rat hearts (35, 160). In ApoE⁻/⁻ mice, BH₄ supplementation reduces vascular immune cell infiltration in atherosclerosis through maintaining eNOS coupling and NO bioavailability, thus preventing the progression of atherosclerosis (115). In addition, exogenous BH₄ given to mice can recouple an already uncoupled eNOS with a subsequent reversing of pressure overload-induced cardiac hypertrophy, fibrosis, and myocardial dysfunction (91). Recent in vitro data demonstrated that the bioavailability of BH₄ is reduced in the early postradiation phase. Berbee et al. (8) demonstrated that the administration of BH₄ decreases radiation-induced vascular peroxynitrite production and that the administration of γ-tocotrienol (GT3, a radioprotective vitamin E analog) 1) reduces the expression of GTPCH feedback regulatory protein, a key negative regulator of BH₄ synthesis, and 2) reverses radiation-induced BH₄-dependent eNOS uncoupling. However, it is not easy to determine BH₄ deficiency in vivo as plasma concentrations of BH₄ do not always provide a reliable indicator of tissue levels or bioavailability (147). A more useful marker may be the ratio of reduced to oxidized biopterins (BH₄: BH₂) ratio in cells (145).

Another limiting factor of the potential clinical use of BH₄ as a pharmaceutical drug is its chemical instability. BH₄ is hydroscopic and easily oxidized to 7, 8-BH₂, and must be frozen for storage. However, 6R-BH₄ is a novel thermo- and photostable BH₄ derivate that is commercially available for use as a phenylketonuria drug (Kuvan, Biomarin, San Francisco, CA). Most recently, 6-hydroxymethyl pterin and 6-acetyl-7,7-dimethyl-7,8-dihydropterin (ADDP), two oxidatively stable analogs of BH₄ that may substitute for BH₄ when the pterin site is unoccupied, were demonstrated to have a protective role in limiting endothelial dysfunction in the pulmonary vasculature (66). ADDP is a stable compound that is soluble in both polar and organic solvents. Also, the administration of sepiapterin, a BH₄ precursor, together with N-acetylcysteine, has been linked to the correction of vasomotor tone in the vasculature of atherosclerotic rabbits by raising BH₄ levels (144, 145). Furthermore, ascorbic acid (vitamin C) has been known to recycle the BH₃ radical to BH₄ (67) and to prevent oxidation of BH₄ (52), thus chemically stabilizing BH₄ and enhancing eNOS coupling. There is also in vivo evidence that ascorbate supplements preserved vascular BH₄ levels and eNOS activity in atherosclerotic mice (31).

With the development of the synthetic BH₄ analog (6R-BH₄), which is already approved by the Food and Drug Administration for the treatment of phenylketonuria, larger trials (unpublished) were set up to study its effect on arterial (NCT00325962, concluded 2008) and pulmonary hypertension (NCT00435331, recruitment stopped in 4/09, no data reported), endothelial dysfunction (NCT00532844, study completed, data unpublished), PAD (NCT00403494, completed 1/09), and sickle cell disease (NCT00445978, completed 6/09). Unfortunately, the overall results were disappointing, not because of adverse effects but because of lacking efficacy, which can be explained in several ways. First, the Km of BH₄ for eNOS is 80 nM. If the stoichiometry with NOS isoforms has to be just right, too much of this synthetic BH₄ analog might activate constitutive NOS [inducible NOS (iNOS, NOS2)]. Also, the intracellular transport of BH₄ is not completely understood. In addition, oral BH₄ may be oxidized and must be re-reduced, so this biochemistry becomes very important for the net result. It has been demonstrated that BH₂ can be more efficiently transported into cells over BH₄, but must then be recycled back to BH₄ (51). Recently, Suckling et al. (129) revealed that a novel BH₄ analog ADDP can diffuse from the plasma across cell membranes and cause vasodilatation by stimulating eNOS activity. This may provide another avenue for clinical translation.

The biochemistry, molecular biology, and experimental translational evidence support an important role of BH₄ as an eNOS modulator to treat myocardial and endothelial dysfunction by stabilizing NOS function and limiting NOS-derived ROS release (29, 30, 62, 91). However, the instability of BH₄ remains a point of concern for the translatability of BH₄ supplementation for clinical disease. Given the many challenging results that have been reported, one would hope that efforts to further enhance the stability of this interesting molecule will continue and some ultimate translation potential be realized.

The Role of Folic Acid for NO Availability

The B-vitamin folic acid (FA) plays an important role in the pathogenesis of different developmental abnormalities, particularly neural tube defects (32), neuropathies (104), neuropsychiatric disorders (127), and the development of neoplasms and preneoplastic conditions (155). FA is chemically stable and considered to be safe (92). As folate is an essential vitamin, humans rely on external sources, i.e., food-derived folates or supplemented FA, to fulfill their needs. The absolute minimal requirement of folate to avoid acute deficiency is at least 50 μg/day (126). It is recommended that women consume 600 mg FA per day during pregnancy to prevent brain and spinal cord birth defects (57).

Importantly, folate deficiency has been associated with cardiovascular diseases (70, 92, 137). The most well-known biological function of FA is its homocysteine-lowering effect mediated by the FA-dependent remethylation of homocysteine to methionine (126). FA also has direct and indirect antioxidant
effects (36, 53, 146) and can either directly interact with eNOS or indirectly by restoring the bioavailability of BH$_4$ (56). 5-Methylenetetrahydrofolate (5-MTHF), the active metabolite of FA, has a similar structure as that of BH$_4$. Dihydrofolate reductase is involved in the reduction of oxidized BH$_2$ back to BH$_4$, whereas dihydropteridine reductase converts quinonoid-BH$_2$ to BH$_4$.

In addition, 5-MTHF can chemically stabilize BH$_4$ and ameliorate the binding affinity of BH$_4$ to eNOS (92). A pteridine-binding domain in eNOS with similarities to the folate binding site of dihydrofolate reductase can serve as a locus through which 5-MTHF can directly bind to eNOS and facilitate the electron transfer by BH$_4$ from the reductase domain of eNOS to its heme (56, 76).

Clinically, FA and 5-MTHF have been shown to restore endothelial function in patients with hypercholesterolemia (146), diabetes mellitus (142), hyperhomocysteinemia (153), and atherosclerosis (5). In animals, FA has been demonstrated to significantly improve myocardial function after myocardial infarction in rats (106) and to reduce myocardial ischemic dysfunction and postreperfusion injury in mice (89). However, recent clinical trials have failed to demonstrate a benefit of long-term use of FA (15, 75) that can be explained by too low of a dosage (89).

In summary, efforts should be made to further understand the mechanism of FA in preventing and/or reversing both endothelial and myocardial dysfunction and how this can be translated to the clinic.

**NO Donors**

Traditional NO donors are predrugs, which rely on the generation of NO for their pharmacological activities. However, there are structurally dissimilar NO donors with different chemical reactivities and metabolic characteristics. More recently, novel signaling molecules, including nitrite (NO$_2^-$) anions; alternative NO delivery strategies, such as NO gas for inhalation; or hybrid NOS gene and progenitor cell transfer approaches showed promising results in preclinical testing and are gradually introduced in clinical trials (61, 86, 100, 148).

Nitrates, together with the organic nitrates and ferrous nitro complexes including sodium nitroprusside, are generally referred to as nitrovasodilators and exert their biological effects via the release of NO or a related S-nitrosothiol. The precise mechanism of organic nitrate bioactivation is incompletely understood but is thought to involve both enzymatic and nonenzymatic mechanisms. The active metabolites following enzymatic reduction by glutathione S-transferases (69), cytochrome P-450 reductases (1), xanthine oxidoreductases (XORs) (79), and mitochondrial aldehyde dehydrogenases (ALDH-2) (23), in turn, activate the intracellular NO receptor enzyme soluble guanylate cyclase in the vessel wall. Activated soluble guanylate cyclase increases tissue levels of the second-messenger cGMP, which ultimately mediates vasorelaxation via a cGMP-dependent protein kinase.

Organic nitrates have been introduced for over a century in cardiovascular therapy, and compounds such as nitroglycerin, isosorbide di- and mononitrate, and pentaerithritol tetranitrate are still widely used in the symptomatic treatment of acute coronary syndromes, chronic angina pectoris, and congestive heart failure. The mechanism of action in these drugs is traditionally believed to result from their arterial vasodilation and venodilation effects and improvement of collateral blood flow, resulting in decreased myocardial oxygen consumption and reduced cardiac workload (98). Recently, it has been recognized that these drugs also have intrinsic antiplatelet and antithrombotic effects, demonstrated both in vitro and in vivo, which adds further rationale for their use in atherothrombotic diseases. However, clinical trials have yielded conflicting results regarding clinical outcome, especially with long-term nitrate use (58).

The major limitation, which renders many organic nitrates unsuitable for long-term therapy, is the induction of tolerance and cross-tolerance, characterized by a decreased sensitivity of the vessel wall to the vasorelaxant effect of organic nitrates and to other endothelium-dependent vasodilators or endogenous NO, respectively (50). The molecular target of organic nitrates, i.e., the NO-cGMP signal transduction, cascades itself in the vessel wall and is often impaired by ROS formation, resulting from the activation and upregulation of vascular NADPH oxidases and XORs (64).

Recently, several novel compounds have been synthesized that are devoid of oxidant-generating properties, including S-nitrosothiols, nitrovasodilator hybrids containing antioxidant moieties, aminoalkyl and aminoethyl nitrates, and diazeniumdilates NONOates, which may offer a more optimal therapeutic index and could prove useful in avoiding tolerance, suppressing oxidative stress in atherosclerosis, potentiating antiplatelet effects, and improving clinical outcome (111). The potential mechanisms for the purported benefit involve the activation of endogenous protective pathways, including heme oxygenase-1, and ferritin, the suppression of NADPH oxidase-dependent oxidative stress (119), or compound-specific ALDH-2- and cytochrome P-450-independent pathways (116). Some of these new compounds, harboring an antioxidant moiety in their drug structure, are already being tested in phase II clinical trials of chronic ischemic cardiovascular disorders for their reduced tendency toward tolerance sensitivity (40). Nevertheless, future large-scale clinical trials need to provide the necessary evidence base before widespread clinical introduction.

Circulating NO$_3^-$ is the largest physiological reservoir of NO in the body and was long considered as an index of NOS activity, with as much as 70% of plasma NO$_3^-$ originating from eNOS. Exogenous sources, principally environmental pollutants and intake of vegetables, also contribute to this NO reserve. While the anion NO$_3^-$ has traditionally been considered a mere oxidative breakdown product of NO and a diagnostic marker of its formation at nanomolar concentrations in biological systems, more recent discoveries indicate that NO$_3^-$ represents a physiologically relevant storage reservoir of NO in blood and tissues with distinct signaling functions at near-physiological concentrations (19). The NO$_3^-$ anion can be readily reduced to bioactive NO along a physiological oxygen and pH gradient either nonenzymatically (167) or by a number of enzymes, including XORs, NOS, mitochondrial cytochromes, and deoxygenated hemoglobin and myoglobin (25, 71, 72, 122, 140). NO$_3^-$ reduction to NO is enhanced under hypoxic conditions, which serves well its potential as a fast and potent pulmonary and systemic vasodilator drug and as a cytoprotective agent in I/R injury (166). The theoretical ease of the administration of NO$_3^-$ anions makes it a potentially inter-
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The mechanisms responsible for these remote effects are likely multiple and include the modification of circulating cells (including leukocytes and platelets) as they pass through the lungs, inhibiting their ability to elicit I/R injury, a reaction with superoxide to form peroxynitrite, which in turn may have dose-dependent cardioprotective effects against I/R (99) or formation of S-nitrosothiols or NO-heme complexes in plasma proteins or in circulating cells with regeneration of NO in systemic vascular beds. S-nitrosylation of proteins, a posttranslational modification process that is redox dependent, thiol based, and reversible is a principal mechanism of NO-based signal transduction in cardiovascular biology and involves the attachment of NO moieties to protein sulfydryl groups. Indeed, S-nitrosylation of eNOS protein has been recently recognized as another level of dynamic posttranslational control of eNOS activity. eNOS is tonically S-nitrosylated at cysteine residues in resting endothelial cells, resulting in a decreased eNOS activity (37). In response to eNOS agonists, eNOS undergoes a rapid transient denitrosylation. Protein nitrosylation is dynamically regulated during physiological and pathophysiological conditions and affects G protein-coupled receptor signaling, mitochondrial metabolic regulation, intracellular calcium handling, and cellular defense against death receptor-mediated apoptosis and oxidative stress in cardiac and vascular cells. The S-nitrosylation mechanism is thought to confer much of the benefit of NO-donor compounds in cardioprotection (132), and its role in NO inhalation protocols remains to be specified. At this early stage, the intricate relations between spermine NONOate, cell death, and cardioprotection are still unclear, and S-nitrosylation of distinct proteins may result in either cytotoxic or cytoprotective phenotypes, depending on the circumstances, subcellular compartmentalization, and cross talk with redox and phosphorylation pathways. Finally, inhaled NO may be converted to NO₂⁻, likely through a type of plasma NO oxidase such as ceruloplasmin (123). NO₂⁻ may in turn be converted back to NO in an acidic/hypoxic environment present in reperfused myocardium by xanthine oxidase (139) or deoxyhemoglobin (26). Alternatively, it may protect myocardium from I/R-induced injury via NO-independent signaling (17).

In summary, one of the major challenges of almost all NO-donor compounds is the rate of NO delivery and the amount of site-specific generated NO, which are critical determinants of cellular effects. The very short half-life of NO and its dose-dependent cellular activity profile remain a formidable challenge with respect to harnessing its full therapeutic potential.

Transcription Enhancers of eNOS

4-Fluoro-N-indan-2-yl-benzamide (AVE9488, CAS no. 291756-32-6; empirical formula, C16H14FNO) and 2,2-difluoro-benzo[1, 3]dioxole-5-carboxylic acid indan-2-yl-amide (AVE3085, CAS no. 450348-85-3; empirical formula, C17H13F2NO3) have been described (152) as two novel transcription enhancers of eNOS. These two small molecules which pharmacological compounds can increase eNOS protein expression and concomitantly maintain eNOS coupling in different cell in vitro and in vivo models. Accordingly, AVE9488 stimulated eNOS promoter activity in a concentration-dependent manner in human endothelial EA.hy 926 cells in vitro, but without changing eNOS mRNA stability (152).

In a rat model of myocardial infarction-induced heart failure, long-term treatment (9 wk) with AVE9488, by upregulating the expression of eNOS, improved left ventricular (LV) remodeling and myocardial dysfunction. Moreover, AVE9488 increased the levels of circulating endothelial progenitor cell levels in rats with myocardial infarction-induced heart failure (41). In primary human umbilical vein endothelial cells, treatment of AVE9488 showed both an increased eNOS mRNA and protein expression as well as an enhanced bradykinin-stimulated release of NO (152). Following these initial observations in cell culture conditions, AVE9488 has been shown to improve eNOS expression levels and confer cardiovascular protective effects in different transgenic animal models. For example, subchronic treatment (17 days) with AVE9488 (30 mg·kg⁻¹·day⁻¹) once a day in adult C57BL/6J mice and at a lower dose (10 mg·kg⁻¹ twice daily) in ApoE⁻/⁻ mice significantly increased eNOS protein levels in femoral arteries and in aortas, respectively. Interestingly, AVE9488-treated ApoE⁻/⁻ mice demonstrated reduced cuff-induced neointima formation. Moreover, this eNOS-mediated inhibiting effect on vascular neointima formation was confirmed using eNOS⁻/⁻ mice. A 12-wk-long-term treatment with AVE9488 reduced the area of atherosclerotic plaque in ApoE⁻/⁻ mice, but not in ApoE/eNOS-double knockout mice, whereas it did not change plasma lipid levels or heart rate in either animal strain. More importantly, aortas from ApoE⁻/⁻ mice, which were treated with AVE9488 (30 mg·kg⁻¹·day⁻¹) for 2 wk showed enhanced vascular BH₄ levels. The administration of l-NAME could not
further reduce ROS production in aortic rings, indicating that AVE9488 reversed eNOS uncoupling (70, 152).

Sasaki et al. (110) demonstrated that pretreatment of AVE9488 can at least in part restore the impaired neovascularization capacity of endothelial progenitor cells from patients with ischemic cardiomyopathy. The enhanced mRNA expression and activity of eNOS was associated with an improved migratory capacity in vitro, which specifically involved NO and improved neovascularization capacity of the infused bone marrow mononuclear cells in an ischemic hindlimb model. Furthermore, it has been demonstrated that AVE9488 has a protective role in cardiac in vivo I/R injury by upregulating eNOS expression and thereby increasing NO production and reducing ROS in mice (43). In addition, AVE9488 reduced platelet activation in parallel to positive modulation of systemic NO bioavailability in rats with severe congestive heart failure (112). A different 12-wk treatment with AVE3085, another eNOS transcription enhancer, reduced atherosclerotic plaque formation in ApoE−/− mice, but not in ApoE/eNOS-double knockout mice as shown with AVE9488 (152). Xue et al. (158) demonstrated that AVE3085 protects against I/R injury in porcine coronary small arteries endothelium through directly enhancing eNOS expression in vitro. In spontaneously hypertensive rats, AVE3085 can improve endothelial function by its acute and chronic transcriptional enhancement of eNOS (159). Chronic treatment for 4 wk with AVE3085 significantly reduced blood pressure. In vitro, it markedly increased acetylcholine-induced relaxation as observed in the acute group (rat aortas treated with 2 h AVE3085). eNOS and phosphorylated eNOS protein levels were significantly higher in AVE3085-treated spontaneously hypertensive rats compared with untreated groups. Although a 48-day chronic treatment with AVE3085 (10 mg·kg−1·day−1) in male diabetic Sprague-Dawley rats did not alter arterial blood pressure or severe hyperglycemia, it significantly increased the expression level of eNOS and phosphorylated vasodilator-stimulated phosphoprotein and cGMP concentration in blood plasma and reduced intracellular and vascular cell adhesion molecules-1 production (109). The same dose of AVE3085 was given over 8 wk to DAHL salt-sensitive rats with diastolic heart failure. Cardiac hypertrophy and fibrosis were attenuated compared with those in untreated animals with diastolic ventricular dysfunction (149). Unfortunately, more clinical data regarding the effectiveness or safety of these eNOS enhancers are not yet available.

**Caveolins and Coexpression Partners**

Caveolins are a family of caveolar proteins composed of three isoforms. Caveolin-1 (Cav-1), a 22-kDa protein of 178 amino acids that oligomerizes and inserts into the cytoplasmic face of the caveolae (88), is expressed in a diverse range of cell types of the cardiovascular system, including endothelial cells, smooth muscle cells, and cardiac fibroblasts. Interestingly, although Cav-2 is dispensable for forming caveolae, it is very often coexpressed with Cav-1 in many of these cells. Cav-3 is a 151 amino acid protein that shares 65% identity and 85% similarity with Cav-1. Its expression is mainly muscular specific and predominant in striated muscle cells. In the heart it is located in cardiac myocytes (150). Mutations in the Cav-3 gene in humans are associated with pathological cardiac hypertrophy, and Cav-3−/− mice also develop a progressive cardiomyopathy (154). Research on the role of caveolins in cardiovascular diseases has mainly focused on Cav-1. In vitro and in vivo studies in Cav-1−/− mice have identified Cav-1 as an inhibitory modulator of eNOS (163, 164). However, the role of Cav-1 in facilitating or preventing eNOS dimerization remains unclear. The NH2-terminal oligomerization domain (amino acids 61–101) and the residues 135–178 located in the COOH-terminal tail are two cytoplasmic domains of Cav-1 interacting with eNOS. Ghosh et al. (47) proposed that the eNOS reductase domain binding sites of Cav-1 antagonize the calmodulin (CaM) binding to eNOS and electron transfer to the eNOS heme, thereby inhibiting heme iron reduction and NO synthesis. eNOS is the bridge holding the eNOS/Cav-1/heat shock protein-90 (Hsp90) heterotrimeric complex together. CaM cannot physically disrupt the eNOS-caveolin complex in vitro, but Hsp90 can facilitate CaM to displace caveolin from eNOS (45).

Understanding the interaction between Cav-1 and eNOS and its impact on eNOS activity is critical for the interpretation of the posttranslational regulations of this enzyme. When the intracellular calcium concentration increases, calcium/CaM interrupts the heteromeric complex of Cav-1 and eNOS. CaM then acts as a direct allosteric competitor of Cav-1 to promote the calcium-dependent activation of eNOS (85). Other groups demonstrated that Cav-1 was not absolutely required for targeting eNOS to caveolae/lipid rafts, suggesting the involvement of other important molecular partners (49, 125). Two other proteins named as eNOS-interacting protein (NOSIP) and eNOS traffic inducer (NOSTRIN) that specifically modulate the caveolin/eNOS interaction have been described (33, 113, 165). NOSTRIN also functions as an adaptor protein to recruit dynamin-2, which is a large GTPase and a positive modulator of eNOS (118) to facilitate internalization of eNOS (113).

GTP cyclohydrolase I (GTPCH I) was found to be colocalized with Cav-1 within caveolae, and its activity was reversely regulated by Cav-1 (105). The coexpression of the arginine transporter-1, Cav-1, and eNOS was demonstrated within caveolae (82). Caveolae were reported to represent the calcium channels of the plasma membrane (44). Thus these flask-shaped vesicular invaginations, caveolae, bring eNOS in close proximity to molecules that are required for its proper function.

The tight interaction between Cav-1 and eNOS and its importance in cardiovascular system lead to numerous investigations in Cav-1−/− mouse models and contributed to a better understanding of the pathogenesis of cardiovascular diseases. eNOS activity is upregulated in Cav-1−/− animals, and this activity can be blunted by using a specific NOS inhibitor, l-NAME (107). Cav-1 deficiency induces a variety of cardiovascular pathological phenotypes, including cardiac hypertrophy, pulmonary arterial hypertension, atherosclerosis, and defective angiogenesis. According to Feron et al. (39), a hypercholesterolemia-induced decrease in NO production is ascribed to an enhanced interaction of Cav-1 and eNOS. Interestingly, the protective role of the complete loss of Cav-1 on the development of aortic atherosclerosis has been demonstrated in ApoE−/−-deficient mice (42). Cav-1−/− mice also showed hyperproliferative and vascular abnormalities (107). Surprisingly, some of these cardiovascular symptoms were attributed to an increased Cav-1 abundance as well. Under basal conditions, Cav-1 maintains eNOS in its inactivated state and limits NO production, whereas in the case of reduced Cav-1 expres-
sion (e.g., Cav-1\(^{-/-}\) animals), eNOS expression and NO release are significantly increased. Agonist-stimulated receptor/effector leads to eNOS activation through a local increase of intracellular calcium, dissociation from Cav-1, and subsequent formation of CaM/eNOS. When Cav-1 abundance falls below a certain threshold level, the coupling between agonist-bound receptor and cytosolic eNOS is lost and NO production is decreased. When the abundance of caveolin is increased, eNOS is inactivated by this excess inhibitory clamping, preventing the activation of the receptor signaling cascade and resulting in reduced NO production (38).

More recently, the inhibition of δ-PKC and the activation of ε-PKC were reported to promote the inactivation of eNOS by Cav-1 (93). This provides a novel perspective on the regulation of Cav-1/eNOS. In addition, an overexpression of Cav-1 enhanced eNOS trafficking to the plasma membrane and NO generation increased in response to shear stress and H\(_2\)O\(_2\) (136). Estrogens mediate rapid eNOS activation via estrogen receptor-α within the plasma membrane of endothelial cells. Cav-1 stimulates 17β-estradiol-induced NO production by promoting estrogen receptor-α to the plasma membrane, which facilitates the activation of the phosphatidylinositol 3-kinase pathway, leading to eNOS activation and NO generation (60, 130). 3-Hydroxy-3-methylglutaryl-CoA reductase inhibitors can improve the impaired endothelial dysfunction of the aorta in spontaneously hypertensive rats and may activate eNOS by phosphorylation and a decrease of Cav-1 abundance (131). In contrast, a 24-h treatment of C-reactive protein increased phosphorylation and a decrease of Cav-1 abundance (141). Methyl-β-cyclohexanecyclodextrin can inhibit caveolaemediated endocytosis by moving cholesterol out of lysosomes and prevent eNOS-dependent NO production at the same time (80). However, no clinical data on the effects of methyl-β-cyclohexanecyclodextrin as an eNOS modulator in cardiovascular diseases are available.

**Phosphorylation of eNOS**

eNOS activity is also posttranslationally regulated by phosphorylations at different residues. The phosphorylation of eNOS at Ser\(^{1177}\) and Thr\(^{895}\) influences its activity in endothelial cells. So far, the knowledge of eNOS phosphorylation in cardiac myocytes is restricted to Ser\(^{1177}\) (94). Hsp90 is thought to be crucial for Akt-induced phosphorylation of eNOS-Ser\(^{1177}\) (16). Thus a binding complex of Hsp90, Akt, and CaM-bound eNOS is proposed to facilitate the proper function of eNOS.

**Other Modulator of eNOS Pathway: Netrin-1**

Netrin-1, a laminin-related secreted protein produced by floor plate cells, is well known for its crucial roles in the pathfinding of axon, cell proliferation, and migration during neuronal development (84). It has been implicated in angiogenic signaling in cardiovascular diseases and tumorigenesis with a controversial role, either pro- or anti-angiogenic (21). Recently, Zhang and Cai (161) demonstrated that netrin-1 activates its “deleted in colorectal cancer” (DCC) receptor in C57BL/6J mouse’s cardiomyocytes and cardiac endothelial cells following I/R, results in the activation of extracellular signal-regulated kinase 1/2 (ERK1/2), and subsequently phosphorylates serine\(^{1177}\) of eNOS (p-eNOSs\(^{1177}\)), thus increasing NO production. NO also contributes to ERK1/2 activation, and netrin-1-induced DCC upregulation can be abolished by U-0126 (MEK1/2 inhibitor), l-NAME, or 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazole-1-oxide (PTIO; NO scavenger), thus forming a feed-forward cycle. Based on these findings, a netrin-1/DCC/ERK1/2/eNOS\(^{1177}\)/NO/DCC feed-forward mechanism was identified as a molecular paradigm subsequently found to also operate in adult aortic endothelial cells. Because in the absence of netrin-1, its receptors can trigger apoptosis, netrin-1 has been suggested as the target in cancer therapy (83). Although studies on netrin-1 as a cardio-protectant are still at a very early stage, these findings highlight its potential as a novel cardioprotective mediator in cardiovascular disease.

**S-glutathionylation of eNOS**

Recently, Zweier et al. (22) elegantly demonstrated that under oxidative stress, the two highly conserved cysteine 689 and 908 residues at the interface between the flavin mononucleotide-binding and FAD-binding domains of eNOS in endothelial cells can be S-glutathionylated, triggering eNOS uncoupling with increased superoxide production. S-glutathionylation is increased in hypertensive vessels with impaired endothelium-dependent vasodilation and can be reversed by thiol-reducing agents. Thus therapeutics with thiol-reducing properties have a great potential to be further developed as potent drugs for cardiovascular disease in which the pathogenetic importance of eNOS uncoupling has been identified (22).

**Experimental (genetic) models for studying eNOS modulation.** The generation and characterization of mice with a targeted disruption of the individual gene products responsible for NO production has greatly advanced our understanding of cardiovascular homeostasis and the pivotal roles of the different NOS isoforms in the pathogenesis of cardiovascular diseases. The inactivation of the neuronal, inducible, and endothelial NOS genes, either individually or in combination, has led to the development of a variety of mutant mice with distinct cardiovascular phenotypes. In concept, these mice have clearly demonstrated the impact of NO on blood pressure regulation, endothelial dysfunction, response to vascular injury, myocardial and brain ischemia, development of atherosclerosis, and cardiac contractility. While the individual knockout models complement and refine pharmacological approaches with (non)selective NOS inhibitors, they can be confounded by developmental abnormalities, secondary adaptations in pathways acting upstream or downstream to the gene product of interest, or reciprocal changes in expression of proteins that compensate for the deleted gene product (121).

Similarly, the generation of genetically engineered mice with cell-specific overexpression of NOS isoforms has enabled a unique appreciation of the ability of NO to modulate vascular and cardiac function. These experimental models represent a powerful tool to study their function in vivo, independent of changes in cardiac contractility and loading conditions, and provide important insights in the significance of NOS isoforms in human cardiovascular diseases (59, 77, 95, 101).

The different exons of mouse eNOS genes had originally been ruled out for study in relation to cardiovascular diseases during the 1990s [exons 24 and 25 (48), exons 24–26 (55), and exon 12 (120)]. The first eNOS overexpressing mouse model...
Table 1. Overview of clinical trials of eNOS modulators

<table>
<thead>
<tr>
<th>Dose</th>
<th>Outcome</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td>3–8 g/day</td>
<td>No acute pharmacological effects. Low ADMA, no effect; high ADMA, normalizes endothelial function</td>
<td>13</td>
</tr>
<tr>
<td>3.0 g po, 3 times daily, for 6 mon</td>
<td>No serious adverse effects, possibly associated with higher mortality, no improvement of vascular stiffness measurements, left ventricular ejection fraction</td>
<td>117</td>
</tr>
<tr>
<td>3 g/day po for 6 mon</td>
<td>No improvement of vascular reactivity, no benefit, possible harm</td>
<td>151</td>
</tr>
<tr>
<td>5-MTHF, 1 μg·100 ml FAV⁻¹·min⁻¹, local forearm intra-arterial</td>
<td>Improvement of endothelial dysfunction</td>
<td>142</td>
</tr>
<tr>
<td>FA, 10 mg/day po for 8 wk</td>
<td>Improvement of endothelium-dependent dilation, higher serum folate levels, lower total plasma homocystine levels</td>
<td>153</td>
</tr>
<tr>
<td>5-MTHF 0.13 mg/kg body wt</td>
<td>Improved NO-mediated endothelium-dependent vasomotor responses, reduced vascular superoxide</td>
<td>5</td>
</tr>
<tr>
<td>5 mg/kg po, twice daily for 8 wk</td>
<td>Not statistically significant. Drop of 6.4 mmHg in patients' SBP</td>
<td>2008 BioMarin Pharmaceutical, NCT00325962 (unpublished)</td>
</tr>
<tr>
<td>2.5 mg·kg⁻¹·day⁻¹ for 2 wk, 5 mg·kg⁻¹·day⁻¹ for 2 wk, 10 mg·kg⁻¹·day⁻¹ for 4 wk, and then 20 mg·kg⁻¹·day⁻¹ for 2 days</td>
<td>Not significant.</td>
<td>2009 BioMarin Pharmaceutical, NCT00532844 (unpublished)</td>
</tr>
<tr>
<td>5 mg/kg po, twice daily for 13.5 days</td>
<td>Improvement of endothelial function</td>
<td>2009 BioMarin Pharmaceutical, NCT00445978 (unpublished)</td>
</tr>
<tr>
<td>16-wk dose escalation phase po, every 4 wk as follows: 2.5, 5, 10 (once daily), and 20 mg·kg⁻¹·day⁻¹ (twice daily), and continued in an optional extension phase at the highest tolerated dose for up to a total of 2 yr</td>
<td>Not significant.</td>
<td>2009 BioMarin Pharmaceutical, NCT00403494 (unpublished)</td>
</tr>
<tr>
<td>400 mg po, twice daily for 24 wk</td>
<td></td>
<td>2009 BioMarin Pharmaceutical, NCT00403494 (unpublished)</td>
</tr>
</tbody>
</table>

eNOS, endothelial nitric oxide (NO) synthase; ADMA, asymmetric dimethyl-L-arginine; MI, myocardial infarction; FA, folic acid; 5-MTHF, 5-methyltetrahydrofolate; FAV, forearm volume; BH4, tetrahydrobiopterin; SBP, systolic blood pressure.
expressing bovine eNOS in the vascular wall by using murine preproendothelin-1 promoter was generated by Ohashi et al. (101), a random insertion of the human eNOS DNA fragment into mouse genes and driven by the native eNOS promoter later generated by R. van Haperen (143).

**Single vs. combined NOS knockout mice.** The targeted deletion of eNOS has revealed a fundamental role of this isoform in the functional and structural responses of the vessel wall to metabolic and mechanical stress and of the heart to ischemic injury and pressure and volume overload. Several strains of eNOS knockout mice have showed significant systemic hypertension, which unequivocally confirmed a critical role of basal NO production in blood pressure regulation (121). In addition, these mice showed an increased neointimal formation and abnormal vascular remodeling in response to vascular injury and an accelerated atherosclerotic lesion formation when crossbred with ApoE−/− mice. When subjected to coronary artery ligation, they showed an impaired survival, a greater contractile dysfunction, and a weakened LV remodeling. Of interest, very similar vascular injury and atherosclerotic phenotypes have been described in neuronal NOS-knockout (nNOS−/−) mice, and the observations that the nNOS gene is upregulated with vascular injury in wild-type mice suggests a compensatory role in the presence of reduced eNOS activity.

In contrast, the targeted deletion of iNOS led to a blunted hypotensive response to sepsis, which in wild-type mice is caused by inappropriate vasodilation when NO is produced in large quantities. The vascular injury phenotype of nNOS−/− mice is more complex with vasculoprotection or exacerbation of the lesion depending on oxidant or antioxidant conditions, which in turn is dictated by the balance between NO and ROS generation and substrate and cofactor availability (121).

Because the phenotypic interpretation of mutant mice with deletion of a single NOS isoform might be confounded by compensatory interactions between residual NOS isoforms, the ultimate role of endogenous NO in cardiovascular disease can best be appreciated in triply deficient NOS1−2−3− mice. The observations that the nNOS gene is upregulated with vascular injury in wild-type mice suggests a compensatory role in the presence of reduced eNOS activity.

**Transgenic eNOS overexpression.** Consistent with observations in eNOS knockout mice, genetically engineered mice with endothelial cell-specific eNOS overexpression showed mild hypertension, variable atherogenesis when backcrossed with ApoE−/− mice, and a reduction in I/R injury and postinfarction heart failure. The underlying mechanisms were proposed to resemble those implicated in the beneficial effects of NO-donor compounds in similar disease conditions, including decreased leukocyte and platelet activation and reduced afterload through potent vasodilation (12). Alternatively, mice with cardiac myocyte-restricted eNOS overexpression showed a blunted chronotropic and inotropic response to catecholamines and a better-preserved systolic and diastolic function after myocardial infarction with reduced dilation of the LV (59).

These studies in NOS-deficient and eNOS-overexpressing mice have emphasized the importance of cellular and even subcellular isoform localization in mouse models of human cardiovascular disease. However, the observations that under conditions of BH4 or l-arginine deficiency and enhanced oxidative stress, eNOS-derived NO may contribute to atherogenesis, deleterious LV remodeling, and vascular (cross-)tolerance to endothelium-dependent relaxation emphasize the critical role of cofactors and substrate in determining the balance between eNOS-mediated protection or harm (102, 133).

**Discussion**

The biochemical, molecular, biological, and experimental evidence supports an important role of eNOS uncoupling in endothelial and myocardial dysfunction associated with many cardiovascular diseases. Accordingly, stabilizing NOS function and suppressing NOS-derived ROS production is a compelling strategy to translational studies in patients with a wide spectrum of cardiovascular disease. There is a great need for significant trials that further explore the importance of eNOS uncoupling in the primary phases of the onset of the different cardiovascular diseases. An overview of selected clinical trials of eNOS modulators are provided in Table 1. In addition, more knowledge is needed to obtain “preferably clinical” data regarding the effect of eNOS modulation on the other parts of NO-signaling pathways. To our knowledge no clinical data exist on the acute and long-term effects of BH4 administration on NO generation in other organs and on iNOS activity.

The different transgenic mouse models provide the necessary foundation for future translation into novel clinical approaches to prevent and treat cardiovascular disease. Whether enhanced NO bioavailability will best be achieved through local NOS gene transfer, or NO inhalation, local delivery of next-generation NO-donor compounds or eNOS cofactors remains to be tested in focused translational studies.

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**DISCLOSURES**

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

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