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

The role of NO synthase substrate: l-Arginine

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
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 NO synthesis (14). Importantly, intracellular L-arginine levels may never decrease to the subsaturating levels for NO, even under nonpathophysiological conditions (28), and L-arginine is intracellularly regenerated (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 BH$_4$ 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 hypertrophic kidneys and excessive crystal deposition, for example, L-arginine administration increases oxidative and nitrosative stress in an $\text{L}^\omega$-nitro-L-arginine methyl ester (L-NAME) reversible manner (54). Thus the supplementation of L-arginine is not a promising strategy to treat cardiovascular diseases.

### The Role of NOS Cofactor BH$_4$

BH$_4$ has been recognized as an essential cofactor of eNOS since it was first described in 1989 (134). In its coupled state, BH$_4$ 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 BH$_4$, 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 BH$_4$ 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 BH$_4$ in eNOS enzymatic function have been demonstrated: (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 BH$_4$ by controlling its protonation state. BH$_4$ 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 BH$_4$ suggests 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 BH$_4$ or an upregulation of the rate-limiting enzyme of BH$_4$ synthesis, guanosine triphosphate cyclohydrolase-1 (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 BH$_4$ in a spontaneously hypertensive rat model (27). The administration of L-arginine to bEnd.3 cells, without an administration of additional BH$_4$, exacerbated ROS generation caused by indicating that eNOS uncoupling induced ROS generation because of relative BH$_4$ deficiency (11). In vivo investigation of eNOS regulation clearly indicates a very tight stoichiometric relation between BH$_4$ and L-arginine, with 3000 molecules of BH$_4$ bound to each arginine molecule (54).

**Fig. 1.** Schematic overview of the different endothelial nitric oxide (NO) synthase (eNOS) modulators. BH$_4$, tetrahydrobiopterin.
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 BH3 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 Ser1177 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 endothelial-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-lactotrienol (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 bipterins (BH2/BH4 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 thermothermal and photo-stable 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 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 properties preserved vascular BH4 levels and eNOS activity in atherosclerotic mice (31).
effects (36, 53, 146) and can either directly interact with eNOS or indirectly by restoring the bioavailability of BH₄ (56). 5-Methyltetrahydrofolate (5-MTHF), the active metabolite of FA, has a similar structure as that of BH₄. Dihydrofolate reductase is involved in the reduction of oxidized BH₂ back to BH₄, whereas dihydrolipid reductase converts quinonoid-BH₂ to BH₄.

In addition, 5-MTHF can chemically stabilize BH₄ and ameliorate the binding affinity of BH₄ 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 BH₄ 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 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 (NO₂⁻) 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 (XO) (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 diazenium-diolate 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 NO₃ 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 NO₂ originating from eNOS. Exogenous sources, principally environmental pollutants and intake of vegetables, also contribute to this NO reserve. While the anion NO₃ 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 NO₃ represents a physiologically relevant storage reservoir of NO in blood and tissues with distinct signaling functions at near-physiological concentrations (19). The NO₃ 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). NO₃ 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 NO₃ anions makes it a potentially inter-
estig drug candidate when NO bioavailability is low. How-
over, the major concern of such a strategy remains the potential
risk of inadvertent systemic vasodilation and hypotension
caused by circulating NO₂ reductases. Currently, there are
several clinical studies underway to investigate safety, i.e.,
methemoglobin formation and inadvertent hypotension, and
potential therapeutic efficacy of NO₂ administration in pa-
patients with acute myocardial infarction, cerebral vasospasm,
and pulmonary hypertension (19). When NO₂ is administered
during noninflammatory states, one of the mechanisms thought
to mediate the beneficial effects is that nitrates are stored in
erotrocytes and can be secondarily reduced to vasoactive NO
by, e.g., XORs under anaerobic conditions (10). Although
NO₂ has been proposed as a therapy to increase NO produc-
tion via heme-containing enzymes, the administration of NO₂
in chronic inflammatory disease or infection states needs to be
avoided. Because under these conditions, myeloperoxidase
in a reaction with NO₂ to form nitrogen dioxide radical, which are
injurious (18).

The prominent function of circulating NO species and trans-
port of bioactivity in some way from the lungs to the periphery
is further supported by a variety of animal and human studies
using NO gas for inhalation (74, 96). In concert, these inves-
tigations have clearly shown that breathing NO gas (up to 80
parts/million) for variable periods of time has not only prom-
inent and selective pulmonary vasodilator effects but also
detectable systemic effects in remote vascular beds. The latter
includes an increase in organ blood flow, an inhibition of
coronary platelet-mediated thrombosis, and a marked protec-
tion against regional I/R injury. Importantly, none of the above
effects was accompanied by prohibitive systemic hypotension.

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
important and selective pulmonary vasodilator effects but also
detectable systemic effects in remote vascular beds. The latter
includes an increase in organ blood flow, an inhibition of
coronary platelet-mediated thrombosis, and a marked protec-
tion against regional I/R injury. Importantly, none of the above
effects was accompanied by prohibitive systemic hypotension.

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 for-
mula, C17H13F2NO3) have been described (152) as two
novel transcription enhancers of eNOS. These two small
molecular-weight 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) remodel-
ing and myocardial dysfunction. Moreover, AVE9488 in-
creased the levels of circulating endothelial progenitor cell
levels in rats with myocardial infarction-induced heart failure
(41). In primary human umbilical ven endothelial cells, treat-
ment of AVE9488 showed both an increased eNOS mRNA and
protein expression as well as an enhanced bradykinin-stimu-
lated release of NO (152). Following these initial observations
in cell culture conditions, AVE9488 has been shown to im-
prove eNOS expression levels and confer cardiovascular pro-
tective effects in different transgenic animal models. For ex-
ample, subchronic treatment (17 days) with AVE9488 (30
mg·kg⁻¹·day⁻¹) once a day in adult C57BL/6j mice and a
lower dose (10 mg/kg twice daily) in ApoE⁻/⁻ mice signifi-
cantly 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 nontreated 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 colocolated 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 H2O2 (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 (131). Methyl-β-cyclohexanecarboxylate can inhibit caveolae-mediated 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-cyclohexanecarboxylate as an eNOS modulator in cardiovascular diseases are available.

**Phosphorylation of eNOS**

eNOS activity is also posttranslationally regulated by phosphorylation at postresidues. The phosphorylation of eNOS at Ser1177 and Thr495 influences its activity in endothelial cells. So far, the knowledge of eNOS phosphorylation in cardiac myocytes is restricted to Ser1177 (94). Hsp90 is thought to be crucial for Akt-induced phosphorylation of eNOS-Ser1177 (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 serine1177 of eNOS (p-eNOSs1177), 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), i-NAME, or 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxide (PTIO; NO scavenger), thus forming a feed-forward cycle. Based on these findings, a netrin-1/DCC/ERK1/2/eNOSs1177/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 therapies 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 confused 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>
<thead>
<tr>
<th>Dose</th>
<th>Outcome</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Arginine</td>
<td>3–8 g/day</td>
<td>No acute pharmacological effects. Low ADMA, no effect; high ADMA, normalizes endothelial function. No serious adverse effects, possibly associated with higher mortality, no improvement of vascular stiffness measurements, left ventricular ejection fraction. No improvement of vascular reactivity, no benefit, possible harm.</td>
</tr>
<tr>
<td>Peripheral arterial disease</td>
<td>3 g/day po for 6 mon</td>
<td>Improvement of endothelial-dependent dilation, higher serum folate levels, lower total plasma homocysteine levels. Not significant.</td>
</tr>
<tr>
<td>FA/5-MTHF</td>
<td>FA, 10 mg/day po for 8 wk</td>
<td>Improvement of endothelial function. Not significant.</td>
</tr>
<tr>
<td>Coronary artery disease</td>
<td>5-MTHF 0.13 mg/kg body wt</td>
<td>Improvement of endothelial function. Not significant.</td>
</tr>
<tr>
<td>6R-BH4 (analog of BH4)</td>
<td>5 mg/kg po, twice daily for 8 wk</td>
<td>Improvement of endothelial dysfunction. Not significant.</td>
</tr>
<tr>
<td>Hypertension</td>
<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 statistically significant. Drop of 6.4 mmHg in patients' SBP. Not significant.</td>
</tr>
<tr>
<td>Endothelial dysfunction</td>
<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>Improvement of endothelial function. Not significant.</td>
</tr>
<tr>
<td>Sickle cell disease</td>
<td>400 mg po, twice daily for 24 wk</td>
<td>Improvement of endothelial function. Not significant.</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 promotor 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 accelerated atherosclerotic lesion formation when crossed 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 triplicy deficient NOS1−2−3−/− mice. The phenotype of combined mutants closely resembles aging, metabolic syndrome in humans with marked activation of the renin-angiotensin system, and nephrogenic diabetes insipidus and manifests marked coronary atherosclerotic lesions, spontaneous myocardial infarction, and sudden death (97). Taken together, so far these observations provide the best evidence for a central role of defective NO production in the pathogenesis of cardiovascular disease.

**Transgenic eNOS overexpression.** Consistent with observations in eNOS knockout mice, genetically engineered mice with endothelial cell-specific eNOS overexpression showed mild hypotension, 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 dilatation 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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