Prevention of Atherosclerosis by Interference with the Vascular Nitric Oxide System

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Abstract: Nitric oxide (NO) produced by endothelial NO synthase (eNOS) represents an anti-atherosclerotic principle. NO bioavailability is decreased in atherosclerosis due to increased NO inactivation by reactive oxygen species and reduced NO synthesis. Various types of vascular pathophysiology are associated with oxidative stress, with NADPH oxidases as the major source of reactive oxygen species. These inactivate NO. Also, oxidative stress is likely to be the main cause for oxidation of the essential NOS cofactor, tetrahydrobiopterin (BH_4). A lack of BH_4 leads to eNOS uncoupling (i.e., uncoupling of oxygen reduction from NO synthesis in eNOS). Based on these pathomechanisms, the therapeutic potential of a number of compounds is discussed in this review: (1) NO donors; (2) L-arginine; (3) folic acid; (4) BH_4 and its precursor sepiapterin; (5) compounds that upregulate eNOS and concomitantly maintain eNOS activity (e.g. midostaurin, betulinic acid, ursolic acid, AVE9488 and AVE3085); (6) compounds that enhance the *de novo* synthesis of BH_4 by stimulating expression or activity of GTP cyclohydrolase I; and (7) 3-hydroxy-3-methylglutaryl-coenzyme A inhibitors (statins) and drugs interrupting the renin-angiotensin-aldosterone system. Statins, angiotensin II type 1 receptor blockers, angiotensin-converting enzyme (ACE) inhibitors, the aldosterone antagonist eplerenone and the renin inhibitor aliskiren enhance NO bioactivity and reduce atherosclerosis progression through multiple mechanisms.

Keywords: Nitric oxide, atherosclerosis, endothelial dysfunction, eNOS uncoupling, tetrahydrobiopterin.

INTRODUCTION

Nitric oxide (NO) is generated from the conversion of L-arginine to L-citrulline by the enzymatic action of an NADPH-dependent NO synthase (NOS), which requires Ca^{2+}/calmodulin, flavin adenine dinucleotide, and flavin mononucleotide, and (6R)-5,6,7,8-tetrahydro-L-biopterin (BH_4) as the cofactors [1]. Three NOS isoforms have been identified: neuronal NOS (nNOS), inducible NOS (iNOS) and endothelial NOS (eNOS) [1, 2].

NO IN ATHEROSCLEROSIS

eNOS-Derived NO is an Anti-Atherosclerotic Principle

In the blood vessels, NO is produced from the endothelium mainly by the constitutively expressed eNOS, which is activated by shear-stress of the flowing blood or agonists such as bradykinin and acetylcholine. Besides its role as endothelium-derived relaxing factor (EDRF), NO protects blood vessels from thrombosis by inhibiting platelet aggregation and adhesion. In addition, endothelial NO possesses multiple anti-atherosclerotic properties, which include (i) prevention of leukocyte adhesion to vascular endothelium and leukocyte migration into the vascular wall; (ii) decreased endothelial permeability, reduced influx of lipoproteins into the vascular wall and inhibition of low-density lipoprotein (LDL) oxidation; and (iii) inhibition of DNA synthesis, mitogenesis, and proliferation of vascular smooth muscle cells [3, 4].

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NO Bioactivity is Decreased in Atherosclerosis

An impairment of endothelium-dependent relaxations is present in atherosclerotic vessels even before vascular structural changes occur. This demonstrates a reduced bioavailability of eNOS-derived NO. Endothelial dysfunction and reduction of eNOS-derived NO markedly contribute to atherogenesis [5].

All major risk factors for atherosclerosis such as hyperlipidemia, diabetes, hypertension, and smoking are associated with endothelial dysfunction [5]. Impaired endothelial function has also been observed in animal models of atherosclerosis [6, 7]. Although the underlining mechanisms of endothelial dysfunction are multifactorial, the major cause is an impairment of the eNOS/NO pathway, which include the reduced NO production by eNOS, increased degradation of NO by reaction with superoxide, and decreased sensitivity to NO [8].

The reduced NO production by eNOS is a result of an inhibition of eNOS enzymatic activity and/or a dysfunction of the enzyme (i.e., eNOS uncoupling, see below). The expression of eNOS, at least in early atherosclerosis, is unchanged or even augmented, despite the presence of endothelial dysfunction [9]. The enzymatic activity of eNOS is inhibited by various mechanisms associated with atherosclerosis and hyperlipidemia. Pro-atherogenic lipids, such as oxidized low-density lipoprotein (oxLDL) and lysophosphatidylcholine, inhibit signal transduction from receptor activation to eNOS activation [5]. Hypercholesterolemic serum and LDL upregulate caveolin abundance, augments caveolin-eNOS heterocomplex, and thereby attenuates NO production from endothelial cells [10]. Endogenous NOS inhibitors such as asymmetrical dimethyl-

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arginine (ADMA) are also likely to be involved in the mechanisms of reduced NO production in atherosclerosis [11, 12]. Mechanisms underlying eNOS uncoupling are discussed separately later in this article.

ROLES OF THE DIFFERENT NOS ISOFORMS IN ATHEROSCLEROSIS

NOS inhibitors like N^G-nitro-L-arginine methyl ester (L-NAME) significantly accelerate atherosclerotic lesion development in rabbits [13, 14] and mice [15], suggesting that inhibition of endogenous NO synthesis facilitates the progression of atherosclerosis.

To study the effects of individual NOS isoforms on atherosclerosis, gene-modified mice have been used. Apolipoprotein E (apoE) knockout mice are a useful model to mimic human atherosclerosis. apoE knockout mice develop spontaneous atherosclerotic lesions in their aortas whose development is accelerated by a high fat “Western” diet [16, 17]. Mice with the gene of one of the NOS isoforms deleted have been crossbred with the apoE knockout mice (creating double knockout mice [2]).

nNOS and Atherosclerosis

Although brain nNOS contributes to tissue damage in the setting of cerebral ischaemia [2], NO produced by vascular nNOS is likely to be atheroprotective. Local adenovirus-mediated nNOS gene transfer to atherosclerotic carotid arteries reduces adhesion molecule expression and inflammatory cell infiltration in cholesterol-fed rabbits [18]. nNOS has been found in atherosclerotic plaques [19], in cells with properties of smooth muscle cells or macrophages [20, 21]. apoE/nNOS double knockout mice on Western diet develop greater atherosclerotic lesion areas than do apoE knockout mice [21]. In the presence of nNOS, lesion area is decreased (by 66% in male mice and by 31% in female mice) [21]. The atheroprotective effect of nNOS seems to be unrelated to blood pressure changes. nNOS knockout mice have blood pressure values similar to their wild-type littermates when awake, and tend to become even hypotensive under anesthesia [2]. In addition to its anti-atherosclerotic effect, nNOS has been shown to decrease mortality in mice [22].

iNOS and Atherosclerosis

In contrast to nNOS, iNOS seems to be a proatherogenic NOS isoform. apoE/iNOS double knockout mice show significantly smaller lesion areas compared to apoE knockout mice [23]. The lipoprotein profile does not differ between apoE knockout mice and apoE/iNOS double knockout mice. The reduction in atherosclerosis in double knockout animals is associated with decreased plasma levels of lipoperoxides, suggesting that reduction in iNOS-mediated oxidative stress may explain the protection from lesion formation in double knockout animals. iNOS-derived NO may largely contribute to vascular peroxynitrite production during early stage of atherogenesis [24]. This in turn may lead to oxidation and depletion of BH_4, resulting in eNOS uncoupling (see below).

eNOS and Atherosclerosis

Two laboratories have shown independently that eNOS deficiency promotes atherosclerosis. Knowles *et al* demonstrated that a genetic lack of eNOS (disrupted at the calmodulin binding site) resulted in enhanced atherosclerosis in apoE/eNOS double-knockout mice [25, 26]. Using another eNOS knockout strain (by disrupting the region that encodes for the NADPH ribose and adenine binding sites), Kuhlencordt *et al* also reported that eNOS deficiency promoted atherosclerosis in apoE/eNOS double-knockout mice [27, 28]. Fed with a "Western-type" diet, apoE/eNOS double-knockout mice showed significant increases in aortic lesion area, which were associated with peripheral coronary atherosclerosis and aortic aneurysm formation [27, 28]. These reports indicate that the absence of endogenous eNOS-derived NO caused by the lack of eNOS gene accelerates atherosclerosis.

In contrast, Shi *et al* reported a paradoxical reduction of atherosclerotic lesion size in high-cholesterol diet-induced atherosclerosis in eNOS knockout mice compared with wild-type mice [29]. The explanation for these contradictory results may be eNOS uncoupling in the latter case.

Controversial results have also been obtained in mice with overexpressed eNOS. Transgenic (eNOS-Tg) mice that overexpress eNOS mainly in the endothelium were crossbred with apoE knockout mice and fed with a "high-cholesterol diet". In these eNOS-overexpressing apoE knockout (apoE-KO/eNOS-Tg) mice, Ozaki *et al* found that the atherosclerotic lesion areas were significantly larger compared with control apoE knockout mice [30]. In contrast, van Haperen *et al* also crossbred apoE knockout mice with another line of eNOS transgenic mice and reported that atherosclerotic lesion size was reduced by eNOS overexpression [31].

It is now clear that eNOS is a Janus-faced enzyme [32]. A functional eNOS produces NO, whereas a dysfunctional eNOS generates superoxide. While NO produced by eNOS is an antiatherogenic factor, eNOS-derived superoxide is a pro-atherogenic molecule. This may explain the discre-pancy between the abovementioned studies. In apoE-KO/eNOS-Tg mice, eNOS was found under a dysfunctional state and producing superoxide [30]. Therefore, chronic over-expression of eNOS does not inhibit, but rather accele-rates atherosclerosis under hypercholesterolemia [5].

eNOS UNCOUPLING IN ATHEROSCLEROSIS

eNOS Uncoupling

Under a number of pathological conditions, the enzymatic reduction of molecular oxygen by eNOS is no longer coupled to L-arginine oxidation, resulting in production of superoxide rather than NO. This phenomenon is referred to as eNOS uncoupling [9, 33].

A number of potential mechanisms have been reported to contribute to eNOS uncoupling [33, 34], these include (i) BH_4 deficiency [35, 36]; (ii) shortage of L-arginine or Hsp90 [37]; (iii) eNOS dephosphorylation on threonine residue 495

[38, 39]; (iv) eNOS redistribution to the cytosolic fraction of the cell [40]; (v) oxidation of the zinc-thiolate cluster in eNOS [41]; or (vi) elevated ADMA levels [42]. Among all of these mechanisms, BH_4 is likely to represent the major "coupling switch" [43], and BH_4 deficiency seems to be the primary cause for eNOS uncoupling in pathophysiology.

Some researchers have postulated that eNOS may exist in two separate pools: a coupled form and an uncoupled form. The coupled enzyme is associated with the membrane and is readily accessible to the "signalome" for activation and NO production, whereas the uncoupled enzyme may reside in the cytosol and produces superoxide [34, 39]. In eNOS-overexpressing mice for example, there is clear evidence for eNOS uncoupling (i.e. eNOS-mediated ROS production). In the same mice, however, NO-generating activity is elevated 2-fold when compared with wild-type mice (the total eNOS protein levels are elevated 8-fold) [35]. Thus, it is possible that coupled eNOS and uncoupled eNOS may exist in the same tissue at the same time.

Role of BH_4 (and BH_2) in eNOS Functionality

BH_4 seems to function as both an allosteric and redox cofactor for eNOS, stabilizes eNOS and improves the binding affinity of L-arginine for eNOS. It participates in the catalytic cycle of NO synthesis by providing the second electron to the heme of eNOS [43, 44].

In BH_4 deficiency, reduction of molecular oxygen still occurs at the heme site of eNOS, but oxidation of the guanidino nitrogen of L-arginine is prevented, so that the reduced oxygen comes off the enzyme as superoxide [43-45].

The partially oxidized BH_4 analog 7,8-dihydrobiopterin (BH_2) has no eNOS cofactor activity and is unable to prevent ROS formation by eNOS [43]. On the contrary, BH_2 may even enhance superoxide formation from purified eNOS in the presence of saturating L-arginine concentration, probably by competition with BH_4 for eNOS binding [46, 47]. Thus, in addition to the absolute availability of BH_4, the ratio of BH_4/BH_2 is important for eNOS activity [48, 49].

A recent study indicates that BH_4/BH_2 ratio may be even more important than the absolute BH_4 for eNOS functionality [50]. BH_4 and BH_2 bind eNOS with equal affinity and BH_2 can rapidly and efficiently replace BH_4 in preformed eNOS-BH_4 complexes. Expose of endothelial cells to diabetic glucose levels does not change the total biopterin pool, whereas BH_2 levels increases from undetectable to 40% of total biopterin. This BH_2 accumulation is associated with eNOS uncoupling. Calcium ionophore-evoked NO synthesis correlates with intracellular BH_4/BH_2 but not with the absolute intracellular levels of BH_4. Reciprocally, eNOS-derived superoxide production has been found to negatively correlate with intracellular BH_4/BH_2. It appears likely that diminished intracellular BH_4/BH_2, rather than BH_4 depletion *per se*, is the molecular trigger for NO insufficiency [50].

eNOS Uncoupling in Atherosclerosis

All major risk factors for atherosclerosis such as hyperlipidemia, diabetes, hypertension, and smoking are associated with endothelial dysfunction. Evidence for uncoupling of eNOS has been obtained in endothelial cells treated with LDL [51], in peroxynitrite-treated rat aorta [52], and in isolated blood vessels from animals with pathophysiological conditions such as spontaneously hypertensive rats (SHR) [53], stroke-prone SHR [54], angiotensin II-induced hypertension [55], hypertension induced with the mineralocorticoid deoxycorticosterone acetate (DOCA) [56], streptozotocin-induced diabetes [57], or nitroglycerin tolerance [58]. Importantly, eNOS uncoupling has also been seen in patients with endothelial dysfunction resulting from hypercholesterolemia [59], diabetes mellitus [60], or essential hypertension [61]; in chronic smokers [62]; and in nitroglycerin-treated patients [63].

eNOS uncoupling has been demonstrated in animal models of atherosclerosis as well. In apoE knockout mice, aortic superoxide production can be reduced by NOS inhibitor L-NAME [6, 64], indicating that eNOS is a source of superoxide in this disease model. Also in eNOS-overexpressing apoE knockout mice, eNOS has been found in an uncoupled state [30]. One major reason for eNOS uncoupling in atherosclerosis seems to be a BH_4 deficiency.

BH_4 Deficiency in Atherosclerosis

BH_4 levels in the aortas from diet-induced hypercholesterolemic rabbits were markedly reduced compared with those from normocholesterolemic rabbits [49]. Aortic BH_4 levels are decreased by 50% in markedly hypercholesterolemic apoE knockout mice compared with wild-type mice [30]. In contrast, d'Uscio *et al* reported that in the aortas of apoE knockout mice with moderate hyper-cholesterolemia, BH_4 levels were increased by 1.8-fold compared with those in control mice [65, 66]. A major component of increased BH_4 synthesis in apoE-deficient mice is localized in the vascular media (thus accessible for iNOS but not for eNOS) due to enhanced protein expression and enzymatic activity of GCH1 [66]. The discrepant results in vascular BH_4 levels in apoE knockout mice may be at least partly explained by the difference in the levels of oxidative stress [5]. The studies of Vasquez-Vivar *et al* and Ozaki *et al* were conducted in animals with severe hypercholesterolemia, which is likely associated with high oxidative stress (and thus extensive oxidation of BH_4 to BH_2), whereas d'Uscio *et al* used animals with mild hypercholesterolemia [30, 49, 65, 66]. This is important, because oxidative stress not only decreases the absolute BH_4 levels, but also increases the levels of BH_2, which may enhance eNOS uncoupling by competition with BH_4.

Of note, it is the stoichiometric relationships between BH_4 and eNOS rather that the absolute BH_4 levels that are important for the functionality of eNOS (coupled or uncoupled). Even in the absence of vascular disease, eNOS overexpression in the endothelium without a concomitant increase in BH_4 levels can result in eNOS uncoupling, as seen in eNOS transgene mice [35].

In normal vascular tissue, the overwhelming majority of vascular BH_4 is present in the endothelium [67-69]. The tissue levels of BH_4 are determined by a balance between its production and degradation. BH_4 is synthesized from guanosine 5'-triphosphate (GTP) via a *de novo* pathway by the rate-limiting enzyme GTP cyclohydrolase I (GCH1). Alternatively, the synthesis of BH_4 can occur via a so-called

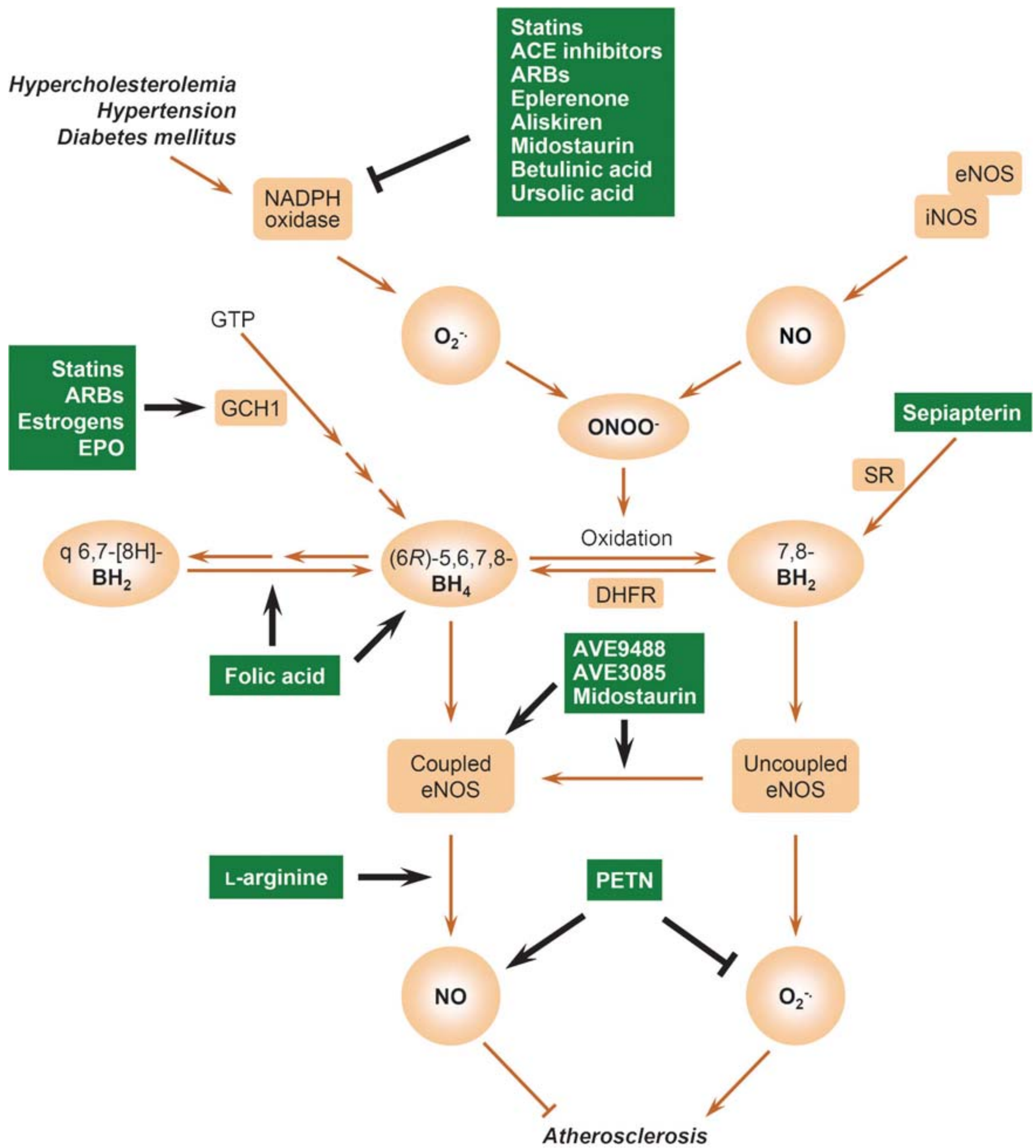

Fig. (1). Potential NO-based therapeutic approaches for atherosclerosis. The essential NOS cofactor (6*R*)-5,6,7,8-tetrahydro-L-biopterin ((6*R*)-5,6,7,8-BH_4) is synthesized from guanosine 5'-triphosphate (GTP) via a *de novo* pathway by the rate-limiting enzyme GTP cyclohydrolase I (GCH1). Alternatively, the synthesis of (6*R*)-5,6,7,8-BH_4 can occur via a so-called salvage pathway, from 7,8-dihydrobiopterin (7,8-BH_2) back to (6*R*)-5,6,7,8-BH_4 by the enzyme dihydrofolate reductase (DHFR); or from quinonoid 6,7-[8H]-BH_2 back to (6*R*)-5,6,7,8-BH_4 by dihydropteridine reductase. Under pathological conditions such as hypercholesterolemia, hypertension or diabetes, NADPH oxidase-derived superoxide ($O_2^{\cdot-}$) may react with nitric oxide (NO) derived from inducible or endothelial NO synthase (iNOS or eNOS). The resulting peroxynitrite ($ONOO^-$) oxidizes (6*R*)-5,6,7,8-BH_4 to 7,8-BH_2. BH_4 deficiency leads to superoxide production by eNOS (eNOS “uncoupling”). While NO generated by eNOS is an atheroprotective factor, superoxide produced by uncoupled eNOS is a pro-atherogenic molecule. Pentaerythritol tetranitrate (PETN) is a NO donor that does not induce significant nitrate tolerance and reduces oxidative stress (probably by inducing heme oxygenase 1). As a substrate, L-arginine stimulates NO release from eNOS. Folic acid may improve eNOS functionality by stabilizing (6*R*)-5,6,7,8-BH_4 and stimulating the endogenous regeneration of quinonoid 6,7-[8H]-BH_2 back to (6*R*)-5,6,7,8-BH_4. Sepiapterin can be reduced in cells by sepiapterin reductase (SR) to 7,8-BH_2, and further by DHFR to form (6*R*)-5,6,7,8-BH_4. Midostaurin, betulinic acid and ursolic acid upregulate eNOS and concomitantly decrease NADPH oxidase expression. AVE9488 and AVE3085 are two eNOS transcription enhancers that reverse eNOS uncoupling and preserve eNOS functionality. Statins, angiotensin II type 1 receptor blockers (ARBs), estrogens and erythropoietin (EPO) enhance (6*R*)-5,6,7,8-BH_4 synthesis by stimulating GCH1 expression or activity. Statins, ARBs, angiotensin-converting enzyme (ACE) inhibitors, the aldosterone antagonist eplerenone and the renin inhibitor aliskiren prevent (6*R*)-5,6,7,8-BH_4 oxidation by decreasing the expression and/or activity of NADPH oxidase.

salvage pathway from BH_2 back to BH_4 by the enzyme dihydrofolate reductase (DHFR) [70]; or from quinonoid BH_2 back to BH_4 by dihydropteridine reductase (DHPR) [71]. The tissue levels of BH_4 are also determined by their degradation, namely by BH_4 oxidation to BH_2 [71]. BH_4 can be rapidly oxidized by reactive oxygen species such as peroxynitrite [41, 52, 56].

The *GCH1* gene, encoding GCH1, is expressed in several cell types such as macrophages [72], hepatocytes [73], and endothelial cells [74, 75]. Pro-atherogenic oxidized LDL reduces *GCH1* gene expression in vascular smooth muscle cells [77]. Several *in vitro* studies suggest that *GCH1* expression in endothelial cells can be induced by cytokines [74-76]. Whether the high concentrations of multiple cytokines required from endothelial *GCH1* induction can be reached *in vivo* (even in atherosclerotic vessels) is questionable, although the systemic inflammatory stimuli may be sufficient to induce *GCH1* in non-vascular tissues. In patients with coronary artery disease, plasma (but not vascular) biopterin levels were correlated with plasma C-reactive protein levels (a marker of systemic inflammation) [69]. However, the plasma BH_4 levels are not relevant for eNOS functionality, because only vascular (but not plasma) BH_4 levels are inversely associated with vascular superoxide production and positively associated with eNOS coupling and NO-mediated endothelial function in human atherosclerosis [69].

Thus, the reason for vascular BH_4 deficiency in atherosclerosis is more likely BH_4 oxidation due to oxidative stress (see below) rather than decreased biosynthesis by GCH1.

NADPH Oxidase-Mediated Oxidative Stress in BH_4 Deficiency

In the vascular wall, ROS can be produced by several enzyme systems, including NADPH oxidases, xanthine oxidase, enzymes of the respiratory chain, and cytochrome P450 monoxygenases [78-80]. Among them, NADPH oxidase plays a major role in vascular cells [81-83]. In atherosclerotic vessels, increased expression of subcomponents of NADPH oxidase has been found [84-87]. In the early stage of atherosclerosis, superoxide seems to be produced from NADPH oxidase localized in the endothelium; in advanced atherosclerosis, vascular smooth muscle cells serve as the major source of NADPH oxidase-derived superoxide [5, 88].

The tissue levels of BH_4 depend on its synthesis and its degradation/oxidation [71]. BH_4 can be rapidly oxidized by the reactive oxygen species peroxynitrite [41, 52, 56]. In DOCA-salt hypertensive mice, superoxide produced by NADPH oxidase leads to the formation of peroxynitrite in reaction with NO, and induces uncoupling of eNOS. With elevated oxidative stress, the oxidation of BH_4 is enhanced and vascular tissue levels of BH_2 increase [56]. Also in animal models of diabetes [57], angiotensin II-induced hypertension [55, 89], nitrate tolerance [58] as well as in spontaneously hypertensive rats [53], we have observed that eNOS uncoupling is associated with increased expression of vascular NADPH oxidases. The increased expression of certain NADPH oxidase components can be partially suppressed *in vivo* by inhibition of protein kinase C (PKC), indicating the involvement of this signalling pathway in NADPH oxidase induction under pathological conditions [90]. Pharmacological suppression of NADPH oxidase expression resulted in enhancement of vascular BH_4 levels, reversal of eNOS uncoupling and improved endothelial function [53].

POTENTIAL CLINICAL INTERVENTIONS

On the basis of the pathophysiology mentioned above, the following pharmacological approaches that potentially increase bioactive NO, or restore eNOS functionality, have been tested.

NO Donors

Organic nitrates such as nitroglycerin (NTG), isosorbide mononitrate (ISMN), and isosorbide dinitrate (ISDN), have been used as therapeutic agents for over a century [91].

At first glance, they appear as an optimal choice to replace endogenous NO in the vasculature. However, epidemiologic evidence indicates that chronic administration of long-acting nitrates increases (rather than decreases) fatal and non-fatal cardiovascular events [92, 93]. In fact, continuous transdermal administration of NTG has been associated with increased vascular production of superoxide anion and endothelial dysfunction [91], a mechanism also involved in nitrate tolerance [94-96]. However, there seem to be substantial differences between the individual organic nitrates [97, 98]. Tolerance to NTG is likely attributable to an increased production of reactive oxygen species and an inhibition of its bioactivating enzyme. Therapy with pentaerythritol tetranitrate (PETN) is devoid of tolerance, at least in part, due to an induction of vascular heme oxygenase 1 [99-101]. Indeed, long-term treatment of hypercholesterolaemic rabbits with a low dose of PETN has been shown to reduce LDL oxidation, endothelial dysfunction, and progression of lesion formation [102-104]. Also ISMN has been shown to decrease superoxide anion and partially prevent intimal lesion formation and endothelial dysfunction in hypercholesterolemic rabbits, despite moderate nitrate tolerance [105, 106].

Bone marrow-derived endothelial progenitor cells (EPCs) play a fundamental role in vascular repair and are regulated by NO. Reduced levels and impaired function of EPCs promote the development and progression of atherosclerotic lesions [107]. A recent study demonstrates that long-acting nitrates increase levels of circulating EPCs, but differ in their effects on EPC function dependent on the induction of intracellular oxidative stress [107]. Treatment of rats with pentaerythritol-trinitrate (PETriN) or ISDN increases circulating EPC levels. EPC from ISDN- but not PETriN-treated animals display impaired migratory capacity and increased reactive oxygen species formation. *In vitro* treatment with ISDN reduces migration and incorporation of human EPCs into vascular structures on matrigel, whereas PETriN improves EPC function [107]. Organic nitrates that improve EPC function may confer long-term cardiovascular protection based on their beneficial effects on EPC biology.

BH_4 and Sepiapterin

As discussed above, BH_4 deficiency is likely to be the primary cause of eNOS dysfunction and BH_4 supplementation can reverse eNOS uncoupling [108]. Administration of BH_4 restores endothelial function in animal models of hypertension [109], diabetes [110] and insulin resistance [111], as well as in patients with diabetes mellitus [60], essential hypertension [61], hypercholesterolemia [59], atherosclerosis [112], and in chronic smokers [62]. Oral administration of BH_4 also slows the progression of atherosclerosis in apoE knockout mice [113].

Sepiapterin, a precursor to BH_4, has been shown to restore endothelial function in acute studies. For example, incubation of vessels from apoE knockout mice with sepiapterin (10 µM for 30-60 min) improves endothelial function and decreases superoxide production [52]. *Ex vivo* incubation with sepiapterin (1 µM for 15 min in organ chamber) improves endothelium-dependent vasodilatation in vessels from humans and pigs with atherosclerosis but not in non-diseased vessels [68]. Similarly, *ex vivo* intraluminal administration of sepiapterin (1 µM for 30 min) restores the impaired flow-dependent dilation in skeletal muscle arterioles of rats with type I diabetes mellitus [114].

However, high concentrations of sepiapterin may have detrimental effects. A 6-hour incubation of hyperlipidemic rabbit vessels with sepiapterin (100 - 500 µM) resulted in enhanced superoxide release, reduced NO formation, and diminished endothelium-dependent relaxation [49]. At high concentrations, sepiapterin may compete with BH_4 for the same binding site in eNOS [5, 46, 47, 115], thereby promoting eNOS uncoupling.

The long-term effects of sepiapterin supplementation on atherogenesis have not been studied yet. In diabetic (db/db) mice, chronic oral supplementation with sepiapterin (10 mg/kg/day) for 8 weeks prevents endothelial dysfunction and reduces oxidative stress [116]. Sepiapterin treatment did not change vascular BH_4 levels in these animals, but reduced BH_2/biopterin concentrations [116]. In another study, *in vivo* knockdown of GCH1 in mice resulted in eNOS uncoupling and elevated blood pressure. Sepiapterin treatment (10 mg/kg/day, 7 days, i.p.) partially reversed these effects [117].

L-Arginine

Infusion of L-arginine into the coronary arteries enhances the blood flow response to acetylcholine in patients with coronary artery disease but not in controls [118]. Chronic treatment with L-arginine inhibits atherosclerotic lesion formation in animal models of atherosclerosis, such as diet-induced atherosclerosis models of rabbits [119] and LDL-receptor knockout mice [120]. In some other studies, however, L-arginine administration shows either no significant improvement of endothelium-dependent vasodilation [121, 122], or even increases vascular superoxide anion production [123]. Beneficial vascular effects of dietary L-arginine are more likely to be reached in patients with L-arginine deficiency or elevated ADMA levels [124].

Although L-arginine concentrations in normal endothelial cells are much higher than the K_M of eNOS for L-arginine [125-127], L-arginine may become limited in hypercholesterolemia [33, 128]. OxLDL leads to activation and upregulation of arginase II and may thus "starve" eNOS [129]. Upregulation of arginase II results in eNOS uncoupling, and arginase inhibition results in eNOS recoupling [130, 131]. Therefore, a relative L-arginine deficiency in the vicinity of eNOS caused by excessive arginase activity is conceivable and may explain part of the beneficial effects of L-arginine supplementation.

Beneficial effects of supplemental L-arginine also could be due to local competition with the endogenous eNOS inhibitor ADMA [128, 132]. The endogenous ADMA levels may determine a subject's response to L-arginine supplementation. L-arginine appears to exert no effect in subjects with low ADMA levels, whereas in subjects with high ADMA levels, L-arginine restores the L-arginine/ADMA ratio and thereby normalizes endothelial function [124].

Folic Acid

The interest in folic acid for the treatment of cardiovascular disease stems from its critical role in converting homocysteine to methionine. Hyperhomocysteinemia has been found associated with a higher risk of cardiovascular disease in epidemiological studies, and dietary folate fortification lowers plasma homocysteine levels [133, 134].

Folic acid and its principal circulating metabolite, 5-methyltetrahydrofolate, have been shown to restore endothelial function in patients with hypercholesterolemia [135], diabetes mellitus [136], or hyperhomocysteinemia [137], and to reduce atherosclerotic lesions in apoE knockout mice [138]. These effects appear to be homocysteine-independent but rather related to their role in eNOS function [134, 139]. Folic acid and 5-methyltetrahydrofolate have been shown to increase vascular BH_4 levels and BH_4/BH_2 ratio and to reverse eNOS uncoupling [140-142]. Several mechanisms may be involved in this action [33, 143]: (i) direct interaction of 5-methyltetrahydrofolate with eNOS; (ii) enhancement of BH_4 binding to eNOS; (iii) chemical stabilization of BH_4; and (iv) augmentation of BH_4 regeneration from quinonoid BH_2.

However, recent clinical trials have failed to demonstrate a benefit of long-term use of folic acid [144, 145], probably due to additional effects of folic acid such as enhancement of cell proliferation, DNA methylation and ADMA formation [146].

GCH1-Upregulating Compounds

Although reduced biosynthesis of BH_4 may not be the principal mechanism of BH_4 loss in vascular disease, GCH1 seems to be a rational target to augment endothelial BH_4 and to normalize eNOS functionality in atherosclerosis.

GCH1 gene transfer to human endothelial cells augments intracellular BH_4 levels in association with an increase in enzymatic activity of eNOS to produce NO [147]. *In vivo GCH1* gene transfer restores vascular BH_4 levels and endothelial function in low renin hypertension [148]. Conversely, treatment of endothelial cells with GCH1 inhibitors or GCH1 small-interference RNA reduces BH_4 levels and induces eNOS uncoupling [117]. Moreover, *in* *vivo* GCH1 knockdown increases aortic superoxide production and elevates blood pressure in wild-type, but not eNOS knockout mice [117].

Endothelial overexpression of GCH1 in apoE knockout mice results in increased aortic BH_4 levels, reduced endothelial superoxide production, reversal of eNOS uncoupling, and preserved NO-mediated endothelium-dependent vasorelaxation. Furthermore, aortic root atherosclerotic plaque is significantly reduced in apoE-KO/GCH-Tg mice compared with apoE knockout controls [6].

In a recent study, eNOS-overexpressing apoE knockout mice were crossed with mice overexpressing GCH1 to generate apoE-KO/eNOS-Tg/GCH-Tg mice. Atherosclerotic lesion formation was increased in apoE-KO/eNOS-Tg mice compared with apoE knockout mice. GCH1 overexpression in ApoE-KO/eNOS-Tg/GCH-Tg mice increased vascular BH_4 levels and reduced plaque area. This reduction was associated with decreased superoxide production from eNOS [149]. These data indicate that eNOS-derived reactive oxygen species play an important role in atherosclerosis progression and restoration of eNOS functionality by upregulation of GCH1 is a rational therapeutic approach.

Therefore, compounds that enhance GCH1 expression and/or activity may be of therapeutic interest. Because of this, GCH1 is currently a hot topic in the research field of vascular biology. GCH1 transcription has been shown to be upregulated by cytokines [72, 74, 75, 150, 151], insulin [152], hydrogen peroxide [153, 154], and 17β-estradiol [155]. Also statins [156, 157] and angiotensin II type 1 receptor blockers (ARBs) [158] have been shown to upregulate GCH1 expression (see below). Interestingly, laminar shear [159-161] and erythropoietin [162] have no effect on GCH1 protein expression but increase its activity.

Compounds Combining eNOS Upregulation with eNOS Recoupling

Strategies to increase eNOS protein without a concomitant augmentation of endothelial BH_4 levels may lead to eNOS uncoupling, enhanced oxidative stress and progression of vascular diseases. Therefore, compounds that increase eNOS protein levels are only beneficial when guaranteeing eNOS functionality.

In the past, we have found compounds that maintain eNOS functionality in disease, and, at the same time, upregulate expression of the enzyme. Midostaurin (4'-N-benzoyl staurosporine, CGP 41251, PKC-412) is a glycosidic indolocarbazole analog of staurosporine. Midostaurin upregulates eNOS expression by PKC-independent mechanisms [163, 164] and reduces NADPH oxidase expression via PKC inhibition [53]. By reducing NADPH oxidase-mediated oxidative stress, midostaurin reverses eNOS uncoupling in spontaneously hypertensive rats and in atherosclerosis-prone apoE knockout mice, which is associated with NO-mediated vasodilation and blood pressure reduction [53, 165]. However, the therapeutic use of PKC inhibitors is limited due to significant side effects *in vivo*.

Recently, we have identified two natural pentacyclic triterpenes - ursolic acid [166] and betulinic acid [167] - that upregulate eNOS, and at the same time, reduce NADPH oxidase expression in human endothelial cells through PKC-independent mechanisms. The therapeutic potential of such compounds in cardiovascular disease needs to be investigated in further studies.

Wohlfart *et al* have discovered two small-molecular-weight eNOS transcription enhancers - AVE9488 (4-fluoro-*N*-indan-2-yl-benzamide) and AVE3085 (2,2-difluoro-benzo [1,3]dioxole-5-carboxylic acid indan-2-ylamide) [64]. These compounds stimulate eNOS transcription in endothelial cells *in vitro* and in vascular tissues *in vivo*. Importantly, treatment of apoE knockout mice with AVE9488 enhances vascular BH_4 levels and reverses eNOS uncoupling. In apoE knockout mice, but not in eNOS-knockout mice, treatment with AVE9488 reduces cuff-induced neointima formation. A 12-week treatment with AVE9488 or AVE3085 reduces atherosclerotic plaque formation in apoE knockout mice, but not in apoE/eNOS-double knockout mice [64]. Moreover, AVE9488 reverses impaired functional activity of EPCs from patients with ischemic cardiomyopathy [168], and improves cardiac remodeling and heart failure after experimental myocardial infarction [169].

ESTABLISHED DRUGS WITH PLEIOTROPIC PROPERTIES RELATED TO THE VASCULAR NO SYSTEM

Statins

Statins are a group of lipid-lowering drugs, 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors, used in the prevention and treatment of cardiovascular diseases. Statins also possess cholesterol-independent or "pleiotropic" effects which include improvement of endothelial function, stabilization of atherosclerotic plaques, inhibition of oxidative stress and inflammation, and a reduction of thrombogenic response [170]. These beneficial effects of statins are, at least in part, mediated by an effect on eNOS because they can be blocked by eNOS inhibitors [171], and are absent in eNOS deficient mice [172]. Statins increase eNOS expression by stabilizing eNOS mRNA [173] and they enhance eNOS activity by decreasing caveolin abundance [174]. Statins ameliorate oxidative stress [175] by reducing the expression and/or activity of NADPH oxidase [176]. These effects may be partly responsible for the anti-atherogenic action of statins [177, 178].

Recently, statins have been shown to increase GCH1 mRNA expression in endothelial cells and to elevate intracellular BH_4 levels [156]. In streptozotocin-induced diabetic rats, atorvastatin normalizes endothelial function, reduces oxidative stress by inhibiting vascular NADPH oxidases, and prevents eNOS uncoupling by an upregulation of GCH1 [157].

Drugs Interrupting the Renin-Angiotensin-Aldosterone System

The renin-angiotensin-aldosterone system is upregulated in atherosclerotic vessels. Angiotensin II and aldosterone both promote endothelial dysfunction and atherosclerosis [179]. Angiotensin-converting enzyme (ACE) inhibitors significantly reduce cardiovascular events in patients with established or at high risk for coronary artery disease [180].

Eplerenone, a selective aldosterone antagonist, attenuates atherosclerosis in cholesterol-fed monkeys [181].

In a recent study, Imanishi *et al* investigated the effect of eplerenone and enalapril, alone or in combination, on atherosclerotic changes in genetically hyperlipidemic rabbits [179]. Both eplerenone and enalapril reduce NADPH oxidase activity, elevate vascular BH_4 levels (and thus limit eNOS uncoupling), and enhance NO bioavailability. Eplerenone also increases eNOS phosphorylation at Ser^{1177}. Both drugs decrease atherosclerotic plaque formation and the combination leads to an additive reduction [179].

Also ARBs may improve eNOS functionality. Losartan restores glomerular NO production by increasing GCH1 protein expression and elevating BH_4 levels in diabetic rats [158].

The renin inhibitor aliskiren (and ARB valsartan) increases eNOS mRNA stability, enhances eNOS phosphorylation at Ser^{1177}, decreases NADPH oxidase expression, augments vascular BH_4 levels, and restores eNOS uncoupling in Watanabe heritable hyperlipidemic rabbits [182]. The anti-atherosclerotic effect of aliskiren [183] is comparable with ARBs valsartan [182] or irbesartan [184]. Combination therapy with aliskiren and valsartan has an additive effect on endothelial function, BH_4 content, NO release, and plaque volume [182].

CONCLUSION

Reduced bioavailability of endothelial NO is involved in the initiation and progression of atherosclerosis. This is due to enhanced NO inactivation by reactive oxygen species, inhibition of eNOS activity and/or an eNOS uncoupling. Pharmacological approaches to improve eNOS functionality may be useful for the prevention and therapy of atherosclerosis.

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CONFLICT OF INTEREST

None.

ABBREVIATIONS

ACE	=	Angiotensin-converting enzyme
ADMA	=	Asymmetric dimethylarginine
apoE	=	Apolipoprotein E
ARBs	=	Angiotensin II type 1 receptor blockers
BH_2	=	7,8-Dihydrobiopterin
BH_4	=	(6R)-5,6,7,8-tetrahydro-L-biopterin, tetrahydrobiopterin
DHFR	=	Dihydrofolate reductase
DHPR	=	Dihydropteridine reductase
DOCA	=	Deoxycorticosterone acetate
EDRF	=	Endothelium-derived relaxing factor
eNOS	=	Endothelial nitric oxide synthase
EPCs	=	Endothelial progenitor cells
EPO	=	Erythropoietin
GCH1	=	Guanosine 5'-triphosphate cyclohydrolase I
GTP	=	Guanosine 5'-triphosphate
HMG-CoA	=	3-Hydroxy-3-methylglutaryl-coenzyme A
iNOS	=	Inducible nitric oxide synthase
ISDN	=	Isosorbide dinitrate
ISMN	=	Isosorbide mononitrate
KO	=	Knockout
L-NAME	=	L-N^G-nitroarginine methyl ester
LDL	=	Low-density lipoprotein
NADPH	=	Nicotinamide adenine dinucleotide phosphate (reduced form)
nNOS	=	Neuronal NOS
NO	=	Nitric oxide
NOS	=	Nitric oxide synthase
NTG	=	Nitroglycerin
oxLDL	=	Oxidized low-density lipoprotein
PETN	=	Pentaerythritol tetranitrate
PETriN	=	Pentaerythritol-trinitrate
PKC	=	Protein kinase C
Tg	=	Transgene
SHR	=	Spontaneously hypertensive rats
SR	=	Sepiapterin reductase

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