

Prevention of Atherosclerosis by Interference with the Vascular Nitric Oxide System

Huige Li* and Ulrich Förstermann

Department of Pharmacology, Johannes Gutenberg University, Mainz, Germany

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 (BH₄). A lack of BH₄ 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) BH₄ 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 BH₄ 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 Ca²⁺/calmodulin, flavin adenine dinucleotide, and flavin mononucleotide, and (6R)-5,6,7,8-tetrahydro-L-biopterin (BH₄) 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].

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-

*Address correspondence to this author at the Department of Pharmacology, Johannes Gutenberg University, Obere Zahlbacher Str. 67, D-55131 Mainz, Germany; Tel: + 49 (6131) 3936929; Fax: + 49 (6131) 3936611; E-mail: huigel@uni-mainz.de

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 BH₄, 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 discrepancy between the abovementioned studies. In apoE-KO/eNOS-Tg mice, eNOS was found under a dysfunctional state and producing superoxide [30]. Therefore, chronic overexpression of eNOS does not inhibit, but rather accelerates 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) BH₄ 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, BH₄ is likely to represent the major "coupling switch" [43], and BH₄ 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 BH₄ (and BH₂) in eNOS Functionality

BH₄ 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 BH₄ 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 BH₄ analog 7,8-dihydrobiopterin (BH₂) has no eNOS cofactor activity and is unable to prevent ROS formation by eNOS [43]. On the contrary, BH₂ may even enhance superoxide formation from purified eNOS in the presence of saturating L-arginine concentration, probably by competition with BH₄ for eNOS binding [46, 47]. Thus, in addition to the absolute availability of BH₄, the ratio of BH₄/BH₂ is important for eNOS activity [48, 49].

A recent study indicates that BH₄/BH₂ ratio may be even more important than the absolute BH₄ for eNOS functionality [50]. BH₄ and BH₂ bind eNOS with equal affinity and BH₂ can rapidly and efficiently replace BH₄ in preformed eNOS-BH₄ complexes. Expose of endothelial cells to diabetic glucose levels does not change the total biopterin pool, whereas BH₂ levels increases from undetectable to 40% of total biopterin. This BH₂ accumulation is associated with eNOS uncoupling. Calcium ionophore-evoked NO synthesis correlates with intracellular BH₄/BH₂ but not with the absolute intracellular levels of BH₄. Reciprocally, eNOS-derived superoxide production has been found to negatively correlate with intracellular BH₄/BH₂. It appears likely that diminished intracellular BH₄/BH₂, rather than BH₄ 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 BH₄ deficiency.

BH₄ Deficiency in Atherosclerosis

BH₄ levels in the aortas from diet-induced hypercholesterolemic rabbits were markedly reduced compared with those from normocholesterolemic rabbits [49]. Aortic BH₄ 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 hypercholesterolemia, BH₄ levels were increased by 1.8-fold compared with those in control mice [65, 66]. A major component of increased BH₄ 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 BH₄ 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 BH₄ to BH₂), 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 BH₄ levels, but also increases the levels of BH₂, which may enhance eNOS uncoupling by competition with BH₄.

Of note, it is the stoichiometric relationships between BH₄ and eNOS rather than the absolute BH₄ 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 BH₄ levels can result in eNOS uncoupling, as seen in eNOS transgene mice [35].

In normal vascular tissue, the overwhelming majority of vascular BH₄ is present in the endothelium [67-69]. The tissue levels of BH₄ are determined by a balance between its production and degradation. BH₄ 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 BH₄ can occur via a so-called

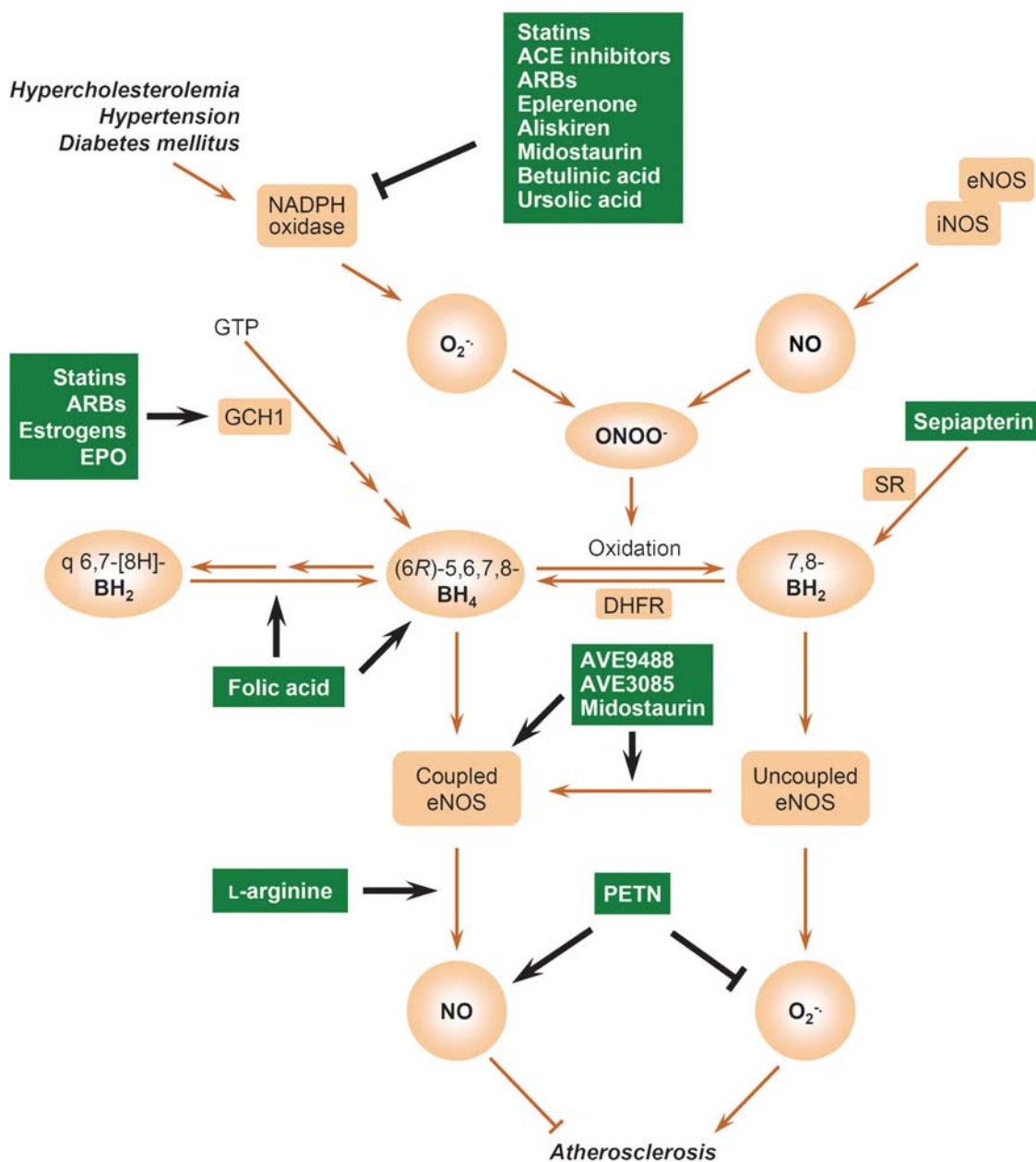


Fig. (1). Potential NO-based therapeutic approaches for atherosclerosis. The essential NOS cofactor (6R)-5,6,7,8-tetrahydro-L-biopterin ((6R)-5,6,7,8-BH₄) is synthesized from guanosine 5'-triphosphate (GTP) via a *de novo* pathway by the rate-limiting enzyme GTP cyclohydrolase I (GCH1). Alternatively, the synthesis of (6R)-5,6,7,8-BH₄ can occur via a so-called salvage pathway, from 7,8-dihydrobiopterin (7,8-BH₂) back to (6R)-5,6,7,8-BH₄ by the enzyme dihydrofolate reductase (DHFR); or from quinonoid 6,7-[8H]-BH₂ back to (6R)-5,6,7,8-BH₄ 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 ($ONOO^{\cdot}$) oxidizes (6R)-5,6,7,8-BH₄ to 7,8-BH₂. BH₄ 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 (6R)-5,6,7,8-BH₄ and stimulating the endogenous regeneration of quinonoid 6,7-[8H]-BH₂ back to (6R)-5,6,7,8-BH₄. Sepiapterin can be reduced in cells by sepiapterin reductase (SR) to 7,8-BH₂, and further by DHFR to form (6R)-5,6,7,8-BH₄. 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 (6R)-5,6,7,8-BH₄ synthesis by stimulating GCH1 expression or activity. Statins, ARBs, angiotensin-converting enzyme (ACE) inhibitors, the aldosterone antagonist eplerenone and the renin inhibitor aliskiren prevent (6R)-5,6,7,8-BH₄ oxidation by decreasing the expression and/or activity of NADPH oxidase.

salvage pathway from BH₂ back to BH₄ by the enzyme dihydrofolate reductase (DHFR) [70]; or from quinonoid BH₂ back to BH₄ by dihydropteridine reductase (DHPR) [71]. The tissue levels of BH₄ are also determined by their degradation, namely by BH₄ oxidation to BH₂ [71]. BH₄ can be rapidly oxidized by reactive oxygen species such as peroxynitrite [41, 52, 56].

The *GCHI* 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 *GCHI* gene expression in vascular smooth muscle cells [77]. Several *in vitro* studies suggest that *GCHI* expression in endothelial cells can be induced by cytokines [74-76]. Whether the high concentrations of multiple cytokines required from endothelial *GCHI* induction can be reached *in vivo* (even in atherosclerotic vessels) is questionable, although the systemic inflammatory stimuli may be sufficient to induce *GCHI* 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 BH₄ levels are not relevant for eNOS functionality, because only vascular (but not plasma) BH₄ 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 BH₄ deficiency in atherosclerosis is more likely BH₄ oxidation due to oxidative stress (see below) rather than decreased biosynthesis by GCH1.

NADPH Oxidase-Mediated Oxidative Stress in BH₄ 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 monooxygenases [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 BH₄ depend on its synthesis and its degradation/oxidation [71]. BH₄ 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 BH₄ is enhanced and vascular tissue levels of BH₂ 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 BH₄ 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.

BH₄ and Sepsipterin

As discussed above, BH₄ deficiency is likely to be the primary cause of eNOS dysfunction and BH₄ supplementation can reverse eNOS uncoupling [108]. Administration of BH₄ 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 BH₄ also slows the progression of atherosclerosis in apoE knockout mice [113].

Sepsipterin, a precursor to BH₄, has been shown to restore endothelial function in acute studies. For example, incubation of vessels from apoE knockout mice with sepsipterin (10 μM for 30-60 min) improves endothelial function and decreases superoxide production [52]. *Ex vivo* incubation with sepsipterin (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 sepsipterin (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 sepsipterin may have detrimental effects. A 6-hour incubation of hyperlipidemic rabbit vessels with sepsipterin (100 - 500 μM) resulted in enhanced superoxide release, reduced NO formation, and diminished endothelium-dependent relaxation [49]. At high concentrations, sepsipterin may compete with BH₄ for the same binding site in eNOS [5, 46, 47, 115], thereby promoting eNOS uncoupling.

The long-term effects of sepsipterin supplementation on atherogenesis have not been studied yet. In diabetic (db/db) mice, chronic oral supplementation with sepsipterin (10 mg/kg/day) for 8 weeks prevents endothelial dysfunction and reduces oxidative stress [116]. Sepsipterin treatment did not change vascular BH₄ levels in these animals, but reduced BH₂/biopterin concentrations [116]. In another study, *in vivo* knockdown of GCH1 in mice resulted in eNOS uncoupling and elevated blood pressure. Sepsipterin 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 BH₄ levels and BH₄/BH₂ 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 BH₄ binding to eNOS; (iii) chemical stabilization of BH₄; and (iv) augmentation of BH₄ regeneration from quinonoid BH₂.

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 BH₄ may not be the principal mechanism of BH₄ loss in vascular disease, GCH1 seems to be a rational target to augment endothelial BH₄ and to normalize eNOS functionality in atherosclerosis.

GCH1 gene transfer to human endothelial cells augments intracellular BH₄ levels in association with an increase in enzymatic activity of eNOS to produce NO [147]. *In vivo* GCH1 gene transfer restores vascular BH₄ levels and endothelial function in low renin hypertension [148]. Conversely, treatment of endothelial cells with GCH1 inhibitors or GCH1 small-interference RNA reduces BH₄ levels and induces eNOS uncoupling [117]. Moreover, *in*

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 BH₄ 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 BH₄ 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 17 β -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 BH₄ 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 BH₄ 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 BH₄ 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 BH₄ levels (and thus limit eNOS uncoupling), and enhance NO bioavailability. Eplerenone also increases eNOS phosphorylation at Ser¹¹⁷⁷. 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 BH₄ levels in diabetic rats [158].

The renin inhibitor aliskiren (and ARB valsartan) increases eNOS mRNA stability, enhances eNOS phosphorylation at Ser¹¹⁷⁷, decreases NADPH oxidase expression, augments vascular BH₄ 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, BH₄ 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.

ACKNOWLEDGEMENTS

This work was supported by the Collaborative Research Center SFB 553 (project A1 to H.L. and U.F.) and by grant LI-1042/1-1 from the DFG (Deutsche Forschungsgemeinschaft), Bonn, Germany. We apologize to those investigators whose work could not be cited in this article due to space limitations.

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

REFERENCES

References 185-187 are related articles recently published.

- [1] Förstermann U, Closs EI, Pollock JS, Nakane M, Schwarz P, Gath I, *et al*. Nitric oxide synthase isozymes. Characterization, purification, molecular cloning, and functions. *Hypertension* 1994; 23: 1121-31.
- [2] Liu VW, Huang PL. Cardiovascular roles of nitric oxide: a review of insights from nitric oxide synthase gene disrupted mice. *Cardiovasc Res* 2008; 77: 19-29.
- [3] Li H, Förstermann U. Nitric oxide in the pathogenesis of vascular disease. *J Pathol* 2000; 190: 244-54.
- [4] Li H, Wallerath T, Förstermann U. Physiological mechanisms regulating the expression of endothelial-type NO synthase. *Nitric Oxide* 2002; 7: 132-47.
- [5] Kawashima S, Yokoyama M. Dysfunction of endothelial nitric oxide synthase and atherosclerosis. *Arterioscler Thromb Vasc Biol* 2004; 24: 998-1005.
- [6] Alp NJ, McAteer MA, Khoo J, Choudhury RP, Channon KM. Increased endothelial tetrahydrobiopterin synthesis by targeted transgenic GTP-cyclohydrolase I overexpression reduces endothelial dysfunction and atherosclerosis in ApoE-knockout mice. *Arterioscler Thromb Vasc Biol* 2004; 24: 445-50.

- [7] d'Uscio LV, Baker TA, Mantilla CB, Smith L, Weiler D, Sieck GC, *et al.* Mechanism of endothelial dysfunction in apolipoprotein E-deficient mice. *Arterioscler Thromb Vasc Biol* 2001; 21: 1017-22.
- [8] Harrison DG. Cellular and molecular mechanisms of endothelial cell dysfunction. *J Clin Invest* 1997; 100: 2153-7.
- [9] Li H, Wallerath T, Münzel T, Forstermann U. Regulation of endothelial-type NO synthase expression in pathophysiology and in response to drugs. *Nitric Oxide* 2002; 7: 149-64.
- [10] Feron O, Dessy C, Moniotte S, Desager JP, Balligand JL. Hypercholesterolemia decreases nitric oxide production by promoting the interaction of caveolin and endothelial nitric oxide synthase. *J Clin Invest* 1999; 103: 897-905.
- [11] Cooke JP. Does ADMA cause endothelial dysfunction? *Arterioscler Thromb Vasc Biol* 2000; 20: 2032-7.
- [12] Miyazaki H, Matsuoka H, Cooke JP, Usui M, Ueda S, Okuda S, *et al.* Endogenous nitric oxide synthase inhibitor: a novel marker of atherosclerosis. *Circulation* 1999; 99: 1141-6.
- [13] Cayatte AJ, Palacino JJ, Horten K, Cohen RA. Chronic inhibition of nitric oxide production accelerates neointima formation and impairs endothelial function in hypercholesterolemic rabbits. *Arterioscler Thromb Vasc Biol* 1994; 14: 753-9.
- [14] Holm P, Korsgaard N, Shalmi M, Andersen HL, Hougaard P, Skouby SO, *et al.* Significant reduction of the antiatherogenic effect of estrogen by long-term inhibition of nitric oxide synthesis in cholesterol-clamped rabbits. *J Clin Invest* 1997; 100: 821-8.
- [15] Kauser K, da Cunha V, Fitch R, Mallari C, Rubanyi GM. Role of endogenous nitric oxide in progression of atherosclerosis in apolipoprotein E-deficient mice. *Am J Physiol Heart Circ Physiol* 2000; 278: H1679-85.
- [16] Nakashima Y, Plump AS, Raines EW, Breslow JL, Ross R. ApoE-deficient mice develop lesions of all phases of atherosclerosis throughout the arterial tree. *Arterioscler Thromb Vasc Biol* 1994; 14: 133-40.
- [17] Plump AS, Smith JD, Hayek T, Aalto-Setälä K, Walsh A, Verstyuyft JG, *et al.* Severe hypercholesterolemia and atherosclerosis in apolipoprotein E-deficient mice created by homologous recombination in ES cells. *Cell* 1992; 71: 343-53.
- [18] Qian H, Neplioueva V, Shetty GA, Channon KM, George SE. Nitric oxide synthase gene therapy rapidly reduces adhesion molecule expression and inflammatory cell infiltration in carotid arteries of cholesterol-fed rabbits. *Circulation* 1999; 99: 2979-82.
- [19] Wilcox JN, Subramanian RR, Sundell CL, Tracey WR, Pollock JS, Harrison DG, *et al.* Expression of multiple isoforms of nitric oxide synthase in normal and atherosclerotic vessels. *Arterioscler Thromb Vasc Biol* 1997; 17: 2479-88.
- [20] Schwarz PM, Kleinert H, Forstermann U. Potential functional significance of brain-type and muscle-type nitric oxide synthase I expressed in adventitia and media of rat aorta. *Arterioscler Thromb Vasc Biol* 1999; 19: 2584-90.
- [21] Kuhlencordt PJ, Hotten S, Schodel J, Rutzel S, Hu K, Widder J, *et al.* Atheroprotective effects of neuronal nitric oxide synthase in apolipoprotein e knockout mice. *Arterioscler Thromb Vasc Biol* 2006; 26: 1539-44.
- [22] Lowenstein CJ. Beneficial effects of neuronal nitric oxide synthase in atherosclerosis. *Arterioscler Thromb Vasc Biol* 2006; 26: 1417-8.
- [23] Kuhlencordt PJ, Chen J, Han F, Astern J, Huang PL. Genetic deficiency of inducible nitric oxide synthase reduces atherosclerosis and lowers plasma lipid peroxides in apolipoprotein E-knockout mice. *Circulation* 2001; 103: 3099-104.
- [24] Upmacis RK, Crabtree MJ, Deeb RS, Shen H, Lane PB, Benguigui LE, *et al.* Profound biopterin oxidation and protein tyrosine nitration in tissues of ApoE-null mice on an atherogenic diet: contribution of inducible nitric oxide synthase. *Am J Physiol Heart Circ Physiol* 2007; 293: H2878-87.
- [25] Hodgins JB, Knowles JW, Kim HS, Smithies O, Maeda N. Interactions between endothelial nitric oxide synthase and sex hormones in vascular protection in mice. *J Clin Invest* 2002; 109: 541-8.
- [26] Knowles JW, Reddick RL, Jennette JC, Shesely EG, Smithies O, Maeda N. Enhanced atherosclerosis and kidney dysfunction in eNOS(-/-)ApoE(-/-) mice are ameliorated by enalapril treatment. *J Clin Invest* 2000; 105: 451-8.
- [27] Kuhlencordt PJ, Gyurko R, Han F, Scherrer-Crosbie M, Aretz TH, Hajjar R, *et al.* Accelerated atherosclerosis, aortic aneurysm formation, and ischemic heart disease in apolipoprotein E/endothelial nitric oxide synthase double-knockout mice. *Circulation* 2001; 104: 448-54.
- [28] Chen J, Kuhlencordt PJ, Astern J, Gyurko R, Huang PL. Hypertension does not account for the accelerated atherosclerosis and development of aneurysms in male apolipoprotein e/endothelial nitric oxide synthase double knockout mice. *Circulation* 2001; 104: 2391-4.
- [29] Shi W, Wang X, Shih DM, Laubach VE, Navab M, Lusis AJ. Paradoxical reduction of fatty streak formation in mice lacking endothelial nitric oxide synthase. *Circulation* 2002; 105: 2078-82.
- [30] Ozaki M, Kawashima S, Yamashita T, Hirase T, Namiki M, Inoue N, *et al.* Overexpression of endothelial nitric oxide synthase accelerates atherosclerotic lesion formation in apoE-deficient mice. *J Clin Invest* 2002; 110: 331-40.
- [31] van Haperen R, de Waard M, van Deel E, Mees B, Kutryk M, van Aken T, *et al.* Reduction of blood pressure, plasma cholesterol, and atherosclerosis by elevated endothelial nitric oxide. *J Biol Chem* 2002; 277: 48803-7.
- [32] Forstermann U. Janus-faced role of endothelial NO synthase in vascular disease: uncoupling of oxygen reduction from NO synthesis and its pharmacological reversal. *Biol Chem* 2006; 387: 1521-33.
- [33] Forstermann U, Münzel T. Endothelial nitric oxide synthase in vascular disease: from marvel to menace. *Circulation* 2006; 113: 1708-14.
- [34] Sullivan JC, Pollock JS. Coupled and uncoupled NOS: separate but equal? Uncoupled NOS in endothelial cells is a critical pathway for intracellular signaling. *Circ Res* 2006; 98: 717-9.
- [35] Bendall JK, Alp NJ, Warrick N, Cai S, Adlam D, Rockett K, *et al.* Stoichiometric relationships between endothelial tetrahydrobiopterin, endothelial NO synthase (eNOS) activity, and eNOS coupling *in vivo*: insights from transgenic mice with endothelial-targeted GTP cyclohydrolase 1 and eNOS overexpression. *Circ Res* 2005; 97: 864-71.
- [36] Bevers LM, Braam B, Post JA, van Zonneveld AJ, Rabelink TJ, Koomans HA, *et al.* Tetrahydrobiopterin, but not L-arginine, decreases NO synthase uncoupling in cells expressing high levels of endothelial NO synthase. *Hypertension* 2006; 47: 87-94.
- [37] Pritchard KA, Jr., Ackerman AW, Gross ER, Stepp DW, Shi Y, Fontana JT, *et al.* Heat shock protein 90 mediates the balance of nitric oxide and superoxide anion from endothelial nitric-oxide synthase. *J Biol Chem* 2001; 276: 17621-4.
- [38] Lin MI, Fulton D, Babbitt R, Fleming I, Busse R, Pritchard KA, Jr., *et al.* Phosphorylation of threonine 497 in endothelial nitric-oxide synthase coordinates the coupling of L-arginine metabolism to efficient nitric oxide production. *J Biol Chem* 2003; 278: 44719-26.
- [39] Gharavi NM, Baker NA, Mouillesseaux KP, Yeung W, Honda HM, Hsieh X, *et al.* Role of endothelial nitric oxide synthase in the regulation of SREBP activation by oxidized phospholipids. *Circ Res* 2006; 98: 768-76.
- [40] Fleming I, Mohamed A, Galle J, Turchanowa L, Brandes RP, Fisslthaler B, *et al.* Oxidized low-density lipoprotein increases superoxide production by endothelial nitric oxide synthase by inhibiting PKC α . *Cardiovasc Res* 2005; 65: 897-906.
- [41] Zou MH, Shi C, Cohen RA. Oxidation of the zinc-thiolate complex and uncoupling of endothelial nitric oxide synthase by peroxynitrite. *J Clin Invest* 2002; 109: 817-26.
- [42] Sud N, Wells SM, Sharma S, Wiseman DA, Wilham J, Black SM. Asymmetric dimethylarginine inhibits HSP90 activity in pulmonary arterial endothelial cells: role of mitochondrial dysfunction. *Am J Physiol Cell Physiol* 2008; 294: C1407-18.
- [43] Gao YT, Roman LJ, Martasek P, Panda SP, Ishimura Y, Masters BS. Oxygen metabolism by endothelial nitric-oxide synthase. *J Biol Chem* 2007; 282: 28557-65.
- [44] Wei CC, Wang ZQ, Tejero J, Yang YP, Hemann C, Hille R, *et al.* Catalytic reduction of a tetrahydrobiopterin radical within nitric-oxide synthase. *J Biol Chem* 2008; 283: 11734-42.
- [45] Xia Y, Tsai AL, Berka V, Zweier JL. Superoxide generation from endothelial nitric-oxide synthase. A Ca²⁺/calmodulin-dependent and tetrahydrobiopterin regulatory process. *J Biol Chem* 1998; 273: 25804-8.
- [46] Vasquez-Vivar J, Martasek P, Whittett J, Joseph J, Kalyanaraman B. The ratio between tetrahydrobiopterin and oxidized tetrahydrobiopterin analogues controls superoxide release from endothelial nitric oxide synthase: an EPR spin trapping study. *Biochem J* 2002; 362: 733-9.

- [47] Vasquez-Vivar J, Kalyanaraman B, Martasek P. The role of tetrahydrobiopterin in superoxide generation from eNOS: enzymology and physiological implications. *Free Radic Res* 2003; 37: 121-7.
- [48] Shinozaki K, Kashiwagi A, Nishio Y, Okamura T, Yoshida Y, Masada M, *et al.* Abnormal biopterin metabolism is a major cause of impaired endothelium-dependent relaxation through nitric oxide/O₂- imbalance in insulin-resistant rat aorta. *Diabetes* 1999; 48: 2437-45.
- [49] Vasquez-Vivar J, Duquaine D, Whitsett J, Kalyanaraman B, Rajagopalan S. Altered tetrahydrobiopterin metabolism in atherosclerosis: implications for use of oxidized tetrahydrobiopterin analogues and thiol antioxidants. *Arterioscler Thromb Vasc Biol* 2002; 22: 1655-61.
- [50] Crabtree MJ, Smith CL, Lam G, Goligorsky MS, Gross SS. Ratio of 5,6,7,8-tetrahydrobiopterin to 7,8-dihydrobiopterin in endothelial cells determines glucose-elicited changes in NO vs. superoxide production by eNOS. *Am J Physiol Heart Circ Physiol* 2008; 294: H1530-40.
- [51] Pritchard KA, Jr., Groszek L, Smalley DM, Sessa WC, Wu M, Villalon P, *et al.* Native low-density lipoprotein increases endothelial cell nitric oxide synthase generation of superoxide anion. *Circ Res* 1995; 77: 510-8.
- [52] Laursen JB, Somers M, Kurz S, McCann L, Warnholtz A, Freeman BA, *et al.* Endothelial regulation of vasomotion in apoE-deficient mice: implications for interactions between peroxynitrite and tetrahydrobiopterin. *Circulation* 2001; 103: 1282-8.
- [53] Li H, Witte K, August M, Brausch I, Godtel-Armbrust U, Habermeier A, *et al.* Reversal of endothelial nitric oxide synthase uncoupling and up-regulation of endothelial nitric oxide synthase expression lowers blood pressure in hypertensive rats. *J Am Coll Cardiol* 2006; 47: 2536-44.
- [54] Kerr S, Brosnan MJ, McIntyre M, Reid JL, Dominiczak AF, Hamilton CA. Superoxide anion production is increased in a model of genetic hypertension: role of the endothelium. *Hypertension* 1999; 33: 1353-8.
- [55] Mollnau H, Wendt M, Szocs K, Lassegue B, Schulz E, Oelze M, *et al.* Effects of angiotensin II infusion on the expression and function of NAD(P)H oxidase and components of nitric oxide/cGMP signaling. *Circ Res* 2002; 90: E58-65.
- [56] Landmesser U, Dikalov S, Price SR, McCann L, Fukui T, Holland SM, *et al.* Oxidation of tetrahydrobiopterin leads to uncoupling of endothelial cell nitric oxide synthase in hypertension. *J Clin Invest* 2003; 111: 1201-9.
- [57] Hink U, Li H, Mollnau H, Oelze M, Matheis E, Hartmann M, *et al.* Mechanisms underlying endothelial dysfunction in diabetes mellitus. *Circ Res* 2001; 88: E14-22.
- [58] Munzel T, Li H, Mollnau H, Hink U, Matheis E, Hartmann M, *et al.* Effects of long-term nitroglycerin treatment on endothelial nitric oxide synthase (NOS III) gene expression, NOS III-mediated superoxide production, and vascular NO bioavailability. *Circ Res* 2000; 86: E7-12.
- [59] Stroes E, Kastelein J, Cosentino F, Erkelens W, Wever R, Koomans H, *et al.* Tetrahydrobiopterin restores endothelial function in hypercholesterolemia. *J Clin Invest* 1997; 99: 41-6.
- [60] Heitzer T, Krohn K, Albers S, Meinertz T. Tetrahydrobiopterin improves endothelium-dependent vasodilation by increasing nitric oxide activity in patients with Type II diabetes mellitus. *Diabetologia* 2000; 43: 1435-8.
- [61] Higashi Y, Sasaki S, Nakagawa K, Fukuda Y, Matsuura H, Oshima T, *et al.* Tetrahydrobiopterin enhances forearm vascular response to acetylcholine in both normotensive and hypertensive individuals. *Am J Hypertens* 2002; 15: 326-32.
- [62] Heitzer T, Brockhoff C, Mayer B, Warnholtz A, Mollnau H, Henne S, *et al.* Tetrahydrobiopterin improves endothelium-dependent vasodilation in chronic smokers: evidence for a dysfunctional nitric oxide synthase. *Circ Res* 2000; 86: E36-41.
- [63] Gori T, Burstein JM, Ahmed S, Miner SE, Al-Hesayen A, Kelly S, *et al.* Folic acid prevents nitroglycerin-induced nitric oxide synthase dysfunction and nitrate tolerance: a human *in vivo* study. *Circulation* 2001; 104: 1119-23.
- [64] Wohlfart P, Xu H, Endlich A, Habermeier A, Closs EI, Hubschle T, *et al.* Antiatherosclerotic effects of small-molecular-weight compounds enhancing endothelial nitric-oxide synthase (eNOS) expression and preventing eNOS uncoupling. *J Pharmacol Exp Ther* 2008; 325: 370-9.
- [65] d'Uscio LV, Milstien S, Richardson D, Smith L, Katusic ZS. Long-term vitamin C treatment increases vascular tetrahydrobiopterin levels and nitric oxide synthase activity. *Circ Res* 2003; 92: 88-95.
- [66] d'Uscio LV, Katusic ZS. Increased vascular biosynthesis of tetrahydrobiopterin in apolipoprotein E-deficient mice. *Am J Physiol Heart Circ Physiol* 2006; 290: H2466-71.
- [67] Katusic ZS. Vascular endothelial dysfunction: does tetrahydrobiopterin play a role? *Am J Physiol Heart Circ Physiol* 2001; 281: H981-6.
- [68] Tiefenbacher CP, Blecke T, Vahl C, Amann K, Vogt A, Kubler W. Endothelial dysfunction of coronary resistance arteries is improved by tetrahydrobiopterin in atherosclerosis. *Circulation* 2000; 102: 2172-9.
- [69] Antoniadou C, Shirodaria C, Crabtree M, Rinze R, Alp N, Cunnington C, *et al.* Altered plasma versus vascular biopterins in human atherosclerosis reveal relationships between endothelial nitric oxide synthase coupling, endothelial function, and inflammation. *Circulation* 2007; 116: 2851-9.
- [70] Chalupsky K, Cai H. Endothelial dihydrofolate reductase: critical for nitric oxide bioavailability and role in angiotensin II uncoupling of endothelial nitric oxide synthase. *Proc Natl Acad Sci USA* 2005; 102: 9056-61.
- [71] Schmidt TS, Alp NJ. Mechanisms for the role of tetrahydrobiopterin in endothelial function and vascular disease. *Clin Sci (Lond)* 2007; 113: 47-63.
- [72] Werner ER, Werner-Felmayer G, Fuchs D, Hausen A, Reibnegger G, Yim JJ, *et al.* Tetrahydrobiopterin biosynthetic activities in human macrophages, fibroblasts, THP-1, and T 24 cells. GTP-cyclohydrolase I is stimulated by interferon-gamma, and 6-pyruvoyl tetrahydropterin synthase and sepiapterin reductase are constitutively present. *J Biol Chem* 1990; 265: 3189-92.
- [73] Geller DA, Di Silvio M, Billiar TR, Hatakeyama K. GTP cyclohydrolase I is coincided in hepatocytes stimulated to produce nitric oxide. *Biochem Biophys Res Commun* 2000; 276: 633-41.
- [74] Katusic ZS, Stelter A, Milstien S. Cytokines stimulate GTP cyclohydrolase I gene expression in cultured human umbilical vein endothelial cells. *Arterioscler Thromb Vasc Biol* 1998; 18: 27-32.
- [75] Hattori Y, Nakanishi N, Kasai K, Shimoda SI. GTP cyclohydrolase I mRNA induction and tetrahydrobiopterin synthesis in human endothelial cells. *Biochim Biophys Acta* 1997; 1358: 61-6.
- [76] Werner-Felmayer G, Werner ER, Fuchs D, Hausen A, Reibnegger G, Schmidt K, *et al.* Pteridine biosynthesis in human endothelial cells. Impact on nitric oxide-mediated formation of cyclic GMP. *J Biol Chem* 1993; 268: 1842-6.
- [77] Dulak J, Polus M, Guevara I, Polus A, Hartwich J, Dembinska-Kiec A. Regulation of inducible nitric oxide synthase (iNOS) and GTP cyclohydrolase I (GTP-CH I) gene expression by ox-LDL in rat vascular smooth muscle cells. *J Physiol Pharmacol* 1997; 48: 689-97.
- [78] Harrison D, Griendling KK, Landmesser U, Hornig B, Drexler H. Role of oxidative stress in atherosclerosis. *Am J Cardiol* 2003; 91: 7A-11A.
- [79] Schnabel R, Blankenberg S. Oxidative stress in cardiovascular disease: successful translation from bench to bedside? *Circulation* 2007; 116: 1338-40.
- [80] Förstermann U. Oxidative stress in vascular disease: causes, defense mechanisms and potential therapies. *Nat Clin Pract Cardiovasc Med* 2008; 5: 338-49.
- [81] Bedard K, Krause KH. The NOX family of ROS-generating NADPH oxidases: physiology and pathophysiology. *Physiol Rev* 2007; 87: 245-313.
- [82] Brandes RP, Kreuzer J. Vascular NADPH oxidases: molecular mechanisms of activation. *Cardiovasc Res* 2005; 65: 16-27.
- [83] Griendling KK. Novel NAD(P)H oxidases in the cardiovascular system. *Heart* 2004; 90: 491-3.
- [84] Sorescu D, Weiss D, Lassegue B, Clempus RE, Szocs K, Sorescu GP, *et al.* Superoxide production and expression of nox family proteins in human atherosclerosis. *Circulation* 2002; 105: 1429-35.
- [85] Hathaway CA, Heistad DD, Piegors DJ, Miller FJ, Jr. Regression of atherosclerosis in monkeys reduces vascular superoxide levels. *Circ Res* 2002; 90: 277-83.
- [86] Azumi H, Inoue N, Takeshita S, Rikitake Y, Kawashima S, Hayashi Y, *et al.* Expression of NADH/NADPH oxidase p22phox in human coronary arteries. *Circulation* 1999; 100: 1494-8.
- [87] Azumi H, Inoue N, Ohashi Y, Terashima M, Mori T, Fujita H, *et al.* Superoxide generation in directional coronary atherectomy

- specimens of patients with angina pectoris: important role of NAD(P)H oxidase. *Arterioscler Thromb Vasc Biol* 2002; 22: 1838-44.
- [88] Warnholtz A, Nickenig G, Schulz E, Macharzina R, Brasen JH, Skatchkov M, *et al.* Increased NADH-oxidase-mediated superoxide production in the early stages of atherosclerosis: evidence for involvement of the renin-angiotensin system. *Circulation* 1999; 99: 2027-33.
- [89] Oelze M, Daiber A, Brandes RP, Hortmann M, Wenzel P, Hink U, *et al.* Nebivolol inhibits superoxide formation by NADPH oxidase and endothelial dysfunction in angiotensin II-treated rats. *Hypertension* 2006; 48: 677-84.
- [90] Xu H, Goetsch C, Xia N, Horke S, Morawietz H, Forstermann U, *et al.* Differential roles of PKCalpha and PKCepsilon in controlling the gene expression of Nox4 in human endothelial cells. *Free Radic Biol Med* 2008; 44: 1656-67.
- [91] Herman AG, Moncada S. Therapeutic potential of nitric oxide donors in the prevention and treatment of atherosclerosis. *Eur Heart J* 2005; 26: 1945-55.
- [92] Ishikawa K, Kanamasa K, Ogawa I, Takenaka T, Naito T, Kamata N, *et al.* Long-term nitrate treatment increases cardiac events in patients with healed myocardial infarction. Secondary Prevention Group. *Jpn Circ J* 1996; 60: 779-88.
- [93] Nakamura Y, Moss AJ, Brown MW, Kinoshita M, Kawai C. Long-term nitrate use may be deleterious in ischemic heart disease: A study using the databases from two large-scale postinfarction studies. Multicenter Myocardial Ischemia Research Group. *Am Heart J* 1999; 138: 577-85.
- [94] Hink U, Daiber A, Kayhan N, Trischler J, Kraatz C, Oelze M, *et al.* Oxidative inhibition of the mitochondrial aldehyde dehydrogenase promotes nitroglycerin tolerance in human blood vessels. *J Am Coll Cardiol* 2007; 50: 2226-32.
- [95] Daiber A, Wenzel P, Oelze M, Munzel T. New insights into bioactivation of organic nitrates, nitrate tolerance and cross-tolerance. *Clin Res Cardiol* 2008; 97: 12-20.
- [96] Munzel T, Daiber A, Mulsch A. Explaining the phenomenon of nitrate tolerance. *Circ Res* 2005; 97: 618-28.
- [97] Jurt U, Gori T, Ravandi A, Babaei S, Zeman P, Parker JD. Differential effects of pentaerythritol tetranitrate and nitroglycerin on the development of tolerance and evidence of lipid peroxidation: a human *in vivo* study. *J Am Coll Cardiol* 2001; 38: 854-9.
- [98] Daiber A, Oelze M, Coldewey M, Bachschmid M, Wenzel P, Sydow K, *et al.* Oxidative stress and mitochondrial aldehyde dehydrogenase activity: a comparison of pentaerythritol tetranitrate with other organic nitrates. *Mol Pharmacol* 2004; 66: 1372-82.
- [99] Wenzel P, Oelze M, Coldewey M, Hortmann M, Seeling A, Hink U, *et al.* Heme oxygenase-1: a novel key player in the development of tolerance in response to organic nitrates. *Arterioscler Thromb Vasc Biol* 2007; 27: 1729-35.
- [100] Mollnau H, Wenzel P, Oelze M, Treiber N, Pautz A, Schulz E, *et al.* Mitochondrial oxidative stress and nitrate tolerance--comparison of nitroglycerin and pentaerythritol tetranitrate in Mn-SOD^{+/−} mice. *BMC Cardiovasc Disord* 2006; 6: 44.
- [101] Dragoni S, Gori T, Lisi M, Di Stolfo G, Pautz A, Kleinert H, *et al.* Pentaerythritol tetranitrate and nitroglycerin, but not isosorbide mononitrate, prevent endothelial dysfunction induced by ischemia and reperfusion. *Arterioscler Thromb Vasc Biol* 2007; 27: 1955-9.
- [102] Hacker A, Muller S, Meyer W, Kojda G. The nitric oxide donor pentaerythritol tetranitrate can preserve endothelial function in established atherosclerosis. *Br J Pharmacol* 2001; 132: 1707-14.
- [103] Kojda G, Stein D, Kottenberg E, Schnaith EM, Noack E. *In vivo* effects of pentaerythritol-tetranitrate and isosorbide-5-mononitrate on the development of atherosclerosis and endothelial dysfunction in cholesterol-fed rabbits. *J Cardiovasc Pharmacol* 1995; 25: 763-73.
- [104] Dikalov S, Fink B, Skatchkov M, Bassenge E. Comparison of glyceryl trinitrate-induced with pentaerythritol tetranitrate-induced *in vivo* formation of superoxide radicals: effect of vitamin C. *Free Radic Biol Med* 1999; 27: 170-6.
- [105] Muller S, Konig I, Meyer W, Kojda G. Inhibition of vascular oxidative stress in hypercholesterolemia by eccentric isosorbide mononitrate. *J Am Coll Cardiol* 2004; 44: 624-31.
- [106] Muller S, Laber U, Mullenheim J, Meyer W, Kojda G. Preserved endothelial function after long-term eccentric isosorbide mononitrate despite moderate nitrate tolerance. *J Am Coll Cardiol* 2003; 41: 1994-2000.
- [107] Thum T, Fraccarollo D, Thum S, Schultheiss M, Daiber A, Wenzel P, *et al.* Differential effects of organic nitrates on endothelial progenitor cells are determined by oxidative stress. *Arterioscler Thromb Vasc Biol* 2007; 27: 748-54.
- [108] Moens AL, Kass DA. Tetrahydrobiopterin and cardiovascular disease. *Arterioscler Thromb Vasc Biol* 2006; 26: 2439-44.
- [109] Cosentino F, Patton S, d'Uscio LV, Werner ER, Werner-Felmayer G, Moreau P, *et al.* Tetrahydrobiopterin alters superoxide and nitric oxide release in prehypertensive rats. *J Clin Invest* 1998; 101: 1530-7.
- [110] Pieper GM. Acute amelioration of diabetic endothelial dysfunction with a derivative of the nitric oxide synthase cofactor, tetrahydrobiopterin. *J Cardiovasc Pharmacol* 1997; 29: 8-15.
- [111] Shinozaki K, Nishio Y, Okamura T, Yoshida Y, Maegawa H, Kojima H, *et al.* Oral administration of tetrahydrobiopterin prevents endothelial dysfunction and vascular oxidative stress in the aortas of insulin-resistant rats. *Circ Res* 2000; 87: 566-73.
- [112] Setoguchi S, Mohri M, Shimokawa H, Takeshita A. Tetrahydrobiopterin improves endothelial dysfunction in coronary microcirculation in patients without epicardial coronary artery disease. *J Am Coll Cardiol* 2001; 38: 493-8.
- [113] Hattori Y, Hattori S, Wang X, Satoh H, Nakanishi N, Kasai K. Oral administration of tetrahydrobiopterin slows the progression of atherosclerosis in apolipoprotein E-knockout mice. *Arterioscler Thromb Vasc Biol* 2007; 27: 865-70.
- [114] Bagi Z, Koller A. Lack of nitric oxide mediation of flow-dependent arteriolar dilation in type I diabetes is restored by sepiapterin. *J Vasc Res* 2003; 40: 47-57.
- [115] Tarpey MM. Sepiapterin treatment in atherosclerosis. *Arterioscler Thromb Vasc Biol* 2002; 22: 1519-21.
- [116] Pannirselvam M, Simon V, Verma S, Anderson T, Triggle CR. Chronic oral supplementation with sepiapterin prevents endothelial dysfunction and oxidative stress in small mesenteric arteries from diabetic (db/db) mice. *Br J Pharmacol* 2003; 140: 701-6.
- [117] Wang S, Xu J, Song P, Wu Y, Zhang J, Chul Choi H, *et al.* Acute inhibition of guanosine triphosphate cyclohydrolase 1 uncouples endothelial nitric oxide synthase and elevates blood pressure. *Hypertension* 2008; 52: 484-90.
- [118] Drexler H, Zeiher AM, Meinzer K, Just H. Correction of endothelial dysfunction in coronary microcirculation of hypercholesterolaemic patients by L-arginine. *Lancet* 1991; 338: 1546-50.
- [119] Candipan RC, Wang BY, Buitrago R, Tsao PS, Cooke JP. Regression or progression. Dependency on vascular nitric oxide. *Arterioscler Thromb Vasc Biol* 1996; 16: 44-50.
- [120] Aji W, Ravalli S, Szabolcs M, Jiang XC, Sciacca RR, Michler RE, *et al.* L-arginine prevents xanthoma development and inhibits atherosclerosis in LDL receptor knockout mice. *Circulation* 1997; 95: 430-7.
- [121] Blum A, Hathaway L, Mincemoyer R, Schenke WH, Kirby M, Csako G, *et al.* Oral L-arginine in patients with coronary artery disease on medical management. *Circulation* 2000; 101: 2160-4.
- [122] Walker HA, McGing E, Fisher I, Boger RH, Bode-Boger SM, Jackson G, *et al.* Endothelium-dependent vasodilation is independent of the plasma L-arginine/ADMA ratio in men with stable angina: lack of effect of oral L-arginine on endothelial function, oxidative stress and exercise performance. *J Am Coll Cardiol* 2001; 38: 499-505.
- [123] Simonet S, Rupin A, Badier-Commander C, Coumilleau S, Behr-Roussel D, Verbeuren TJ. Evidence for superoxide anion generation in aortas of cholesterol-fed rabbits treated with L-arginine. *Eur J Pharmacol* 2004; 492: 211-6.
- [124] Boger RH. The pharmacodynamics of L-arginine. *J Nutr* 2007; 137: 1650S-5S.
- [125] Pollock JS, Forstermann U, Mitchell JA, Warner TD, Schmidt HH, Nakane M, *et al.* Purification and characterization of particulate endothelium-derived relaxing factor synthase from cultured and native bovine aortic endothelial cells. *Proc Natl Acad Sci USA* 1991; 88: 10480-4.
- [126] Closs EI, Scheld JS, Sharafi M, Forstermann U. Substrate supply for nitric-oxide synthase in macrophages and endothelial cells: role of cationic amino acid transporters. *Mol Pharmacol* 2000; 57: 68-74.
- [127] Simon A, Plies L, Habermeyer A, Martine U, Reining M, Closs EI. Role of neutral amino acid transport and protein breakdown for

- substrate supply of nitric oxide synthase in human endothelial cells. *Circ Res* 2003; 93: 813-20.
- [128] Cooke JP. Asymmetrical dimethylarginine: the Uber marker? *Circulation* 2004; 109: 1813-8.
- [129] Ryoo S, Lemmon CA, Soucy KG, Gupta G, White AR, Nyhan D, *et al.* Oxidized low-density lipoprotein-dependent endothelial arginase II activation contributes to impaired nitric oxide signaling. *Circ Res* 2006; 99: 951-60.
- [130] Lim HK, Lim HK, Ryoo S, Benjo A, Shuleri K, Miriel V, *et al.* Mitochondrial arginase II constrains endothelial NOS-3 activity. *Am J Physiol Heart Circ Physiol* 2007; 293: H3317-24.
- [131] Ryoo S, Gupta G, Benjo A, Lim HK, Camara A, Sikka G, *et al.* Endothelial arginase II: a novel target for the treatment of atherosclerosis. *Circ Res* 2008; 102: 923-32.
- [132] Maas R. Pharmacotherapies and their influence on asymmetric dimethylarginine (ADMA). *Vasc Med* 2005; 10 (Suppl 1): S49-57.
- [133] Moens AL, Champion HC, Claeys MJ, Tavazzi B, Kaminski PM, Wolin MS, *et al.* High-dose folic acid pretreatment blunts cardiac dysfunction during ischemia coupled to maintenance of high-energy phosphates and reduces postreperfusion injury. *Circulation* 2008; 117: 1810-9.
- [134] Tian R, Ingwall JS. How does folic acid cure heart attacks? *Circulation* 2008; 117: 1772-4.
- [135] Verhaar MC, Wever RM, Kastelein JJ, van Dam T, Koomans HA, Rabelink TJ. 5-methyltetrahydrofolate, the active form of folic acid, restores endothelial function in familial hypercholesterolemia. *Circulation* 1998; 97: 237-41.
- [136] van Etten RW, de Koning EJ, Verhaar MC, Gaillard CA, Rabelink TJ. Impaired NO-dependent vasodilation in patients with Type II (non-insulin-dependent) diabetes mellitus is restored by acute administration of folate. *Diabetologia* 2002; 45: 1004-10.
- [137] Woo KS, Chook P, Lolini YI, Sanderson JE, Metreweli C, Celermajer DS. Folic acid improves arterial endothelial function in adults with hyperhomocysteinemia. *J Am Coll Cardiol* 1999; 34: 2002-6.
- [138] Carnicer R, Navarro MA, Arbones-Mainar JM, Acin S, Guzman MA, Surra JC, *et al.* Folic acid supplementation delays atherosclerotic lesion development in apoE-deficient mice. *Life Sci* 2007; 80: 638-43.
- [139] Moat SJ, Lang D, McDowell IF, Clarke ZL, Madhavan AK, Lewis MJ, *et al.* Folate, homocysteine, endothelial function and cardiovascular disease. *J Nutr Biochem* 2004; 15: 64-79.
- [140] Stroes ES, van Faassen EE, Yo M, Martasek P, Boer P, Govers R, *et al.* Folic acid reverts dysfunction of endothelial nitric oxide synthase. *Circ Res* 2000; 86: 1129-34.
- [141] Antoniadou C, Shirodaria C, Warrick N, Cai S, de Bono J, Lee J, *et al.* 5-methyltetrahydrofolate rapidly improves endothelial function and decreases superoxide production in human vessels: effects on vascular tetrahydrobiopterin availability and endothelial nitric oxide synthase coupling. *Circulation* 2006; 114: 1193-201.
- [142] Shirodaria C, Antoniadou C, Lee J, Jackson CE, Robson MD, Francis JM, *et al.* Global improvement of vascular function and redox state with low-dose folic acid: implications for folate therapy in patients with coronary artery disease. *Circulation* 2007; 115: 2262-70.
- [143] Moens AL, Vrints CJ, Claeys MJ, Timmermans JP, Champion HC, Kass DA. Mechanisms and potential therapeutic targets for folic acid in cardiovascular disease. *Am J Physiol Heart Circ Physiol* 2008; 294: H1971-7.
- [144] Lonn E, Yusuf S, Arnold MJ, Sheridan P, Pogue J, Micks M, *et al.* Homocysteine lowering with folic acid and B vitamins in vascular disease. *N Engl J Med* 2006; 354: 1567-77.
- [145] Bona KH, Njolstad I, Ueland PM, Schirmer H, Tverdal A, Steigen T, *et al.* Homocysteine lowering and cardiovascular events after acute myocardial infarction. *N Engl J Med* 2006; 354: 1578-88.
- [146] Loscalzo J. Homocysteine trials--clear outcomes for complex reasons. *N Engl J Med* 2006; 354: 1629-32.
- [147] Cai S, Alp NJ, McDonald D, Smith I, Kay J, Canevari L, *et al.* GTP cyclohydrolase I gene transfer augments intracellular tetrahydrobiopterin in human endothelial cells: effects on nitric oxide synthase activity, protein levels and dimerisation. *Cardiovasc Res* 2002; 55: 838-49.
- [148] Zheng JS, Yang XQ, Lookingland KJ, Fink GD, Hesslinger C, Kapatos G, *et al.* Gene transfer of human guanosine 5'-triphosphate cyclohydrolase I restores vascular tetrahydrobiopterin level and endothelial function in low renin hypertension. *Circulation* 2003; 108: 1238-45.
- [149] Takaya T, Hirata K, Yamashita T, Shinohara M, Sasaki N, Inoue N, *et al.* A specific role for eNOS-derived reactive oxygen species in atherosclerosis progression. *Arterioscler Thromb Vasc Biol* 2007; 27: 1632-7.
- [150] Huang A, Zhang YY, Chen K, Hatakeyama K, Keaney JF, Jr. Cytokine-stimulated GTP cyclohydrolase I expression in endothelial cells requires coordinated activation of nuclear factor-kappaB and Stat1/Stat3. *Circ Res* 2005; 96: 164-71.
- [151] Schoedon G, Schneemann M, Blau N, Edgell CJ, Schaffner A. Modulation of human endothelial cell tetrahydrobiopterin synthesis by activating and deactivating cytokines: new perspectives on endothelium-derived relaxing factor. *Biochem Biophys Res Commun* 1993; 196: 1343-8.
- [152] Ishii M, Shimizu S, Nagai T, Shiota K, Kiuchi Y, Yamamoto T. Stimulation of tetrahydrobiopterin synthesis induced by insulin: possible involvement of phosphatidylinositol 3-kinase. *Int J Biochem Cell Biol* 2001; 33: 65-73.
- [153] Shimizu S, Hiroi T, Ishii M, Hagiwara T, Wajima T, Miyazaki A, *et al.* Hydrogen peroxide stimulates tetrahydrobiopterin synthesis through activation of the Jak2 tyrosine kinase pathway in vascular endothelial cells. *Int J Biochem Cell Biol* 2008; 40: 755-65.
- [154] Shimizu S, Shiota K, Yamamoto S, Miyazaki Y, Ishii M, Watabe T, *et al.* Hydrogen peroxide stimulates tetrahydrobiopterin synthesis through the induction of GTP-cyclohydrolase I and increases nitric oxide synthase activity in vascular endothelial cells. *Free Radic Biol Med* 2003; 34: 1343-52.
- [155] Miyazaki-Akita A, Hayashi T, Ding QF, Shiraishi H, Nomura T, Hattori Y, *et al.* 17beta-estradiol antagonizes the down-regulation of endothelial nitric-oxide synthase and GTP cyclohydrolase I by high glucose: relevance to postmenopausal diabetic cardiovascular disease. *J Pharmacol Exp Ther* 2007; 320: 591-8.
- [156] Hattori Y, Nakanishi N, Akimoto K, Yoshida M, Kasai K. HMG-CoA reductase inhibitor increases GTP cyclohydrolase I mRNA and tetrahydrobiopterin in vascular endothelial cells. *Arterioscler Thromb Vasc Biol* 2003; 23: 176-82.
- [157] Wenzel P, Daiber A, Oelze M, Brandt M, Closs E, Xu J, *et al.* Mechanisms underlying recoupling of eNOS by HMG-CoA reductase inhibition in a rat model of streptozotocin-induced diabetes mellitus. *Atherosclerosis* 2008; 198: 65-76.
- [158] Satoh M, Fujimoto S, Arakawa S, Yada T, Namikoshi T, Haruna Y, *et al.* Angiotensin II type 1 receptor blocker ameliorates uncoupled endothelial nitric oxide synthase in rats with experimental diabetic nephropathy. *Nephrol Dial Transplant* 2008; 23: 3806-13.
- [159] Lam CF, Peterson TE, Richardson DM, Croatt AJ, d'Uscio LV, Nath KA, *et al.* Increased blood flow causes coordinated upregulation of arterial eNOS and biosynthesis of tetrahydrobiopterin. *Am J Physiol Heart Circ Physiol* 2006; 290: H786-93.
- [160] Widder JD, Chen W, Li L, Dikalov S, Thony B, Hatakeyama K, *et al.* Regulation of tetrahydrobiopterin biosynthesis by shear stress. *Circ Res* 2007; 101: 830-8.
- [161] De Bono JP, Channon KM. Endothelial cell tetrahydrobiopterin: going with the flow. *Circ Res* 2007; 101: 752-4.
- [162] d'Uscio LV, Katusic ZS. Erythropoietin increases endothelial biosynthesis of tetrahydrobiopterin by activation of protein kinase B alpha/Akt1. *Hypertension* 2008; 52: 93-9.
- [163] Li H, Förstermann U. Structure-activity relationship of staurosporine analogs in regulating expression of endothelial nitric-oxide synthase gene. *Mol Pharmacol* 2000; 57: 427-35.
- [164] Li H, Oehrlin SA, Wallerath T, Ihrig-Biedert I, Wohlfart P, Ulfshofer T, *et al.* Activation of protein kinase C alpha and/or epsilon enhances transcription of the human endothelial nitric oxide synthase gene. *Mol Pharmacol* 1998; 53: 630-7.
- [165] Li H, Hergert SM, Schafer SC, Brausch I, Yao Y, Huang Q, *et al.* Midostaurin upregulates eNOS gene expression and preserves eNOS function in the microcirculation of the mouse. *Nitric Oxide* 2005; 12: 231-6.
- [166] Steinkamp-Fenske K, Bollinger L, Voller N, Xu H, Yao Y, Bauer R, *et al.* Ursolic acid from the Chinese herb danshen (*Salvia miltiorrhiza* L.) upregulates eNOS and downregulates Nox4 expression in human endothelial cells. *Atherosclerosis* 2007; 195: e104-11.
- [167] Steinkamp-Fenske K, Bollinger L, Xu H, Yao Y, Horke S, Förstermann U, *et al.* Reciprocal regulation of endothelial nitric-

- oxide synthase and NADPH oxidase by betulinic acid in human endothelial cells. *J Pharmacol Exp Ther* 2007; 322: 836-42.
- [168] Sasaki K, Heeschen C, Aicher A, Ziebart T, Honold J, Urbich C, *et al.* Ex vivo pretreatment of bone marrow mononuclear cells with endothelial NO synthase enhancer AVE9488 enhances their functional activity for cell therapy. *Proc Natl Acad Sci USA* 2006; 103: 14537-41.
- [169] Fraccarollo D, Widder JD, Galuppo P, Thum T, Tsikas D, Hoffmann M, *et al.* Improvement in left ventricular remodeling by the endothelial nitric oxide synthase enhancer AVE9488 after experimental myocardial infarction. *Circulation* 2008; 118: 818-27.
- [170] Liao JK, Laufs U. Pleiotropic effects of statins. *Annu Rev Pharmacol Toxicol* 2005; 45: 89-118.
- [171] John S, Schlaich M, Langenfeld M, Weihprecht H, Schmitz G, Weidinger G, *et al.* Increased bioavailability of nitric oxide after lipid-lowering therapy in hypercholesterolemic patients: a randomized, placebo-controlled, double-blind study. *Circulation* 1998; 98: 211-6.
- [172] Landmesser U, Engberding N, Bahlmann FH, Schaefer A, Wiencke A, Heineke A, *et al.* Statin-induced improvement of endothelial progenitor cell mobilization, myocardial neovascularization, left ventricular function, and survival after experimental myocardial infarction requires endothelial nitric oxide synthase. *Circulation* 2004; 110: 1933-9.
- [173] Laufs U, La Fata V, Plutzky J, Liao JK. Upregulation of endothelial nitric oxide synthase by HMG CoA reductase inhibitors. *Circulation* 1998; 97: 1129-35.
- [174] Feron O, Dessy C, Desager JP, Balligand JL. Hydroxymethylglutaryl-coenzyme A reductase inhibition promotes endothelial nitric oxide synthase activation through a decrease in caveolin abundance. *Circulation* 2001; 103: 113-8.
- [175] Wagner AH, Kohler T, Ruckschloss U, Just I, Hecker M. Improvement of nitric oxide-dependent vasodilatation by HMG-CoA reductase inhibitors through attenuation of endothelial superoxide anion formation. *Arterioscler Thromb Vasc Biol* 2000; 20: 61-9.
- [176] Wassmann S, Laufs U, Muller K, Konkol C, Ahlbory K, Baumer AT, *et al.* Cellular antioxidant effects of atorvastatin *in vitro* and *in vivo*. *Arterioscler Thromb Vasc Biol* 2002; 22: 300-5.
- [177] Nissen SE, Nicholls SJ, Sipahi I, Libby P, Raichlen JS, Ballantyne CM, *et al.* Effect of very high-intensity statin therapy on regression of coronary atherosclerosis: the ASTEROID trial. *JAMA* 2006; 295: 1556-65.
- [178] Patel TN, Shishehbor MH, Bhatt DL. A review of high-dose statin therapy: targeting cholesterol and inflammation in atherosclerosis. *Eur Heart J* 2007; 28: 664-72.
- [179] Imanishi T, Ikejima H, Tsujioka H, Kuroi A, Kobayashi K, Muragaki Y, *et al.* Addition of eplerenone to an angiotensin-converting enzyme inhibitor effectively improves nitric oxide bioavailability. *Hypertension* 2008; 51: 734-41.
- [180] Bauersachs J, Fraccarollo D. More NO - no more ROS: combined selective mineralocorticoid receptor blockade and angiotensin-converting enzyme inhibition for vascular protection. *Hypertension* 2008; 51: 624-5.
- [181] Takai S, Jin D, Muramatsu M, Kirimura K, Sakonjo H, Miyazaki M. Eplerenone inhibits atherosclerosis in nonhuman primates. *Hypertension* 2005; 46: 1135-9.
- [182] Imanishi T, Tsujioka H, Ikejima H, Kuroi A, Takarada S, Kitabata H, *et al.* Renin inhibitor aliskiren improves impaired nitric oxide bioavailability and protects against atherosclerotic changes. *Hypertension* 2008; 52: 563-72.
- [183] Verma S, Gupta MK. Aliskiren improves nitric oxide bioavailability and limits atherosclerosis. *Hypertension* 2008; 52: 467-9.
- [184] Nussberger J, Aubert JF, Bouzourene K, Pellegrin M, Hayoz D, Mazzolai L. Renin inhibition by aliskiren prevents atherosclerosis progression: comparison with irbesartan, atenolol, and amlodipine. *Hypertension* 2008; 51: 1306-11.
- [185] Aversa A, Caprio M, Rosano GM, Spera G. Endothelial effects of drugs designed to treat erectile dysfunction. *Curr Pharm Des* 2008; 14(35): 3768-78.
- [186] Chung HT, Pae HO, Cha YN. Role of heme oxygenase-1 in vascular disease. *Curr Pharm Des* 2008; 14(5): 422-8.
- [187] Yamagishi S, Ueda S, Nakamura K, Matsui T, Okuda S. Role of asymmetric dimethylarginine (ADMA) in diabetic vascular complications. *Curr Pharm Des* 2008; 14(25): 2613-8.