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INVITED REVIEW

Nitric oxide and oxidative stress in vascular disease

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Abstract Endothelium-derived nitric oxide (NO) is a paracrine factor that controls vascular tone, inhibits platelet function, prevents adhesion of leukocytes, and reduces proliferation of the intima. An enhanced inactivation and/or reduced synthesis of NO is seen in conjunction with risk factors for cardiovascular disease. This condition, referred to as endothelial dysfunction, can promote vasospasm, thrombosis, vascular inflammation, and proliferation of vascular smooth muscle cells. Vascular oxidative stress with an increased production of reactive oxygen species (ROS) contributes to mechanisms of vascular dysfunction. Oxidative stress is mainly caused by an imbalance between the activity of endogenous pro-oxidative enzymes (such as NADPH oxidase, xanthine oxidase, or the mitochondrial respiratory chain) and anti-oxidative enzymes (such as superoxide dismutase, glutathione peroxidase, heme oxygenase, thioredoxin peroxidase/peroxiredoxin, catalase, and paraoxonase) in favor of the former. Also, small molecular weight antioxidants may play a role in the defense against oxidative stress. Increased ROS concentrations reduce the amount of bioactive NO by chemical inactivation to form toxic peroxynitrite. Peroxynitrite—in turn—can "uncouple" endothelial NO synthase to become a dysfunctional superoxide-generating enzyme that contributes to vascular oxidative stress. Oxidative stress and endothelial dysfunction can promote atherogenesis. Therapeutically, drugs in clinical use such as ACE inhibitors, AT_1 receptor blockers, and statins have pleiotropic actions that can improve endothelial function. Also, dietary polyphenolic antioxidants can reduce oxidative stress, whereas clinical trials with antioxidant vitamins C and E failed to show an improved cardiovascular outcome.

Keywords Endothelial NO synthase · Reactive oxygen species · AT_1 receptor antagonists · ACE inhibitors · Statins · Vitamin C · Vitamin E · Polyphenols

Introduction

The endothelial cells of our vasculature—if functional—protect us against thrombosis and atherosclerosis. A key regulator of endothelial function is endothelium-derived nitric oxide (NO) generated by endothelial NO synthase (eNOS) [28]. Vascular NO relaxes blood vessels, prevents platelet aggregation and adhesion, limits oxidation of low-density lipoprotein (LDL) cholesterol, inhibits proliferation of vascular smooth muscle cells, and decreases the expression of pro-inflammatory genes that advance atherogenesis [26–28].

Cardiac risk factors and cardiovascular disease impair endothelial function. An increased production and/or impaired inactivation of reactive oxygen species (ROS), i.e., oxidative stress, leads to reduced bioactivity of NO. A dominant mechanism reducing bioavailability of vascular NO is rapid oxidative inactivation by the ROS superoxide ($O_2^{-}\cdot$). In addition, there is evidence that persisting oxidative stress will render eNOS dysfunctional such that it no longer produces NO, but $O_2^{-}\cdot$ [26, 27]. This review discusses the interrelationship between vascular disease and vascular oxidative stress, as well as therapeutic approaches, to reduce oxidative stress and restore endothelial function.

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Structure and enzymatic function of eNOS

Structurally, all NOS isozymes are homodimers (Fig. 1). In a functional NOS, the C-terminal reductase domain of one monomer (with binding sites for NADPH, FMN, and FAD) is linked to the N-terminal oxygenase domain of the opposite monomer (Fig. 1). This oxygenase domain carries a prosthetic heme group. Theoxygenase domain also binds (6*R*-)5,6,7,8-tetrahydrobiopterin (BH_4), molecular oxygen, and the substrate L-arginine [1, 19]. All NOS isoforms show a zinc thiolate cluster formed by a zinc ion that is tetrahedrally coordinated to two CXXXXC motifs (one contributed by each monomer) at the interface of the NOS dimer [46, 73, 86, 107]. All NOS isozymes catalyze flavin-mediated electron transfer from the C-terminally bound NADPH to the heme in the N-terminal oxygenase domain. Calmodulin (CaM), upon calcium-induced binding to the NOS, increases both the electron transfer within the reductase domain (from NADPH to the flavins; Fig. 1) and the electron transfer from the reductase domain to the heme center in the oxygenase domain (Fig. 1) [45]. At the heme, the electrons are used to reduce and activate O_2. In a first step, NOS hydroxylates L-arginine to N^G-hydroxy-L-arginine (which remains largely bound to the enzyme). In a second step, NOS oxidizes N^G-hydroxy-L-arginine to citrulline and NO [96, 123]. In human eNOS, cysteine99 (Cys99, which is part of the zinc thiolate cluster) is thought to represent (or largely contribute to) the binding site for BH_4. Mutation of the homologous Cys331 in nNOS to alanine (Cys331Ala) led to an enzyme that lost its binding affinity for BH_4 and became catalytically inactive [81]. Interestingly, the estimated K_d for L-arginine binding to the Cys331Ala-mutated nNOS was >100 μmol/L [81] compared with 2–3 μmol/L for the wild-type enzyme, suggesting cooperativity between L-arginine and BH_4. binding (Fig. 1) [81, 85].

Regulation of eNOS activity

When intracellular Ca^{2+} is enhanced, eNOS activity markedly increases; eNOS synthesizes NO in a pulsatile, Ca^{2+}/CaM-dependent manner. Ca^{2+} induces the binding of

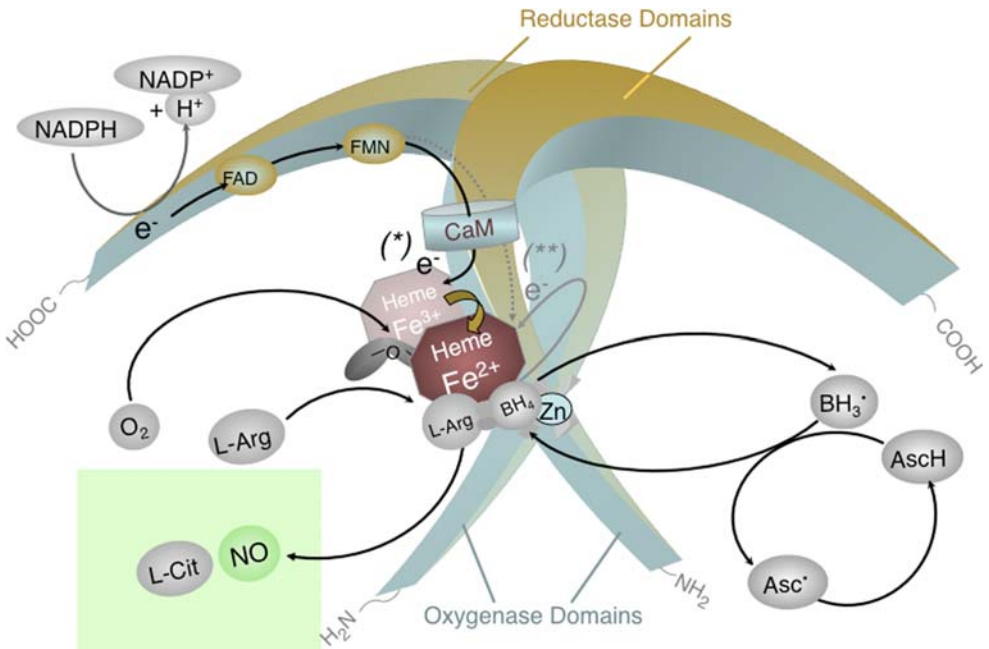


Fig. 1 Scheme of a functional eNOS. eNOS is a homodimeric enzyme; each subunit consists of a reductase domain and an oxygenase domain. NADPH donates electrons for NOS; the flow of electrons is only shown from the reductase domain of one monomer (*left*) to the oxygenase domain of the other monomer. The enzyme contains a zinc (Zn) thiolate cluster at the dimer interface. This site is of major importance for the binding of the cofactor (6*R*-)5,6,7,8-tetrahydrobiopterin (*BH_4*) and the substrate L-arginine (*L-Arg*). Interestingly, there is evidence for a mutual enhancement of BH_4 and L-arginine binding, which may be of therapeutic relevance in vivo. Electron transfer from the reductase domain *(*)* enables NOS ferric (Fe^{3+}) heme to bind O_2 and form a ferrous (Fe^{2+})-dioxy species. This species may receive a second electron preferentially from BH_4 (or alternatively from the reductase domain) *(**)*. This activates oxygen and allows the catalysis of L-arginine hydroxylation. The oxidized species formed from BH_4 is trihydrobiopterin radical ($BH_3\cdot$) or trihydropterin radical cation protonated at N5 ($BH_3\cdot H^+$). The $BH_3\cdot$ radical (or radical cation) can be recycled to BH_4 by eNOS itself (using an electron supplied by the flavins) or by L-ascorbic acid (which is present in cells in millimolar concentrations). This function of L-ascorbic acid can explain its stimulatory effect on eNOS activity. The $BH_3\cdot$ radical can be converted to the quinonoid 6,7-[8H]-H2-biopterin (BH2), which can also be reduced by L-ascorbic acid back to BH_4. BH_4 is likely to also play a redox role in the second reaction cycle, i.e., the conversion of N^{ω}-hydroxy-L-arginine to NO. *AscH* ascorbic acid, *Asc* ascorbate radical

CaM to the enzyme, which in turn increases the rate of electron transfer from NADPH to the heme center [45].

However, eNOS can also be activated by stimuli that do not produce sustained increases in intracellular Ca^{2+}, but still induce a long-lasting release of NO. The best established such stimulus is the shear stress of the flowing blood, which can increase enzyme activity at resting Ca^{2+} levels. This activation is mediated by phosphorylation of the enzyme [25] (Fig. 2).

The eNOS protein can be phosphorylated on several serine (Ser), threonine (Thr), and tyrosine (Tyr) residues; however, major changes in enzyme function have been reported for the phosphorylation of amino acid residues Ser1177 and Thr495 (in the human eNOS sequence). Phosphorylation of Ser1177 stimulates the flux of electrons within the reductase domain, increases the Ca^{2+} sensitivity of the enzyme, and represents an additional and independent mechanism of eNOS activation [25]. Several protein kinases can phosphorylate eNOS at Ser1177 can participate in eNOS activation following mechanical and/or hormonal stimulation of endothelial cells. These kinases include Akt/protein kinase B, protein kinase A (PKA), 5′-AMP-activated protein kinase (AMPK), and calmodulin-dependent kinase II (CAMKII) (Fig. 2).

The amino acid residue Thr495, on the other hand, is a negative regulatory site whose phosphorylation decreases enzymatic activity. This site tends to be phosphorylated under non-stimulated conditions (most probably by protein kinase C, PKC). Phosphorylation of Thr495 is likely to interfere with the binding of CaM to the CaM-binding domain. In fact, dephosphorylation of Thr495 is associated with stimuli such as histamine or bradykinin that elevate intracellular Ca^{2+} concentrations and increase eNOS activity markedly. In endothelial cells stimulated with such Ca^{2+}-elevating agonists, substantially more CaM binds to eNOS when Thr495 is dephosphorylated [25] (Fig. 2).

In vascular disease, oxidative stress reduces the bioavailability of vascular NO

Oxidative stress in vascular disease (i.e., a relative overproduction of ROS), contributes markedly to endothelial

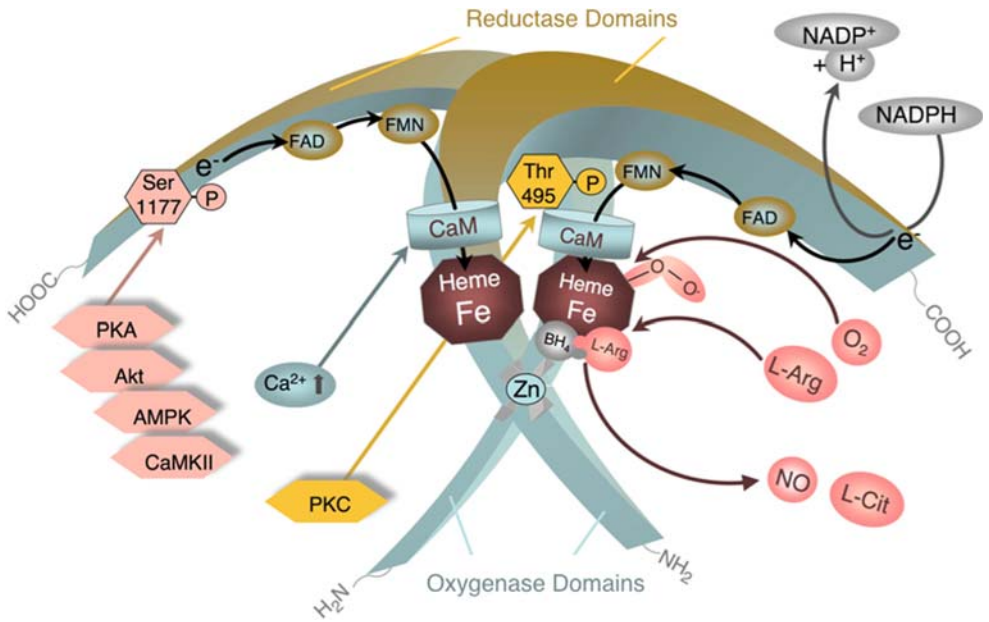


Fig. 2 Regulation of eNOS activity by intracellular Ca^{2+} and phosphorylation. An increase in intracellular Ca^{2+} (as produced by agonists such as histamine or bradykinin) leads to an enhanced binding of CaM to the enzyme, which in turn displaces an auto-inhibitory loop and facilitates the flow of electrons from NADPH in the reductase domain to the heme in the oxygenase domain. There are several potential phosphorylation sites in eNOS, but most is known about the functional consequences of phosphorylation of Ser1177 (human eNOS sequence) in the reductase domain and Thr495 (human eNOS sequence) within the CaM-binding domain. In resting endothelial cells, Ser1177 is usually not phosphorylated. Phosphorylation is induced when the cells are exposed to fluid shear stress, estrogens, VEGF, insulin, or bradykinin. The kinases responsible for phosphorylation depend on the primary stimulus. Shear stress elicits the phosphorylation of Ser1177 by activating protein kinase A (*PKA*), estrogen and VEGF phosphorylate eNOS mainly via Akt, insulin probably activates both Akt and the AMP-activated protein kinase (*AMPK*), and the bradykinin-induced phosphorylation of Ser1177 is mediated by CaMKII. Phosphorylation of the Ser1177 residue increases the flux of electrons through the reductase domain and thus enzyme activity. The Thr495 residue of human eNOS tends to be constitutively phosphorylated in endothelial cells. Thr495 is a negative regulatory site, and its phosphorylation is associated with a decrease in enzyme activity. The constitutively active kinase that phosphorylates eNOS Thr495 is most probably protein kinase C (*PKC*). The phosphatase that dephosphorylates Thr495 appears to be protein phosphatase1

dysfunction. In the state of oxidative stress, the production of ROS exceeds the available antioxidant defense systems. As a consequence, bioactivity of NO is reduced. A dominant mechanism reducing bioavailability of vascular NO relates to its rapid oxidative inactivation by the ROS superoxide (O_2^{-}). In addition, there is evidence that persisting oxidative stress will render eNOS dysfunctional such that it no longer produces NO, but $O_2^{-}\cdot$ (see below).

Cardiovascular risk factors and vascular disease are associated with an increased production of reactive oxygen species in the vascular wall

Risk factors for cardiovascular disease include (but are not limited to) hypertension [75], diabetes mellitus [48], hypercholesterolemia [142], and atherosclerosis [115]. These risk factors lead to dramatic increases in reactive ROS in the vascular wall, a situation that culminates into oxidative stress. ROS include free oxygen radicals, oxygen ions, and peroxides (Fig. 3a). There are several enzyme systems that can potentially produce ROS in the vessel wall, with four systems being of major importance. These include NADPH oxidase, xanthine oxidase, a dysfunctional eNOS (in which oxygen reduction is uncoupled from NO synthesis), and enzymes of the mitochondrial respiratory chain [92] (Fig. 3a).

NADPH oxidases

NADPH oxidases are multicomponent enzymes functional in membranes of various cell types including endothelial cells, smooth muscle cells, and fibroblasts. Several isoforms of $O_2^{-}\cdot$-producing NADPH oxidase exist in the vascular wall. Evidence for an activation of NADPH oxidase in the vasculature has been provided in animal models of vascular disease such as angiotensin II-induced hypertension [31, 83], genetic hypertension [76], diabetes mellitus [48], and hypercholesterolemia [142]. In atherosclerotic arteries, there is evidence for increased expression of the NADPH oxidase subunits gp91phox (Nox2) and Nox4 [115]; angiotensin II leads to an overexpression of Nox1 [83]. A confirmation of the role of NADPH oxidase-derived ROS in hypertension and atherosclerosis came from studies with genetic disruption of subunits of the enzyme. Knockout of the p47phox subunit reduced blood pressure responses to angiotensin II and diminished atherogenesis in apolipoprotein E $(apoE)^{-/-}$ mice [6, 65]. Similarly, Nox1-deficient mice show smaller blood pressure increases to angiotensin II [83], whereas mice overexpressing Nox1 in smooth muscle showed greater blood pressure responses to angiotensin II and increased $O_2^{-}\cdot$ production [21].

Involvement of the renin–angiotensin system

The inflammatory cells present in the atherosclerotic vessel wall are capable of producing angiotensin II. Angiotensin-converting enzyme activity, as well as local angiotensin II concentrations, is increased in atherosclerotic plaques [20, 100]. In addition, in hypercholesterolemia, local renin–angiotensin systems may be activated. The stimulating effects of angiotensin II on the activity of NADPH oxidases suggests that an activated renin–angiotensin system could cause increased vascular $O_2^{-}\cdot$ production and thus vascular dysfunction [35]. In vessels from hypercholesterolemic animals [133] and in platelets from hypercholesterolemic patients [95], the angiotensin II receptor subtype AT1 has been found to be upregulated.

Xanthine oxidase

The enzyme xanthine oxidase is generated from xanthine dehydrogenase by proteolysis. Xanthine oxidase readily donates electrons to molecular oxygen, thereby producing $O_2^{-}\cdot$ and hydrogen peroxide. Oxypurinol, an inhibitor of xanthine oxidase, has been shown to reduce $O_2^{-}\cdot$ production and improve endothelium-dependent vascular relaxations to acetylcholine in blood vessels from hyperlipidemic animals [98]. This suggests a contribution of xanthine oxidase to endothelial dysfunction in early hypercholesterolemia. The source of xanthine oxidase is not completely clear, but increased cholesterol levels have been shown to stimulate the release of the enzyme from the liver into the circulation. This circulating xanthine oxidase can then associate with endothelial glycosaminoglycans [147]. Unlike NADPH oxidases, the relative importance of xanthine oxidase for endothelial dysfunction is less certain. Recent experimental evidence has suggested that endothelial cells themselves can express xanthine dehydrogenase (and thus xanthine oxidase) and that this expression is regulated in a redox-sensitive way, dependent on the endothelial NADPH oxidase [84]. However clinical data relative to xanthine oxidase are controversial. Whereas some investigators reported an improvement of endothelial dysfunction in hypercholesterolemic and diabetic patients with xanthine oxidase inhibitors such as oxypurinol and allopurinol [14, 15], others failed to show an effect with allopurinol [97].

Respiratory chain of the mitochondria

About 1% of the O_2 consumed by mitochondria is reduced by only a single electron thereby forming $O_2^{-}\cdot$. $O_2^{-}\cdot$ can be produced in at least two locations within the respiratory chain; in the NADH dehydrogenase (complex I) and in the ubiquinone–cytochrome b–c_1 region (complex III) [129]. However, the actual amount of $O_2^{-}\cdot$ released by the

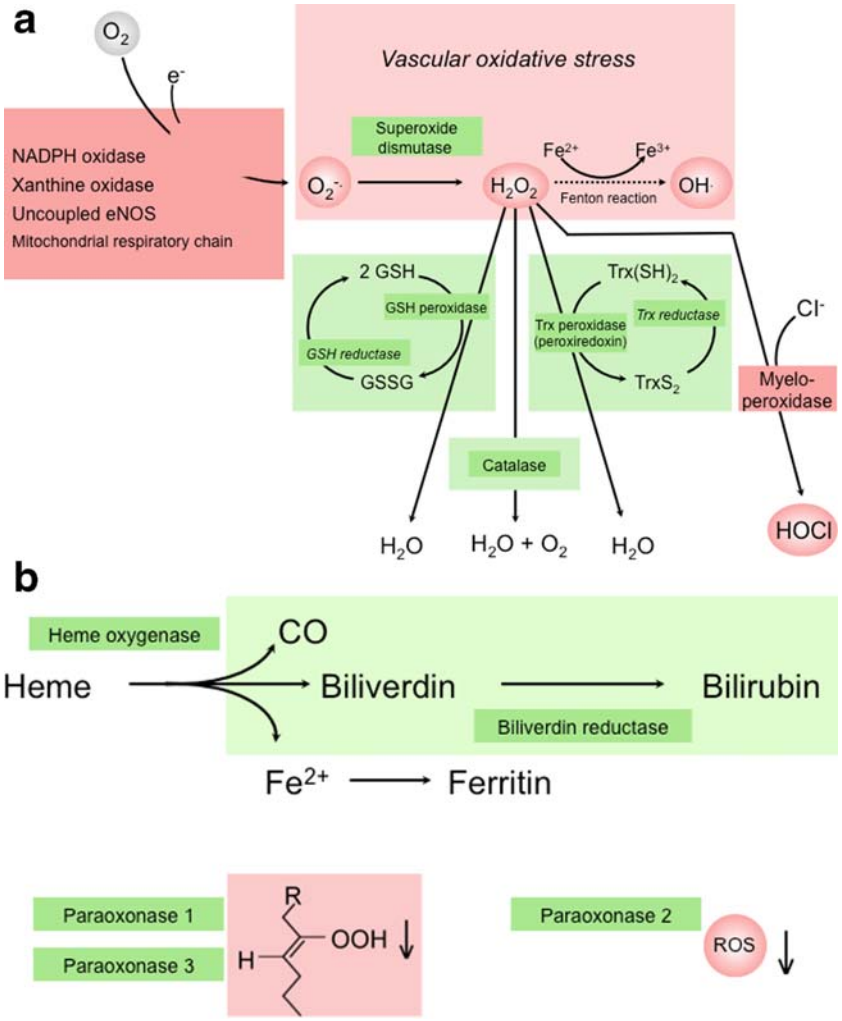


Fig. 3 Enzymes involved in the generation and inactivation of reactive oxygen species (*ROS*). There are numerous enzyme systems that generate and degrade ROS; a relative overproduction of ROS results in oxidative stress (*light red box*, **a**). Molecular oxygen (O_2) reacts with an impaired electron (e^-) to form the superoxide anion ($O_2^{-\cdot}$). Numerous studies implicate NADPH oxidases, xanthine oxidase, "uncoupled" eNOS (in which oxygen reduction has been uncoupled from NO synthesis), and the "leakage" of activated oxygen from mitochondria during oxidative respiration as important sources of $O_2^{-\cdot}$ in the cardiovascular system (*red boxes*). $O_2^{-\cdot}$ can be converted to hydrogen peroxide (H_2O_2, the two-electron reduction state of O_2) by the enzyme superoxide dismutase. H_2O_2 can undergo spontaneous conversion to hydroxyl radical (OH·, the three-electron reduction state of O_2) via the Fenton reaction. OH· is extremely reactive and will attack most cellular components. H_2O_2 can be detoxified via glutathione peroxidase, catalase, or thioredoxin (*Trx*) peroxidase to H_2O and O_2 (*green boxes*). The enzyme myeloperoxidase can use H_2O_2 to oxidize chloride to the strong oxidizing agent hypochlorous acid (*HOCl*). Blood levels of myeloperoxidase have been found to correlate with endothelial dysfunction and the risk for myocardial infarction. HOCl can chlorinate and thereby inactivate various biomolecules, including lipoproteins and the eNOS substrate L-arginine. Besides HOCl generation, MPO can oxidize NO to nitrite (NO_2^-) in the vasculature. Also inducible heme oxygenase 1 and its products function as adaptive molecules against oxidative stress (**b**). Heme oxygenase is the first rate-limiting enzyme in heme breakdown to generate equimolar quantities of carbon monoxide (CO), biliverdin, and free ferrous iron. For mechanisms by which heme oxygenase reduces oxidative stress, see text. Other enzymes with antioxidant properties are paraoxonases. Paraoxonase isoforms 1 and 3 are circulating in serum residing on high-density lipoprotein, decreasing high- and low-density lipoprotein lipid peroxidation. Paraoxonase 2 is cell-associated and seems to represent a novel endogenous defense mechanism against vascular oxidative stress. The biochemical mechanism is still largely unclear

mitochondria also depends on the activity of Mn-containing superoxide dismutase2 (SOD2) located in the mitochondrial matrix (see below). There is evidence to suggest that some cardiovascular diseases are associated with mitochondrial dysfunction [106] and that mitochondrial production of ROS may be linked to the development of early atherosclerotic lesions [5]. Mitochondrial dysfunction, e.g., resulting from SOD2 deficiency, increases mitochondrial DNA (mtDNA) damage and accelerates atherosclerosis in apoE$^{-/-}$ mice [99]. However, a causal

role of mitochondrial $O_2^{-}\cdot$ for vascular disease in vivo remains to be established.

eNOS itself can be a source of superoxide

NOS enzymes contain four redox active prosthetic groups (FAD, FMN, heme, and BH_4) that could—in principle—pass electrons to O_2 [40, 82, 104, 150]. Thus, electron transfer in NOS enzymes needs to be tightly controlled to prevent uncoupling of O_2 reduction from NO synthesis that will turn a functional NOS into a dysfunctional $O_2^{-}\cdot$-generating enzyme [123].

Evidence for eNOS uncoupling has been obtained in peroxynitrite-treated rat aorta [69], in endothelial cells treated with low-density lipoprotein [105], and in isolated blood vessels from animals with pathophysiological conditions such as SHR [18], stroke-prone spontaneously hypertensive rats (SHRSP) [60], angiotensin II-induced hypertension [90], deoxycorticosterone acetate (DOCA)–salt hypertension [66], streptozotocin-induced diabetes [48], or nitroglycerin tolerance [43].

eNOS uncoupling has also been seen in patients with endothelial dysfunction due to hypercholesterolemia [121], diabetes mellitus [41] or essential hypertension [47], and in chronic smokers [43].

Antioxidant enzymes potentially protecting against vascular oxidative stress

Important antioxidant enzymes include superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase, heme oxygenase (HO), and the thioredoxin (Trx) peroxidase (Fig. 3a, b) and perhaps also paraoxonases (PON).

Superoxide dismutase

SOD catalyzes the dismutation of $O_2^{-}\cdot$ into oxygen and hydrogen peroxide, thereby serving a key antioxidant role (Fig. 3a). In humans, three forms of the enzyme are present. SOD1 (Cu–Zn–SOD) is located in the cytoplasm, SOD2 (Mn–SOD, see above) in the mitochondria, and SOD3 (Cu–Zn–SOD) is extracellular. In the cardiovascular system, the action of SOD3 lowers $O_2^{-}\cdot$ and maintains vascular NO levels [59]. Mice with a deleted SOD1 gene develop normally and show no marked phenotype under normal conditions. However, they are more susceptible to myocardial ischemia–reperfusion injury [155]. A common gene variant of human SOD3, present in approximately 5% of humans, shows decreased SOD activity, oxidative stress, and accelerated NO inactivation. This variant is indeed associated with an increased risk of ischemic heart disease [55].

Catalase

The enzyme catalase catalyzes the decomposition of hydrogen peroxide to water and oxygen (Fig. 3a). The overall biological significance of catalase is not completely clear. Homozygous catalase knockout mice develop normally and show no gross abnormalities, indicating that this enzyme is dispensable for animal life [50]. Interestingly, overexpression of catalase has protective effects in the cardiovascular system such as delayed development of atherosclerosis [154] and inhibition of angiotensin II-induced aortic wall hypertrophy [161].

Glutathione peroxidase

GPx reduces free hydrogen peroxide to water and lipid hydroperoxides to their corresponding alcohols (Fig. 3a). There are several isozymes, and GPx1 is the most abundant version, found in the cytoplasm of many mammalian tissues. Mice genetically engineered to lack GPx1 are apparently healthy and fertile, but exhibit a pronounced susceptibility to myocardial ischemia–reperfusion injury [156]. In patients with coronary artery disease, the activity of red blood cell GPx1 is inversely associated with the risk of cardiovascular events [10]. In apoE-deficient mice, the deficiency of GPx1 accelerates and modifies atherosclerotic lesion progression [127]. GPx4 knockout mice are not viable; they die during early embryonic development.

Heme oxygenase

HO catalyzes the first step in heme breakdown to generate equimolar quantities of carbon monoxide, biliverdin, and free ferrous iron (Fig. 3b). There is ample evidence that HO1 can protect against vascular remodeling and atherogenesis [119]. HO1 is induced by oxidative stress, probably as an adaptive response. The proposed mechanisms by which HO1 may protect cells include its abilities to degrade the pro-oxidative heme to biliverdin. This gets subsequently converted to bilirubin, which has radical-scavenging properties. Moreover, bilirubin seems to directly inhibit fully functional NADPH oxidase and can also interrupt assembly and activation of the enzyme [57]. In addition, a decrease in heme content caused by HO1 limits heme availability for maturation of the Nox2 subunit of NADPH oxidase prevents assembly of a functional enzyme and reduces cellular ROS generation [126]. Carbon monoxide generated by HO has antiproliferative and anti-inflammatory as well as vasodilator properties [91]. The potential cytotoxic effects of iron (e.g., stimulation of the Fenton reaction; Fig. 3a) are limited by the simultaneous enhancement of intracellular ferritin [91]. Genetic models of HO1 deficiency or overexpression of HO1 suggest that the actions of

HO1 are important in modulating the severity of atherosclerosis [51].

Thioredoxin

Also Trx has been recognized as critical protective system acting via direct (antioxidant) and indirect (regulation of signal transduction) effects [153]. Trx is present in endothelial cells and vascular smooth muscle. Trx seems to exert most of its ROS-scavenging properties through Trx peroxidase (peroxiredoxin), which uses endogenous SH groups as reducing equivalents (Fig. 3a). Trx reduces the oxidized form of Trx peroxidase and the reduced Trx peroxidase scavenges ROS (such as H_2O_2; Fig. 3a) and also $ONOO^-$ [153].

Paraoxonase

The PON family of enzymes (Fig. 3b) seems to contribute to vascular antioxidant defense and protect against coronary artery disease [3]. The PON1 and PON3 enzymes are synthesized in the liver and circulate in plasma associated with the high-density lipoprotein (HDL) fraction. The capacity of HDL in decreasing HDL and LDL lipid peroxidation largely depends on its PON1 content [3]. Deletion of the PON1 gene increases oxidative stress in mouse macrophages and aortae [110], and $apoE^{-/-}$ mice overexpressing PON1 developed fewer atherosclerotic lesions [130]. PON2 is expressed in many cell types. Polymorphisms of the PON2 gene have been reported to be associated with cardiovascular diseases [70]. The enzyme has been shown to reduce ROS in human endothelial cells, vascular smooth muscle cells, and fibroblasts [53]. The ROS species removed by PON2 is O_2^-, not H_2O_2 (Horke and Förstermann, unpublished). PON2-deficient mice with an $apoE^{-/-}$ background developed more atherosclerotic lesions, whereas PON2-overexpressing mice were protected against those lesions [93].

Molecular mechanisms contributing to reduced levels of bioactive NO in vascular disease

Endothelial dysfunction is largely equivalent to the inability of the endothelium to generate adequate amounts of bioactive NO (and to produce NO-mediated vasodilation). Endothelial dysfunction has been shown for patients with all common types of cardiovascular risk factors. Several possible defects could account for reductions in bioactive NO. These include an increased depletion of NO due to its reaction with O_2^-, a decreased NO production due to changes in the activity of eNOS (e.g., eNOS uncoupling), or a decreased NO production due to changes in the expression of the enzyme. Also, a depletion of the NOS substrate L-arginine, e.g., due to enhanced arginase activity, could contribute to endothelial dysfunction.

Oxidative stress produced by NADPH oxidase induces eNOS uncoupling

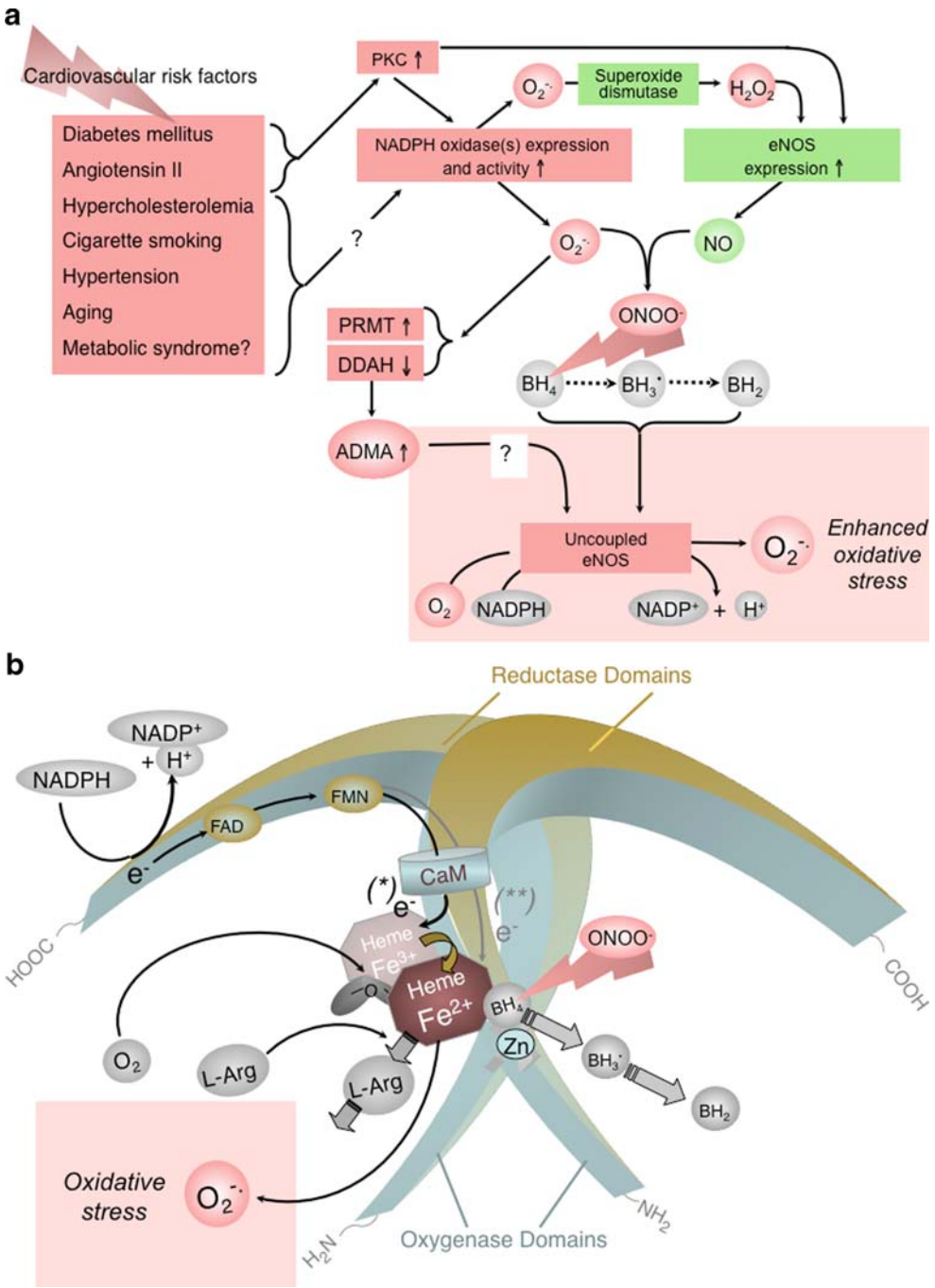
There is a growing body of evidence that vascular NADPH oxidase plays a crucial role in the phenomenon of eNOS uncoupling (Fig. 4a). An important hint came from experiments with NADPH oxidase (p47phox) knockout animals [66]. When hypertension was induced in normal mice with a combination of the mineralocorticoid DOCA and salt, these animals showed an increased production of vascular ROS. This was significantly reduced by the NOS inhibitor L-NAME, demonstrating a marked contribution of uncoupled eNOS to oxidative stress in vascular tissue. p47phox knockout animals showed much less oxidative stress upon DOCA–salt treatment, and levels of ROS could no longer be reduced with L-NAME [66]. These findings demonstrate that NADPH oxidase-derived ROS can indeed represent the trigger leading to eNOS uncoupling and that uncoupled eNOS significantly contributes to oxidative stress [66]. The detailed mechanism of how NADPH oxidase-derived ROS can trigger eNOS uncoupling is described below (Fig. 4a).

Molecular mechanisms triggering eNOS uncoupling

Oxidation of BH_4 is likely to represent a major mechanism for eNOS uncoupling

NO and L-citrulline production by eNOS in endothelial cells correlates closely with the intracellular concentration of BH_4 [108, 146]. In isolated arteries [17] or rats in vivo [152], a BH_4 depletion produced endothelial dysfunction. Conversely, supplementation with BH_4 is capable of correcting eNOS dysfunction in several types of pathophysiology: In isolated aortas from prehypertensive SHR, BH_4 supplementation diminished the NOS-dependent generation of $O_2^{-}\cdot$ [18]. Administration of BH_4 restored endothelial function in animal models of diabetes [102] and insulin resistance [113], as well as in patients with hypercholesterolemia [121], diabetes mellitus [41], essential hypertension [47], and in chronic smokers [43].

Intracellular BH_4 levels depend on the balance of its de novo synthesis and its degradation or oxidation. BH_4 is one of the most potent naturally occurring reducing agents. It is therefore reasonable to hypothesize that oxidative stress may lead to excessive oxidation and depletion of BH_4 [69, 87]. As oxidative stress occurs in cardiovascular pathophysiology (see above), oxidation of BH_4 may be the



common cause of eNOS dysfunction in these situations. In agreement with this concept, BH_4 levels have been found decreased in the aorta from insulin-resistant rats [112], in plasma of SHR compared with age-matched WKY rats [52], in aorta of hypercholesterolemic–apoE-knockout mice [69] and in DOCA–salt-treated hypertensive rats [66]. Conversely, an infusion of the eNOS cofactor BH_4 can restore eNOS functionality as demonstrated by studies in chronic smokers [43], diabetics [41], hypercholesterolemic patients [121], and hypertensive individuals [47].

$ONOO^-$—the direct reaction product of NO. and O_2^{-}—is able to oxidize BH_4 (Fig. 4b). Published data show that $ONOO^-$ oxidizes BH_4 to the BH_3· radical, which is re-reduced to BH_4 by NOS itself or when enough reducing equivalents such as vitamin C are available [63, 145] (Fig. 1). BH_3. radical can also disproportionate to the quinonoid 6,7-[8H]-H2-biopterin, which again can be reduced by vitamin C back to BH_4 [44, 145]. The exact mechanism of action of L-ascorbic acid is unknown, but as detailed below, the improvement of endothelial dysfunction

◀ **Fig. 4** **a** Potential mechanisms by which risk factors for atherosclerosis and various cardiovascular diseases lead to eNOS uncoupling and endothelial dysfunction. In many types of vascular disease, NADPH oxidases are upregulated in the vascular wall and generate superoxide ($O_2^{-}\cdot$). In experimental diabetes mellitus and angiotensin II-induced hypertension, this has been shown to be mediated by protein kinase C (*PKC*) [48, 90]. Expression of eNOS is also increased in vascular disease. H_2O_2, the dismutation product of $O_2^{-}\cdot$, can increase eNOS expression via transcriptional and post-transcriptional mechanisms (*SOD* superoxide dismutase) [23]. In addition, also PKC activation can enhance eNOS expression [74], and PKC inhibitors reduce eNOS expression levels in vascular disease [48]. The products of NADPH oxidases and eNOS, $O_2^{-}\cdot$ and NO, rapidly recombine to form peroxynitrite ($ONOO^{-}$). This can oxidize the essential cofactor of eNOS (6*R*-)5,6,7,8-tetrahydrobiopterin (BH_4) to trihydrobiopterin radical (BH_3.) [7, 33]. BH_3. can disproportionate to the quinonoid 6,7-[8H]-H_2-biopterin (BH_2). As a consequence, oxygen reduction and O_2 reduction by eNOS are uncoupled from NO formation, and a functional NOS is converted into a dysfunctional $O_2^{-}\cdot$-generating enzyme that contributes to vascular oxidative stress. The enhanced eNOS expression (see above) aggravates the situation. Under oxidative stress, concentrations of asymmetric dimethyl-L-arginine (*ADMA*) increase in cells because arginine N-methyltransferase (*PRMT*, type I) is upregulated [11], and the ADMA-degrading enzyme dimethylarginine dimethylaminohydrolase (*DDAH*) is downregulated [78]. ADMA in turn may contribute to eNOS uncoupling [124]. **b** Scheme of an endothelial NO synthase (*eNOS*) whose oxygen reduction is uncoupled from NO synthesis. Oxidative stress is associated with endothelial dysfunction. $ONOO^{-}$ can oxidize BH_4 to biologically inactive products such as trihydrobiopterin radical ($BH_3\cdot$) or trihydropterin radical cation protonated at N5 ($BH_3\cdot H^{+}$). The $BH_3\cdot$ radical can be converted to the quinonoid 6,7-[8H]-H_2-biopterin (BH_2), which also lacks biological activity. When $ONOO^{-}$ overwhelms the cell's capacity to re-reduce these products to BH_4, eNOS "uncouples" and reduces oxygen to $O_2^{-}\cdot$, but does not synthesize NO anymore. eNOS then contributes to oxidative stress in the cell

by vitamin C may be due to enhanced regeneration of BH_4 rather than the scavenging of $O_2^{-}\cdot$ (see Fig. 1).

Potential role of L-arginine levels for eNOS uncoupling

Beneficial effects of L-arginine supplementation have been documented in both animal studies and humans under pathophysiological conditions such as hypercholesterolemia and hypertension [22, 49, 56, 109]. This raises the question as to whether L-arginine concentrations can become critical as a substrate in vivo. At first glance, this appears unlikely. The K_m of eNOS for L-arginine is ~3 μmol/L [103], normal L-arginine plasma concentrations are ~100 μmol/L (even in pathophysiology they hardly fall below 60 μmol/L), and there is an up to 10-fold accumulation of L-arginine within cells [16]. In addition, human endothelial cells are not even dependent on L-arginine uptake from the extracellular space; they can effectively recycle L-citrulline to L-arginine and can also obtain L-arginine from proteolysis [38, 114].

Endothelial cells, however, also express arginases that can compete with eNOS for substrate and—if highly expressed—"starve" eNOS. In porcine coronary and rat aortic endothelial cells, arginase I is constitutively expressed [13, 159], whereas arginase II can be induced by lipopolysaccharide [13]. In human endothelial cells, arginase II seems to be the predominant isozyme [4, 88]. An upregulated expression and activity of arginase II has been found in human diabetic corpus cavernosum [9] and in endothelium from patients with pulmonary hypertension [151]. Evidence for a role of increased enzymatic activity of arginase in endothelial dysfunction has also been provided in animal models of cardiovascular disease such as aging [8], atherosclerosis [88], endothelial dysfunction following ischemia–reperfusion [39], and hypertension induced by aortic coarctation or high salt [58, 160]. In apoE-knockout mice, the expression of arginase II was unchanged compared with wild-type mice, but the activity of the enzyme was markedly increased [88]. Similarly, in human umbilical vein endothelial cells, arginase II enzymatic activity, but not protein expression, was enhanced after an 18- to 24-h exposure to thrombin [88] or a 24-h stimulation with inflammatory cytokines [4].

Thus, a relative L-arginine deficiency in the vicinity of eNOS caused by excessive arginase activity is conceivable and could explain part of the beneficial effects of L-arginine supplementation. However, also non-substrate effects of L-arginine could contribute to these effects. These include potential direct radical-scavenging properties of the guanidino nitrogen group, the cooperativity between L-arginine and BH_4 binding sites of NOS [34, 81] (see Fig. 1), or the competition of L-arginine with the derivative asymmetric dimethyl-L-arginine (ADMA), which is an endogenous inhibitor of eNOS activity [125].

Contribution of ADMA to eNOS uncoupling?

ADMA represents an independent predictor for all-cause cardiovascular mortality. The activities (not the expression) of both protein arginine *N*-methyltransferase (PRMT), type I [11], and of the ADMA-degrading enzyme dimethylarginine dimethylaminohydrolase (DDAH) [78] are redox sensitive (Fig. 4a). In cultured endothelial cells, rat models and man, oxidative stress has been shown to increase the activity of PRMT(s) and decrease that of DDAH, thereby leading to increased ADMA concentrations [11, 78, 125]. Thus, an increased production of ROS could be the reason for increased ADMA levels. Elevated ADMA inhibits NO synthesis by eNOS or could even uncouple the enzyme [125] (Fig. 4). However, it remains to be established whether ADMA concentrations reached in vivo (even in pathophysiology) are sufficient to effectively interact with eNOS.

A reduced eNOS expression is unlikely to contribute to endothelial dysfunction

Several studies have shown that cardiovascular risk factors are associated with an increase rather than a decrease in eNOS expression [37, 48, 69, 72, 76, 90, 132]. The increased expression of eNOS in vascular disease is likely to be a consequence of an increased production of the ROS H_2O_2, the dismutation product of $O_2^{-}\cdot$. H_2O_2. can increase eNOS expression through transcriptional and post-transcriptional mechanisms [23] (see Fig. 4a).

Vascular oxidative stress and endothelial dysfunction predispose to atherosclerosis

Most risk factors favoring the development of atherosclerosis (such as hypertension, hypercholesterolemia, diabetes, cigarette smoking, or a positive family history of premature coronary artery disease) are associated with vascular oxidative stress and endothelial dysfunction [137]. Pathophysiological mechanisms are probably best established for hypertension. Angiotensin II—through stimulation of AT_1 receptors—leads to an upregulation and activation of NADPH oxidases in the vascular wall resulting in oxidative stress [90, 94, 158]. Angiotensin II can increase NADPH oxidase activity by inducing a rapid translocation of the small GTPase rac1 to the cell membrane [143] or by phosphorylation and translocation of the NADPH oxidase subunit p47phox to the membrane [128]. Also, mechanical stretch, characteristic of hypertension, can induce p47phox membrane translocation and NADPH oxidase activation [36]; stretch-induced NADPH oxidase activation was absent in p47phox-deficient cells. A critical role for oxidative stress has been demonstrated in hypertensive rats. Treatment of these rats with statins decreased $O_2^{-}\cdot$ production and reduced blood pressure [144].

Endothelial dysfunction is already found in the preclinical stage of atherosclerosis and can be detected much earlier than angiographic or ultrasound evidence of structural coronary artery disease. In fact, coronary endothelial vasodilator dysfunction has been described as an independent predictor of the progression of atherosclerosis and the risk of cardiovascular events [111]. Interestingly, patients with impaired endothelium-dependent vasodilation in a peripheral vascular bed (the human forearm) have a higher risk for cardiovascular events over a follow-up period of 4.5 years [42].

However, it ought to be mentioned that hard evidence that oxidative stress is both necessary and sufficient for atherosclerosis has been difficult to find. Only if tools become available that limit oxidative stress at its source (and ameliorate its secondary phenomena) will we be able to finally decide what components of atherosclerosis are directly caused by oxidative stress.

Pharmacological approaches to reduce oxidative stress and prevent or reverse eNOS uncoupling

Angiotensin-converting enzyme inhibitors and AT_1 receptor antagonists

Angiotensin II activates NADPH oxidases via AT_1 receptor stimulation [35]. In addition, the AT_1 receptor is upregulated in vitro by LDL [142]. Therefore, ACE inhibitors and AT_1 receptor antagonists may have indirect antioxidant effects by preventing the activation of NADPH oxidase [61, 142] and/or increasing the activity of SOD3 [54]. Indeed, there is clinical evidence for a beneficial effect of inhibition of the renin–angiotensin system. ACE inhibitors improve endothelial function in patients with coronary artery disease and in hypertensive patients. Further, the Heart Outcome Prevention Evaluation (HOPE) trial [157] showed that treatment with ramipril greatly reduced the incidence of death, myocardial infarction, and stroke in high risk patients without heart failure. Also, the European Trial on Reduction of Cardiac Events with Perindopril in Stable Coronary Artery Disease (EUROPA) [29] demonstrated a 20% reduction with perindopril of the relative risk for cardiovascular endpoints in a patient population with stable coronary heart disease. In contrast, the Prevention of Events with Angiotensin Converting Enzyme Inhibition (PEACE) trial [12] could not show that patients with stable coronary artery disease and largely intact ventricular function have a therapeutic benefit from the addition of ACE inhibitors to modern conventional therapy. The failure of ACE inhibitors to reduce the cardiovascular events in this trial may have been attributable to the low overall event rate of hard endpoints, such as myocardial infarction or death in this patient population.

Statins (3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors)

Much of the clinical benefit obtained with statins is clearly related to their cholesterol-lowering properties, but effects unrelated to this effect seem to be beneficial on various aspects of cardiovascular disease [77]. Statins can inhibit endothelial $O_2^{-}\cdot$ formation by preventing the isoprenylation of p21 Rac, which is critical for the assembly of NADPH oxidase after activation of PKC [138]. In addition, SOD3 activity was more than doubled by simvastatin, and simvastatin treatment also increased the number of functionally active endothelial progenitor cells [67]. Finally, statins increase the expression of eNOS by inhibition of

Rho isoprenylation [68], and statins can also directly activate eNOS via post-translational mechanisms involving activation of the phosphatidylinositol 3-kinase/protein kinase Akt pathway [62]. Taken together, statin treatment improves endothelial function at least in part by reducing oxidant stress and improving eNOS function.

Folic acid and 5-methyltetrahydrofolate

Folic acid and its active form 5-methyltetrahydrofolate have been used successfully to restore endothelial function in patients with hypercholesterolemia [135], diabetes mellitus [131], or hyperhomocysteinemia [149] and in healthy volunteers with postprandial endothelial dysfunction [148].

In cultured endothelial cells and with recombinant eNOS enzyme, 5-methyltetrahydrofolate, NO synthesis was enhanced and $O_2^{-}\cdot$ production was reduced [122]. This provides a reasonable explanation for the clinical reports of positive effects of 5-methyltetrahydrofolate on endothelial function in vivo. The molecular mechanism by which folate or 5-methyltetrahydrofolate increase eNOS activity is not completely clear. It has been reported that folic acid reverses endothelial dysfunction by direct effects on the eNOS enzyme and independently of either the regeneration or stabilization of BH_4 [89, 122], whereas others have postulated that folates possess antioxidant properties and may recouple eNOS by BH_4 stabilization or BH_4 regeneration from quinonoid BH2 [134].

Polyphenolic antioxidants

Dietary polyphenolic antioxidants are likely to assist in preventing ROS damage. Epidemiological evidence suggests a negative correlation between the consumption of polyphenol-rich foods (fruits, vegetables, and cocoa contained in chocolate) or beverages (wine, especially red wine, grape juice, green tea, etc.) and the incidence of cardiovascular disease [2, 80, 120, 136]. Most polyphenols are only mild antioxidants, but some can reduce the activity of pro-oxidative NADPH oxidases, and others can stimulate anti-oxidative enzymes and eNOS [71, 117, 118, 139–141]. Current evidence suggests that small molecular weight compounds with indirect mechanisms of action, such as some dietary antioxidants, may offer more long-term benefit than directly acting radical scavengers or antioxidant vitamins (see below).

(6*R*-)5,6,7,8-tetrahydrobiopterin (BH_4)

Acute intraarterial infusion of the eNOS cofactor BH_4 can restore eNOS functionality (i.e., “recouple” eNOS) in clinical situations as demonstrated by studies in chronic

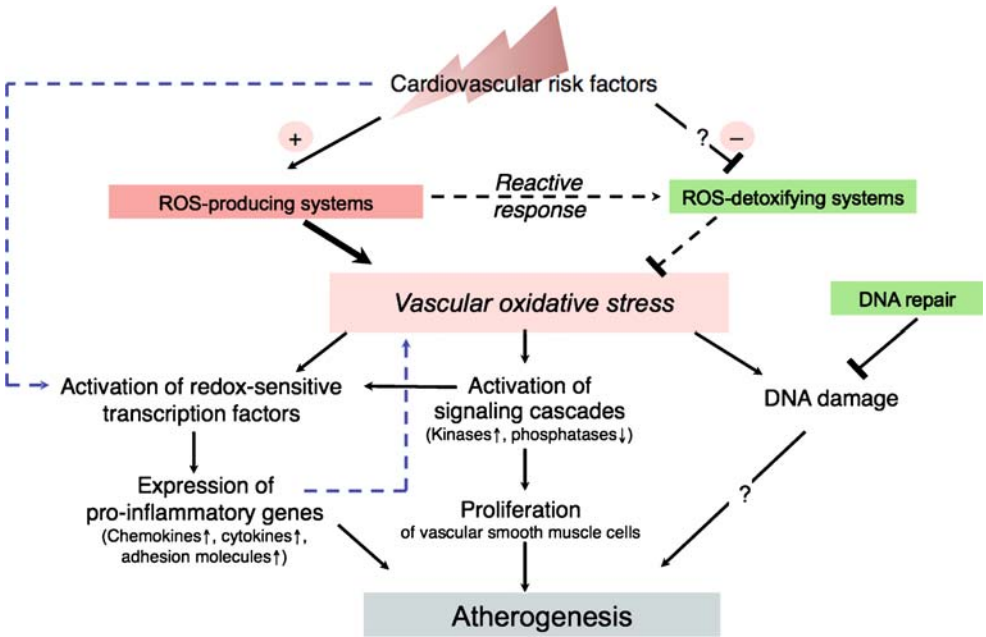


Fig. 5 Scheme viewing vascular oxidative stress as a cause of atherogenesis. Cardiovascular risk factors activate ROS-producing enzyme systems and/or weaken ROS-detoxifying systems. Many of the underlying mechanisms are still unclear. The resulting oxidative stress promotes atherogenesis through a number of different mechanisms, including the activation of redox-sensitive transcription factors (which stimulate the expression of pro-inflammatory genes) and the activation of signaling cascades (activation of kinases, inhibition of phosphatases). Elevated levels of ROS can also stimulate mitochondrial and nuclear DNA damage (and activate reactive DNA repair systems). Enhanced DNA damage is found associated with various types of vascular disease and may be involved in atherogenesis. However, despite important evidence in favor of the above hypothesis, the possibility remains that inflammation is the primary process of atherogenesis and oxidative stress the detrimental secondary response. The *dashed blue arrows* indicate this possibility

smokers [43], diabetics [41], hypercholesterolemic [121], and hypertensive [47] patients. Also, in coronary arteries of patients with hypercholesterolemia, BH_4 restored endothelial function [30]. However, the need for parenteral administration, the short half-life, and the high cost of BH_4 restrict its therapeutic use.

Vitamin C

In cultured endothelial cells, ascorbic acid (vitamin C) increased eNOS enzyme activity via regeneration of BH_4. Long-term in vivo treatment with vitamin C restored endothelial function and endothelial NOS activity in aortae of apoE-deficient mice. Also, in patients, acute infusions of high doses of vitamin C have been found to improve endothelial function [42, 64]. Some studies in patients also demonstrated improvements of endothelial function/endothelial NO production [32] or reduced levels of ROS in the vascular wall [24] after longer-term oral treatment with vitamin C. The exact mechanism of action of L-ascorbic acid in improving endothelial dysfunction is not clear, but the most important mechanism may be the enhanced regeneration or stabilization of BH_4 and eNOS "recoupling" rather than the scavenging of $O_2^{-}\cdot$ (Fig. 1). Disappointingly, however, long-term epidemiological trials with oral vitamin C have been ambiguous at best and do not support an important role for vitamin C in reducing the risk of coronary disease or other types of cardiovascular morbidity or mortality [101].

Vitamin E

Because of its antioxidant properties, also vitamin E has been believed to help prevent diseases associated with oxidative stress. However, large-scale randomized clinical trials undertaken to prove this hypothesis failed to verify a consistent benefit in terms of prevention of coronary heart disease and death [116]. In fact, there is enough evidence from large, well-designed studies to discourage the use of vitamin E in primary prevention of cardiovascular disease [79].

Conclusions

Endothelial dysfunction and oxidative stress has been identified as a common denominator of many cardiovascular risk factors. They support pro-inflammatory, prothrombotic, proliferative, and vasoconstrictor mechanisms that are involved in the initiation, progression, and complications of atherosclerosis. Diagnostically, a deficiency in bioactive NO in coronary or peripheral arteries appears to be predictive of future cardiovascular events. The pathophysiologic causes of oxidative stress are likely to involve changes in a number of different enzyme systems; most importantly, there is an upregulation of NADPH oxidases and eNOS (Fig. 5). Together, they lead to an increased production of $ONOO^{-}$. This conveys oxidative damage to eNOS and/or its cofactor BH_4 leading to "uncoupling" of the enzyme (Figs. 4 and 5). As a consequence, an increased production of ROS by uncoupled eNOS is likely to contribute significantly to vascular oxidative stress and endothelial dysfunction (Fig. 4).

Therapeutically, cardiovascular drugs in clinical use such as ACE inhibitors, AT_1 receptor blockers, and statins have pleiotropic actions that improve endothelial function. In addition, dietary polyphenolic antioxidants may prevent or reverse eNOS uncoupling and restore its normal function. Such compounds either block pathways leading to oxidative stress and/or upregulate antioxidant enzymes. Despite promising initial observations, clinical trials with antioxidant vitamins C and E failed to show an improved cardiovascular outcome.

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