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Forum Review Article

Nitric Oxide, NAD(P)H Oxidase, and Atherosclerosis

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Abstract

The endothelial cell layer plays a major role in the development and progression of atherosclerosis. Endothelial NO synthase (eNOS) produces nitric oxide (NO) from L-arginine. NO can rapidly react with reactive oxygen species to form peroxynitrite. This reduces NO availability, impairs vasodilatation, and mediates proinflammatory and prothrombotic processes such as leukocyte adhesion and platelet aggregation. In the vessel wall, specific NAD(P)H oxidase complexes are major sources of reactive oxygen species. These NAD(P)H oxidases can transfer electrons across membranes to oxygen and generate superoxide anions. The short-lived superoxide anion rapidly dismutates to hydrogen peroxide, which can further increase the production of reactive oxygen species. This can lead to uncoupling of eNOS switching enzymatic activity from NO to superoxide production. This review describes the structure and regulation of different NAD(P)H oxidase complexes. We will also focus on NO/superoxide anion balance as modulated by hemodynamic forces, vasoconstrictors, and oxidized low-density lipoprotein. We will then summarize the recent advances defining the role of nitric oxide and NAD(P)H oxidase-derived reactive oxygen species in the development and progression of atherosclerosis. In conclusion, novel mechanisms affecting the vascular NO/superoxide anion balance will allow the development of therapeutic strategies in the treatment of cardiovascular diseases. *Antioxid. Redox Signal.* 11, 1711–1731.

Introduction

Endothelial cells play a major role in the development and progression of atherosclerosis. Acetylcholine requires an intact endothelial cell layer for the release of nitric oxide (NO) leading to dilatation of adjacent vascular smooth muscle cells (66). Endothelial dysfunction represents an impaired vasodilatation in response to acetylcholine or bradykinin. Endothelial NO synthase (eNOS) produces NO from L-arginine amino acid. NO can rapidly react with reactive oxygen species (ROS) to form peroxynitrite, while reducing the amount of available NO (78). This reduced NO availability impairs endothelial function (Fig. 1), reducing vasodilatation and mediating proinflammatory and prothrombotic processes such as leukocyte adhesion and platelet aggregation (126). Specific NAD(P)H oxidase complexes are major sources of ROS in the vessel wall, and can transfer electrons across membranes to oxygen and produce superoxide anions ($\cdot O_2^-$). The short-lived superoxide anion rapidly dismutates spontaneously or by superoxide dismutase to hydrogen peroxide, which can increase an uncoupling of NO synthase dimers and the production of superoxide. Furthermore, vascular NAD(P)H oxidase-derived H_2O_2 amplifies its own production, resulting in self-propagation and prolongation of redox-sensitive signaling, thus contributing to vascular diseases (25). This review describes the structure and regulation of different NAD(P)H oxidase subunits. Second, this review will focus on NO/superoxide anion balance as modulated by hemodynamic forces, vasoconstrictors, and oxidized low-density lipoprotein, summarizing recent advances defining the role of nitric oxide and NAD(P)H oxidase-derived reactive oxygen species in the development and progression of atherosclerosis. Finally, we will discuss NO/superoxide anion balance regulation using a variety of therapeutic strategies in treating atherosclerosis and associated cardiovascular diseases.

Reactive Oxygen Species in the Vessel Wall

Reactive oxygen species (ROS) include oxygen-derived molecules transformed into radicals such as superoxide

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This review is dedicated to Professor Jürgen Holtz for his achievements in cardiovascular research on the occasion of his 65th birthday.

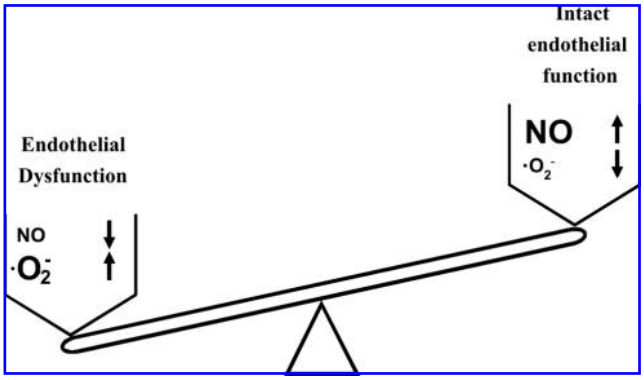


FIG. 1. Balance of nitric oxide and superoxide in endothelial function. Locally increased NO availability and reduced superoxide anion ($\cdot O_2^-$) generation results in intact endothelial function. This balance is disturbed in endothelial dysfunction.

anions ($\cdot O_2^-$), hydroxyl radicals ($HO^\bullet$), peroxyl radicals ($RO_2^\bullet$), or alkoxyl radicals ($RO^\bullet$). $\cdot O_2^-$ has a short half-life, and is rapidly converted into other ROS species such as hydrogen peroxide (H_2O_2) or peroxynitrite (139).

Several enzymatic sources of vascular ROS have been described, and include NAD(P)H oxidase, uncoupled endothelial NO synthase, mitochondrial electron transport enzymes, xanthine oxidase, cyclooxygenase, lipoxygenase, myeloperoxidase, and cytochrome P450 enzymes (Fig. 2). Specific NAD(P)H oxidase complexes are major molecular sources of $\cdot O_2^-$ (18); xanthine oxidase also contributes to increased ROS production (84), affecting the impaired endothelial vasodilator function of hypercholesterolemic patients as shown by its inhibition using oxypurinol (28, 169). An important source of ROS has been identified in uncoupled endothelial NO synthase (eNOS), which arises due to the limited availability of its cofactor tetrahydrobiopterin (BH_4) (127). The importance of BH_4 has been demonstrated in mice with eNOS overexpression with a genetic apolipoprotein E (apo E) knockout background. These animals developed severe atherosclerotic plaques due to the exaggerated production of ROS, while mice overexpressing GTP-cyclohydrolase I (GCH), the rate-limiting enzyme in BH_4 synthesis, show a decreased plaque area with an apoE-KO background (216). In addition, dysfunctional respiratory chain proteins lead to mitochondrial overproduction of ROS with increased LDL oxidation, apoptosis in vascular cells, and plaque rupture (145). Cyclooxygenase-derived prostaglandins and ROS are important mediators of inflammation processes during development of atherosclerosis (133), while lipoxygenases contribute to the pathogenesis of atherosclerosis by producing leukotrienes, inflammatory mediators, and ROS, thus causing phagocyte chemotaxis and increased vascular permeability (178). Myeloperoxidase (MPO) and products of MPO-mediated reactions are present in atherosclerotic lesions, and have been proposed as playing a key role in low-density and high-density lipoprotein oxidation, thereby contributing to atherosclerosis and counteracting HDL-mediated antiatherosclerotic effects. Furthermore, MPO causes oxidative modifications of lipids and lipoproteins via generation of reactive nitrogen species contributing to atherosclerosis, reduced NO availability, and endothelial dysfunction (37). Cytochrome P450 monooxygenase promotes atherosclerosis by metabolic activation of polycyclic aromatic hydrocarbons, which suppresses liver X receptor-mediated signal transduction (103). Cytochrome P450 enzymes are considerable sources of reactive oxygen species in the vessel wall, an interesting issue that has been recently addressed in an excellent review (59).

ROS play an important physiological role in signal transduction as second messengers (51). ROS formation is balanced by antioxidative mechanisms that include enzymatic degradation by superoxide dismutases, catalase, and glutathione peroxidase, or scavenging by α-tocopherol, β-carotene, ascorbate, glutathione, and thioredoxin. Disturbed equilibrium with increased ROS formation or reduced antioxidative capacity is termed oxidative stress, and exaggerated ROS production leading to oxidative stress has been considered as risk factor in the early development of atherosclerosis and cardiovascular diseases. Due to their high reactivity, ROS can interact with proteins, lipids, and nucleic acids, and affect or diminish their function (31). In addition to these deleterious effects, increased ROS formation due to an oxidative burst in phagocytes stimulates antimicrobial defense mechanisms (198).

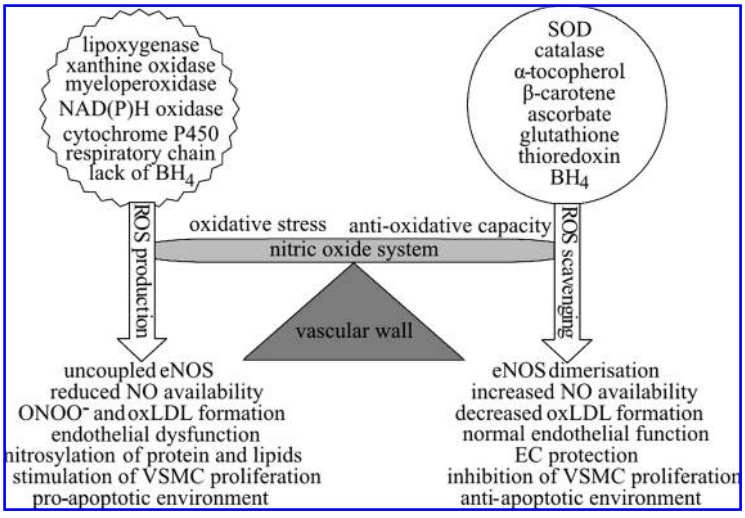


FIG. 2. Interactions between oxidant and antioxidant, and nitric oxide systems. Several sources of vascular production of ROS and different antioxidants can affect the nitric oxide system. This results in specific responses in the regulation of vascular tone, endothelial cell (EC) protection, vascular smooth muscle cell (VSMC) proliferation, and apoptosis.

NAD(P)H Oxidase Complexes and NOS

Several NAD(P)H oxidase complexes have been identified as important sources of $\cdot O_2^-$ formation over the past few years (124). The NAD(P)H oxidase complex first discovered in granulocytes consists of four essential subunits, membrane-bound subunits gp91phox and p22phox and initially cytosolic subunits p47phox and p67phox; in addition, a cytosolic subunit, p40phox, has also been described. Activation of NAD(P)H oxidase complex leads to phosphorylation of cytosolic subunits and translocation from the cytosol to the membrane. Of special importance in the complex is the subunit gp91phox, which mediates the electron transfer from NADH/NADPH to oxygen. Several novel isoforms of gp91phox have been described in the Nox family of NAD(P)H oxidase subunits (124, 125, 189). The novel specific Nox complexes contain up to five additional subunits, while cytosolic proteins can also be replaced by different isoforms in other Nox complexes. Furthermore, activation may require binding of small G proteins such as Rac. A common feature of Nox family members is their transmembrane domain allowing electron transport across the membrane to generate $\cdot O_2^-$; all Nox isoforms have NADPH and FAD binding sites at the C-terminus. Transmembrane helices with conserved histidines accommodate two heme-binding sites, and there is an additional transmembrane helix at the Duox1/2 protein N-terminus (18). Seven proteins belonging to the Nox family have been discovered so far (18, 124, 125, 189).

Nox1

Nox1 encodes a protein of 564 amino acids, and has a molecular weight of 55–60 kDa (39). Nox1 may be generated by gene duplication (10), and its exon/intron structure shows a high similarity to Nox2 (14). In the vasculature, Nox1 is expressed in smooth muscle cells (88); weak expression has also been described in endothelial cell lines or endothelium from specific vascular beds (119, 130, 175), while other studies have not detected Nox1 in any considerable amount in endothelial cells (73). Nox1 can assemble with p22phox and cytosolic subunits including p47phox and the p67phox homolog Nox activator 1 (NOXA1) (Fig. 3A) (10).

Nox2

The prototype of all other Nox isoforms was identified as the membrane-bound subunit gp91phox (188), which is mainly found in phagocytes and is named as Nox2 in the novel Nox nomenclature (125). The human Nox2 gene encodes a protein of 570 amino acids and a molecular weight about 55 kDa in the unmodified Nox2 protein, while glycosylation extends the molecular weight up to 91 kDa (229). Nox2 expression is distributed in many tissues, which could partially reflect infiltration with phagocytes. The Nox2 complex consists of five NAD(P)H oxidase subunits (Fig. 3B), where Nox2 and p22phox represent the membrane-bound subunits, and the cytosolic subunits of this complex are p47phox, p67phox, and p40phox. Phosphorylation of p47phox at serine residues accelerates interaction with p22phox and association with p67phox and p40phox, and binding GTPase Rac to the complex activates NAD(P)H oxidase and $\cdot O_2^-$ formation. Nox2 is colocalized with p22phox in the plasma membrane and in intracellular membranes of granules (110). Activation of the plasma membrane-localized NAD(P)H oxidase is regulated by Ca^{2+} signaling (74); the resulting release of neutrophil oxidants is an important step in microbicidal activity (110), and is finally mediated by redox-sensitive activation of proteases by K^+ flux (183). Nox2 can be induced by interferon-γ or angiotensin II at transcriptional and posttranscriptional level (18).

Nox3

The human gene of Nox3 encodes a protein of 568 amino acids with 56% identity to Nox2. Nox3 is mainly expressed in the inner ear including cochlear, vestibular sensory epithelia, and the spiral ganglion; a role in the maintenance of equilibrium sense has been proposed (11), while weak Nox3 expression has been described in fetal kidney, spleen, and brain. Nox3 forms a complex with p22phox (Fig. 3C), and can be regulated by Nox organizers and activators (223).

Nox4

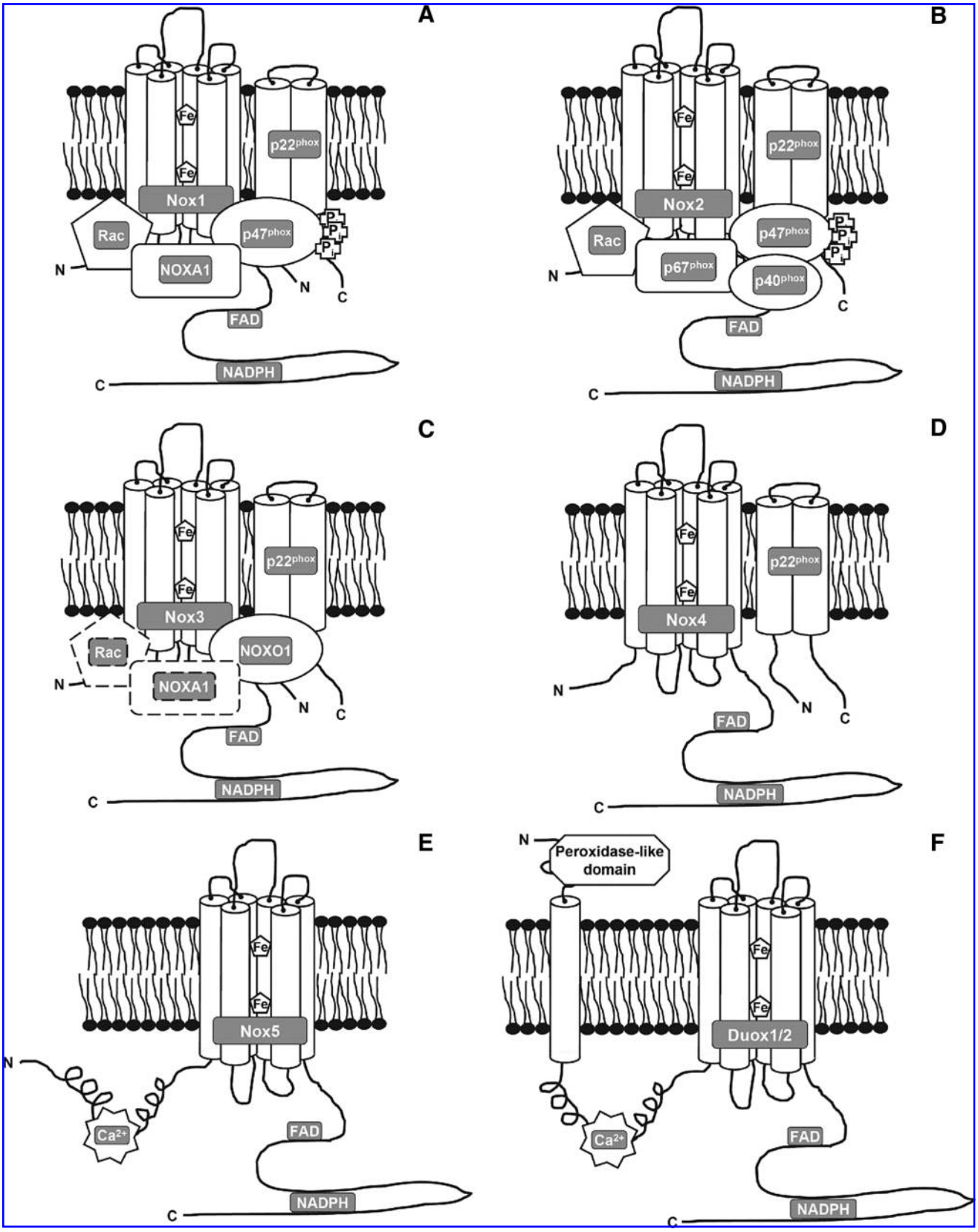
Nox4 was identified in the kidney and initially named "renox" (69), and has also been localized in the endoplasmic reticulum (224). The Nox4 gene encodes a protein of 578 amino acids that shows 39% identity with Nox2, and has a predicted molecular weight of 64 kDa (204). An active Nox4 complex (Fig. 3D) consists of the subunits Nox4 and p22phox (5), and can produce considerable amounts of superoxide anions in a rather constitutive manner (147). Nox4 seems to play an important role in the vasculature; a Nox4-containing NAD(P)H oxidase is a major source of $\cdot O_2^-$ in endothelial cells (2, 211), with recent data suggesting that the type of ROS released from Nox4-expressing cells is mainly H_2O_2 (200). Angiotensin II induces Nox4 in vascular smooth muscle cells (235), while oscillatory shear stress mediates a transient increase of Nox4 expression in bovine endothelial cells (98); its expression can be reduced by peroxisome proliferator-activated receptor-gamma (PPAR-γ) ligands (97). VEGF-induced Nox4 upregulation involves PKCα and is associated with enhanced proliferation and angiogenesis in human endothelial cells (238).

Nox5

The human gene of Nox5 encodes a protein of 747 amino acids with a molecular weight at about 85 kDa (23). Several Nox5 splice variants have been described in endothelial and vascular smooth muscle cells (19, 36). The Nox5 protein has an intracellular N-terminus with a Ca^{2+}-binding EF hand domain (12, 13) (Fig. 3E), and is activated in response to PKC stimulation or to Ca^{2+} binding; it does not need any additional subunits for activation (199). A novel role of Nox5 and its variants in the control of ROS-dependent formation of capillary structures, proliferation or the response to thrombin in the vasculature has been proposed (19).

Duox1 and Duox2

Human genes Duox1 and Duox2 are localized in tight vicinity on chromosome 15 with a 16 kb spacer region (171), and are mainly expressed in the thyroid (44). The Duox1 gene encodes a protein of 1,551 amino acids, while the Duox2 protein contains 1,548 amino acids. Both Duox proteins (Fig. 3F) have transmembrane α-helices with an N-terminal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peroxidase-like domain (187), and can generate $\cdot O_2^-$ that rapidly dismutates to hydrogen peroxide (6); mutations in the N-terminal peroxidase-like region can lead to hypothyroidism (227). Cytokines interleukin 4 and 13 can induce Duox1 expression, while Duox2 is induced by interferon-γ (83). The Duox1 promoter has several putative SP-1 binding sites that are missing in the Duox2 promoter (171). An active Duox2 complex does not need interaction with cytosolic subunits, but Ca^{2+} binding to the EF hand domain is considered to play a crucial role in enzymatic activity (6).

Thox1, another multidomain oxidase/peroxidase, has been found in primary thyroid cells. It shows prominent mRNA expression in human aortic fibrofatty lesions and its expression is increased with lesion severity (108).

p22phox

The human p22phox gene encodes a protein of 195 amino acids and a molecular weight of 22 kDa (173), which has two transmembrane α-helices. Angiotensin II and TNFα induce p22phox mRNA expression in vascular smooth muscle cells (45, 76), while expression of p22phox is upregulated in experimental hypertension (154, 243). The p22phox promoter contains consensus sequences for TATA and CCAC boxes and binding sites for SP-1, Elk1, GAGA, and NF-κB (160). The p22phox subunit stabilizes several Nox subunits in the membrane, protects complexes from degradation, and mediates interaction with initially cytosolic subunits (47).

p47phox

The p47phox gene encodes a protein of 390 amino acids; protein–protein interactions with the other subunits are mediated by its Src homology 3 (SH3) domains (137). The p47phox promoter has a PU.1 transcription factor binding site essential for its expression in phagocytes (140). Homocysteine-stimulated superoxide anion production in monocytes is regulated via PKC-dependent phosphorylation of the p47phox and p67phox subunits of NAD(P)H oxidase, and plays an important role in homocysteine-induced inflammatory responses during atherogenesis (207). The p47phox subunit can be found in NAD(P)H oxidase complexes containing Nox1 and Nox2; these complexes can be found in vascular smooth muscle cells, endothelial cells, and monocytes. Increased formation of reactive oxygen species in these vascular cells types may reduce NO availability while increasing endothelial dysfunction, uptake of oxidized lipoproteins in the vessel wall, vascular hypertrophy and inflammation, thus accelerating the formation of atherosclerotic plaques.

NoxO1

The novel p47phox homolog NoxO1/p41phox encodes a protein of 370 amino acids at 41 kDa (10, 218). NoxO1/p41phox interacts with p22phox via the two SH3 domains, while the phox domain (PX) of NoxO1 binds to phosphatidylinositols (34). In contrast to p47phox, NoxO1 has no autoinhibitory region (218). Alternative mRNA splicing results in NoxO1 isoforms with different tissue-specificity and interactions with Nox1 and Nox3 (35).

p67phox

The p67phox gene encodes a protein of 526 amino acids with 67 kDa. The protein has domains such as a tetratricopeptide repeat (TPR) at the N-terminus, an activation domain, a "Phox and Bem 1" (PB1) domain, and a SH3 domain at the C-terminus (18). Protein–protein interaction with p47phox is mediated by its SH3 domains. Furthermore, TPR motifs of p67phox interact with Rac leading to activation of the NAD(P)H oxidase in phagocytes. The p67phox promoter contains binding sites of transcription factors PU.1 and AP-1 (56, 68). The p67phox subunit is part of the classical Nox2-containing complex found mainly in monocytes and, at low levels, in endothelial cells. Inflammatory processes, endothelial dysfunction, and oxLDL uptake may especially be promoted by p67phox-containing complexes, thus leading to atherosclerotic changes in the vessel wall.

NoxA1

NoxA1/p51phox has been recently identified as a p67phox homolog (10, 218). NoxA1 contains an N-terminal TPR, an activation domain, a "Phox and Bem 1" (PB1) domain, and a C-terminal SH3 domain (18), and encodes a protein of 483 amino acids with 51 kDa in humans. Protein–protein interaction between NoxA1 and NoxO1 or p47phox involves SH3 domains and proline-rich regions; in comparison to p67phox, the PB1 domain of NoxA1 is unable to bind to p40phox. NoxA1 can interact with Rac and replace p67phox in the Nox1-containing complex in VSMC from large vessels (4, 129).

FIG. 3. Structure of Nox complexes. (**A**) Structure of the complex containing novel Nox isoform Nox1. The transmembrane domains, the heme-binding residues, and the C-terminal cytosolic binding sites for FAD and NADPH of the Nox1 protein are indicated. Additional subunits of the proposed vascular Nox1 complex involve p22phox, p47phox (with phosphorylation sites), NOXA1, and Rac. (**B**) Structure of the classical gp91phox/Nox2 complex. The transmembrane domains, heme-binding residues, and C-terminal cytosolic binding sites for FAD and NADPH of the Nox2 protein are shown. Additional subunits of the vascular Nox2 complex involve p22phox, p47phox (with phosphorylation sites), p67phox, p40phox, and Rac. (**C**) Proposed schematic structure of the Nox3 complex. The transmembrane domains, heme-binding residues, and C-terminal cytosolic binding sites for FAD and NADPH of the Nox3 protein are summarized. Additional subunits of the proposed Nox3 complex are p22phox, NOXO1, NOXA1, and Rac. (**D**) Structure of the complex containing the Nox isoform Nox4. The transmembrane domains, heme-binding residues, and C-terminal cytosolic binding sites for FAD and NADPH of the Nox4 protein are summarized. In addition, only subunit p22phox is needed to generate superoxide anions and H_2O_2. (**E**) Structure of Nox5. The transmembrane domains, heme-binding residues, N-terminal Ca^{2+} binding EF-hand domain, and C-terminal cytosolic binding sites for FAD and NADPH are indicated. No further subunits are necessary for superoxide anion generation. (**F**) Proposed structure of the Nox isoforms Duox1 or 2. The transmembrane domains, heme-binding residues, N-terminal Ca^{2+} binding, and peroxidase-like domain, and C-terminal cytosolic binding sites for FAD and NADPH of the Duox1 or 2 proteins are summarized. No additional subunits are needed for generation of superoxide anions.

It is considered to play a central role in the activation of the NAD(P)H oxidase complex.

p40phox

The human gene of p40phox encodes a 40 kDa-protein of 339 amino acids. The p40phox protein contains SH3, PX, and PB1 domains. The PX domain can bind phosphatidylinositol 3-phosphate (109). PB1-mediated PX regulation occurs without preventing the PB1–PB1 association with p67phox (92). The complex contains p47phox, p67phox, and p40phox at a ratio of 1:1:1 (128). Angiotensin II induces p40phox mRNA expression in vascular smooth muscle cells (222), and the p40phox gene promoter has three potential binding sites for transcription factor PU.1 (141).

Nitric oxide synthases

The three isoforms of nitric oxide synthases (NOS) are known as neuronal or nNOS (NOS I), inducible or iNOS (NOS II), and endothelial or eNOS (NOS III) (164). All three isoforms generate NO using amino acid L-arginine as a substrate. Under physiological conditions the enzyme is in a coupled state as a NOS dimer, and generates NO, which can be reduced in availability for four main reasons: decreased expression and/or activity of the NOS enzymes, NOS uncoupling, enhanced breakdown or scavenging of NO, and impaired transmission of NO-mediated signaling events (failure of the effector mechanisms) (22). Decreased bioavailability of the essential cofactor, tetrahydrobiopterin (BH_4), or main substrate, L-arginine, switches the NOS dimers from coupled to uncoupled state (127), increasing production of superoxide anions instead of NO (62). Furthermore, NO can be considered as a double-edged sword in atherosclerosis. While sustained low concentrations of NO are mainly vasoprotective, excessive cytotoxic formation of NO (*e.g.*, by a highly active iNOS complex in VSMC or macrophages) may also promote atherosclerotic lesion formation.

Vascular ROS Regulation by Hemodynamic Forces

Specific NAD(P)H oxidase complexes have been identified as major sources of $\cdot O_2^-$ formation (Fig. 4) in every cell type in the vessel wall. In endothelial cells, a NADPH oxidase similar to the complex in granulocytes was initially shown to be a source of $\cdot O_2^-$ formation (53, 73, 106, 191). More recently, Nox4-containing NAD(P)H oxidase complexes have been recognized as the major source of $\cdot O_2^-$ in endothelial cells (2, 211). Complexes containing Nox1 and Nox4 are dominant in vascular smooth muscle cells (88), while NAD(P)H oxidase complexes of vascular fibroblasts and macrophages containing Nox2 and Nox4 are considered to be a barrier to nitric oxide bioactivity in the adventitia (184), and can be activated at different stages of cardiovascular disease.

The endothelial cell layer *in vivo* is constantly exposed to hemodynamic forces such as shear stress and cyclic strain by flowing blood, but the amount and degree of biomechanical forces can differ at specific sites of the vasculature. The local biomechanical force profile is considered to be a predictor for the development of atherosclerotic plaques (Fig. 5); a correlation between the localization of the atherosclerotic plaques in the cardiovascular system and regions exposed to low, oscillatory, or disturbed flow has been hypothesized, especially at bifurcations or vessel branches. Areas without these disturbed flow patterns mainly exposed to laminar shear stress are thought to be more resistant to the development of atherosclerosis (71), which may explain the differences in the heavy susceptibility to atherosclerosis in the human coronary artery exposed to complex and disturbed flow patterns compared to the lower rate of atherosclerosis in the internal mammary artery, where there are less discontinuities in flow patterns exposed to endothelial cells (225).

This review focuses on the impact of shear stress on endothelial cells *in vitro*. The type and duration of shear stress has an important impact on endothelial ROS formation, as short-term application of pulsatile stretch increases NAD(P)H oxidase-dependent superoxide production in human aortic endothelial cells (90) and rabbit aorta (135). Furthermore, continuous oscillatory shear stress can induce endothelial ROS formation (46), and can shift the balance between NO and ROS levels. This could be a key step in the initial development of atherosclerosis—exposing endothelial cells to oscillatory shear stress increases the activity of $\cdot O_2^-$-generating NAD(P)H oxidase and expression of redox-sensitive genes such as c-*fos* and heme oxygenase-1, while increased ROS

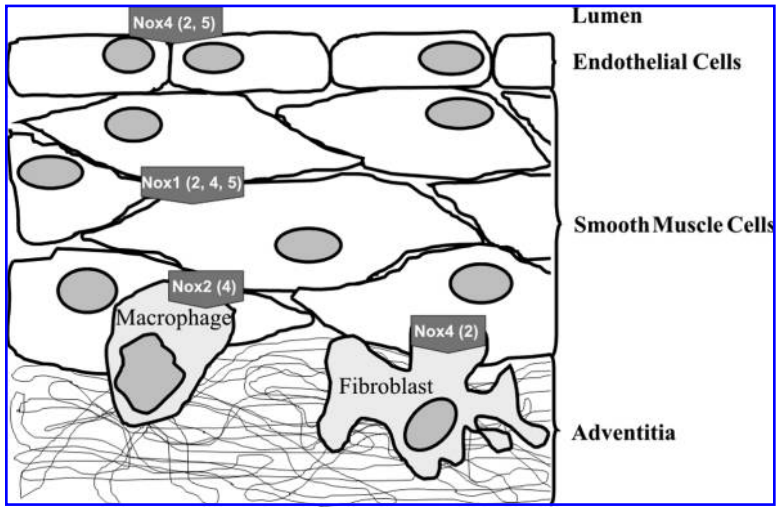

FIG. 4. NOX complexes in the vessel wall. The expression of main and weakly or vessel-specifically expressed Nox isoformes (*in brackets*) is indicated for the different parts of the vessel wall.

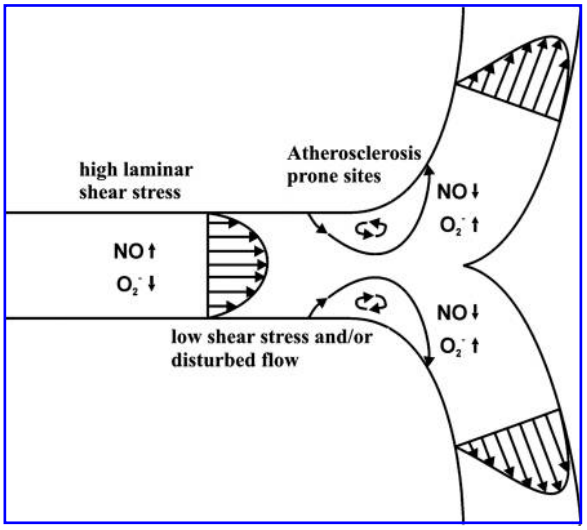

FIG. 5. Flow pattern and NO/$\cdot O_2^-$ balance at bifurcations. Regions in the vasculature with high laminar shear stress are characterized by increased NO generation and reduced superoxide anion formation. In contrast, regions of low shear stress and/or disturbed flow have a reversed NO/$\cdot O_2^-$ balance thus promoting an endothelial phenotype prone to atherosclerosis at bifurcations.

production and c-*fos* gene expression can be blocked by catalase (46, 95). Accelerated endothelial $\cdot O_2^-$ formation after application of oscillatory shear stress involves p47phox-containing NAD(P)H oxidase complexes (98, 99) and xanthine oxidase (152). Oscillatory shear stress significantly upregulates Nox4 expression accompanied by an increase in $\cdot O_2^-$ production in bovine aortic endothelial cells, whereas pulsatile shear stress upregulates eNOS expression and NO production. $\cdot O_2^-$ and NO have been implicated in vascular nitrative stress by $ONOO^-$ formation, and LDL added to endothelial-cell medium under oscillatory shear stress conditions shows higher levels of 3-nitrotyrosine, dityrosine, and o-hydroxyphenylalanine as compared with pulsatile shear stress. This $ONOO^-$-mediated nitration occurs at specific apo-B-100 tyrosine residues in the alpha and beta helices (93). Furthermore, a p47phox-containing complex plays a crucial role in flow-dependent vascular remodeling (30). These findings further support a crucial role of hemodynamic changes in ROS/NO balance in the pathogenesis of atherosclerosis.

An important role of the endothelium has been suggested in the vasodilator response to increased flow *in vivo* (176); flow-dependent generation of NO plays a major role in this context (24), but shear stress can also affect endothelial NO/$\cdot O_2^-$ balance. Several studies have shown increased eNOS expression by long-term laminar shear stress in endothelial cells (60, 168). Endothelial NO formation by short-term and long-term application of shear stress seems to involve different mechanisms—shear stress induces NO production of an endothelium-intact arterial segment and changes the tone of a preconstricted endothelium-denuded detector ring in a biphasic manner. An initial transient Ca^{2+}-dependent phase accompanied by a functional activation of eNOS protein is followed by a second Ca^{2+}-independent plateau phase characterized by increased eNOS expression (8). This upregulation in NO formation is a major cause of vasoprotective and antiatherosclerotic potential in laminar shear stress (Fig. 6). In contrast, locally disturbed flow pattern potentiates mechanisms promoting the development and progression of atherosclerosis (Fig. 7).

Whether NO and $\cdot O_2^-$ can interact during exposure to shear stress is less well understood. Recently, we described a short-term induction of superoxide anion formation by laminar shear stress in human endothelial cells (54), which was inhibited by NAD(P)H oxidase inhibitor gp91ds-*tat*, while NAD(P)H oxidase subunit expression remained unchanged. This increased $\cdot O_2^-$ generation in response to short-term shear stress most probably represents an activation of NAD(P)H oxidase complexes using preformed subunits. In contrast, we observed downregulation of $\cdot O_2^-$ formation, mRNA, and protein expression of NAD(P)H oxidase subunits Nox2 and p47phox after application of long-term arterial laminar shear stress in the same study. The expression of NAD(P)H oxidase subunits p22phox and p67phox protein was not affected by long-term laminar shear stress. Downregulating superoxide anion formation by long-term laminar shear stress would result in increased flow-dependent NO availability in human endothelial cells, which may be seen as a beneficial effect on endothelial function. In parallel, endothelial NO formation and eNOS, but not Cu/Zn SOD, protein expression was increased. Downregulation of $\cdot O_2^-$ formation, Nox2, and p47phox expression by long-term laminar shear stress was blocked by NOS-inhibitor L-NAME, while NO donor DETA-NO even downregulated $\cdot O_2^-$ formation, Nox2, and p47phox expression in static cell cultures. Further studies from our lab further support downregulation of Nox4 by long-term laminar shear stress (52). NO does not seem to be involved in the shear stress-dependent downregulation of this Nox isoform. These data suggest transient activation of $\cdot O_2^-$ formation by short-term laminar shear stress, followed by downregulated endothelial NAD(P)H oxidase in response to long-term laminar shear stress. NO-mediated downregulation by shear stress preferentially affects the Nox2/p47phox-containing NAD(P)H oxidase complex, which might

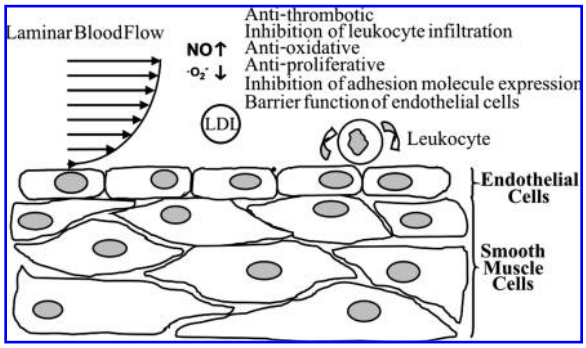

FIG. 6. Antiatherosclerotic potential of laminar blood flow. Laminar blood flow increases NO and reduces superoxide anion formation. This mediates antithrombotic, antioxidative, and antiproliferative properties of the endothelial cell layer, inhibition of adhesion molecule expression, reduced leukocyte infiltration, and intact barrier function of endothelial cells.

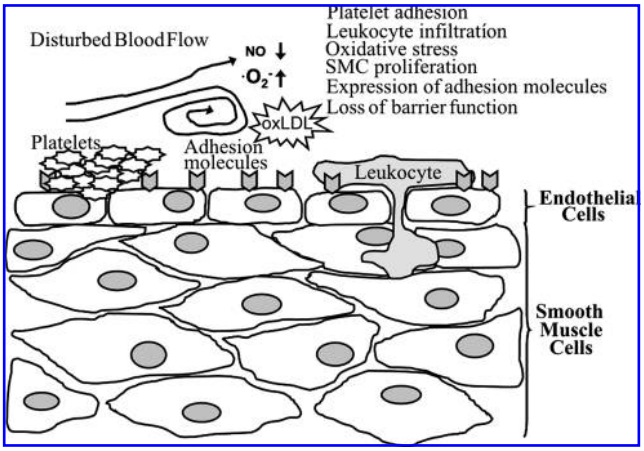

FIG. 7. Proatherosclerotic potential of disturbed blood flow. Disturbed blood flow reduces local NO generation and accelerates superoxide anion formation. This can promote thrombotic, oxidative, and proliferative properties of the endothelial cells, increase adhesion molecule expression, and promote leukocyte infiltration and loss of barrier function of the endothelial cell layer.

contribute to the regulation of endothelial $NO/\cdot O_2^-$ balance and the vasoprotective potential of physiological levels of laminar shear stress (156).

As an *in vivo* model of increased shear stress, voluntary running increases blood flow and reduces superoxide release, Nox1 and p47phox expression in murine vessels (132). A recent study supports the clinical importance of these findings, where chronic exercise training in patients with coronary artery disease increased flow and decreased reactive oxygen species generation and Nox2 expression in internal mammary arteries (1). Therefore, the flow-dependent endothelial $NO/\cdot O_2^-$ balance regulation may modulate the endothelial cell layer's antiatherosclerotic and vasoprotective properties as well as endothelial function regulation. ROS and RNS generation in response to modifications in hemodynamic forces initiates a diversity of signaling processes that control vascular smooth muscle proliferation, inflammatory phenotype changes, and extracellular matrix homeostasis that underlies atherosclerotic processes (134, 237).

Nitric Oxide, NAD(P)H Oxidase, and Atherosclerosis

NO is an important mediator of endothelium-derived relaxation. Furthermore, NO has been shown to mediate antiatherosclerotic effects such as the inhibition of thrombocyte aggregation, endothelial adhesion molecule expression, and smooth muscle cell proliferation (67, 114, 179). Inhibition of eNOS or accelerated superoxide anion generation increases endothelial cell proliferation, while NO donors or NAD(P)H oxidase inhibitors attenuated coronary endothelial cell growth (17). Exogenous superoxide mediates prooxidative, proinflammatory, proapoptotic, and procoagulatory mechanisms in endothelial cells (104), while superoxide anions rapidly react with nitric oxide (NO), leading to peroxynitrite formation (220). This reduced NO availability accelerates endothelial dysfunction and development of atherosclerosis (63, 136). The effects of increased vascular ROS production and impaired NO bioactivity may affect proatherogenic factors and contribute to the development and progression of atherosclerosis at all stages of the disease (Fig. 8).

NO and superoxide anions show further interactions in the vessel wall. Where the tetrahydrobiopterin cofactor is limited, eNOS does not produce NO; instead, the eNOS protein starts to generate superoxide anions. Oxidation of tetrahydrobiopterin leads to uncoupling of endothelial cell nitric oxide synthase in hypertension (127). As a putative therapeutic consequence, oral administration of tetrahydrobiopterin improves endothelial function and slows the progression of atherosclerosis in $apoE^{-/-}$ mice (86). As an alternative mechanism, hypochlorous acid, the major leukocyte-derived myeloperoxidase oxidant, uncouples eNOS by peroxynitrite generated from NAD(P)H oxidase (239). Furthermore, peroxynitrite may inhibit vasodilator prostacyclin, thus reducing endothelial function (249). Uncoupled eNOS leads from a protective enzyme to a contributor to ROS production as has been described in several *in vitro* models, animal models of cardiovascular diseases, and patients with cardiovascular risk factors (62). Increased superoxide generation by NAD(P)H oxidase has been associated with clinical risk factors of atherosclerosis (79); indeed, the severity of atherosclerosis has been associated with expression of NAD(P)H oxidase subunits (209), indicating an involvement of ROS in the initiation and progression of hypertension and atherosclerosis (75).

Role of Oxidized LDL in Atherosclerosis

Increased oxidative modification of low-density lipoprotein (LDL) is a proatherosclerotic effect of augmented vascular $\cdot O_2^-$ formation (41). Inhibitors of flavoproteins such as NAD(P)H oxidase prevent macrophage-mediated oxidation of LDL (234), while oxidized LDL (oxLDL) contributes to the pathogenesis of atherosclerosis. OxLDL accelerates chemotactic factors, adhesion molecules, and scavenger receptors on macrophages (40, 122, 242), triggering hypoxia-inducible factor-1α accumulation in macrophages by a redox-sensitive mechanism (202) while promoting the infiltration of macrophages into the intima. Unlimited uptake of oxLDL by these macrophages via scavenger receptors leads to foam-cell formation and development of atherosclerotic plaques (236). In respect to vascular function, oxLDL interferes with the endothelium-dependent relaxation by reducing expression of eNOS (131). Increased plasma levels of native low-density lipoprotein (nLDL) can be oxidized by ROS to oxLDL; LDL-cholesterol is considered as a significant predictor of both oxidative stress and endothelial dysfunction, while LDL-cholesterol and oxidized LDL may affect eNOS trafficking to caveolae (203) and the uncoupling of eNOS, leading to increased $\cdot O_2^-$ production (226). OxLDL itself has been described as a potent inducer of $\cdot O_2^-$, and therefore as a cause of oxidative stress (191). This induction of endothelial radical formation may be blocked by the novel Nox inhibitor, VAS2870 (212). Another new antioxidative substance is aspirin-triggered lipoxin A_4 analog ATL-1, which leads to a reduction in vascular oxidative stress (155, 166). Beside oxidative modification of lipoproteins, glycosylation of high-density lipoprotein can increase NAD(P)H oxidase-dependent ROS formation and reduce eNOS expression (150) that may contribute to endothelial dysfunction and atherosclerosis in diabetic patients.

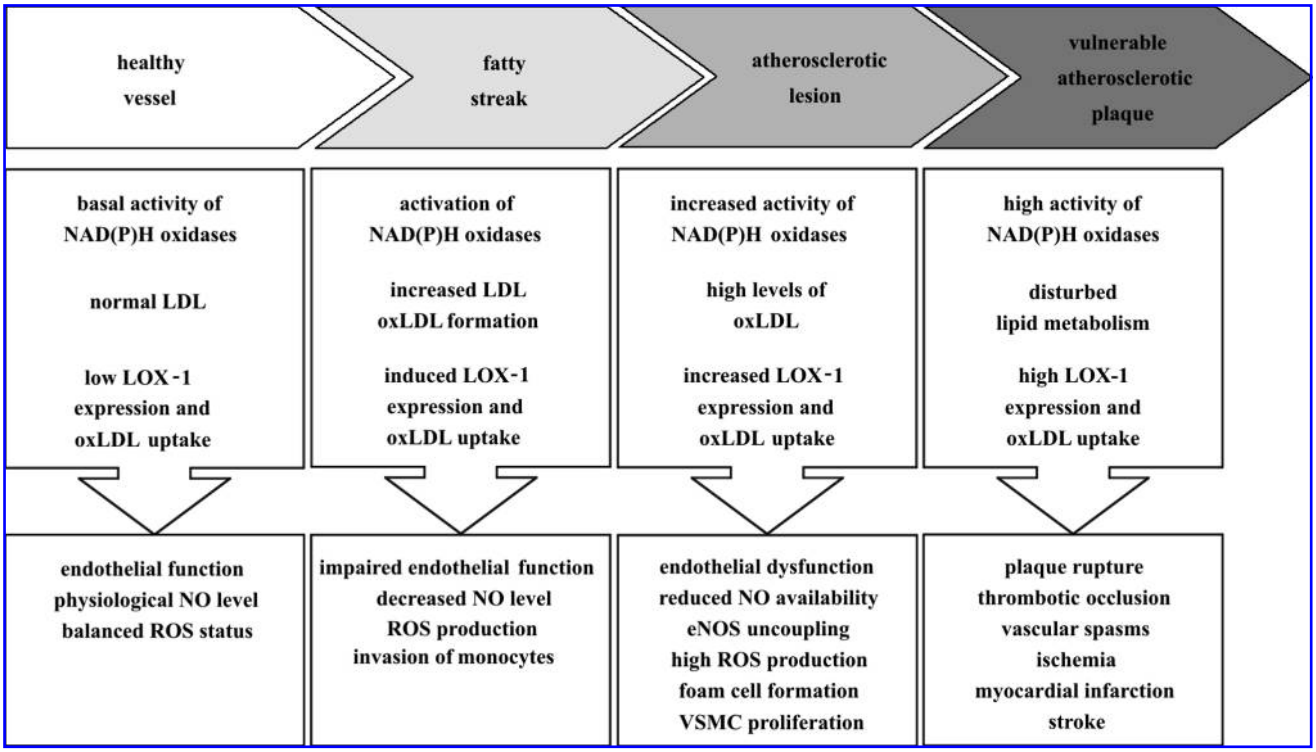

FIG. 8. Effects of exaggerated vascular production of ROS and impaired NO bioactivity in response to proatherogenic factors, their contribution to the development and progression of atherosclerosis at all stages of the disease. The figure summarizes the changes in the NO/ROS balance from healthy vessels to vulnerable atherosclerotic plaque rupture.

Antioxidants and Atherosclerosis

The role of the ROS/NO balance in atherosclerosis is not specific to the oxidation of LDL. Modulation of ROS/NO balance is also important for endothelial and vascular smooth muscle cells integrity; indeed, antioxidant systems have been shown to be of major importance in atherosclerosis, specifically with regard to endothelium integrity. Among these, mitochondrial thioredoxin (Trx2) is one of the major systems regulated in endothelial cells, and is also implicated in apoptosis and inflammation—two major mechanisms occurring in the atherosclerotic plaque (247). Compared to their control littermates, transgenic Trx2 mice showed an increased level of total antioxidants, reduced ROS formation, and increased NO levels in serum, leading to reduced vasoconstriction and enhanced vasodilation of aortic vessels. Endothelial cells from transgenic Trx2 mice have an increased capacity to scavenge reactive oxygen species generated from mitochondria, which enhances the bioavailability of NO. Furthermore, Trx2 improves endothelial function and reduces the formation of atherosclerotic lesions in apolipoprotein E-deficient mice (247).

Thioredoxin also seems to be upregulated by oxLDL (82); human macrophage uptake of oxidized LDL induces a coordinated upregulation of genes of the glutathione and thioredoxin systems such as thioredoxin, thioredoxin reductase 1, glutathione reductase, manganese superoxide dismutase, and catalase, suggesting that these systems may participate in the cellular defense against oxidized LDL, possibly modulating the development of atherosclerosis. Glutathione peroxidase-1 (Gpx1) seems to play a crucial role in this atherosclerotic process (221) by causing more atherosclerotic lesions. Deficiency of this antioxidative enzyme accelerates atherosclerotic lesion progression in double Gpx-1/apoE knockout mice in comparison to apoE knockout animals, which is accompanied by increased ROS generation, lower levels of bioactive NO, and higher levels of peroxynitrite as shown by increased tyrosine nitration. Therefore, antioxidant systems are important modulators of vascular ROS formation, and can affect development and progression of atherosclerosis.

Vasoconstrictors, Oxidative Stress, and Atherosclerosis

Vasoactive and growth promoting peptide angiotensin II is an important mediator in the development and progression of atherosclerosis, as it has has been shown to induce the formation of reactive oxygen species and the expression of NAD(P)H oxidase subunits in vascular smooth muscle cells (76). Chronic infusion of angiotensin II results in augmented $\cdot O_2^-$ formation, endothelial dysfunction, and hypertension (181). Furthermore, angiotensin II increases NAD(P)H oxidase-derived superoxide anion formation in human endothelial cells (20, 190, 246).

The identification of mineralocorticoid receptors in heart, vasculature, and brain raised speculations that aldosterone may mediate direct effects in these target organs independently of angiotensin II. Aldosterone increases vascular tone, induces endothelial dysfunction, and enhances pressor response to catecholamines and upregulation of angiotensin II receptors (194), while also inducing electrolyte transport in the cell membrane of vascular smooth muscle cells and playing a crucial role in arterial vascular remodeling. Moreover,

aldosterone promotes collagen synthesis that leads to increased arterial stiffness and elevation of blood pressure. Endothelial and vascular smooth muscle cells can synthesize aldosterone, and tissue aldosterone may play a more important role in resistant hypertension and target organ damage than circulating aldosterone. A selective aldosterone receptor antagonist has been shown to improve endothelial function and reduces $\cdot O_2^-$ generation in diet-induced atherosclerosis in rabbits (180). Furthermore, aldosterone receptor antagonist eplerenone reduce oxidative stress and p22phox expression, enhance eNOS expression, and normalize vascular function in spontaneously hypertensive rats (192). Recent evidence further supports a role of aldosterone in the pathogenesis of atherosclerosis (215), which may involve induced expression of adhesion molecules and subsequent adhesion of leukocytes to endothelial cells in response to aldosterone (121). Thus, targeting aldosterone by blocking its receptor has potential antiatherosclerotic effects.

Another mechanism may involve endothelin-1 (ET-1), a potent vasoconstrictor. The role of ET-1 in atherosclerosis can also be seen as a double-edged sword. In endothelial cells, low doses of ET-1 even induce NO release via the ET_B receptor, causing initial vasodilation (89, 241). ET-1 significantly enhances ROS generation by an endothelin receptor B-mediated mechanism in human endothelial cells (53). In further studies, ET-1 was shown to enhance oxidative stress, cell proliferation, and reduced apoptosis in human umbilical vein endothelial cells (50). Therefore, this may even be considered as a vasoprotective mechanism that helps to maintain endothelial cell integrity in healthy vasculature without additional risk factors. On the other hand, ET-1 decreases eNOS expression and activity (232). In arterial vascular smooth muscle cells, ET-1 stimulates cell proliferation by inducing reactive oxygen species (233), which may lead to endothelial dysfunction and vascular hypertrophy in later stages of atherosclerosis. In line with this hypothesis, endothelin ET_A receptor blockade restores NO-mediated endothelial function and inhibits atherosclerosis in apolipoprotein E-deficient mice (16). Intimal thickening further potentiates endothelial dysfunction, hypertension, and atherosclerosis, while increased levels of ROS may induce apoptosis of vascular smooth muscle cells, resulting in aneurysms (151). In addition, Nox2-dependent $\cdot O_2^-$ induction of matrix metalloproteinase-9 in monocytes promotes atherothrombotic events by decreasing plaque stability (244).

The data are less clear regarding a putative link between NAD(P)H oxidase and atherosclerosis using transgenic animals. Initial reports using gp91phox $^{-/-}$ or p47phox $^{-/-}$ and apoE $^{-/-}$ mice failed to inhibit atherosclerosis (94, 116). In contrast, p47phox was required for atherosclerotic lesion progression in apoE $^{-/-}$ mouse aorta (15). In addition, lower NADPH production in glucose-6-phosphate dehydrogenase deficiency reduces NAD(P)H oxidase-derived superoxide anions and aortic lesion growth (149). These more recent reports further substantiate a putative link between vascular superoxide anion formation and atherosclerosis.

Vasoconstrictors and OxLDL Uptake

Endothelial oxLDL receptor LOX-1 is a putative proatherosclerotic link between the renin–angiotensin system and the uptake of oxLDL (117). LOX-1 is induced by angiotensin II in human endothelial cells (138, 158), and its upregulation by high-cholesterol diet in the neointima of rabbit aortas decreases after treatment with AT_1 receptor blocker losartan (33). AT_1 receptor blockade and NAD(P)H oxidase inhibitor gp91ds-*tat* normalizes endothelial dysfunction while increasing ROS production, LOX-1 and MCP-1 expression in Dahl salt-sensitive rats (197, 248). Furthermore, apart from the role of angiotensin II-induced LOX-1, a recent study has implicated LOX-1 in endothelial dysfunction and atherosclerosis (240), further suggesting that oxLDL impairs endothelium-dependent NO-mediated dilation of coronary arterioles by activating a signaling cascade involving LOX-1 and NAD(P)H oxidase expression. In addition, beta 1-antagonism may reduce increased vascular p47phox and LOX-1 expression and accelerate decreased eNOS expression in Dahl salt-sensitive rats (118).

Several findings support a connection between the endothelin system and oxLDL uptake; endothelin-1 (ET-1) stimulates LOX-1 expression and oxLDL uptake in human endothelial cells (157). We have demonstrated that oxLDL itself induces endothelin-converting enzyme-1, prepro-endothelin-1, and the release of ET-1 peptide (167). Furthermore, human endothelial cells show a transient induction of the endothelin receptor type B (ET_B) in response to nLDL and oxLDL (162), supporting the hypothesis that LDL-cholesterol and the endothelin system potentiate each other in the uptake of oxidized lipoproteins and pathogenesis of atherosclerosis. These data strongly indicate a vicious cycle involving locally increased angiotensin II (Ang II) and endothelin-1 (ET-1) levels, augmented oxidative stress, increasing oxidation of low-density lipoprotein (LDL) to oxidized LDL (oxLDL), augmented uptake of oxLDL by endothelial oxLDL receptor LOX-1 in response to Ang II and ET-1, and further potentiation of oxidative stress in response to oxLDL in the vessel wall, thus promoting atherosclerosis (Fig. 9).

Impact of NAD(P)H Oxidase-Derived Superoxide Anions on Endothelial Function

Several lines of evidence support a role for the NAD(P)H oxidase in the regulation of NO availability and endothelial function (113, 163). Endothelial dysfunction occurs in conjunction with increased levels of $\cdot O_2^-$ and markers of oxidative stress in human vessels (38, 57). Endothelial function may be improved by scavenging superoxide anions with superoxide dismutase under the involvement of protein kinase C inhibitors (87). Therefore, NAD(P)H oxidase has been proposed to play a central role in vascular redox-sensitive signaling and function (77).

NAD(P)H oxidase also has an impact on endothelial dysfunction since superoxide anions reduce the half-life of NO, possibly due to the accumulation of asymmetric dimethylarginine (ADMA), a major endogenous NO synthase inhibitor thought to be a key contributor to endothelial dysfunction. Oxidative stress-dependent decrease under the influence of dimethylarginine dimethylaminohydrolase (DDAH), a major hydrolase of ADMA, causes ADMA to accumulate in the presence of some risk factors of atherosclerosis, including hypercholesterolemia. Thus, ADMA may not only be a marker but also an active player in cardiovascular disease, which makes it a potential target for therapeutic intervention (144).

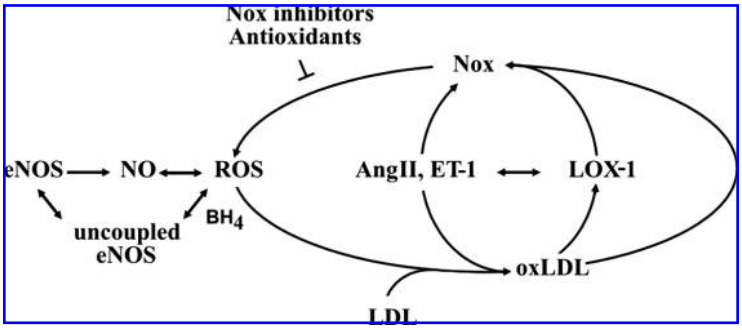

FIG. 9. Proposed vicious cycle summarizing the role of oxLDL and the interaction with NO/ROS balance. Vicious cycle of locally increased angiotensin II (Ang II) and endothelin-1 (ET-1) levels, augmented oxidative stress with uncoupling of eNOS, increasing oxidation of low-density lipoprotein (LDL) to oxidized LDL (oxLDL), augmented uptake of oxLDL by endothelial oxLDL receptor LOX-1 in response to Ang II and ET-1, and further potentiation of oxidative stress in response to oxLDL in the vessel wall thus promoting atherosclerosis.

Several well-known risk factors also increase vascular $\cdot O_2^-$ formation. Hydrosoluble components of cigarette smoke can activate the vascular NAD(P)H oxidase (170). Obesity can increase vascular NAD(P)H oxidase expression (182). Even obese human subjects free of clinical disease have increased vascular p47phox expression and oxidative stress (206). Whether this vascular $\cdot O_2^-$ formation affects arteries or veins to the same extent, is currently not well understood (214). A different $\cdot O_2^-$ production and expression pattern of Nox subunits has been described in veins and arteries (81). In a recent study, oxidative stress and endothelial function in patients with coronary artery disease (CAD) undergoing coronary artery bypass graft surgery was compared to patients undergoing surgery for removal of varicose veins (3). The CAD patients showed an increase $\cdot O_2^-$ production and a decreased relaxation in saphenous veins compared to control patients.

Angiotensin II infusion can alter vascular reactivity by enhancing $\cdot O_2^-$ generation and p22phox expression in large (64, 181) and resistance vessels (231). NO synthase inhibitor-treated rats show exaggerate production of ROS while maintaining contractile function in response to ET-1 (219). Endothelial ROS formation can also be increased by risk factors such as homocysteine and high glucose levels (201). Furthermore, depolarization is an important stimulus of endothelial superoxide production involving tyrosine phosphorylation-dependent translocation of the small G-protein, Rac (208). Vascular endothelial growth factor also induces endothelial $\cdot O_2^-$ release (120). Furthermore, pressure-induced, NAD(P)H oxidase-derived ROS generation increases Ca^{2+} sensitivity, which is necessary for full myogenic vasoconstriction (112). A role of the Nox2 complex in the development of endothelial dysfunction in Nox2 knockout mice (177) has been demonstrated in the 2-kidney, 1-clip model of renovascular hypertension (107). In conclusion, growing evidence supports the impact of the NAD(P)H oxidase on endothelial function. Cardiovascular risk factors increase Nox expression, $\cdot O_2^-$ formation, and impair endothelial function. Therefore, Nox inhibitors may have beneficial effects on endothelial function by reducing vascular oxidative stress.

Therapeutic Implications

The exaggerated production of ROS is considered as an important therapeutic target in cardiovascular diseases (26, 100), renal dysfunction (111), and diabetes (31); however, it is important to note that the NO/$\cdot O_2^-$ balance is tightly regulated under physiological conditions. Mutations in NAD(P)H oxidase subunits severely affecting NAD(P)H oxidase activity may lead to impaired oxidative burst in leukocytes and chronic granulomatous disease (CGD). CGD patients show high incidence of cognitive dysfunction, and often die within the first three decades of life due to recurrent infections. Four genetic forms of CGD correspond to mutations in subunits gp91phox, p22phox, p47phox, and p67phox (198). Most cases affect the gp91phox subunit and termed X-linked CGD (146). The CYBA C242T polymorphism in subunit p22phox has been associated with reduced NAD(P)H oxidase activity in human blood vessels (80). However, reduced formation of ROS may not only have detrimental effects—improved NO-dependent arterial flow-mediated dilatation was recently described in X-CGD patients (228). Platelets from gp91phox-deficient patients produced only a small amount of ROS, and showed weak proatherosclerotic oxidation of LDL (29). These recent observations further support an impact of NAD(P)H oxidase-derived reactive oxygen species on endothelial function. Several therapeutic strategies target the accelerated superoxide anion formation in vascular disorders. Statins, angiotensin-converting enzyme inhibitors, and AT_1 receptor blockers have the potential to reduce vascular oxidative stress (61). Statins are inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A reductase, and reduce serum cholesterol levels. Statins have proven beneficial in primary and secondary prevention of coronary heart disease in clinical trials (142). Experimental studies support this concept as well. In dogs with tachycardia-induced congestive heart failure, statins were found to normalize increased vascular superoxide production, NAD(P)H oxidase activity, Nox4 and p47phox expression (217). The benefits observed with statin treatment appear to be greater than that may be expected from reduction in lipid levels alone; the pleiotropic effects of statins involve upregulation of eNOS (131) and inhibition of NAD(P)H oxidase activity (230). In particular, inhibition of small GTP-binding proteins, Rho, Ras, and Rac, which are regulated by isoprenoids, seems to play an important role in mediating the pleiotropic effects of statins. This contributes to the statin-mediated improvement of endothelial function, stability of atherosclerotic plaques, decreased inflammation, inhibition of thrombogenic response, and lower ROS production (123).

The renin–angiotensin system (RAS) is significantly activated in the pathogenesis of cardiovascular disease, and specifically atherosclerosis (196). There is strong evidence that the RAS has effects on the mechanisms of action in atherosclerosis, including endothelial function, fibrinolytic balance, and plaque stability. Pharmacological inhibition of the renin–angiotensin system includes angiotensin converting enzyme (ACE) inhibitors, angiotensin receptor blockers (ARBs), and more recently, direct renin inhibitors (91). In particular, ACE

inhibitors and ARBs have demonstrated clinical benefits in reducing morbidity and mortality in the management of hypertension, congestive heart failure, and acute myocardial infarction (48). Whereas ACE inhibitors reduce the proteolytic cleavage of angiotensin I to angiotensin II, ARBs selectively block the binding of angiotensin II to AT_1 receptors. In addition, both therapies have the potential to increase the bradykinin levels, as ACE inhibitors can block the bradykinin degradation; ARBs can elevate the systemic angiotensin II levels with subsequent binding to the AT_2 receptor, stimulating bradykinin synthesis. We have analyzed the expression of NAD(P)H oxidase subunits in internal mammary arteries from patients undergoing elective coronary artery bypass grafting. Preoperative treatment with AT_1 receptor antagonists, but not with ACE inhibitors, reduced expression of gp91phox (190), while Ang II-induced expression of endothelial oxidized low-density lipoprotein (oxLDL) receptor LOX-1 was reduced in internal mammary arteries in patients undergoing ACE inhibitor therapy (158). In this context, the prescribed ACE inhibitor dosage seems to be crucial in reducing proatherosclerotic oxidative stress and uptake of oxLDL; higher doses of ACE inhibitors show beneficial effects in patients with heart failure (172). In a HOPE sub study (SECURE), ACE inhibitors dose-dependently reduced the progression of atherosclerosis (143). ARBs have the potential to inhibit the early atherogenesis in diet-induced hypercholesterolemia (213), and improve endothelium-dependent relaxation (70, 195). In our recent Endothelial Protection, AT_1 blockade and Cholesterol-Dependent Oxidative Stress (EPAS) trial, we tested whether statin and AT_1 receptor blocker therapies independently or in combination influence endothelial expression of anti- and proatherosclerotic genes and endothelial function in arteries of patients with coronary artery disease in a clinical trial according to the PROBE (Prospective Randomized Open Label and Blinded Evaluation) design (159). Statin and AT_1 blocker therapy independently and in combination improved endothelial expression quotient of anti- and proatherosclerotic genes (including NAD(P)H oxidase subunit and eNOS expression) and endothelial function. However, a significant interaction between both therapies was not observed. These findings support beneficial effects of both therapies in the treatment of coronary artery disease.

However, therapy with glucocorticoids can lead to vascular complication and atherosclerosis by accelerated vascular superoxide and peroxynitrite production (102). Apart from these agents, there are other components that improve endothelial function: mineralocorticoid receptor blockers, calcium channel blockers, antioxidants (vitamin C, vitamin E), PPARγ and α activators (fibrates), endothelin receptors blockers, folic acid, and finally aspirin.

The roles found for aldosterone and mineralocorticoid receptors in cardiovascular disease have expanded over the past decade (65). Selective aldosterone receptor blockers may have beneficial effects in resistant hypertension (55), while peripheral blood monocytes and vascular smooth muscle cells are both influenced by aldosterone to produce reactive oxygen species, contributing to activation of NF-κB and genes regulated by this transcription factor. Aldosterone therefore plays an important role in atherosclerosis and hypertension-induced vascular injury (58).

Long-acting calcium channel blockers are part of first-line therapy for diastolic and/or systolic hypertension (115). Antioxidant effects of calcium channel blockers include rather indirect effects by reduction of blood pressure, reduced angiotensin II and endothelin-1 levels, and increased NO release. On the other hand, a direct scavenging effect has been proposed for high lipophilic calcium channel blockers when their chemical structure facilitates proton-donating and resonance-stabilization mechanisms that quench the free radical reaction (148). Furthermore, preservation of the SOD activity by calcium channel blockers has been described (72).

Research on dietetic regimes has shown that consumers of large amounts of fruit and vegetables show a lower incidence of cardiovascular diseases, stroke, and tumors, but the protective mechanisms of these foods are still not completely clear (186). Possible reasons include a greater consumption of vitamins and an increased consumption of dietetic fibers. Research has put forward a hypothetical mechanism by which antioxidant substances may reduce the risk of atherosclerosis via the inhibition of oxidative damage. In a primate model of atherosclerosis, regression diet normalized increased superoxide anion formation and NAD(P)H oxidase expression, and improved endothelial function (85). Equol, a phytoestrogen, inhibits superoxide anion formation, leading to decreased LDL oxidation and increased NO availability (96). Antioxidant probucol decreases accelerated oxidative stress and p22phox expression and improved aortic stiffness in hypercholesterolemic rabbits (101). Some descriptive and case-control studies suggest that the consumption of antioxidant vitamins (A, C, and E) reduces the risk of cardiovascular diseases. In contrast, the use of dietary antioxidants in randomized clinical trials for the prevention of cardiovascular diseases has not yet been resolved (105). Many studies involving a great number of participants have not confirmed this hypothesis, and the results are often contradictory (185). Therefore, further clinical studies are necessary to further substantiate the link between vitamins and endothelial dysfunction.

Peroxisome Proliferator-Activated Receptors (PPARs) are key regulators of metabolic pathways. PPARs are members of the nuclear hormone receptor superfamily of ligand-activated transcription factors; fibrates act via PPARα as lipid-lowering agents. Furthermore, PPARα may increase the stability of atherosclerotic plaques and limit plaque thrombogenicity. PPARα and PPARγ dual ligands have proposed glucose, triglyceride, cholesterol lowering, HDL elevating, and body weight reducing activity (42), while PPARγ agonists reduce insulin resistance and show beneficial effects in the treatment of type 2 diabetes. In later stages of atherosclerosis, activation of PPARα inhibits the formation of macrophage foam cells by regulating expression of genes involved in reverse cholesterol transport, formation of ROS, and associated lipoprotein oxidative modification (245). Furthermore, PPARγ ligands increase release of nitric oxide from endothelial cells (27). Therefore, several experimental and clinical data support beneficial effects of PPAR ligands on the NO/ROS balance in the prevention and treatment of cardiovascular diseases (9).

Endopeptidase inhibitors and receptor antagonists have been developed as therapeutic strategies counteracting the actions of ET-1 (43). The first dual ET_A/ET_B receptor blocker, bosentan, has already been approved by the Food and Drug Administration for the treatment of pulmonary arterial hypertension. Even though a certain amount of experimental data support a role for activated endothelin system in heart

failure, clinical trials using endothelin receptor antagonists have not so far been successful in improving clinical symptoms of patients with mainly terminal heart failure (161). As an example, ET_A blockade with darusentan did not improve cardiac remodeling or clinical symptoms or outcomes in patients with chronic heart failure receiving angiotensin-converting-enzyme inhibitors, beta blockers, or aldosterone antagonists (7). However, dual ET_A/ET_B receptor blockade improves endothelial function and exerts direct vasodilator effects in atherosclerosis patients also undergoing treatment with ramipril, suggesting that ET receptor blockade may have important therapeutic effects when added to ACE inhibition in these patients (21). These changes could involve beneficial effects on the vascular NO/ROS balance as well.

Although dietary folate fortification decreases plasma homocystein and may reduce cardiovascular risk, high-dose folic acid therapy does not appear to alter clinical outcome. Folic acid and its principal circulating metabolite, 5-methyltetrahydrofolate, improve vascular function, but mechanisms relating folate dose to vascular function remain unclear. In a recent randomized clinical study, low-dose folic acid increased nitric oxide-mediated endothelium-dependent vasomotor responses, reduced vascular superoxide production, and improved enzymatic coupling of endothelial nitric oxide synthase through availability of the cofactor tetrahydrobiopterin in patients with coronary artery disease (205). These direct vascular effects are related to vascular tissue levels of 5-methyltetrahydrofolate; high-dose folic acid treatment likely confers no further benefit in subjects already receiving folate supplementation.

Aspirin remains the gold standard of antiplatelet therapy, and shows beneficial effects in the prevention of cardiovascular disease (174). Aspirin interferes with arachidonic acid metabolism in platelets and endothelial cells, thus reducing thromboxane A2 and prostacyclin. It also has other mechanisms of action, including antiinflammatory roles, enhancement of fibrinolysis, suppression of plasma coagulation, platelet-dependent inhibition of thrombin generation, and protection from oxidative stress (153). Aspirin may help to decrease the progression of atherosclerosis by protecting low-density lipoprotein from oxidative modification while also improving endothelial function in atherosclerotic vessels (210). Chronic treatment with nitric oxide-releasing aspirin has been shown to reduce low-density lipoprotein oxidation (LDL), oxidative stress and atherosclerosis in hypercholesterolemia (165). Aspirin can also directly scavenge hydroxyl radicals to form dihydroxybenzoate derivatives, which serve as markers of oxidative stress, quench oxy-radical flux, and acetylate amino groups of lysine residues in proteins, which prevents their oxidation (193). This antioxidant effect on proteins may be important in limiting both lipoprotein and fibrinogen oxidation; in the latter case, oxidation enhances fibrin formation, and lysine acetylation enhances fibrinolysis. Therefore, apart from its benefits in antiplatelet therapy, aspirin has antioxidative capacity and a positive impact on endothelial function and the progression of atherosclerosis.

Finally, L-arginine oral supplementation also seems to improve endothelial function and coronary circulation in hypercholesterolemic subjects (32, 49). In summary, the clinical importance of the NO/ROS balance for cardiovascular diseases will accelerate the development of novel antioxidative strategies in the next years. This will improve our experimental and therapeutic options in substantiating the proposed beneficial effects of increased antioxidative defense for the prevention of cardiovascular diseases.

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Abbreviations

ACE, angiotensin converting enzyme; ADMA, asymmetric dimethylarginine; apo E, apolipoprotein E; ARBs, angiotensin receptor blockers; BH_4, tetrahydrobiopterin; CAD, coronary artery disease; CGD, chronic granulomatous disease; DDAH, dimethylarginine dimethylaminohydrolase; Duox, dual oxidase; eNOS, endothelial NO synthase; ET-1, endothelin-1; ET_A, endothelin receptor A; ET_B, endothelin receptor B; GCH, GTP-cyclohydrolase I; Gpx1, glutathione peroxidase-1; H_2O_2, hydrogen peroxide; HDL, high-density lipoprotein; $HO^{\bullet}$, hydroxyl radical; iNOS, inducible NO synthase; LDL, low-density lipoprotein; LOX-1, lectin-like oxLDL receptor-1; MCP-1, monocyte chemoattractant protein-1; MPO, myeloperoxidase; nNOS, neuronal NO synthase; nLDL, native low-density lipoprotein; NO, nitric oxide; Nox, NAD(P)H oxidase; NOXA1, NADPH oxidase activator 1; NOXO1, NADPH oxidase organizer 1; $\cdot O_2^-$, superoxide anion; $ONOO^-$, peroxynitrite; oxLDL, oxidized low-density lipoprotein; PB1, Phox and Bem 1 domain; PKC, protein kinase C; PPAR, peroxisome proliferator-activated receptor; PX, phox domain; RAS, renin-angiotensin system; $RO^{\bullet}$, alkoxyl radical; $RO_2^{\bullet}$, peroxyl radical; ROS, reactive oxygen species; SH3, Src homology 3 domain; SOD, superoxide dismutase; TPR, tetratricopeptide repeat; Trx, thioredoxin; VEGF, vascular endothelial growth factor; VSMC, vascular smooth muscle cells.

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