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Vascular NADPH oxidases as drug targets for novel antioxidant strategies

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Reactive oxygen species (ROS) play important roles in the pathogenesis of cardiovascular disease. Surprisingly, large clinical trials have shown that ROS scavenging by antioxidant vitamins is ineffective or harmful. Therefore, prevention of ROS formation, by targeting specific sources of superoxide anion and other ROS, might prove beneficial. Potential targets include the NADPH oxidases (Nox enzymes), xanthine oxidase, endothelial nitric oxide synthase and mitochondrial oxidases. Nox enzymes play a central role because they can regulate other enzymatic sources of ROS. Statins, angiotensin-converting enzyme inhibitors and angiotensin receptor antagonists block upstream signaling of Nox activation, which contributes to their clinical effectiveness. Here, we discuss novel possibilities where drugs that directly inhibit Nox activation could successfully inhibit oxidative stress.

In recent years our understanding of the role reactive oxygen species (ROS) play in disease pathophysiology has increased substantially. The production and effects of ROS depend on the expression and proper function of numerous enzymes involved in ROS handling by the cell (see Box 1 and Figure 1) [1]. Moreover, the cell contains numerous antioxidant defenses that detoxify ROS or reduce their effects. The sites of ROS production and the distribution of antioxidant enzymes are highly localized within the cell, and therefore the cellular redox status (the balance between these pro- and anti-oxidant factors) should not be considered uniform. As an example, ROS production can occur predominantly in the nucleus, the mitochondria or at the cell membrane, and ROS have minimal effects on other portions of the cell. Antioxidant interventions have been mainly targeted to change the overall redox balance in the cell in a nontargeted, nonspecific fashion. For many years, interest has focused on strategies that enhance removal of ROS, either using antioxidants or drugs that enhance endogenous antioxidants such as glutathione or superoxide dismutase (SOD) mimetics [2,3]. Although those strategies have been effective in the laboratory, several large trials have shown that they do not reduce cardiovascular events and in some cases have actually worsened the outcome. An attractive alternative approach to reducing oxidative stress is inhibiting ROS production by blocking the enzymes involved in their synthesis. In this article we will critically review current and potential approaches that could be employed to inhibit vascular oxidases, we will also discuss the clinical potential of such therapies.

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Endothelial function, ROS and vascular diseases

The normal endothelium has anticoagulant and anti-inflammatory properties, and promotes vasodilatation by production of nitric oxide (NO), prostacyclin and other vasodilators. In various diseases the endothelium can become dysfunctional and can promote thrombosis, inflammation and lose its vasodilator influences [4]. A major mechanism responsible for such endothelial dysfunction is excessive production of ROS (Figures 1 and 2). ROS can alter production of NO, stimulate proinflammatory gene expression and can increase procoagulant mechanisms. Endothelial dysfunction has been shown to precede vascular diseases and atherosclerotic lesion formation [5].

A key role of NO in the regulation of vascular function has been defined by many studies during the past two decades [6] (Figure 2). Endothelial NO is produced by endothelial NO synthase (eNOS), in response to physiological stimuli like shear-stress (force of

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BOX 1

Reactive oxygen species

Reactive oxygen species (ROS) are metabolites of oxygen that, through their high reactivity, are prone to participation in oxidation–reduction reactions. Some ROS contain an unpaired electron in their outer orbital, which leads to their extreme chemical reactivity [e.g. superoxide ($O_2^{\bullet -}$), hydroxyl ($O^{\bullet}H$), nitric oxide radical ($NO^{\bullet}$), alkoxyl radical ($R\text{-}O^{\bullet}$), etc.]. Other ROS do not contain an unpaired electron and, therefore, are not radicals but are prone to exchanging electrons with other molecules. These include hydrogen peroxide (H_2O_2), peroxynitrite ($ONOO^-$), singlet oxygen ($^1O_2$), hypochlorous acid (HOCl) and lipid peroxides (LOOH).

flowing blood acting on a vessel wall) or agonists like bradykinin. NO exerts many beneficial antiatherogenic effects. Acting on vascular smooth muscle cells (VSMCs), NO activates soluble guanylate cyclase (sGC), leading to elevation of cGMP and vasorelaxation [1]. This is the primary basis for blood-flow and -pressure regulation. NO also reacts with a variety of other targets (i.e. modulates ion channel function, intracellular signal transduction and gene expression). It can inhibit vascular leukocyte recruitment and, therefore, exert anti-inflammatory properties, and is important in inhibiting platelet adhesion and aggregation. Therefore, loss of NO bioavailability is a key feature of endothelial dysfunction. Multiple pathways can reduce NO bioavailability by altering its synthesis or biodegradation. Excessive production of ROS seems to be a major mechanism of reduced vascular NO levels.

Effects of ROS on NO bioavailability and synthesis

There are several mechanisms through which NO bioavailability can be modified by ROS. First, superoxide ($O_2^{\bullet -}$) rapidly reacts with NO leading to the production of the strong oxidant peroxynitrite ($ONOO^-$) [7]. This reaction is likely to be very important in common conditions leading to endothelial dysfunction, including hypercholesterolemia, hypertension, diabetes and aging, in which the vascular production of $O_2^{\bullet -}$ is increased. Importantly, correction of

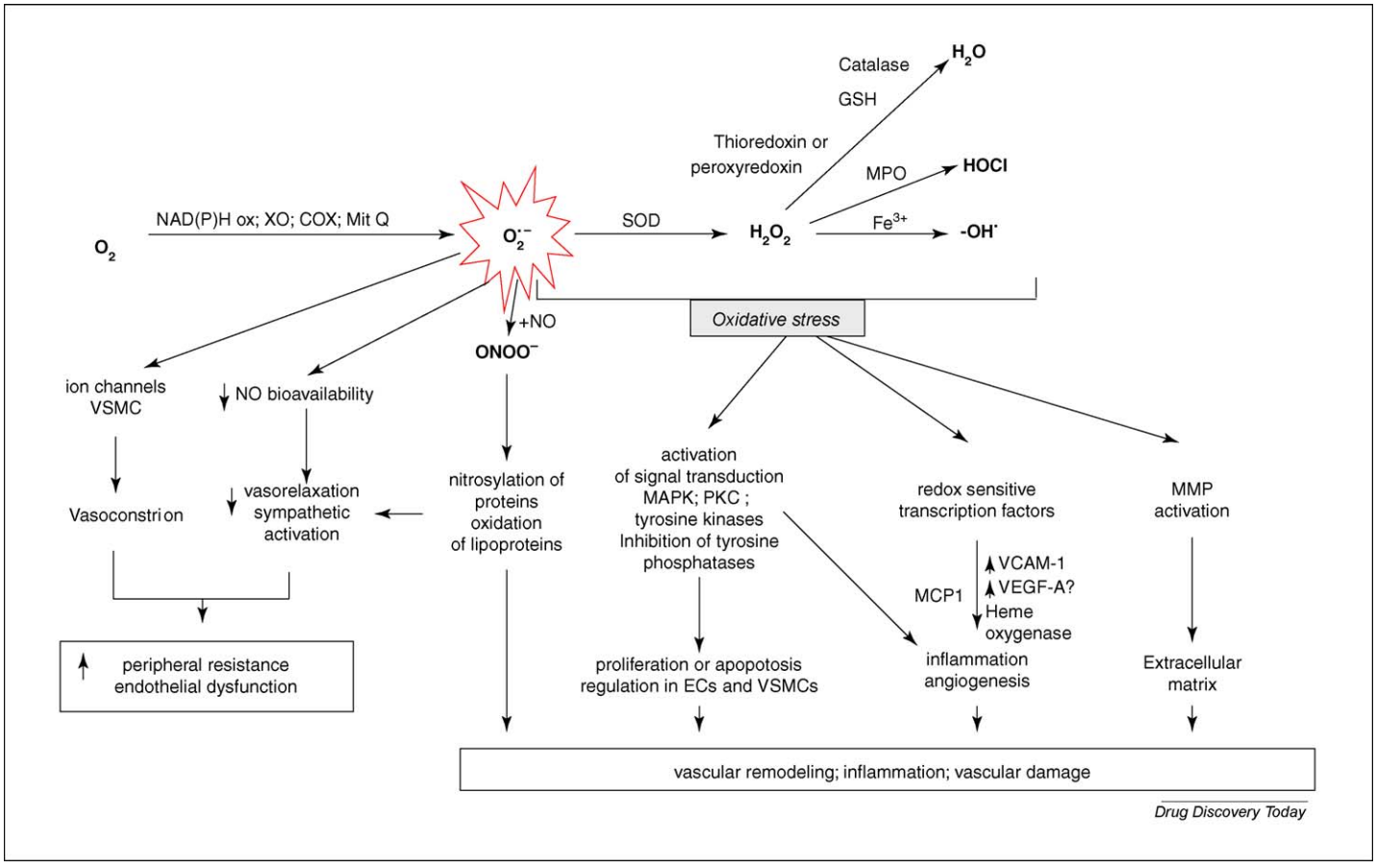

FIGURE 1

Major reactive oxygen species (ROS), their interactions and biological consequences. Superoxide ($O_2^{\bullet -}$) can be produced by numerous oxidoreductases [NADPH oxidase, xanthine oxidase (XO), cyclooxygenase (COX) or mitochondrial enzymes (Mit)]. Superoxide can then rapidly react with nitric oxide (NO), forming peroxynitrite ($ONOO^-$) and leading to loss of NO bioavailability and impaired endothelium-dependent vasodilatation. Superoxide can be converted by superoxide dismutase (SOD) to H_2O_2. ROS can stimulate mitogenesis in vascular smooth muscle cells (VSMC), activate other redox-sensitive signaling pathways and transcription factors and also oxidize cellular proteins. Activation of these transcription factors leads to redox-sensitive changes in expression of proinflammatory genes, such as vascular cellular adhesion molecule 1 (VCAM-1), monocyte chemotactic protein 1 (MCP-1) and intercellular adhesion molecule 1 (ICAM-1). H_2O_2 can also lead to the activation of protein kinase C (PKC), which can further activate NADPH oxidase leading to further propagation of ROS production. In addition to those mechanisms ROS modulate ion channels and, therefore, influence intracellular Ca^{2+} and K^+ concentrations. Finally, $O_2^{\bullet -}$ and H_2O_2 can activate matrix metalloproteinases (e.g. MMP-13, MMP-2 and MMP-9). The ultimate actions of ROS in the vasculature also depend on levels and function of scavenging enzymes including SOD, catalase, glutathione (GSH) peroxidase, thioredoxin and others.

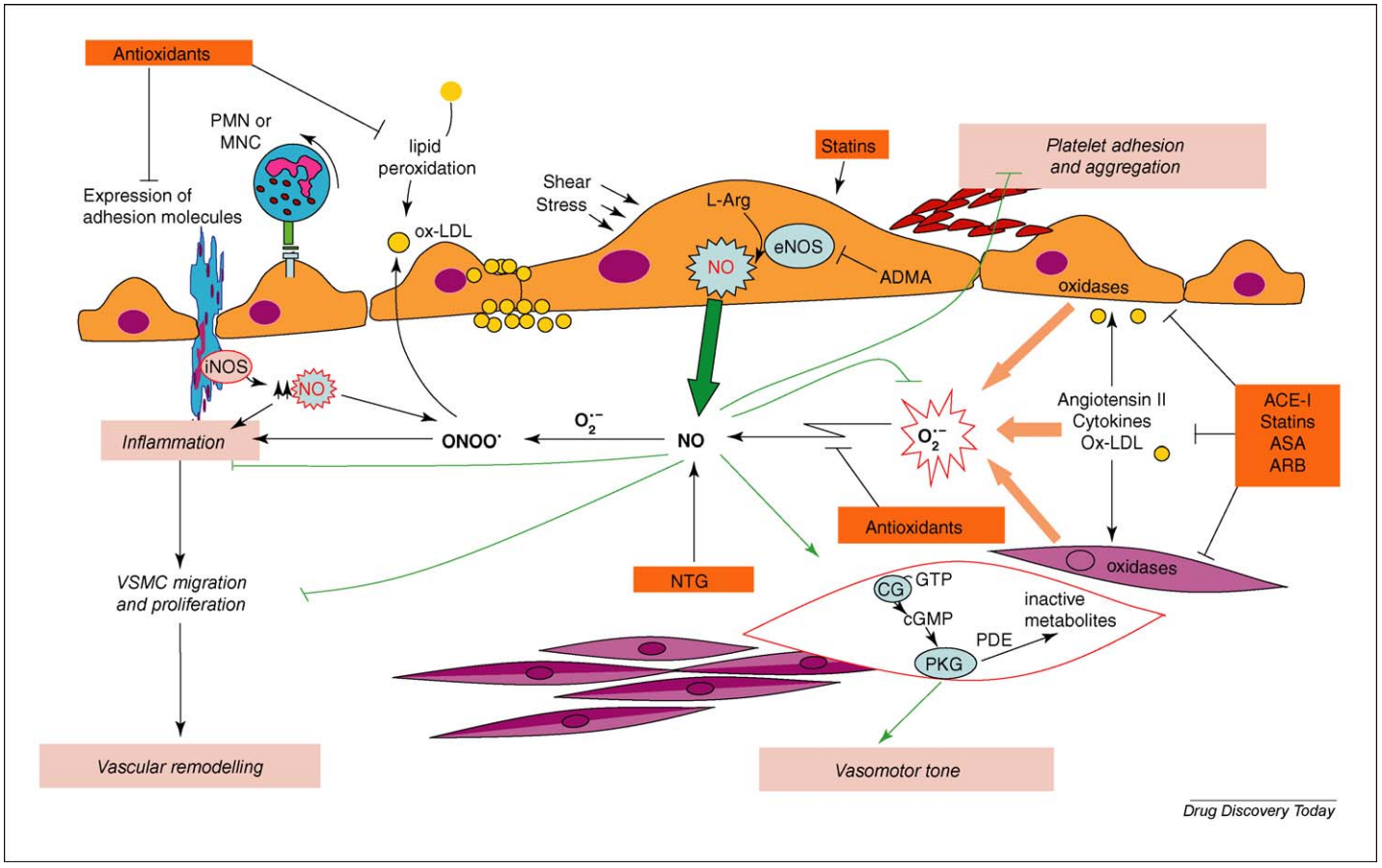

FIGURE 2

Nitric oxide–superoxide interactions in the pathogenesis of atherosclerosis. Nitric oxide (NO) exerts numerous beneficial effects. The loss of NO bioavailability can lead to inflammation, vascular remodeling, altered vasomotor tone and increased platelet adhesion. This loss of NO can be caused by decreased endothelial NO synthase (eNOS) activity or expression, lack of eNOS substrates (L-Arg) or cofactors (BH_4), or the presence of the endogenous NOS inhibitor asymmetric dimethyl-L-arginine (ADMA). A predominant cause of endothelial dysfunction is the overproduction of superoxide anion ($O_2^{\bullet -}$) and its interference with NO-related pathways. Increased $O_2^{\bullet -}$ can result from increased activity of vascular oxidases caused by angiotensin II, cytokines or ox-LDL (ox-low-density lipoprotein). NO– $O_2^{\bullet -}$ interaction additionally generates peroxynitrite ($ONOO^-$), another strong oxidant that can perpetuate endothelial dysfunction, lipid peroxidation and inflammation. The shaded boxes show that drugs currently used in the treatment of coronary artery disease and hypertension [statins, angiotensin-converting enzyme (ACE) inhibitors] can increase NO production and eNOS expression, as can exercise and increased endothelial cell shear-stress. Antioxidant agents and drugs that directly inhibit vascular oxidases can also enhance NO bioavailability.

hypercholesterolemia [8] and treatment with SOD or SOD mimetics [9] reduce vascular $O_2^{\bullet -}$ levels and restore endothelial function. In angiotensin-II-mediated hypertension vascular $O_2^{\bullet -}$ production is increased and treatment with membrane-targeted forms of SOD reduce blood pressure [10]. In line with these findings, acute infusion of high concentrations of vitamin C improves endothelium-dependent vasodilatation in humans with atherosclerosis, probably via $O_2^{\bullet -}$ scavenging [11].

A second effect of ROS on NO biology is the oxidation of the NO synthase cofactor tetrahydrobiopterin (BH_4) (Figure 3). BH_4 plays an essential role in transfer of electrons from the prosthetic heme group of NOS to L-arginine, ultimately leading to NO and citrulline production. In the absence of BH_4, the $Fe^{II}OO$ intermediate (formed during NOS catalysis) yields $O_2^{\bullet -}$, a condition referred to as NOS uncoupling, which will be discussed in more detail.

A third mechanism through which ROS can modify endothelial NO production is via oxidation of the zinc-thiolate center of NOS by $ONOO^-$ (the product of NO–$O_2^{\bullet -}$ interaction) [12]. Acute exposure of endothelial cells to peroxynitrite leads to a loss of zinc from eNOS disrupting the BH_4 binding domain. In keeping with this, eNOS purified from diabetic, low-density lipoprotein (LDL) receptor-deficient mice contained less zinc and was present in the endothelium preferentially in a monomer form [12].

A fourth mechanism whereby NO bioavailability is altered by ROS involves a reaction between hydrogen peroxide (H_2O_2) and myeloperoxidase (MPO). H_2O_2 has no direct reaction with NO; however, it has recently been demonstrated that H_2O_2 reacts with the heme center of myeloperoxidase to produce Fe^{IV}, which in turn can oxidize NO to NO_2^-, which can be further oxidized to the nitrogen dioxide radical ($NO_2^{\bullet}$) [13]. MPO is not normally present in vascular cells; however, in inflammation granulocytes can release MPO, which can be taken up by endothelial cells [13].

Another mechanism whereby ROS could alter NO production relates to post-translational modification of calmodulin. It has recently been shown that oxidation of methionine in calmodulin by hypochlorous acid decreases its binding activity [14]. This has not been studied in the context of eNOS; however, calmodulin binding is essential for appropriate eNOS activation, and oxidation of calmodulin can almost certainly reduce endothelial NO production [14].

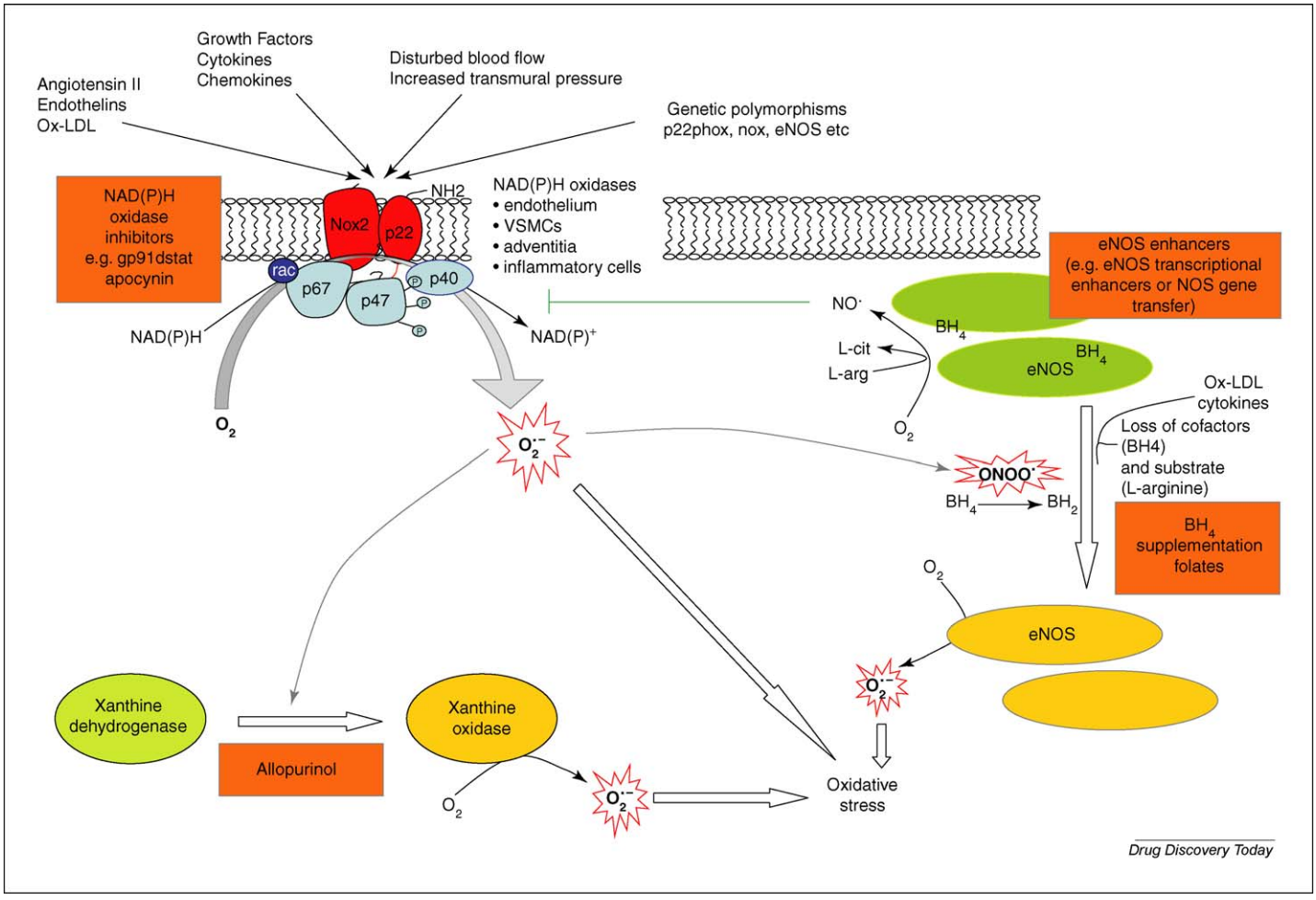

FIGURE 3

Crucial role of NADPH oxidases (Nox enzymes) in the pathogenesis of vascular oxidative stress. NADPH oxidase activity can be increased by humoral factors, cytokines or by disturbed flow conditions and can be affected by genetic factors. Excessive amounts of superoxide ($O_2^{\bullet -}$) produced by this oxidase (schematic exemplification shown on the graph is of a classical Nox2-based enzyme although other Nox enzymes can have similar effects) can stimulate other enzymes such as endothelial NO synthase (eNOS) and xanthine oxidase (XO) to produce $O_2^{\bullet -}$. The orientation of $O_2^{\bullet -}$ production in vascular cells (intra- or extracellular) is still unclear.

Finally, ROS can interfere with the actions of NO on vascular smooth muscle cells. Irreversible oxidation of relevant cysteine thiols blocks S-glutathiolation and activation of sarco-endoplasmic-reticulum calcium ATPase (SERCA), increasing cytoplasmic Ca^{2+} levels and impairing vascular relaxations and endothelial function [15].

Direct effects of ROS

Reactions with NO and reduction of NO synthesis is only one mechanism whereby ROS contribute to the atherosclerotic process [16]. ROS contribute to lipid oxidation, enhance vascular smooth muscle proliferation, promote endothelial cell apoptosis, activate and increase expression of matrix metalloproteinases and impair cholesterol reverse-transport by apolipoprotein A1 (ApoA1). These mechanisms have been extensively reviewed elsewhere [16–18] and are summarized in Figure 1.

Clinical relevance of ROS in atherosclerosis

Clinical studies have confirmed the importance of ROS and their interaction with NO in human atherosclerosis. Heitzer *et al.* [11] found that patients with the most abnormal endothelium-dependent vasodilatation had the largest number of cardiovascular events. Among patients with endothelial dysfunction, cardiovascular prognosis (acute coronary events and survival) were worst in patients where endothelial dysfunction was ROS-dependent. Another study has recently shown that oxidative stress, defined by serum levels of organic peroxides, correlated with C-reactive protein levels in otherwise healthy subjects without overt cardiovascular disease [19].

Antioxidants and treatment of atherosclerosis

Although numerous experimental studies have indicated that antioxidants and ROS scavenging could prevent events leading to atherosclerosis, extending this concept into the treatment of human disease has been problematic. Two small trials, CHAOS and ATBC, showed the benefit of vitamin E in reducing myocardial infarction [20,21]. Unfortunately, several larger studies, including the HOPE trial [22], the heart protection study [23], and the GISSI prevenzione [24] (involving many thousands of subjects), have failed to show any benefits of antioxidant vitamins in prevention of cardiovascular disease. Surprisingly, some studies have shown that vitamin E is harmful. The incidence of heart failure and hospitalization for heart failure was increased in subjects treated

with vitamin E in the HOPE-TOO trial [25]. A recent large meta-analysis has suggested that high-dose (>400 IU/d) vitamin E might increase mortality and should be avoided [26]. The reasons for the lack of beneficial clinical effects of vitamin E supplementation are unclear [27]. There are several potential explanations for these results. One is that the oxidative-stress hypothesis of atherosclerosis is wrong. Given the wealth of information from human and experimental studies, this seems improbable. A second explanation is that when vitamin E and similar antioxidants scavenge a radical they become radicals that have their own untoward effects [28]. For example, the tocopheroxyl radical can paradoxically contribute to LDL oxidation under certain circumstances. Vitamin C can reduce the tocopheroxyl radical back to active vitamin E. This has led to the use of combined antioxidant interventions, including vitamin C, vitamin E and β-carotene, within the Heart Protection Study (HPS) [23]. Administration of other vitamins alone, mainly vitamin C or β-carotene, has not proven to be effective either [27,29]. It might be related to the poor penetration (of orally supplemented vitamins) into the vascular wall [30]. A final, highly likely reason is that ROS have important signaling properties, and the nonselective approach of scavenging all ROS could have deleterious effects. Scavenging the 'pathologic' radicals and not interfering with ROS that have important signaling properties is difficult. A final, highly likely reason Moreover, as discussed above, ROS production can be especially localized, and antioxidants, particular vitamin E, might not effectively reach these sites.

Whatever the explanation for the results they clearly show that commonly employed antioxidants are ineffective in treating cardiovascular diseases. Moreover, recent studies have shown that vitamin E should actually be avoided in anything but the small doses present in multivitamins. This has focused attention to the use of specific inhibitors of enzyme systems involved in ROS generation.

Mechanisms of ROS production in human vasculature

Understanding the mechanisms of oxidative stress in the human cardiovascular system is crucial for the development of specific interventions. It is interesting that those mechanisms in humans can differ depending on the clinical condition. Potential major cellular sources of vascular superoxide include NADPH-dependent oxidases [31], xanthine oxidase [32], lipoxygenases, mitochondrial oxidases [16] and NO synthases [33]. A large body of evidence based on animal models suggests that a membrane-bound NADPH oxidase, xanthine oxidase and dysfunctional eNOS are the major sources of the superoxide anion in various preatherosclerotic conditions (Figure 3) [31,32]. These data have recently been confirmed in human peripheral conduit arteries and veins [34], as well as in coronary arteries [35–37].

NADPH oxidases

Variations of the phagocytic NADPH oxidase, referred to as the Nox enzymes, have been found in all vascular cells including endothelial cells, VSMCs and fibroblasts [38]. Several protein components form the classic NADPH oxidase complex: $p22^{phox}$ and $gp91^{phox}$ (the membrane-bound cytochrome b558, crucial for the activity) and $p47^{phox}$, $p67^{phox}$ (regulatory cytosolic proteins) and the low molecular weight G protein Rac. There are at least five variations of the NADPH oxidase, differentiated by their catalytic subunits known as the Nox proteins (reviewed in detail by Lambeth [39]). These catalytic subunits possess flavin- and heme-binding regions and generate $O_2^{\bullet-}$ via one electron transfer from NADH or NADPH to oxygen. An exception to this is Nox4, which seems to produce H_2O_2 predominantly. The complexity of structure and protein–protein interactions leading to the activation of NADPH oxidase is exemplified in Figure 4.

Of the various Nox isoforms, Nox1, Nox2 and Nox4 are the most important in vascular cells. With the exception of Nox5 all the Nox isoforms require $p22^{phox}$ as a docking subunit. Nox4 functions constitutively and does not require cytosolic subunits. Interestingly, Nox homologs can be differentially associated with various vascular disease phenotypes. Changes in Nox1 expression directly alter cell proliferation [40] and treatment of VSMCs with angiotensin II or platelet-derived growth factor (PDGF) upregulates Nox1, at the same time downregulating Nox4 [41]. Vascular injury increases expression of Nox1, Nox2 and $p22^{phox}$, and Nox4 expression increases later [42], coinciding with a reduction in the rate of VSMC proliferation. These findings suggest that although Nox1 and Nox2 are involved in acute response to injury, or to angiotensin II stimulation, Nox4 is involved in maintaining the quiescent phenotype [42]. $p22^{phox}$, Nox1, Nox2 and Nox4 are expressed in humans [34,35]. Azumi *et al.* [43] were first to show the presence of a $p22^{phox}$-based NADPH oxidase in human coronary artery atherosclerotic plaques. Nox2 and $p22^{phox}$ are greatly increased with the progression of human atherosclerosis [44], whereas Nox4 is increased in early lesions and decreased in severe lesions [35]. A direct spatial relationship between NADPH oxidase-generated ROS and oxidized LDL was demonstrated in carotid plaques and $O_2^{\bullet-}$ production was increased in lesions associated with unstable angina [45]. The role of the NADPH oxidase in lesion instability is further illustrated by the finding that the 'shoulder regions' of plaques have intense ROS and increased $p22^{phox}$ and Nox2 [35].

The activity and expression of NADPH oxidase can be regulated by cytokines [tumor necrosis factor-α (TNF-α), transforming growth factor-β (TGF-β), PDGF] and agonists like angiotensin II and thrombin [38]. Hemodynamic forces, such as oscillatory shear-stress and stretch, can also activate the NADPH oxidase and increase Nox expression. Importantly, angiotensin II is one of the most potent stimuli of vascular NADPH oxidase activity and expression [38,46]. This property clearly links ROS production with activation of the rennin–angiotensin system in hypertension, in early stages of atherosclerosis [47] and in heart failure [48]. In rats made hypertensive by chronic angiotensin II infusion the expression of Nox1 and $p22^{phox}$ mRNA is elevated [49]. Moreover, the increase in blood pressure caused by angiotensin II is markedly reduced in $p47^{phox-/-}$ mice [50].

In summary, the NADPH oxidases have been indicated to be a main source of $O_2^{\cdot-}$ in the majority of vascular disease models. In humans it has been confirmed that NADPH oxidase activity is inversely correlated with endothelial function. This relationship exists even when corrected against other major risk factors for atherosclerosis, including diabetes and hypercholesterolemia [51].

Xanthine oxidase

As well as the NADPH oxidases, xanthine oxidase (XO) can be an additional source of vascular superoxide [36,37]. Stimuli such as

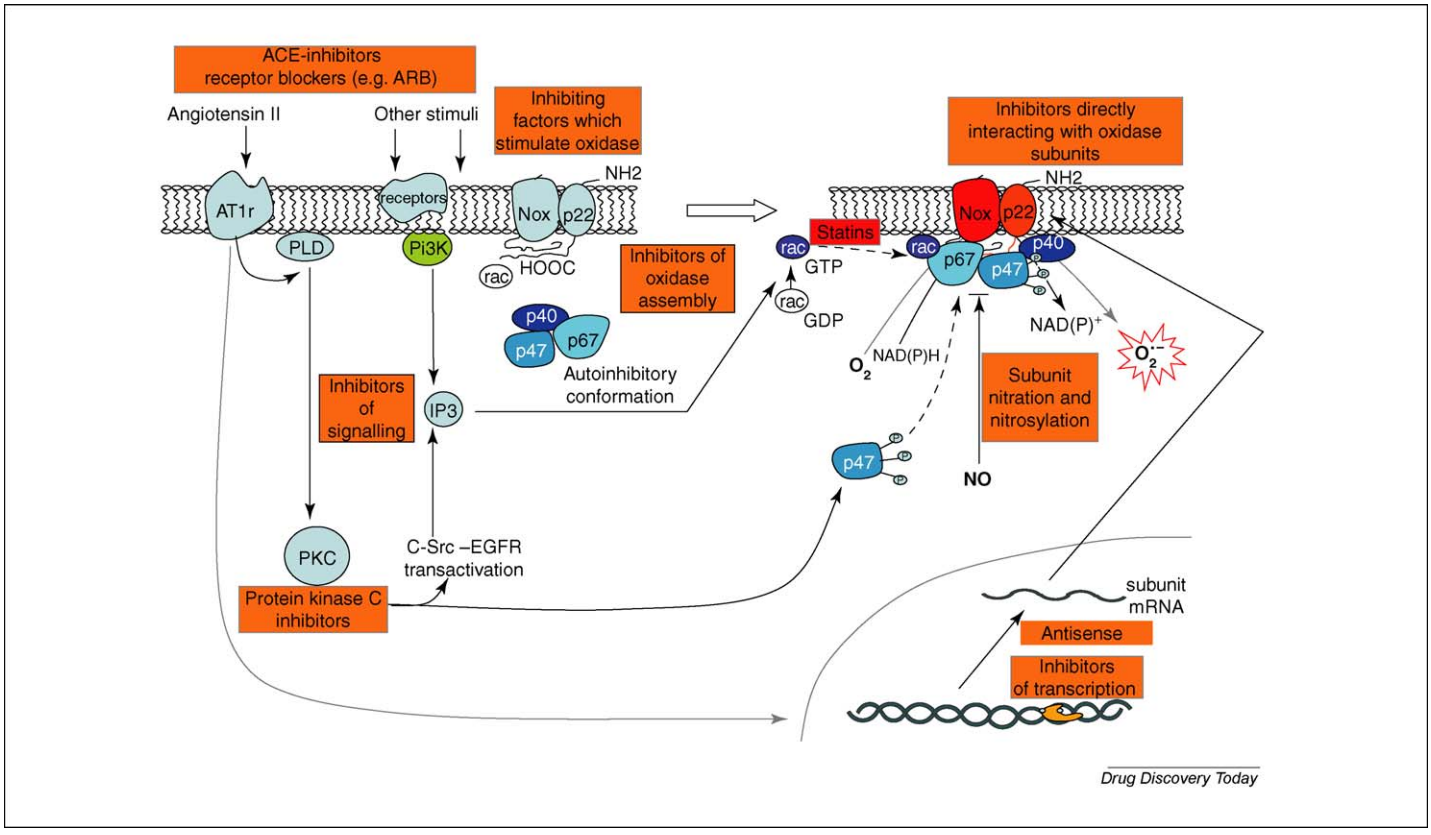

FIGURE 4

NADPH oxidase activity regulation and potential drug targets for current, novel oxidase-inhibiting therapies. Superoxide ($O_2^{\bullet-}$) production by the NADPH oxidases (Nox enzymes) can be induced by several distinct mechanisms that stimulate assembly of cytosolic regulatory subunits ($p47^{phox}$, $p67^{phox}$ and $p40^{phox}$) with the membrane subunit complex ($p22^{phox}$ and $gp91^{phox}$ or Nox). These mechanisms include protein kinase C (PKC) activation leading to phosphorylation of the $p47^{phox}$ autoinhibitory region allowing it's binding to $p22^{phox}$ or activation of an IP3-dependent pathway leading to Rac translocation. Statins can inhibit this by preventing isoprenylation of Rac. Vascular oxidases are also regulated at the level of expression of subunit mRNA. As an example, Nox enzymes can be induced by angiotensin II, cytokines and disturbed-flow profiles. Potential drug targets for inhibition of NAD(P)H oxidase are shown in the shaded boxes.

hypoxia and reoxygenation, cytokines, and oscillatory shear-stress [52] increase endothelial XO activity. XO is an important source of ROS in a variety of pathophysiological states, including hypertension [53], atherosclerosis, ischemia–reperfusion and heart failure [37]. In humans with heart failure and in subjects with CAD the endothelial levels of XO are increased and correlate with the degree of impairment in endothelium-dependent vasodilatation [36,50].

Endothelial NO synthase

In the absence of its cofactor (BH_4) or its substrate (L-arginine), the endothelial NO synthase generates $O_2^{\bullet-}$ instead of NO [33]. The oxygenase domain of each eNOS monomer binds BH_4, which stabilizes the dimer and donates electrons to the ferrous-dioxygen complex in the oxygenase domain to yield an iron–oxy complex that participates in L-arginine hydroxylation [33]. In BH_4 deficiency the ferrous-dioxygen complex dissociates yielding superoxide [54]. BH_4 could play a role in stabilizing the dimeric conformation of eNOS, crucial for NO production [33], although there is still considerable debate as to whether it is the eNOS dimer or monomer that produces superoxide. $ONOO^-$ and other strong oxidants rapidly react with BH_4 [55], leading to the formation of a $BH_3^{\bullet}$-radical intermediate, which rapidly degrades to BH_2. Extensive oxidation of BH_4 can lead to disruption of the pterin structure. Exposure of endothelial cells to $ONOO^-$ rapidly leads to NOS uncoupling, in part via this mechanism [56]. BH_4 oxidation and NOS uncoupling have been demonstrated in hypertension, diabetes and hypercholesterolemia [33,56] and administration of BH_4 improves endothelium-dependent vasodilatation in experimental animals and in humans with these conditions.

Other sources

Other potential sources of superoxide include enzymes involved in the metabolism of arachidonic acid (cyclooxygenases or lipooxygenases) and the mitochondrial electron transport chain. These have been reviewed elsewhere [53]. In addition, in porcine vascular cells, the cytochrome p450 Cyp2C9 subtype seems to be an important source of $O_2^{\bullet-}$.

Interactions of NADPH oxidases with other oxidase systems

Data obtained using NADPH oxidase $p47^{phox}$ knockout mice and cells have shown that the NADPH oxidase is crucial in the regulation of superoxide production from eNOS [56] and XO [57] (Figure 3). Peroxynitrite, the product of NO–$O_2^{\bullet-}$ interaction, oxidizes BH_4 and leads to eNOS uncoupling [56]. In endothelial cells lacking $p47^{phox}$, $O_2^{\bullet-}$ production was depressed and XO protein and activity were minimal, and $p47^{phox}$ transfection

restored XO protein levels [57]. Similarly eNOS uncoupling was prevented in the absence of NADPH oxidase [56].

These findings make NADPH oxidases particularly important drug targets for specific antioxidation, which modulates ROS production from other sources as well.

NADPH oxidase – new drug target for old drugs

Several studies have shown that NADPH oxidase activity can be modified by drugs that decrease cardiovascular events in the clinical setting. For example, the 3-hydroxy-3-methylgluatryl coenzyme A (HMG-CoA) reductase inhibitors (statins) inhibit both the activity and expression of Nox1 [58] and Nox2 [59] in vascular cells. Membrane association of the GTPase Rac1, required for NADPH oxidase activation, is dependent on geranylgeranylation, which is prevented by HMG-CoA reductase inhibitors [58]. Spontaneously hypertensive rats treated with atorvastatin have reduced aortic ROS production, $p22^{phox}$ expression and Nox1 expression, as well as Rac1 translocation [58]. Similar observations have been described in mammary arteries from atherosclerotic patients [59]. To date, atorvastatin, simvastatin, rosuvastatin and fluvastatin have all been shown to inhibit NADPH oxidase activity.

As discussed earlier, angiotensin II plays an important role in activation of the vascular NADPH oxidases. Accordingly, drugs affecting the rennin–angiotensin system reduce oxidase activity. In diseases such as atherosclerosis and hypertension marked activation of the NADPH oxidase by angiotensin II has been reported and angiotensin receptor blockers (ARBs) and angiotensin-converting enzyme (ACE) inhibitors have proven effective in reducing vascular $O_2^{\bullet-}$ production and improving endothelial function by inhibiting NADPH oxidase expression and activity [60].

NADPH oxidase – drug target for novel drugs

In addition to the accepted therapies for cardiovascular disease, newer and more-specific agents have been employed to inhibit the NADPH oxidases. Peptide inhibitors that interfere with binding of the regulatory subunits to the cytochrome b558 have been developed. One such agent, Gp91ds-tat, consists of nine amino acids from $gp91^{phox}$ that normally bind to $p47^{phox}$ (linked to the amino acid sequence from the coat-protein of HIV), allowing cell permeability. As such, Gp91ds-tat acts as a decoy that binds $p47^{phox}$ and prevents its interaction with Nox proteins. It probably prevents activation of Nox1 as well as Nox2. Because Nox4 functions independently of $p47^{phox}$ Gp91ds-tat has no effect on function of this isoform. This peptide has been shown to attenuate the hypertension caused by angiotensin II infusion and to reduce vascular $O_2^{\bullet-}$ production [61]. Gp91ds-tat also inhibits intimal hyperplasia following vascular injury [62]. However, because Gp91ds-tat is a peptide its use is limited to parenteral administration. Its effectiveness has yet to be studied in humans.

PR39 is a naturally occurring 39-amino-acid proline- and arginine-rich peptide, originally isolated from pig intestine and originally identified in neutrophil azurophilic granules and macrophages [63]. PR39 binds to the Src homology domain 3 (SH3) of $p47^{phox}$ and, like Gp91ds-tat, prevents the association between $p47^{phox}$ and Nox proteins [64]. This peptide has been successfully used in reducing ROS in ischemia–reperfusion models [65]. PR39 is not specific for NADPH oxidase and exerts other properties related to its binding to SH3 homology domains of proteins. Like Gp91ds-tat, it cannot be given orally.

Apocynin is an orally active agent, originally isolated from the roots of *Picrorhiza kurroa*, that can block NADPH oxidase assembly [66]. Apocynin requires reaction with a peroxidase for its activation and, therefore, does not work immediately. Apocynin reduces ROS production when administered in animal models of arthritis and asthma. Oral treatment of apocynin blunted the development of hypertension, abolished the increase in vascular $O_2^{\bullet-}$ and prevented endothelial dysfunction in DOCA-salt hypertensive rats [67]. Apocynin is not entirely specific. It has been reported to affect arachidonic acid metabolism [68], increase glutathione synthesis and to activate the AP-1 transcription factor [69]. Two other chemical inhibitors of the NADPH oxidase under investigation include aminoethyl benzenesulphonyl-fluoride (AEBSF) [63] and the benzo(b)pyran-4-one derivative S17834 [70], however their effects and specificity have not been adequately defined to date.

An important early step in NADPH oxidase activation is phosphorylation of $p47^{phox}$ by protein kinase C (PKC). Because of this, PKC inhibitors can serve as NADPH oxidase inhibitors [71,72]. This has been recently confirmed by *in vitro* studies in animals and in humans [34,72,73]. A recent placebo-controlled trial in humans has shown that the selective PKC-β inhibitor LY333531 can prevent endothelial dysfunction caused by hyperglycemia, probably because of a reduction in vascular $O_2^{\bullet-}$ production [74]. Because the activation of the NADPH oxidase by angiotensin II is partly mediated by PKC, drugs that inhibit PKC might be effective in conditions where angiotensin II is increased [72]. In keeping with this, inhibition of PKC in humans with atherosclerotic risk factors decreased $O_2^{\bullet-}$ production by the NADPH oxidase [34]. Additional studies of the effects of PKC inhibitors on NADPH oxidase activity in humans are warranted.

Polyphenols and anthocyanins, apart from their direct $O_2^{\bullet-}$ scavenging properties, can inhibit NADPH oxidases. They have been shown to prevent increased expression of NADPH oxidase subunits and to reduce oxidase activity in some animal models [75].

Vascular superoxide production has been reduced by 17-β-estradiol in cardiovascular diseases [76]. Likewise, ghrelin, a novel peptide involved in appetite control, can also decrease vascular $O_2^{\bullet-}$ production, probably by inhibiting the NADPH oxidase [77].

Finally, nitration and nitrosylation of the NADPH oxidase might lead to the inhibition of the enzyme. We have observed that NOS gene transfer significantly inhibited activity and expression of vascular NADPH oxidase and prevented intimal hyperplasia in a NO-dependent manner [78].

Inhibiting eNOS dysfunction or XO activity

As discussed earlier, in the absence of BH_4 the NO synthases become dysfunctional (uncoupled) and begin to produce $O_2^{\bullet-}$ rather than NO. The intra-arterial administration of BH_4 has been shown to improve endothelial function in humans with atherosclerosis. Oral administration of BH_4 has been shown to lower blood pressure, reduce vascular $O_2^{\bullet-}$ and increase NO production in hypertension and insulin resistance [33]. A recent preliminary study in humans with hypertension has shown that oral BH_4 therapy is effective in lowering blood pressure and improving endothelial function. Sepiapterin is a stable cell-permeable pre-

cursor of BH_4 that prevents endothelial dysfunction and oxidative stress in diabetic animals when given orally [79]. It is possible that tetrahydrofolate can substitute for BH_4 and support NOS catalysis [80], however its use in the vascular setting has not been demonstrated yet.

XO is an important source of ROS in a variety of pathophysiological states. Because of this there has been interest in using allopurinol or its active metabolite oxypurinol to reduce ROS production in these conditions. Allopurinol has been shown to improve endothelial function in humans with atherosclerosis and heart failure [81]. Surprisingly, there have been no long-term studies of allopurinol in the treatment of humans with diseases thought to be related to oxidative stress; however, there is currently an ongoing study to examine the effect of oxypurinol in humans with heart failure [82].

Conclusion

Research conducted in recent years has enhanced our understanding of oxidative stress and its role in vascular disease. It has become clear that oxidative stress, particularly at early stages of disease, is related to slight disturbances of oxidation–reduction potentials localized to selected compartments within the cell, rather than changes in the overall redox status of the cell (Box 2). Such disturbances in redox signaling within vascular cells play important roles in the pathogenesis of numerous cardiovascular diseases, including atherosclerosis, hypertension, heart failure and diabetic vascular dysfunction. These disturbances are also unlikely to be corrected with simple free-radical scavenging. The disappointing results from large clinical trials of classical antioxidant vitamins have prompted investigators to seek alternative approaches. A potentially important, novel treatment strategy is selective inhibition of specific Nox-based NADPH oxidases. Much remains to be learned before we can embark on this route in clinical practice. The precise role of the various Nox isoforms needs to be better defined. The development of specific, orally active NADPH oxidase inhibitors is necessary. Finally, recent studies have shown that clinically available drugs like the statins, ACE inhibitors and angiotensin receptor antagonists inhibit the vascular NADPH oxidases, and reduction of vascular ROS production might explain some of their beneficial effects.

BOX 2

Changing concepts of oxidative stress

Traditionally, oxidative stress was defined as an imbalance between the cellular antioxidant mechanisms (e.g. superoxide dismutases, glutathione peroxidases, thioredoxins, etc.) and pro-oxidant mechanisms (e.g. superoxide-producing systems, etc.), see Figure 1. Recent studies have suggested that, although these profound changes are observed in advanced stages of disease, in the early stages the alterations occur predominantly within individual cellular compartments or even within individual enzymes, without modification of total cellular redox status. Such changes of electron flow in certain enzyme systems result in the formation of pathological, or excessive, amounts of a correct product leading to the disturbance of redox signaling. Endothelial nitric oxide synthase (eNOS) uncoupling (leading to production of superoxide instead of nitric oxide) or superoxide 'leakage' from a mitochondrial chain might be examples of such local changes. Therapeutic interventions on the level of total cellular redox status (like antioxidant vitamins) might not be sufficient to correct these disturbances. Novel strategies should instead focus on repairing the function of individual molecules involved in reactive oxygen species (ROS) generation.

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