Mechanisms for suppressing NADPH oxidase in the vascular wall

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Oxidative stress underlies many forms of vascular disease as well as tissue injury following ischemia and reperfusion. The major source of oxidative stress in the artery wall is an NADPH oxidase. This enzyme complex as expressed in vascular cells differs from that in phagocytic leucocytes both in biochemical structure and functions. The crucial flavin-containing catalytic subunits, Nox1 and Nox4, are not found in leucocytes, but are highly expressed in vascular cells and upregulated with vascular remodeling, such as that found in hypertension and atherosclerosis. The difference in catalytic subunits offers the opportunity to develop "vascular specific" NADPH oxidase inhibitors that do not compromise the essential physiological signaling and phagocytic functions carried out by reactive oxygen and nitrogen species. Nitric oxide and targeted inhibitors of NADPH oxidase that block the source of oxidative stress in the vasculature are more likely to prevent the deterioration of vascular function that leads to stroke and heart attack, than are conventional antioxidants. The roles of Nox isoforms in other inflammatory conditions are yet to be explored.

Key words: atherosclerosis - gp91phox - hypertension - NADPH oxidase - nitric oxide - Nox1 - Nox4 - reactive oxygen species

Oxidative stress and cardiovascular disease

There is growing evidence that oxidative stress, meaning an excessive production of reactive oxygen and nitrogen species, underlies many forms of cardiovascular and other age-related diseases. Indeed, increased production in vascular tissues of reactive species (RS), particularly superoxide anions ($O_2^{-\lambda}$), has been implicated as playing an important role in hypertension, vascular remodeling after angioplasty, atherosclerosis, myocardial infarction, and ischemic stroke (Wattanapitayakul & Bauer 2001). Excess superoxide or superoxide-derived RS have multiple pathophysiological actions in the artery wall. For example, RS, directly or indirectly, promote lipid peroxidation and low-density lipoprotein (LDL) oxidation, which are necessary steps in subendothelial lipid accumulation and atherosclerotic lesion formation (White et al. 1994, Steinberg 2002). Superoxide rapidly inactivates endothelium-derived nitric oxide (NO), the important endogenous vasodilator, thereby reducing NO bioavailability, elevating vascular resistance and promoting vasoconstriction (Dusting et al. 1998, Dusting & Dart 1999, Zicha et al. 2001). Moreover, impairment of NO function by superoxide may result in vascular smooth muscle cell proliferation and migration, as well as promoting leucocyte and platelet adhesion (see below) (Dusting & Dart 1999). RS are also known to act as intracellular messengers in proinflammatory signaling, leading to the activation of the redox sensitive transcription factor nuclear factor kappa B (NF-κB) and expression of adhesion molecules [such as selectins, vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1)] and chemokines [monocyte chemoattractant protein-1 (MCP-1)] in the vascular endothelium (Kunsch & Medford 1999), all of which have key roles in atherogenesis and restenosis. In endothelial cells, RS may also play a crucial role in angiogenesis (Ushio-Fukai et al. 2002). Thus the enzymatic sources of oxidative stress are appealing therapeutic targets for many cardiovascular conditions and for cancers that are dependent upon angiogenesis for tumour growth.

NADPH oxidase as a source of oxidative stress in the vessel wall

Several oxidant enzyme systems, such as xanthine oxidase, cyclooxygenases, lipoxygenase, cytochrome P450 and substrate-uncoupled nitric oxide synthase (NOS), have been proposed as sources of vascular superoxide. However none of these systems can satisfactorily explain the bulk of intracellular production of superoxide in vascular tissues under either normal or pathophysiological conditions (Munzel et al. 1999). Recently, a superoxide-producing NADPH oxidase similar to the enzyme complex in phagocytic leucocytes (Babior 1999), has been identified in vascular tissues. The enzyme complex in phagocytes comprises at least 5 components: two cytosolic subunits p47phox and p67phox, a cell membrane bound cytochrome b558 which consists of gp91phox (renamed Nox2) and p22phox, and a small G protein Rac. Upon assembly of these subunits in the membrane, this enzyme generates a burst of superoxide on the extracellular side of the membrane by one-electron reduction of oxygen via its gp91phox subunit, using reduced β-nicotinamide adenine dinucleotide phosphate (NADPH) as the electron donor (Babior 1999). The vascular NADPH oxidase in endothelial and smooth muscle cells differs from this in several respects, although the details of precisely how it is activated are still emerging. Notwithstanding, several distinguishing characteristics of the vascular NADPH oxi-

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dase are clear. First, the vascular enzyme produces superoxide at lower levels over a longer period of time, and much of this is generated intracellularly where it is ideally placed for cell signaling roles (Cai et al. 2003, Fig. 1). Second, the gp91phox catalytic subunit to which NADPH and oxygen bind is substituted by Nox1 or Nox4 homologues, particularly in smooth muscle (Lassegue et al. 2001, Bengtsson et al. 2003, Ellmark et al. 2004). Third, whilst the Nox homologue and the membrane-bound p22phox subunit are essential to maintain a stable unit capable of supporting electron transfer for superoxide generation (Ambasta et al. 2004), it remains unclear what role the cytosolic components play in the vascular NADPH oxidase: this latter point has implications for the action and specificity of NADPH oxidase inhibitors (see below).

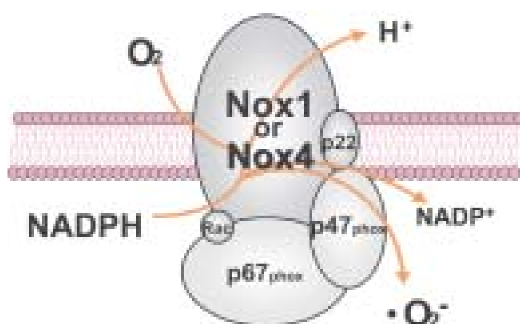

Fig. 1: the vascular NADPH oxidase complex may contain Nox1 or Nox4 as substitutes for the catalytic gp91phox subunit of the phagocytic oxidase. Superoxide (lO_2^-) is produced intracellularly. The Nox subunit is bound to p22phox in the plasma membrane, and they stabilise each other. The cytosolic subunits shown may also be required for full and sustained activation of the complex in vascular cells, although there is evidence that alternative subunits may be involved for Nox1 activation.

We and others have shown that increased activity of NADPH oxidase makes an important contribution to the pathogenesis of experimental models of vascular disease including intimal hyperplasia induced by periarterial collars (Paravicini et al. 2002) and arterial balloon injury (Souza et al. 2000, Chen et al. 2004), cholesterol-induced atherosclerosis (Warnholtz et al. 1999), vein graft intimal hyperplasia (West et al. 2001) and hypertension (Zalba et al. 2000). Gene disruption of the p47phox component has been shown to significantly reduce superoxide production by vascular smooth muscle cells and importantly to reduce the development of atherosclerotic lesions (Barry-Lane et al. 2001). Importantly, increased superoxide generation by NADPH oxidase in vessels has been linked to the clinical risk factors for atherosclerosis and impaired endothelial NO function in patients with coronary artery disease (Guzik et al. 2000).

Nox homologues in vascular cells

The subunit gp91phox, expressed in phagocytic leucocytes, is the NADPH-binding, flavin adenine dinucleotide (FAD)-containing and electron transporting subunit of the active NADPH oxidase. The expression of gp91phox, however, cannot be readily detected in certain vascular cells such as smooth muscle (Gorlach et al. 2000, Lassegue et al. 2001). This led to the cloning of gp91phox homologues, termed Nox, in both human (Cheng et al. 2001) and animal tissues (Cheng et al. 2001, Yang et al. 2001). In humans, Nox1 and Nox4 are expressed in vascular smooth muscle and endothelial cells (Nox4 predominates) but not in peripheral blood leucocytes (Suh et al. 1999, Cheng et al. 2001, Yang et al. 2001, Ago et al. 2004). Recently, we have demonstrated that Nox4 is the predominant homologue expressed in mouse vascular smooth muscle cells (Bengtsson et al. 2002, Drummond et al. 2003, Ellmark et al. 2004) where Nox1 expression is almost undetectable.

In aortic smooth muscle cells derived from C57BL/6J mouse, gene silencing with a specific antisense oligonucleotide to Nox4 significantly reduced NADPH-dependent superoxide generation (Fig. 2). This strongly suggests that Nox4 has an important role in NADPH oxidase activation and superoxide generation in mouse vascular smooth muscle cells (Drummond et al. 2003, Ellmark et al. 2004). However, in rat vascular smooth muscle it has been found that Nox1 was responsible for the increased superoxide formation and subsequent redox-sensitive signaling induced by angiotensin II (Lassegue et al. 2001). The importance of Nox proteins in vascular superoxide generation is underlined by in vivo studies showing that superoxide production by vascular smooth muscle cells from gp91phox gene knockout mice is not different from that in

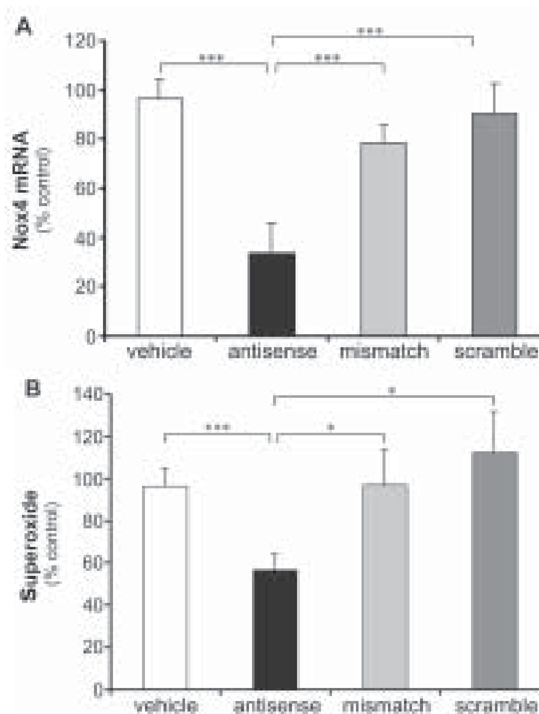

Fig. 2: Nox4 gene silencing in mouse vascular smooth muscle cells. Nox4 mRNA (A, by real time PCR) and superoxide production (B, by lucigenin chemiluminescence) is reduced by 50-60% by a Nox4 antisense oligomer, but is not affected by mismatch or scrambled oligonucleotide control sequences. See Ellmark et al. 2004 for details.

wild type controls (Kirk et al. 2000). Moreover, the finding that deficiency in gp91phox failed to affect the development of atherosclerosis (Kirk et al. 2000) could be taken as evidence that the NADPH oxidase of leucocytes does not have a major role in atherogenesis. Conversely, the observation of reduced lesions in p47phox knockouts (Barry-Lane et al. 2001), suggests that Nox proteins in vascular cells, substituting for gp91phox, do make important contributions to the development of cardiovascular disorders associated with increased oxidative stress.

Activation and regulation of vascular NADPH oxidase

The vascular NADPH oxidases are activated and regulated by a variety of hormones and factors known to be important players in vascular remodeling and disease. These include thrombin, platelet-derived growth factor (PDGF), tumor necrosis factor (TNFα), lactosylceramide, interleukin-1, and oxidized LDL (for review see Griendling et al. 2000). Oxidized LDL increases RS production and specifically increases expression of Nox4 in human endothelium (Thum & Borlak 2004). Changes in shear stress are very relevant stimuli in endothelial cells: oscillatory shear stress (zero net forward flow) increases RS production and upregulates Nox4 as well as gp91phox, while pulsatile shear (net forward flow) may actually downregulate these components as compared to static cells (Hwang et al. 2003). The most studied stimulus of the vascular NADPH oxidase is angiotensin II. Angiotensin increases the activity of the NADPH oxidase at three or more levels. First there is rapid activation of c-Src and other kinases, leading to phosphorylation of p47phox which translocates to the membrane cytochrome complex (Touyz et al. 2003) In vascular smooth muscle, the EGF receptor transactivation is also involved, leading to sequential activation of PI3 kinase and the small G-protein Rac, all of which occurs within minutes of angiotensin AT_1 receptor activation (Seshiah et al. 2002). There is also some evidence for the involvement of Rho family GTPases in RS generation in vascular smooth muscle, and this is involved in suppression of NADPH oxidase by statins (see below). All these events following stimulation with angiotensin serve to activate, promote and sustain electron flow through the cytochrome complex. A further level of action of angiotensin, and some other stimuli, is to increase the expression of NADPH oxidase subunits over hours to days (Cai et al. 2003). The importance of angiotensin for activation of NADPH oxidase is underlined by studies showing that oxidative stress, NADPH oxidase activation and some of the pathological features of hypertension and atherosclerosis are abrogated by angiotensin AT_1 receptor antagonists (Warnholtz et al. 1999, Baykal et al. 2003).

Recently a direct molecular link between NADPH oxidase and endotoxin-induced activation of innate immunity and inflammation has been proposed. Several lines of evidence indicate that activation of the crucial NF-κB transcription factor can be controlled by RS (Janssen-Heininger et al. 2000). One way that the endotoxin lipopolysaccharide (LPS, an integral component of the outer membrane of gram-negative bacteria) can activate NADPH oxidase is through binding to the Toll-like receptor (TLR4), the cytoplasmic tail of which was recently found to interact with the carboxy terminal of Nox4 in HEK293 kidney cells (Park et al. 2004). Knockdown of Nox4 by siRNA in HEK 293 and a Nox4-expressing U937 monocytic cell line virtually abolished LPS-induced superoxide production, and also reduced or abolished NF-κB activation. Whether this interaction occurs in vascular cells expressing Nox4 has yet to be determined, but the observation provides a mechanism whereby LPS activate inflammatory reactions and adhesion molecule expression in vascular endothelium (acutely in septic shock, or longer term in atherogenesis, vascular remodeling etc), pathogenic processes that could be targeted by blocking NADPH oxidase activation.

Interaction of NO with RS and suppression of NADPH oxidase

NO derived from the vascular endothelium is a crucial mediator of vasodilation and may also play a role in aggregation and adhesion of platelets and leucocytes, as well as maintaining the balance between smooth muscle growth, migration and differentiation (Dusting & Dart 1999). NO is also a free radical, and when produced in concert with superoxide it reacts extremely rapidly (rate constant $7x10^9$ mol l^{-1} s^{-1}) to form a further RS, the highly reactive molecule peroxynitrite (ONOO-) (Beckman et al. 1990). This results in endothelial dysfunction, failure of endothelium-dependent vasodilation and a propensity for vasoconstriction, as well as promoting vascular remodeling. Indeed, peroxynitrite is an important mediator of lipid peroxidation and protein nitration, including oxidation of LDL which is crucial for atherogenesis, and might induce apoptosis of endothelium or cells of the fibrous cap of unstable plaques.

NO donor drugs (also known as nitrovasodilators) have been used for more than a century to treat coronary artery disease, hypertension, and heart failure. Apart from vasodilation, these drugs have anti-inflammatory actions in the blood vessel wall such as suppression of lipoprotein oxidation, inhibition of vascular smooth muscle cell migration and proliferation, and inhibition of platelet aggregation (Dusting & Dart 1999). In addition, NO donors have been demonstrated to suppress expression of adhesion molecules and chemokines after stimulation with proinflammatory factors, thereby preventing infiltration of inflammatory cells into the vessel wall (Dusting & Dart 1999). These actions clearly oppose the pro-inflammatory actions of RS on the blood vessel wall. We have recently discovered that NO may exert an anti-inflammatory effect in the artery wall by suppressing RS generating enzymes, particularly NADPH oxidase. Three chemically distinct NO donor compounds (DETA-NONOate, sodium nitroprusside, sodium acetylpenicillamine) were shown to inhibit the production of superoxide by NADPH oxidase in human cultured endothelial cells in a concentration- and time-dependent manner (Selemidis et al. 2003). Furthermore, suppression of superoxide production persists for more than 6 h after washing NO donor from the cells (Fig. 3). This action does not appear to involve cyclic GMP nor alterations in transcription of NADPH oxidase subunits, but preliminary data indicates that NO may disrupt acti-

vation and assembly of the vascular NADPH oxidase complex. That NO is able to suppress NADPH oxidase activation would allow NO to perform its physiological functions in vascular homeostasis when required by removing a major source of its inactivation (Fig. 4). Therapeutically, the routine administration of NO donor drugs for coronary artery disease and angina pectoris may also be treating an underlying cause of the disease - increased RS in the blood vessel wall.

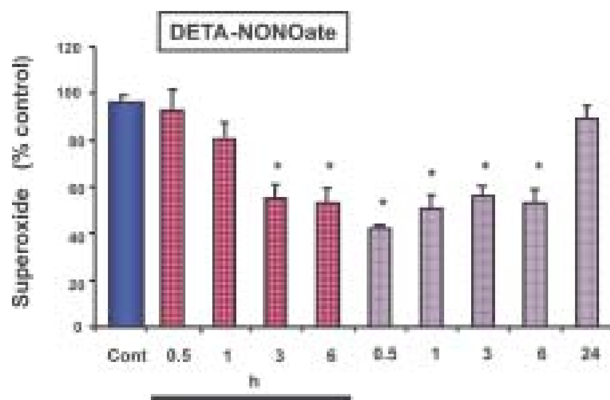

Fig. 3: NADPH-dependent superoxide produced by human endothelial cells is suppressed by an nitric oxide (NO) donor compound. DETA-NONOate (100 µM) is incubated with cells for the periods indicated by the bar, and washed from the cells immediately before assay. The effect of NO is sustained for more than 6 h after removing the compound from the cells.

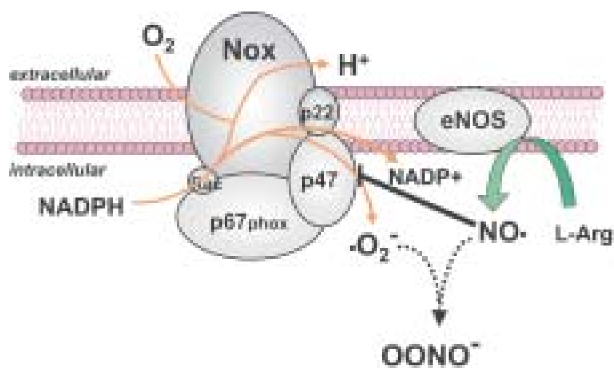

Fig. 4: interactions of nitric oxide (NO) with NADPH oxidase in vascular cells. NO produced by eNOS not only reacts with superoxide (lO_2^-) to produce the reactive species peroxynitrite (OONO-), but it also may act to suppress NADPH oxidase activation.

Inhibition of NADPH oxidase

Pharmacological inhibitors of NADPH oxidase that directly block the catalytic activity of this enzyme have also been identified, comprising both nonpeptide and peptide inhibitors. Among the former, diphenyleneiodonium (DPI) and 4'-hydroxy-3'-methoxyacetophenone (apocynin) have been widely used as experimental tools to block NADPH oxidase activity in vitro (Cai et al. 2003). In the neutrophil it has been suggested that DPI abstracts an electron from the reduced redox centre of NADPH oxidase to form a radical, which then forms covalent adducts with the flavin cofactor (FAD), resulting in a shunt of the electron to molecular oxygen (O'Donnell et al. 1993). The usefulness of this compound is limited because DPI interacts with all flavoenzymes, as exemplified by its potent inhibition of NO synthases (Stuehr et al. 1991). In neutrophils, apocynin has been demonstrated to prevent the translocation of p47phox and p67phox subunits from cytoplasm to membrane, and is therefore thought to prevent the assembly of NADPH oxidase (Stolk et al. 1994). The inhibitory effects of DPI and apocynin were initially characterized in the phagocytic NADPH oxidase, and it is assumed that the same mechanisms operate in vascular cells, although it is much less certain how the cytosolic components are involved in superoxide production by vascular NADPH oxidases. Moreover, apocynin, in common with other polyphenolic derivatives, has multiple biological actions in addition to its antioxidant effects (Jiang & Dusting 2003). For example, it has been reported that apocynin attenuated ischemia-reperfusion lung injury in sheep while DPI worsened the injury, although both compounds produced similar inhibition of NADPH oxidase (Dodd & Pearse 2000). This discrepancy indicates that great care needs to be taken in interpreting the actions of these compounds in vivo, and that DPI in particular is of little value as an experimental tool in vivo. It is also clear that these NADPH oxidase inhibitors inhibit both vascular and phagocytic NADPH oxidases, and the latter is vital for maintaining a normal immune defence against infection. Therefore, systemic administration of either of these compounds will undoubtedly cause severe side effects.

Recently, a novel flavonoid derivative S17834 [6,8-diallyl 5,7-dihydroxy 2-(2-allyl 3-hydroxy 4-methoxyphenyl)1-H benzo(b)pyran-4-one] has been reported to directly inhibit vascular NADPH oxidase in vitro (Cayatte et al. 2001). The authors measured superoxide production from tumor necrosis factor-stimulated human umbilical endothelial cells and NADPH oxidase activity in the membrane fraction from these cells, and found S17834 suppressed superoxide generation and NADPH oxidase activity by > 50%, although the compound did not directly scavenge superoxide. Given in vivo, S17834 reduced tumor necrosis factor-stimulated VCAM, ICAM-1 and E-selectin expression, and reduced aortic atherosclerosis by 60% in apolipoprotein E-deficient mice. However, no other information about this compound is available in the public domain and the mechanism of its suppression of NADPH oxidase is unknown.

As mentioned above, the fully activated NADPH oxidase contains the small G protein Rac1. Post-translational isoprenylation of Rac1 facilitates its translocation to the cell membrane and association with other subunits of the enzyme complex. Rac1 isoprenylation (geranylgeranylation) appears to be essential for a full activation of the phagocytic enzyme (Gorzalczany et al. 2000). In vascular smooth muscle cells, we (Jiang et al. 2002) and others (Boota et al. 2000) have obtained evidence that inhibitors of protein geranylgeranyltransferase (GGTI-286 and GGTI-298), which catalyzes the transfer of the geranylgeranyl group to the target protein (*eg*. Rac), sup-

pressed superoxide production by NADPH oxidase. These studies suggest that pharmacological intervention in the isoprenylation of small G proteins may be a useful approach to suppress vascular NADPH oxidase activity. Indeed, this mechanism is known to contribute to the pleiotropic actions of 3-hydroxyl-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (statins), which prevent the synthesis of precursors of the isoprenyl groups, and have been shown to block oxidant stress-related endothelial dysfunction in cardiovascular disease (Wagner et al. 2000, Maack et al. 2003).

Several peptide-based inhibitors of NADPH oxidase have also been reported. One of these peptidoinhibitors is the antibiotic PR-39, a proline/arginine-rich peptide secreted endogenously by human and/or porcine intestinal tissues and neutrophils (Shi et al. 1996). In neutrophils, PR-39 inhibits NADPH oxidase activity by binding to the Src homology 3 domain of the p47phox subunit, thereby blocking its interaction with the membrane bound p22phox (Shi et al. 1996). PR-39 also has inhibitory actions on nonphagocytic NADPH oxidase. For example, PR-39 inhibited RS production in cultured bovine pulmonary artery endothelial cells exposed to high potassium, and in the endothelium of isolated perfused rat lungs after ischemic injury (Al-Mehdi et al. 1998). Recently, Pagano and others (Rey et al. 2001) developed a novel competitive peptidoinhibitor of NADPH oxidase (gp91ds-tat), which is a chimeric peptide comprising a conserved sequence from gp91phox (gp91 docking sequence) linked to a specific 9-amino acid peptide from the human immunodefective viral coat proteins (termed *tat*) that facilitates cellular internalization of the chimeric peptide (Rey et al. 2001). The gp91 docking sequence competitively binds to the cytosolic NADPH oxidase subunits, thereby preventing the assembly of the enzyme, whether composed of gp91phox or another Nox catalytic unit. More interestingly, the authors have shown that systemic infusion of this peptide in C57Bl/6 mice prevented the elevation of systolic blood pressure induced by in vivo angiotensin II treatment, and this hypotensive effect was accompanied by decreased superoxide production in the aorta (Rey et al. 2001). Subsequently, this group showed that in vivo treatment with this peptide decreased superoxide production and neointima formation after angioplasty in rat carotid arteries (Jacobson et al. 2003). Reinforcing this, we have recently shown that local application of apocynin to carotid arteries reduces myo-intimal thickening, endothelial dysfunction and adhesion molecule expression induced by periarterial collars in rabbits (Dusting et al. 2004). These emerging data indicate the importance of NADPH oxidase in vascular remodeling of both large and small arteries, and illustrate the potential utility of other compounds designed to block Nox function.

Finally, considering the therapeutic potential for NADPH oxidase inhibitors, it is noteworthy that all three of the most widely used classes of cardiovascular drugs, the cholesterol-lowering HMG-CoA reductase inhibitors (statins) and drugs that block the renin-angiotensin system [angiotensin-converting enzyme (ACE) inhibitors and AT_1 receptor antagonists] indirectly suppress the vascular NADPH oxidase system. It is interesting to speculate that the clearly demonstrated benefits in clinical outcomes with these therapeutic agents might derive partly from their ability to suppress oxidative stress in the cardiovascular system.

Conclusions

NADPH oxidase has emerged as a major source of oxidative stress in the artery wall, particularly in artery disease. While animal data suggest the vascular NADPH oxidase has a fundamental, causative role in the development and outcomes of artery disease, clarifying its relevance to human disease awaits the outcomes of targeted antisense approaches and future trials of specific small molecule NADPH oxidase inhibitors that are under development. Targeting specifically the vascular Nox4 or Nox1 isoforms might well have advantages over non-specific suppression of the NADPH oxidase. Our finding that NO also suppresses vascular NADPH oxidase activity has implications not only for the development of vascular disease, but also for acute recovery from ischemia and reperfusion injury to which NADPH-dependent oxidative stress makes a major contribution.

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