Suppression of Oxidative Stress in the Endothelium and Vascular Wall

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There is growing evidence that oxidative stress, meaning an excessive production of reactive oxygen and nitrogen species, underlies many forms of cardiovascular disease. The major source of oxidative stress in the artery wall is an NADPH oxidase. This enzyme complex in vascular cells, including endothelium, differs from that in phagocytic leucocytes in both biochemical structure and functions. The crucial flavin-containing catalytic subunits Nox1 and Nox4 are not present in leucocytes, but are highly expressed in vascular cells and upregulated in vascular remodeling, such as that found in hypertension and atherosclerosis. This offers the opportunity to develop “vascular specific” NADPH oxidase inhibitors that do not compromise the essential physiological signaling and phagocytic function carried out by reactive oxygen and nitrogen molecules. Although many conventional antioxidants fail to significantly affect outcomes in cardiovascular disease, 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.

Keywords: Atherosclerosis, gp91phox, Hypertension, NADPH Oxidase, Nox1, Nox4, Reactive Oxygen Species

NADPH OXIDASE AS A SOURCE OF OXIDATIVE STRESS IN THE VESSEL WALL

Several ROS are thought to play prominent roles in vascular homeostasis and disease. These include nitric oxide (NO) as well as superoxide (·O2−), hydrogen peroxide (H2O2), and peroxynitrite (ONOO−). NO has a pivotal role in vascular homeostasis, and can be formed for discrete signaling purposes by a constitutive enzyme in the endothelium (endothelial NO synthase or eNOS), or it can be formed by three related superoxide dismutases) to the more stable oxidant H2O2 and thence inactivated to water via catalase or glutathione peroxidase. The actions and roles of these radicals in vascular function depend on the amounts produced. Generated in low amounts intracellularly, ROS may act as second messengers, modulating the function of biochemical pathways involved in growth and proliferation of vascular smooth muscle cells and fibroblasts. Higher levels of ROS may cause toxicity, damaging DNA, or promoting apoptosis in cells such as endothelium and smooth muscle.

There are several potential enzymatic sources of ROS in vascular cells, each with its own cellular and subcellular compartmentalisation. Vascular enzymes capable of producing superoxide include complexes I and IV of the mitochondrial respiratory chain, xanthine oxidase (extracellular membrane), cyclooxygenases, heme oxygenase, and lipoxygenases (microsomal...
membrane fractions), as well as eNOS. Two of the prominent sources that have received considerable attention in recent years are a cytochrome P450 monoxygenase homologous to CYP2C9, and NADPH oxidases associated with the cell membrane. The relative importance of these enzymatic sources varies with the cellular or subcellular circumstances and environment. For example, activation of eNOS in endothelial cells normally produces NO from L-arginine, provided there is sufficient of the essential cofactor tetrahydrobiopterin (BH4). However, this complex may become uncoupled when BH4 levels are low, and under those circumstances eNOS produces superoxide instead (Vasquez-Vivar et al. 1998). This review focuses on the NADPH oxidase source of superoxide in vascular cells. However, it is important to bear in mind that the availability of substrates and cofactors for each of the enzyme complexes in their microenvironment determines their relative importance, and these mechanisms have not been well defined in physiology and pathophysiology.

**STRUCTURE OF NADPH OXIDASE IN VASCULAR CELLS**

All vascular cells appear to express NADPH oxidase. The structure, regulation, mechanism of activation, and function of vascular NADPH oxidases differ considerably from the classical leucocyte enzyme (Babior 1999; Griendling et al. 2000). Whereas the NADPH oxidase of “professional” phagocytes, upon stimulation, produces large amounts of superoxide within seconds in the respiratory burst, the enzyme complex of vascular cells produces smaller amounts over minutes to hours, and appears to be active constitutively. NADPH oxidases are multimeric protein complexes consisting of up to three cytosolic subunits (p47phox, p67phox, and p40phox), a regulatory G-protein (Rac1 or Rac2), and a membrane-bound cytochrome b558 reductase domain (Babior 1999; Griendling et al. 2000; Figure 1). The cytochrome b558 domain is made up of two proteins: a small α-subunit, p22phox, and a larger, catalytic β-subunit that contains binding sites for NADPH and molecular oxygen, as well as flavin and heme groups to allow electron transport between the two substrates (Babior 1999). It is well established that in neutrophils and other phagocytic cells such as macrophages, the electron transport function of NADPH oxidase resides in gp91phox (Babior 1999). A gp91phox-containing NADPH oxidase also appears to be involved in ROS production in endothelial cells and adventitial fibroblasts because cultures of these cells derived from gp91phox-deficient (gp91phox−/−) mice fail to generate superoxide in response to known stimuli of leucocyte NADPH oxidase (Gorlach et al. 2000; Frey et al. 2002; Rey et al. 2002). However, although gp91phox was shown to be expressed in vascular smooth muscle cells (VSMCs) from resistance arteries (Touyz et al. 2002), studies on VSMCs isolated from aortas and other large arteries demonstrate that gp91phox is either absent or expressed at very low levels (Ushio-Fukai et al. 1996; Sorescu et al. 2002). Moreover, aortic tissue and VSMCs from gp91phox-deficient mice do express a functional NADPH oxidase, indicating that this subunit of the phagocytic oxidase may not be responsible for superoxide generation in smooth muscle (Barry-Lane et al. 2001; Souza et al. 2001).

Recently, two novel homologues of gp91phox were shown to be coexpressed in rat VSMCs (Lassegue et al. 2001). The first of these, termed Nox1, is a 563-amino acid protein (564 amino acids in humans) that shares ~55% homology with gp91phox and contains binding sites for NADPH, flavin adenine dinucleotide (FAD), and heme, making it a strong candidate for the alternative catalytic subunit of the NADPH oxidase of VSMCs (Suh et al. 1999; Lambeth et al. 2000). Stimuli that increase superoxide production in rat VSMCs such as angiotensin II and platelet-derived growth factor (PDGF) also up-regulate expression of Nox1 in these cells (Lassegue et al. 2001). Furthermore, transfection of rat VSMCs with a full-length antisense against Nox1 inhibited superoxide production in response to these mitogens, but had no effect on basal ROS levels, suggesting that this subunit may be important for agonist-stimulated superoxide production (Lassegue et al. 2001). By contrast, we have recently demonstrated that in the absence of mitogenic stimuli, Nox4 is critical for VSMC superoxide production (Drummond et al. 2003). Nox4, a 578-amino acid protein that exhibits 39% identity to gp91phox with special conservation in membrane-spanning regions and binding sites for NADPH, FAD, and heme, was first identified as an essential subunit of the NADPH oxidase in kidney cells (Geiszt et al. 2000; Shiose et al. 2001). Nox4 mRNA expression in unstimulated mouse VSMCs was markedly higher than that of the other gp91phox homologues and short phosphorothioate antisense oligonucleotides against this subunit markedly attenuated basal superoxide production in these cells. Most recently, in human endothelial cells, Nox4 was again found to be more highly expressed than either gp91phox or Nox1, and antisense to Nox4 blocked superoxide production (Ago et al. 2004).

**FIG. 1.** Protein subunits of vascular NADPH oxidases. How the subunits are assembled has not been defined precisely, and it may vary for different cell types. The membrane-bound gp91phox and its homologues contain the flavins (FAD) and is the binding site of NADPH and molecular oxygen.
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, PDGF, tumor necrosis factor alpha (TNFα), lactosylceramide, interleukin 1, and, in endothelial cells, changes in laminar shear stress (for review see Griendling et al. 2000). The most studied stimulus of NADPH activity is angiotensin, and the increase of activity occurs at two or more levels, including rapid activation of c-Src, phosphorylation of p47phox, and its translocation to the membrane (Touyz et al. 2003). The second 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 prevented by angiotensin AT1 receptor antagonists (Warnholtz et al. 1999; Baykal et al. 2003).

NADPH OXIDASE IN VASCULAR DISEASE

Animal studies have provided much evidence that ROS and NADPH oxidase in particular have fundamental roles in several forms of vascular disease. We and others have shown that NADPH oxidase activity is up-regulated with intimal hyperplasia induced by periartrial collars (Paravicini et al. 2002), hypercholesterolemia (Warnholtz et al. 1999), arterial balloon injury (Patterson et al. 1999; Shi et al. 2001), vein grafting (West et al. 2001), and several forms of experimental hypertension (Berry et al. 2000; Zalba et al. 2000; Beswick et al. 2001; Chen et al. 2001). Increased NADPH oxidase activity has also been linked to clinical risk factors for atherosclerosis in humans and to impaired endothelial NO function in patients with coronary artery disease (Guzik et al. 2000). However, the most convincing evidence that NADPH oxidase–derived superoxide is critically involved in vascular disease comes from studies on mice that are genetically deficient in p47phox (p47phox−/−). Aortas and endothelial and VSMC cultures derived from p47phox−/− mice show diminished mitogen-stimulated superoxide production as compared to those from wild-type animals (Patterson et al. 1999; Brandes et al. 2002; Landmesser et al. 2002; Li et al. 2002; Li and Shah 2003). Importantly, thrombin-mediated activation of cellular markers of growth and proliferation (e.g., p38 mitogen-activated protein kinase and vascular endothelial growth factor) is blunted in VSMCs from p47phox−/− mice. Recently, Barry-Lane et al. (2001) showed that crossing genetically hypercholesterolemic apolipoprotein E-knockout (ApoE−/−) mice with p47phox−/− mice reduces the area of the descending aorta covered by lesions by as much as 80%, regardless of whether the animals were fed a standard chow or high-fat diet. This demonstrates that increased NADPH oxidase activity is not just a symptom of atherosclerosis, but makes a major contribution to the pathogenesis of the disease. However, because p47phox appears to be an essential subunit of all NADPH oxidase isoforms, it is unclear from these studies which NADPH oxidase–endowed cells, and which NADPH oxidase isoforms, are most important in vascular remodelling and lesion development.

Several studies have addressed the vascular expression of NADPH oxidase subunits during atherogenesis, and they consistently demonstrate that although gp91phox is up-regulated, Nox4 expression remains relatively unchanged throughout lesion development. For example, we have shown that induction of neointimal lesions by periartrial collars in rabbits, which mimics early-stage human atheroma, causes an increase in NADPH oxidase–dependent superoxide production in both the endothelial and adventitial layers of the blood vessel wall (Figure 2). Increased superoxide production in this model was accompanied by marked up-regulation of gp91phox expression, with no change in expression of the Nox4 subunit (Paravicini et al. 2002). Likewise, development of atherosclerosis in aortas from ApoE−/− mice, which is associated with elevated endothelial superoxide production (Laursen et al. 2001; Jiang et al. 2003), is accompanied by an increase in gp91phox mRNA expression, with expression of Nox4 remaining unchanged (Drummond et al. 2001). In human atherosclerotic arteries, Sorescu et al. (2002) demonstrated a strong correlation between gp91phox expression and lesion severity, and the majority of gp91phox was associated with intimal macrophages. Gp91phox was also expressed, but to a lesser extent, in endothelial cells (Sorescu et al. 2002) and in a subset of α-actin–positive cells residing in the neointima (Kalinina et al. 2002). Whether these α-actin–positive cells are VSMCs or adventitial fibroblasts that migrated across the medial layer and into the developing intima remains to be determined. In contrast, Nox4 mRNA expression, which is markedly higher than that of all other gp91phox homologues in normal human arteries, remains relatively unchanged throughout most stages of atherosclerosis but is much lower in
FIG. 2. Localization of superoxide production in sections of carotid artery of a rabbit. Sections are control (left panels) or after 14 days with a periarterial collar (right panels), which has induced a neointima, and all are incubated with dihydroethidium. The lower panels were also incubated with tiron to remove superoxide. Note the highest superoxide signal is from the endothelium and outer layer of adventitia. (Reproduced, with permission, from Paravicini et al. 2002.)

the most advanced lesions (Sorescu et al. 2002). Nox4 is primarily expressed in medial smooth muscle cells underlying the intima as well as in a small population of \(\alpha\)-actin–positive cells surrounding the central core of the plaque (Sorescu et al. 2002; Kalinina et al. 2002).

Although it remains to be determined what roles Nox1 and Nox4 play in VSMC physiology/pathophysiology, studies on NIH3T3 fibroblasts may provide some clues. Overexpression of Nox4 cDNA in these cells retarded their growth and induced phenotypic changes characteristic of cellular senescence (Geiszt et al. 2000; Shiose et al. 2001). Conversely, transfection of NIH3T3 cells with Nox1 increased proliferation rate in a ROS-dependent manner (Suh et al. 1999). The reason for the opposing effects of Nox4 and Nox1 on cell growth is unclear. One possibility is that Nox1 and Nox4 are localized in separate cellular compartments such that the ROS they generate affects different signalling pathways.

As indicated above, there is considerable evidence that increased activity of NADPH oxidase is a contributor to many forms of experimental hypertension, both angiotensin dependent and independent (Griendling and FitzGerald 2003). It has been suggested that superoxide and hydrogen peroxide contribute to hypertension through different mechanisms: excess superoxide leads to endothelial dysfunction (perhaps partly by uncoupling eNOS) and hydrogen peroxide contributes to vascular remodelling (Griendling and FitzGerald 2003). Similarly in diabetes mellitus there is much evidence of increased vascular oxidative stress, endothelial dysfunction, and NADPH oxidase involvement. However, the real contribution of NADPH oxidase to the gamut of vascular consequences has not been elucidated.

Endothelial Dysfunction

A major hallmark of vascular disease of many forms is a defect in endothelium-dependent vasodilatation, often referred to as “endothelial dysfunction.” It is has been well established over more than a decade that impaired endothelium-dependent vasodilatation associated with vascular disease is almost always attributable to compromised bioavailability of endothelium-derived NO, and increased vascular superoxide production at least partly underlies this defect (Mugge et al. 1991; Dusting and Dart 1999). However it is important to note that endothelial dysfunction may be manifest in other ways, which may or may not derive from reduced NO or increased ROS generation. Leaving aside the issue of whether endothelial dysfunction is
a cause or an effect of chronic vascular disease, because this pharmacodynamic response can be measured in vivo in patients or animals, it is often used as a surrogate marker for the short-term benefits of classical antioxidants and other agents directed at relieving oxidant stress in the vasculature.

SUPPRESSION OF NADPH OXIDASE–DERIVED OXIDATIVE STRESS

Suppression of oxidant molecule generation by the NADPH oxidase system can be achieved by either removing its product (superoxide) or by a direct inhibition of its enzymatic activity. The former goal can be targeted by using antioxidant compounds, whereas the latter can be achieved by different strategies including pharmacological modulators of this enzyme and molecular tools. Although the clinical findings in trials of antioxidants have been disappointing in terms of outcomes in cardiovascular disease, it is premature to conclude that oxidant stress does not contribute to cardiovascular damage in these conditions, for reasons detailed below.

Antioxidant Vitamins

Antioxidant vitamins such as ascorbic acid (Vitamin C) and tocopherol (Vitamin E) are among the most widely used medicines in the population. Although there is evidence that acute administration of these vitamins results in reversal of endothelial dysfunction in conditions associated with increased oxidant stress, e.g., coronary artery disease and hyperlipidemia (Kugiyama et al. 1999; Erbs et al. 2003), a direct link between the observed endothelial protective effects and NADPH oxidase–derived oxidant stress has been addressed only recently. In vitro studies using isolated thoracic aortas from spontaneously hypertensive rats have consistently found endothelial dysfunction in that hypertensive aortas showed impaired relaxant responses to acetylcholine despite an increase in eNOS activity as compared to normotensive controls. Hypertensive aortas also showed a two-fold increase in NADPH oxidase activity and treatments with vitamin C and vitamin E reduced superoxide production and improved endothelial function (Ulker et al. 2003). In a guinea pig model of left ventricular hypertrophy, it has been demonstrated that the myocardial protein expression of gp91phox and p67phox and the NADPH oxidase activity were significantly increased in hypertrophic heart, and this contributed to the impaired left ventricular relaxation to endothelial NO. Short-term treatment with vitamin C restored the relaxant responses to the endothelial NO–releasing agonists bradykinin and substance P (MacCarthy et al. 2001). More interestingly, it has been shown that the vascular activities of NADPH oxidase in patients with coronary artery disease was increased as compared with healthy controls, and acute infusion of vitamin C improved flow-induced vasodilation, a measure of vascular endothelial function (Spiekermann et al. 2003). Although the authors concluded that xanthine oxidase might have a more prominent role than NADPH oxidase in the increased vascular oxidant stress and endothelial dysfunction observed in this study, taken together, these studies suggest that antioxidant vitamins, to some degree, may reduce NADPH oxidase–derived ROS and thereby preserve endothelial NO function.

Notwithstanding, the results of population studies and clinical trials on the long-term benefits of antioxidant vitamin treatment on major cardiovascular outcomes, such as ischemic stroke, progression of atherosclerotic plaques, and ischemic coronary events, are clearly disappointing (Stephens et al. 1996; Daviglus et al. 1997; Leppala et al. 2000; Heart Protection Study Collaborative Group 2002; Waters et al. 2002; Salonen et al. 2003). Likewise, the therapeutic effects of vitamins on hypertension, which is partly a result of increased vascular oxidative stress, are controversial (Palumbo et al. 2000; Boshtam et al. 2002; Darko et al. 2002; Kim et al. 2002). All these data indicate that reduction of NADPH oxidase–derived oxidants by antioxidant compounds and the subsequent improvement of endothelial function is not readily translated into clinical benefits.

There are several reasons why conventional antioxidants failed to improve outcomes in these studies (Griendling and FitzGerald 2003). Apart from factors in trial design, vitamins E and C are weak antioxidants and neither completely suppresses elevated biomarkers of oxidant stress in humans (Griendling and FitzGerald 2003). Much higher doses of these vitamins have been used in animal models than are administered in clinical trials. Moreover, the rate constant for the reaction of vitamin E with superoxide is 5 orders of magnitude slower than the rate of reaction with NO and superoxide dismutase (SOD) (Afanas’ev et al. 1989). A second mechanistic reason for their ineffectiveness may be that conventional superoxide scavengers have dismutation activity and convert superoxide to hydrogen peroxide. Hydrogen peroxide itself has direct regulatory effects on cellular pathophysiological processes such as stimulation of proinflammatory gene expression (Lo et al. 1993), and also facilitates the production of more harmful oxidant molecules such as hypochlorous acid and hydroxyl radicals. Third, such antioxidants may not reach appropriate cellular compartments to eliminate downstream effects of ROS, especially where ROS are produced intracellularly. These and other lines of evidence prompt the argument that a direct inhibition of NADPH oxidase at the enzyme level may produce greater therapeutic benefits than attempting to remove the enzymatic product (superoxide) after its generation.

Non-specific Superoxide Scavengers

The free radical spin trap reagents tiron (4,5-dihydroxy-1,3-benzenedisulfonic acid) (Ledenev et al. 1986) and tempol (4-hydroxy-2,2,6,6-tetramethylpiperidine 1-oxyl) (Weiss et al. 1993) have been tested in vitro as cell-permeable superoxide scavengers that target superoxide production by NADPH oxidase, and these have been shown to improve endothelial function (Gorlach et al. 2000; Adler et al. 2003; Didion and Faraci 2003). In most experimental studies these compounds have been used at concentrations in the millimolar range. Tempol has also been used in vivo in animal studies to reduce NADPH
oxidase–derived oxidant stress. For example, systemic administration of tempol has been shown to attenuate angiotensin II–induced acute renal vasoconstrictor and antinatriuretic actions by reducing NADPH oxidase–derived superoxide in a rat model, and to normalize hyperglycemia-induced oxidative stress and endothelial dysfunction in dog coronary arteries (Gross et al. 2003; Lopez et al. 2003). We have assayed the superoxide scavenging activity of tiron and tempol using a cell-free biochemical system comprising xanthine plus xanthine oxidase, and found that significant (>50%) superoxide scavenging activity was only observed at concentrations of >1 mM (Jiang et al. 2003). It is unlikely that systemic administration of these compounds can reach such high concentrations at the tissue level, and this makes it difficult to evaluate the specificity of the observed effects of tiron or tempol in vivo, so proper control of the local drug concentration (Lopez et al. 2003) is a necessity.

Other less specific antioxidants, including N-acetyl-L-cysteine (NAC) and probucol, have been used experimentally to target NADPH oxidase–derived ROS (Ushio-Fukai et al. 2002; Matsunaga et al. 2003; Nakagami et al. 2003). Probucol, notably, has been shown to produce some therapeutic benefits in oxidative stress–related cardiovascular conditions such as atherosclerosis and arterial restenosis (Lee et al. 1996; Sawayama et al. 2002). Moreover, NAC has been shown to reduce oxidative stress during acute myocardial infarction in humans (Sajkowska et al. 1999). However, direct measurement of an inhibitory effect of NAC or probucol on NADPH oxidase–dependent superoxide production has not been reported in human subjects, and there is some doubt about whether the protective effect of probucol in atherosclerosis is actually derived from an antioxidant action (Witting et al. 2000).

**Specific SOD Mimetics**

Dismutation by endogenous SOD is the major route for removing superoxide generated in the body. The therapeutic potential of native SOD enzyme is, however, restricted because of the limited stability and permeability of this large peptide. However, it has been demonstrated that polyethylene-glycolated SOD (Mugge et al. 1991), which has a longer half-life and higher cellular permeability than native SOD, and adenovirus-mediated SOD gene transfer (Arai et al. 1998; Zanetti et al. 2001; Chu et al. 2003), which induced intracellular SOD protein expression, are effective in reversing redox-sensitive cellular events, whereas native SOD had limited efficacy (Arai et al. 1998; Pueyo et al. 2000). Therefore, development of cell-permeable small-molecule SOD mimetics has been a focus of research, exemplified by the manganic porphyrins (e.g., MnTBAP, MnTMPyP) (Day et al. 1997), a manganese-bis(cyclohexyl)pyridine macrocyclic complex (M40403) (Salvemini et al. 1999), and synthetic salen-manganese complexes (e.g., EUK-8, EUK-134) (Bayne and Sohal 2002) (for structures see Figure 3).

We have characterized the effects of M40403 on NADPH oxidase–dependent superoxide generation in cultured VSMCs and aortic tissue (Jiang et al. 2003). M40403 was much more effective than native Cu/Zn SOD at reducing NADPH-stimulated superoxide either in intact smooth muscle cell or in cell homogenates, where the impermeability of SOD across cytoplasmic membrane could not explain the difference. M40403 was also capable of reversing endothelial dysfunction in ApoE-deficient aortas. Clearly, small-molecule SOD mimetics have potential therapeutic advantages over the native SOD enzyme. In other studies, some SOD mimetic compounds, such as MnTMPyP (Xiao et al. 2002) and EUK-8 (Wang et al. 1998), have also been used as experimental tools to investigate the role of NADPH oxidase. However, the real therapeutic efficacies of these synthetic SOD mimetics remain to be tested in appropriate animal models and clinical trials.

**PHARMACOLOGICAL INHIBITORS OF NADPH OXIDASE**

Pharmacological inhibitors of NADPH oxidase that directly block the catalytic activity of this enzyme have been identified, and these can be divided into 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. It was reported in the neutrophil 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 by the nonselectivity of DPI toward all flavoproteins, as exemplified by its potent inhibition of nitric oxide 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). Although the inhibitory effects of DPI and apocynin were initially characterized in the phagocytic NADPH oxidase, it is assumed that the same mechanisms are also operative in vascular cells. However, apocynin, in common with other polyphenolic derivatives, has multiple biological actions in addition to its antioxidant effects (Jiang and Dusting 2003). Recently, it has been reported that apocynin attenuated ischemia-reperfusion lung injury in sheep whereas DPI worsened the injury, although both compounds produced similar inhibition of NADPH oxidase (Dodd and 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. Moreover, these nonselective 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 direct inhibitory action against vascular NADPH oxidase has been reported for 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] (Cayatte et al. 2001). The authors measured superoxide production from TNF-stimulated human umbilical endothelial cells and NADPH oxidase activity in the membrane fraction from these cells, and found S17834 at 50 μM suppressed superoxide generation and NADPH oxidase activity by >50%, although the compound did not directly scavenge superoxide. Given in vivo, S17834 reduced TNF-stimulated vascular cell adhesion molecule-1 (VCAM-1), in-tercellular cell adhesion molecule-1 (ICAM-1), and E-selectin expression, and reduced aortic atherosclerosis by 60% in ApoE-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 assembled 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 (Gorzalczyzny et al. 2000). In vascular smooth muscle cells, we (Jiang et al. 2002) and others (Boota et al. 2000) have obtained evidence that treatment with inhibitors (GGTI-286 and GGTI-298, respectively) of protein geranylgeranyltransferase, which catalyzes the transfer of the geranylgeranyl group to target proteins (e.g., Rac), suppressed superoxide production by NADPH oxidase. These studies suggest that pharmacological intervention in the isoprenylation of small G-proteins may represent a novel strategy to suppress vascular NADPH oxidase activity. Indeed, this mechanism is known to contribute to the nonhypolipidemic effects 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 reactive oxygen 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 developed a novel competitive peptidoinhibitor of NADPH oxidase, 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 immunodefectorive 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. 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). These emerging data indicate the importance of NADPH oxidase in vascular remodeling of both large and small arteries.

As discussed above, 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 AT1 receptor antagonists) indirectly suppress the vascular NADPH oxidase system. It is interesting to speculate that the clearly demonstrated clinical benefits of these therapeutics 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. Although
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 inhibitors of the system that are under development. The shortcomings of the unsuccessful trials with antioxidants leave open the question of whether blocking the major source of ROS will have therapeutic benefits that cannot be achieved by attempting to inactivate superoxide.

REFERENCES


