REVIEW

NADPH oxidase and endothelial cell function

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ABSTRACT

Intracellular ROS (reactive oxygen species) such as superoxide and H$_2$O$_2$ have been increasingly appreciated to have a role in endothelial pathophysiology. Of the several sources within the vasculature, a family of multi-subunit NADPH oxidases appears to be a predominant contributor of endothelial superoxide. More importantly, this enzyme system is activated by numerous stimuli and is involved in triggering diverse intracellular signalling pathways (‘redox-sensitive’ signalling pathways) that have a central role in conditions such as endothelial activation and inflammation, cell growth, apoptosis and hypertrophy. Furthermore, NADPH oxidase-derived superoxide contributes to the impairment of endothelium-dependent vasodilatation by inactivating nitric oxide; the resultant endothelial dysfunction is implicated in the pathophysiology of diseases such as atherosclerosis, hypertension, diabetic vasculopathy and heart failure. A detailed understanding of the regulation of NADPH oxidases and their modulation and downstream effects may define novel therapeutic targets for cardiovascular disease prevention and treatment in the clinical setting, in contrast with global antioxidant therapy which has to date been disappointing.

INTRODUCTION

The endothelium is a single layer of cells that lines the inner surface of all blood vessels and the heart, forming an important interface with circulating blood. Gone are the days when this lining was thought to be simply a non-reactive mechanical barrier that allowed passive diffusion of biomolecules. This highly specialized dynamic tissue now has well-established roles in cardiovascular homeostasis, including autocrine, paracrine, endocrine and immunological functions, the initiation of the inflammatory process through increases in cell adhesiveness and permeability, and the maintenance of blood fluidity [1,2]. Furthermore, endothelial cells can sense changes in local blood-borne signals, haemodynamic forces and ambient $P_{O_2}$ (partial pressure of oxygen) and respond by appropriately modulating the equilibrium between potentially opposing processes such as: (i) vasodilatation and vaso-constriction, thereby regulating vascular tone, (ii) anti-thrombotic and pro-coagulant effects, thereby influencing haemostasis, and (iii) cell proliferation and apoptosis, thereby modulating cell growth and number in the vessel wall. These endothelial functions are mediated through the regulated production and release of paracrine mediators such as NO (nitric oxide), ET-1 (endothelin-1), prostacyclin and growth factors; through the activity of surface enzymes such as ACE (angiotensin-converting enzyme) and tPA (tissue plasminogen activator); and through the expression of surface proteins such as adhesion molecules. Endothelial dysfunction refers to a breakdown in these normal functions of the endothelium and is characterized by imbalance in one or more of the

Key words: endothelium, NADPH oxidase, nitric oxide, reactive oxygen species, redox signalling.

Abbreviations: ACE, angiotensin-converting enzyme; AGE, advanced glycation end-product; AT$_1$, angiotensin II type 1; CABG, coronary artery bypass graft; ET-1, endothelin-1; H$_4$B, tetrahydrobiopterin; LDL, low-density lipoprotein; MAPK, mitogen-activated protein kinase; MCP-1, monocyte chemotactic protein-1; NEFA, non-esterified fatty acid; NF-κB, nuclear factor κB; NO, nitric oxide; NOS, NO synthase; eNOS, endothelial NOS; Nox, NADPH oxidase; ox-LDL, oxidized LDL; O$_2^•−$, superoxide radical; ONOO$^{−−}$, peroxynitrite radical; PKC, protein kinase C; ROS, reactive oxygen species; SOD, superoxide dismutase; TNFα, tumour necrosis factor α; TRAF4, TNF-receptor-associated factor 4; VEGF, vascular endothelial growth factor; VSM, vascular smooth muscle.

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above processes [1]. Most commonly, the presence of impaired endothelium-dependent vasodilatation is taken as a marker of endothelial dysfunction, and is implicated in the pathophysiology of several cardiovascular disorders, including atherosclerosis, hypertension, heart failure and diabetes mellitus. Moreover, recent studies have confirmed that clinical endothelial dysfunction (impaired endothelium-dependent vasodilatation) is a powerful independent predictor of adverse cardiovascular outcomes in patients with ischaemic heart disease, hypertension or heart failure [3,4]. A more specific alteration in endothelial function that is also implicated in the pathophysiology of several conditions is endothelial activation, which refers to regulated changes in endothelial phenotype characterized by the expression of cell-surface adhesion molecules and other proteins involved in cell–cell interactions. Endothelial activation is important physiologically in the context of the inflammatory response as well as pathophysiologically in ischaemia/reperfusion, sepsis and early atherosclerosis [1,5].

In view of the importance of normal endothelial function in cardiovascular homeostasis and the involvement of endothelial dysfunction and activation in disease pathogenesis, the mechanisms underlying endothelial activation and the development of endothelial dysfunction are of great interest. In this regard, a large body of evidence indicates that the generation of ROS (reactive oxygen species) both within endothelial cells and in adjacent cells [e.g. VSM (vascular smooth muscle), adventitial fibroblasts and inflammatory cells] has a major role in the genesis of endothelial activation and dysfunction [5]. Furthermore, it is also becoming clear that ROS may have important roles in regulating the normal function of the endothelium. In this review, we briefly consider the main sources of ROS generation in endothelial cells and then focus on the role of NADPH oxidases, a relatively recently recognized ROS source that appears to be especially important with regard to the regulated production of ROS in endothelial cells.

GENERAL EFFECTS OF ROS

ROS include free radicals such as O$_2^•$− (superoxide), NO$^•$ (nitric oxide), ONOO$^{−}$− (peroxynitrite) and OH$^•$ (hydroxyl), and non-radicals such as H$_2$O$_2$ [6]. In this review, we focus mainly on O$_2^•$− and H$_2$O$_2$, which are the main species relevant to the current review. O$_2^•$− is formed by the univalent reduction of molecular oxygen, has a half-life of only a few seconds, and is rapidly converted into H$_2$O$_2$ in a process that is markedly accelerated by SOD (superoxide dismutase) isoenzymes. Whereas O$_2^•$− is generally restricted to its site of production due to its poor cell membrane permeability, H$_2$O$_2$ is more freely diffusible and therefore much more likely to exert more distant effects. O$_2^•$− can react with NO at a much faster rate than with SOD so that, when both O$_2^•$− and NO levels are in the high nanomolar range, the former reaction will generate ONOO$^{−}$− while at the same time inactivating NO. The levels of O$_2^•$− and other ROS within cells depend not only upon the rates of formation, but also on the activities of antioxidant systems designed to strictly regulate the redox state [5–7]. In particular, the three SOD isoenzymes (CuZnSOD, MnSOD and ecSOD) are pivotal in regulating O$_2^•$− levels, whereas glutathione peroxidase and catalase are the major antioxidants involved in H$_2$O$_2$ degradation [6,8].

ROS, classically, were regarded as accidental by-products of metabolism and were considered to be usually detrimental through their reaction with membranes, DNA, macromolecules and proteins [9]. This mechanism is recognized to be important in some disease settings, for example reperfusion injury, where the generation of more toxic radicals such as OH$^•$ downstream of O$_2^•$− and H$_2$O$_2$ is implicated. However, more recently, it has been recognized that ROS (notably, O$_2^•$−, H$_2$O$_2$ and NO) can exert much more subtle effects on cellular function, acting as (patho)physiological regulators of intracellular signalling cascades [5,6]. Such redox signalling leading to alterations in gene transcription and protein activities (and therefore cell phenotype) is implicated in the pathophysiology of many diseases. A third general mode of action of O$_2^•$− relevant to endothelial biology is through its rapid inactivation of NO, thereby causing endothelial dysfunction.

ENDOTHELIAL SOURCES OF O$_2^•$− AND H$_2$O$_2$

Several potential sources of O$_2^•$− are implicated in endothelial physiology and pathophysiology, including the mitochondrial electron transport chain, xanthine oxidase, cytochrome P-450 enzymes, uncoupled NOSs (NO synthases), the phagocytic myeloperoxidase system and NADPH oxidases (for comprehensive reviews, see [1,5,10–12]). Although the present review focuses on NADPH oxidases, a brief discussion of some of the other sources is included as they may potentially be modulated by NADPH-oxidase-derived ROS (Figure 1).

The mitochondrial electron transport chain can be a significant ROS source and increased mitochondrial ROS generation is implicated in diabetic vasculopathy and in ischaemia/reperfusion. Interestingly, mitochondria are quite susceptible to oxidative damage which can result in enhanced mitochondrial ROS production [11]. Xanthine oxidase is expressed on the luminal surface of the endothelium in many organs and catalyses the conversion of hypoxanthine into urate in a process that generates O$_2^•$−. The enzyme is normally present as xanthine dehydrogenase, which does not generate O$_2^•$−, but is converted into xanthine oxidase either through oxidation or by proteolytic cleavage of a segment of xanthine dehydrogenase.
Figure 1 Interplay between NADPH oxidase and other sources of ROS

$O_2^-$ generated from NADPH oxidase can potentially influence ROS production by other enzymatic sources of $O_2^-$. For example, xanthine dehydrogenase is converted into $O_2^-$-generating xanthine oxidase through oxidation, which may result in augmentation of $O_2^-$ production. Similarly, mitochondria are susceptible to oxidative damage which can result in enhanced mitochondrial ROS production. Finally, $O_2^-$ or ONOO$^-$ can degrade the essential NOS cofactor H$_4$B and thereby promote NOS uncoupling, which leads to $O_2^-$ generation by the enzyme.

The former mode of activation is notable in that it provides a mechanism for an increase in xanthine oxidase activity in response to oxidative stress, i.e. a mechanism that may potentially amplify oxidative stress originally arising from other sources. Increased xanthine-oxidase-derived $O_2^-$ production may be involved in ischaemia/reperfusion and in endothelial dysfunction in several diseases [12].

NO synthases normally generate NO, but can become ‘uncoupled’, usually in the context of deficiency of the essential NOS cofactor H$_4$B (tetrahydrobiopterin) [1]. When this occurs, eNOS (endothelial NOS) becomes a significant source of $O_2^-$. ROS generation by uncoupled eNOS is reportedly involved in the pathophysiology of diabetic vasculopathy, atherosclerosis, hypertension and hypercholesterolaemia. Of interest, H$_4$B is highly susceptible to oxidative degradation by $O_2^-$ or ONOO$^-$; thus, initial degradation of H$_4$B by ROS derived from another source can induce NOS uncoupling and amplification of oxidative stress [13,14]. In experimental hypertension, it has been convincingly demonstrated that initial ROS generation by NADPH oxidase leads to eNOS uncoupling and amplification of ROS production [14].

The above discussion illustrates how there may be complex interactions among different sources of ROS. Accordingly, it is increasingly clear that, rather than an individual enzymatic source being the only one implicated in a particular disease setting, in many cases there may be involvement of multiple sources. Nevertheless, the NADPH oxidases may be considered as rather special sources of ROS generation in that they appear to be the only enzymes whose primary function is regulated ROS production. Furthermore, they appear to be especially important with regard to redox signalling.

NADPH OXIDASE STRUCTURE AND ISOFORMS

The NADPH oxidase complex was originally identified and characterized in phagocytes, where it plays an essential role in non-specific host defence against microbial organisms [15]. The phagocytic enzyme is normally quiescent, but becomes activated during the neutrophil oxidative burst to generate large amounts of $O_2^-$. The classical neutrophil NADPH oxidase comprises a catalytic subunit, gp91phox (91 kDa apparent molecular mass on gels when glycosylated; where phox is phagocyte oxidase), which in conjunction with a p22phox subunit forms a membrane-bound flavocytochrome $b_{558}$. A number of cytosolic regulatory subunits are required for activation of the enzyme, namely p67phox, p47phox, p40phox and the small GTPase Rac2. Upon enzyme activation, these are translocated to and assembled with the membrane cytochrome in a highly regulated process that involves post-translational modification of several of the cytosolic subunits and specific protein–protein binding through tandem SH3 (Src homology 3) domains. In the activated enzyme complex, the flavin-containing catalytic subunit functions as...
The classical NADPH oxidase comprises a membrane-bound gp91phox/p22phox heterodimer and other subunits (p67phox, p47phox, p40phox and Rac) which associate with this complex in the activated enzyme. The NADPH-binding domain is predicted to be on one side of the membrane, whereas O$_2^•^-$ generation is predicted to occur on the other [15,16]. The oxidase may be located either on the plasma membrane or on intracellular membranes depending upon the cell type.

Table 1  NADPH oxidase (Nox) homologues

<table>
<thead>
<tr>
<th>Binding partners</th>
<th>Tissue distribution</th>
<th>Homology with Rac</th>
<th>References</th>
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<tr>
<td></td>
<td></td>
<td>p22phox</td>
<td>p47phox</td>
</tr>
<tr>
<td>Nox 1 (mox 1)</td>
<td>Colon, VSM, uterus and prostate.</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Nox 2</td>
<td>Phagocyte, endothelium, cardiomyocytes,</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td></td>
<td>VSM (?) and lung.</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Nox 3</td>
<td>Inner ear, kidney, liver, lung and spleen.</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Nox 4 (Renox)</td>
<td>Kidney, VSM, endothelium, cardiomyocytes,</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td></td>
<td>bone, ovary, eye, placenta and skeletal muscle.</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Nox 5</td>
<td>Lymphoid tissue, testis, prostate, breast and brain.</td>
<td>✓</td>
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</table>

Despite a relatively high degree of conservation in the overall topology of the Noxs, studies to date indicate that they differ greatly in their tissue distribution and are also likely to be differentially activated and regulated (Table 1) [24–30]. It is thought that all the Nox homologues may bind to p22phox in a similar manner to the gp91phox/p22phox complex, although this needs to be definitively demonstrated [23]. However, the requirement for other cytosolic subunits for an active oxidase complex may vary among the Noxs. In the case of Nox1, homologues of p47phox and p67phox (termed NOXO1 and NOXA1 respectively) have been found to be important for its activation [26,31]. Interestingly, NOXO1 may exhibit significant differences in function compared with p47phox. On the other hand, it is reported that Nox4 activation does not require either p47phox and p67phox or NOXO1 and NOXA1 [23,27]. The detailed mechanisms...
responsible for the activation and regulation of these homologues remain to be established.

**ENDOTHELIAL NADPH OXIDASE STRUCTURE**

Early studies from our laboratory and others showed that all the classical NADPH oxidase subunits are expressed in endothelial cells both at the mRNA and protein levels [19,20,32,33]. Functionally, however, there are significant differences between the phagocyte oxidase and the enzyme in endothelial cells (as well as other non-phagocytic cells). Firstly, the endothelial oxidase continuously generates comparatively small amounts of \( \text{O}_2^- \) even in unstimulated cells, but its activity can be augmented by specific agonists (although not to the same levels as in neutrophils). The reduced activity is reflected by the finding of considerably lower levels of mRNA encoding neutrophil \( \text{O}_2^- \) than being plasma membrane-bound as in neutrophils [17]. Secondly, a large proportion of the \( \text{O}_2^- \) generated in endothelial cells is intracellular, whereas neutrophil \( \text{O}_2^- \) generation is mainly in the extracellular compartment.

In unstimulated cultured endothelial cells, studies from our laboratory found, using multiple complementary approaches, that a substantial proportion of the Nox2-based oxidase was predominantly located in a perinuclear distribution in association with the cytoskeleton, rather than being plasma membrane-bound as in neutrophils [34]. Furthermore, there was evidence for the presence of many fully pre-assembled ROS-generating oxidase complexes. These results can explain both the continuous low-level \( \text{O}_2^- \) generation found in unstimulated endothelium as well as the intracellular site of ROS production. However, endothelial cells also express substantial amounts of Nox4 (indeed, greater than Nox2 levels) and it has been suggested that this may contribute to the basal constitutive \( \text{O}_2^- \) generation discussed above [35]. The relative roles of these two isoforms in endothelial cells remain to be elucidated; most of the information on regulation of the endothelial oxidase discussed below probably relates to Nox2.

**REGULATION OF THE ENDOTHELIAL NADPH OXIDASE AND REDOX SIGNALLING**

An important reason why interest in the endothelial (and more generally vascular) NADPH oxidase has grown in the last few years is the finding that enzyme activity is sensitively regulated by a wide range of (patho)physiologically relevant factors [17]. Stimuli found to increase endothelial NADPH oxidase activity include (i) agonists of G-protein-coupled receptors such as angiotensin II and ET-1 [36–38 ]; (ii) growth factors such as thrombin and VEGF (vascular endothelial growth factor) [39 ]; (iii) cytokines such as TNF\( \alpha \) (tumour necrosis factor \( \alpha \) ) [40,41 ]; (iv) metabolic factors such as increased glucose, insulin, NEFAs (non-esterified fatty acids) or AGEs (advanced glycation end-products) [42–44 ]; (v) oxidized lipids [45 ]; (vi) oscillatory shear stress [46 ]; and (viii) hypoxia/reoxygenation and nutrient deprivation [47,48 ] (Table 2). The two general mechanisms underlying an increase in oxidase activity are either an acute increase in oxidase complex formation secondary to post-translational modification of regulatory subunits (p47phox and Rac1) or a chronic increase in the expression and abundance of component subunits. Studies from several laboratories, including ours, have begun to define the pathways responsible for acute activation of the endothelial oxidase.

<table>
<thead>
<tr>
<th>Activators of NADPH oxidase</th>
<th>Regulatory subunit involved</th>
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<tbody>
<tr>
<td>G-protein-coupled-receptor agonist</td>
<td>p47phox</td>
</tr>
<tr>
<td>Angiotensin II</td>
<td>✔</td>
</tr>
<tr>
<td>ET-1</td>
<td>✔</td>
</tr>
<tr>
<td>Growth factors</td>
<td>p47phox</td>
</tr>
<tr>
<td>Thrombin</td>
<td>✔</td>
</tr>
<tr>
<td>VEGF</td>
<td>✔</td>
</tr>
<tr>
<td>Cytokines</td>
<td>p47phox</td>
</tr>
<tr>
<td>TNF( \alpha )</td>
<td>✔</td>
</tr>
<tr>
<td>Metabolic factors</td>
<td>p47phox</td>
</tr>
<tr>
<td>ox-LDL</td>
<td>✔</td>
</tr>
<tr>
<td>NEFAs</td>
<td>✔</td>
</tr>
<tr>
<td>Glucose</td>
<td>✔</td>
</tr>
<tr>
<td>Insulin</td>
<td>✔</td>
</tr>
<tr>
<td>AGEs</td>
<td>✔</td>
</tr>
<tr>
<td>Mechanical factors</td>
<td>Rac</td>
</tr>
<tr>
<td>Shear stress</td>
<td>✔</td>
</tr>
<tr>
<td>Flow cessation</td>
<td>✔</td>
</tr>
<tr>
<td>Nutrient deprivation</td>
<td>✔</td>
</tr>
<tr>
<td>Hypoxia re-oxygenation</td>
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</table>

A major mechanism that is involved is the PKC (protein kinase C)-dependent phosphorylation of the p47phox regulatory subunit and its translocation to the Nox2/p22phox heterodimer to form more fully assembled complexes, just as in neutrophils. A requirement for p47phox phosphorylation has been convincingly demonstrated for acute activation by angiotensin II, phorbol esters, TNF\( \alpha \), VEGF and oscillatory shear, using multiple specific molecular approaches such as the use of p47phox-deficient cells and p47phox transfection [36,40,41,49]. Interestingly, p47phox can also bind to other signalling molecules and potentially participate in the formation of signalling complexes. In this regard, we have recently shown that TNF\( \alpha \)-induced endothelial oxidase activation and downstream phosphorylation of ERK1/2 (extracellular-signal-regulated kinase 1/2) involves not only the
phosphorylation of p47phox, but also its binding to TRAF4 (TNF-receptor-associated factor 4) [50]. TRAF4/p47phox binding in this case may serve to spatially confine the ROS-mediated signal and hence selectively activate a subfamily of MAPKs (mitogen-activated protein kinases). The role of p47phox in the endothelial oxidase is probably a bit more complex since, interestingly, depletion of p47phox results in an increase in basal oxidase activity [36,41]. Consistent with this finding, vascular rings from p47phox-deficient mice had evidence of mild endothelial dysfunction which was normalized by O2•− scavengers [51]. One interpretation of this finding would be that unphosphorylated p47phox may exert inhibitory effects on basal oxidase activity, which are relieved upon its phosphorylation (which at the same time increases oxidase activity).

Many studies have also demonstrated an important role for the small GTPase Rac1 in endothelial NADPH oxidase activation [52]. Activated Rac in its GTP-bound state is thought to bind to the cytosolic p67phox subunit and activate the oxidase. Activation of the oxidase by Rac1 is therapeutically relevant because Rac1 activation requires its post-translational modification by isoprenylation, a process that is inhibited by HMG-CoA (3-hydroxy-3-methylglutaryl-CoA) reductase inhibitors (statins). Basal oxidase activity in endothelial cells is inhibited by a dominant-negative Rac mutant as is oxidase activation by shear stress, hypoxia/re-oxygenation, depolarization, nutrient deprivation or VEGF [53]. The relative roles of p47phox phosphorylation versus Rac1 activation in the stimulation of the endothelial enzyme in vivo remain unclear.

The ROS-dependent modulation of signal transduction pathways is the example par excellence of an effect of oxidative stress where NADPH oxidase appears to be especially important. Efficient signal transduction through such a pathway depends upon both the ligand-specific stimulation of oxidase activation (as discussed above) and the specific interactions of the ROS so generated with individual downstream targets. The precise mechanisms involved in imparting this specificity remain the subject of intense investigation. In general, many such effects are probably mediated by H2O2, rather than O2•−, with the targets including the activity of protein phosphatases and kinases, and transcription factors such as NF-κB (nuclear factor κB) [5,6]. Activation of the endothelial NADPH oxidase has been shown to be required for MAPK activation by many agonists such as angiotensin II and TNFα [51]; the redox-dependent expression of cell-surface adhesion molecules and MCP-1 (monocyte chemoattractant protein-1) by cytokines, oscillatory shear stress or hypercholesterolaemia through a mechanism involving NF-κB [54,55]; and redox-dependent alterations in the expression of a large number of genes [17]. NADPH-oxidase-dependent redox signalling is also likely to account for its involvement in many of the disease processes discussed in the subsequent sections of this review.

INvolvement in endothelial activation

Endothelial activation is essential for the inflammatory response in which the recruitment of leucocytes and their adhesion and emigration into the affected tissues requires the regulated expression of adhesion molecules and other proteins such as MCP-1. A large body of data implicate ROS production as being involved in the regulation of expression of adhesion molecules such as ICAM-1 (intercellular cell-adhesion molecule-1), VCAM-1 (vascular cell adhesion molecule-1) and selectins on endothelial cells in response to stimuli, including cytokines, lipopolysaccharide (endotoxin), hypercholesterolaemia and oscillatory shear stress [56]. NADPH oxidases appear to be important sources of these ROS based on experiments with pharmacological inhibitors of these enzymes, studies using dominant-negative Rac1 mutants, and experiments in NADPH-oxidase-deficient mice [57–59]. In the in vivo setting, studies using bone marrow chimaeras to dissect out the roles of circulating inflammatory cells compared with resident vascular cells have clearly shown that both types of cell are important for the NADPH-oxidase-dependent responses [58]. NADPH-oxidase-dependent expression of endothelial adhesion molecules is also reported to occur in response to angiotensin II and AGE, whereas, in the context of ischaemia/reperfusion, xanthine-oxidase-derived ROS may be more important [12,44,59].

With regard to early atherogenesis, the endothelial expression of adhesion molecules and MCP-1 is a key step in the process of monocyte adhesion and emigration to form macrophages and then foam cells. Atherosclerotic levels of LDL (low-density lipoprotein) stimulate NADPH-oxidase-generated O2•− [45] which, in turn, may contribute to LDL oxidation; ox-LDL (oxidized LDL) acts as an especially potent stimulus for oxidase activation, both in the vasculature and in macrophages, thereby accentuating the process. A role for AT1 (angiotensin II type 1)-receptor-driven increases in NADPH oxidase activity has also been reported [60]. It is therefore clear that oxidase-derived ROS may be involved at multiple levels; indeed, studies in p47phox−/− mice crossed on to an ApoE (apolipoprotein E)−/− background have shown that NADPH oxidase deficiency retards the development of atherosclerotic lesions in the mouse aorta [61].

Role in endothelial proliferation, migration and angiogenesis

Recent studies suggest that NADPH-oxidase-derived ROS may be important in the signal transduction of
processes involved in angiogenesis. Early studies found that the proliferation and migration of cultured endothelial cells in response to angiotensin II, ox-LDL, hypoxia or VEGF required NADPH-oxidase-derived ROS [62]. Several components of the cellular processes required for cell migration appear to require ROS, e.g., cytoskeleton reorganization and cell polarization. More direct evidence for an involvement in angiogenesis was provided by Ushio-Fukai et al. [39], who found that angiogenesis induced by VEGF in an in vivo sponge implant model was markedly inhibited in Nox2-deficient mice.

NADPH-oxidase-derived ROS may also be involved in apoptosis (programmed cell death) or in a special form of programmed cell death associated with cell detachment from the extracellular matrix, termed anoikis. Ox-LDL and high-glucose-induced NADPH oxidase activation promoted endothelial cell apoptosis [63], whereas anoikis associated with growth factor deprivation also involves ROS generation [64].

**NADPH OXIDASE AND IMPAIRED ENDOTHELIUM-DEPENDENT VASODILATION**

As outlined in the Introduction, an increase in oxidative stress appears to be a major mechanism underlying the development of vascular endothelial dysfunction in a wide range of cardiovascular diseases (although other mechanisms such as reduced eNOS expression or activity may also contribute) [1]. In the last decade, numerous studies have found that major sources of ROS responsible for this increased oxidative stress are vascular NADPH oxidases. It is important to emphasize that, in many (if not most) cases, the increased NADPH oxidase activity emanates from several different cell types within the vessel wall, i.e. endothelial cells, VSM, adventitial fibroblasts and/or infiltrating inflammatory cells [17]. One important question yet to be answered is whether the generation of ROS by different NADPH oxidase isoforms in these different cell types is a factor that can be used to therapeutic advantage. For example, Nox2-based ROS production is of major importance in the endothelium and adventitia, whereas Nox1 and/or Nox4 may be much more important in VSM. In this review, we focus mainly on the effects of NADPH oxidases on the endothelium; the roles of NADPH oxidases on the rest of the vessel wall (e.g. in atherogenesis and VSM hypertrophy) have been addressed by excellent recent reviews [17,65,66].

The traditional risk factors for the development of atherosclerotic disease, namely dyslipidaemia, hypertension, diabetes mellitus and cigarette smoking, are all associated with endothelial dysfunction that is, at least in part, attributable to increased ROS production [1]. Several experimental studies have found that each of these factors is capable of increasing NADPH oxidase activity and thereby impairing endothelium-dependent (NO-dependent) vasodilatation [5,17,60,67]. With regard to hypertension, this has been shown to be the case not only for angiotensin-dependent hypertension, but also for genetic hypertension [68], renovascular hypertension and low renin [DOCA (deoxycorticosterone acetate)-salt] hypertension [14]. In the latter case, ET-1 is reported to be an important pathogenic factor responsible for the increased oxidase activity [69].

In the clinical setting, studies in vascular tissue from patients undergoing CABG (coronary artery bypass graft) surgery confirmed that NADPH-oxidase-derived ROS were a major source of ROS contributing to endothelial dysfunction [70]. Furthermore, ROS production was shown to increase in proportion to risk factor load in this study. Interestingly, in another study that used vascular tissue from patients with coronary artery disease, increased ROS generation was shown to emanate not only from NADPH oxidase, but also xanthine oxidase, re-emphasizing the point that multiple ROS sources may often be involved, most likely through direct ROS–ROS interactions as discussed earlier [71]. Likewise, ROS production by uncoupled NOS may also be involved in some of these settings.

Diabetes appears to be an especially potent stimulus for NADPH oxidase activation leading to endothelial dysfunction, both experimentally and in patients. In a model of streptozotocin-induced Type I diabetes in rats, both NADPH oxidase and uncoupled NOS were found to contribute to endothelial dysfunction, with the increased oxidase activity being attributable to enhanced PKC activation and increased Nox2 expression [67]. Similar data have been reported in other experimental models of diabetes. In vessels from diabetic patients undergoing CABG, NADPH oxidase activity and subunit expression were also significantly increased compared with matched non-diabetics, again at least partly driven by PKC [72]. Multiple factors may be responsible for the increased NADPH oxidase activation in diabetes, including hypercholesterolaemia, hyperglycaemia, elevated NEFAs, hyperinsulinaemia, increased AGEs and activation of the renin–angiotensin system [5,17].

**HEART FAILURE**

Vascular endothelial dysfunction is an important feature of heart failure which contributes to increased peripheral vascular resistance in this condition. Oxidative stress appears to be a major underlying mechanism; for example, clinical studies have shown that acute infusion of the antioxidant vitamin C improved endothelial function in subjects with heart failure [73]. In experimental models of heart failure induced by coronary ligation, NADPH-oxidase-derived ROS have been shown to be a major
source of the ROS responsible for endothelial dysfunction [74]. Interestingly, endothelial NADPH-oxidase-derived ROS may also impact on myocardial contractile function through inactivation of NO, as suggested by studies from our laboratory in an experimental model of pressure overload left ventricular hypertrophy [75,76]. Recent studies clearly demonstrate that increased NADPH oxidase activity is of wider pathogenic significance in heart failure in that increased activity is involved also in cardiac myocyte hypertrophy and interstitial fibrosis [22,77]. Furthermore, evidence of increased myocardial NADPH oxidase activity has been confirmed in human heart failure [78,79].

**CONCLUSIONS AND THERAPEUTIC CONSIDERATIONS**

The importance of oxidative stress in the development of endothelial activation and dysfunction is now well recognized, as is the long-term significance for future cardiovascular morbidity and mortality. However, this knowledge has so far not resulted in the introduction of new therapies. Clinical trials that have tested various antioxidants (e.g. vitamin C, vitamin E and the carotenoids) for the prevention of cardiovascular end points have generally been unsuccessful [80,81]. With a better understanding of the roles that oxidative stress may play in disease pathogenesis, it seems likely that a therapeutic approach based on blanket scavenging of free radicals is probably flawed. Instead, it may be more appropriate to focus on the inhibition of specific ROS-generating enzymes. The NADPH oxidases may be especially important in this regard in view of their highly specific regulation and involvement in ROS production. It is of interest that several classes of proven existing therapeutic agents may have significant antioxidant actions through the inhibition of NADPH oxidase. For example, ACE inhibitors and AT1-receptor blockers both efficiently reduce angiotensin II-dependent activation of NADPH oxidase. For example, ACE inhibitors and AT1-receptor blockers both efficiently reduce angiotensin II-dependent activation of NADPH oxidase. Similarly, statins can also potently inhibit NADPH oxidase via their disruption of Rac activation. Nevertheless, the complexity of roles played by ROS and the highly specific regulation of the NADPH oxidases suggest that there may be significant potential for the development of novel therapies that target redox pathways in cell-, tissue- and pathway-specific manners at appropriate time points in the disease process. Further studies to dissect out detailed mechanisms of action of enzymes such as the NADPH oxidases in redox signalling will be invaluable in this aim.

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