Redox signalling involving NADPH oxidase-derived reactive oxygen species

R. Dworakowski, N. Anilkumar, M. Zhang and A.M. Shah

Cardiovascular Division, Department of Cardiology, King’s College London School of Medicine, Bessemer Road, London SE5 9PJ, U.K.

Abstract

Increased oxidative stress plays an important role in the pathophysiology of many diseases such as atherosclerosis, diabetes mellitus, myocardial infarction and heart failure. In addition to the well-known damaging effects of oxygen-free radicals, ROS (reactive oxygen species) also have signalling roles, acting as second messengers that modulate the activity of diverse intracellular signalling pathways and transcription factors, thereby inducing changes in cell phenotype. NADPH oxidases appear to be especially important sources of ROS involved in redox signalling. Seven NADPH oxidase isoforms, known as Noxs (NADPH oxidases), are expressed in a cell- and tissue-specific fashion. These oxidases are thought to subserve distinct functions as a result of their tightly regulated activation (e.g. by neurohormonal and growth factors and mechanical stimuli) and their specific coupling with distinct downstream signalling pathways. In the present paper, we review the structure and mechanisms of activation of NADPH oxidases and consider their involvement in redox signalling, focusing mainly on the cardiovascular system.

Introduction

Reactive oxygen species (ROS) are characterized by high chemical reactivity and have emerged as important in many pathophysiological processes. ROS include free radicals, such as superoxide (O$_2^-$) and hydroxyl (OH), and non-radical species such as H$_2$O$_2$. Their biological effects depend upon the specific moiety generated, its localization and the relative balance between levels generated and the activity of antioxidant systems that reduce ROS levels. Three general types of ROS-dependent biological action may be considered. First, in settings of redox imbalance (oxidative stress) where large amounts of radicals are generated, these may induce oxidation and damage of macromolecules, membranes and DNA and thus be detrimental for cellular function and viability. Secondly, the ROS superoxide interacts with the signalling molecule NO (nitric oxide), resulting in a reduction in NO bioavailability and the generation of another reactive species, peroxynitrite, which itself has biological activity. ROS-mediated reduction in NO bioavailability is a key mechanism that contributes to the development of endothelial dysfunction. Thirdly, it is recognized that tightly regulated ROS production modulates the activity of diverse intracellular molecules and signalling pathways, thereby inducing highly specific acute and chronic changes in cell phenotype (commonly termed ‘redox signalling’). In most cases, the main ROS involved in redox signalling is probably H$_2$O$_2$, which is generated upon dismutation of superoxide (a process catalysed by superoxide dismutases).

There are several potential sources of ROS in most cells, including the mitochondria, NADPH oxidases, cytochrome P450-based enzymes, xanthine oxidase and uncoupled NO synthases. Among these, the NADPH oxidases appear to be especially important for redox signalling and indeed possess several biochemical properties that make them well suited for involvement in signal transduction.

NADPH oxidase isoforms

The NADPH oxidase enzyme complex was first described in neutrophils where it is normally quiescent but generates a large quantity of superoxide upon activation during phagocytosis and plays a vital role in non-specific host defence against ingested pathogens [1]. The phagocytic oxidase comprises a membrane-associated cytochrome b$_{558}$, composed of one p22$_{phox}$ subunit and one gp91$_{phox}$ subunit. Enzyme activation requires the translocation of several regulatory subunits (p47$_{phox}$, p67$_{phox}$, p40$_{phox}$ and Rac) to the membrane where they associate with cytochrome b$_{558}$ and facilitate the transfer of electrons from NADPH via FAD and two haem moieties to molecular oxygen, resulting in superoxide formation (Figure 1).

In the last 18 years, many non-phagocytic cells have been found to contain NADPH oxidase type ROS-generating
activity. In the cardiovascular system, these include VSMCs (vascular smooth-muscle cells) [2], endothelial cells [3], adventitial and cardiac fibroblasts [4] and cardiomyocytes [5]. These cells usually constitutively generate low-level NADPH-dependent ROS but production is significantly augmented by various specific stimuli. Recently, it has been shown that there is in fact a family of non-phagocytic NADPH oxidases based on seven different isoforms of gp91<sub>phox</sub> or Nox (for NADPH oxidase), each encoded by different genes [1,6]. The classical gp91<sub>phox</sub> isoform is known as Nox2 in this new terminology. The Nox family may be classified into three groups based on predicted domain structures (Figure 2): (i) Nox1–Nox4 have up to 60% homology and are predicted to contain six transmembrane α-helices and an NADPH-binding domain towards the C-terminus; (ii) Nox5 has the same basic structure as Nox1–Nox4 but with an additional N-terminal calmodulin-like Ca<sup>2+</sup>-binding domain; (iii) Duox1 and Duox2 are similar to Nox5 but include an additional N-terminal peroxidase homology domain [1].

Nox isoform expression varies in a cell-specific manner [5,7–10]. Nox1 is expressed in many epithelia (e.g. colon) as well as in VSMCs. Nox2 is expressed in endothelial cells, cardiomyocytes and fibroblasts in addition to its classical expression in phagocytes. Nox3 is primarily expressed in foetal tissues and adult inner ear. Nox4 was first identified in kidney but is in fact widely expressed in many tissues including placentae, endothelial cells, VSMCs, cardiomyocytes, fibroblasts, ovary, testis and skeletal muscle. Nox5 is expressed in foetal
tissues and adult testis, spleen, ovary, placenta and pancreas. It is of interest that several cell types can co-express more than one Nox subunit. For example, cultured VSMCs express both Nox1 and Nox4 [11], while endothelial cells [9] and cardiomyocytes [10,12] co-express Nox2 and Nox4.

**Activation mechanisms of non-phagocytic NADPH oxidases**

NADPH oxidase activity in non-phagocytic cells, such as cardiovascular cells, is acutely increased by diverse pathophysiological stimuli including: (i) G-protein-coupled receptor agonists, e.g. angiotensin II and endothelin-1; (ii) cytokines, e.g. TNFα (tumour necrosis factor α) and TGFβ (transforming growth factor β); (iii) growth factors, e.g. thrombin, VEGF (vascular endothelial growth factor) and insulin; (iv) ‘metabolic’ factors, e.g. oxidized low-density lipoprotein, non-esterified (‘free’) fatty acids and glycated proteins; (v) hypoxia-reoxygenation or ischaemia-reperfusion; and (vi) mechanical stimuli, e.g. oscillatory shear [13].

The molecular events at the level of the oxidase that are involved in its acute activation in cardiovascular cells are best characterized for the classical Nox2-containing oxidase and Nox1. In general, Nox2 oxidase activation in these cells involves a similar process to that in neutrophils, namely the association of cytosolic oxidase components (p47^phox, p67^phox and Rac1) with cytochrome b^558. Binding of p67^phox to an activation site on Nox2 initiates the electron transfer process but the key post-translational modifications involved in oxidase activation are the phosphorylation of p47^phox and Rac activation [1]. Phosphorylation of p47^phox allows its interaction with p22^phox and facilitates the binding of p67^phox to cytochrome b^558.

In endothelial cells, p47^phox phosphorylation and binding to the cytochrome have been shown to be involved in oxidase activation in response to angiotensin II, TNFα, VEGF and oscillatory shear stress [14–16]; similar results are available in other cell types. PKC (protein kinase C) isoforms are believed to be the major kinases responsible for p47^phox phosphorylation although other kinases such as Akt (or protein kinase B), p38 MAPK (mitogen-activated protein kinase) and PAK (p21-activated kinase) may also play a role depending on the stimulus [1,13,17]. Translocation and activation of Rac require its geranylgeranyl modification and conversion from a GDP- into GTP-bound state, catalysed by guanine nucleotide-exchange factors. Rac translocation is implicated in NADPH oxidase activation in response to altered shear stress [18], VEGF [19], TNFα [20] and ischaemia-reperfusion [21]. The agonist-stimulated signalling events that lead to p47^phox phosphorylation and Rac activation and thus oxidase activation in cardiovascular cells have been very well studied for some agonists (e.g. angiotensin II) and have been reviewed in [22].

Recently, isoforms of p47^phox and p67^phox were discovered. These were termed NoxO1 (for Nox organizer 1) and NoxA1 (for Nox activator 1), which substitute for p47^phox and p67^phox respectively [1]. An important difference between p47^phox and NoxO1 is that the latter lacks the p47^phox domain that is regulated by phosphorylation; therefore NoxO1 may influence oxidase activity quite differently from p47^phox. In colon epithelial cells, Nox1 oxidase activity requires NoxO1 and NoxA1 instead of p47^phox and p67^phox. However, it remains unclear whether these analogues influence Nox1 activity in VSMCs. Pending such a demonstration, it would appear that the general mode of activation of Nox1 in VSMCs may be quite similar to that described for Nox2.

In contrast with Nox1 and Nox2, activation mechanisms for the Nox4-based oxidase are poorly defined. Several studies indicate that Nox4 does not require either p47^phox, p67^phox (or analogues) or Rac for its activity, suggesting that its mode of activation may be significantly different [23,24].

**Cardiovascular redox signalling involving NADPH oxidases**

ROS may influence signal transduction pathways in several ways: (i) through changes in the activity of redox-sensitive protein kinases [such as members of the MAPK family, Akt, PKC, PKD and JAK (Janus kinase)] either indirectly via inactivation of tyrosine phosphatases or in some cases direct activation; (ii) by altering the activity of redox-sensitive transcription factors [e.g. AP-1 (activator protein 1), NF-κB (nuclear factor κB), HIF-1 (hypoxia-inducible factor 1) and STAT (signal transducer and activator of transcription)] either directly or secondary to altered activity of upstream kinases; (iii) via changes in activity of redox-sensitive molecules such as thioredoxin; and (iv) through direct effects on enzymes, receptors or ion channels [13].

Good evidence implicates NADPH oxidases as mediators of redox signalling in conditions such as endothelial activation, angiogenesis, atherosclerosis, VSMCs and cardiac hypertrophy, and vascular and cardiac remodelling [13,22]. The surface expression of adhesion molecules such as ICAM-1 (intercellular adhesion molecule 1) and VCAM-1 (vascular cell adhesion molecule 1) on endothelial cells in response to increased oscillatory shear, TNFα, renin–angiotensin system activation or hypercholesterolaemia is reported to involve NADPH oxidase-derived ROS, which may act through the activation of MAPKs [e.g. ERK1/2 (extracellular-signal-regulated kinase 1/2)] and/or NF-κB [17,25]. NADPH oxidase is also involved in TNFα-induced JNK-mediated phosphorylation of VE cadherin and subsequent increases in endothelial permeability [20]. VEGF-induced endothelial cell migration involves ROS generation by Nox2 NADPH oxidase and the subsequent activation of Akt [19]. The role of NADPH oxidase in activating redox-sensitive kinases (e.g. p38 MAPK and Akt) involved in VSMC growth in response to agonists such as angiotensin II and thrombin has been thoroughly delineated [22]. NADPH oxidase is also implicated in the activation of MAPKs (e.g. ERK1/2) during cardiomyocyte hypertrophy in response to α-adrenergic stimulation [26]. Recently, cardiomyocyte Rac1 was shown to be involved in the angiotensin II-induced activation of ASK1 (apoptosis signal-regulating kinase 1) and NF-κB, which
were implicated in the development of NADPH oxidase-dependent cardiac hypertrophy [27].

Remodelling of the extracellular matrix is critical in several cardiac and vascular conditions and it is well established that this process is redox-sensitive; for example, both the expression and activation of MMPs (matrix metalloproteinases) are increased by ROS. In VSMC subjected to cyclical mechanical stretch, the increase in MMP2 expression and activity has been shown to be p47phox-dependent [28]. Similarly, p47phox was implicated in the MMP activation and flow-induced vascular remodelling in an experimental model of chronically increased arterial blood flow [29].

Alterations in activity of transcription factors are an important component of NADPH oxidase-dependent redox signalling. NADPH oxidase involvement in TNFa-induced increases in NF-κB activation in endothelial cells was referred to earlier [17]. Similarly, endothelial cell NF-κB activation induced by advanced glycation end-products is NADPH oxidase-dependent [30]. In cultured cardiomyocytes, NF-κB activation by glycated albumin was also dependent on Nox2 oxidative activation [31]. Similarly, Nox2 oxidative is required for in vivo myocardial NF-κB activation in an experimental model of angiotensin II-induced cardiac fibrosis [32]. In cultured rat cardiomyocytes, angiotensin II-induced increases in AP-1 activity were suggested to be NADPH oxidase-dependent [33], while in cultured cardiac fibroblasts, TGFβ1-induced activation of Smad 2/3 was Nox4 oxidase-dependent [7]. In cultured VSMC, angiotensin II-induced JAK/STAT signalling and synthesis of IL-6 (interleukin 6) were p47phox-dependent [34].

Downstream of kinase activation and/or changes in transcription factor activity, NADPH oxidase-dependent signalling alters the transcription of many genes. For example, transfection of NIH 3T3 fibroblasts with Nox1 induced upregulation of numerous genes critical to cell growth [35]. In a recent study in VSMC, it was demonstrated that p47phox was involved in thrombin-induced changes in the expression of distinct subsets of genes (notably the CD44 and BMP4-Id signalling pathway) which may be involved in atherosclerosis and restenosis [36]. Similar results are also evident in vivo. For example, chronic infusion of angiotensin II in mice induces an increase in the myocardial expression of connective tissue growth factor, pro-collagen 1, pro-collagen 3 and fibronectin mRNAs, all of which are dependent on Nox2 since these changes are inhibited in Nox2-deficient animals [32].

An intriguing question in the field for several years has been the mechanisms through which NADPH oxidase signalling achieves the required specificity. Several recent studies are beginning to address this question. One important mechanism may involve the interaction of oxidase subunits such as p47phox and Rac with non-oxidase proteins, serving to spatially confine NADPH oxidase-derived ROS signals in the vicinity of signalling targets. For example, VEGF-induced JNK activation and membrane ruffle formation in endothelial cells were found to involve interaction of p47phox with WAVE1 [Wiskott–Aldrich syndrome protein] verprolin homologous 1], an important regulator of the cytoskeleton; the WAVE1-dependent complex also contained Rac1 and the kinase PAK1 [37]. In human microvascular endothelial cells, TNFα-induced ROS-dependent activation of ERK1/2 required the association of phosphorylated p47phox with the signalling molecule TRAF-4 (TNF-receptor-associated factor-4) [25]. In VSMC, angiotensin II-stimulated oxidative activation and downstream phosphorylation of p38 MAPK and JNK involved an interaction of p47phox with cytoskeletal elements [38]. In endothelial cells induced to migrate in response to VEGF, the interaction of Nox2 and Rac1 with the molecule IQGAP1 (IQ motif-containing GTPase-activating protein 1) was found to be critical [39]. Recently, it was also shown that in response to IL-1β stimulation of MCF-7 epithelial cells, Nox2 was internalized in endosomes in a Rac1-dependent manner together with the IL receptor, and that H2O2 generation within the endosomes played a critical role in subsequent activation of an IκB (inhibitory κB) kinase complex and NF-κB [40]. These results could explain how cells might use Nox-derived ROS at the plasma membrane to selectively influence signal transduction in response to specific agonists.

Summary
The important role of redox signalling in many pathophysiological settings is increasingly recognized. Recent studies indicate that tightly regulated ROS production by a family of NADPH oxidases may be especially important in redox signalling. These enzymes appear to be involved in the pathophysiology of several cardiovascular (and other) diseases. A better understanding of their regulation and the mechanisms through which they modulate redox signalling may lead to novel therapeutic possibilities.

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References