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Review Article

Detection of reactive oxygen species derived from the family of NOX NADPH oxidases

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ABSTRACT

NADPH oxidases (NOX) are superoxide anion radical ($O_2^{-\bullet}$)-generating enzymes. They form a family of seven members, each with a specific tissue distribution. They function as electron transport chains across membranes, using NADPH as electron donor to reduce molecular oxygen to $O_2^{-\bullet}$. NOX have multiple biological functions, ranging from host defense to inflammation and cellular signaling. Measuring NOX activity is crucial in understanding the roles of these enzymes in physiology and pathology. Many of the methods used to measure NOX activity are based on the detection of small molecules that react with NOX-generated $O_2^{-\bullet}$ or its direct dismutation product hydrogen peroxide (H_2O_2) to form fluorescent, luminescent, or colored products. Initial techniques were developed to measure the activity of the phagocyte isoform NOX2 during the oxidative burst of stimulated polymorphonuclear leukocytes, which generate large quantities of $O_2^{-\bullet}$. However, other members of the NOX family generate much less $O_2^{-\bullet}$ and hence H_2O_2, and their activity is difficult to distinguish from other sources of these reactive species. In addition, $O_2^{-\bullet}$ and H_2O_2 are reactive molecules and most probes are prone to artifacts and therefore should be used with appropriate controls and the data carefully interpreted. This review gives an overview of current methods used to measure NOX activity and NOX-derived $O_2^{-\bullet}$ and H_2O_2 in cells, tissues, isolated systems, and living organisms, describing the advantages and caveats of many established methods with emphasis on more recent technologies and future perspectives.

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Contents

Introduction: NADPH oxidases 1904
What types of ROS are derived from NOX? 1904
Systems in which NOX activity can be measured 1905
  Intact cells 1905
  Cell-free assays 1905
  Tissues and organs 1905
Evaluation of the specificity of measurement of NOX activity 1905
Measurement of NOX-derived $O_2^{-\bullet}$ and H_2O_2 1908
Electron spin resonance (ESR) spectroscopy. 1908
Cytochrome *c* reduction 1908
Tetrazolium salts. 1908

Abbreviations: AGT, alkylguanine-DNA alkyltransferase; CGD, chronic granulomatous disease; cpYFP, circularly permuted yellow fluorescent protein; DCFH, 2′, 7′-dichlorofluorescein; DPI, diphenyleneiodonium; DUOX, Dual oxidase; ER, endoplasmic reticulum; E^+, ethidium; ESR, electron spin resonance; GSH, glutathione; HPLC, high performance liquid chromatography; HE, hydroethidine; H_2O_2, hydrogen peroxide; 2-OH-E^+, 2-hydroxyethidium; HOCl, hypocholorous acid; L-012, 8-amino-5-chloro-7-phenylpyridol[3,4–d]pyridazine-1,4-(2*H*,3*H*)dione sodium salt; MCLA, 2-methyl-6-[*p*-methoxyphenyl]-3,7-dihydroimidazo-[1,2–a]pyrazine-3-one; MRI, magnetic resonance imaging; MS, mass spectrometry; NOX, NADPH oxidase; NBT, nitroblue tetrazolium; PG1, Peroxy Green 1; $ONOO^-$, peroxynitrite; PET, photo-induced electron transfer; ROS, reactive oxygen species; $O_2^{-\bullet}$, superoxide anion radical; SOD, superoxide dismutases; TPO, thyroperoxidase; WST-1, (2-(4-iodophenyl)-3-(4-nitropenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium monosodium salt

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Ferrous oxidation – xylenol Orange (FOX) assay . . . 1908
Aconitase inhibition assay. . . . 1909
Hydroethidine (dihydroethidium, HE) . . . 1909
Hydrocyanines. . . . 1910
Lucigenin, and other chemiluminescent probes. . . . 1911
L-012 1911
MCLA 1911
Dichlorofluorescein diacetate (DCFH-DA). . . . 1911
Amplex red 1911
Nonredox-based chemical probes/biosensors. . . . 1912
Benzenesulfonyl fluorescein probes 1912
Boronate probes for H_2O_2 1912
Photo-induced electron transfer-based probes. . . . 1913
Genetically encoded biosensors 1914
roGFPs 1914
Hyper biosensors. . . . 1914
Circularly permuted YFP 1914
Future prospects: targeted probes for $O_2^{-\bullet}$ and H_2O_2 1915
Conclusions 1915
Acknowledgments. . . . 1916
References 1916

Introduction: NADPH oxidases

NADPH oxidases (NOX) are a family of transmembrane proteins comprising seven members (NOX1, NOX2, NOX3, NOX4, NOX5, DUOX1, and DUOX2). Each member of the NOX family presents a particular pattern of tissue expression and regulation (for review, see [1,2]). NOX function as transmembrane electron transport chains using cytosolic NADPH as electron donor and oxygen in the proximal cellular or extracellular environment as electron acceptor (Fig. 1). The electron donor, NADPH, is formed principally by the oxidation of glucose via the pentose phosphate pathway.

Among all NOX isoforms, NOX2 (formerly known as gp91^{phox} or phagocyte oxidase) is the best described in terms of structure, activation, redox potential, and transmembrane electron transfer mechanism (for review, see [3]). All redox centers are conserved among the NOX family [4], and it is assumed that all NOX share a similar mechanism and generate $O_2^{-\bullet}$ as a primary product. In DUOX and NOX4 expression systems, however, only H_2O_2 is generated without apparent $O_2^{-\bullet}$ detection [5,6]. Because of the uncertainty of the primary product generated by certain NOX, we will refer hereafter to NOX-derived $O_2^{-\bullet}$ and H_2O_2 as NOX-derived reactive oxygen species (ROS).

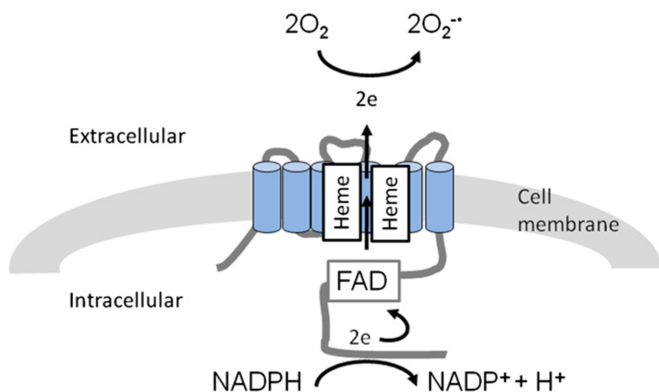

Fig. 1. Schematic representation of the prototypical catalytic subunit of NOX enzymes. A typical NOX NADPH oxidase contains 6 (7 for DUOXes) alpha helix transmembrane domains. NOX redox centers include two molecules of intramembranous heme and a long C terminal region containing NADPH and FAD binding sites. NOX catalyze the transfer of electrons through the membrane using cytosolic NADPH as electron donor and molecular oxygen as electron acceptor. Adapted from [123].

NOX activity is a tightly regulated process, involving other membrane proteins, cytosolic proteins, and regulating domains for complete activity. For example, NOX1, NOX2, and NOX3 require cytosolic factors for full activation while NOX4 generates ROS constitutively; NOX5 and DUOX enzymes contain N-terminal EF-hand domains and require elevation in cellular Ca^{2+} concentrations.

NOX2 is highly expressed in neutrophils, where, on activation, it is the key enzyme responsible for the oxidative burst (or respiratory burst) that leads to microbicidal activity. For NADPH oxidase activity, NOX2 requires the presence of two transmembrane proteins, NOX2 and p22^{phox}, the cytosolic factors p47^{phox}, p67^{phox}, and p40^{phox}, and the small GTP binding protein rac1. The complete loss of function of *NOX2* results in chronic granulomatous disease (CGD), a hereditary disease characterized by the development of granulomas and by a susceptibility to certain fungal and bacterial infections due to impaired killing of these microorganisms [7].

Other NOX are structurally related to, but functionally distinct from, NOX2. The most striking differences are that (1) they often generate constitutively low levels of $O_2^{-\bullet}$ even in nonstimulated cells; (2) although enzyme activity can be up-regulated in several pathologic settings, measurable $O_2^{-\bullet}$ production is still much lower than that found in activated neutrophils, suggesting a role in cellular signaling; and (3) a substantial proportion of the NOX-derived ROS generated in nonphagocytic cells occurs in intracellular organelles, whereas ROS generation by phagocytes is extracellular or occurs in the extracellular space contained in the phagosome formed during phagocytosis. Although the function of many NOX is still unclear, the identification of loss of function mutations and the generation of knock-out animals has shed light on diverse functions, which are often crucial for the physiology of the tissue where they are expressed [8].

What types of ROS are derived from NOX?

The primary product of most NOX is $O_2^{-\bullet}$, according to the following reaction:

$$NADPH + 2O_2 \rightarrow NADP^+ + H^+ + 2O_2^{-\bullet}.$$

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