The function of the NADPH oxidase of phagocytes, and its relationship to other NOXs

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Abstract
The NADPH oxidase of ‘professional’ phagocytic cells transfers electrons across the wall of the phagocytic vacuole, forming superoxide in the lumen. It is generally accepted that this system promotes microbial killing through the generation of reactive oxygen species and through the activity of myeloperoxidase. An alternative scenario exists in which the passage of electrons across the membrane alters the pH and generates a charge that drives ions into, and out of, the vacuole. It is proposed that the primary function of the oxidase is to produce these pH changes and ion fluxes, and the issues surrounding these processes are considered in this review. The neutrophil oxidase is the prototype of a whole family of NOXs (NADPH oxidases) that exist throughout biology, from plants to humans, which might function, at least in part, in a similar fashion.

The neutrophil NADPH oxidase and the roles, and consequences, of ion fluxes into the phagocytic vacuole
Phagocytosis by neutrophils is accompanied by a burst of oxygen consumption known as the ‘respiratory burst’. This is non-mitochondrial respiration that is accomplished by the NADPH oxidase, a protein complex that assembles in the wall of the phagocytic vacuole and produces O2− (superoxide) in the vacuole [1,2]. Electron transfer occurs through gp91phox, a flavocytochrome b that contains a transmembrane domain in which there are two low-potential haemns, located near the internal and external surfaces of the membrane, and cytosolic domains that bind FAD and the substrate, NADPH.

CGD (chronic granulomatous disease) is an immuno-deficiency syndrome caused by failure of this oxidase system, which is completely deficient in the vast majority of cases, but, in a small number of cases, mutations in the components of the NADPH oxidase, usually in regulatory regions of the proteins, result in partial loss of the protein or of its function [3]. Cells from these patients, with ‘variant CGD’, can produce up to 10–20% of normal amounts of oxidase activity, and yet these patients present clinically because they are predisposed to infections and other manifestations of CGD. This ‘variant’ condition becomes very relevant when considering the function of the oxidase, as will be discussed below.

How does NADPH oxidase activity promote microbial killing?
The NADPH oxidase transfers electrons across the wall of the phagocytic vacuole where it is accepted by O2, forming O2− in the vacuole. There is a major difference of opinion as to the role of this electron transport. One theory is that the oxygen free radicals and the products of the activity of MPO (myeloperoxidase) on H2O2 are directly toxic. The respiratory burst generates H2O2 [4], O2− [5] and various reaction products, including H2O2, OH−, singlet oxygen and ozone, were all considered to be microbicidal, either singly or in combination [1].

In 1967, MPO was shown to be microbicidal when combined with H2O2 and halides [6], and MPO-mediated halogenation was readily accepted as a primary microbicidal mechanism. Catalase-negative organisms rarely infect CGD patients [7], and it was proposed that these bacteria generated enough H2O2 to catalyse their own MPO-mediated halogenation within the vacuole of the phagocytosing neutrophil [8–10].

However, these theories have been challenged [11,12]. Previous studies have not taken into account the conditions pertaining in the phagocytic vacuole where the amounts of O2− produced are enormous, in the order of approx. 1–4 mol/l [11,13], as is the concentration of granule proteins at approx. 500 mg/ml [11], and the pH is between 7.4 and 8.0 [14].

In a study to examine the antibacterial action of O2− and H2O2, and products of chloride oxidation (HOCl), under these conditions [15], it was found that, although these reactive species did kill bacteria when incubated with them in a protein-free salt solution, in the physiological situation in which granule proteins were also present, this microbicidal effect was abolished [15].

The theory that bacterial mutants that were deficient in catalase might lead to their own destruction by producing H2O2 was attractive. However, catalase-deficient Aspergillus nidulans [16] and Staphylococcus aureus [17] have subsequently been shown to be as virulent as the catalase-positive variety in mouse models of CGD.

Patients with variant CGD show that the production of approx. 100 mM O2− in the vacuole, an amount that
is considerably greater than would be produced by a xanthine/xanthine oxidase-generating system, or by catalase-deficient organisms, is insufficient to kill the microbes.

The strongest, irrefutable, evidence against the microbicidal effect of ROS (reactive oxygen species) and oxidized halides came from mice in which the genes for the neutral granule proteases, cathepsin G and elastase, were removed by gene targeting [11]. These mice demonstrated a normal respiratory burst, indicating the normal production of ROS, and normal iodination, as a consequence of normal MPO activity, but showed a marked inability to kill *S. aureus* and *C. albicans*, with a killing defect at least as severe as that in the CGD mouse lacking p47<sub>phox</sub>.

Thus both the neutral proteases and adequate activity of the oxidase are required; what is the relationship between these two seemingly diverse systems?

**The oxidase elevates the pH in the phagocytic vacuole and induces ion fluxes across the vacuolar membrane**

An alternative concept is that the main function of electron transport is to drive ion fluxes across the vacuolar membrane and to adjust the pH within the vacuole, so as to optimize conditions for the killing and digestive functions of the granule enzymes.

The oxidase is electrogenic [18], and it has been believed for many years that the charge across the vacuolar membrane is compensated for completely by the passage of protons into the vacuole from the cytosol, through specific proton channels [19], or through the flavocytochrome itself [20,21].

A voltage-gated proton channel, H<sub>I</sub>, has been identified which is thought to represent the proton channel that compensates for the charge in neutrophils [22]. Interestingly, the expression pattern was unlike one that might be expected from a protein strongly associated with a highly specialized process in professional phagocytes.

**Charge compensation by K<sup>+</sup> passing through BK (large-conductance Ca<sup>2+</sup>-activated K<sup>+</sup>) channels**

Proton channels provide an attractive mechanism of charge compensation because, in addition to compensating for the charge across the membrane, this would achieve the effects of removing the protons left behind in the cytosol when electrons are passed across the membrane. However, the pH within the phagocytic vacuoles rises to approx. 7.8–8.0 [14], despite having approx. 200 mM protons released into them from the granules, in which the pH is maintained at approx. 5.5 [23], which must mean that there is a net consumption of protons in the vacuole by the protonation of O<sub>2</sub><sup>2-</sup> (peroxide), which could not occur if each electron were accompanied across the membrane by a proton:

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4O_2 + 4e^- \rightarrow 4O_2^- \rightarrow 2O_2 + 2O_2
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**NHE (Na<sup>+</sup>/H<sup>+</sup> exchanger) 1 has a role in regulating vacuolar pH**

We have found that NHEs play an important role in regulating vacuolar pH by exchanging Na<sup>+</sup> for cytosolic H<sup>+</sup>. The general NHE inhibitor EIPA [5-(N-ethyl-N-isopropyl) amiloride], and HOE-694 (3-methylsulfonyl-4-piperidino-benzoxy guanidine mesylate) [26], the specific inhibitor of NHE1, both inhibit 22Na<sup>+</sup> uptake by PMA-activated neutrophils (J. Ahluwalia, J. Young and A.W. Segal, unpublished work). This uptake was highly dependent upon the extracellular pH, as would be expected if Na<sup>+</sup> were being exchanged for protons.

If the concentration of Na<sup>+</sup> is measured with the fluorophore Sodium Green, the vacuolar concentration of Na<sup>+</sup> is dramatically reduced by the active oxidase, an effect that is inhibited by DPI (diphenyleneiodonium) or EIPA. As expected, this inhibition also has an important effect on pH, with vacuolar pH rising to above 9.0, while cytosolic pH drops to approx. 6.0, although this cytosolic acidity could also be due to inhibition of plasma membrane NHEs, which have been shown to be important for the regulation of cytoplasmic pH in neutrophils [27].

If NHE1 plays an important role in the regulation of vacuolar pH, there must be some means of regenerating Na<sup>+</sup> in the vacuole because the levels of this ion in the granules is only approx. 100 mM, much less than the amount of alkali secreted into the vacuole.

**Role of MPO**

If the predominant function of the oxidase is to optimize conditions for the efficient function of the granule enzymes in the phagocytic vacuole, then the current dogma regarding the antimicrobial role of MPO (see the review by Klebanoff [28]), needs to be reconsidered.

Besides its HOCl-generating capacity, MPO can act as a catalase, a peroxidase and a generator or consumer of O<sub>2</sub><sup>2-</sup>, O<sub>2</sub><sup>-</sup> and O<sub>2</sub>. The balance of these reactions will be very dependent upon ambient conditions of pH, ionic strength and enzyme and substrate concentrations, and these have been modelled recently [13].
The NOX family of NADPH oxidases

A large family of NADPH oxidase have been identified in fungi, plants, fruitflies, nematode worms and sea urchins, and in multiple organs in higher animals [29] by screening databases for proteins homologous with gp91phox. The central electron-transporting molecules all have a similar structure to that we described for gp91phox. They have NADPH- and FAD-binding sites in the cytosolic C-terminal tail and six transmembrane helices with ligands for the two haem, one near each surface of the membrane.

DUOXs (dual oxidases) produce H$_2$O$_2$ as substrate for peroxidase reactions

DUOXs generate H$_2$O$_2$ as a substrate for peroxidase-mediated reactions. This H$_2$O$_2$ is required in nematode worms [30] and fruitflies for the cross-linking of tyrosines in the cuticle and wing membrane respectively.

The generation of H$_2$O$_2$ by the sea urchin egg following fertilization, which is catalysed by the DUOX Udx1 [31], has been studied in some detail. The H$_2$O$_2$ acts as substrate for ovoperoxidase which cross-links the envelope into a hardened matrix that is insensitive to biochemical and mechanical challenges, providing a permanent physical obstruction to polyspermy [32].

Duox2 and possibly also Duox1 are expressed in the thyroid gland where they generate H$_2$O$_2$ as substrate for thyroperoxidase-mediated iodination of tyrosine residues within thyroglobulin [33]. The mechanism responsible for iodide efflux across the apical membrane into the lumen of the follicle is unknown, but it involves pendrin, a protein that is abnormal in Pendred’s syndrome, characterized by goitre, sometimes associated with hypothyroidism, and sensorineural deafness. Pendrin [SLC (solute carrier) 26A4] is a member of the SLC26 family of anion exchangers [34] and its loss results in abnormalities in the cochlea in which the endolymph is abnormally acidic and contains high levels of Ca$^{2+}$ [35].

NOXs as pH modulators and drivers of ion fluxes

NOXs are widely distributed throughout the biological world in which they undoubtedly play many different roles. An important, if not primary, role of these molecules might be to couple electron transport to pH modulation and ion fluxes, as has been demonstrated in the paradigm of the neutrophil vacuole.

NOX3 in the inner ear

NOX3 is strongly expressed in the inner ear. The phenotype of the mouse in which this system is defective has been very revealing [36]. The mice adopt a ‘tilted’ position of the head and show abnormal performance in several motor co-ordination tests. The reason for this is that they failed to calcify their otocinia, which signal movement of the head by pressure of the relatively inert mass on the hair cells.

In the pendrin-knockout mouse, the endolymph is excessively acidic, and the Ca$^{2+}$ concentration is abnormally high [35], and there is an almost complete absence of normal otocinia, with frequent giant otocinia, indicative of abnormal endolymph [37]. In addition, the inner ears are dilated, suggesting an osmotic effect of Cl$^-$ trapped in the absence of this anion exchanger.

The coexistence of pendrin with DUOX in the thyroid, and with NOX3 in the inner ear, with clear linkage of dysfunction of these two proteins to grossly similar phenotypes, suggests that they might be closely linked mechanistically. It seems very possible that pendrin could be involved in ion transport coupled to charge compensation of electron transport through the DUOX or NOX.

Root hairs

Plant roots produce small hairs which are long thin tubular outgrowths from epidermal cells that are produced in the differentiating zone of the root [38].

Arabidopsis contains various respiratory burst oxidase homologues, which also contain EF-hand Ca$^{2+}$-binding motifs in their extended N-terminal regions [39]. One such homologue, RHD2 (root hair defective 2), is important for root development, and, when the gene coding for it is knocked out [40], the roots are stunted and the root hairs that develop are very short. RHD2 produces ROS at the root-hair tip, and it was suggested that the ROS induce root hair extension by opening hyperpolarization-activated Ca$^{2+}$ channels. It is also possible that a transmembrane electron-transport system is responsible for membrane depolarization [41], driving K$^+$, Cl$^-$ [42] and other ion fluxes that are necessary for increasing the osmotic pressure in the region of the tip of the root/hair, thereby driving it forward.

NOX enzymes in mammals

In addition to NOX2 in phagocytes and NOX3 in the inner ear, described above, mammals contain NOXs 1, 3 and 5. NOX1 is expressed most highly in the colon, and NOX 4 in the kidney and blood vessels. These are sites in which there is an interface between cells and a surface across which major fluxes of ions occur, in which the NOX system might be mechanistically involved.

Vasculature

NOXs are expressed in endothelial, adventitial and smooth muscle cells of the vasculature (reviewed in [29]), and there is a large literature on the role of NOXs and ROS in the regulation of blood pressure. The general opinion is that NOXs do have an important regulatory role on the vasculature, including an effect on vascular tone and blood pressure regulation [43].

It is generally believed that blood pressure is regulated by contraction of the muscular wall of the resistance vessels. Another possibility is that blood pressure is regulated, at least in part, by the osmotic swelling and shrinkage of cells in response to fluxes of ions driven by NOX-induced charge compensation and associated NHE activity. This could be driven, and regulated, by NOXs, and could occur in any of
the cells in the vessel walls in which these electron transport chains are expressed. On the basis of the model system described above, the efflux of electrons would be balanced by the influx of Cl\textsuperscript{-}, and the exchange of intracellular protons for extracellular Na\textsuperscript{+} through NHEs, followed by the osmotic movement of water. The inhibition of the influx of Na\textsuperscript{+} through NHEs, which are inhibited by thiazide diuretics, could explain the antihypertensive effect of these drugs, which appear to exert their action through vasodilatation rather than saluresis or loss of free water [44,45].

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References


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