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The role of PI3Ks in the regulation of the neutrophil NADPH oxidase

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Abstract

The NADPH oxidase complex of neutrophils and macrophages is an important weapon used by these cells to kill microbial pathogens. The regulation of this enzyme complex is necessarily complicated by the diverse receptor types that are needed to trigger its activation and also the tight control that is required to deliver this activation at the appropriate time and place. As such, several signalling pathways have been established to regulate the NADPH oxidase downstream of cell surface receptors. Central amongst these are PI3K- (phosphoinositide 3-kinase)-dependent pathways, blockade of which severely limits activation of the oxidase to several soluble and particulate stimuli. The precise roles of the phosphoinositide products of PI3K activity in regulating NADPH oxidase assembly and activation are still unclear, but there is emerging evidence that they play a key role via regulation of guanine nucleotide exchange on Rac, a key component in the oxidase complex. There is also very strong evidence that the PI3K products PtdIns(3,4)P_2 and PtdIns3P can bind directly to the PX (Phox homology) domains of the core oxidase components p47^{phox} and p40^{phox} respectively. However, the significance of these interactions in terms of membrane localization or allosteric consequences for the oxidase complex remains to be established.

The NADPH oxidase is critical in innate immunity

The NADPH oxidase of neutrophils and macrophages plays a key role in our defense against a variety of microbial pathogens [1,2]. This enzyme complex can transfer electrons from NADPH on one side of the membrane to molecular

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oxygen on the other side, to generate the superoxide anion (O_2^-). O_2^- can then be converted by both enzymic and non-enzymic processes into a variety of other ROS (reactive oxygen species), e.g. H_2O_2, HOCl. This process is electrogenic and the build up of an inhibitory membrane-potential is avoided by charge compensation; there is currently much controversy concerning the nature of this compensation, with evidence to suggest both H^+ and K^+ channels are involved [3,4]. ROS directed into the phagosome play a key role in microbial killing in this organelle via a combination of direct ROS toxicity and indirect activation of proteases [3,5]. The importance of the NADPH oxidase in our immune system is illustrated by recurrent, life threatening infections in cases of CGD (chronic granulomatous disease), a disease caused by mutations in components of the oxidase complex [6,7].

Pathogens can be engulfed by professional phagocytes, such as macrophages and neutrophils, by a variety of cell surface receptors that can recognize intrinsic molecules on the pathogen surface or host-derived opsonins (e.g. integrins, Fcγ receptors, Toll-like receptors and dectins). It is probably true that in all natural circumstances several receptor types are co-operatively activated to regulate engulfment and activation of subsequent oxidase activity in the phagosome (see Figure 1A for an overview).

While it is clear that the NADPH oxidase plays a critical role in phagosomal killing, its role in other physiological processes is less clear. It has been known for many years that the NADPH oxidase can also be activated at the plasma membrane of neutrophils, macrophages, eosinophils and also other, non-myeloid lineage cells, such as lymphocytes and endothelial cells [1,8,9]. Many different types of stimuli have been shown to elicit production of extracellular ROS through this mechanism. The best studied examples are the activation of neutrophils by several soluble, inflammatory stimuli e.g. bacterial peptide fragments, components of the complement cascade, PAF (platelet-actvating factor) or LtB4 (leukotriene B4; see Figure 1B). This type of stimulation is complex, requiring a substantial period of priming by either lower concentrations of the primary stimuli themselves or, other inflammatory mediators, which do not themselves elicit an oxidase response e.g. GM-CSF (granulocyte/macrophage colony-stimulating factor), TNF-α (tumour necrosis factor-α) or LPS (lipopolysaccharide) [10,11]. It is thought that this type of co-incidence signalling is used to concentrate ROS production in time and space to the site of inflammation, reducing potential collateral damage to surrounding tissue by toxic ROS [12]. It is still far from clear however, whether this production of extracellular ROS is more important in inflammatory pathologies [e.g. in ARDS (acute respiratory distress syndrome), where neutrophil accumulation in the lung can cause severe damage to the endothelial and mucosal barriers] or has a physiological role (possibilities here include extracellular killing, particularly of larger organisms e.g. fungal hyphae or helminths where phagocytosis appears impossible, destruction of nitric oxide or pericellular signalling).

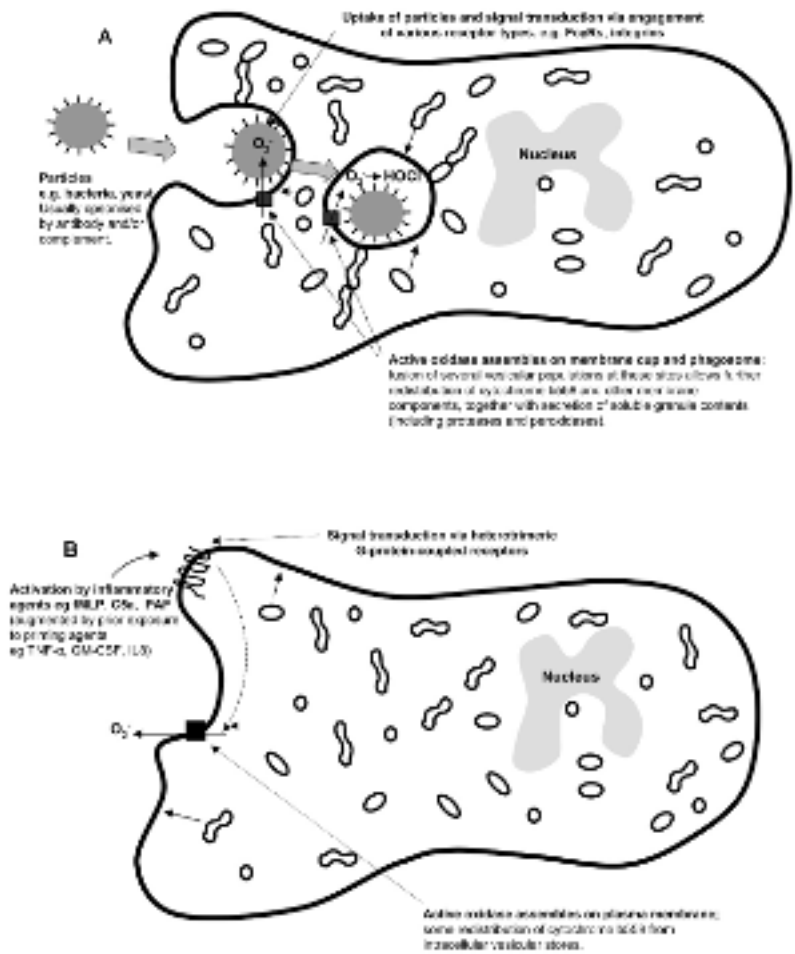

Figure 1 Activation of NADPH oxidase. (**A**) Activation of NADPH oxidase by particulate stimuli. (**B**) Activation of NADPH oxidase by soluble stimuli. IL8, interleukin 8.

The components of the NADPH oxidase complex

The core components of the NADPH oxidase complex in phagocytic cells have been identified by a combination of genetic and biochemical means [1,2,10]. Under the appropriate conditions, *in vitro* re-constitution of a complex containing a two subunit, membrane bound, cytochrome b558 (gp91^{phox}/p22^{phox}) and four soluble proteins (p67^{phox}, p47^{phox}, p40^{phox} and GTP-loaded Rac) is able to effectively transfer electrons from NADPH to molecular O_2 to generate $O_2^{\cdot-}$. Cytochrome b558 in neutrophils is found partioned between the membranes of specific and secretory granules and also the plasma membrane. p40^{phox}/p67^{phox} are found as a complex of uncertain stoichiometry in the cytosol. p47^{phox} is found in the cytosol. GDP-Rac is found in a cytosolic complex with GDI (guanine nucleotide dissociation inhibitor). There is some

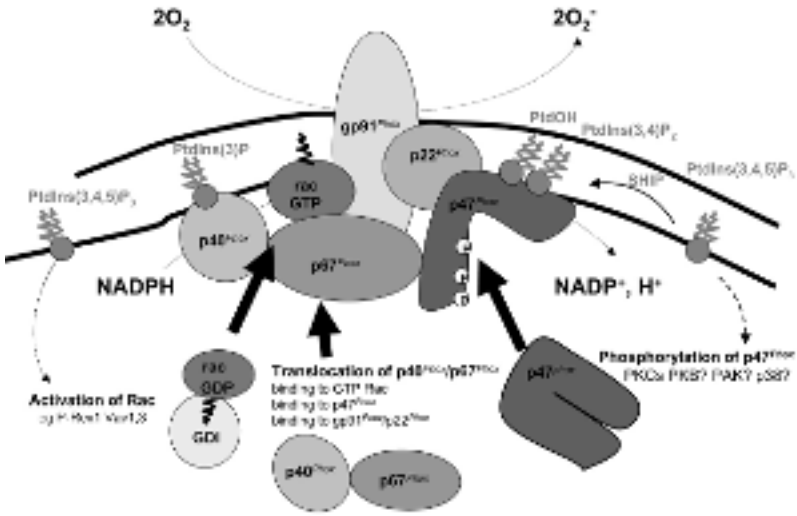

Figure 2 Pathways leading to activation of the NADPH oxidase.

evidence that the cytosolic components can also be found, to some degree, associated with each other and/or the cytoskeleton, but the extent to which this reflects a step in the activation process is uncertain. Upon activation, a proportion of the soluble components p40^{phox}/p67^{phox}, p47^{phox} and GTP-Rac are found associated with cytochrome b558, and the formation of this complex at a particular membrane location is taken as synonomous with oxidase activation (Figure 2).

There is now a substantial body of evidence that has started to delineate some key elements in the activation process that leads to the formation of the active enzyme complex described above [1,2,10]. One element is the activation of the small GTPase Rac. The activation mechanism here draws on knowledge of how this GTPase participates in other signalling pathways and involves guanine nucleotide exchange of GDP for GTP catalysed by GEFs (guanine nucleotide exchange factors) and dissociation from GDI, leading to the delivery of GTP-Rac to the membrane. GTP-Rac is associated with the membrane via its isoprenyl lipid modification (which is thought to be hidden in the complex with GDI) and Rac has been shown to bind directly to p67^{phox} via its TPR (tetratrico peptide repeat) motif and also to make contacts with gp91^{phox}.

Another key step is the conversion of p47^{phox} from an auto-inhibited conformation to an active one which can bind both p67^{phox} and p22^{phox}. This involves a transition from a state in which the SH3 domains of p47^{phox} are locked up with its own C-terminus and PP (polyproline) motif to one where the SH3 domains and PP are available to make contact with p22^{phox} and p67^{phox}. This transition is mediated in *in vitro* reconstitution experiments by the presence of anionic lipids or detergents, but there is good evidence that it is mediated *in vivo* by phosphorylation of the C-terminus at multiple sites. Thus through a combination of protein–protein interactions that link all of the components, an active oxidase complex is thought to assemble around cytochrome b558 (Figure 2) [1,2,10].

Signalling pathways regulating the NADPH oxidase

Many different signalling pathways are thought to be involved in the mechanism by which cell surface receptors regulate the assembly of the active oxidase complex described above [1,2,10]. This complex signalling web is both a consequence of the flexibility that this system must have to be able to deliver active oxidase in response to a huge variety of different cell-surface receptor types and yet also the tight control that must exist to limit the release of potentially damaging ROS to the appropriate time and place. It is also likely that a certain redundancy in the system exists to combat potential escape mechanisms used by the pathogens themselves. Thus the signalling literature for the NADPH oxidase is characterized by a very large number of studies which have suggested partial involvement for many of the signalling pathways known to operate in neutrophils, without a clear perspective on their relative roles or importance. For the remainder of this review we shall focus on the role of PI3K (phosphoinositide 3-kinase) in these pathways.

PI3K involvement in the regulation of the oxidase

PI3Ks are a large family of enzymes that can be classified according to their structure and functional properties into three classes, I, II and III [13] (see Figure 3 for an overview of PI3K signalling pathways). The class I enzymes are activated by many different types of cell-surface receptor, operating via both G-protein and tyrosine kinase transduction mechanisms, and are thought to primarily phosphorylate PtdIns(4,5)P_2 in the inner leaflet of the plasma membrane to generate the lipid-messenger PtdIns(3,4,5)P_3. PtdIns(3,4,5)P_3 can be dephosphorylated by 3-phosphatases [e.g. PTENs (phosphatase and tensin homologue deleted on chromosome 10)] to form PtdIns(4,5)P_2 and 5-phosphatases [e.g. SHIPs (SH2-containing inositol phosphatase)] to form PtdIns(3,4)P_2,

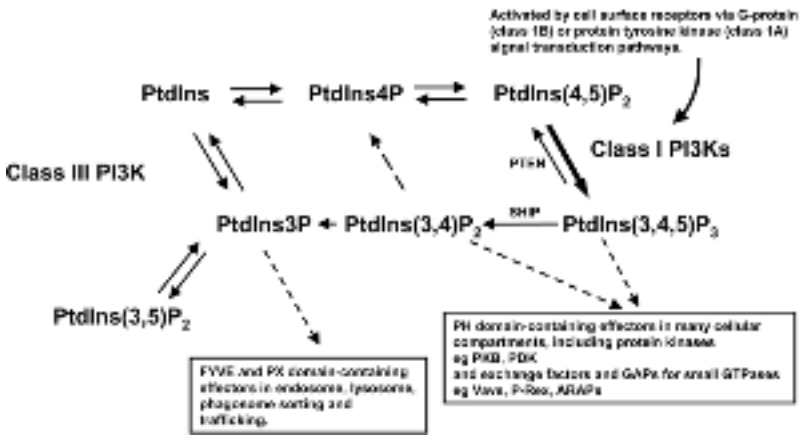

Figure 3 PI3K signalling pathways.

a lipid with increasing credentials as a further messenger molecule. Class III enzymes are thought to be primarily responsible for phosphorylating PtdIns in the lysosomal/endosomal network to form PtdIns3*P*, a lipid messenger which is known to influence the activity of many proteins involved in trafficking through these compartments. Importantly with respect to phagocyte function, PtdIns3*P* is also synthesized on phagosomal membranes during macrophage engulfment of IgG-opsonized particles, yeast and bacteria where it is thought to play a key role in phagosomal maturation [14,15]. Class II enzymes are far less well studied but probably synthesize PtdIns3*P* downstream of receptor activation; thus far there is very little evidence describing their function in phagocytes.

PI3Ks were identified as important components of the signalling pathways controlling oxidase activation when the fungal metabolite wortmannin, a compound isolated on the basis of its ability to inhibit several neutrophil functions, was shown to be a specific catalytic-site inhibitor of PI3K family enzymes [16]. Wortmannin is now well characterized as a potent inhibitor of oxidase responses elicited in primed human and mouse neutrophils by activation of heterotrimeric G-protein coupled receptors [e.g. receptors for C5a, PAF, fMLP (*N*-formyl methionyl-leucyl-phenylalanine)], leading in most cases to ≥95% inhibition of extracellular ROS production. A key role of PI3Ks in this response has been confirmed by hugely reduced ROS production in bone-marrow derived neutrophils from PI3K$\gamma^{-/-}$ mice in response to this type of stimulation [17–19]. PI3Kγ is a class I PI3K isoform which can be directly activated by G$\beta\gamma$ subunits and thus appears adapted to deliver rapid synthesis of PtdIns(3,4,5)P_3 in response to this type of stimulation. Recent work however has suggested that the production of a PtdIns(3,4,5)P_3 signal relevant to oxidase activation in this context is more complex, with good evidence that in human neutrophils at least, the initial PI3Kγ-derived PtdIns(3,4,5)P_3 elicits secondary, priming-dependent and substantially PI3Kδ-dependent PtdIns(3,4,5)P_3 formation that is important for oxidase activation [20].

Given the amount of work done characterizing the importance of PI3Ks in oxidase activation downstream of G-protein coupled receptors, it is perhaps surprising that so little has been done characterizing the role of PI3Ks in other contexts of oxidase-activation, where the physiological purpose is clearer, for example in ROS generation in response to phagocytosis of bacteria or yeast. The problems have been that the variety of cell-surface receptors involved and the confounding issues of phagocytosis or adhesion make it very difficult to ascribe specific effects of PI3Ks on oxidase regulation [21].

How do the lipid products of PI3Ks regulate oxidase activity?

The lipid products of PI3K activity are now well established signalling molecules that characteristically interact with a subset of effector proteins via specific interaction with a relatively small number of conserved target domains. The best characterized of these domains are the PH (pleckstrin homology) domain for binding PtdIns(3,4,5)P_3/PtdIns(3,4)P_2 and the PX and FYVE domains for PtdIns3*P* [22–24].

As described above, guanine nucleotide exchange on Rac is an important event in oxidase activation. This is illustrated by large deficiencies in oxidase activation by several stimuli in Rac-2 deficient mouse neutrophils [25,26]. There are many different GEFs that couple different receptors to the activation of Rac and several are known to possess PtdIns(3,4,5)P_3-binding PH domains (e.g. Vav, P-Rex, Tiam), suggesting that this is a point of possible PI3K-intervention [27]. Indeed recent data from several transgenic mouse models suggest that P-Rex-1 and Vav-1 play significant, possibly redundant, roles in oxidase activation by G-protein coupled receptors [28–30].

Phosphorylation of the C-terminal tail of p47^{phox} is known to be important for oxidase activation (see above; [1,3,10,31]). There is overwhelming evidence that PKC (protein kinase C) family enzymes play a major role in this process, though the particular isoforms, sites of phosphorylation and modes of regulation in any particular incidence of oxidase activation are less clear. There is some evidence that the PI3K signalling pathway may sometimes be involved in PKC activation, alongside classical activation by PLC-mediated formation of DAG (diacylglycerol) and Ins(1,4,5)P_3/Ca^{2+}. The molecular mechanism of this activation is unclear but may involve direct PtdIns(3,4,5)P_3-binding to PKC isoforms or, indirect effects of PtdIns(3,4,5)P_3/PtdIns(3,4)P_2 on PDK-1 (phosphoinositide-dependent kinase 1)-mediated phosphorylation and maturation of PKCs. There is also some evidence that PtdIns(3,4,5)P_3/PtdIns(3,4)P_2 may influence p47^{phox} phosphorylation via direct activation of PKB (protein kinase B) or indirect activation of PAK (p21-activated kinase) via Rac, but the precise sites, context and physiological relevance of these phosphorylations have yet to be described [1,2,10,31].

A significant advance in our understanding of how PI3K lipid products might regulate oxidase assembly and activation is the discovery that PtdIns(3,4)P_2 can bind synergistically with PA (phosphatidic acid) to the PX (phox homology) domain of p47^{phox} and that PtdIns3*P* can bind with high affinity and specificity to the PX domain of p40^{phox} [31]. The PX domain was initially defined by sequence conservation in phox proteins and these discoveries pointed to a widespread role for phosphoinositide binding, particularly PtdIns3*P*, to other PX domain-containing proteins, especially those involved in vesicle trafficking [23]. This observation did not in itself however, immediately point to an explanation of how PI3Ks regulate oxidase activation. By analogy with how these lipids are used as 'regulatable scaffolds' in other signalling contexts, often activating effectors by a combination of membrane-localization and allosteric effects, these interactions are predicted to be important in regulating oxidase assembly and activation at a particular membrane location [31]. PtdIns(3,4)P_2 is predicted to be formed subsequent to class I PI3K-dependent synthesis of PtdIns(3,4,5)P_3 at the plasma membrane or phagosomal cup and thus may be a messenger linking receptors for soluble or particulate agonists to oxidase activation at this location. PtdIns3*P* is predicted to be formed around the phagosome soon after closure from the plasma membrane and thus may be a messenger regulating ROS delivery into this organelle. There have not yet been any clear reports of important allosteric changes in p47^{phox} or p40^{phox} subsequent to lipid binding, and it may

be that they act predominantly as membrane localization signals, though this has yet to be shown definitively [31].

There are substantial problems assigning physiological roles for PI3K lipid products in the regulation of the oxidase. Attempts to define their involvement by limiting their synthesis (e.g. via PI3K inhibition) suffer from the potentially confounding effects of inhibition of other PI3K-dependent processes and, while important, the demonstration that they can bind to certain effectors *in vitro* does not define their contribution *in vivo*. This situation is exacerbated by the difficulties in manipulating the expression of target proteins in fully differentiated neutrophils by classical cell biological techniques (they are for example highly refractory to transfection). Thus it is likely that specific knock-in mutations in the phosphoinositide binding domains of target proteins and subsequent characterization of differentiated neutrophil responses (technology currently only really available for the mouse) will be required before significant progress can be made in this area.

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