**Review**

**Molecular basis of activation and regulation of the phagocyte respiratory burst**

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**Summary** The molecular basis of activation and regulation of the phagocyte respiratory burst is discussed with particular reference to the role of inositol phospholipid hydrolysis, guanine nucleotide coupling proteins, and activation of protein kinase C.

**Phagocyte ‘respiratory burst’**

Phagocytic cells—neutrophil and eosinophil polymorphs, and mononuclear phagocytes—possess a unique membrane bound flavoprotein/b-type cytochrome complex (NADPH-oxidase) which enables these cells, when activated, to generate large amounts of superoxide anion (O$_2^-$) at their surface. Oxy radical production by phagocytes is believed to have a direct role in their microbicidal and cytocidal activities$^{1,2}$ and may also have a role in modulating the immune response.$^{3,4}$ The inappropriate stimulation of phagocyte oxy radical production by immune complexes, complement, and other inflammatory mediators may also contribute to the tissue damage and abnormal immune function seen in immune complex disease such as rheumatoid arthritis.$^5$ Congenital deficiency of the NADPH-oxidase or abnormalities of its activation are associated with a predisposition to severe pyogenic infection.$^6$ and, interestingly, the X linked carrier state for one form of NADPH-oxidase deficiency is associated with ‘autoimmune-like’ phenomena.$^7$ The role and function of the NADPH-oxidase in microbicidal mechanisms and the genetic basis for defects in this system have been well reviewed.$^{6-8}$

In view of the increasing interest in the role of phagocyte oxy radical production in the pathogenesis of disease$^9$ and in the effect of drugs on this function$^9$ this review will examine some of the molecular mechanisms which may lead to activation of the NADPH-oxidase in phagocytes.

**Metabolic pathways leading to phagocyte superoxide anion release**

A variety of soluble and particulate agents are capable of activating phagocytes and triggering a ‘respiratory burst’, which is characterised by increased oxygen consumption, increased anaerobic glycolysis, and the generation of superoxide and other oxy radicals (Fig. 1). These events are associated with activation of a membrane NADPH-oxidase, which reduces molecular oxygen to superoxide by electron transfer from NADPH.$^{10,11}$ Agents which activate phagocytes can be further divided into those which act through specific surface membrane receptors and those which act at metabolic sites within the cell. The latter group includes a number of ‘artificial’ stimuli such as phorbol esters, calcium ionophores, and fluoride ion, whereas the former are mainly physiological agents which are generated during inflammatory reactions (Fig. 1).

**Role of guanine nucleotide-coupling protein (G protein) in surface receptor dependent superoxide release**

Interaction of certain ligands, notably formyl-methionyl-leucyl-phenylalanine (FMLP),$^{12-14}$ leucotriene B$_4$,$^{12}$ and phagocytosable particles,$^{15}$ with membrane receptors provokes a breakdown, dependent on phosphoinositidase C, of membrane phosphatidyl inositol-4,5-bisphosphate to liberate diacylglycerol and inositol-1,4,5-trisphosphate (IP$_3$).$^{14}$

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Accumulating evidence suggests that the linkage of some receptors to phosphoinositidase C is dependent on coupling via a pertussis toxin (PT) sensitive G protein with properties similar to coupling G proteins involved in the adenyl cyclase system. But does not alter FMLP receptor binding. Similarly, in human PMN, PT inhibits FMLP induced inositol lipid hydrolysis, mobilisation of intracellular Ca\(^{++}\), secretion, and aggregation. More direct evidence for the association of FMLP receptors with a G protein is that in PMN membranes, PT inhibits FMLP mediated guanine nucleotide exchange and nucleotide mediated regulation of FMLP receptors. Furthermore, treatment of PMN with PT results in adenosine diphosphate-riboylation of a single 41 kD protein believed to be the alpha subunit of Ni. Another study has provided evidence that the G protein is also involved in the linkage of receptors for platelet activating factor and C5a to mechanisms leading to superoxide release.

**Role of IP\(_3\) and diacylglycerol in phagocyte activation**

Release of IP\(_3\) and diacylglycerol from inositol lipids induced by ligands is believed to cause activation of two independent but synergistic pathways which lead to cell activation events such as superoxide release. IP\(_3\), an acidic hydrophilic molecule, is released into the cytoplasm where it triggers a transient rise in cytosol Ca\(^{++}\) by releasing Ca\(^{++}\) from the endoplasmic reticulum. The rise in cytosol Ca\(^{++}\) triggers several processes, which probably include phospholipase A\(_2\)-dependent arachidonate release from phospholipid and protein phosphorylation dependent on calmodulin. The role of these IP\(_3\) dependent events in the phagocyte respiratory burst, however, remains to be investigated more fully. Diacylglycerol, the other molecule released by inositol lipid hydrolysis, binds and activates the Ca\(^{++}\)/phospholipid dependent protein kinase C (PKC). In the resting cell PKC appears to be cytosolic or only loosely associated with the cell membrane, but during activation it becomes tightly bound to the phagocyte cell membrane (Fig. 3), bringing it into close

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**Fig. 1** Activators of the respiratory burst. Three groups of stimuli activate the phagocyte NADPH-oxidase. Activation of the oxidase is associated with increased oxygen consumption, generation of superoxide, and increased anaerobic glycolysis via the hexose monophosphate (HMP) shunt which provides substrate NADPH. FMLP=formyl-methionyl-leucyl-phenylalanine; PAF=platelet activating factor; LTB\(_4\)=leucotriene B\(_4\).
association with its cofactor, phosphatidylserine.23 Recent evidence suggests that the translocation and binding of PKC to cell membranes is catalysed by Ca++ 27 and that Ca++ enhances the cofactor activity of phospholipid.23 The role of Ca++ in translocation, activation, and subsequent proteolysis of PKC is discussed in greater detail below. Once activated, PKC catalyses phosphorylation of a number of endogenous proteins,28-30 including the transferrin31 and interleukin 2 receptors,32 lipocortin,33 and HLA class I antigens,34 in temporal association with sustained activation of the NADPH-oxidase.29 35

Mechanism of action of calcium ionophore A23187, phorbol esters, and fluoride
Agents which have proved invaluable tools for investigating Ca++/calmodulin and PKC dependent pathways are the calcium ionophore A23187 and the biologically active phorbol esters. A23187 mimics the action of IP3, by raising cytosol Ca++, whereas the phorbol esters are believed to act as structural analogues of diacylglycerol which, in association with phospholipid, bind and activate PKC (Fig. 3).22-24 36 For example, phorbol-12,13-myristate acetate (PMA) and other synthetic analogues of diacylglycerol (e.g., 1-oleoyl-2-acetylglycerol) directly activate PKC and superoxide generation independently of inositol lipid turnover or Ca++ fluxes.22 37 A23187 causes a rise in cytosol Ca++ and in concentrations between 1 and 10 μmol/l stimulates rapid activation of the NADPH-oxidase.38 Possible mechanisms for the action of A23187 include calmodulin dependent phosphorylation39 or Ca++ dependent proteolysis of PKC to generate a cytosolic Ca++/phospholipid independent kinase. The latter is discussed in greater detail below.

Fluoride ion is another activator of the respiratory burst whose mechanism of action until recently has been obscure. Current evidence suggests that fluoride stimulates the respiratory burst by directly activating the G protein, triggering inositol lipid hydrolysis, and activation of PKC.40 41

Other types of receptor linkage
Not all reports have confirmed a role for inositol lipid hydrolysis during signal transduction by surface receptors in phagocytes. Leucotriene B4, which is a strong chemoattractant but a relatively weak secretagogue, has been reported to mobilise Ca++ without a concomitant breakdown of inositol lipids41 in rabbit PMN. The failure to detect inositol lipid hydrolysis may reflect insensitivity of the detection system, inadequate purification of leucotriene B4,42 or a different transduction mechanism. These studies conflict with others reporting that leucotriene B4 does stimulate inositol lipid hydrolysis.12 The Fc receptor (IgGl/2b) in macrophages may activate ligand gated Ca++ channels53 or have intrinsic phospholipase A2 activity,44 but there have been no reports to date linking Fc receptors to inositol lipid hydrolysis.

Further evidence for the existence of alternative

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**Fig. 2** Receptor induced hydrolysis of inositol phospholipids. Stimulation of surface receptors provokes a breakdown, dependent on phosphoinositidase C (PIC), of phosphatidyl inositol-4,5-bisphosphate (PIP₂) to release two key second messengers—inositol-1,4,5-trisphosphate (IP₃) and diacylglycerol (DG). Surface receptors are linked to PIC via a G protein sensitive to pertussis toxin. IP₃ stimulates release of Ca++ from the endoplasmic reticulum and DG activates protein kinase C. Ca++ and protein kinase C act synergistically to activate the NADPH-oxidase. LTB₄=leucotriene B₄; PAF=platelet activating factor.
Role of calcium and diacylglycerol in activation of NADPH-oxidase. The rise in Ca\(^{++}\) stimulated by IP\(_3\) facilitates translocation and binding of protein kinase C (PKC) to the cell membrane. This brings it into association with its phospholipid cofactor and enhances its activation by diacylglycerol. Activated PKC phosphorylates a number of cellular proteins (PROT-P), which may include a component(s) of the NADPH-oxidase. Ca\(^{++}\) also activates phospholipase A\(_2\) (PLA\(_2\)) dependent release of arachidonic acid (AA) from phospholipid (PL) and calmodulin dependent events. PIC=phosphoinositidase C; PROT-P=phosphatidyl inositol-4,5-biphosphate.

Pathways independent of PKC and leading to activation of NADPH-oxidase is suggested by recent experiments in our laboratory with the myelomonocytic cell line U937. This cell line, after in vitro differentiation with dimethyl sulfoxide, responds with a respiratory burst to both PMA and serum opsonised zymosan. We have identified a subclone which responds to serum opsonised zymosan but fails to respond to stimulation with PMA (Table 1). These data suggest the existence of a distinct pathway leading to superoxide release which does not involve PKC. Furthermore, macrophages which have been desensitised with phorbol ester, and are refractory to stimulation by phorbol esters, still respond to serum opsonised zymosan.

**Final common pathway leading to activation of NADPH-oxidase**

The final steps leading to activation of the NADPH-oxidase remain unclear but probably involve phosphorylation of a component of the NADPH-oxidase. The NADPH-oxidase has been shown to contain both an unusual b-type cytochrome\(^{46}\) and a flavoprotein containing flavin adenine dinucleotide (FAD).\(^{47}\) The cytochrome has a sufficiently low oxidation-reduction potential (\(E_{m,70}=-245\) mV) to enable it to rapidly reduce \(O_2\) to \(O_2^\cdot\).\(^{46}\) The cytochrome is absent from phagocytes in patients with X linked, chronic granulomatous disease.\(^{5-8}\) Studies by Cross, Jones, and others\(^{46-48}\) clearly support the schema shown in Fig. 4, in which (a) all electrons from NADPH pass to the cytochrome via the FAD-containing flavoprotein and (b) activation of the oxidase occurs either before or at the level of the flavoprotein. Two recent independent observations suggest that the flavoprotein is a 44-45 kD polypeptide. Segal et al have reported that there is failure of phosphorylation of a 44 kD polypeptide during activation of neutrophils from patients with

**Table 1  Superoxide production (nmol/10\(^6\)/30 min)**

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>Clone A (mean (SD))</th>
<th>Clone B</th>
</tr>
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<tbody>
<tr>
<td>STZ (1 (\mu)g/ml)</td>
<td>9.0 (0.6)</td>
<td>6.6 (1.0)</td>
</tr>
<tr>
<td>PMA (10 (\mu)g/ml)</td>
<td>41.1 (5.2)</td>
<td>0</td>
</tr>
</tbody>
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Mean (SD) superoxide release by two clones of U937 cells (10\(^6\)/ml) stimulated by opsonised zymosan (STZ) or phorbol-12,13-myristate acetate (PMA). Superoxide release was measured as superoxide dismutase inhibitable reduction of cytochrome \(c\) (n=3).

**Fig. 4  Possible structure of the NADPH-oxidase.** The NADPH-oxidase probably consists of at least two components—a b-type cytochrome and a flavoprotein. The cytochrome (cytochrome \(b_{44-45}\)) has a low midpoint potential and binds oxygen directly, transferring electrons from NADPH via a flavoprotein dehydrogenase containing flavin adenine dinucleotide (FAD).
autosomal recessive chronic granulomatous disease. This peptide is phosphorylated during activation of PMN from normal subjects or from patients with X linked chronic granulomatous disease who lack the b-type cytochrome. Secondly, Cross and Jones have developed a specific inhibitor of the oxidase—diphenylene iodonium—which appears to act at the level of the flavoprotein. Furthermore, [125I] diphenylene iodonium specifically labels a polypeptide of 45 kD, and this labelling is inhibited by NADPH.

Assuming that the 45 kD polypeptide represents the flavoprotein, a further question is the nature of the kinase responsible for phosphorylating the flavoprotein. Partially purified dormant NADPH-oxidase in a light membrane fraction of PMN can be activated by the addition of purified PKC, PMA, adenosine triphosphate, phosphatidylserine, and NADPH. This, in conjunction with the evidence that the 45 kD protein is phosphorylated in PMN stimulated by PMA, suggests that PKC may directly activate the oxidase.

A further important feature of the final activation process is that, at least in PMN, there appear to be two pools of NADPH-oxidase: one membrane bound and one which requires translocation to the cytoplasmic membrane from cytoplasmic specific granules.

**Regulation of phagocyte respiratory burst by calcium and protein kinase C**

**Synergistic effect of Ca++ on membrane translocation, activation, and proteolysis of protein kinase C**

It has been shown that A23187 functions synergistically with activators of PKC such as phorbol ester to elicit physiological responses from a wide variety of cells, including neutrophils, lymphocytes, platelets, pancreatic islets, and many other. In both human PMN and monocytes, A23187 shortens the initial ‘lag time’ and increases the initial rate of response to PMA without affecting total superoxide production. Thus A23187 appears to affect only early activation events. Recent evidence suggests that the basis for this synergism may be that the rise in calcium induced by A23187 facilitates the initial translocation and binding of PKC to the cell membrane, thereby shortening the activation process (Fig. 3). This model is further supported by the observation that pretreatment of leucocytes with A23187 increases the affinity of binding of [3H]phorbol dibutyrate to its receptor (i.e., PKC). The binding of phorbol esters to PKC is dependent on phospholipid and the association of PKC with the plasma membrane would therefore increase the availability of phospholipid and its binding affinity for phorbol ester.

The potential physiological significance of these observations is twofold. Firstly, calcium fluxes generated in response to ligands which stimulate inositol lipid hydrolysis may accelerate the activation of PKC by diacylglycerol. Secondly, they also suggest a possible mechanism whereby a rise in cytoplasmic calcium, stimulated by an initial signal, may ‘prime’ PMN for responses to second signals. For example, incubation of PMN with low concentrations of A23187 augments their response to FMLP. Other examples include the ‘priming’ of PMN by various cytokines, including tumour necrosis factor and interferon-gamma, granulocyte/macrophage colony stimulating factor, or chemotactic factors (FMLP, C5a), which results in augmented phagocytic and respiratory burst activity. Mononuclear phagocytes are similarly activated and ‘primed’ by a wide variety of agents including macrophage colony stimulating factor, interferon-gamma, and bacterial products such as lipo polysaccharide. Although the mechanism for each of these examples of ‘priming’ is uncertain, there is increasing evidence that they may be mediated by a rise in cytosol calcium levels.

Calcium also has a role in proteolysis of activated PKC and the generation of a Ca++/phospholipid independent kinase (PKM). Earlier studies showed that activation of platelets by phorbol esters or phospholipase C resulted in the appearance of a cytosolic 50 kD Ca++/phospholipid independent protein kinase. More recent studies on PMN have shown that Ca++ promotes binding of both PKC and a Ca++ requiring proteinase (calpain) to the cell membrane. After binding of PKC to the membrane, calpain cleaves the catalytic domain of PKC from its regulatory domain, releasing a peptide of approximately 50 kD. This peptide, which shows Ca++/phospholipid independent kinase activity is released into the cytosol, thus allowing access to protein substrates unavailable to activated membrane bound PKC. This kinase may be important in the PMN degranulation response and therefore might be involved in translocation of the specific granule pool of NADPH-oxidase. The release of membrane serine proteases requires the participation of native membrane bound PKC rather than PKM, but it is not known whether PKM is involved in activation of the membrane NADPH-oxidase. It is possible that the PMN respiratory burst elicited by A23187 is mediated by PKM rather than PKC. Involvement of PKM in activation of the respiratory burst could explain why certain inhibitors of PKC, which block the PMN response to PMA, fail to inhibit the response to C5a or FMLP.
Role of protein kinase C in inhibition of cell activation

While calcium signals appear to amplify some mechanisms of cell activation there is also increasing evidence that PKC has a regulatory or inhibitory role during cell activation which may serve to limit the cellular response to signals dependent on surface receptors.

Stimulation of cells via surface receptors which activate phosphoinositide turnover elicit relatively short lived responses (less than five minutes). For example, inositol lipid hydrolysis and superoxide production in PMN stimulated with FMLP is rapid but terminates within five minutes. This suggests that there are regulatory mechanisms to terminate the cell response. In contrast, stimulation of cells with phorbol ester results in prolonged cell activation, which is not associated with inositol lipid hydrolysis and shows no evidence of feed back control. This suggests that PKC lies distal to a metabolic site(s) regulating the cellular response to surface receptor dependent stimuli. Mechanisms of regulating responses to surface receptor stimulation include destruction of the ligand or internalisation of receptors.

Another mechanism is suggested by the observation that pretreatment of cells with phorbol ester renders them refractory to stimulation by ligands acting on surface receptors. Thus PMN pretreated briefly with stimulatory concentrations of phorbol myristate acetate (10 ng/ml) no longer show enzyme secretion or calcium fluxes in response to stimulation by FMLP, even though the FMLP receptors retain their normal binding characteristics.

This 'uncoupling' action resembles the effect of pertussis toxin and suggests that PKC may modify either the receptor or an associated coupling protein (Fig. 5). Evidence for both mechanisms exists: for example, activation of PKC by phorbol ester results in phosphorylation and internalisation of the transferrin receptor in HL60 myeloid leukaemia cells, whereas in human platelets PKC may uncouple adrenergic receptors from adenyl cyclase by phosphorylating the coupling protein (Ni). Recent studies suggest that phosphorylation of the coupling G protein also occurs in PMN.

Conclusions

A more complete picture of the metabolic pathways controlling activation of the phagocyte respiratory burst is beginning to emerge and much of this new information will be relevant to other aspects of phagocyte function. A similar picture is emerging from studies of transmembrane signalling in other types of leucocyte. Although the picture is still incomplete, it should now be possible to apply this new knowledge to studies of the mechanism of action of anti-inflammatory drugs and to the study of phagocyte activation during inflammatory disease.

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