Neutrophil priming: pathophysiological consequences and underlying mechanisms

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1. Neutrophil priming by agents such as tumour necrosis factor-α, granulocyte/macrophage colony-stimulating factor and lipopolysaccharide causes a dramatic increase in the response of these cells to an activating agent; this process has been shown to be critical for neutrophil-mediated tissue injury both in vitro and in vivo.

2. The principle consequence of priming, aside from a direct effect on cell polarization, deformability and integrin/selectin expression, is to permit secretagogue-induced superoxide anion generation, degranulation and lipid mediator (e.g. leukotriene B4 and arachidonic acid) release. It is now recognized that most priming agents also serve an additional function of delaying apoptosis and hence increasing the functional longevity of these cells at the inflamed site.

3. The potential mechanisms underlying priming are discussed; current data suggest a dissociation between priming and changes in receptor number and/or affinity, G-protein expression, phospholipase C and phospholipase A2 activation and changes in intracellular Ca2+ concentration. However, more recent studies support a key role for protein tyrosine phosphorylation and enhanced phospholipase D and phosphoinositide 3-kinase activity in neutrophil priming.

4. Recent work has also revealed the potential for neutrophils to spontaneously and fully ‘de-prime’ after an initial challenge with platelet-activating factor. This ability of neutrophils to undergo a complete cycle of priming-de-priming (and re-priming) reveals a previously unrecognized flexibility in the control of neutrophil behaviour at an inflamed site.

INTRODUCTION

Neutrophils comprise a fundamental component of the non-specific immune response, being recruited rapidly to sites of inflammation. These cells respond to injurious agents by phagocytosis, the release of pre-formed granular enzymes and proteins, and by the de novo production of a range of potentially damaging, but ephemeral, reactive oxygen intermediates. That these functions are essential for host defence against invading micro-organisms is illustrated by the propensity of patients with neutrophil deficiency syndromes such as neutropenia, chronic granulomatous disease and leucocyte adhesion deficiency to develop recurrent and often overwhelming infections. Paradoxically, it is now equally clear that inappropriate or excessive activation of neutrophils may contribute to inflammatory tissue injury in a variety of clinical scenarios (e.g. the acute respiratory distress syndrome (ARDS), rheumatoid arthritis and ischaemia–reperfusion injury). Thus modulation of the activation status of the neutrophil is of key importance in determining the balance between defence and injury. The aim of this review is to outline the functional consequences and in vivo significance of neutrophil priming and thereafter discuss in detail the current controversy that exists regarding the mechanistic basis of priming.

NEUTROPHIL PRIMING

Priming refers to a process whereby the response of neutrophils to an activating stimulus is potentiated, sometimes greatly, by prior exposure to a priming agent (Figure 1). Normal circulating neutrophils do not express anywhere near their full microbicidal capacity when challenged with biological activating agents such as the bacterial formylated peptide N-formylmethionyl-leucyl-phenylalanine (fMLP) unless they have first been primed. The other key concept regarding priming is that these agents do not elicit the effector function(s) on their own (although they may

Key words: neutrophil priming, phosphoinositidase C, phosphoinositide 3-kinase, phospholipase D, superoxide anions.

Abbreviations: AA, arachidonic acid; ARDS, acute respiratory distress syndrome; fMLP, N-formylmethionyl-leucyl-phenylalanine; GM-CSF, granulocyte-macrophage colony-stimulating factor; Ins(1,4,5)P3, inositol 1,4,5-trisphosphate; LPS, lipopolysaccharide; LTBr, leukotriene B4; PAF, platelet-activating factor; PI3K, phosphoinositide 3-kinase; PKC, protein kinase C; PJA2, phospholipase A2; PLC, phospholipase C; PLD, phospholipase D; PtdIns(4,5)P2, phosphatidylinositol 4,5-bisphosphate; PtdIns(3,4,5)P3, phosphatidylinositol 3,4,5-trisphosphate; TNF-α, tumour necrosis factor-α.

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do so when applied at very high concentrations), and by definition, to be effective, they must be presented to the cell for a variable period before the cell is exposed to the activating stimulus. Hence, the neutrophil respiratory burst that occurs in response to a secretagogue agonist and results in the release of superoxide anions \( \left( \text{O}_2^\cdot \right) \) may be enhanced up to 20-fold by prior exposure of cells to a priming agent [1]. Likewise, substantial priming of agonist-induced degranulation [2] and the generation of lipid mediators (principally arachidonic acid (AA), leukotriene \( \text{B}_4 \) (LTB\(_4\)) and platelet-activating factor (PAF); [3,4]) has also been described. A wide variety of substances, both physiological and pharmacological, have now been shown to act as priming agents (see Table 1), and the differing signal transduction routes utilized by these agents reflect the varying preincubation times required to initiate maximal priming, which range from a few seconds (e.g. ATP) to over an hour [e.g. lipopolysaccharide (LPS), interferon-\( \gamma \)] (Table 1).

### Table 1 Variability of neutrophil priming agents

<table>
<thead>
<tr>
<th>Priming agent</th>
<th>Time required to induce maximal priming</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP</td>
<td>15 s</td>
<td>[5]</td>
</tr>
<tr>
<td>Substance P</td>
<td>1 min</td>
<td>[6]</td>
</tr>
<tr>
<td>Ionomycin</td>
<td>2 min</td>
<td>[7]</td>
</tr>
<tr>
<td>Inositol hexakisphosphate</td>
<td>2 min</td>
<td>[8]</td>
</tr>
<tr>
<td>L-selectin cross-linking</td>
<td>3 min</td>
<td>[9]</td>
</tr>
<tr>
<td>PAF</td>
<td>5 min</td>
<td>[10]</td>
</tr>
<tr>
<td>CD18 cross-linking</td>
<td>5 min</td>
<td>[11]</td>
</tr>
<tr>
<td>TNF-( \alpha )</td>
<td>10 min</td>
<td>[12]</td>
</tr>
<tr>
<td>Interleukin-( \beta )</td>
<td>10 min</td>
<td>[13]</td>
</tr>
<tr>
<td>Orthovanadate</td>
<td>10 min</td>
<td>[14]</td>
</tr>
<tr>
<td>Influenza A virus</td>
<td>30 min</td>
<td>[15]</td>
</tr>
<tr>
<td>LPS</td>
<td>120 min</td>
<td>[16]</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>120 min</td>
<td>[17]</td>
</tr>
<tr>
<td>Interferon-( \gamma )</td>
<td>120 min</td>
<td>[18]</td>
</tr>
</tbody>
</table>

**Figure 1** Neutrophil priming by inflammatory mediators results in enhanced superoxide production

**Importance of priming in vivo**

Although priming was initially described as an *in vitro* phenomenon, many priming agents have clear biological relevance *in vivo* and are released in response to infection, trauma and haemorrhage. For example, circulating endotoxin has been associated with the development of ARDS [18], and persistent high levels of plasma tumour necrosis factor-\( \alpha \) (TNF-\( \alpha \)) and interleukin-6 have been linked to poor outcome in septic shock [19]. Although such cytokines are detectable in the bloodstream only in extreme circumstances, locally generated mediators serve to upregulate the functional responses of extravasated neutrophils; indeed, since cross-linking of neutrophil adhesion receptors is itself a priming stimulus [9,11], the process of extravasation *per se* may result in a degree of priming.

Two approaches have been used to study priming *in vivo*: firstly, investigators have infused priming agents into laboratory animals or human volunteers and studied granulocyte responses; and secondly, the functional responses of neutrophils isolated from patients with a variety of infectious or inflammatory conditions have been assessed and compared with control cells. Continuous endotoxin infusion has been found to prime the respiratory burst of neutrophils isolated from rat liver at 3 h and to a lesser extent at 30 h [20]. Intravenous injection of TNF-\( \alpha \) into healthy human volunteers resulted either in systemic neutrophil activation with elastase and lactoferrin release [21] or in neutrophil priming resulting in enhanced hypochlorous acid release [22]. Primed neutrophils have also been identified in the peripheral blood of patients after blunt trauma [23], ARDS [24], bacterial (Gram-positive and Gram-negative) and fungal infections [25] and in the joints of patients with active rheumatoid arthritis [26].

Neutrophil priming has been shown to be critical for the induction of endothelial injury, both *in vitro* and *in vivo*. Smedley et al. [27] found that neutrophils stimulated with activating agents alone (fMLP, the complement component C5a, or very high concentrations of LPS) produced minimal damage to endothelial monolayers, but when the neutrophils were first primed with lower concentrations of LPS and subsequently stimulated with fMLP or C5a, extensive endothelial cell injury ensued. Moreover, in a rabbit model, intravascular administration of LPS in addition to fMLP greatly enhanced neutrophil vascular sequestration within the lung, and the combination of priming and activating agents led to lung damage, an effect not seen when either substance was used alone; this effect was completely abrogated by prior depletion of neutrophils with nitrogen mustard [28]. Thus priming appears to represent one of the key processes regulating the functional responsiveness of neutrophils, both *in vitro* and *in vivo*, and may play a crucial role in dictating the appropriateness of the neutrophil's response at an inflamed site.
FUNCTIONAL CONSEQUENCES OF NEUTROPHIL PRIMING

Enhancement of the neutrophil respiratory burst activity

Enhancement of the $O_2^-$ response to fMLP has been viewed as the 'gold standard' priming response. Full activation of 'respiratory burst' activity in the neutrophil results from translocation and assembly of the cytosolic components of the NADPH oxidase enzyme system ($p47^\text{phox}$, $p67^\text{phox}$, $p21^\text{ras}$) with the membrane-associated flavocytochrome, cytochrome $b_558$. This translocation is dependent on the co-operative interaction of the various components, and involves multiple $p47^\text{phox}$-directed phosphorylation events [29] and dissociation of the small GTP-binding protein $p21^\text{ras}$ from complexation with its chaperone inhibitor, GDP-dissociation inhibitor. The activated NADPH oxidase then catalyses the following reaction:

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

Hence the NADPH produced by the cytosolic hexose monophosphate shunt functions as an electron donor to reduce two atoms of molecular oxygen [30]. The $O_2^-$ formed in this reaction then rapidly dismutates to form hydrogen peroxide, a reaction catalysed by superoxide dismutase:

$$2O_2^- + 2H^+ \rightarrow O_2 + H_2O_2$$

Thereafter, in the presence of halide ions (preferentially chloride ions), myeloperoxidase released from neutrophil azurophilic granules can catalyse the conversion of $H_2O_2$ into hypohalous acids, such as hypochlorous acid:

$$H_2O_2 + HCl \rightarrow HOCl + H_2O$$

This set of sequential reactions triggered by the NADPH oxidase is essential for efficient bacterial killing. Deficiency of any of the oxidase components results in chronic granulomatous disease; neutrophils from patients with this disease migrate and phagocytose normally but fail to generate a respiratory burst with the resultant failure of intracellular killing leading to recurrent bacterial (especially Staphylococcal) and fungal infection, and early death. Since agonist-stimulated $O_2^-$ generation is the most 'primable' neutrophil function, with the potential to be up-regulated at least 20-fold, the effects of priming agents on the signalling pathways required for competent assembly and activation of the NADPH oxidase represent the obvious target to investigate the mechanisms underlying neutrophil priming.

Shape-change and deformability

When neutrophils are exposed to inflammatory mediators (including priming agents) in a non-gradient manner, they undergo a polarization or shape-change response which is believed to represent frustrated chemotaxis [31], and in many studies the extent of shape-change has been shown to correlate closely with priming of $O_2^-$ generation [31,32]. Shape-change occurs as a result of modifications in cytoskeletal actin and has the key effect of making neutrophils less deformable; this 'stiffening' of the cell has been shown to increase sequestration of neutrophils within the pulmonary capillary bed, which in the lung represents the main anatomical site of neutrophil efflux [33,34]. Even if these structural changes are not integral to priming, prolonged retention of primed neutrophils within the pulmonary capillary bed clearly increases the risk of neutrophil-mediated damage to the vascular endothelium.

Adhesion molecule expression and function

Intravascular sequestration of neutrophils at inflamed sites, and their subsequent trans-endothelial migration, requires precise bipolar regulation of cell adhesion molecule expression and function. These cell adhesion molecules include members of the immunoglobulin superfamily, integrins and selectins. The vascular selectins CD62-P (P-selectin) and CD62-E (E-selectin) and the constitutively expressed leucocyte antigen CD62-L (L-selectin) mediate the initial, low-affinity 'rolling' interaction of neutrophils along the vascular endothelium [35]. The firm adhesion that follows to allow arrest of neutrophil movement is principally a function of the leucocyte $\beta_2$-integrins, particularly CD11b/CD18 (Mac-1), and their endothelial cell ligand intercellular adhesion molecule-1 (ICAM-1). Priming agents, in common with full secretagogue agonists, have the capacity to enhance neutrophil integrin expression and function and induce CD62-L shedding; moreover, each priming agent appears to produce a unique spectrum of effects, with differing and preferential effects on integrin and selectin expression and variable time-courses for the manifestation of these effects [36]. Priming may therefore modulate the adhesive and migratory potential of neutrophils with the effect of promoting recruitment to an inflamed focus.

Degranulation and lipid mediator release

In contrast to the massive enhancement of secretagogue-induced $O_2^-$ release produced by priming, the effects of this process on degranulation are far more modest. For example, although a degree of priming of elastase and myeloperoxidase release was demonstrated by Fittschen et al. [2] in response to LPS, for other measured enzymes the effects of priming and activating agents were simply additive. A more significant priming effect, however, has been shown for LTB$_4$ and AA release [3,4], but the reasons for the differences in 'primability' of these responses have not been explored.
Inhibition of neutrophil apoptosis

It has now become apparent that many priming agents, particularly LPS and granulocyte–macrophage colony-stimulating factor (GM-CSF), in addition to upregulating neutrophil responsiveness, also delay the onset of programmed cell death [37]. Prolongation of the functional lifespan of the neutrophil is now thought to represent a further important mechanism whereby priming may enhance the neutrophil-mediated response to injury or infection. However, it should be noted that priming is not universally associated with delayed apoptosis since the potent priming agent TNF-α has a bimodal effect on this process with accelerated neutrophil apoptosis at early time points (< 8 h) and delayed apoptosis at later times, while PAF has no effect on the rate of constitutive cell death [38].

Neutrophil priming: potential mechanisms

The great diversity of agents capable of inducing neutrophil priming (see Table 1) raises the possibility that a single common priming mechanism may not exist, indeed that there may be class- or even agent-specific variations in the signalling mechanisms whereby the primed state is achieved. The variable potency displayed by different agents for priming (for example, inositol hexakisphosphate induces a very weak priming signal while TNF-α can augment O₂⁻ release 20-fold) also suggests that this process is not an all-or-none phenomenon; in fact, priming agents may well act primarily by recruiting additional cells into an agonist-responsive pool [39]. Nevertheless, the involvement of the following signalling pathways in priming has been the subject of intense study and although considerable controversy remains, certain common themes are now emerging.

Modulation of agonist receptors

Priming-induced upregulation of the number and/ or affinity of agonist receptors that trigger activation represents an obvious potential mechanism to augment subsequent responses to a secretagogue agonist, and indeed some priming agents have been shown to have such effects on the G-protein-coupled fMLP receptor (Table 2). However, such results need to be interpreted with caution, not least because the receptor-generated component of the signal may not be the rate-limiting part of the response, and increasing the receptor number may serve only to increase ‘receptor reserve’ and hence the sensitivity rather than the magnitude of the agonist response. Furthermore, O’Flaherty et al. [40] demonstrated that while TNF-α increased fMLP binding to neutrophils, this effect lagged behind the cytokine’s ability to prime fMLP-induced degranulation and hence could not be responsible for priming. Again, Roberts et al. [44] showed that priming of the respiratory burst was maximal with an interleukin-8 concentration of 10–50 ng/ml, whereas no increase in fMLP receptor number was detectable at concentrations below 100 ng/ml. Thus effects on fMLP receptor number can be clearly dissociated from priming, and since neutrophils possess granule-associated fMLP receptors that are mobilized to the cell surface on degranulation, the presence of increased surface fMLP-binding may simply reflect stimulated exocytosis.

Few investigators have quantified the effects of priming on other agonist receptors; however, O’Flaherty et al. [40] showed that TNF-α priming of the degranulation response to PAF and LTB₄ was associated with a reduction in surface LTB₄ receptors and only a transient increase in PAF receptors, again arguing against a critical role for receptor modulation in priming.

Heterotrimeric GTP-binding proteins (G-proteins)

In neutrophils the predominant G-protein α-subunit is G₁₂ [45], although the closely related G₁₃ is also present. The identity of the Gᵦ₁ subunits, and the specific G-protein(s) coupling the fMLP receptor to its various effectors are unknown. Both G₁₂ and G₁₃ are substrates for pertussis toxin, which inactivates them by ADP-ribosylation. As pertussis toxin abolishes fMLP-induced O₂⁻ production it cannot be used to dissect the potential involvement of G-proteins in priming this response. However, functional responses to phorbol myristate acetate or AA are not suppressed by pertussis toxin, and Berkow and Dodson [46] demonstrated that the slight priming effect of TNF-α on phorbol myristate acetate-induced O₂⁻ production was not altered by pretreatment with this toxin. Variable data have been obtained regarding GM-CSF priming of AA-induced oxidase activity, which has been reported to be either unaffected [47] or abolished [48] by pertussis toxin: hence this does not appear to be an ideal model system or tool for investigating the role of G-proteins in priming.

An alternative approach to address the potential role of G-proteins in mediating the primed response has been to assess the effects of priming agents on G-protein localization within the cell. Translocation of G₁₂ to the neutrophil membrane has been reported in response to priming concentrations of LPS [49], GM-
CSF [50] and TNF-α [51], and the time-course of \( \Delta G_{\text{int}} \) translocation after treatment with LPS, PAF and TNF-α appears to correlate closely with that for priming of the \( O_2^- \) response [52]. However, significant amounts of \( \Delta G_{\text{int}} \) is associated with the plasma membrane fraction under basal conditions where no \( O_2^- \) generation is observed in response to agonist stimulation, and the priming-associated augmentation of membrane-associated G-protein in these studies was at most 3-fold compared with a 10–20-fold increase in \( O_2^- \) release. Finally, the most compelling evidence against a central role for G-proteins as effectors of the primed response is that certain key G-protein-linked signalling events (notably inositol 1,4,5-trisphosphate \( \text{Ins}(1,4,5)P_3 \) generation, see below) are not enhanced in primed cells, suggesting that increased G-protein availability plays a minor, if any, role in priming.

### Phospholipase C activation

The \( \beta \) isoform of phospholipase C (PLC) is the major phosphoinositidase C (PIC) activity present in neutrophils, and is activated predominantly by \( G_{\beta \gamma} \) rather than \( G_{\alpha} \) subunits [53] to hydrolyse its membrane phospholipid substrate phosphatidylinositol 4,5-bisphosphate \( \text{PtdIns}(4,5)P_2 \). This reaction generates \( \text{Ins}(1,4,5)P_3 \), which induces \( Ca^{2+} \) release from intracellular stores and diacylglycerol, which activates protein kinase C. Diacylglycerol can also be generated, often in a more sustained fashion, by agonist stimulation of phospholipase D (PLD), which hydrolyses phosphatidylinosamine.

**\( \text{Ins}(1,4,5)P_3 \) accumulation.** The application of fMLP to a neutrophil population results in a rapid but transient elevation of \( \text{Ins}(1,4,5)P_3 \), with peak levels at 10–15 s and thereafter a rapid return to basal levels. Despite the clear demonstration that the resultant calcium transient is necessary for activation of the respiratory burst [54], remarkably few studies have examined the potential for this second-messenger pathway to be upregulated in the priming process. In the three studies that have been published, GM-CSF either had no effect on [48, 55], or caused a small increase in [56], resting and stimulated \[^{3}P\]Ins(1,4,5)P\(_3\) levels. Unfortunately the \[^{3}P\]Ins labelling protocols used in these studies that allow identification and quantification of the separate inositol polyphosphates do not label the phosphoinositides to isotopic equilibrium and may also have induced a degree of basal priming. Using the \( \text{Ins}(1,4,5)P_3 \) mass assay described by Challiss et al. [57] to avoid these problems, we have demonstrated (A. M. Condliffe, E. R. Chilvers, P. Hawkins and L. Stephens, unpublished work) that the classic priming agents PAF and TNF-α have no effect on baseline or fMLP-stimulated \( \text{Ins}(1,4,5)P_3 \) accumulation, which appears to be a fully competent response even in unprimed cells which produce little or no \( O_2^- \) on agonist challenge. Although in these studies fMLP-stimulated \( \text{Ins}(1,4,5)P_3 \) levels returned to baseline values by 30 s in both primed and unprimed cells, an effect of priming on \( \text{Ins}(1,4,5)P_3 \) flux cannot be excluded. This would require an analysis of total \[^{32}P\]//[^{3}H]InsP\(_x\) accumulation measured in the presence of lithium to block the inositol monophosphatase and hence prevent recycling of the inositol headgroup.

**Elevation of \( Ca^{2+} \).** Binding of \( \text{Ins}(1,4,5)P_3 \) to its intracellular receptor induces a conformational change in the receptor resulting in \( Ca^{2+} \) release from intracellular stores which in turn activates \( Ca^{2+} \) influx from the external medium (capacitative \( Ca^{2+} \) entry). While it is now well established that a rise in \( Ca^{2+} \), is an essential step in neutrophil activation and \( O_2^- \) generation in that neutrophils depleted of \( Ca^{2+} \) do not undergo a respiratory burst [54] and oxidase activation only occurs if a threshold \([Ca^{2+}]/\) is exceeded [58], its role in priming is less clear.

Previous studies have demonstrated that calcium ionophores such as ionomycin can act as priming agents, and that elevation of \( [Ca^{2+}] \), correlates closely with the degree of priming [7]. However, as can be seen in Table 3, there are conflicting reports on the effects of individual priming agents on resting and stimulated \( [Ca^{2+}] \),. For example, it is generally agreed that the archetypal priming agent TNF-α does not mobilize \( Ca^{2+} \) or augment the \( Ca^{2+} \) transient generated in response to other agonists, and similar results have been reported for other agents such as substance P. Likewise, despite consistent reports that PAF mobilizes \( Ca^{2+} \), and may augment subsequent agonist-induced \( Ca^{2+} \) transients, Koenderman et al. [68] reported that PAF-induced priming was only partially inhibited under \( [Ca^{2+}] \)-buffering conditions. Furthermore, in experiments by Wymann et al. [69], application of fMLP 2 min after a low ‘priming’ concentration of phorbol myristate acetate resulted in a rapid (2 s) enhancement of chemiluminescence that preceded the rise in fura-2 fluorescence, again suggesting that priming of the oxidative burst does not rely on enhanced calcium fluxes. Taken together, these results imply that while an elevation in \([Ca^{2+}]\), can induce priming and indeed is essential for full receptor-mediated NADPH oxidase assembly and activation, it is not an obligate step for priming and hence is unlikely

### Table 3 Effect of priming agents on basal- and agonist-stimulated neutrophil calcium transients

<table>
<thead>
<tr>
<th>Priming agent/( \text{C}_2^{2+} ) indicator</th>
<th>( \text{Ca}^{2+} ) (Basal)</th>
<th>( \text{Ca}^{2+} ) (Stimulated)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>LPS/fura2</td>
<td>↑</td>
<td>↑</td>
<td>[59]</td>
</tr>
<tr>
<td>LPS/fura2</td>
<td>↑</td>
<td>↑</td>
<td>[3]</td>
</tr>
<tr>
<td>LPS/fluoro-3</td>
<td>↑</td>
<td>↑</td>
<td>[60]</td>
</tr>
<tr>
<td>LPS/fura2</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>TNF-α/fura2</td>
<td>→</td>
<td>→</td>
<td>[61]</td>
</tr>
<tr>
<td>TNF-α/fura2</td>
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<td>→</td>
<td>[62]</td>
</tr>
<tr>
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<td>↑</td>
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<td>[63]</td>
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<tr>
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<td>↑</td>
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<tr>
<td>Substance P/fura2</td>
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</tr>
<tr>
<td>ATP/fura2</td>
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<tr>
<td>Vanadate/fura2</td>
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<td>↑</td>
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</tr>
</tbody>
</table>
to represent a ‘final common pathway’ mediating priming.

Diacylglycerol and protein kinase C. Diacylglycerol produced by the action of PLC and/or PLD activates members of the protein kinase C (PKC) family [70], inducing phosphorylation of several substrates (including p47phox and activation of the NADPH oxidase. Neutrophils have been shown to possess at least two PKC isoforms, the conventional \( \beta_1 \)-PKC (the most abundant isoform) and the novel \( \gamma \)-PKC [71]. While it is important to emphasize that phorbol esters (pharmacological activators of classical PKCs) used alone can fully activate the NADPH oxidase, in sub-activating concentrations these agents prime neutrophils, indicating a possible role for PKC in the priming process.

The effects of priming agents on the PKC-activator diacylglycerol are somewhat controversial. It has been shown that GM-CSF alone produces only a small increase in neutrophil diacylglycerol mass but can markedly increase its accumulation in response to fMLP [55,72]; this effect presumably reflects enhanced fMLP-induced PLD activation as PIC-mediated PtdIns(4,5)P_2 hydrolysis is not increased. In contrast, Bauldry et al. [73] found that treatment of polymorphonuclear cells with TNF-\( \alpha \) alone could not enhance the fMLP-induced diacylglycerol response in the absence of cytochalasin B.

Whereas some of these studies suggest that priming may enhance agonist-stimulated diacylglycerol generation, subsequent studies have failed to show translocation or activation of PKC in primed cells: LPS [59], TNF-\( \alpha \) [46] and GM-CSF [65] all fail to induce PKC translocation under priming conditions. Thus the weight of evidence remains against a significant role for augmented PKC activation in priming.

PLD activation

PLD catalyses the cleavage of the terminal phosphodiester bond of phosphatidylcholine to yield phosphatic acid and an inactive choline molecule, and fMLP stimulation of a phosphatidylcholine-specific PLD activity is a well-documented response in neutrophils [74]. Phosphatic acid is converted to diacylglycerol by phosphatidate phosphohydrolase [75], and this results in a second, often more sustained phase of diacylglycerol generation than that resulting from the activation of PIC [75,76]. Inhibition of PLD-mediated phosphatidic acid production prevents neutrophil fMLP-induced \( O_2^- \) release [77].

Bourgoin et al. [55] and Bauldry et al. [73] both studied the effects of priming (with GM-CSF and TNF-\( \alpha \) respectively) on PLD activity and demonstrated that while treatment of neutrophils with the priming agent alone did not result in accumulation of phosphatic acid, the subsequent fMLP-induced accumulation was augmented and prolonged in primed cells. In the latter study, phosphatic acid production was found to correlate closely with \( O_2^- \) generation. While other priming agents have not been examined in this model, these two studies provide good evidence that regulation of agonist-induced PLD activity may represent an important target for TNF-\( \alpha \) and GM-CSF priming activity. Moreover, Waite et al. [78] have recently identified a novel phosphatidic acid-stimulated protein kinase activity in human neutrophils which is capable of phosphorylating (among other substrates) p47phox.

Phospholipase A_2 (PLA_2) activation

PLA_2 releases free AA from cellular phospholipids [79] in what is believed to be the rate-limiting step in the synthesis of biologically active eicosanoids. Priming agents can enhance the synthesis of bioactive lipids (AA, PAF, LTB_4) in response to activating stimuli [3,47,80]. In eosinophils, in marked contrast to the situation in neutrophils, PLA_2 plays a key role in activating the NADPH oxidase, a response that is both Ca\(^{2+}\)- and phosphoinositide 3-hydroxykinase (PI3K)-independent. Hence, activation and/or translocation of PLA_2 has been suggested as a potential mechanism of priming. However, agonist-stimulated PLA_2 activation and AA release, unlike \( O_2^- \) generation, are events that are entirely dependent on Ca\(^{2+}\) influx and are unaffected by the PI3K inhibitor wortmannin (see later): hence PLA_2 activity, although ‘primable’, appears to be mediated via a discrete set of signalling pathways not involved in NADPH oxidase assembly [81].

Investigations into the mechanism(s) of PLA_2 priming have demonstrated that a PLA_2 activity is translocated from the cytosolic to the plasma-membrane fraction in LPS-primed neutrophils [82], and Doerfler et al. [4] in confirming this finding also identified that LPS induced the phosphorylation of PLA_2, an event previously correlated with PLA_2 activation [83]. However, LPS priming of LTB_4 release appeared to be agonist-selective and was not observed using fMLP as the secretagogue agonist [3], and again none of these studies was able to link PLA_2 activation with priming of the respiratory burst.

Worthen et al. [64] correlated LPS-induced synthesis of intracellular PAF with priming of the respiratory burst, and suggested that PAF could be responsible. However, Stewart et al. [84] demonstrated that neither blockade of PAF receptors (with WEB 2086) nor inhibition of LTB_4 synthesis (with CGS 8515) influenced the priming of fMLP-mediated \( O_2^- \) generation by TNF-\( \alpha \) or GM-CSF.

Finally, Ely et al. [85] have now published data demonstrating that the release of physiological amounts of AA in the neutrophil does not induce \( O_2^- \) generation or degranulation, or influence fMLP or primed fMLP responses. It therefore seems unlikely that mobilization of AA from cellular phospholipids plays a major role in neutrophil priming activation.

Protein phosphorylation and neutrophil priming

The cytosolic oxidase component p47phox becomes extensively phosphorylated on cellular activation with
the phosphorylation targets consisting of a group of serines in the carboxy-terminus of the peptide [29]. The enzyme(s) responsible have not been definitively identified. Activation of neutrophils by fMLP and other stimuli is associated with increased tyrosine phosphorylation of multiple protein substrates [86]. Tyrosine phosphorylation has also been described in response to several priming agents, including GM-CSF [67], TNF-α [46,63], substance P [63] and PAF [87]. One of the targets appears to be mitogen-activated protein kinases [88]. In several of these studies, the time-course of tyrosine phosphorylation was consistent with a role in priming, and manipulation of tyrosine phosphorylation levels using tyrosine phosphatase or tyrosine kinase inhibitors resulted in priming and inhibition of priming respectively [14]. It therefore seems likely that tyrosine phosphorylation plays an important, if as yet uncharacterized, role in priming.

PI3K

Many neutrophil secretagogue agonists stimulate PI3K with resultant formation of phosphatidylinositol 3,4,5-trisphosphate [PtdIns(3,4,5)P3] from PtdIns(4,5)P2 [89]. Indeed, agonist-stimulated PtdIns(3,4,5)P3 accumulation was first identified in the neutrophil and much of the cardinal biochemistry regarding PI3K activation and PtdIns(3,4,5)P3 signalling has been undertaken in this cell. The ability of wortmannin, a relatively specific and irreversible inhibitor of neutrophil PI3K activity, to abolish both fMLP- and phagocytosis-induced respiratory burst activity without any effect on agonist-induced [Ca2+]i flux, PKC-mediated NADPH-oxidase activation or granule exocytosis [54,90] has provided strong evidence to support a second-messenger role for PtdIns(3,4,5)P3 in O2− generation. In addition to the well-characterized and seemingly ubiquitous p110α/p85 PI3K, neutrophils also possess a novel Gαq-regulated p120/p101 PI3K activity [91]. Moreover, we have recently demonstrated that priming of human neutrophils by TNF-α results in a substantial enhancement of fMLP-stimulated PtdIns(3,4,5)P3 accumulation (A. M. Condiffe, E. R. Chilvers, P. Hawkins and L. Stephens, unpublished work), further implicating PtdIns(3,4,5)P3 as a messenger critical to signalling the primed state. This finding fits well with the recent data indicating that PtdIns(3,4,5)P3 can activate the small GTP-binding protein p21Rac [92], which is an essential component of the NADPH oxidase, and can activate a p21-activated kinase which phosphorylates p47phox [93].

A MODEL OF NEUTROPHIL PRIMING

The variety of priming agents, and the complexity of the data reviewed above, suggest that priming is not a simple event, and that several complementary signal transduction processes are involved in the overall response. The final common pathway of priming of the neutrophil respiratory burst is the activation of the NADPH oxidase, and many signals may impinge on this process (Figure 2). For example, the phosphorylation of p47phox appears to require the co-ordinated activity of a number of kinases including PKC, p42/p44MAPK, p21-activated kinase and a phosphatidic acid-activated protein kinase, and this event may allow conformational changes promoting membrane translocation and oxidase activation. Rac must also dissociate from its GDP-dissociation inhibitor to access the other oxidase components, and this dissociation may be promoted by signalling molecules derived from PI3 and PI3K and perhaps PLA2 activation [99]. Likewise, the increased membrane expression of agonist receptors and G-proteins may be involved in enhancing the sensitivity of the cell to stimulating agonists and in combating receptor desensitization by supplying new signalling units to the plasma membrane. It is clear therefore that the redundancy and cross-talk that exist within this system allow widely differing agents to modulate and arrive at a final common effector pathway, although the disparate routes taken may explain the variable and selective priming properties of these agents. It is also likely that priming of responses other than O2− generation may rely on different mechanisms; for example, phosphorylation and Ca2+-dependent translocation of PLA2 may be more important in priming the release of lipid mediators, and Ca2+ transients may dominate degranulation responses.

NEUTROPHIL DEPRIMING

Studies of human peripheral blood neutrophils primed with LPS or G-CSF [94] suggest that the capacity for enhanced respiratory burst activity is maintained for at least 24 h; in vivo infusion of endotoxin also engenders a primed state in sheep peripheral blood and bone marrow-derived neutrophils that lasts for at least 24 h [95]. However, the assumption that neutrophil priming is an irreversible process was recently challenged by the demonstration that both hypotonic shock and cell-swelling could induce a hyperresponsive state of the human neutrophil NADPH oxidase that was only temporary [96,97]. A similar phenomenon was subsequently identified after exposure of neutrophils to the highly charged Ca2+-chelator inositol hexakisphosphate [98]. Recently, it has been demonstrated that receptor (PAF)-mediated priming of neutrophils is also fully reversible, with cells able to return to a non-polarized morphology with low CD11b/CD18 activity and minimal O2− release to fMLP. Furthermore, the cells which had 'deprimed' could subsequently be fully 'reprimed' by either TNF-α or PAF [32]. This ability of neutrophils to undergo a complete cycle of priming/depriming/repriming permits a previously unrecognized flexibility in the control of neutrophil behaviour at an inflammatory site, and provides a potential mechanism for damage limitation and 'cell rescue' at an inflamed site.
CONCLUSION

This review has outlined the importance of priming as a key regulator of neutrophil function and highlighted the crucial role priming plays in dictating neutrophil-mediated tissue damage in vivo. The recent finding that priming is reversible further underlines the potential for therapeutic intervention which, if successful, could offer the possibility of modulation rather than ablation of neutrophil activity, and facilitate the safe and timely removal of these cells from the inflamed site via apoptosis. Finally, although the signalling pathways orchestrating NADPH oxidase priming remain elusive, upregulated PLD and PI3K activity appear to be attractive candidate targets.

ACKNOWLEDGMENTS

E.R.C. was supported by a Wellcome Trust Senior Research Fellowship in Clinical Science. A.M.C. was supported by an MRC Training Fellowship, and E.K. by a Wellcome Trust Prize Studentship.

REFERENCES


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