Modulation of Neutrophil Oxidative Responses to Soluble Stimuli by Platelet-Activating Factor

By James C. Gay, Jeffrey K. Beckman, Karen A. Zaboy, and John N. Lukens

The role of platelet activating factor (PAF) as a regulator of human neutrophil superoxide (O$_2^-$) generation in response to soluble and particulate stimuli was examined. At concentrations $>$10$^{-7}$ mol/L, PAF alone induced a brief burst of O$_2^-$ production. When cells were exposed to PAF and either the chemotactic peptide n-formylmethionyl-leucyl-phenylalanine (FMLP 10$^{-7}$ mol/L) or the tumor promoter phorbol myristate acetate (PMA 10 ng/mL), a marked synergistic augmentation of O$_2^-$ release was noted when compared to control cells stimulated with FMLP or PMA alone. Mean percentage of enhancement by 10$^{-8}$ mol/L of PAF was 297% ± 35% (n = 9) of control responses to FMLP and 185% ± 16% (n = 3) of control responses to PMA. Consistent enhancement occurred with PAF concentrations of as low as 10$^{-9}$ mol/L. Enhancement could be demonstrated when neutrophils were exposed to PAF either at the same time as, or up to 60 minutes prior to, the second stimulus, and was neither reversed by removal of PAF from the medium prior to addition of FMLP or PMA nor dependent on the presence of extracellular divalent cations. Continuous recordings revealed that the enhancement was due to an increased maximal rate of O$_2^-$ production. In contrast, PAF concentrations up to 10$^{-6}$ mol/L had only a minimal effect on the response to neutrophils to opsonized zymosan. Analysis of the enhancing properties of lipids structurally related to PAF revealed that the critical moiety was the saturated fatty acid at position 1. These results indicate the presence of a PAF-mediated positive feedback loop whereby the oxidative burst induced by some soluble stimuli is augmented. Modulation of neutrophil O$_2^-$ production by PAF may serve to amplify neutrophil oxidative responses at sites of inflammation.

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Neutrophil suspensions. Human neutrophils were isolated from heparinized venous blood from healthy adult volunteers by Ficoll-Hypaque gradient centrifugation, as previously described.  

Neutrophil superoxide release. Superoxide (O$_2^-$) was assayed by measuring the O$_2^-$-dependent (SOD-inhibitable) reduction of ferricytochrome c, as previously described.  

RESULTS  

PAF-induced O$_2^-$ production. As reported by others, PAF is a direct stimulus of neutrophil O$_2^-$ generation (Fig 1A). However, at the cell concentrations used in these studies (1.5 x 10$^6$ PMNs), only a small amount of O$_2^-$ was released in response to PAF and only at concentrations >10$^{-4}$ mol/L. The direct response to PAF was decreased to 68% of control values when calcium and magnesium were omitted from the suspending medium (data not shown). 

Effects of PAF on neutrophil responses to other oxidative stimuli. Also illustrated in Fig 1 is the effect of PAF on neutrophil oxidative responses to FMLP, PMA, and opsonized zymosan. Responses to FMLP and PMA (Fig 1B and C) were consistently enhanced by PAF in concentration-dependent fashion. At the highest PAF concentration used (10$^{-3}$ mol/L), enhancement averaged 297% ± 9% of controls for FMLP (10$^{-7}$ mol/L) and 185% ± 16% of controls for PMA (10 ng/mL). Consistent slight enhancement was evident at PAF concentrations as low as 10$^{-9}$ mol/L (143% ± 12% of controls with FMLP and 126% ± 7% of controls for PMA). The concentration of FMLP used in these experiments (10$^{-7}$ mol/L) represents an optimal oxidative concentration of the peptide. In the presence of higher PMA concentrations, the percentage of enhancement by PAF decreased as the control response to PMA increased such that no enhancement could be demonstrated with a PMA concentration of 3.2 x 10$^{-7}$ mol/L (200 ng/mL; Table 1). Maximal absolute increase in O$_2^-$ production seen in the presence of PAF was noted at a PMA concentration of 10 ng/mL, although the percentage of increase was greater at lower PMA concentrations. PAF, therefore, was a more potent enhancer at submaximal concentrations of the phorbol ester. The effect of PAF on the O$_2^-$ response to FMLP and low concentrations of PMA was synergistic rather than additive. In contrast, PAF concentrations up to 10$^{-4}$ mol/L had only a minimal enhancing effect on the response to zymosan (250 μg/mL) (Fig 1D). 

Characteristics of PAF-enhanced responses. PAF enhancement of the neutrophil oxidative response to FMLP did not require incubation of neutrophils with the lipid prior to exposure to the peptide (Table 2). In our experience and that of other investigators, the duration of O$_2^-$ production in

![Image](www.bloodjournal.org)
response to PAF alone is brief. To determine whether the enhancing effect of PAF could be demonstrated after neutrophils had reached the plateau phase of response to PAF alone, cells were incubated with PAF for periods up to 60 minutes prior to addition of FMLP. As shown in Table 2, neutrophils exposed to PAF retained an enhanced responsiveness to FMLP even after 60 minutes of preincubation, although the effect tended to diminish with longer incubation times. Thus, PAF not only had synergistic effects with FMLP, but its enhancing effect persisted after the direct stimulating effect of PAF had reached completion. Similar effects were noted when PMA was used in place of FMLP as the secondary stimulus (data not shown).

Table 2. Effect of Preincubation Time on PAF-Mediated Enhancement of FMLP-Induced O2 Generation

<table>
<thead>
<tr>
<th>Preincubation Time (min)</th>
<th>O2 Production† (% Control Response)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>52.45 ± 0.36 (203)</td>
</tr>
<tr>
<td>1</td>
<td>52.90 ± 1.60 (204)</td>
</tr>
<tr>
<td>5</td>
<td>56.60 ± 0.15 (219)</td>
</tr>
<tr>
<td>10</td>
<td>50.11 ± 0.67 (194)</td>
</tr>
<tr>
<td>20</td>
<td>48.34 ± 1.05 (187)</td>
</tr>
<tr>
<td>30</td>
<td>47.12 ± 1.85 (182)</td>
</tr>
<tr>
<td>40</td>
<td>45.48 ± 0.94 (176)</td>
</tr>
<tr>
<td>60</td>
<td>37.67 ± 0.81 (146)</td>
</tr>
<tr>
<td>FMLP alone</td>
<td>25.87 ± 0.68 (100)</td>
</tr>
</tbody>
</table>

PAF, platelet-activating factor; FMLP, formyl-methionyl-leucyl-phenylalanine.

*Neutrophils (1.5 × 10⁶) were exposed to PAF (10⁻⁶ mol/L) at 37 °C for 0 to 60 minutes prior to stimulation with FMLP (10⁻⁷ mol/L) for 5 minutes.
†Neutrophils (nmols O₂ per 1.5 × 10⁶); mean ± SE of triplicate determinations in a single representative experiment.

Because we had demonstrated that optimal direct stimulation of O₂⁻ production by PAF was dependent on extracellular calcium, we sought to determine the calcium requirement for PAF enhancement of the response to FMLP and PMA. As noted by other investigators,¹⁷ the response of neutrophils to FMLP was decreased in the absence of extracellular calcium (Table 3). However, in the absence of calcium, PAF-treated cells still exhibited enhanced responsiveness to the peptide, in a degree similar to enhancement seen in cells maintained in calcium-containing buffer. Enhanced responses were also noted when cells were exposed to PMA despite the absence of extracellular cations. Therefore, augmentation of FMLP-stimulated and PMA-stimulated O₂⁻ production by PAF did not require the presence of the divalent cation.

It is well known that the fungal metabolite cytochalasin B enhances measurable O₂⁻ generation in response to chemotactic peptides. The mechanism for this effect is not entirely clear but may be related to failure to internalize O₂⁻ produced because of impaired microfilament assembly or to aggregation or uncovering of peptide receptors.¹⁸ Despite their already enhanced responsiveness to FMLP in the presence of cytochalasin B, neutrophils generated even greater amounts of O₂⁻ anion when simultaneously exposed to PAF (Table 3).

To determine whether the cellular response to PAF that resulted in enhanced responsiveness required the continued presence of PAF in the medium, neutrophils were exposed to PAF and washed twice prior to stimulation with FMLP or PMA. After being washed, PAF-treated cells continued to exhibit greater O₂⁻ production than did buffer-treated controls (Table 3).

Because other investigators have shown that PAF induces LTB₄ production in neutrophils,¹²,¹³ we sought to determine whether the enhancing effect of PAF was dependent on formation of the leukotriene. As previously demonstrated¹⁹ 10 μmol/L of ETYA, a lipoxigenase inhibitor, inhibited the response to FMLP by 59% ± 1% [FMLP alone = 16.54 ± 0.50 nmols O₂⁻ (n = 2); ETYA + FMLP = 6.77 ± 0.19 nmols O₂⁻ (n = 2)]. Despite the presence of the lipoxigenase inhibitor, PAF continued to enhance oxidative responses to FMLP (ETYA + PAF + FMLP = 33.23 ± 0.90 nmols O₂⁻). Similarly, the presence of ETYA did not alter PAF enhancement of responses to PMA (data not shown). Thus, it is unlikely that the enhancing effect of PAF on neutrophils is mediated by LTB₄.

Effect of PAF on the kinetics of O₂⁻ production. We next examined the effects of PAF on the kinetics of the oxidative response by the continuous recording method of Cohen and Chovaniec.¹⁵ The neutrophil oxidative response to the chemotactic peptide is characterized by a three- to six-second lag phase before O₂⁻ production is detected. This is followed by a progressive increase in O₂⁻ release which
terminates three to four minutes after onset. The addition of PAF prior to addition of FMLP altered neither the activation time nor the total period of time during which the $O_2^-$ forming oxidase was active (Fig 2). PAF-treated cells exhibited a markedly enhanced rate of $O_2^-$ production, however, with a mean maximal rate ($V_{\text{max}}$) 205% ± 42% of control ($n = 3$). Thus, a markedly enhanced rate of $O_2^-$ generation accounted for the augmentation of cumulative $O_2^-$ measurements in FMLP-stimulated cells. An increase in $V_{\text{max}}$ could be demonstrated even when PAF was added after FMLP. When PAF was added one minute after FMLP, the time when the rate of $O_2^-$ production is constant, the subsequent rate was increased although the total time of oxidase activity was unchanged. When PAF was added to cells two minutes after FMLP, when $O_2^-$ production is beginning to diminish, an immediate increase in $V_{\text{max}}$ still occurred, although the magnitude of the increase was less than when PAF was added earlier.

PAF also had striking effects on the kinetics of the response to PMA. The cellular response to PMA is characterized by a lag period prior to the onset of measurable $O_2^-$ generation which is dependent on the PMA concentration. Figure 3 shows that when PAF was added simultaneously with PMA, the lag time was drastically shortened and the rate of $O_2^-$ production increased markedly (mean percentage of enhancement 152% ± 11%, $n = 7$). When PAF was added two minutes prior to PMA, the lag time was unchanged (data not shown) although the $V_{\text{max}}$ to $O_2^-$ generation remained enhanced. It appears likely that the shortened activation time seen when both agents were added simultaneously is due to the direct effect of PAF, since the lag time in response to this agent is very short. When PMA was added after the direct effect of PAF was completed, the contribution of PAF to the shortened lag time was not seen. Even after preincubation of up to 20 minutes with PAF, an enhanced rate of $O_2^-$ production was seen after addition of PMA.

Effect of structurally similar lipids on oxidative response. To determine the specificity of PAF in enhancing $O_2^-$ production, we studied the ability of several structurally-related lipids to enhance responses to FMLP. Because PAF consists of a glycerol backbone with an ether-linked C16 or C18 saturated fatty acid at position 1, an acetyl group at position 2, and a phosphorylcholine at position 3, we chose compounds with side chains similar to PAF in at least one of three positions. Figure 4 demonstrates dose-response curves for representative lipids. Removal of the acetyl group from position 2 of PAF yielded a compound (lyso-PAF) with 52% of the enhancing activity of PAF at 10⁻⁵ mol/L. Lyso-PC (substitution of a palmitoyl group for the ether-linked moiety of lyso-PAF) and monopalmitin (removal of the phosphorylcholine group from lyso-PC) had enhancing potency roughly equal to that of lyso-PAF at 10⁻⁵ mol/L (data not shown). Diolein, a diacylglycerol, had no significant enhancing capabilities. Neither did glycerophosphorylcholine have any effect (data not shown). In contrast, another diacylglycerol, 1-oleyl, 2-acetyl glycerol, had enhancing activity of magnitude similar to PAF at 10⁻⁵ mol/L, although PAF was a more potent enhancer at lower concentrations. These results
suggest that the fatty acid moiety and diacylglycerol-like activity are important components of the enhancing properties of PAF.

**DISCUSSION**

PAF triggers neutrophil aggregation, degranulation, and chemotaxis, and is a weak stimulus of $O_2^-$ production. In addition to these direct effects, PAF acts as a regulator of neutrophil responses to other stimuli. Lin et al.\(^2\) have shown that PAF desensitizes neutrophils to aggregation induced by LTB\(_4\). Jouvin-Marche et al. demonstrated that PAF enhanced the $O_2^-$ response of neutrophils to zymosan particles.\(^2\) The degree of enhancement was consistent with an additive effect of the two oxidative stimuli, in agreement with our own studies using the same particulate stimulus. Ingraham et al.\(^2\) demonstrated that PAF enhanced oxygen uptake in neutrophils exposed to FMLP. The present studies suggest that PAF modulates neutrophil $O_2^-$ production in response to the chemotactic peptide FMLP and the phorbol ester PMA and synergistically enhances the oxidative response to these soluble stimuli. Enhancement could be demonstrated without a preincubation period and without the presence of extracellular calcium or magnesium. It was not reversed by removal of PAF from the medium prior to FMLP or PMA exposure.

The mechanism through which PAF accomplishes enhancement remains unclear, although several potential hypotheses seem unlikely in light of our data. Because PAF stimulates LTB\(_4\) production in neutrophils, some investigators have suggested that cellular responses to the phospholipid may be mediated by LTB\(_4\).\(^2\) Our demonstration that both LTB\(_4\) and PAF enhance the oxidative response to FMLP is in keeping with this hypothesis.\(^10\) Although an attractive explanation, it seems unlikely for several reasons. First, enhancement of the response to FMLP by LTB\(_4\) is seen only after cells are exposed to the leukotriene for several minutes prior to addition of the peptide.\(^10\) In contrast, no preincubation period is necessary using PAF as the enhancing agent. Second, we noted marked augmentation of the response to PMA in PAF-treated cells, whereas LTB\(_4\) had no effect on the response of cells to the phorbol ester.\(^10\) Third, the lipoxigenase inhibitor ETYA did not eliminate PAF-mediated enhancement. Thus, it seems likely that PAF exerts its enhancing effect on neutrophil $O_2^-$ production by a mechanism independent of the generation of LTB\(_4\). Alternatively, it is possible that enhancement is mediated by an increase in cellular uptake of calcium. The finding that enhancement of FMLP-induced and PMA-induced oxidative responses by PAF occurred even in the absence of extracellular Ca\(^{2+}\) suggests that the mechanism of PAF enhancement does not involve transport of Ca\(^{2+}\) into the cell. From our data, however, we cannot rule out an effect on intracellular Ca\(^{2+}\) metabolism which could result in a heightened response to soluble stimuli.

It has been shown that preincubation of neutrophils with any of a number of oxidative stimuli results in potentiation of subsequent responses to heterologous oxidative stimuli.\(^11\) McPhail et al.\(^2\) studied this phenomenon and described the cellular response to the first stimulus as "priming." These authors suggested that although oxidative stimuli differ in the transductional mechanisms, they share a common intermediate pathway. One attractive intermediate is protein kinase C, which is activated during neutrophil stimulation by several oxidative stimuli.\(^22-24\) Such stimuli may act through protein kinase C-mediated phosphorylation of neutrophil proteins involved in the generation of $O_2^-$. A priming stimulus may activate protein kinase C in such a way that response to a second stimulus also working through the kinase is augmented. PAF may be another primary oxidative stimulus acting through this intermediate to enhance responses to other stimuli. In our studies, 1-oleoyl, 2-acyetyl glycerol, a diacylglycerol known to stimulate protein kinase C activity, also enhanced $O_2^-$ production, in agreement with a recent report by DeWald et al.\(^23\) Therefore, it is also possible that the enhancing effect of PAF is also mediated through the kinase. Our failure to demonstrate enhancement with diolein, a long-chain diacylglycerol, is consistent with reports showing that this compound does not activate protein kinase C in intact cells, presumably because of the lipid's inability to intercalate into the plasma membrane.\(^26\)

The priming effect of PAF appears to be virtually instantaneous, as shown by continuous assays of $O_2^-$ generation. Such rapid responses may be mediated through direct effects on the neutrophil plasma membrane. The fact that many lipids, including lyso-PC (structurally similar to PAF) are detergents suggests that PAF may also have detergent-like properties. Such properties may alter membrane configuration in such a way that the activity of the $O_2^-$-forming NADPH oxidase system is augmented.

PAF is produced by activated neutrophils and thus may be present in extracellular fluid in inflammatory foci. The lipid may serve to increase oxygen metabolite release at these sites, potentially enhancing both the microbicidal and tissuedamaging properties of neutrophils. The modulation of neutrophil $O_2^-$ generation by PAF is just one of many described instances of regulation of the oxidative response. These regulatory points, whether they be enhancing or inhibitory, provide multiple checks and balances on the inflammatory response. Such mechanisms ensure that this potent immunologic weapon can be held in check until the time when the rapid, powerful neutrophil metabolic burst offers maximal benefit to the host.

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