Activation and priming of neutrophil nicotinamide adenine dinucleotide phosphate oxidase and phospholipase A2 are dissociated by inhibitors of the kinases p42ERK2 and p38SAPK and by methyl arachidonyl fluorophosphonate, the dual inhibitor of cytosolic and calcium-independent phospholipase A2

Elahe Mollapour, David C. Linch, and Pamela J. Roberts

Arachidonic acid (AA) generated by phospholipase A2 (PLA2) is thought to be an essential cofactor for phagocyte nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity. Both enzymes are simultaneously primed by cytokines such as granulocyte-macrophage colony-stimulating factor (GM-CSF) and tumor necrosis factor-α (TNF-α). The possibility that either unprimed or cytokine-primed responses of PLA2 or NADPH oxidase to the chemotactic agents formyl-methionyl-leucyl-phenylalanine (FMLP) and complement factor 5a (C5a) could be differentially inhibited by inhibitors of the mitogen-activated protein (MAP) kinase family members p42ERK2 (PD98059) and p38SAPK (SB203580) was investigated. PD98059 inhibited the activation of p42ERK2 by GM-CSF, TNF-α, and FMLP, but it did not inhibit FMLP-stimulated superoxide production in either unprimed or primed neutrophils. There was no significant arachidonate release from unprimed neutrophils stimulated by FMLP, and arachidonate release stimulated by calcium ionophore A23187 was not inhibited by PD98059. In contrast, PD98059 inhibited both TNF-α- and GM-CSF-primed PLA2 responses stimulated by FMLP. On the other hand, SB203580 inhibited FMLP-superoxide responses in unprimed as well as TNF-α- and GM-CSF-primed neutrophils, but failed to inhibit TNF-α- and GM-CSF-primed PLA2 responses stimulated by FMLP, and additionally enhanced A23187-stimulated arachidonate release, showing that priming and activation of PLA2 and NADPH oxidase are differentially dependent on both the p38SAPK and p42ERK2 pathways. Studies using C5a as an agonist gave similar results and confirmed the findings with FMLP. In addition, methyl arachidonoyl fluorophosphonate (MAFP), the dual inhibitor of c and iPLA2 enzymes, failed to inhibit superoxide production in primed cells at concentrations that inhibited arachidonate release. These data demonstrate that NADPH oxidase activity can be dissociated from AA generation and indicate a more complex role for arachidonate in neutrophil superoxide production. (Blood. 2001;97:2469-2477)
oxidase. Although not directly activating superoxide production, 1 µM arachidonate caused enhanced release of NADPH oxidase activity when the cells were subsequently stimulated by the chemotactic peptide FMLP. In addition, inhibition of arachidonate release by mecapirine inhibited the priming of NADPH oxidase by GM-CSF without inhibiting superoxide production by unprimed cells. At present it is unclear which PLA2 enzymes are activated during cytokine-mediated priming of arachidonate release, although it was recently reported that both Group IV cPLA2 and Group II sPLA2 are activated by TNF priming of neutrophils. Furthermore, different PLA2 may be involved in arachidonate release for eicosanoid production rather than for superoxide generation.

The mechanisms by which cytokines regulate the different PLA2 have not been fully elucidated. Serine phosphorylation is important for regulating cPLA2. Several kinases are reported to phosphorylate cPLA2 including protein kinase C, and 12-O-tetradecanoylphorbol 13-acetate (TPA) (the potent agonist of protein kinase C) in combination with calcium ionophore induces massive arachidonate release. The kinases p42/44ERK2 and p38SAPK can also induce phosphorylation of cPLA2. In platelets stimulated with collagen or thrombin, both serine (S)505 and S727 residues of cPLA2 are phosphorylated, and inhibition of p38SAPK was shown to partially inhibit the phosphorylation of PLA2 on both S505 and S727. As only the S505 residue lies within a MAPK consensus sequence, the involvement of another kinase downstream of p38SAPK is suggested. Both GM-CSF and TNF- 

Measurement of p38 MAP kinase activity

Purified human neutrophils (2 

Materials and methods

Materials

Cytokines. Stock solutions of recombinant human (rh)GM-CSF (expressed in Escherichia coli) (Hoechst, Hounslow, England) and rhTNF- 

Agonists. FMLP, C5a, TPA, and calcium ionophore (A23187) (all from Sigma Chemical, Poole, England) were used. A stock solution of A23187, prepared in 5 mg/mL dimethyl sulfoxide (DMSO) and stored at -20°C, was diluted to 100 µM in PBS immediately prior to the experiment.

Inhibitors. Inhibitors included the 5-lipoxygenase activating protein inhibitor MK886 (gift from Merck-Frosst, Kirkland, Quebec, Canada). A 100 µM stock solution in DMSO was prepared immediately prior to use. A stock solution of N-ethylmaleimide (NEM) (Sigma) in 100 mM PBS was prepared daily. Stock solutions of 30 mM PD98059 (Calbiochem-Novabiochem, La Jolla, CA) and 30 mM SB203580 (Alexis, Nottingham, England) in DMSO were stored at -20°C and diluted 1000-fold into reaction mixtures. Methyl arachidonyl fluorophosphonate (MAFP) was supplied in solution in methyl acetate (Cayman Chemical, Ann Arbor, MI). The solvent was evaporated under nitrogen, and MAFP was reconstituted with DMSO at 50 mM and stored at -80°C.

Purification of neutrophils

Peripheral venous blood from healthy adult donors was anticoagulated with 2 mM ethylenediamine tetraacetic acid (EDTA) (pH 7.4), and the neutrophils were purified by dextran sedimentation of erythrocytes, centrifugation through Ficoll-Paque (Pharmacia Biotech, Uppsala, Sweden), and hypo-osmotic lysis of the remaining erythrocytes as described previously. Sterile preparations and procedures were used throughout to minimize contact of cells with endotoxin and to reduce inadvertent priming. Cells were resuspended in PBS supplemented with 0.9 mM calcium, 0.5 mM magnesium, and 5 mM glucose (PBSG).

Detection of p38 phosphorylation by immunoblotting

Neutrophils (1 

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Immunodetection of phosphorylated cPLA2 was performed according to the protocol described. Stimulation with either GM-CSF or FCS diluent for 5 minutes at 37°C. After 2 washes with TBS-T and one wash with TBS, the phosphorylated p38 and total p38 MAP kinase bands were detected by enhanced chemiluminescence (ECL) (Amersham Pharmacia).

**Measurement of superoxide production**

Superoxide generation was measured at 37°C by the dihydrorhodamine 123 (DHR) assay as previously described. Purified human neutrophils (1 × 10⁶ cells per mL) were stimulated with 10 ng/mL TNF-α, 10 ng/mL GM-CSF, or 0.01% FCS for 10 minutes. The pellet was resuspended to 2 × 10⁶ cells/mL in PBS, 5 mM glucose, and left at room temperature for 1 hour to allow spontaneous chemiluminescence. After 2 washes with TBS-T and one wash with TBS, the samples were resuspended in 200 mM MOPS-NaOH buffer at pH 7.4 and left at room temperature for 1 hour. The membrane was washed 3 times with wash buffer and incubated with 10 ng/mL NBT and 10 μg/mL CrO₃ for 1 hour. The superoxide was measured as previously described and confirmed using thin-layer chromatography (TLC). The results were expressed as the mean ± SE of the number of experiments given in the text. Analyses to determine the statistical significance employed the Student paired t-test.

**Results**

**Activation of neutrophil p38MAPK by agonists and cytokines**

Purified neutrophils were stimulated with 1 μM chemotactic peptide FMLP; p38MAPK immunoprecipitates were prepared, and their kinase activity was measured by phosphorylation of a MAPKAPK-2 substrate, as described in “Materials and methods.” Figure 1A shows that p38MAPK was rapidly and transiently stimulated by FMLP. Activity was detected within 30 seconds, maximal at one minute, but was no longer detectable at 5 minutes. Figure 1B-C shows that the cytokines TNF-α and GM-CSF also stimulate the activation of p38MAPK. The optimum inhibitory concentration of p38MAPK was detectable within 2 minutes and was sustained for 5 minutes (Figure 1C), whereas TNF-α–induced phosphorylation was slightly slower in onset and more transient (Figure 1B). The kinetics of p38MAPK phosphorylation stimulated by FMLP, when determined by immunoblotting with the phosphospecific antibody, was similar to the activation of p38MAPK activity (data not shown). The optimum inhibitory concentration of the p38MAPK inhibitor SB203580 was determined using the p38 MAP kinase assay. The data in Figure 1D show that SB203580 inhibited the phosphorylation of MAPKAPK-2 in a dose-dependent manner with approximately 1 μM IC₅₀ and complete inhibition at 30 μM.

**Effect of inhibition of p38MAPK on neutrophil superoxide production and AA release**

Studies with SB203580 were performed to determine whether p38MAPK has a role in mediating the priming effects of GM-CSF and TNF-α on either PLA2 or NADPH oxidase activity stimulated with FMLP. Neutrophils were preincubated with SB203580, and NADPH oxidase activity was measured by the superoxide dismutase–inhibitable reduction of cytochrome c as described in “Materials and methods.” Figure 2A-B shows that SB203580 inhibited supraxical 10.00 µg/mL aprotinin, 50 mM NaF, 200 µM NaN₃, 0.75 mM microcystin, 10 nM NADPH, 10 M microcystin for 15 minutes. The cell lysates were subjected to centrifugation at 12 000g for 15 minutes at 4°C. The supernatants were incubated with either rabbit anti-cPLA2 antibody (N-216, Santa Cruz Biotechnology) at a 1:500 dilution for 2 hours followed by 25 μL protein A Sepharose beads for 30 minutes at 4°C. The immunoprecipitates were washed 4 times with 1 mL wash buffer (0.5% Triton X-100 and 150 mM NaCl [pH 7.4]), twice with wash buffer containing 750 mM NaCl, and finally twice with the initial wash buffer. The samples were resuspended in 2 times Laemmli sample buffer, incubated for 10 minutes at 60°C, and subjected to 10% SDS-PAGE. Proteins were transferred to nitrocellulose membranes (Hybond C extra, Amersham Pharmacia). The membranes were blocked overnight in 3% bovine serum albumin and then incubated with rabbit anti-cPLA2 antibody at a dilution of 1:5000 for 1 hour. The membrane was washed 3 times with wash buffer, and immunoreactive cPLA2 bands were detected using ECL.
unprimed as well as GM-CSF– and TNF–primed superoxide production stimulated by FMLP. SB203580, at doses that inhibited p38SAPK, did not inhibit NADPH oxidase activity stimulated by the receptor-independent agonist TPA and thus did not inhibit the assembly and activation of the oxidase stimulated via PKC (Figure 2C).

PLA2 activity was determined as the extracellular release of 3H-AA from prelabeled phospholipid stores in neutrophils whose 5-lipoxygenase activity had been fully inhibited by the highly specific inhibitor MK886.42 This allowed maximal detection of PLA2 rather than 5-lipoxygenase activity and allowed the effect of the kinase inhibitors on PLA2 to be determined without interference from any possible effect on the downstream metabolism of AA. In 14 experiments the amount of AA released from non-primed neutrophils stimulated with FMLP was not significantly greater than background activity (2340 ± 239 cpn/10⁶ unstimulated cells and 2474 ± 230 cpn/10⁶ FMLP-stimulated cells), as we previously reported,7 but AA release was significantly greater than background in FMLP-stimulated cells primed with either TNF-α (4294 ± 667 cpn/10⁶ cells) (n = 7, P = .02) or GM-CSF (4660 ± 539 cpn/10⁶ cells) (n = 7, P = .001).

The data presented in Table 1 show that when neutrophils were preincubated with SB203580 under the conditions that gave complete inhibition of p38SAPK and significant inhibition of NADPH oxidase activity, there was no significant inhibition of either GM-CSF– or TNF-primed FMLP-stimulated AA release. To confirm these data, neutrophils treated with SB203580 was also stimulated with 1 μM calcium ionophore A23187, and AA release was measured. Table 1 shows that no inhibition of AA release from either unprimed or primed cells was apparent; in fact, SB203580 significantly enhanced AA release from ionophore-stimulated cells that had been primed with TNF-α.

**Activation of neutrophil p42ERK2 by agonists and cytokines**

Neutrophils were stimulated with FMLP, GM-CSF, and TNF-α, and analysis of p42ERK2 phosphorylation was by gel retardation assay as described in “Materials and methods.” Figure 3 shows that FMLP, GM-CSF, and TNF-α activate p42ERK2 in neutrophils in addition to activating p38 SAPK. FMLP stimulated the phosphorylation of p42ERK2 within 30 seconds, and the activation was sustained for at least 40 minutes (Figure 3A). TNF-α stimulation

![Figure 1. Activation of p38SAPK in purified neutrophils.](image1)

![Figure 2. The effect of SB203580 and PD98059 on neutrophil superoxide production.](image2)
Table 1. Effect of the p38 MAP kinase inhibitor SB203580 on cytokine-mediated priming of arachidonate release in neutrophils

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<th>Stimulus, μM</th>
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<tr>
<td>SB203580, μM</td>
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<tr>
<td>FMLP</td>
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<tr>
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<tr>
<td>20</td>
<td>97 ± 21</td>
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<td>30</td>
<td>112 ± 31</td>
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To investigate whether the target class of PLA₂ that was inhibited by PD98059 was cPLA₂, we investigated the effects of PD98059

Table 2. Effect of the MEK kinase inhibitor PD98059 on cytokine-mediated priming of arachidonate release in neutrophils

<table>
<thead>
<tr>
<th>Stimulus, μM</th>
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<tr>
<td>SB203580, μM</td>
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<td>1</td>
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<td>30</td>
<td>80 ± 21</td>
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</table>

Figure 3. Effect of PD98059 on the phosphorylation of p42ERK. Purified neutrophils were incubated with either DMSO or 30 μM PD98059 for 30 minutes before stimulation with either (A) 1 μM FMLP, (B) 500 U/mL TNF-α, or (C) 10 ng/mL GM-CSF or FCS diluent for the times indicated. Phosphorylation of p42ERK was measured by gel retardation as described in "Materials and methods." The data shown are from a single experiment that was performed twice with similar results.

3. Data from dose-response studies showed that complete inhibition of p42<sup>ERK2</sup> activation in neutrophils stimulated by GM-CSF was achieved at 10 μM PD98059 (data not shown). Inhibition of p42<sup>ERK2</sup> kinase by PD98059 was achieved rapidly, a preincubation of 5 minutes was sufficient to fully inhibit the enzyme, and inhibition was sustained for at least 60 minutes after GM-CSF stimulation (data not shown).

Effect of inhibition of p42ERK2 MAP kinase on neutrophil superoxide production and AA release

Figure 2D-E shows that under the conditions where p42ERK2 activation was completely blocked, there was no observable concomitant inhibition of either unprimed (n = 4), GM-CSF–primed (n = 3), or TNF-α-primed (n = 3) NADPH oxidase activity stimulated by FMLP. Neither did PD98059 at any dose inhibit TPA-stimulated NADPH oxidase activity (n = 3) (Figure 2F).

However, the data given in Table 2 show that PD98059 did partially inhibit both GM-CSF- and TNF-α-primed FMLP-stimulated PLA₂ responses. To confirm the inhibitory effect of PD98059 on arachidonate release, studies were performed using 1 μM calcium ionophore A23187 as stimulant. In 4 experiments, AA release from unprimed neutrophils stimulated by A23187 was not significantly inhibited by PD98059 at any concentration, whereas GM-CSF and TNF-α priming of AA release stimulated by A23187 was inhibited in a dose-dependent fashion (Table 2).
on cPLA₂ phosphorylation as determined by gel retardation. The data in Figure 4 show that phosphorylation of cPLA₂ was stimulated by GM-CSF and that this was not inhibited by either 20 or 30 μM PD98059, doses that completely inhibit p42ERK2 activity (Figure 3A-C).

The effect of the PLA₂ inhibitor MAFP on superoxide production and arachidonate release

The data so far presented suggest that superoxide production and arachidonate release can be dissociated by selective inhibition of either p42ERK2 or p38SAPK. To further investigate whether superoxide production can occur independently from arachidonate production, the effect of PLA₂ inhibitors on the respiratory burst and AA release was measured. Neutrophils were preincubated with the dual c and iPLA₂ inhibitor MAFP before priming and stimulation with FMLP, and the effect on NADPH oxidase and arachidonate release was measured. Figure 5 shows that MAFP inhibited FMLP-stimulated AA release primed by GM-CSF or TNF-α with approximately 0.1 μM IC₅₀, whereas this compound did not inhibit either unprimed or primed FMLP-stimulated NADPH oxidase activity unless used at a much higher concentration of 5 μM. In control experiments measuring the activation of a different signal transduction pathway, we showed that these high doses of MAFP did not inhibit signal transducer and activator of transcription (STAT)5b activation in neutrophils stimulated with GM-CSF (data not shown), thus the inhibitory effects of MAFP were not due to generalized cellular toxicity.

Studies with C5a

Neutrophil superoxide production and AA release stimulated by C5a were also measured in samples that had been preincubated with either PD98059, SB203580, or MAFP. Although C5a was a weaker agonist than FMLP, similar results were found with regard to the sensitivity of neutrophils to the MAP kinase and phospholipase inhibitors. Figure 6A-B shows that superoxide production was inhibited by SB203580 in a dose-dependent manner, but not by PD98059, whereas these compounds had the reverse effect on arachidonate release (Figure 6C). In addition, 1 μM MAFP enhanced both unprimed and GM-CSF–primed C5a-stimulated superoxide production (Figure 6D), but inhibited to basal levels C5a-stimulated AA release (Figure 6E).

Figure 4. Effect of PD98059 on the phosphorylation of cytosolic PLA₂. Purified neutrophils were preincubated with PD98059 or DMSO vehicle at the doses indicated in the figure for 30 minutes before stimulation with 10 ng/mL GM-CSF for 5 minutes. Migration of cPLA₂ immunoprecipitates was determined by SDS-PAGE followed by Western blotting with an anti-cPLA₂ antibody.

Figure 5. Effect of MAFP on neutrophil arachidonate release and superoxide production. Neutrophils were preincubated with MAFP at the range of concentrations indicated in the text, followed by priming with either (A, C) 10 ng/mL GM-CSF (●) or (B, D) 500 U/mL TNF-α (●) or FCS diluent (○) and stimulated with either 1 μM FMLP (●) or PBS (○). (A, B) Arachidonate release was measured as described in “Materials and methods.” Stimulation with FMLP was for 30 minutes. (C, D) Superoxide production was measured by the reduction of cytochrome c in a dual-beam spectrophotometer. Stimulation with FMLP was for 10 minutes.

Figure 6. The effect of SB203580, PD98059, and MAFP on neutrophil responses stimulated by C5a. (A, B) Superoxide production following incubation with either SB203580 or PD98059 at the doses indicated in the figure, followed by priming with GM-CSF or cytokine diluent and stimulation with 100 ng/mL C5a. (C) AA release from GM-CSF–primed cells preincubated with either 30 μM SB203580, PD98059, or DMSO diluent and stimulated with 100 ng/mL C5a. (D) Superoxide production from GM-CSF– or diluent-primed cells, preincubated with either MAFP at the doses indicated or DMSO diluent, followed by stimulation with 100 ng/mL C5a. (E) AA release from neutrophils incubated with or without 1 μM MAFP and then primed by incubation with 10 ng/mL GM-CSF or cytokine diluent, followed by stimulation with 100 ng/mL C5a.
**Discussion**

Our data extend previous findings that FMLP \(^{50-52}\) GM-CSF \(^{53-55}\) and TNF-\(\alpha\) \(^{54-56}\) can activate p38SAPK in human neutrophils. The kinetics of activation of p38SAPK by GM-CSF and TNF-\(\alpha\) were broadly similar to, but more prolonged than, that of FMLP. The p38 kinase inhibitor SB203580 had approximately 1 \(\mu M\) IC\(_{50}\) in the p38SAPK kinase assay and inhibited FMLP- and C5a-stimulated superoxide production both in unprimed and GM-CSF– or TNF-\(\alpha\)–primed cells. This suggests that p38SAPK has a role in activating the neutrophil respiratory burst stimulated by FMLP (in confirmation of previous reports \(^{54,55,57}\)) and by C5a. However, there was no detected effect of p38SAPK inhibition on superoxide production stimulated by TPA, which is in contrast to the recent findings of Lal et al. \(^{57}\)

Parallel experiments measuring arachidonate release stimulated by FMLP and C5a showed little inhibition attributable to blocking p38SAPK, and in fact, arachidonate release was enhanced by 1-20 \(\mu M\) SB203580. The enhancing effect of SB203580 on arachidonate release was further confirmed in cells stimulated with calcium ionophore. These results show that (1) SB203580 was not a global inhibitor of either FMLP or C5a signaling upstream of the NADPH oxidase, and thus the site of action of p38SAPK is likely to be the oxidase itself; (2) priming and activation of neutrophil AA release is not dependent on p38SAPK; and (3) the signal transduction pathways important for activating the NADPH oxidase via FMLP or C5a receptors in either primed or unprimed cells are not the same as those for activating PL\(A_2\).

Our observations are in agreement with those of Syrbu et al. \(^{38}\) who showed that SB203580 does not inhibit the phosphorylation of cPL\(A_2\) in FMLP-stimulated neutrophils, as determined by a gel-shift assay. However, Syrbu et al. \(^{38}\) report that SB203580 did partially inhibit FMLP-stimulated AA release from intact cells. The differences between these findings and ours may be due to differences in techniques used, as Syrbu et al did not use an inhibitor of 5-lipoxygenase in these assays and therefore measured the additive effects of SB203580 on both PL\(A_2\) and 5-lipoxygenase. Indeed, we have shown that SB203580 does partially inhibit 5-lipoxygenase, \(^{39}\) but it fails to inhibit AA release in cells where 5-lipoxygenase is blocked with MK886. SB203580 similarly inhibits other enzymes, such as cyclo-oxygenase 2, which are downstream of PL\(A_2\). \(^{60}\)

In contrast, inhibition of p42ERK2 had no effect on superoxide production in unprimed or primed cells. Unprimed cells stimulated with calcium ionophore, but not FMLP or C5a, released arachidonate, but this release was not inhibited significantly by the p42ERK2 pathway inhibitor. A dose-dependent, but partial, inhibition of arachidonate release was only seen in cytokine-primed cells. The data support other reports that activation of the FMLP-stimulated respiratory burst is not dependent on p42ERK2, 61-63 but indicate that primed PL\(A_2\) responses are dependent on this kinase. Our data show that activation of NADPH oxidase and PL\(A_2\) in GM-CSF– or TNF-\(\alpha\)–primed PMN can be dissociated by either blocking p38SAPK or p42ERK2 kinase pathways and suggest that arachidonate release is not required for the activation of the respiratory burst by FMLP or C5a. It should be noted that inhibitors, such as the MAP kinase inhibitors, may have other effects when used in complex biological systems than when used in single enzyme assays. Thus, differential effects may also reflect different affinities and concentration dependence of the end points that are studied.

To examine further the dissociation of the activation pathways from the FMLP receptor to NADPH oxidase and arachidonate release, the effect of the PL\(A_2\) inhibitor MAFP \(^{48,49}\) on these enzyme systems was studied. We previously showed that both GM-CSF–primed oxidase and PL\(A_2\) activity were inhibited by mepacrine, a relatively nonspecific inhibitor of PL\(A_2\), which suggests a possible role for this enzyme in activating the primed oxidase. \(^7\) Superoxide production by unprimed neutrophils was not inhibited by mepacrine, suggesting that only the enhanced superoxide production in response to priming was dependent on concomitant arachidonate production. However, concentrations of MAFP that inhibited FMLP- and C5a-stimulated arachidonate release primed by GM-CSF and TNF-\(\alpha\) did not inhibit the NADPH oxidase. This supports the hypothesis that arachidonate production in neutrophils is not exclusively required for respiratory burst activity. Our experiments with MAFP do not exclude the possibility that a small amount of AA is produced by other noncytosolic PL\(A_2\), which is sufficient to allow superoxide production to occur. Of relevance, bromoenol lactone, which is reported to be a more potent inhibitor of iPL\(A_2\) than MAFP, \(^64,65\) did inhibit both PL\(A_2\) and NADPH oxidase activities (data not shown). However, it was recently reported that BEL has the property of directly binding to the p67\(^{phox}\) protein \(^{66}\) and thus may inhibit NADPH oxidase by a mechanism which is independent from that inhibiting PL\(A_2\) activity.

The ability to dissociate FMLP- and C5a-stimulated NADPH oxidase activity from arachidonate release has important implications for the role of arachidonate in respiratory burst activity. Arachidonate is thought to interact with the NADPH oxidase in several ways, \(^{52,53,54}\) and specific binding sites for arachidonate on the p67\(^{phox}\) protein have been recently elucidated. \(^{66}\) However, recent work with leukemic cells lines induced to differentiate to mature cells have revealed conflicting results. Lowenthal and Levy \(^{31}\) showed that human myeloid PLB-958 cells transfected with antisense cPL\(A_2\) lack respiratory burst activity following differentiation with either retinoic acid or vitamin \(D_3\), but that activity was restored by addition of exogenous arachidonate. In contrast we previously showed that U937 cells differentiated with interferon-\(\gamma\) develop a robust superoxide response to the agonists TPA, FMLP, and cross-linking FcyRII, all in the absence of detectable AA release or leukotriene production. \(^67\) More recently others confirmed the lack of AA release in U937 cells, unless they are differentiated with vitamin \(D_3\), and showed that the development of oxidase activity with cell maturation depended on the cosynthesis of PLD. \(^68\) The data support the hypothesis that there are alternative pathways for activating the NADPH oxidase other than via AA generated by PL\(A_2\).

Our data reveal a greater sensitivity of AA release stimulated by calcium ionophore to PD98059 in primed versus unprimed neutrophils; thus it is possible that a different PL\(A_2\) enzyme may be responsible for AA release in primed cells compared to unprimed cells. The PD98059-sensitive PL\(A_2\) in primed cells may not be PL\(A_2\); as Syrbu et al. \(^{38}\) have shown that PD98059 inhibits neither FMLP- nor TNF-\(\alpha\)-stimulated phosphorylation of this enzyme. Similarly, we find that PD98059 does not inhibit GM-CSF–mediated phosphorylation of cPL\(A_2\).

The aim of this work was to investigate the signaling pathway responsible for priming PL\(A_2\) activity and to compare and contrast it with those pathways responsible for the priming and activation of the respiratory burst. Our results indicate that superoxide production is dependent on p38SAPK, which is in agreement with previous work \(^{54,55,57}\) By contrast, when we looked at PL\(A_2\), there was no dependence on the p38SAPK pathway, but there was a dependence on p42ERK2 MAP kinase activity only in cytokine-primed cells. Blocking arachidonate release with either a p42ERK2 pathway inhibitor or PL\(A_2\) inhibitor did not
completely reduce NADPH oxidase activity. Thus the signaling pathways from the GM-CSF and TNF-α receptor for priming PL2 activity differ from those responsible for priming the NADPH oxidase, thereby emphasizing the heterogeneity and complexity of both growth factor receptor signaling and the requirement of arachidonate for NADPH oxidase activation.

Acknowledgments

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