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 5α (C5α) 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 C5α as an agonist gave similar results and confirmed the findings with FMLP. In addition, methyl arachidonyl 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 enhancement of NADPH oxidase activity when the cells were subsequently stimulated by the chemotactic peptide FMLP. In addition, inhibition of arachidonate release by mepacrine 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/ERK, p38, and p38APK 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 p38APK 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 p38APK is suggested. Both GM-CSF and TNF-α induce phosphorylation of cPLA2 as well as activating p42/ERK and p38APK, respectively. Therefore, these kinases may play a role in cytokine-driven, arachidonate-mediated priming of NADPH oxidase. However, GM-CSF also activates PI-3 kinase as well as members of the src kinase family, lyn and yes, which could also be involved in priming events.

The aim of this study was to investigate the role of p42/ERK and p38APK as well as AA production in the activation and priming of the neutrophil respiratory burst. We show that NADPH oxidase and PLA2 have a differential dependence on p42/ERK and p38APK activity and that cPLA2 activation is not obligatory for eliciting the priming of FMLP- or C5a-stimulated superoxide production.

Materials and methods

Materials

Cytokines. Stock solutions of recombinant human (rh)GM-CSF (expressed in Escherichia coli) (Hoechst, Hounslow, England) and rhTNF-α (R&D Systems, Europe Abingdon, England) were prepared in sterile phosphate-buffered saline (PBS, pH 7.4) (Gibco-BRL, Life Technologies, Paisley, Scotland) containing 1% (vol/vol) fetal calf serum (FCS) (Gibco) and stored at −20°C.

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 sulphoxide (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, Upplands Väsby, Sweden), and hypotonic 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).

Measurement of p38 MAP kinase activity

Purified human neutrophils (2 × 10⁶ cells per mL) were preincubated for 30 minutes at 37°C with either 1, 5, 10, 20, 30, or 40 μM SB203580 or with DMSO diluent and then stimulated for 1 minute with either 1 μM FMLP or with PBS diluent. The cells were pelleted by a brief centrifugation step at 12 000g for 30 seconds and lysed by incubation for 30 minutes in 0.5 mM ice-cold lysis buffer—20 mM Tris-Cl (tris(hydroxymethyl)aminomethane–hydrochloride) (pH 8.0), 137 mM sodium chloride (NaCl), 1 mM magnesium dichloride (MgCl2), 1 mM calcium dichloride (CaCl2), 10% glycerol (vol/vol), 1% Nonidet P40 (Sigma) (vol/vol), 2 mM EDTA, 1 mM sodium orthovanadate, 5 mM sodium pyrophosphate, 1 mM sodium fluoride (NaF), and 1 mM β-glycerophosphate—containing the following protease inhibitors: 1 mM phenylmethylsulphonyl fluoride (PMSF), 10 μg/mL leupeptin, 10 μg/mL aprotinin, 10 μg/mL pepstatin A, 1 mM diisopropylfluorophosphosphate (DFP), and 2 mM Pefabloc SC (4-(2-aminoethyl)-benzenesulfonyl fluoride, hydrochloride) (Roche Diagnostics, Lewes, England).

The lysates were centrifuged at 12 000g for 10 minutes at 4°C, and the supernatants were incubated on ice for 30 minutes with 2 μg/mL of a polyclonal anti-p38 MAPK antibody (C-20; Santa Cruz Biotechnology, Santa Cruz, CA) and then with an equal volume of protein G Sepharose beads (Pharmacia Biotech) for 30 minutes at 4°C with rotation. The immune complexes were washed twice with wash buffer—0.5% Triton X-100, 1 mM EDTA, 50 mM Tris-HCl (pH 8.0), and 100 mM NaCl—and finally with kinase buffer—25 mM Tris-HCl (pH 8.0), 10 mM MgCl2, and 2 mM MnCl2—and finally resuspended in 10 μL assay dilution buffer containing 200 ng GST-MAPKAPK-2 (residues 46–400 of rhMAPKAPK-2 with an N-terminal glutathione S-transferase tag and a C-terminal myc epitope) (Upstate Biotechnology, Lake Placid, NY).

We added 2 μL magnesium/ATP (adenosine 5’-triphosphate) cocktail (prepared according to the manufacturer’s instructions) and 0.185 MBq (5 μCi) γ-phosphorous-32 (γ-32P)-ATP (specific activity, 11 × 10⁶ Bq/mM (3000 Ci/mM)) (Amersham Pharmacia Biotech, Little Chalfont, England) to give a final volume of 36 μL, and the reaction mixtures were incubated for 15 minutes at 30°C. Next, 9 μL of 4 times Laemmli sample buffer was added, and the samples were boiled for 10 minutes. Proteins were separated by 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), the gel was dried, and MAPKAP kinase-2 phosphorylation was detected by autoradiography. Aliquots of the samples equivalent to 1-2 × 10⁶ cells were analyzed for total p38 content by immunoblotting as described below.

Detection of p38 phosphorylation by immunoblotting

Neutrophils (1 × 10⁶ cells per mL) were stimulated with either diluent (0.01% FCS), 1 μM FMLP, 500 U/mL TNF-α, or 10 ng/mL GM-CSF at 37°C. At timed intervals 100-μL samples were taken, and the reaction was terminated by addition of 2 mM NEM followed by rapid centrifugation at 12 000g for 30 seconds. The pellet was resuspended in 50 μL ice-cold lysis buffer (composition as given above, but without Pefabloc). We added 50 μL of 2 times Laemmli sample buffer, and the samples were boiled for 10 minutes. Proteins were separated by 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), the gel was dried, and MAFKAP kinase-2 phosphorylation was detected by autoradiography. Aliquots of the samples equivalent to 1-2 × 10⁶ cells were analyzed for total p38 content by immunoblotting as described below.
stimulated with either GM-CSF or FCS diluent for 5 minutes at 37°C. Cells were then washed 3 times with TBS-T and then incubated for 1 hour at room temperature with a 1:2000 dilution of either secondary horseradish peroxidase–conjugated antirabbit or antigoat immunoglobulin G (IgG) antibodies (Dako, High Wycombe, England). After 2 washes with TBS-T and one wash with TBS, the phosphorylated p38 and total p38 MAPK kinase bands were detected by enhanced chemiluminescence (ECL) (Amersham Pharmacia).

Measurement of superoxide production
Superoxide generation was measured at 37°C by the superoxide dismutase–inhibitable reduction of ferricytochrome c in a dual-beam spectrophotometer as previously described.43 Purified human neutrophils (1 × 10⁶ cells per mL) were incubated in 1-mL cuvettes with or without inhibitors (PD98059 for 10 minutes or SB203580 for 30 minutes) prior to addition of either 500 U/mL TNF-α, 10 ng/mL GM-CSF, or 0.01% FCS for 30 minutes. The samples were stimulated with 1 μM FMLP or 100 ng/mL C5a for 10 minutes, and the reactions were stopped with NEM at a final concentration of 2 mM.

Release of hydrogen-3–AA
Release of hydrogen-3 (³H)–AA from purified human neutrophils was measured as previously described and confirmed using thin-layer chromatography.3 We incubated 5 × 10⁶ cells per mL in PBS, 5 mM glucose, and 0.01% vol/vol FCS with 0.38 Mbq (0.5 μCi) 5,6,8,9,11,12,14,15-³H-AA (specific activity, 7.33 TBq/mM (202 Ci/mM) (Amersham Pharmacia) for 2 hours at room temperature. The radio-labeled cells were then centrifuged at 300g for 7 minutes to remove unincorporated radioactivity and then washed 3 times in PBS. The pellet was resuspended to 2 × 10⁶ cells per mL in PBSG. Neutrophil samples (0.5 mL in duplicate) were incubated with 200 nM MK886 (to inhibit metabolism of AA by the 5-lipoxygenase pathway) for 5 minutes at 37°C. Then cells were incubated with either 0.01% FCS as diluent control, 500 U/mL TNF-α, or 10 ng/mL GM-CSF for 20 minutes followed by stimulation with 1 μM calcium ionophore A23187, 1 μM FMLP, or 100 ng/mL C5a for 20 minutes at 37°C. The reaction was stopped by placing the samples on ice and then centrifuged at 12,000 g for 4 minutes; 0.4-mL aliquots of the supernatants were assayed for radioactivity by scintillation spectroscopy.

Phosphorylation of p42ERK2 measured by gel retardation assay
Samples of stimulated neutrophils were prepared for Western blot analysis as described above. Proteins equivalent to 1-2 × 10⁶ cells per lane were separated by 15% SDS-PAGE (acrylamide:bis percentage, 15:0.075 [200:1]) for 15 minutes at 120 V. After electrophoresis, proteins were electrophoretically transferred onto PVDF membranes (Millipore). Non-specific binding sites on the membrane were blocked in TBS-T/5% (wt/vol) nonfat dried milk for 1 hour at room temperature. Membranes were incubated with either GM-CSF or FCS diluent for 5 minutes at 37°C. Immunodetection of phosphorylated cPLA₂ was performed according to the method of Kramer et al.44 The reaction was terminated by addition of 1 mL ice-cold lysis buffer (final concentrations, 1% Triton X-100, 0.5% SDS, 0.75% deoxycholate, 10 mM EDTA, 1 mM PMFS, 10 μg/mL leupeptin, 10 μM pepstatin A, 100 μg/mL aprotinin, 50 mM NaF, 200 μM Na₃VO₄, 10 mM Na₂P₂O₇, and 1 μM microcystin) for 15 minutes. The cell lysates were subjected to centrifugation at 12,000 g for 15 minutes at 4°C. The supernatants were incubated with a rabbit antihuman 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]), then 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 antihuman cPLA₂ antibody at 1:500 dilution for 2 hours at room temperature. The membranes were washed 3 times with wash buffer and incubated with a secondary horseradish peroxidase–conjugated goat antirabbit IgG antibody at a dilution of 1:2000 for 1 hour. The membrane was washed 3 times with wash buffer, and immunoreactive cPLA₂ bands were detected using ECL.

Statistical analysis
The data presented are 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 the 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, was 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 in purified neutrophils, and phosphorylation of this molecule is determined by immunoblotting of whole cell lysates with a p38MAPK phosphospecific antibody. GM-CSF–induced phosphorylation of p38MAPK was detectable within 2 minutes and was sustained for 15 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 (15.5 μM) was determined using the p38 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...
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 cpm/10⁶ unstimulated cells and 2474 ± 230 cpm/10⁶ FMLP-stimulated cells), as we previously reported, but AA release was significantly greater than background in FMLP-stimulated cells primed with either TNF-α (4294 ± 667 cpm/10⁶ cells) (n = 7, P < 0.02) or GM-CSF (4660 ± 539 cpm/10⁶ cells) (n = 7, P < 0.001).

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 p38SAPK. 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)
of p42ERK2 was only transient, with a weak band being detected at 10 minutes after stimulation (Figure 3B), whereas GM-CSF induced more sustained activation (Figure 3C). Activation of p42ERK2 was inhibited by preincubation of neutrophils with the noncompetitive MEK1 inhibitor PD98059, as shown in Figure 3. Data from dose-response studies showed that complete inhibition of p42ERK2 activation in neutrophils stimulated by GM-CSF was achieved at 10 μM PD98059 (data not shown). Inhibition of p42ERK2 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 PLAD2 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).

### Effect of the MAP kinase inhibitors on the phosphorylation of cPLA2

To investigate whether the target class of PLA2 that was inhibited by PD98059 was cPLA2, we investigated the effects of PD98059

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**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, 1 μM</th>
<th>SB203580, μM</th>
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<th>FCS</th>
<th>TNF-α</th>
<th>GM-CSF</th>
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Purified neutrophils were incubated with SB203580 for 30 minutes at 37°C before priming with either 500 U/mL TNF-α, 10 ng/mL GM-CSF, or 0.01% FCS diluent for 20 minutes followed by stimulation with agonist as indicated for 15 minutes. Arachidonate release was measured as described in “Materials and methods.” Basal release of arachidonate in unstimulated samples was subtracted from the stimulated value, and the data were expressed as a percentage of the DMSO diluent control. Absolute values were expressed as cpm/106 cells for (1) FMLP stimulation: FCS, 118 ± 100; TNF, 1493 ± 461; and GM-CSF, 1317 ± 321 and (2) A23187 stimulation: FCS, 2584 ± 786; TNF, 4856 ± 1094; and GM-CSF, 11306 ± 3332. In all cases the release of arachidonate from unprimed FMLP-stimulated cells was not significantly different from background. The data shown are the mean ± SE of 4 experiments. ND indicates not determined; FCS, fet al calf serum; TNF-α, tumor necrosis factor-α; GM-CSF, granulocyte-macrophage colony-stimulating factor; FMLP, formyl-methionyl-leucyl-phenylalanine.

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

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Purified neutrophils were incubated with PD98059 for 10 minutes at 37°C before priming and stimulation with agonist and measurement of arachidonate release as described in the legend to Table 1. Basal release of arachidonate in unstimulated samples was subtracted from the stimulated value, and the data were expressed as a percentage of DMSO diluent control. Absolute values were expressed as cpm/106 cells for (1) FMLP stimulation: FCS, 153 ± 142; TNF, 2563 ± 952; and GM-CSF, 1130 ± 30; and (2) A23187 stimulation: FCS, 2472 ± 779; TNF, 5266 ± 1251; and GM-CSF, 10505 ± 524. Release of arachidonate from unprimed FMLP-stimulated cells was not significantly different from background, therefore the percent of control values was not determined. The data shown are the mean ± SE of 4 experiments.

See Table 1 for abbreviations.*0.01 > P > .001 indicates significant difference between samples with inhibitor. †0.05 > P > .01 indicates significant difference between samples without inhibitor.
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 p42 ERK2 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 p38 SAPK. 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 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, GM-CSF, and TNF-α can activate p38SAPK in human neutrophils. The kinetics of activation of p38SAPK by GM-CSF and TNF-α were broadly similar to, but more prolonged than, that of FMLP. The p38 kinase inhibitor SB203580 had approximately 1 μM IC50 in the p38SAPK kinase assay and inhibited FMLP- and C5a-stimulated superoxide production both in unprimed and GM-CSF- or TNF-α–primed cells. This suggests that p38SAPK has a role in activating the neutrophil respiratory burst stimulated by FMLP (in confirmation of previous reports) 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.

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 μM 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 PL2.

Our observations are in agreement with those of Syrbu et al., who showed that SB203580 does not inhibit the phosphorylation of cPLA2 in FMLP-stimulated neutrophils, as determined by a gel-shift assay. However, Syrbu et al. 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 PLA2 and 5-lipoxygenase. Indeed, we have shown that SB203580 does partially inhibit 5-lipoxygenase, 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 PLA2.

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 arachidonic acid. However, calcium ionophore did not inhibit FMLP-stimulated AA release from intact cells. The results also 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 PLA2.

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, and specific binding sites for arachidonate on the p47phox protein have been recently elucidated. Recent work with leukemic cells lines induced to differentiate to mature cells have revealed conflicting results. Lowenthal and Levy showed that human myeloid PLB-985 cells transfected with antisense cPLA2 lack respiratory burst activity following differentiation with either retinoic acid or vitamin D3, but that activity was restored by addition of exogenous arachidonate. In contrast we previously showed that U937 cells differentiated with interferon-γ develop a robust superoxide response to the agonists TPA, FMLP, and cross-linking FcγRII, all in the absence of detectable AA release or leukotriene production. More recently others confirmed the lack of AA release in U937 cells, unless they are differentiated with vitamin D3, and showed that the development of oxidase activity with cell maturation depended on the cosynthesis of PLD. The data support the hypothesis that there are alternative pathways for activating the NADPH oxidase other than via AA generated by PLA2.

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 PLA2 enzyme may be responsible for AA release in primed cells compared to unprimed cells. The PD98059-sensitive PLA2 in primed cells may not be cPLA2, as Syrbu et al. have shown that PD98059 inhibits neither FMLP- nor TNF-α–stimulated phosphorylation of this enzyme. Similarly, we find that PD98059 does not inhibit GM-CSF–mediated phosphorylation of cPLA2.

The aim of this work was to investigate the signaling pathway responsible for priming PL2 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. By contrast, when we looked at PL2, 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 PLA2 inhibitor did not...
concomitantly reduce NADPH oxidase activity. Thus the signaling pathways from the GM-CSF and TNF-α receptor for priming PLA₂ 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.

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