Phosphorylation of Cytosolic Phospholipase A \(_2\) and the Release of Arachidonic Acid in Human Neutrophils

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Kinases mediating phosphorylation and activation of cytosolic phospholipase A2 (cPLA2) in intact cells remain to be fully characterized. Platelet-activating factor stimulation of human neutrophils increases cPLA2 phosphorylation. This increase is inhibited by PD 98059, a mitogen-activated protein (MAP)/extracellular signal-regulating kinase (erk) 1 inhibitor, but not by SB 203580, a p38 MAP kinase inhibitor, indicating that this action is mediated through activation of the p42 MAP kinase (erk2). However, platelet-activating factor-induced arachidonic acid release is inhibited by both PD 98059 and SB 203580. Stimulation by TNF-α increases cPLA2 phosphorylation, which is inhibited by SB 203580, but not PD 98059, suggesting a role for p38 MAP kinase. LPS increases cPLA2 phosphorylation and arachidonic acid release. However, neither of these actions is inhibited by either PD 98059 or SB 203580. PMA increases cPLA2 phosphorylation. This action is inhibited by PD 98059 but not SB 203580. Finally, FMLP increases cPLA2 phosphorylation and arachidonic acid release. Interestingly, while the FMLP-induced phosphorylation of cPLA2 is not affected by the inhibitors of the p38 MAP kinase or erk cascades, both inhibitors significantly decrease arachidonic acid release stimulated by FMLP. SB 203580 or PD 98059 has no inhibitory effects on the activity of coenzyme A-independent transacylase. The Journal of Immunology, 1999, 162: 2334–2340.

Any different cell types, including neutrophils, release arachidonic acid in response to stimulation. This important fatty acid is the precursor of biologically active eicosanoids such as prostaglandins, prostacyclin, thromboxane, and leukotrienes. Moreover, the rate-limiting step in eicosanoid biosynthesis is the liberation of arachidonic acid. Although the process involved in the release of arachidonic acid is complex and not fully understood, the main step involves phospholipase A2 (PLA2)3 activation and translocation to the compartments where its phospholipid substrate is located. The cytosolic form of PLA2 (cPLA2) mediates the production of agonist-induced arachidonic acid release, and it plays an essential role in the release of platelet-activating factor (PAF), also an important inflammatory lipid mediator (1–8). Activation of cPLA2 has been shown to require the phosphorylation of the enzyme and an increase in the concentration of intracellular free calcium (7). Although it is generally agreed that extracellular signal-regulating kinase (erk) 2 phosphorylates cPLA2 on serine 505 in vitro (7), recent evidence, using human neutrophils, human platelets, and resident mouse macrophages, suggest that kinases other than erk1 and erk2 can phosphorylate and activate cPLA2 (9–16). The identities of these kinases remain to be determined.

Until recently, the mitogen-activated protein (MAP) kinases (MAPK), also referred to as erk1 and erk2, were the only cloned and well-characterized mammalian MAPKs. Recently, two other MAPK subtypes, the c-Jun N-terminal kinase (JNK) stress-activated protein kinase (SAPK) and p38/reactivating kinase (mammalian equivalent of high osmolality glycerol response-1 (HOG-1) in yeast) were discovered (17–21). Currently, there are three parallel kinase cascades involved in agonist-induced signal transduction: the erk1/2, the JNK/SAPK, and the p38. These three kinases are themselves activated by phosphorylation on threonine and tyrosine residues, and the upstream kinases that can phosphorylate these enzymes are MAP/erk kinase (MEK) (threonine glutamic acid tyrosine kinase), SAP/erk kinase (SEK) (threonine proline tyrosine kinase), and reactivating kinase (TGY kinase), respectively (17–19). In proliferating cells, substrates for the erk cascade include ribosomal S6 kinase (p90Rsk), and transcription factors, such as c-jun and c-fos (17–21). The substrates for the JNK/SAPK and p38 cascades in proliferating cells are just beginning to be identified. The substrates and the roles of all three MAPK subtypes in differentiated cells such as human neutrophils are unknown.

Recently, we and others demonstrated the presence of a novel 38-kDa protein that is tyrosine phosphorylated and activated in human neutrophils and terminally differentiated cells upon stimulation with various agonists (12, 22, 23). This 38-kDa protein was identified as the mammalian homologue of HOG-1 in yeast, the p38 MAPK. The present studies were undertaken to determine the roles of various kinases in the phosphorylation of cPLA2 and the release of arachidonic acid in human neutrophils stimulated by FMLP, PAF, LPS, PMA, and TNF-α. This was done using three recently developed compounds; SB 203580, a highly specific inhibitor of the p38 MAPK activity, which does not affect erk2 or Jun kinase subtypes; PD 98059, a specific inhibitor of the erk cascade; and

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Materials and Methods

Isolation of neutrophils

Blood was obtained from human volunteers, and neutrophils were isolated using a Ficoll/Hypaque gradient. Contaminating RBC were lysed by hypotonic shock (27, 28). The neutrophils were resuspended in modified HBSS containing 0.1% BSA and 10 mM HEPES, pH 7.35.

Fractionation of cells into soluble and particulate fractions

Fractionation of cells into soluble and particulate fractions was conducted as described previously (29, 30). Briefly, 3 ml of cells (1 x 10^6 cells/ml) were treated with inhibitor or diluent for 30 min following the addition of buffer or agonist. Following reaction termination, the cells were resuspended at 10^6 cells/ml in iced buffered sucrose solution (10 mM HEPES, pH 7.5, 100 mM sucrose, 1 mM EGTA, 0.5 mM EDTA, 50 µg/ml leupeptin, 2 mM sodium orthovanadate, 5 mM sodium pyrophosphate, and 100 µM PMSE). Cells were disrupted by sonication, then centrifuged at 100,000 x g for 30 min at 4°C. The cytosolic fraction protein was measured, adjusted, and used in subsequent assays.

Immunoblotting

Immunoblotting was performed as described previously (28–30). Equivalent amounts of protein (100 µg) were loaded onto SDS-PAGE (8% for cPLA2 detection or 12% for p38 MAPK and erk2 detection) gels. After electrophoresis, proteins were transferred from the gel to polyvinylidene difluoride membranes in transfer buffer (20 mM Tris base, 150 mM glycerol). The samples were boiled for 5 min and electrophoresed on 12% polyacrylamide gels. Phosphorylation of hsp 27 protein was measured under conditions where it was linear with respect to the time of incubation and enzyme dilution.

Arachidonic acid release

Labeling the cells with [3H]arachidonic acid and the release of radiolabeled arachidonic acid were conducted as described previously (32) with slight modifications. Briefly, the cells (1 x 10^6/ml) were added to 100 mM Tris, pH 7.4, 20 mM MgCl2, 40 mM ATP, 16 mM okadaic acid, 33 mg/ml leupeptin, 2 mM sodium orthovanadate, 5 mM sodium pyrophosphate, and 100 µM PMSE), centrifuged at 100,000 x g for 40 min. The microsomes were pelleted from the supernatant by centrifugation and were washed once with PBS (138 mM NaCl, 10 mM Na2HPO4, 2.7 mM KCl) containing 1 mM EGTA, pH 7.4, pelleted by centrifugation, and resuspended in PBS and stored at -70°C until assayed. CoA-IT activity in microsome prepeation was measured as described by Winkler et al. (33). Briefly, microsomes were diluted in PBS containing 1 mM EGTA, and reaction was started by the addition of 0.1 µCi [3H]1-alkyl-2-lyso-GPC/ tube and 1 µM final unlabeled 1-alkyl-2-lyso-glycerol-3-phosphorylcholine (GPC) and run for 10 min at 37°C. Lipids were extracted according to the method of Bligh and Dyer (34), and aliquots of the chloroform phase were separated by TLC. The [3H]-1-alkyl-2-lyso-GPC and [3H]-1-alkyl-2-acetyl-GPC bands were scraped and quantified by liquid scintillation spectroscopy.

Results

Tyrosine phosphorylation of p38 MAPK and erk2 in control, PD 98059-, and SB 203580-treated human neutrophils stimulated with various agonists

The effects of SB 203580, the p38 MAPK inhibitor, and PD 98059, the MEK inhibitor, on the agonist-stimulated tyrosine phosphorylation of the p38 MAPK and erk2 in human neutrophils were examined. In these experiments, the cells were incubated with diluent, SB 203580, or PD 98059 and then stimulated with FMLP, PAF, TNF-α, or LPS. After reaction termination, cells were sonicated and cytosolic fractions subjected to SDS-PAGE. Immunoblot analysis was performed using anti-phospho-MAPK or anti-phospho-p38 kinase. The results are summarized in Fig. 1. These data show several points. First, PAF and FMLP stimulate the tyrosine phosphorylation of both erk2 and p38 MAPK. Second, as previously reported (13, 15, 22), TNF-α and LPS increase the tyrosine phosphorylation of the p38 MAPK and erk2 in human neutrophils. The effects of SB 203580, the p38 MAPK inhibitor, and PD 98059, the MEK inhibitor, on the agonist-stimulated tyrosine phosphorylation of the p38 MAPK and erk2 in human neutrophils were examined. In these experiments, the cells were incubated with diluent, SB 203580, or PD 98059 and then stimulated with FMLP, PAF, TNF-α, or LPS. After reaction termination, cells were sonicated and cytosolic fractions subjected to SDS-PAGE. Immunoblot analysis was performed using anti-phospho-MAPK or anti-phospho-p38 kinase. The results are summarized in Fig. 1. These data show several points. First, PAF and FMLP stimulate the tyrosine phosphorylation of both erk2 and p38 MAPK. Second, as previously reported (13, 15, 22), TNF-α and LPS increase the tyrosine phosphorylation only of p38 MAPK. Third, PD 98059 inhibits the stimulated phosphorylation of erk2 but has no effect on the phosphorylation of p38 MAPK. Fourth, SB 203580 does not affect the phosphorylation of either erk2 or p38 MAPK. The inhibitory action of SB 203580 is on the enzymatic activity of the p38 MAPK so its phosphorylation is not affected. On the other hand, the inhibitory effect of PD 98059 on the erk2 cascade is through mek1 inhibition and, therefore, erk2 tyrosine phosphorylation. Also note that SB 203580 alone causes a slight increase in p38 MAPK tyrosine phosphorylation.
Activity of p38 MAPK in control and cells treated with the inhibitor SB 203580 following stimulation by various agonists

An important in vivo substrate for p38 MAPK is MAPK/activated protein kinase-2 (MAPKAPK-2). The activated MAPKAPK-2 increases the phosphorylation of the small m.w. hsp 27. Accordingly, the activity of p38 MAPK was determined using the small m.w. hsp 27 as substrate. In these experiments, designed to show the efficacy of SB 203580, human neutrophils in suspension were incubated with diluent, SB 203580, or PD 98059 before stimulation by PAF (A), FMLP (B), TNF-α (C), or LPS in combination with 1% serum (C). Upon reaction termination, cells were sonicated and soluble fractions were subjected to SDS-PAGE. The resulting immunoblots were probed with anti-phospho p38 MAPK and anti-phospho erk2 Abs, which recognize the phosphorylated forms of p38 MAPK and erk2, respectively. The exposures shown are representative of three similar results.

Phosphorylation of PLA₂ in control and PD 98059-treated human neutrophils stimulated with PAF and FMLP

It is generally agreed that erk2 increases the phosphorylation of cPLA₂ on serine 505 in an in vitro assay (7). However, it is not known if the phosphorylation of cPLA₂ in intact cells by physiological stimuli is mediated by erk2. To examine this point, we measured the phosphorylation of cPLA₂ in control and PD 98059-treated human neutrophils in suspension stimulated by PAF and FMLP. Both of these stimuli increase the tyrosine phosphorylation of erk2, and this phosphorylation is inhibited by PD 98059 (see FIGURE 3. Effect of PD 98059 on PAF- and FMLP-stimulated cPLA₂ phosphorylation in human neutrophils. Cells were treated with diluent or PD 98059 before stimulation by PAF (A) or FMLP (B). After reaction termination, cells were sonicated and soluble fractions were subjected to SDS-PAGE. Immunoblot analysis was performed using anti-cPLA₂ Ab. cPLA₂ phosphorylation is detected as a decrease in the electrophoretic mobility. These exposures are representative of a single experiment conducted at least three times.
In these experiments, the cells were treated with PD 98059 and then stimulated with PAF or FMLP. After reaction termination, cells were sonicated and cytosolic fractions subjected to SDS-PAGE. Immunoblot analysis was performed using anti-cPLA2. The phosphorylation of cPLA2 is detected by the retarded mobility of the phosphorylated enzyme. The results summarized in Fig. 3 clearly show that the PAF-induced, but not FMLP-induced, phosphorylation of cPLA2 is inhibited by PD 98059. As expected, PD 98059 had no effect on the phosphorylation of cPLA2 in human neutrophils in suspension stimulated by TNF-α or LPS (data not shown).

**Phosphorylation of cPLA2 in control and SB 203580-treated human neutrophils stimulated with LPS, PAF, TNF-α, and FMLP**

Recently, we and others (8–12) have presented evidence that indicates that the phosphorylation of cPLA2 can be achieved independently of erk2. To address this point further, the phosphorylation of cPLA2 in control and SB 203580-treated human neutrophils upon stimulation with LPS, PAF, FMLP, or TNF-α was examined. In these experiments, human neutrophils in suspension were incubated with either diluent or SB 203580, and cells were then stimulated with FMLP, LPS, TNF-α, or PAF. Cytosolic fractions were prepared, electrophoresed, transferred, and the resulting immunoblot probed with anti-cPLA2 Ab. The data summarized in Fig. 4 clearly show that only the TNF-α-induced (Fig. 4A) phosphorylation of cPLA2 is inhibited in cells pretreated with SB 203580. Also, the FMLP-stimulated phosphorylation was not affected in cells pretreated with both SB 203580 and PD 98059 added together (Fig. 4B).

**Role of PKC in the phosphorylation of cPLA2 in human neutrophils stimulated by FMLP**

As shown in the previous sections, the FMLP-induced phosphorylation of cPLA2 is not inhibited by PD 98059 or SB 203580. This strongly suggests that FMLP can phosphorylate cPLA2 independently of the activation of erk2 and p38 MAPK. The chemotactic tripeptide FMLP is known to activate the PKC system and calcium-activated kinases. To examine the possibility that the PKC system may phosphorylate cPLA2 directly, we measured the effects of the PKC inhibitor, GO 6850, on the phosphorylation of cPLA2 in human neutrophils stimulated by FMLP and PMA, an activator of the PKC system. The results summarized in Fig. 5 show three main points. First, stimulation of human neutrophils with PMA induces the phosphorylation of cPLA2, and this phosphorylation is greatly diminished by the PKC inhibitor GO 6850 (Fig. 5A). Second, while the PKC inhibitor decreases the phosphorylation of cPLA2 in cells stimulated with PMA, it has no effect on the actions of FMLP (Fig. 5A). Third, stimulation of human neutrophils with PMA increases the tyrosine phosphorylation of erk1 and erk2 (Fig. 5B) and p38 MAPK (Fig. 5C). However, while GO 6850 inhibits the PMA-induced tyrosine phosphorylation of both p38 and the erk kinases, it has no effect on the tyrosine phosphorylation of these kinases induced by FMLP. Note that in Fig. 5A, the effect of GO 6850 on the phosphorylation of cPLA2 is visible, indicating that the PKC system is involved in this process.
SB 203580 and PD 98059 on PMA-stimulated cPLA2 phosphorylation. Cells were treated before stimulation with either diluent, PD 98059 (A), or SB 203580 (B). Cells were then stimulated with 3 nM PMA for 5 min. After reaction termination, cells were sonicated and the soluble fraction was subjected to SDS-PAGE. Immunoblot analysis was performed using anti-cPLA2 Ab. cPLA2 phosphorylation is detected as a decrease in the electrophoretic mobility. These exposures are representative of a single experiment conducted at least three times.

In a separate set of experiments, we also examined the effects of SB 203580 and PD 98059 on the phosphorylation of cPLA2 in human neutrophils stimulated with PMA. The results summarized in Fig. 6 clearly show that the PMA-induced phosphorylation of cPLA2 is inhibited by PD 98059 (Fig. 6A). Also note that PMA-induced phosphorylation of cPLA2 is not affected by SB 203580 (Fig. 6B). On the other hand, SB 203580 in combination with PD 98059 inhibits this phosphorylation (data not shown). When examining the action of PD 98059 on PMA-induced effects, it is extremely important to control for concentrations of PMA. Because of the nature of the inhibitory action of PD 98059 on the tyrosine phosphorylation of erk1 and 2, it is possible to overcome this inhibition by using relatively high concentrations of PMA (data not shown).

Effect of SB 203580 and PD 98059 on agonist-induced arachidonic acid release in human neutrophils

Because cPLA2 has been shown to mediate the production of agonist-induced arachidonic acid release and phosphorylation of the enzyme is necessary for its activation (1–8), the relationship between inhibition of cPLA2 phosphorylation and arachidonic acid release in human neutrophils was examined. To determine this, neutrophils were treated with SB 203580 or PD 98059, stimulated with either LPS, TNF-α, PAF, FMLP, LPS plus PAF, LPS plus

<table>
<thead>
<tr>
<th>Condition</th>
<th>Arachidonic Acid Release (relative to control)</th>
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<tr>
<td>No addition</td>
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<tr>
<td>LPS</td>
<td>2.3 ± 0.1</td>
</tr>
<tr>
<td>TNF-α</td>
<td>1.1 ± 0.1</td>
</tr>
<tr>
<td>PAF</td>
<td>2.1 ± 0.2</td>
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<tr>
<td>FMLP</td>
<td>2.4 ± 0.2</td>
</tr>
<tr>
<td>LPS + PAF</td>
<td>6.3 ± 0.8</td>
</tr>
<tr>
<td>LPS + FMLP</td>
<td>8.7 ± 0.7</td>
</tr>
<tr>
<td>TNF-α + PAF</td>
<td>4.0 ± 0.2</td>
</tr>
<tr>
<td>TNF-α + FMLP</td>
<td>3.6 ± 0.6</td>
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*Neutrophils treated either with diluent, 15 μM SB 203580, or 15 μM PD 98059 for 30 min were then stimulated with LPS (100 ng/ml plus 1% serum) for 30 min; TNF-α (10 ng/ml) for 5 min; PAF (1 μM) for 2 min; or FMLP (500 nM) for 2 min. When LPS or TNF-α were used together with PAF or FMLP, the cells were first incubated with LPS plus serum for 30 min or TNF-α for 5 min and then stimulated with either PAF or FMLP for 2 min.

Effect of SB 203580 and PD 98059 on the activity of CoA-IT in microsomes prepared from human neutrophils

One possible explanation for the observed inhibition of FMLP-induced arachidonic acid release by SB 203580 is that this agent inhibits CoA-IT activity. This enzyme selectively remodels arachidonate between different phospholipids (33). Consistent with this possibility is the recent finding that β-lactams SB 212047 and other similar compounds are irreversible inhibitors of this enzyme (33). These authors have reported that the inhibition of CoA-IT blocks the release of arachidonic acid in stimulated neutrophils (33). To examine this possibility, we measured the effects of SB 203580 and PD 98059 on the activity of CoA-IT in microsomes isolated from human neutrophils. The results summarized in Fig. 7 clearly show that these two inhibitors have no effect on the activity of CoA-IT. Several concentrations (as high as 80 μM) were tested, and the results were the same. Note that tosylamido-2-phenylethyl chloromethyl ketone (TPCK) (1 mM), a known inhibitor of this enzyme, reduces the activity of this enzyme greatly (33). Also PMSF (1 mM), which is known to inhibit this enzyme by 40%, produces similar inhibition (33). We also found that 1-alkyl-2-acyl-GPC exhibits a negative feedback on CoA-IT (Fig. 7). This effect was concentration dependent (data not shown).

Discussion

Arachidonic acid release and the generation of biologically important eicosanoids in human neutrophils and other cell types require the activation of cPLA2. Phosphorylation of cPLA2 is a key step in
The activation of this enzyme and the release of arachidonic acid from phospholipids (7, 36). Depending on the cell type, the released arachidonic acid is then metabolized to generate one or more important lipid mediators such as leukotrienes, prostacyclin, prostaglandins, and thromboxane (1–8). While there are several sites on cPLA₂ that can be phosphorylated (36), it is generally agreed that cPLA₂ can be phosphorylated on serine 505 by erk2 in vitro. However, there is strong evidence suggesting that kinases other than erk1/2 may be involved in the phosphorylation of cPLA₂ (9–16). At the present, the kinases that phosphorylate this enzyme in vivo remain to be identified.

The data presented here show several new and interesting interrelated points concerning the kinases that phosphorylate cPLA₂ in human neutrophils, and probably other cell types. First, in intact cells, cPLA₂ can be phosphorylated by several kinases. These include erk1/2, p38 MAPK, and as yet unidentified kinases. Second, the kinase involved in the phosphorylation of cPLA₂ depends on the stimulus used. For example, erk1/2 and p38 MAPK mediate the phosphorylation of cPLA₂ in cells stimulated by PAF and TNF-α, respectively. On the other hand, other kinases can also mediate the phosphorylation of cPLA₂ in cells stimulated by LPS or FMLP. Third, while PMA-induced phosphorylation of cPLA₂ is inhibited by the PKC inhibitor GO 6850 in intact neutrophils, the effect of PKC on cPLA₂ is mediated through erk1/2. One possible kinase that may mediate the action of FMLP on cPLA₂ is the calcium calmodulin protein kinase(s). However, utilization of a potent inhibitor of calcium calmodulin protein kinase II, K-252a (37), failed to inhibit the FMLP-induced phosphorylation of cPLA₂ in human neutrophils (data not shown).

Interestingly, while the FMLP-induced phosphorylation of cPLA₂ is not affected by inhibitors of the p38 MAPK cascade or erk, the FMLP-stimulated arachidonic acid release is greatly reduced by inhibitors of the p38 MAPK and erk cascades. This is not due to possible action of these inhibitors on CoA-IT, because, unlike β-lactams SB 212047 (33), SB 203580 or PD 98059 does not inhibit the activity of CoA-IT (Fig. 7). It is possible that these inhibitors affect the translocation of cPLA₂ to where its substrates are located and/or reduce the rise in the intracellular concentration of free calcium. In preliminary experiments, we found that the rise in calcium is qualitatively similar in both control and SB 203580-treated cells, which were stimulated with FMLP (data not shown). The determination of the kinases that mediate the phosphorylation of cPLA₂ in human neutrophils stimulated by LPS and FMLP, the site(s) that are phosphorylated by each stimulus, and the effects of these inhibitors on the basal and stimulated distribution of cPLA₂ remain to be examined and are the subjects of future studies.

**FIGURE 7**. CoA-IT activity in microsomes isolated from human neutrophils. A, The effect of protein concentrations on CoA-IT activity is shown. Varying amounts of protein from human neutrophils microsomal fraction were preincubated for 40 min at 37°C with 20 µM SB 203580 and CoA-IT activity measured as described in Materials and Methods. The reaction was conducted for 10 min at 37°C. The ordinates represents the phosphorylation of cPLA2 in cells stimulated by PAF and the stimulus used. For example, erk1/2 and p38 MAPK mediate the phosphorylation of cPLA₂ (9–16). At the present, the kinases that phosphorylate this enzyme and the release of arachidonic acid from phospholipids (7, 36). Depending on the cell type, the released arachidonic acid is then metabolized to generate one or more important lipid mediators such as leukotrienes, prostacyclin, prostaglandins, and thromboxane (1–8). While there are several sites on cPLA₂ that can be phosphorylated (36), it is generally agreed that cPLA₂ can be phosphorylated on serine 505 by erk2 in vitro. However, there is strong evidence suggesting that kinases other than erk1/2 may be involved in the phosphorylation of cPLA₂ (9–16). At the present, the kinases that phosphorylate this enzyme in vivo remain to be identified.

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**References**