Role of p38 in the Priming of Human Neutrophils by Peritoneal Dialysis Effluent

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Peritoneal dialysis effluent (PDE) contains a low-molecular-weight substance that is able to prime human neutrophils for the release of arachidonic acid and superoxide anion. Conventional priming agents, such as tumor necrosis factor alpha (TNF-α), are known to signal via mitogen-activated protein (MAP) kinases; at least one possible substrate for MAP kinases is cytosolic phospholipase A₂ (cPLA₂). Phosphorylation of this enzyme results in arachidonic acid release, and this fatty acid is a potent primer and activator of the human neutrophil NADPH oxidase. Because of the striking similarities between the priming of neutrophils with agents such as TNF-α and PDE, we have investigated the signalling pathways evoked by PDE and explored the possibility that cPLA₂ is a target for activated MAP kinases. Our results show that PDE treatment of human neutrophils results in the phosphorylation of the p38 kinase rather than the p42 and p44 kinases. Phosphorylation of p38 is transient with maximal activity being observed 1 min after exposure to PDE. We were unable to demonstrate that activation of p38 resulted in phosphorylation of cPLA₂; furthermore, translocation of this enzyme to a membrane-containing fraction was not enhanced in PDE-treated neutrophils. Taken together, these data suggest that, in a manner similar to that of TNF-α, PDE primes human neutrophils by the activation of the p38 kinase. However, unlike the cytokine, the activation of this protein does not result in phosphorylation or activation of cPLA₂.

The intracellular signalling pathways utilized by priming agents, such as lipopolysaccharide (LPS), tumor necrosis factor alpha (TNF-α), and granulocyte-macrophage colony-stimulating factor (GM-CSF), have recently become an area of intense study. An increasing body of evidence has been presented to suggest that all of these priming agents act by signalling through the mitogen-activated protein (MAP) kinase cascade (10, 24, 32, 33, 37). MAP kinases are proline-directed serine-threonine protein kinases that are activated by phosphorylation upon threonine and tyrosine residues in a Thr-X-Tyr motif that is found in an activation loop proximal to the ATP and substrate binding sites. There are three main classes of MAP kinases: the erk, c-jun N-terminal, and p38 kinases. All three groups differ in size of the activation loop and nature of the X amino acid in the Thr-X-Tyr motif (i.e., Glu in the erk kinases, Pro in the c-jun kinases, and Gly in p38). Signalling through this cascade by priming agents appears to involve one of two pathways: treatment of cells with GM-CSF appears to phosphorylate erk I/II (32), while LPS and TNF-α treatment results in phosphorylation of p38 (10, 24, 32, 33, 37). Although many substrates for erk I/II and p38 have been identified in vitro (for a review, see reference 21), the exact pathway from MAP kinase activation to a functional cellular response (e.g., priming) has yet to be elucidated. One suggested route has been via phosphorylation and/or translocation of the 85-kDa cytosolic phospholipase A₂ (cPLA₂) (16). Phosphorylation and subsequent translocation of this enzyme results in arachidonic acid release (9), and it is well documented that this fatty acid is able to both prime and directly activate the NADPH oxidase in human neutrophils (5, 12, 27). We have previously reported a novel priming agent present in peritoneal dialysis effluent (PDE) that is a potent primer of NADPH oxidase activity and arachidonic acid release in human neutrophils (6, 17). In this study, we have investigated the MAP kinase cascade utilized by this priming agent and explored the possibility that its effects upon the NADPH oxidase are mediated through the phosphorylation of cPLA₂.

**MATERIALS AND METHODS**

**Reagents.** Polyclonal anti-p38 and anti-erk II MAP kinases were purchased from Santa Cruz Biotechnology Inc., Santa Cruz, Calif. and anti-phospho-specific p38 and anti-phospho-specific erk I/II were purchased from Cellbiochem, Nottingham, England. Polyclonal cPLA₂ was a kind gift from Astra Charnwood, Leics, England, and SB203580, SB203590, PD-098,098, and genistein were purchased from Alexis Corporation, Nottingham, England. All other reagents, unless otherwise stated, were purchased from Sigma Chemical Company, Poole, Dorset, England.

**Preparation of neutrophils.** Human peripheral blood neutrophils were prepared by standard methods (3). Fresh venous blood was taken into EDTA (dipotassium salt) to give a final concentration of 3.5 mM. Ertyhocytes were sedimented on dextran, and the leukocyte-rich plasma was further purified over a Ficoll gradient (lymphocyte separation medium; Flow Laboratories, Herts, England). The neutrophil-rich pellet was subjected to hypotonic lysis to remove remaining erythrocytes and washed twice in phosphate-buffered saline (PBS; pH 7.4). Purification of cells by this method routinely gave preparations of >99% viability, as assessed by trypan blue exclusion, and >97% purity, as assessed by examination of stained cytospin preparations. Cells were counted in a hemocytometer and suspended at a concentration of 1 × 10⁶ ml⁻¹.

**PDE.** Six PDEs (1.36%; Dianecel; Baxter Travenol Inc., Chicago, Ill.) were obtained from patients receiving continuous ambulatory peritoneal dialysis (CAPD) after an intraperitoneal dwell of 4 h. All patients had been established on CAPD for more than 2 months, were not suffering infection, and had not received antibiotic therapy over the preceding 4 weeks. PDE was stored at −70°C until required. Before use, PDE was filtered through a 0.2-μm-pore-size membrane and the pH was adjusted to 7.4 by the addition of HEPES, to give a final concentration of 20 mM. The concentrations of creatinine and urea in PDE were determined by autoanalysis; the concentration of protein was determined by the method of Lowry et al. (19); the concentration of endotoxin was determined by the limulus amoebocyte lysate assay (Sigma diagnostic kit; Sigma Chemical Company); and the concentrations of TNF-α and interleukin 1β were determined by enzyme-linked immunosorbent assay (ELISA) (R & D Systems, Abingdon, Oxon, England).

**Assay for superoxide.** Superoxide production by neutrophils was determined by lucigenin-enhanced chemiluminescence in a plate-reading luminometer (Lumiscan; Labsystems, Basingstoke, England). The reaction mixture (200 μl) contained 25 μM lucigenin, PBS (pH 7.2), 1 mM CaCl₂, 0.7 mM MgCl₂, 0.1%
low endotoxin bovine serum albumin, inhibitor and/or vehicle and neutrophils to give a final concentration of 1 × 10^6 ml⁻¹. PDEs were routinely used at a concentration of 50% (vol/vol). Reactions were initiated by the addition of 1 μM N-formyl-Met-Leu-Pho (fMLP). Superoxide anion formation was taken as the integral of superoxide dismutase inhibitable light output over the initial 30 min of the reaction.

**Assay of inhibitor toxicity.** The toxicities of PD-098,059, SB203580, SB203590, genistein, and the vehicle upon the neutrophils were determined over a 60-min period by ATP bioluminescence (4). The ability of these compounds to scavenge superoxide anions was determined by using a xanthine-xanthine oxidase cell-free system (17).

**Preparation of neutrophils for cPLA₂ and MAP kinase analysis.** Neutrophils were incubated with end-over-end rotation at 37°C in either PBS (pH 7.4) containing 1 mM CaCl₂, 0.7 mM MgCl₂, and 0.1% (vol/vol) low endotoxin bovine serum albumin or PDE (50% [vol/vol]). In some experiments, cells were also stimulated with 1 μM fMLP or 100 ng of phorbol myristate acetate (PMA) ml⁻¹. Neutrophils (2 × 10⁶ cell equivalents) were removed at various time intervals, plunged into 30 ml of ice-cold PBS, and rapidly pelleted by centrifugation at 4°C (250 × g for 5 min). The pellet was suspended in 400 μl of ice-cold lysis buffer (50 mM HEPES [pH 7.2] containing 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 1 mM sodium orthovanadate, 1 mM sodium pyrophosphate, and 100 μM of mammalian cell extract protease inhibitor cocktail [Sigma Chemical Company] ml⁻¹) and disrupted by sonication on ice (two 10-s bursts/power setting 2; Rapidis 50 cell disrupter; Ultrasonics, London, England). Cell disruption was confirmed by light microscopy. Cell homogenates were centrifuged at 250 × g for 15 min at 4°C to remove unbroken cells and cell debris before further centrifugation at 100,000 × g for 60 min at 4°C in a Beckman L8M ultracentrifuge. The supernatant (cytosolic fraction) was retained, and the membrane-containing pellet was suspended in 400 μl of ice-cold lysis buffer containing 0.5% [vol/vol] Triton X-100. Membrane proteins were solubilized by further sonication on ice (two 10-s bursts) before filtration through a 0.45-μm-pore-size membrane to remove particulate material (ultrafilter units; Millipore, Bedford, Mass.). Protein concentrations were determined in both cytosol and membrane fractions by the method of Lowry et al. (19).

**Immunoblotting.** Fifty microliters (equivalent to 50 μg of protein) of cytosol or 100 μl (100 μg of protein) of membrane were mixed with an equal volume of 4× concentrated Laemmli stopping solution (62.5 mM Tris-HCl [pH 6.8], 20% [vol/vol] glycerol, 5% [vol/vol] concentrated Laemmli stopping solution (62.5 mM Tris-HCl [pH 6.8], 20% [vol/vol] glycerol, 5% [vol/vol] SDS, 50% [vol/vol] sucrose, trace bromophenol blue) and heated at 95°C for 10 min. Samples were further sonication on ice (two 10-s bursts/power setting 2; Rapidis 50 cell disrupter; Ultrasonics, London, England). Once visualized, blots were stripped of the primary antibody-secondary antibody complex by exposure of the plates to iodine vapors. The area of silica corresponding to the fatty acid composition was calculated by the computer multifunction statistics library package NWAStatpak (mean ± SEM) (range 57 to 114 pg ml⁻¹); again, these levels are below that reported to prime neutrophils (35). Furthermore, LPS priming requires preincubation of neutrophils with endotoxin while PDE priming is immediate (6, 35). Further, biochemical analysis of the fluids employed revealed protein levels for both PDE-primed and unprimed cells. This inhibitory concentration did not affect cell viability, as determined by measurement of ATP release (Fig. 1), and did not affect superoxide measurement in a cell-free xanthine-xanthine oxidase system (data not shown), demonstrating that it did not act as a free radical scavenger. The effect of SB203580 [4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1H-imidazole], a p38 kinase inhibitor, upon fMLP-induced superoxide release from PDE-primed and unprimed neutrophils is shown in Fig. 2. SB203580 was a moderately good inhibitor of fMLP superoxide anion generation, displaying an estimated IC₅₀ of 1.5 μM.

**RESULTS**

Because both LPS and TNF-α treatments of neutrophils are known to result in the priming of the NADPH oxidase and the activation of MAP kinases, we directly measured the relative levels of these two agents in the six dialysis fluids employed in this study. TNF-α levels were below that detectable by ELISA; furthermore, the molecular weight of the priming agent in PDE is considerably lower than that of the cytokine (6). Endotoxin levels in PDE were 71.25 ± 9.22 pg ml⁻¹ (mean ± SEM) (range 57 to 114 pg ml⁻¹); again, these levels are below that reported to prime neutrophils (35). Furthermore, LPS priming requires preincubation of neutrophils with endotoxin while PDE priming is immediate (6, 35). Further, biochemical analysis of the fluids employed revealed protein levels (mean ± SEM) of 3.18 ± 0.37 mg ml⁻¹ (range 2.25 to 4.90 mg ml⁻¹), urea concentrations of 18.18 ± 1.56 mM (range 14.0 to 25.4 mM), and creatinine concentrations of 900 ± 70 μM (range 707 to 1290 μM). As for TNF-α, the levels of interleukin 1β were below that detectable by ELISA.

**Effects of inhibition of MAP kinases and protein tyrosine kinase upon NADPH oxidase activity.** Figure 1 shows the effects of treating neutrophils with PD-098,059, a potent and specific inhibitor of MAP kinase kinase (MEK) which is reported to catalyze the phosphorylation of erkII upon threo-nine202 and tyrosine204. PD-098,059 (0 to 100 μM) upon superoxide generation in neutrophils challenged with fMLP (1 μM). Neutrophils were incubated in buffer (open circles) or PDE (closed circles). Results are expressed as percent inhibition from the control (no PD-098,059) ± SEM (n = 6; six dialysis effluents upon six donor neutrophils). Absolute control values (100%) were 1,777 ± 42 and 948 ± 52 relative light units (RLU) for primed and unprimed cells, respectively. The inset shows the effect of PD-098,059 (0 to 100 μM) upon ATP release from neutrophils (open squares) and from ATP standard control (closed squares).
When neutrophils were primed with PDE, the concentration response curve of the inhibitor was significantly left-shifted, reducing the effective IC\textsubscript{50} by sixfold to 0.25 m\textsubscript{M}. In a similar manner to PD-098,059, the effectiveness of SB203580 at inhibiting NADPH oxidase activity increased with increasing incubation times (up to 60 min) (data not shown). However, significant inhibition was evident when oxidase activity was measured immediately after the addition of the inhibitor (i.e., no incubation period) (data not shown). In our hands, an incubation period of 30 min prior to stimulation by fMLP produced consistent results for both SB203580 and PD-098,059. Figure 3 demonstrates typical results from an individual representative experiment showing that when the concentration of SB203580 was 1.5 m\textsubscript{M}, the priming effect of PDE to fMLP was completely lost. However, for both primed and unprimed neutrophils the generation of superoxide in the presence of SB203580 could not be completely abolished, even at concentrations approaching 100 m\textsubscript{M}, suggesting that the inhibitor has little effect upon spontaneous, unstimulated superoxide generation. Further experiments were conducted with the recently developed pyridinyl imidazole inhibitor SB203590 \[4-(4-fluorophenyl)-2-(4-hydroxyphenyl)-5-(4-pyridyl)1H-imidazole\], a compound that is reportedly more selective for the \(\beta\) isoform of p38 (p38\(\beta\)) (13). These experiments demonstrated that SB203590, like SB203580, was a highly potent inhibitor of the priming response of PDE (IC\textsubscript{50} = 1 \textmu M and 0.2 \textmu M for unprimed and primed neutrophils, respectively) (Fig. 4).

In view of the results obtained with specific inhibitors of MAP kinase pathways, we also treated neutrophils with the broad-range tyrosine kinase inhibitor genistein (Fig. 5). Incubation of neutrophils with 10 \textmu M genistein resulted in a >80% inhibition of fMLP-stimulated superoxide generation. We estimated the IC\textsubscript{50} of this compound in our system to be 1 \textmu M. Priming of neutrophils by PDE after treatment with genistein did not effect the potency of the inhibitor. As with the MAP kinase inhibitors PD-098,059 and SB203580, the observed loss of oxidative activity was not attributable to cell death, since incubation of neutrophils with concentrations of genistein up to 100 \textmu M did not effect the release of ATP (Fig. 5). Furthermore, genistein did not effect the measurement of superoxide generated by a cell-free system (data not shown).

**Phosphorylation of erkI/II and p38.** In an attempt to confirm that p38 and not erkI/II was the MAP kinase signalling pathway utilized by PDE in its priming of human neutrophil NADPH oxidase, we also subjected whole neutrophil homogenates to SDS-PAGE and immunoblotting with antibodies to phospho-ERK1/2 and phospho-p38 (14). As shown in Figure 6, PDE priming of human neutrophils resulted in a marked increase in phosphorylation of both erkI/II and p38, consistent with our previous observations from assays of superoxide generation (14). These experiments confirmed that the priming effect of PDE was mediated, at least in part, by activation of erkI/II and p38.
oxidase activity, we examined the phosphorylation status of these proteins in neutrophils exposed to PDE. Both p38 and erkI/II require phosphorylation upon threonine and tyrosine residues to become fully functional enzymes (28). This change in phosphorylation status can be detected either by the use of antibodies specific for the phosphorylated enzyme or by a mobility shift of the protein on polyacrylamide gels (i.e., the phosphorylated form of the protein migrates more slowly).

Figure 6 shows the effect of exposure of neutrophils to PDE for various time periods. Western blots were probed with a polyclonal antibody to erkII (p42), the predominant erk form found in human neutrophils (22). The antibody demonstrated slight cross-reactivity with erkI (as indicated by the suppliers and as shown in Fig. 6A, lanes 3 to 7); however, no mobility shift of the protein was observed (lanes 1 to 7), suggesting that phosphorylation had not occurred. As a positive control for this experiment, some cells were stimulated with 100 ng of PMA ml\(^{-1}\) (Fig. 6A, lane 8), a known activator of erk (38). In these cells, the erkII band appeared as a doublet, with the slower-migrating band representing the phosphorylated protein. These results were further confirmed by stripping the blot and reprobing it with an antibody specific for phosphorylated erkII. In this case, only the PMA-treated samples showed a positive band (Fig. 6B, lane 8).

Figure 7A shows the effect of probing the same blot with an antibody to p38. The results indicate that all of the sample lanes contained a similar total amount of this protein. Further reprobing of this blot with an antibody specific for the phosphorylated protein (Fig. 7B) reveals that not only PMA stimulation of cells results in p38 phosphorylation (lane 8) but also exposure of cells to PDE. Phosphorylation by PDE was transient, appearing after 30 s (Fig. 7B, lane 3), peaking at 60 s (lane 5), and diminishing by 5 min (lane 7). Some phosphorylation of p38 was observed in cells in buffer; this phosphorylation appeared to increase with time (Fig. 7B, lanes 2, 4, and 6), suggesting that some activation of neutrophils had occurred during sample handling. The majority of both erkII and p38...
PDE. Figure 8 demonstrates that exposure of neutrophils to PDE for periods of up to 5 min failed to induce a measurable increase in cPLA2 activity in cytosolic neutrophil fractions (Fig. 8A); however, despite this vigorous response these cells appear unable to function normally. We have previously reported that PDE removed from end-stage renal failure patients following a 4-h intraperitoneal dwell contains a potent priming agent of both fMLP-stimulated arachidonic acid release (17) and NADPH oxidase activity (6) in human neutrophils. We have speculated that the excessive release of neutrophil reactive oxygen species due to this priming agent may scar the peritoneal membrane, compromising its efficiency during dialysis and promoting peritonitis (17). In this study, we have investigated the MAP kinase signalling pathway(s) utilized by PDE and investigated the possibility that MAP kinase activation results in cPLA2 phosphorylation, causing release of arachidonic acid and superoxide anion.

To initially investigate the relationship between the priming of human neutrophils and oxidase activity, we studied the effects of two recently developed specific inhibitors of the erk and p38 MAP kinase pathways upon fMLP-induced superoxide release (PD-098,059 and SB203580, respectively). In agreement with other authors, we established that the selective inhibition of MAP kinase kinase (MEK) by PD-098,059 has little effect upon fMLP-stimulated oxidase activity (29, 37, 38). Our data would suggest that erk plays only a very limited role in mediating the oxidase response in human neutrophils. This observation is further supported by the fact that the fMLP-induced gel shift of erk II (indicative of phosphorylation) (38) is completely abolished by 30 μM PD-098,059, while the same inhibitory concentration will reduce only the oxidase response by 20% (29, 38). Avdi et al. (1) have reported inhibition of the fMLP response approaching 45% with 10 μM PD-098,059, while the same inhibitor, but the activity of the p38 kinase is markedly reduced by 20% (29, 38). Schnyder et al. report an IC50 for SB203580 of approximately 1.5 μM, which is in good agreement with other authors who have used similar imidazole compounds (e.g., Schnyder et al. report an IC50 of approximately 2 μM for SB203580).

In contrast to PD-098,059, treatment of human neutrophils with either SB203580 or SB203590 markedly reduced fMLP-induced oxidase activity. Both of these pyridinyl imidazole inhibitors are thought to inhibit p38 kinase activity by binding to the ATP binding site (36). However, tyrosine phosphorylation of p38 appears not to be effected by this compound since TNF-α-induced phosphorylation of p38 is unaffected by the inhibitor, but the activity of the p38 kinase is markedly reduced at about 15 μM (34). In our hands, we report an IC50 for SB203580 of approximately 1.5 μM, which is in good agreement with other authors who have used similar imidazole compounds.

**DISCUSSION**

CAPD is a popular form of treatment for patients with end-stage renal failure that is frequently complicated by episodes of recurrent bacterial peritonitis. Peritonitis is charac-

**TABLE 1. Activity of cPLA2 in cytosolic fractions of neutrophils exposed to PDE for various times**

| Time (min) | Buffer alone | Buffer and PDE |
|------------|--------------|----------------|
| 0          | 8.0 ± 2.0    |               |
| 0.5        | 9.3 ± 1.9    |               |
| 1          | 7.0 ± 3.0    |               |
| 5          | 10.0 ± 1.8   |               |

**Note:** Enzyme activity was measured by the release of 14C-labelled phosphatidylcholine (PC) as described in Materials and Methods. In some instances, neutrophils were stimulated with 100 ng of PAM ml⁻¹ to provide a positive control. The results represent the means ± SEM for three separate experiments.

**Phosphorylation and translocation of cPLA2.** One mechanism by which p38 activation may result in a primed oxidase response is through the phosphorylation and/or membrane translocation of cPLA2. We therefore investigated the activation and translocation of this enzyme in neutrophils exposed to PDE. Figure 8 demonstrates that exposure of neutrophils to PDE for periods up to 5 min failed to phosphorylate cPLA2, compared to PMA-treated cells (Fig. 8, lanes 8). The majority of cPLA2 was present within the cytosolic preparation of neutrophils (Fig. 8A); however, we were able to detect small amounts of PLA2 in solubilized membrane preparations (Fig. 8B), but exposure of neutrophils to PDE did not result in greater translocation of the enzyme to the membrane.

To confirm that the PDE-induced activation of p38 did not give rise to the subsequent activation of cPLA2, we directly measured the activity of this enzyme in both cytosol and membrane preparations of human neutrophils exposed to PDE by using 14C-labelled phosphatidylcholine as the substrate. The results shown in Table 1 demonstrate that the exposure of neutrophils to PDE for periods of up to 5 min failed to induce a measurable increase in cPLA2 activity in cytosolic neutrophil fractions. We were unable to access the activation of the enzyme in membrane preparations under these conditions, possibly because of a dilution of labelled substrate by membrane phospholipids (data not shown).
μM for Smith Kline and French compound 86002 [29] while Nick et al. report a value of between 0.5 and 1.0 μM for the same compound [25]). Interestingly, when neutrophils were primed with PDE the concentration response curve to SB203580 was significantly left-shifted, reducing the IC50 to approximately 0.25 μM. Indeed, concentrations of SB203580 that exceeded 1.5 μM completely destroyed the ability of PDE to prime cells for fMLP-induced oxidase activity. These data would strongly suggest that the priming effect of PDE on human neutrophils is mediated via the p38 kinase. It is well documented that the tyrosine-directed phosphorylation of proteins is an essential prerequisite for activation of the NADPH oxidase, particularly during the early phase of superoxide anion release (18). Since both erk and p38 require phosphorylation (threonine202/tyrosine182 and threonine180/tyrosine182, respectively) for their complete activation, it would seem reasonable to assume that both of these enzymes would act as potential targets for genistein, a potent inhibitor of tyrosine specific kinases. This, however, does not appear to be the case. GM-CSF-enhanced erk activity and the subsequent phosphorylation of cPLA2 are both blocked by genistein in a time- and concentration-dependent manner (11, 23). Yet the compound is ineffective at preventing cPLA2 phosphorylation by agents that signal via the p38 kinase pathway, i.e., TNF-α and LPS (10, 33). In agreement with others (27, 29, 38), we found that genistein was a good inhibitor of fMLP-induced superoxide release. However, it was equally as effective against PDE-primed neutrophils, suggesting that the agent inhibits tyrosine kinases that are essential for oxidase activity but are also common to both the p38 and erk pathways.

To further confirm the involvement of the p38 kinase in the priming of human neutrophils by PDE, we directly examined the phosphorylation of MAP kinases in cell lysates. In agreement with other authors (2, 31), we found that the majority of both erk and p38 proteins resided in the cytosolic fraction of neutrophils. Furthermore, by using phospho-specific antibodies and electrophoretic mobility shifts, we were able to clearly establish that only p38 was phosphorylated in the presence of PDE. The phosphorylation of this protein was transient, with maximal phosphorylation being apparent after 60 s of exposure to effluent.

Although over the past several years it has become apparent that both direct stimuli, such as fMLP, PMA, and platelet-activating factor (1, 15, 25, 29, 30, 38), and priming agents, such as GM-CSF, LPS, and TNF-α (10, 24, 32, 33, 37), result in the activation of MAP kinase cascades in human neutrophils, the pathway from these cascades to physiological responses has yet to be determined. Several substrates of both erk and p38 have recently been identified (21). One such substrate that has generated recent interest is the 85-kDa cPLA2 (16). In human neutrophils, cPLA2 is thought to translocate from the cytosol to the membrane as intracellular Ca2+ concentrations rise. This translocation, coupled with phosphorylation upon serine175 results in a fully activated enzyme. Once at the membrane, cPLA2 selectively cleaves arachidonoyl-containing phospholipids resulting in the liberation of arachidonic acid and a lyso-phospholipid (9). Since arachidonic acid is a potent activator and primer of human neutrophil oxidase activity (5, 12, 17, 27) and since exposure of neutrophils to PDE results in the release of the fatty acid (17), it is tempting to speculate that cPLA2 is the substrate for PDE-activated p38. However, we were unable to demonstrate either increased phosphorylation or translocation of this enzyme upon exposure of neutrophils to PDE.

Although the target of PDE-activated p38 remains elusive, several possibilities are apparent. The NADPH oxidase in human neutrophils is a multicomponent enzyme that is functional only when a number of cytosolic and granule components combine at the membrane. One important cytosolic component of this enzyme complex is p47phox. For activation, this protein requires phosphorylation upon a number of serine residues, several of which are recognized by proline-directed kinases, such as erk and p38 (2). Furthermore, the locations of both activated p38 and p47phox, i.e., the cytoplasm, are consistent with the latter being a potential substrate for p38.

Another important component of the neutrophil NADPH oxidase is the terminal electron donor b558. This flavohemoprotein is believed to be located within the specific granules of neutrophils (26). The observation that imidazole inhibitors, such as SB203580 (rather than MEK inhibitors, such as PD-098059), inhibit the expression of the neutrophil adhesion molecule CD11/11b, also located within the specific granules (29), is a clear indication that p38 is involved in the control of the release of these granules. We have previously demonstrated that PDE augments specific granule release in neutrophils (8). The observation that this release precedes oxidase activity (8) and mimics the time course of p38 activation by PDE, i.e., peaks at approximately 1 min, would suggest that control of degranulation resulting in enhanced release of b558 and hence oxidase activity is a more likely route for PDE priming. If this is the case, arachidonic acid release seen in PDE-treated neutrophils may be secondary to oxidase activity and arise as a result of release of the 14-kDa secretory PLA2, also located within the specific granules (20). Until effective and specific cytosolic and secretory PLA2 inhibitors are developed, the contribution of these two enzymes to oxidase activity remains difficult to determine (5).

In summary, our studies suggest that priming of human neutrophils with PDE, in a manner similar to that of LPS and TNF-α, is via the p38 kinase and not erk. However, unlike these conventional priming agents, we were unable to show that activation of this cascade resulted in phosphorylation or activation of cPLA2. The possibility remains that p38 activation by PDE may result in direct phosphorylation of oxidase components or may simply increase release of secondary granules to potentiate superoxide release. These possibilities are currently under investigation in our laboratory.

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