Examination of the signal transduction pathways leading to activation of extracellular signal-regulated kinase by formyl-methionyl-leucyl-phenylalanine in rat neutrophils

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Abstract The signaling pathways leading to extracellular signal-regulated kinase (ERK) activation in formyl-methionyl-leucyl-phenylalanine (fMLP)-stimulated rat neutrophils were examined. fMLP-stimulated ERK activation based on immunoblot analysis with antibodies against the phosphorylation form of ERK was attenuated by the pretreatment of cells with pertussis toxin but not with a dual cyclo-oxygenase/lipoxygenase inhibitor BW755C. Exposure of cells to the tyrosine kinase inhibitor genistein, phosphatidylinositol 3-kinase (PI3K) inhibitors wortmannin and LY294002, or protein kinase C (PKC) inhibitors Gö6976, Gö6983, and GF109203X inhibited fMLP-stimulated ERK phosphorylation in a concentration-dependent manner. In addition, both the phospholipase C (PLC) inhibitor U73122 and the Ca^{2+} chelator BAPTA attenuated ERK activation. These results indicate that $G_{i/o}$ protein, tyrosine kinase, PI3K, PKC, and PLC/Ca^{2+}, but not arachidonate metabolites, act upstream of fMLP-stimulated ERK activation.

Key words: Neutrophil; Extracellular signal-regulated kinase; Tyrosine kinase; Protein kinase C; Intracellular calcium; Phosphatidylinositol 3-kinase

1. Introduction

Neutrophils constitute the first line of defense against invading microorganisms and they are activated in response to a variety of soluble and particle stimuli. After activation, neutrophils engage in migration, phagocytosis, granule release, and the production of reactive oxygen species [1]. Although the signaling pathways responsible for neutrophil activation have not been fully delineated, a rapid induction of protein phosphorylation is thought to be crucial to the regulation of neutrophil effector functions [2,3]. Although the identities of many of the phosphorylated substrates present in activated neutrophils remain unknown, it has been demonstrated that extracellular signal-regulated kinase (ERK), also known as p44/42 mitogen-activated protein kinase (MAPK), can be phosphorylated [4]. Several isoforms of ERK have been described, and at least two of them, ERK1 (p44 MAPK) and ERK2 (p42 MAPK), are expressed in neutrophils [4]. ERK1 shares 85% homology with ERK2, and both are activated as a result of a cascade of different upstream kinases [5]. ERK has been proposed to play roles in cell growth and differentiation in mitotic cells [6]. Studies of ERK in neutrophils appear to have revealed some non-mitotic signaling functions for these enzymes, such as adhesion and superoxide anion generation [7,8]. These results suggest that ERK may be the component of signal transduction pathways leading to crucial neutrophil functional responses during inflammation and infection.

Cell stimulation induces a signaling cascade that leads to the activation of ERK via phosphorylation on both tyrosine and threonine residues within the TEY motif [9]. Structural analysis of ERK indicates that phosphorylation induces a conformational change that exposes the active site for substrate binding. Phosphorylation of the TEY motif on ERK is catalyzed by a dual specificity kinase termed MAPK kinase (MEK). MEK is itself activated by the phosphorylation of serine residues by Raf or MEK kinase (MEKK) [10]. The bacterial signal peptide formyl-methionyl-leucyl-phenylalanine (fMLP) and other chemoattractants activate ERK with kinetics concordant with the rapid responses of neutrophils [4]. Although ERK activation of neutrophils by fMLP has been suggested via the activation of Ras, Raf, and MEK [11,12], the signaling pathways responsible for fMLP-stimulated ERK activation have not been fully delineated. In this study, to understand the proximal signaling pathways involved in fMLP-stimulated ERK activation, the effects of pharmacological inhibitors of several signaling pathways on the phosphorylation of ERK were examined.

2. Materials and methods

2.1. Neutrophil isolation

Rat blood was collected from the abdominal aorta and the neutrophils were purified by dextran sedimentation, centrifugation through Ficoll-Hypaque, and the hypotonic lysis of erythrocytes [13]. Purified neutrophils containing >95% viable cells were normally resuspended in Hanks' balanced salt solution (HBSS) containing 10 mM HEPES, pH 7.4, and 4 mM $NaHCO_3$, and kept in an icebath before use.

2.2. Immunoblotting analysis

Cells were preincubated with test drugs at 37°C for the indicated time before stimulation with fMLP plus dihydrocytochalasin B (CB). One minute later, reactions were quenched by the addition of stopping solution (20% trichloroacetic acid, 1 mM PMSF, 2 mM *N*-ethylmaleimide, 10 mM NaF, 2 mM Na_3VO_4, 2 mM *p*-nitrophenyl phosphate, 7 μg/ml of leupeptin and pepstatin). Proteins were electrophoresed on 10% SDS-polyacrylamide gels and then transferred to polyvinylidene difluoride membrane. The membranes were blocked with 5% non-fat dried milk in TBST buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl and 0.1% Tween 20) and probed with rabbit polyclonal anti-phospho-p44/42 MAPK antibodies (1:2500 dilution in TBST buffer with 0.1% non-fat dried milk). To standardize for protein loading in each lane, blots were stripped by incubating them in Tris buffer (62.5 mM Tris-HCl, pH 6.8, 100 mM 2-mercaptoethanol, 2% SDS) at 50°C for 30 min, washed extensively, and followed by reprobing with antibodies against ERK1 and ERK2, or pan-ERK (1:1000 to 1:5000

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dilution in TBST buffer with 0.1% non-fat dried milk). Detection was made using enhanced chemiluminescence reagent.

2.3. Materials

All chemicals were purchased from Sigma (USA) except for the following: dextran T-500 (Pharmacia, Sweden); Hanks' balanced salt solution (Gibco, USA); pertussis toxin (Research Biochemicals International, USA); U73122 and LY294002 (Biomol, USA); GF109203X, Gö6976, Gö6983, and wortmannin (Calbiochem, USA); U0126 (Promega, USA); BAPTA-AM (Molecular Probes, USA); enhanced chemiluminescence reagent (Amersham, UK); polyvinylidene difluoride membrane (Millipore, USA); mouse monoclonal antibodies to ERK1, ERK2, and pan-ERK (Transduction, USA); and rabbit polyclonal antibodies to phospho-p44/42 MAPK (New England, USA). BW755C was provided by Wellcome Research (UK).

3. Results and discussion

3.1. Phosphorylation of ERK via a G protein-dependent and arachidonate metabolite-independent pathway

ERK-mediated signaling pathways convert extracellular stimulation into a variety of cellular functions. However, the signal transduction events upstream to ERK occurring in neutrophils in response to fMLP have been incompletely delineated. Based on immunoblot analysis with anti-phospho-p44/42 MAPK antibodies, we found that exposure of neutrophils to fMLP rapidly induced the activation of ERK, reconcile the earlier reports [14,15], and these responses were attenuated by 0.3 μM U0126 (Fig. 1) as well as 3 μM PD98059 (data not shown), both selective MEK inhibitors [16,17].

Heterotrimeric G protein-coupled receptors are able to induce a variety of responses including the activation of several intracellular kinase cascades. Recent data have shown that the activation of ERK induced by G protein-coupled receptors in mammalian cells is mediated by tyrosine phosphorylation of Shc, leading to increased functional association among the adapter proteins Shc, Grb2, and Sos, and then Ras/ERK activation [18]. Preincubation of rat neutrophils with pertussis toxin (PTX), a potent endotoxin that catalyzes ADP-ribosylation of $G_{i/o}$, greatly inhibited ERK activation by fMLP (Fig. 1), consistent with the previous reports in human neutrophils [11,14].

fMLP operates via PTX-sensitive G protein-coupled receptors to activate phospholipase A_2, which in turn releases arachidonate (AA) from membrane phospholipids [19]. The endogenous AA released is subject to conversion to the metabolites of cyclo-oxygenase (COX) and lipoxygenase (LO). A recent report indicates that AA, acting as a second messenger, is able to stimulate the phosphorylation and activity of ERK in a variety of cell types including human neutrophils [20]. The activation of ERK by AA through an LO-dependent but COX-independent pathway has been reported in vascular smooth muscle cells [21] and in human neutrophils [22]. The inability of BW755C, a dual inhibitor of COX and LO [23], to prevent ERK activation by fMLP in rat neutrophils (Fig. 1) eliminated this possibility. In addition, the pretreatment of cells with 30 nM MK886, a leukotriene biosynthesis inhibitor [24], or 1 μM indomethacin had no effect on the fMLP-induced response (data not shown). Thus, these data indicate that the activation of ERK by fMLP is not regulated by the AA metabolites of COX and LO in rat neutrophils.

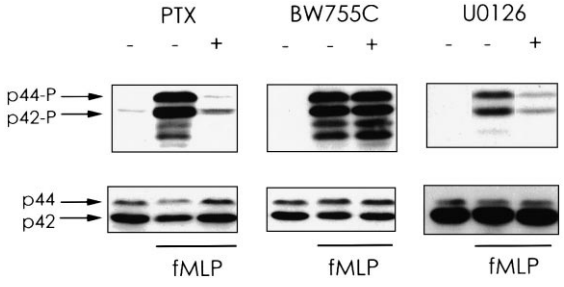

Fig. 1. Effect of PTX, BW755C and U0126 on fMLP-stimulated ERK phosphorylation. Cells were preincubated at 37°C with the vehicle, 1 μg/ml of PTX for 2 h, 30 μM BW755C or 0.3 μM U0126 for 10 min, either before stimulation with 0.1 μM fMLP plus 5 μg/ml CB or without stimulation as a control. One minute later, phosphorylation of ERK was detected by immunoblot analysis using anti-phospho-p44/42 MAPK antibodies. The lower blot was generated by reprobing the blot above with anti-ERK1 and anti-ERK2 (for PTX and BW755C experiments), or anti-pan ERK (for the U0126 experiment) antibodies as a measure of total ERK. The data presented are representative of three independent experiments with similar results.

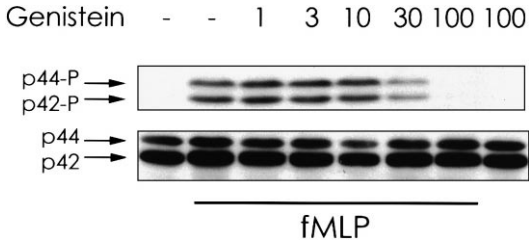

Fig. 2. Involvement of tyrosine kinase activation in fMLP-stimulated ERK phosphorylation. Cells were pretreated with the vehicle or indicated concentrations (μM) of genistein for 30 min at 37°C either before stimulation with 0.1 μM fMLP plus 5 μg/ml CB or without stimulation as a control. One minute later, phosphorylation of ERK was detected by immunoblot analysis using anti-phospho-p44/42 MAPK antibodies. The lower blot was generated by reprobing the blot above with anti-pan ERK antibodies as a measure of total ERK. The data presented are representative of three independent experiments with similar results.

3.2. Tyrosine kinase acts upstream of ERK phosphorylation

A previous study indicated that G protein-mediated Shc phosphorylation is sensitive to tyrosine kinase inhibitors [25]. PTX-sensitive activation of the Src family kinase Lyn in human neutrophils has been reported. Activation of Lyn is associated with binding to the Shc adapter protein and allows the G protein-coupled receptors to modulate the activity of the Ras/ERK cascade [26]. The treatment of rat neutrophils with genistein, a general protein tyrosine kinase inhibitor [27], concentration-dependently reduced the phosphorylation of ERK (Fig. 2), suggesting an involvement of tyrosine kinase in fMLP-stimulated activation. These results are similar to earlier observations of human neutrophils [4], in which the effect of genistein was analyzed by immunoblot with anti-phosphotyrosine antibodies, and on HL60 cells [28], in which 30 μM genistein inhibited ERK activity.

3.3. Phosphorylation of ERK is dependent on PI3K

The participation of phosphatidylinositol 3-kinase (PI3K) in ERK cascade activation after the stimulation of G protein-coupled receptors has been reported in a variety of cell types [29,30]. Complexes of phosphorylated Lyn and Shc with PI3K are rapidly formed in fMLP-stimulated neutrophils [26]. Neutrophils contain two classes of PI3K, the classical tyrosine kinase-regulated PI3Kα (p85/p110α) and a novel Gβγ-regu-

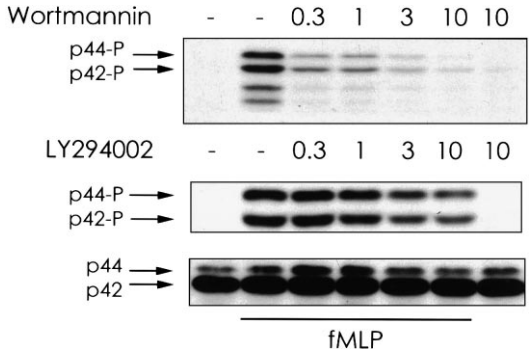

Fig. 3. PI3K acts upstream of ERK activation by fMLP. Cells were pretreated with the vehicle or indicated concentrations (μM) of wortmannin (upper blot) or LY294002 (middle blot) for 10 min at 37°C either before stimulation with 0.1 μM fMLP plus 5 μg/ml CB or without stimulation as a control. One minute later, phosphorylation of ERK was detected by immunoblot analysis using anti-phospho-p44/42 MAPK antibodies. Data are representative of three similar experiments. The blots above were then stripped and reprobed with anti-pan ERK antibodies as a measure of total ERK. The lower blots shown are representative of similar results.

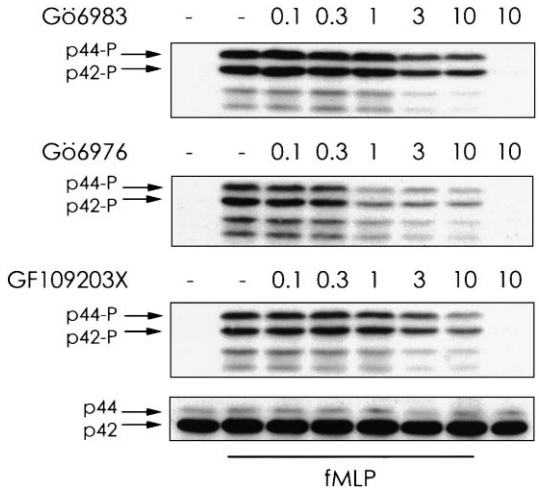

Fig. 4. The role of PKC in fMLP-stimulated ERK phosphorylation. Cells were pretreated with the vehicle or indicated concentrations (μM) of Gö6983 (upper blot), Gö6976 (2nd blot), or GF109203X (3rd blot) for 10 min at 37°C either before stimulation with 0.1 μM fMLP plus 5 μg/ml CB or without stimulation as a control. One minute later, phosphorylation of ERK was detected by immunoblot analysis using anti-phospho-p44/42 MAPK antibodies. Data are representative of three similar experiments. The blots above were then stripped and reprobed with anti-pan ERK antibodies as a measure of total ERK. The lower blots shown are representative of similar results.

lated PI3Kγ (p101/p110γ) [31]. In order to evaluate the role of PI3K in fMLP-stimulated ERK activation in rat neutrophils, wortmannin, an irreversible inhibitor of PI3K [32], was used. Wortmannin concentration-dependently inhibited the activation of ERK, as demonstrated by immunoblot using anti-phospho-p44/42 MAPK antibodies (Fig. 3). Wortmannin at 0.3 μM significantly reduced ERK phosphorylation, with results similar to previous observations of inhibiting PAF-stimulated ERK activity in guinea pig neutrophils [33], and at 10 μM it inhibited the fMLP-induced response to non-detectable levels, suggesting an involvement of PI3K in fMLP-stimulated activation. However, wortmannin has also been reported to inhibit phospholipase D and other kinases [34]. Therefore, the involvement of PI3K in fMLP-stimulated ERK activation was further tested using a specific PI3K inhibitor, LY294002 [35]. LY294002 also inhibited ERK phosphorylation in a concentration-dependent manner (Fig. 3). However, the inhibitory activity of LY294002 was significantly less potent than that of wortmannin. A similar observation has been reported in HL60 cells [28]. The inhibition of ERK activation by wortmannin and LY294002 suggests that PI3K acts as one component of upstream regulators of fMLP-stimulated ERK activation.

3.4. PKC acts as a proximal signaling pathway to activate ERK

Activated protein kinase C (PKC) has been reported to stimulate ERK, Ras, and Raf in a number of cell types [36,37]. In addition, PMA stimulates neutrophil ERK activity, and this effect has been inhibited by PKC inhibitors [22]. In this study, we used several PKC inhibitors to evaluate the role of PKC on fMLP-stimulated ERK activation in rat neutrophils. Pretreatment of cells with Gö6983, Gö6976, or GF109203X significantly attenuated ERK phosphorylation in a concentration-dependent manner (Fig. 4). These results support the recent reports that fMLP-stimulated ERK activity is inhibited by GF109203X and calphostin C in human neutrophils and HL60 cells, respectively [12,28]. Since the discovery of PKC, several PKC isoforms have been identified. Our previous report demonstrated that rat neutrophils express PKCα, β, gamma;, δ, ε, θ, μ, ι/λ, and ζ, although λ and ζ are barely detected [38]. Our recent study indicated that PKCγ is not expressed in rat neutrophils (data not shown). The different results are due to the anti-PKCγ antibody that we used in our previous report also cross-reacting with PKCα.

The pivotal role of PKC in cellular signaling instigated the search for potent and selective PKC inhibitors. Gö6976 preferentially inhibits PKCα, β, and μ, Gö6983 inhibits PKCα, β, γ, δ and ζ, and GF109203X inhibits PKCα, β, δ, ε, ζ and μ in in vitro kinase assays [39,40]. Both Gö6983 and GF109203X inhibited PKC isoforms with specificity broader than Gö6976, however, their effects on the inhibition of ERK activation were not more pronounced. These results suggest that Ca^{2+}-dependent PKC probably acts as the major PKC isoforms on

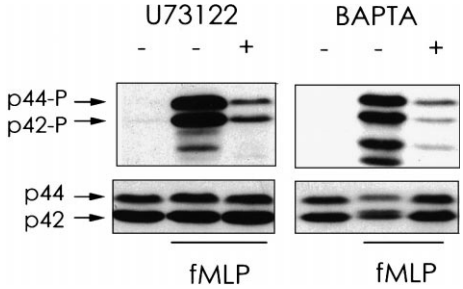

Fig. 5. Elevation of $[Ca^{2+}]_i$ is necessary for ERK activation by fMLP. Cells were stimulated with 0.1 μM fMLP plus 5 μg/ml CB or its diluent for 1 min. Where indicated, the cells had been pretreated at 37°C with the vehicle or 10 μM U73122 for 10 min or were loaded with BAPTA by incubation with 10 μM BAPTA-AM for 1 h. After stimulation, phosphorylation of ERK was detected by immunoblot analysis using anti-phospho-p44/42 MAPK antibodies. The lower blot was generated by reprobing the blot above with anti-ERK1 and anti-ERK2 antibodies as a measure of total ERK. The data presented are representative of three independent experiments with similar results.

the regulation of fMLP-stimulated ERK phosphorylation, whereas the inhibition of novel and atypical PKC isoforms failed to improve but partially reduced the efficacy. It has been reported that fMLP induces G-dependent activation of Ras, Raf, and MEKK in human neutrophils in a PKC-independent manner [11,12]. On the other hand, GF109203X and staurosporine inhibit the activities of ERK and MEK, respectively, in human neutrophils [12,41]. Therefore, PKC may probably act downstream of Raf/MEKK to regulate the fMLP-stimulated ERK cascade.

3.5. PLC/Ca^{2+} pathway couples to the activation of ERK

The stimulation of neutrophils by receptor-binding ligands can activate phospholipase C (PLC) with the formation of inositol trisphosphate, which increases in $[Ca^{2+}]_i$, and diacylglycerol, which activates PKC [42]. We next investigated the role of PLC/Ca^{2+} on ERK activation. PAF activates ERK through a Ca^{2+}-dependent pathway in guinea pig neutrophils [33], and fMLP-stimulated ERK activity in HL60 cells was dependent on PLC [28]. On the contrary, experiments using electropermeabilized human neutrophils indicated that elevation of $[Ca^{2+}]_i$ is not required for activation of MEK by fMLP [41]. Our results using the PLC inhibitor U73122 [43] and the intracellular Ca^{2+} chelator BAPTA [44] to prevent the increase of $[Ca^{2+}]_i$ showed the attenuation of fMLP-stimulated ERK phosphorylation (Fig. 5) and suggest that fMLP activates ERK also through a PLC/Ca^{2+}-dependent pathway.

In summary, our results demonstrated that fMLP activates ERK via PTX-sensitive G protein, not through AA metabolites, and is regulated by tyrosine kinase, PI3K, PKC, and PLC/Ca^{2+} signaling pathways in rat neutrophils.

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