The Mitogen-Activated Protein Kinase Extracellular Signal-Regulated Kinase 1/2 Pathway Is Involved in formyl-Methionyl-Leucyl-Phenylalanine-Induced p47phox Phosphorylation in Human Neutrophils

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*J Immunol* 2000; 165:5238-5244; doi: 10.4049/jimmunol.165.9.5238

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*The Journal of Immunology* is published twice each month by The American Association of Immunologists, Inc., 1451 Rockville Pike, Suite 650, Rockville, MD 20852

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Print ISSN: 0022-1767 Online ISSN: 1550-6606.
The Mitogen-Activated Protein Kinase Extracellular Signal-Regulated Kinase 1/2 Pathway Is Involved in Formyl-Methionyl-Leucyl-Phenylalanine-Induced \( p47^{\text{phox}} \) Phosphorylation in Human Neutrophils

Cédric Dewas, Michèle Fay, Marie-Anne Gougerot-Pocidalo, and Jamel El-Benna

Phosphorylation of \( p47 \) phagocyte oxidase, \( (p47^{\text{phox}}) \), one of the NADPH oxidase components, is essential for the activation of this enzyme and for superoxide production. \( p47^{\text{phox}} \) is phosphorylated on multiple serine residues, but the kinases involved in this process in vivo remain to be characterized. We examined the role of extracellular signal-regulated kinase (ERK1/2) and p38 mitogen-activated protein kinase in \( p47^{\text{phox}} \) phosphorylation. Inhibition of ERK1/2 activation by PD98059, a specific inhibitor of ERK kinase 1/2, inhibited the fMLP-induced phosphorylation of \( p47^{\text{phox}} \). However, PD98059 weakly affected PMA-induced \( p47^{\text{phox}} \) phosphorylation, even though ERK1/2 activation was abrogated. This effect was confirmed using U0126, a second ERK kinase inhibitor. Unlike PD98059 and U0126, the p38 mitogen-activated protein kinase inhibitor SB203580 did not inhibit the phosphorylation of \( p47^{\text{phox}} \) induced either by fMLP or by PMA. Two-dimensional phosphopeptide mapping analysis showed that, in fMLP-induced \( p47^{\text{phox}} \) phosphorylation, PD98059 affected the phosphorylation of all the major phosphopeptides, suggesting that ERK1/2 may regulate \( p47^{\text{phox}} \) phosphorylation either directly or indirectly via other kinases. In PMA-induced \( p47^{\text{phox}} \) phosphorylation, GF109203X, a protein kinase C inhibitor, strongly inhibits \( p47^{\text{phox}} \) phosphorylation. However, in fMLP-induced \( p47^{\text{phox}} \) phosphorylation, PD98059 and GF109203X partially inhibited the phosphorylation of \( p47^{\text{phox}} \) when tested alone, and exerted additive inhibitory effects on \( p47^{\text{phox}} \) phosphorylation when tested together. These results show for the first time that the ERK1/2 pathway participates in the phosphorylation of \( p47^{\text{phox}} \). Furthermore, they strongly suggest that \( p47^{\text{phox}} \) is targeted by several kinase cascades in intact neutrophils activated by fMLP and is therefore a converging point for ERK1/2 and protein kinase C.

Human neutrophils play a key role in host defenses against invading pathogens and are the major effector of the acute inflammatory reaction. In response to a variety of agents, neutrophils release large quantities of superoxide anion \( (O_2^-) \) in a phenomenon known as the respiratory burst. \( O_2^- \) is the precursor of other reactive oxygen species, which are essential for bacterial killing and amplify inflammatory reactions (1). Neutrophil production of \( O_2^- \) is dependent on the respiratory burst oxidase, or NADPH oxidase, a multicomponent enzyme system that catalyzes NADPH-dependent reduction of oxygen to \( O_2^- (2, 3) \). NADPH oxidase is activated by a variety of agents, including fMLP, which is representative of bacteria-derived \( N \)-formyl peptides and the protein kinase C (PKC)\(^3\) activator PMA. These stimuli trigger biochemical cascades leading to the phosphorylation of several proteins of the NADPH-oxidase system, such as \( p47 \) phagocyte oxidase \( (p47^{\text{phox}}) \), a key component in the assembly and activation of this \( O_2^- \)-producing machinery \((4–6)\). In addition to the well-documented PKC pathway, one of these cascades involves activation of members of the mitogen-activated protein kinase (MAPK) family. Although many authors have implicated PKC in \( p47^{\text{phox}} \) phosphorylation \((7–11)\), no data are available on the possible involvement of the MAPK pathway in this process.

MAPK enzymes are proline-directed serine/threonine kinases activated by phosphorylation of tyrosine and threonine residues. The MAPK superfamily is believed to be an important signaling pathway in many cell types \((12, 13)\). Although the role of MAP kinases in proliferation and differentiation is well established, their roles and targets in terminally differentiated nonproliferating cells such as neutrophils are not known. Several recent studies have demonstrated that MAPK pathways such as extracellular signal-regulated kinases (ERK1/2) and p38 MAPK, but not c-Jun N-terminal kinase, are activated in human neutrophils by fMLP, PMA, GM-CSF, and TNF \((14–19)\), but the targets of these kinases remain to be identified.

The NADPH oxidase component \( p47^{\text{phox}} \) is phosphorylated on several serine residues, two of which are serines recognized by proline-directed kinases \((20, 21)\). \( p47^{\text{phox}} \) is an in vitro substrate for activated MAPK (ERK and p38 kinases), PKC, PKA, p21-activated kinase, and a phosphatidic acid-activated kinase \((16, 20, 22, 23)\), but which of these enzymes phosphorylate \( p47^{\text{phox}} \) in vivo, and at which site, is unknown. Several studies have shown that inhibitors of the ERK pathway and p38 MAPK inhibit the respiratory burst \((14, 15, 24, 25)\), but the mechanisms underlying this inhibitory effect and the identity of the MAPK involved in \( p47^{\text{phox}} \) phosphorylation in intact cells are unknown.

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Received for publication January 28, 2000. Accepted for publication August 7, 2000.

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This work was supported by a grant from the Association pour la Recherche sur le Cancer (ARC). C. D. is a recipient of a fellowship from the Association de Recherche sur la Polyarthrite (ARP).

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3 Abbreviations used in this paper: PKC, protein kinase C; MAPK, mitogen-activated protein kinase; \( p47^{\text{phox}} \), \( p47 \) phagocyte oxidase; ERK, extracellular signal-regulated protein kinase; hsp27, heat shock protein 27.

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Myeloid cells such as neutrophils are terminally differentiated short-lived cells resistant to transfection. An alternative strategy to study the role of specific enzymes is to use cell-permeant pharmacologic inhibitors. In this study we used PD98059 and U0126, which are MEK1/2 inhibitors (26, 27), and SB203580, the p38 MAPK inhibitor (28), to analyze the role of these two MAPK pathways in p47<sup>phox</sup> phosphorylation in intact neutrophils.

We found that only the inhibitors of the ERK1/2 pathway inhibited p47<sup>phox</sup> phosphorylation in human neutrophils. In addition, we obtained evidence that the PKC and ERK1/2 pathways cooperate in p47<sup>phox</sup> phosphorylation triggered by fMLP.

Materials and Methods

Materials

fMLP, PMA, and lucigenin were obtained from Sigma (St. Louis, MO); SDS-PAGE reagents were purchased from Bio-Rad (Richmond, CA); SB203580, PD98059, U0126, and GF109203X were obtained from Calbiochem (La Jolla, CA); 32Pi was obtained from NEN (Boston, MA); anti-p47<sup>phox</sup> Ab (The Scripps Research Institute, Division of Biochemistry, La Jolla, CA). Anti-active phosphorylated ERK1/2 Abs were purchased from New England Biolabs (Beverly, MA) and Promega (Madison, WI). Anti-ERK1 and anti-ERK2 Abs were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Recombinant human heat shock protein 27 (hsp27) was purchased from StressGen Biotechnologies (Victoria, Canada).

Neutrophil preparation

Human neutrophils were obtained from healthy volunteers by Dextran sedimentation and Ficoll-Hypaque fractionation of freshly drawn blood, as previously described (28).

32P-labeling of neutrophils

Neutrophils were resuspended at a density of 10<sup>8</sup> cells/ml in phosphate-free buffer (20 mM HEPES pH 7.4, 140 mM NaCl, 5.7 mM KCl, 0.8 mM MgCl<sub>2</sub>, and 0.025% BSA), treated with dispotyrophylliphosphate (2.5 mM) for 15 min, and washed twice. Neutrophils were then loaded with 0.5 mCi/ml 32Pi for 1 h at 30°C (29) and incubated for 45 min with inhibitors or DMSO at 37°C. They were then activated with fMLP (0.1 μM) for 3 min or with PMA (500 ng/ml) for 6 min. Activation was stopped with ice-cold buffer, and cells were lysed as previously described (29). p47<sup>phox</sup> was immunoprecipitated with a specific Ab (1/200 dilution) complexed with Sepharose G beads (Pharmacia Biotech, Uppsala, Sweden). Proteins were analyzed by 10% SDS-PAGE and autoradiography.
PMA (0.5 μg/ml), p47phox was immunoprecipitated with a specific Ab and then analyzed by SDS-PAGE, Western blot, and autoradiography. The results (Fig. 1A, 32P-p47phox) showed that PD98059, unlike SB203580, clearly inhibited p47phox phosphorylation triggered by the chemotactic peptide fMLP (51.2 ± 6.9% of the control, n = 5, p < 0.05 and 101.7 ± 0.3% of the control, n = 5, for PD98059 and SB203580, respectively, as determined by radioactivity counting). However, the same concentration of PD98059 only weakly inhibited the phosphorylation of p47phox when the latter was induced by PMA, a direct PKC activator (Fig. 1B). Specific detection of p47phox with an Ab (Fig. 1, p47 phox) showed that the same amount of p47phox was immunoprecipitated from each sample. Fig. 2A and B, clearly shows that in the conditions used above, PD98059 inhibited the phosphorylation of ERK1/2 as measured with a phosphospecific Ab; SB203580 inhibited the phosphorylation of hsp27, as described in Materials and Methods. Data are representative of three experiments.

A new MEK inhibitor, U0126, decreases p47phox phosphorylation and the respiratory burst in fMLP-stimulated neutrophils

The results obtained above by the use of PD98059 strongly suggest the involvement of ERK1/2 in fMLP-induced p47phox phosphorylation. Because chemical compounds may affect cellular functions unrelated to their intended targets, we sought to confirm our observations by using a chemically unrelated inhibitor of MEK1/2, namely U0126 (27). As shown in Fig. 3, the phosphorylation of p47phox was effectively inhibited by incubation of neutrophils for 30 min with 10 μM U0126 (61.8 ± 6.6% of the control, n = 6, p < 0.01). Like PD98059, U0126 also inhibited superoxide production of fMLP-activated neutrophils (53.2 ± 5, 32.8 ± 5.3, and 21.4 ± 4.8% of the control for 10, 25, and 50 μM U0126, respectively; n = 3, p < 0.01). Under these conditions U0126 completely blocks ERK1/2 activation (data not shown).

Concentration-dependent effect of PD98059 on p47phox phosphorylation and ERK1/2 activation

To link the inhibitory effect of PD98059 on p47phox phosphorylation and ERK1/2 activation, we performed parallel concentration-effect studies of these two processes. As shown in Fig. 4, the inhibitory effect of PD98059 on fMLP-induced p47phox phosphorylation (Fig. 4A) was concentration dependent, running parallel to the inhibitory effect on MAPK activation (Fig. 4B). This

![Figure 2](http://www.jimmunol.org/) Effect of PD98059 and SB203580 on MAPK activation in human neutrophils. Neutrophils were incubated in the presence or absence of SB203580 (SB; 10 μM) or PD98059 (PD; 50 μM) for 45 min, then stimulated with fMLP (10^{-7} M) or PMA (500 ng/ml). ERK1/2 activation (A) was measured by SDS-PAGE and Western blot with an anti-phospho-ERK1/2 Ab. P38 MAPK activation (B) was measured by in vitro phosphorylation of hsp27, as described in Materials and Methods. Data are representative of three experiments.

![Figure 3](http://www.jimmunol.org/) Effect of PD98059 and U0126 on fMLP-induced p47phox phosphorylation in human neutrophils. 32P-labeled neutrophils were incubated in the presence or absence of PD98059 (50 μM) or U0126 (10 μM) for 30 min, then stimulated with fMLP (10^{-7} M) for 3 min. p47phox was immunoprecipitated and analyzed by SDS-PAGE transfer and revealed by autoradiography (32P-p47phox) and anti-p47phox Ab (p47phox). Data are representative of six experiments.
inhibitory effect reached a plateau at a concentration of 50 μM (48.8 ± 6.8% of the control, n = 6, p < 0.01, determined by densitometric analysis). In contrast, PD98059 weakly inhibited PMA-induced p47phox phosphorylation (Fig. 4C) (20.2 ± 7.8% inhibition, n = 5, p < 0.01) at concentrations that completely inhibit ERK1/2 activation (Fig. 4D). These results suggest that ERK1/2, directly or indirectly, play a major role in p47phox phosphorylation in fMLP-activated neutrophils. However, in PMA-activated neutrophils, although ERK1/2 was activated, it appeared to have a minimal role in p47phox phosphorylation.

Effect of MAPK inhibitors on individual p47phox phosphorylated peptides analyzed by two-dimensional tryptic phosphopeptide mapping

p47phox is phosphorylated on several serine residues, which can be phosphorylated in vitro by PKC, PKA, and MAPKs (ERK1/2 and p38). The peptides containing these serines can be analyzed by tryptic phosphopeptide mapping. In vitro, MAPK phosphorylates only one peptide but PKC phosphorlates several other peptides. The possibility that PD98059 or SB203580 might inhibit the phosphorylation of one or more individual sites in p47phox was studied by using two-dimensional peptide mapping. Fig. 5 shows that when neutrophils were stimulated with PMA, phosphorylation of p47phox occurred on several peptides; some major peptides were strongly phosphorylated and a few minor peptides were less phosphorylated. PD98059 inhibited the phosphorylation of three minor peptides (dotted lines) and minimally affected the phosphorylation of other major peptides. However, when fMLP was the stimulus (Fig. 6), the phosphopeptides were located in the same position on the map but were much less phosphorylated, except for one peptide. PD98059 strongly inhibited the phosphorylation of all the major peptides (dotted lines), including those phosphorylated in PMA-stimulated conditions. As mentioned in Materials and Methods, in each experiment we ensured by immunodetection techniques that the same quantities of p47phox were immunoprecipitated from each sample. In addition, all the plates from the same experiment were analyzed by autoradiography for the same period of time. These results suggest that, in fMLP-stimulated cells, ERK1/2 plays an important role in p47phox phosphorylation by regulating the phosphorylation of all the major sites.
PD98059, SB203580 had no effect on PMA-induced phosphorylation, but had a moderate stimulatory effect on fMLP-induced phosphorylation when analyzed by two-dimensional phosphopeptide mapping (Figs. 5 and 6).

The ERK1/2 pathway inhibitor PD98059 and the PKC inhibitor GF109203X exert additive effects on fMLP-induced p47phox phosphorylation and the fMLP-induced respiratory burst

The chemotactic peptide fMLP is known to activate several PKC isoforms that participate in the phosphorylation of p47phox. We postulated that the PKC system and ERK1/2 pathway could converge to phosphorylate p47phox. Therefore, we analyzed the effect of PKC and MAPK inhibitors alone, and in combination, on the phosphorylation of p47phox. Fig. 7A shows that when PD98059 (50 μM) and GF109203X (5 μM) were added alone, they partially inhibited fMLP-induced p47phox phosphorylation (59.3 ± 7.4 and 67.2 ± 1.2% of the control, respectively), and that their combination resulted in a clearly additive inhibitory effect (lanes 3 and 4 compared with lane 5) (35.5 ± 2.2% of the control). In keeping with the above results, PMA-induced p47phox phosphorylation was more sensitive to GF109203X (Fig. 7C), with only a weak additional effect of the MEK inhibitor PD98059 (24.4 ± 14.4, 108.5 ± 17.7, and 21.3 ± 19% of the control for GF109203X, PD98059, and GF109203X + PD98059, respectively). The MAPK activation blots clearly show that GF109203X does not inhibit ERK1/2 activation in fMLP-activated cells (Fig. 7B) while inhibiting this process when it was induced with PMA, thus explaining the strong effect of GF109203X on PMA-induced p47phox phosphorylation (Fig. 7D). The two-dimensional peptide map in these conditions showed that all of the sites were inhibited (data not shown). Taken

**FIGURE 7.** Effects of GF109203X, a PKC inhibitor, and PD98059 on fMLP- and PMA-induced p47phox phosphorylation and ERK1/2 activation. 32P-labeled neutrophils were incubated in the presence or absence of PD98059 (PD; 50 μM for 45 min) and/or GF109203X (GFX; 5 μM for 15 min), then stimulated with fMLP (10⁻⁷ M) (A and B) or PMA (500 ng/ml) (C and D). p47phox was immunoprecipitated and analyzed by SDS-PAGE transfer and autoradiography or immunodetection (C and D). Cells were lysed and proteins were analyzed by SDS-PAGE transfer and detection with an anti-phospho-ERK1/2 Ab (B and D). Data are representative of three experiments.
indicates a PKC-dependent Raf/ERK1/2 activation. This step is minor for PKC-dependent pathway (PMA-activatable isoforms). The dashed arrow but independent of the p38 MAPK pathway. PMA essentially stimulates a phosphorylation: a PKC (unidentified isoforms) and an ERK1/2 pathway.

Together, these results suggest that after PMA stimulation, PKC plays the major role in p47phox phosphorylation, whereas in more physiological conditions (fMLP stimulation), ERK1/2 and PKC participate together, directly or indirectly, in the phosphorylation of p47phox.

It is well established that the phosphorylation of p47phox correlates with NADPH oxidase activation. We thus checked whether the additive effect of the PKC inhibitor and the ERK1/2 pathway inhibitor on p47phox phosphorylation correlated with their effect on the activation of the enzyme. PD98059 and GF109203X alone partially inhibited fMLP-induced superoxide production (40.7 ± 9.6 and 62.5 ± 0.6% of the control, respectively, n = 3), whereas when added together they had an additive inhibitory effect on the fMLP-triggered response (18.1 ± 5.8% of the control, n = 3, p < 0.05). Experiments using both inhibitors were not performed with PMA as stimulus, as the same concentration of GF109203X (5 µM) as that used in fMLP experiments completely abrogated the PMA-induced response.

Discussion

p47phox phosphorylation is considered to be a key event in NADPH oxidase activation, but the kinase cascades controlling this process are poorly documented. In this study we show that under physiological conditions (fMLP stimulation), ERK1/2 and PKC cooperate to phosphorylate p47phox, whereas PMA-induced phosphorylation of p47phox is mainly dependent on conventional PKC activation.

Under physiological conditions, represented here by fMLP stimulation of human neutrophils, several kinase pathways (PKC, p21-activated kinase, MAPK) are activated via the serpine receptor. However, the kinases involved in p47phox phosphorylation have not yet been identified, although it has been reported that PKC (7–11) and some PKC isoforms may be involved in the process (32–34). Here we provide clear evidence that ERK1/2 are implicated in the phosphorylation cascade of p47phox in intact cells. The data also suggest cooperation between PKC and ERK1/2 in this process. It is noteworthy that p47phox is phosphorylated on multiple serine residues that could be targeted by different kinases, thus explaining the tight control of NADPH oxidase.

Two-dimensional phosphopeptide mapping analysis of p47phox isolated from EBV-transformed B lymphocytes and human neutrophils shows that the same peptides are phosphorylated in both cells. The use of site-directed mutagenesis of p47phox in B lymphocytes revealed that serines 303–370 are phosphorylated and that each peptide on the map contains one or several phosphorylated serines (21). The results reported here show that in the case of fMLP, PD98059 clearly inhibits the peptides containing serines 303 + 304, 315, 320, and 345 + 348. ERK1/2 phosphorylate p47phox in vitro on only one peptide containing serines located in the MAPK recognition sequence, i.e., 345 + 348 (16, 21). As the other p47phox serines have not been identified as ERK substrates (21), the observation that PD98059 inhibits all the major phosphopeptides in fMLP-stimulated cells suggests that ERK1/2 regulates non-MAPK sites by an unknown mechanism. One possibility is that p47phox phosphorylation by ERK1/2 induces conformational changes that make the other sites more accessible to their protein kinases. Indeed, it was recently shown that p47phox phosphorylation in vitro induces conformational changes of the protein (35, 36). The other possibility is that ERK1/2 phosphorylate p47phox on their specific serines and that, in addition, they may be upstream regulators of other kinases such as MAPK-activated protein kinase which, in turn, could phosphorylate p47phox.

A hierarchical organization of p47phox phosphorylation has previously been suggested (37–39). In addition, we recently demonstrated that an early phosphorylation step took place under conditions of priming by proinflammatory cytokines such as GM-CSF (40). Whether or not MAPK are involved in this process is currently under investigation.

The results obtained here also confirm that PKCs play a predominant role in p47phox phosphorylation in PMA-activated cells, and suggest that PKCs could simultaneously activate the ERK1/2 pathway, which might also contribute to p47phox phosphorylation. However, the role of ERK1/2 MAPK seems to be minor in PMA activation conditions, as PD98059 only slightly inhibited p47phox phosphorylation and the respiratory burst (data not shown). This is in keeping with previous results showing that mutation of p47phox at Ser345 and Ser348 (sites within MAPK recognition sequence) moderately decreases the respiratory burst in transfected B lymphoblasts stimulated with PMA (41).

Unlike PD98059, the p38 inhibitor SB203580 did not decrease p47phox phosphorylation induced by PMA or fMLP, while nonetheless inhibiting the respiratory burst (Refs. 14, 19 and results not shown). This result is in agreement with a recent publication (42) that shows that SB203580 was without effect on p47phox phosphorylation. p38 MAPK could affect other events necessary for NADPH activation such as the phosphorylation of other oxidase components or proteins involved in NADPH oxidase activation, such as cytoskeleton reorganization or phospholipase A2 activity. It has been reported that MAPK-activated kinase phosphorylates hsp27 (43) and lymphocyte-specific protein 1 (44), which are both involved in actin polymerization (44, 45). Fig. 8 illustrates a summary of the different pathways possibly activated by fMLP or PMA leading to p47phox phosphorylation and NADPH oxidase activation.

In summary, we show that ERK1/2 are involved in p47phox phosphorylation and in the regulation of this process in fMLP-stimulated cells. Moreover, the additive effects of GF109203X and PD98059 suggest that fMLP triggers both the ERK1/2-MAPK and PKC pathways to phosphorylate p47phox. In addition to PKC,
ERK1/2 could thus be a candidate target for novel therapeutic agents in inflammatory diseases involving abnormal neutrophil
peroxide production.

Acknowledgments
We thank Dr. B. Babior for the anti-p47phox Ab and for his continuous support, and Dr B. Rouot (Institut National de la Santé et de la Recherche Médicale, Unité 431) for his advice on the assay of MAPK activities.

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