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Extracellular Signal-regulated Kinase 1/2 is Involved in the Activation of NADPH Oxidase Induced by FMLP Receptor but not by Complement Receptor 3 in Rat Neutrophils

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This experiment was performed to clarify the role of extracellular signal-regulated kinase, ERK1/2, in NADPH oxidase-dependent O_2^- production in rat peritoneal neutrophils. When neutrophils were exposed to *N*-formyl-methionyl-leucyl-phenylalanine (fMLP) to stimulate an *N*-formyl peptide receptor, not only the production of O_2^- but also the activation of ERK1/2 was observed. The translocation of an NADPH oxidase component, $p47^{phox}$, from cytosol to membrane also occurred in neutrophils stimulated with fMLP. U0126, an ERK1/2 kinase inhibitor, inhibited both the production of O_2^- and the translocation of $p47^{phox}$ elicited by fMLP. On the other hand, when complement receptor 3 of neutrophils was stimulated with opsonized zymosan (OZ), weaker activation of ERK1/2 than that by fMLP was observed. In this case, U0126 showed no inhibition against the production of O_2^- and slight inhibition against the translocation of $p47^{phox}$. Large inhibition against the OZ-induced production of O_2^- was only observed in neutrophils treated with GF109203X, a PKC inhibitor. The present study indicates that receptor dependence exists in the ERK1/2 signaling pathway leading to the activation of NADPH oxidase.

Keywords: CR3; ERK1/2; fMLP; NADPH oxidase; Neutrophil; $p47^{phox}$

INTRODUCTION

Neutrophils play a pivotal role in the first stage of the host defense against invading microorganisms. In response to various stimuli, for example, bacteria, phagocytic particles and chemoattractants, neutrophils demonstrate several functional reactions including phagocytosis of pathogens, generation of reactive oxygen species (ROS) and release of bactericidal proteins to phagosomes. ROS include OCl^-, $^{\bullet}OH$ and H_2O_2, which are derivatives of O_2^- generated from oxygen by NADPH oxidase.[1]

NADPH oxidase is a multicomponent enzyme producing O_2^-, which is essential for bactericidal activity.[2] This oxidase consists of at least two membrane proteins ($gp91^{phox}$ and $p22^{phox}$) and three cytosolic proteins ($p47^{phox}$ $p67^{phox}$ and Rac). When resting cells are exposed to various stimuli, cytosolic components of the oxidase migrate to the membrane and associate with membrane components followed by its activation.[2] The phosphorylation of $p47^{phox}$ mediated by protein kinase C, (PKC), is thought to be an initial event to trigger the translocation of cytosol components of NADPH oxidase.[3,4]

Three receptors, *N*-formyl-methionyl-leucyl-phenylalanine (fMLP) receptor, an Fcγ receptor (FcγR) and complement receptor 3, CR3, are known to participate in the activation of NADPH oxidase of neutrophils. The fMLP receptor, which is known to be a seven transmembrane-spanning G-protein-linked receptor, is activated by fMLP, a chemoattractant.[5] FcγR is activated by binding with the Fcγ region of immunoglobulin[6] and CR3, a member of the integrin family of adhesion molecules, is activated by serum-opsonized

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zymosan (OZ).[7] These receptors are considered to activate PKC at the upstream of $p47^{phox}$ phosphorylation.[7–10] Recently, in addition to PKC, extracellular signal-regulated kinase, ERK1/2, belonging to the mitogen-activated protein kinase (MAPK) family has been focused on as an important molecule in neutrophil functions.[11] However, details of its role in the activation of NADPH oxidase are still uncertain.

In this study, to obtain a better understanding of the roles of ERK1/2 in the signaling pathway leading to the activation of NADPH oxidase, we examined the effects of U0126, an ERK1/2 kinase inhibitor, on the activation of ERK1/2, the translocation of $p47^{phox}$ from cytosol to membrane and the production of O_2^- in rat peritoneal neutrophils stimulated with fMLP and OZ.

MATERIALS AND METHODS

Reagents

Zymosan, horseradish peroxidase, cytochalasin B and fMLP were purchased from Sigma Chemical Co. (St. Louis, MO). Casein, luminol and U0126 were from Wako Pure Chemicals Industries, Ltd. (Osaka, Japan). GF109203X was from Calbiochem. Ca^{2+}- and Mg^{2+}-free Hanks' balanced salt solution (HBSS [−]) was from Invitrogen Co., (Carlsbad, CA). Anti-phosphorylated ERK1/2 antibody was from Cell Signaling Technology, Inc. (Beverly, MA). Anti-actin antibody was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-$p47^{phox}$ antibody was a kind gift by Dr B.M. Babior, The Scripps Research Institute, CA.

Isolation of Peritoneal Neutrophils

Neutrophils were obtained from the peritoneal cavities of male Wistar rats. Briefly, each rat was intraperitoneally administered 10 ml of 3% casein in physiological saline. Animals were killed and cells collected by rinsing the abdominal cavity with HBSS (−) 15 h after the administration of 3% casein. Cell viability was estimated by the trypan blue exclusion test. The present experiments adhered to the Guide for the Care and Use of Laboratory Animals, Graduate School of Veterinary Medicine, Hokkaido University.

Preparation of Serum-opsonized Zymosan (OZ)

Zymosan was suspended in fresh rat serum at a concentration of 10 mg/ml and incubated for 30 min at 37°C. After incubation, the suspension was washed twice with HBSS (−) and resuspended in HBSS (−) at a concentration of 10 mg/ml.

Assay of the Production of O_2^-

The production of O_2^- was measured by chemiluminescence with luminol. HBSS with 0.5 mM $CaCl_2$ and 1 mM $MgCl_2$ (HBSS [+]) containing 3×10^6 neutrophils, 10 μM luminol and 50 μg/ml of horseradish peroxidase (5 μg/ml of cytochalasin B in the case of fMLP stimulation) was prepared in each well of a 96-well microplate. The suspension, with a volume of 315 μl, was incubated for 5 min at 37°C. After incubation, neutrophils were activated by adding 35 μl of fMLP (1 μM) or OZ (10 mg/ml) and then chemiluminescence from each well was measured with a luminometer (Luminescencer-JNR; ATTO Co., Tokyo, Japan) for 0.5 s at 37°C.

Immunoblotting for Activated ERK1/2

Neutrophils (5×10^6) were preincubated in HBBS (+) at 37°C for 5 min before adding fMLP at 100 nM or OZ at 1 mg/ml. After stimulation at 37°C for the indicated times, reactions were terminated by adding 1 ml of ice-cold HBSS (−) and subsequent centrifugation at 6000 rpm for 30 s at 4°C. Cell pellets were suspended in 50 μl of lysis buffer (1% Triton X-100, 20 mM Tris–HCl [pH 8.0], 100 mM NaCl, 10 mM $Na_2P_2O_7$, 2 mM EDTA, 50 mM NaF, 10% [v/v] glycerol, 1 mM Na_3VO_4, 10 μg/ml of aprotinin, 10 μg/ml of leupeptin, 1 mM phenylmethylsulfonyl fluoride [PMSF]) and incubated on ice for 30 min. After centrifugation at 12,000 rpm for 15 min at 4°C, 20 μl of 3 × Laemmli's sample buffer was added to 40 μl of the supernatant. Proteins in the solution were separated by 10% SDS-PAGE after boiling for 5 min and transferred onto a nitrocellulose membrane (ADVANTEC Toyo, Ltd., Tokyo, Japan). The membrane was incubated with anti-phosphorylated ERK1/2 antibody diluted 1/2000 in TBST buffer (10 mM Tris–HCl [pH 7.4], 0.1 M NaCl, 0.1% Tween-20) containing 5% bovine serum albumin, and then with HRP-conjugated anti-rabbit IgG. After washing three times with TBST, bound antibody was detected with a chemiluminescence detection kit (NEN Life Science Products, Inc. Boston, MA).

Translocation of $p47^{phox}$

Neutrophils (4×10^7) were stimulated with fMLP or OZ as described above. The reaction was then terminated by adding ice-cold relaxation buffer (100 mM KCl, 3 mM NaCl, 3.5 mM $MgCl_2$, 10 mM PIPES [pH7.4], 1.25 mM EGTA, 1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 10 μg/ml pepstatin) and immediately poured into liquid nitrogen. After thawing, the suspension was sonicated 10 s × 3) on ice and centrifuged at 3300 rpm for 10 min at 4°C. The supernatant was loaded on 1 ml of discontinuous sucrose gradient 15 and 34%

[w/w]) and then centrifuged at 50,000 rpm for 30 min at 4°C. The interface between 15 and 34% sucrose cushions was collected as the membrane fraction, diluted with four volumes of relaxation buffer, and then pelleted at 60,000 rpm for 30 min at 4°C. The membrane pellet was resuspended in Laemeli's sample buffer, separated by 12% SDS-PAGE, and transferred onto a nitrocellulose membrane. Blocking, treatment with primary and secondary antibodies and visualization were similar to the procedures used for ERK1/2.

RESULTS

Production of O_2^- Induced by fMLP and OZ Stimulation

We first measured fluorescence enhanced by luminol to examine the kinetics of O_2^- production evoked by the activation of NADPH oxidase in neutrophils exposed to fMLP and OZ. As shown in Fig. 1A, fMLP quickly induced the production of O_2^-, which peaked at 1 min followed by a prompt decrease. This response in chemiluminescence was due to the extracellular production of O_2^-, since the presence of Zn, Cu-superoxide dismutase (Zn, Cu-SOD) completely abolished it (data not shown). In neutrophils exposed to OZ, the production of O_2^-, which peaked at 5 min followed by a gradual decrease, was observed (Fig. 1B). It is generally considered that the main opsonins in serum activating neutrophils are IgG and complement iC3b. To examine which receptor was involved in the production of O_2^- in neutrophils stimulated with OZ, we employed the zymosan opsonized by heated serum since the complement in serum was inactivated by heat treatment for 30 min at 56°C.[12] The production of O_2^- from neutrophils stimulated with zymosan opsonized by heated serum was reduced to one-ninth in comparison with that by zymosan opsonized by unheated serum (Fig. 1B). This fact indicated that serum-OZ mainly stimulated the iC3b receptor, CR3, but not the IgG receptor, FcγR.

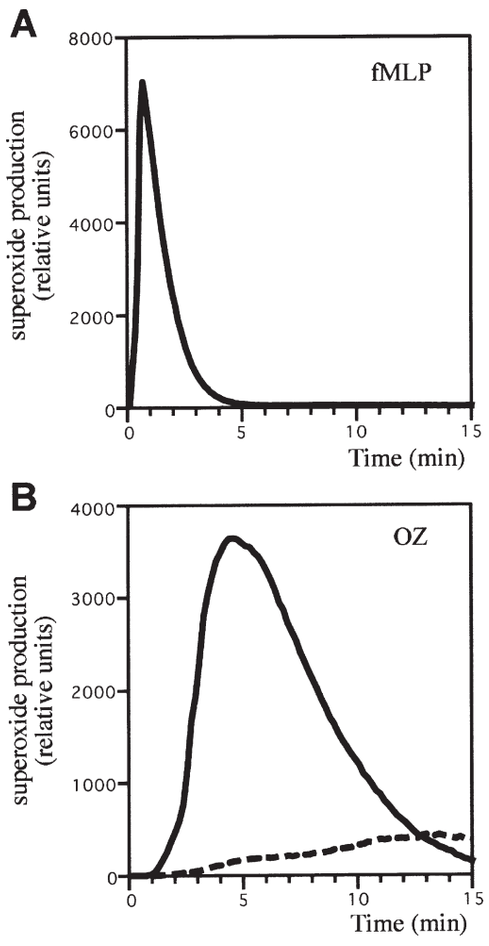

FIGURE 1 Time course of O_2^- production in fMLP- and OZ-stimulated neutrophils. The production of O_2^- was measured by chemiluminescence as described in the text. After preincubation for 5 min at 37°C, neutrophils were stimulated with 100 nM fMLP and 1 mg/ml of OZ at 37°C. (A) Time course of O_2^- production in fMLP-stimulated neutrophils, and (B) time course of O_2^- production in OZ-stimulated neutrophils. Solidline: zymosan opsonized by unheated serum, broken line: zymosan opsonized by heated serum.

Effect of U0126, an ERK1/2 Kinase Inhibitor, on the Production of O_2^-

To clarify whether ERK1/2 was involved in the production of O_2^-, we examined the effects of U0126 on fMLP- and OZ-induced O_2^- production. Figure 2 shows that U0126 inhibited the production of O_2^- elicited by fMLP stimulation in a concentration-dependent manner and completely abolished the production of O_2^- at concentrations over 5 μM. However, it did not inhibit the production of O_2^- evoked by OZ stimulation. These results suggested that ERK1/2 might be involved in the activation of NADPH oxidase when stimulated with fMLP, but not with OZ.

Effect of U0126, an ERK1/2 Kinase Inhibitor, on the Activation of ERK1/2

We next examined the activation of ERK1/2 elicited by fMLP and OZ. fMLP stimulation evoked rapid and strong activation of ERK1/2 that reached the maximum at 1 min (Fig. 3A, upper panel). The time course was similar to that of the production of O_2^- elicited by fMLP stimulation. On the other hand, OZ stimulation activated ERK1/2 less than fMLP stimulation did. The activation peaked at about 2 min. The time course was different from that

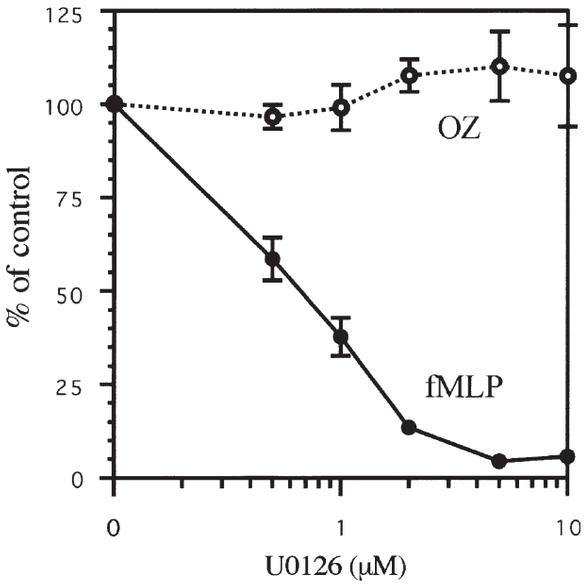

FIGURE 2 Effects of U0126 on the production of O_2^- in fMLP- and OZ-stimulated neutrophils. Neutrophils were preincubated with various concentrations of U0126 for 5 min at 37°C before stimulation with 100 nM fMLP or 1 mg/ml of OZ. Closed circle: fMLP stimulation, open circle: OZ stimulation. The peak value of O_2^- production induced by each stimulus in the absence of the inhibitor was defined as 100%. Each point and bar represent the mean ± S.E. of eight independent measurements.

of the production of O_2^- (Fig. 3A, bottom panel). Five micromolars U0126, which inhibited the production of O_2^- elicited by fMLP, but not by OZ (Fig. 2), abolished the phosphorylation of ERK1/2 caused by either fMLP or OZ (Fig. 3B). Thus, it was found that U0126 had an inhibitory effect on the OZ-induced activation (phosphorylation) of ERK1/2, whereas it showed no inhibitory effect on the OZ-induced production of O_2^-.

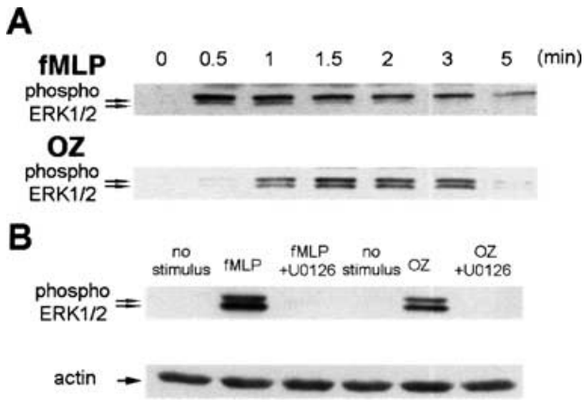

FIGURE 3 Time courses of ERK1/2 activation and the effects of U0126 on it in fMLP- and OZ-stimulated neutrophils. (A) Time courses of ERK1/2 activation elicited by fMLP (upper panel) and OZ (bottom panel), and (B) effects of U0126 on the activation of ERK1/2. Neutrophils were preincubated with or without 5 μM U0126 for 5 min at 37°C before stimulation. Reaction times were 1 min for fMLP stimulation and 2 min for OZ stimulation. Upper and bottom panels indicate western blots detected by anti-phospho ERK1/2 and anti-actin, respectively.

Effect of U0126, an ERK1/2 Kinase Inhibitor, on the Translocation of p47^{phox}

Since the activation of NADPH oxidase was known to be triggered by the translocation of p47^{phox} to plasma membrane as described above, we investigated the relationship between the translocation of p47^{phox} and the activation of ERK1/2. As shown in Fig. 4, the translocation of p47^{phox} occurred as a result of stimulation with fMLP and OZ. A moderate inhibitory effect (about 31% reduction) of U0126 on the translocation of p47^{phox} was observed during stimulation with fMLP, but little inhibitory effect (about 7% reduction) was observed under stimulation with OZ. These results indicated that the activation of ERK1/2 deeply participated in the translocation of p47^{phox} induced by fMLP, but not by OZ.

Contribution of PKC to the Activation of NADPH Oxidase in fMLP- and OZ-stimulated Neutrophils

Since the importance of PKC in the activation of NADPH oxidase has been well documented, we examined the contribution of PKC to the production of O_2^- in fMLP- and OZ-stimulated neutrophils, and whether PKC was correlated to the activation of ERK1/2. The production of O_2^- from neutrophils stimulated with fMLP and OZ in the presence of a PKC inhibitor, GF109203X (GFX), is shown in Fig. 5A. Similar concentration-dependent inhibition was observed in the fMLP- and OZ-stimulated neutrophils, indicating that PKC contributed to the activation of NADPH oxidase in fMLP- and OZ-induced O_2^- production in neutrophils. However, neither fMLP- nor OZ-induced activation of ERK1/2 was inhibited by 1 μM GFX as shown in Fig. 5B. This meant that PKC was not correlated to the activation of ERK1/2 mediated by the fMLP receptor and CR3.

DISCUSSION

In neutrophils stimulated with fMLP, activation of the Ras/Raf/MEK/ERK cascade has been reported.[13] Concerning the relationship between the Ras/Raf/MEK/ERK cascade and the activation of NADPH oxidase, the present study showed that U0126, an ERK1/2 kinase inhibitor, remarkably suppressed the production of O_2^- and the translocation of p47^{phox} caused by fMLP, and the time course of O_2^- production was comparable to that of the phosphorylation of ERK1/2 elicited by fMLP. These results meant that ERK1/2 certainly participated in the activation of NADPH oxidase by fMLP at

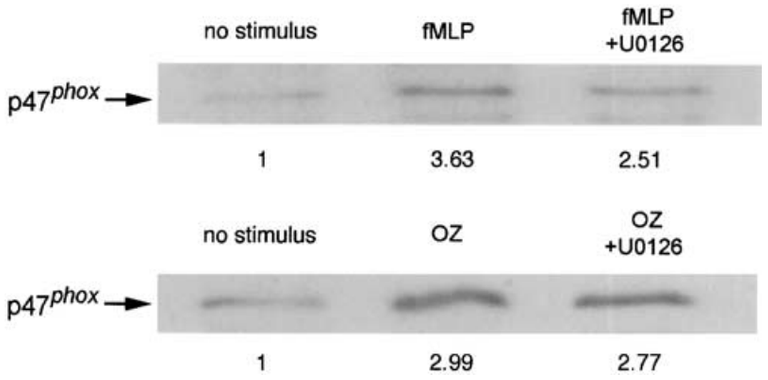

FIGURE 4 Effects of U0126 on the translocation of $p47^{phox}$. Neutrophils were preincubated with or without 5 μM U0126 for 5 min at 37°C before stimulation. After stimulation, membrane fractions of neutrophils were separated by ultracentrifugation using a discontinuous 15/34% sucrose gradient as described in the text. Reaction times were 45 s for fMLP stimulation and 5 min for OZ stimulation. Upper and bottom panels indicate western blots detected by anti-$p47^{phox}$ when neutrophils were stimulated by fMLP and OZ, respectively. The ratio of the intensity of translocated $p47^{phox}$ to that of unstimulated cells is calculated from the densitometric trace and the relative value is given under each lane.

the upstream of the translocation of $p47^{phox}$. Participation of ERK1/2 in the activation of NADPH oxidase was also proved by our recent study using human HL60 cells stimulated with fMLP.[14] fMLP appears to activate not only ERK1/2 but also PKC in connection with the activation of NADPH oxidase because GFX, a PKC inhibitor, effectively abrogated the production of O_2^- by fMLP. From the findings that the translocation of $p47^{phox}$ was regulated by PKC in fMLP-stimulated neutrophils[15] and fMLP-induced activation of ERK1/2 was not influenced by GFX (Fig. 5B), it was inferred that PKC acted as a regulator of the production of O_2^- regardless of the activation of ERK1/2 or existed in the downstream signal of ERK1/2.

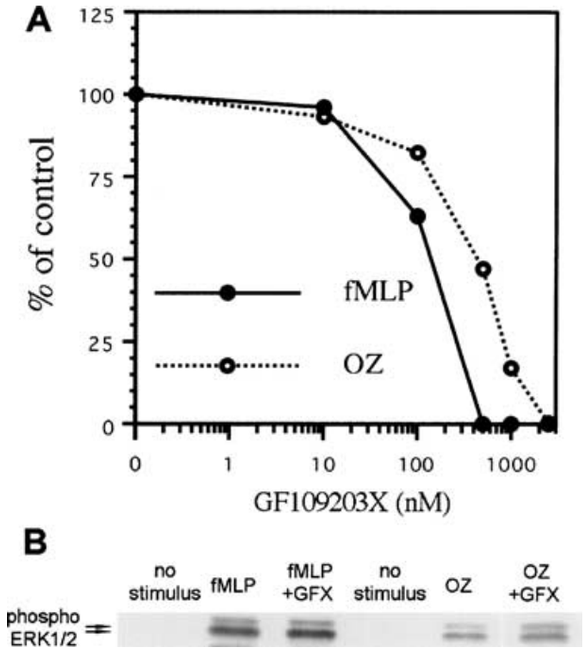

FIGURE 5 Effects of GF109203X (GFX) on the production of O_2^- and the activation of ERK1/2. Neutrophils were preincubated with or without GFX for 5 min at 37°C before stimulation. (A) Effects of various concentrations of GFX on the production of O_2^- in fMLP- and OZ-stimulated neutrophils. Closed circle: fMLP stimulation, open circle: OZ stimulation. The maximum value of O_2^- production induced by each stimulus in the absence of the inhibitor was defined as 100%, and (B) effects of 1 μM GFX on the activation of ERK1/2. The reaction times were 1 min for fMLP stimulation and 2 min for OZ stimulation.

From experiments using heated serum, CR3 was found to be the receptor responding to the stimulation with OZ in rat neutrophils. In OZ-stimulated neutrophils, the inhibition of ERK1/2 by U0126 had no effect on the activation of NADPH oxidase or on the translocation of $p47^{phox}$. A discrepancy in the time course between the maximum production of O_2^- and the maximum phosphorylation of ERK1/2 was also observed. In addition, the level of ERK1/2 phosphorylation by OZ was lower than that by fMLP. These results supported the idea that the OZ-induced activation of ERK1/2 might not necessarily play an essential role in the activation of NADPH oxidase in rat neutrophils. On the other hand, GFX, a PKC inhibitor, could inhibit the activation of NADPH oxidase mediated by CR3 in a fashion similar to that by the fMLP receptor as shown in Fig. 5A. These data implied that the CR3-induced activation of NADPH oxidase might mainly depend on the activation of PKC but not on that of ERK1/2.

There are many reports about the roles of the MAPK family, including p38 MAPK and ERK1/2, in the activation of NADPH oxidase of neutrophils.[12,16–22] Our previous studies using bovine neutrophils demonstrated that OZ-induced activation of p38 MAPK participated in the signaling at the upstream of $p47^{phox}$ phosphorylation and Rac activation.[16,17] Several reports about human

neutrophils also showed that p38 MAPK regulated the production of O_2^- when they were stimulated with LPS, TNFα, fNLLP, fMLP and phorbol myristate acetate (PMA).[18–20] Dewas *et al.* demonstrated that ERK1/2 participated in the phosphorylation of $p47^{phox}$ and the activation of NADPH oxidase in human neutrophils stimulated with fMLP.[11] In contrast, PD98059, an ERK1/2 kinase inhibitor, showed no effects on the production of O_2^- in human neutrophils stimulated with plasma opsonized *Staphylococcus aureus*[21] or in mouse bone marrow neutrophils stimulated with PMA.[22] Tardif *et al.* reported that PD98059 had no effects on the production of O_2^- induced by co-stimulation with PMA and fMLFK in dibutyryl-cAMP-differentiated HL60 cells.[23]

The controversial results about the roles of ERK1/2 in the activation of NADPH oxidase observed in these reports led us to assume that the upstream signal of $p47^{phox}$ translocation to the plasma membrane was largely dependent on the receptor type, though p38 MAPK has been generally accepted to participate in the activation of NADPH oxidase. The present study demonstrated that receptor dependence existed in the ERK1/2 signaling leading to the activation of NADPH oxidase. That is, ERK1/2 regulated the activation of NADPH oxidase mediated by an *N*-formyl peptide receptor but not by CR3. It was proved that the ERK1/2 pathway was dominant in the signaling for the production of O_2^- in rat neutrophils in the *N*-formyl-peptide reaction system, but not responsible for the bactericidal respiratory burst during phagocytosis.

Acknowledgements

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