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Involvement of p38 MAP Kinase in Not Only Activation of the Phagocyte NADPH Oxidase Induced by Formyl-methionyl-leucyl-phenylalanine but Also Determination of the Extent of the Activity

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Activated NADPH oxidase in neutrophils produces superoxide. We investigated the role of p38 MAP kinase in activating NADPH oxidase stimulated by the bacteria-derived peptide fMLP. fMLP-stimulated superoxide production was completely abolished by SB203580, a p38 MAP kinase inhibitor, whereas anisomycin, a p38 MAP kinase activator, did not induce superoxide production, indicating that p38 MAP kinase was essential, but not sufficient, for NADPH oxidase activation. Anisomycin pretreatment strongly activated p38 MAP kinase in fMLP-stimulated cells, accompanied by greatly increased superoxide production, suggesting that p38 MAP kinase determines the extent of the fMLP-stimulated NADPH oxidase activity. Furthermore, superoxide production was remarkably reactivated by cytochalasin B addition after fMLP-stimulated production had disappeared, and this was correlated with highly activated p38 MAP kinase. These results suggest that p38 MAP kinase is involved not only in activating NADPH oxidase stimulated by fMLP but also in determining the extent of its activity.

Key words: fMLP, neutrophil, p38 MAPK, phagocyte NADPH oxidase, superoxide.

Abbreviations: AM, anisomycin; CytB, cytochalasin B; fMLP, formyl-methionyl-leucyl-phenylalanine; MAPK, mitogen-activated protein kinase; PI3K, phosphatidylinositol 3-kinase; PKC, protein kinase C; SB, SB203580; SOD, superoxide dismutase.

Professional phagocytes, including neutrophils, eosinophils and monocytes, play crucial roles in host defense against microbial infections (*1*, *2*). During phagocytosis of invading microorganisms or upon cell stimulation with soluble agents, such as the bacteria-derived peptide formyl-methionyl-leucyl-phenylalanine (fMLP), phagocytes produce superoxide, a precursor of microbicidal oxidants, via catalysis by activated phagocyte NADPH oxidase (*3–5*). The reactive oxygen species subsequently derived from superoxide are essential for microbial killing (*6*). The importance of phagocyte NADPH oxidase is emphasized by a genetic disorder known as chronic granulomatous disease in which neutrophils cannot produce superoxide, with the result that the patients suffer from severe recurrent bacterial and fungal infections (*6*, *7*). The oxidase is composed of a membrane-spanning glycoprotein, cytochrome b_{558}, and cytosolic proteins such as $p47^{phox}$, $p67^{phox}$, $p40^{phox}$ and the small G-protein Rac (*8–11*). Cytochrome b_{558}, a heterodimeric protein composed of a 91-kDa glycoprotein ($gp91^{phox}$) and a 22-kDa protein ($p22^{phox}$), is located in the plasma membrane of phagocytes (*12*, *13*). When the cells are stimulated, Rac exchanges GDP for GTP, and $p47^{phox}$ is phosphorylated to become an active form. Those cytosolic proteins translocate to the membrane and form a complex with cytochrome b_{558} to produce superoxide (*4*, *8*, *14*, *15*).

p38 mitogen–activated protein kinase (MAPK), one of the stress-activated protein kinases, was first identified as a phosphorylated protein in response to lipopolysacchalide and hyperosmolarity in mammalian cells (*16*). In a signaling cascade, the kinase is activated by tyrosine kinases, phosphatidylinositol 3-kinase (PI3K), protein kinase C (PKC) and phospholipase C, which are activated by a set of trimeric G-proteins coupled with the fMLP receptor (*17*, *18*). p38 MAPK is activated by dual phosphorylation in a TGY motif, which is recognized by a specific anti-active form antibody (*19*, *20*). SB203580 (SB), a competitive inhibitor of p38 MAPK against ATP binding, does not prevent p38 MAPK phosphorylation, *i.e.* its activation, but rather specifically blocks its kinase activity (*21*, *22*). On the contrary, anisomycin (AM), which was originally identified as a protein synthesis inhibitor, was found to stimulate p38 MAPK at much lower concentrations than it inhibits protein synthesis (*23*, *24*).

To date, the role of p38 MAPK in the process of NADPH oxidase activation has remained unknown. Indeed, $p47^{phox}$ is phosphorylated through p38 MAPK by fMLP stimulation, although there is no data indicating correlation between activity of p38 MAPK and superoxide production (*25*). Others have shown that SB mostly suppresses the oxidase activation (*26–28*), while another group has shown that SB does not inhibit the activation very well (*29*). Thus far AM, p38 MAPK activator, has not been

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applied to study the signaling mechanism of the kinase in the activation of human neutrophil NADPH oxidase. Therefore, the aim of the present study was to clarify the role of p38 MAPK in the activation of the NADPH oxidase following stimulation with fMLP, by evaluating the effects of AM and SB as well as cytochalasin B (CytB), an actin network disrupter which reactivates the oxidase following the disappearance of the fMLP-stimulated activity (*30*). Our results suggest that the p38 MAPK pathway is not only involved in the activation process of the phagocyte NADPH oxidase but also essential for the regulatory process that determines the extent of its activity.

MATERIALS AND METHODS

Materials—fMLP, cytochrome *c*, superoxide dismutase (SOD), AM, SB, CytB and phosphate-buffered saline (PBS) were purchased from Sigma-Aldrich (St. Louis, MO). All other chemicals used were of the highest purity commercially available.

Neutrophil Preparation—Neutrophils were purified from fresh citrate-buffered whole blood from healthy adult donors after informed consent was obtained, as described previously (*4*, *14*). Briefly, the blood samples were subjected to dextran sedimentation, hypotonic lysis and Conray/Ficoll density gradient centrifugation and then suspended in PBS, pH 7.4. More than 95% of the cells in each preparation were neutrophils.

Assay of Superoxide Production—Superoxide production by neutrophils was determined by a SOD-sensitive cytochrome *c* reduction assay as described previously (*11*). Typically, 1.0×10^6 cells were resuspended in 1 ml of HEPES-buffered salt solution (120 mM NaCl, 5 mM KCl, 5 mM glucose, 1 mM $MgCl_2$, 0.5 mM $CaCl_2$ and 17 mM HEPES, pH 7.4) containing cytochrome *c* (75 μM) and preincubated at 37°C for 2 min before the addition of SB (10 μM) or vehicle. At 10 min after the addition, AM (300 ng/ml) or vehicle was added, and the cell suspension was incubated for another 40 min before stimulation with fMLP (300 nM). The reduction of cytochrome *c* was measured using a 557S spectrophotometer (Hitachi, Ibaragi), and superoxide production was calculated from the absorbance difference at 550–540 nm by using an absorption coefficient of 21,000 $M^{-1} \cdot cm^{-1}$. The incubation was continued for further specified times before measurable cytochrome *c* reduction was halted by the addition of SOD. In cases of reactivation with CytB, the cell suspension was preincubated at 37°C in a cuvette, and the cytochrome *c* reduction was monitored in the spectrophotometer for 2 min before stimulation with fMLP. At 10 min after the fMLP stimulation, CytB (5 μg/ml) or vehicle was added, and the reduction was further monitored.

Detection of Activated p38 MAPK—The reactions of neutrophils were terminated by addition of 10% trichloroacetic acid to the cell suspension at the indicated times, and the cells were lysed in SDS-PAGE sample buffer containing 8.0 M urea, 5% 2-mercaptoethanol, 1% Triton-X100 and 2% sodium dodecylsulfate (*11*). The samples (1.0×10^5 cells/lane) were subjected to western blot analysis with an anti-phosphorylated, *i.e.* active, p38 MAPK antibody (pTGpY; Promega) (*20*), followed by a horseradish peroxidase–conjugated goat anti-mouse or anti-rabbit secondary antibody. Detection of the bound antibodies was carried out using an enhanced chemiluminescence method (GE Health care Bio-Sciences). The blots were reprobed with an anti–p38 MAPK antibody (sc-7972; Santa Cruz Biotechnology Inc., Santa Cruz, CA).

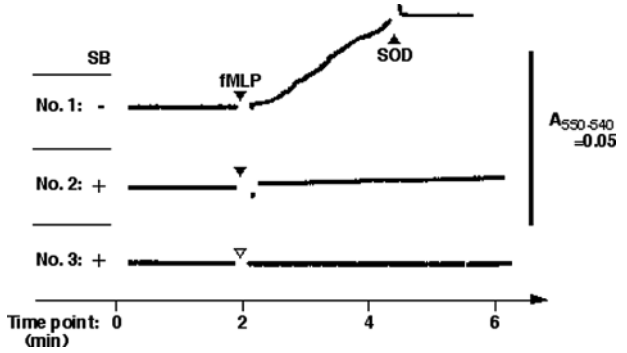

Fig. 1. **Effect of SB on fMLP-stimulated superoxide production.** Neutrophils were preincubated at 37°C for 2 min before the addition of SB (Nos. 2 and 3) or buffer alone (No. 1). After 8 min incubation at time point 0, *i.e.* 2 min before the addition of fMLP (inverted solid triangles, Nos. 1 and 2) or vehicle (inverted open triangle, No. 3), the cuvettes were placed in a spectrophotometer and superoxide production was measured by monitoring the reduction of cytochrome *c*. In trace No. 1, the reduction was halted by SOD (solid triangle) at time point 4.

RESULTS

p38 MAPK Is Essential, but Not Sufficient, for Activation of the fMLP-Stimulated NADPH Oxidase—First, we studied the effect of SB, a p38 MAPK–specific inhibitor, on superoxide production by human peripheral blood neutrophils stimulated with fMLP because we wanted to define the role of p38 MAPK in NADPH oxidase activation. The superoxide production mediated by the oxidase was measured by SOD-sensitive cytochrome *c* reduction (Fig. 1). SB addition at 10 min before fMLP addition completely inhibited fMLP-stimulated superoxide production (Fig. 1, No. 2), indicating that p38 MAPK was essential for activating the fMLP-stimulated oxidase. If p38 MAPK was involved in the signal transduction pathway from the fMLP receptor to the oxidase as an essential and sufficient component, its activation alone would be expected to activate the oxidase. Therefore, we treated neutrophils with AM, a specific p38 MAPK activator (*23*, *24*). AM gradually increased the amount of phosphorylated, i.e. activated, p38 MAPK (Fig. 2A), but did not induce superoxide production (Fig. 2B). These findings indicate that activation of p38 MAPK alone is not sufficient for NADPH oxidase activation.

p38 MAPK Is Sufficient for Enhancing the NADPH Oxidase Activity Stimulated by fMLP—Next, we tried to elucidate the role of p38 MAPK in regulating the fMLP-stimulated NADPH oxidase activity by activating p38 MAPK with AM. p38 MAPK was significantly phosphorylated after AM pretreatment for 40 min (Fig. 3A, upper panel, lane 5) compared to that incubated without AM (lane 2), indicating that p38 MAPK was indeed activated by AM. When AM-pretreated neutrophils were stimulated with fMLP, the amount of phosphorylated p38 MAPK greatly increased within 2 min (lane 6), and even

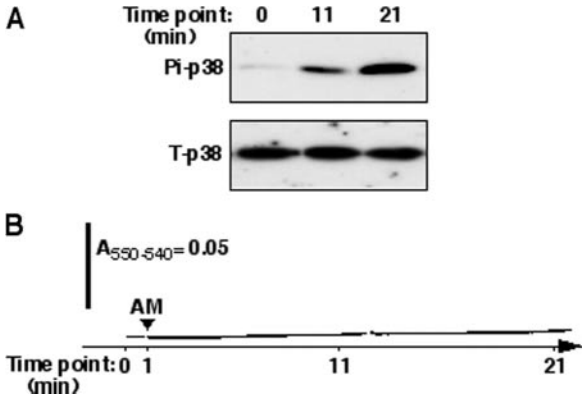

Fig. 2 **AM activates p38 MAPK but does not induce superoxide production.** (A) AM activates p38 MAPK. Neutrophils were preincubated at 37°C for 1 min before the addition of AM at time point 1. The cells were harvested at the indicated time points and subjected to western blot analyses. Phosphorylated p38 MAPK (Pi-p38) and total p38 MAPK (T-p38) were visualized with specific antibodies, as described in the "MATERIALS AND METHODS." (B) AM alone does not induce superoxide production. Neutrophils were stimulated with AM at time point 1 (inverted solid triangle), and the superoxide production was monitored by cytochrome *c* reduction. The trace is a representative result of 3 independent experiments.

exceeded the sum of the amounts observed in cells treated individually with fMLP or AM (lanes 3 and 5). Based on the strong phosphorylation of p38 MAPK with both fMLP and AM, we studied the effects of AM on superoxide production stimulated with fMLP in relation to dose or preincubation time. Maximal superoxide production was observed at concentrations of 0.1–1 μg/ml AM and more than 30 min preincubation times (Fig. 3B and C), consistent with previous findings obtained when AM was used under similar conditions to activate p38 MAPK in mammalian cells (*23*). Corresponding to the large amount of phosphorylated p38 MAPK in AM-preincubated and fMLP-stimulated cells and small amount of phosphorylated p38 MAPK in cells treated with fMLP alone (Fig. 3A, lanes 3 and 6, respectively), superoxide production was dramatically higher in the former cells (4.3 ± 0.6 nmol/min/10^6 cells) than in the latter cells (0.6 ± 0.2 nmol/min/10^6 cells) (Fig. 3D, Nos. 1 and 2). The enhanced and basal superoxide productions were both completely abolished by addition of SB (Fig. 3D, Nos. 3 and 4, and 3E, columns 4 and 5). To our knowledge, this is the first demonstration that a p38 MAPK activator, such as AM, strongly enhances the NADPH oxidase activity stimulated with fMLP. No superoxide production was observed with AM alone, even if the neutrophils were incubated for more than 40 min (Fig. 3E, column 6), and the results were similar to the case of the 20 min incubation (Fig. 2B). These results suggest that the increase in p38 MAPK activity was sufficient to enhance the fMLP-stimulated NADPH oxidase activity.

p38 MAPK Is Required for Enhancement of the NADPH Oxidase Activity Stimulated with fMLP—Finally, we addressed the question of whether or not the increased NADPH oxidase activity accompanied the enhancement of p38 MAPK activity, by utilizing a previously reported NADPH oxidase reactivation system involving CytB (*30*) (Fig. 4). CytB alone did not activate neutrophils (Fig. 4A, No. 3 and 4B, column 3) but reactivated fMLP-stimulated neutrophils to produce superoxide dramatically (Fig. 4A, No. 1 and 4B, column 4). The extent of the reactivated superoxide production was comparable to the extent of the AM-enhanced production (Fig. 3D, No. 1), indicating that CytB acted like AM although their orders of addition were opposite relative to the addition of fMLP. The CytB-reactivated superoxide production was 5.4 ± 0.7 nmol/min/10^6 cells (Fig. 4B, column 4) and close to that calculated for the AM-enhanced superoxide production (Fig. 3E, column 3). Whereas the total amount of p38 MAPK remained constant regardless of fMLP treatment with or without CytB (Fig. 4C, bottom panel), phosphorylated p38 MAPK was strongly detected only in both fMLP-stimulated and CytB-reactivated cells (Fig. 4C, upper panel, lane 4). Thus, the highly increased NADPH oxidase activity was correlated with the greatly increased amount of active p38 MAPK. Although a small amount of phosphorylated p38 MAPK was detectable in the late phase of fMLP stimulation (Fig. 4C, upper panel, lane 3), its extent was consistent with the barely detectable superoxide production (Fig. 4A, No. 2). These results suggest that p38 MAPK is essential for enhancing the fMLP-stimulated NADPH oxidase activity.

DISCUSSION

In signaling pathways from the fMLP receptor to NADPH oxidase, p38 MAPK is involved in activating the phagocyte NADPH oxidase stimulated by fMLP and determining the extent of its activity. This is supported by the finding that a calcium chelator, ethylenediaminetetraacetic acid (EDTA) decreased fMLP-stimulated superoxide production in a parallel motion with phosphorylation of p38 MAPK (data not shown). Furthermore, the observation by Yan *et al.* (*28*) also supports the argument. They showed that phosphorylation of p38 MAPK highly correlates with fMLP-stimulated superoxide production of neutrophils primed by lipopolysaccharide. The mechanism for how p38 MAPK stimulates NADPH oxidase has recently been studied. Phosphorylation of $p47^{phox}$ is important for NADPH oxidase activation, since its SH3 domains are masked in the resting state, *i.e.* these domains are not accessible to the proline-rich region of $p22^{phox}$ in the resting state. However, upon activation with a stimulant such as fMLP or PMA, the C-terminal Ser and Thr are phosphorylated, and the SH3 domains become unmasked and bind with the proline-rich region of $p22^{phox}$ (*15*). This binding is essential for the oxidase activation, and $p47^{phox}$ is certainly phosphorylated by p38 MAPK upon stimulation with fMLP (*25*). Both p38 MAPK and Rac are activated by PI3K, and Rac activation is also essential for the oxidase activation (*4*). The PI3K inhibitors wortmannin and LY294002 block not only p38 MAPK activation but also Rac activation. Furthermore, SB only abolishes Rac activation but not PI3K activation (*26*), suggesting that p38 MAPK stimulated by PI3K acts upstream of Rac.

Interestingly, the extent of superoxide production by neutrophils stimulated with phorbol myristate acetate, a PKC activator, was similar to that of superoxide production by neutrophils stimulated by fMLP in the presence of AM or CytB (data not shown). Since PKC acts upstream of

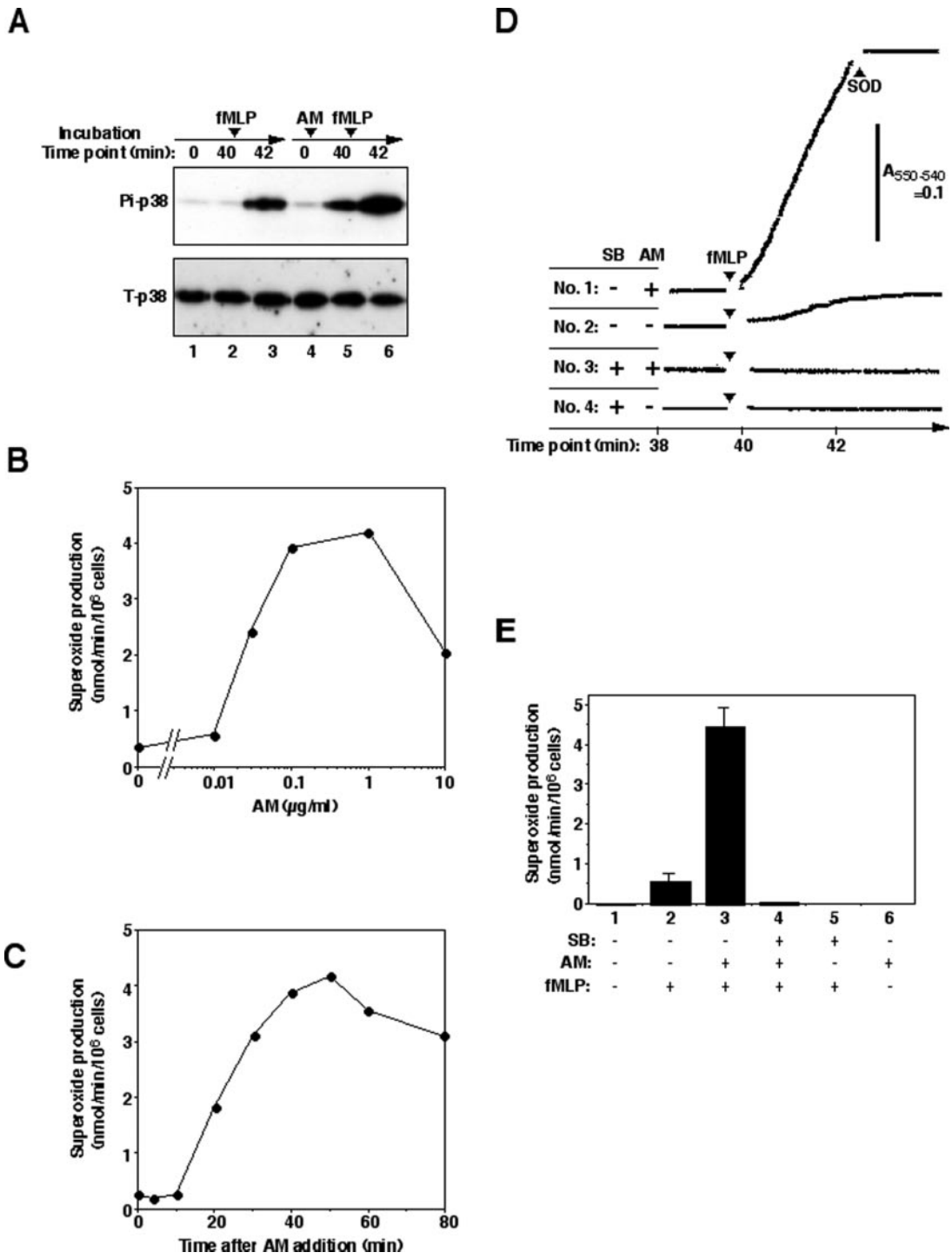

Fig. 3. **Effects of AM on p38 MAPK and fMLP-stimulated superoxide production.** (A) Activation of p38 MAPK. AM or buffer was added to the cell suspension at time point 0, and the cells were incubated for 40 min before stimulation with fMLP. Two minutes after fMLP stimulation with or without AM, the incubation was finally terminated. At the indicated time points, the cell lysates were subjected to western blot analyses, as described in the legend for Fig. 2A. (B) Superoxide production induced by fMLP-stimulation after preincubation with different concentrations of AM for 40 min. (C) Effects of preincubation with AM on superoxide production stimulated by fMLP. After preincubation with AM (0.3 μg/ml) for the indicated times, the cells were stimulated with fMLP, and cytochrome *c* reduction was measured. (D) Effects of AM or SB on fMLP-stimulated superoxide production. Neutrophils were preincubated at 37°C for 2 min before the addition of SB (Nos. 3 and 4) or diluent alone (Nos. 1 and 2). After preincubation for 10 min, AM (Nos. 1 and 3) or diluent alone (Nos. 2 and 4) was added, and the cells were incubated for 40 min prior to stimulation with fMLP (inverted solid triangles). Two minutes before fMLP addition, the cuvettes were placed in a spectrophotometer at time point 38, and the superoxide production was measured. In the case of No. 1, the cytochrome *c* reduction was halted at time point 42 by SOD (solid triangle). (E) Neutrophils were stimulated with fMLP, and the superoxide production was calculated by determining the rate of cytochrome *c* reduction. The experimental conditions were identical to (D). The data represent means ± SD of at least 3 separate experiments.

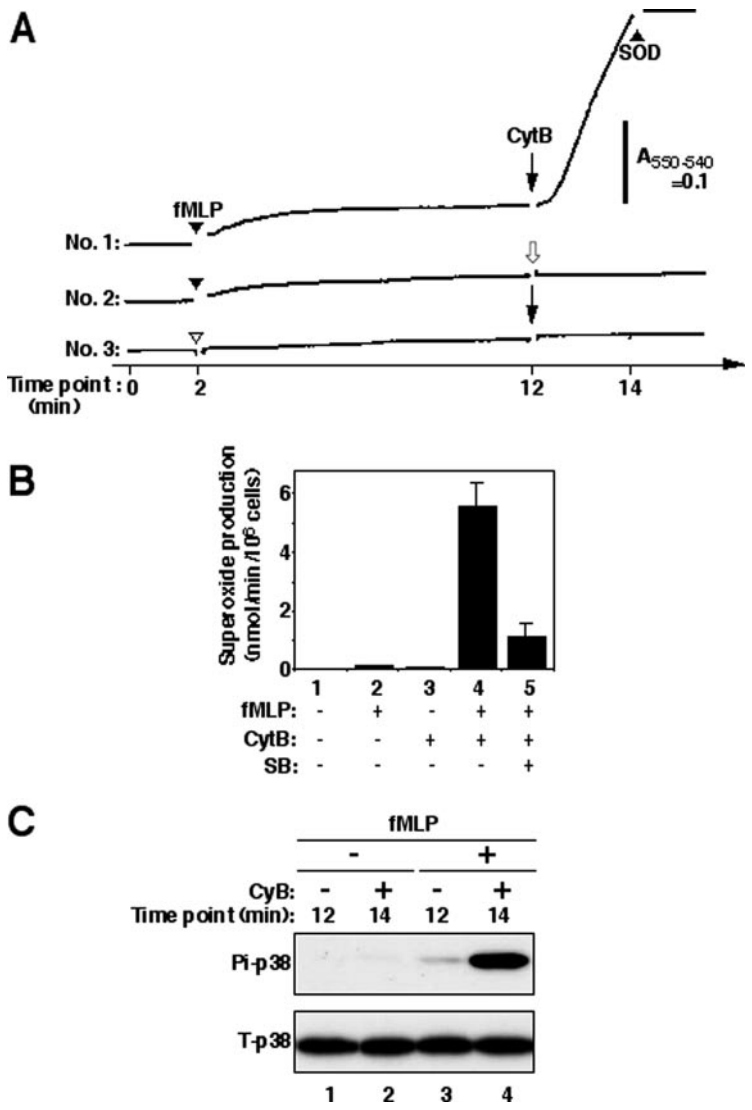

Fig. 4. **Correlation between CytB-dependent reactivation of fMLP-stimulated superoxide production and p38 MAPK activation.** (A) Reactivation of fMLP-stimulated superoxide production. Cuvettes containing neutrophils were placed in a spectrophotometer (time point 0). After 2 min, fMLP (inverted solid triangles) or vehicle (inverted open triangle) was added (time point 2). The superoxide production of the cells was measured as described in the legend for Fig. 1, and the oxidase was reactivated by the addition of CytB (Nos. 1 and 3) at time point 12 (↓) and halted by SOD (solid triangle) (No. 1). Instead of reagents, media were added at the time points indicated by the open symbols (⇓) (No. 2). (B) Superoxide production measured under various conditions. The experimental conditions were identical to (A). The data represent means ± SD of at least 3 separate experiments. (C) Western blot analyses of fMLP-stimulated and unstimulated neutrophils obtained before and after reactivation with CytB. Samples were obtained at the indicated time points shown in (A). Phosphorylated p38 (Pi-p38) and total p38 (T-p38) were visualized as described in the legend for Fig. 2A.

p38 MAPK (*17*), p38 MAPK may be essential and sufficient for maximizing NADPH oxidase activity.

The present results show that CytB-dependent reactivation of NADPH oxidase, whose activity was previously stimulated by fMLP and then terminated, is dependent on p38 MAPK. The molecular basis for this reactivation of fMLP-stimulated NADPH oxidase is suggested to be a direct effect of cytoskeleton-dependent signaling, since the ligated fMLP receptor binds to the cytoskeleton, an event known to occur shortly after binding of a chemoattractant to its receptor (*31*, *32*). In fact, the association of the fMLP receptor with cytoskeletal components induces physical segregation of the trimeric G-proteins and the receptor into different plasma membrane regions, leading to termination of the oxidase activation (*31*). This physical separation of the components is achieved through direct binding of the ligand-receptor complex to the cytoskeleton. This binding is disrupted by CytB, resulting in reactivation of the oxidase. Immunoprecipitation analyses of actin with an anti-actin antibody in differentiated L6E9 skeletal muscle cells revealed direct binding between actin and p38 MAPK (*33*). In neutrophils, actin may also affect p38 MAPK activation which, in turn, affects NADPH oxidase activation and the regulation of its activity.

It is important to clarify the molecular mechanism by which p38 MAPK participates in phagocyte NADPH oxidase activation, since p38 MAPK inhibitors are likely to be available for clinical use to control neutrophil-mediated autoinflammatory tissue injury facilitated by the activated oxidase (*34*, *35*).

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