Primining of the Neutrophil Respiratory Burst Involves p38 Mitogen-activated Protein Kinase-dependent Exocytosis of Flavocytochrome b558-containing Granules*

Richard A. Ward†‡§, Michio Nakamura¶, and Kenneth R. McLeish¶**

From the †Molecular Signaling Group, Department of Medicine and the ‡Department of Biochemistry and Molecular Biology, University of Louisville, Louisville, Kentucky 40202-1718, the ¶Department of Host-defense Biochemistry, Institute of Tropical Medicine, Nagasaki University, 1-12-4 Sakamoto, Nagasaki 852-8523, Japan, and the **Veterans Affairs Medical Center, Louisville, Kentucky 40204

The respiratory burst of human neutrophils is primed by a number of pro-inflammatory stimuli, including tumor necrosis factor-α (TNFα) and lipopolysaccharide (LPS); however, the mechanism of priming remains unknown. LPS has been shown previously to increase membrane expression of flavocytochrome b558, a component of the NADPH oxidase. This study shows that TNFα also increases membrane expression of flavocytochrome b558. Mitogen-activated protein kinase (MAPK) modules have been implicated in the action of priming agents. Pharmacologic inhibitors of MAPKs, SB203580 and PD98059, revealed that priming of the respiratory burst and up-regulation of flavocytochrome b558 are dependent on p38 MAPK but not on extracellular-signal regulated kinase (ERK). TNFα and LPS primed respiratory burst activity and increased membrane expression of CD35 and CD66b, specific markers of secretory vesicles and specific granules that contain flavocytochrome b558, with similar time courses and concentration dependences. These processes also required p38 MAPK but were independent of ERK. TNFα failed to prime respiratory burst activity or to increase membrane CD35 expression in enucleated neutrophil cytoplasts. These data suggest that one mechanism by which TNFα and LPS prime neutrophil respiratory burst activity is by increasing membrane expression of flavocytochrome b558 through exocytosis of intracellular granules in a process regulated by p38 MAPK.

Polymorphonuclear leukocytes (PMNs) play a central role in innate immunity through their ability to kill bacteria, in part, by generating toxic oxygen radicals in a process known as the respiratory burst. A multicomponent enzyme complex, called the NADPH oxidase, produces the respiratory burst of PMNs. In resting PMNs, this enzyme complex consists of unassembled plasma membrane and cytosolic components (1). Following activation, the cytosolic components p40 Phox, p47 Phox, p67 Phox, and Rac-2 translocate to the membrane where they associate with flavocytochrome b558, a heterodimer composed of gp22 Phox and gp91 Phox, and Rap1A to form the active oxidase (1). In resting cells 10–25% of flavocytochrome b558 is located in the plasma membrane, with the remaining 75–90% distributed among secretory vesicles and gelatinase and specific granules (2, 3).

Normally, circulating PMNs possess a limited NADPH oxidase response to phagocytosis and chemotactic stimulation. Their respiratory burst response is enhanced by a number of pro-inflammatory stimuli, such as tumor necrosis factor-α (TNFα) (4), granulocyte-macrophage colony-stimulating factor (GM-CSF) (5), and bacterial lipopolysaccharide (LPS) (6), that generally do not stimulate respiratory burst activity on their own. This enhancement of respiratory burst activity is referred to as priming. Priming occurs in vivo during infection (7, 8) or following intravenous infusion of TNFα into human volunteers (4). Priming enhances the capacity of PMNs to kill microorganisms as well as damage normal tissue. For example, intravenous infusion of LPS into rabbits prior to the infusion of fMLP resulted in PMN sequestration in the lungs and lung injury, which was not produced by either agent alone (9). Similarly, priming of PMNs prior to their infusion into the renal artery of isolated rat kidneys enhanced renal injury following an ischemic insult (10). Thus, priming contributes both to protection against infection and to organ damage during inflammation and ischemia-reperfusion injury.

The mechanisms by which PMNs are primed are not fully understood. A number of studies suggest that increased plasma membrane expression of key components of PMN responses to external stimuli may play a role. The membrane density of adhesion molecules, chemotactic receptors, and heterotrimetric G proteins have all been reported to be increased in models of PMN priming (6, 11–17). The increased expression of these components, however, cannot explain the increased respiratory burst response to a variety of stimuli. Recently, DeLeo et al. (3) reported that LPS stimulated an increase in plasma membrane expression of flavocytochrome b558 in PMNs, and subsequent stimulation of the cells with fMLP resulted in a 3–5-fold increase in translocation of p47 Phox, p67 Phox, and Rac-2 to the plasma membrane compared with cells not exposed to LPS. They concluded that redistribution of the

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§ To whom correspondence should be addressed: Dept. of Medicine, University of Louisville, 615 S. Preston St., Louisville, KY 40202-1718. Tel.: 502-852-5757; Fax: 502-852-7643; E-mail: richard.ward@louisville.edu.

† The abbreviations used are: PMN, polymorphonuclear leukocyte; ERK, extracellular signal-regulated kinase; fMLP, formylmethionyl-leucyl alanine; GM-CSF, granulocyte-macrophage colony-stimulating factor; JNK, c-Jun amino-terminal kinase; KRPB, Krebs-Ringer phosphate buffer; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; mcf, mean channel number of fluorescence intensity; MKK, MAPK kinase; MKKK, MAPK kinase kinase; PMA, phorbol myristate acetate; TNFα, tumor necrosis factor-α; FITC, fluorescein isothiocyanate.
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NADPH oxidase components might be the basis for LPS-induced priming.

Mitogen-activated protein kinase (MAPK) modules are activated by a variety of PMN stimuli, and they have been reported to participate in a number of PMN functional responses (18–26). MAPK modules are three-member protein kinase cascades that link a variety of extracellular signals to cellular events, such as growth, differentiation, apoptosis, and stress and inflammatory responses (27). MAPK modules begin with a serine/threonine kinase, known as a MAPK kinase kinase (MKKK). When activated, MKKKs phosphorylate and activate a dual specificity kinase known as MAPK kinase (MKK). MKKs, in turn, activate the third member of the module, a serine/threonine protein kinase, known as a MAPK, by recognition and phosphorylation of a TXY motif (27). Three MAPK modules have been identified in PMNs, p38 MAPK, extracellular signal-regulated kinase (ERK), and c-Jun amino-terminal kinase (JNK) (25, 26, 28, 29). Chemoattractants TNFα, GM-CSF, LPS, phagocytosis, and FcyR cross-linking stimulate the activity of p38 MAPK and ERK, but not JNK, in human PMNs (18, 24, 26, 28–32). Pharmacologic inhibition of p38 MAPK and ERK attenuates chemotaxis, adherence, phagocytosis, and/or respiratory burst activity (19, 21, 26, 29, 33). El Benna et al. (34) reported that both ERK and p38 MAPK phosphorylated p47phox. Based on the ability of MAPKs to regulate these PMN functions, we examined previously the participation of p38 MAPK and ERK in TNFα and GM-CSF priming of FMLP-stimulated respiratory burst activity, and we demonstrated that inhibition of ERK or p38 MAPK significantly attenuated priming by either TNFα or GM-CSF (28).

To define further the molecular basis for priming, the present study examined the hypothesis that, similar to LPS, TNFα increases membrane expression of flavocytochrome b558 and that increased expression is dependent on ERK and/or p38 MAPK activation. Our results indicate that TNFα and LPS stimulate a significant increase in flavocytochrome b558 expression in the plasma membrane of human PMNs. TNFα and LPS also stimulate increased plasma membrane expression of CD35 and CD66b, markers of secretory vesicles and specific granules, respectively, suggesting that the increased expression of flavocytochrome b558 is due to exocytosis of these intracellular granules. TNFα- and LPS-stimulated exocytosis of secretory vesicles and specific granules and increased expression of flavocytochrome b558 were dependent on p38 MAPK, but not ERK, activation. These results define a role of p38 MAPK in the regulation of intracellular granule exocytosis in PMNs that is, in part, responsible for priming the respiratory burst response.

EXPERIMENTAL PROCEDURES

Materials—Endotoxin-free reagents and plastics were used in all experiments. TNFα was obtained from R & D Systems (Minneapolis, MN). LPS (Salmonella minnesota Re595) was from Sigma. The MAPK inhibitors, SB203580 and PD98059, were obtained from Calbiochem. FITC-labeled monoclonal anti-CD35, FITC-labeled mouse IgG1, and FITC-labeled goat anti-mouse IgG1 were obtained from PharMingen (San Diego, CA). FITC-labeled monoclonal anti-CD66b was obtained from Accurate Chemical and Scientific (Westbury, NY). Monoclonal antibody against the extracellular domain of flavocytochrome b558 (7DS) was produced and characterized as described previously (35). Other reagents were obtained from Sigma unless otherwise specified.

PMNs—Blood was obtained from healthy donors in accordance with a protocol approved by the University Human Studies Committee at the University of Louisville. PMNs were isolated using plasma-Percoll gradients as described by Haselett et al. (36). After isolation, PMNs were washed in LPS-free Krebs-Ringer phosphate buffer (KRPB) (pH 7.2) containing 0.2% dextrose. Microscopic evaluation of isolated cells indicated that greater than 97% were PMNs and viability was always greater than 95% by trypan blue exclusion.

Cytoplasts—Encuclated PMN cytoplasts were prepared according to the method of Roos et al. (37). Briefly, PMNs were resuspended in 4.5 ml of 12.5% Ficoll containing 20 μM cytochalasin B and warmed at 37°C for 5 min before being layered onto a prewarmed step gradient consisting of 4.5 ml of 16% Ficoll, each containing 20 μM cytochalasin B. The gradient was centrifuged at 800 x g for 30 min at 34°C using a SW28 rotor in a Beckman model L-70K ultracentrifuge (Beckman Instruments, Fullerton, CA). Cytoplasts were recovered from the 12.5/16% interface and washed three times in KRPB at room temperature.

Respiratory Burst Activity—Respiratory burst activity was determined as H2O2 production stimulated by the phagoctyosis of Staphylococcus aureus using a previously described flow cytometric assay (8, 38). Briefly, PMNs were resuspended in KRPB containing calcium and magnesium to a concentration of 2 x 106 cells/ml and incubated with 2',7'-dichlorofluorescein diacetate (final concentration 0.5 μM) for 10 min at 37°C. Fifty microliters of cell suspension was then sampled before, and 10 min after, the addition of 50 μl of opsonized, propidium iodide-labeled S. aureus (final concentration approximately 109 bacteria/ml). The sampled cells were washed in KRPB and fixed in 1% paraformaldehyde. Samples were analyzed for phagocytosis and H2O2 production by flow cytometry (Epics Profile II, Coulter, Hialeah, FL). The mean channel number of fluorescence intensity (mcf) was used as a quantitative index of phagocytosis and H2O2 production. The flow cytometer was calibrated before the analysis of each set of samples using Standard-Brite beads (Coulter).

Alternatively, respiratory burst activity was determined as superoxide release in the presence of 10-7 M fMLP. Superoxide release was measured spectrophotometrically by ferricytochrome c reduction as described previously (39).

Expression of Plasma Membrane Proteins—Expression of plasma membrane proteins was determined by flow cytometry. For determination of CD35 and CD66b, PMNs were suspended in ice-cold KRPB and incubated at 4°C for 30 min with FITC-conjugated monoclonal anti-CD35 or FITC-conjugated monoclonal anti-CD66b. FITC-conjugated mouse IgG1 was used as an isotype control. Membrane expression of flavocytochrome b558 was determined using a monoclonal antibody (7DS) against its extracellular domain (35). After blocking by incubation with 2% goat serum for 10 min at 4°C, PMNs were incubated for 30 min at 4°C with 7DS or mouse serum. The cells were then washed with ice-cold KRPB and incubated for a further 30 min at 4°C with FITC-conjugated goat anti-mouse IgG. Labeled cells were washed in KRPB and analyzed for fluorescence intensity by flow cytometry.

Degranulation—Degranulation was assessed by measuring the release of lysozyme from specific and gelatinase granules. PMNs (106/ml) were incubated with TNFα (200 units/ml), PMA (100 ng/ml), or KRPB (control) for 10 min at 37°C. The lysozyme content of the supernatant was determined by measuring the rate of lysis of Micrococcus lysodeikticus using a spectrophotometric assay (40). Release of lysozyme was expressed as a percent of total cell content, determined by lysing cells with 1% Triton X-100.

Statistical Analysis—Differences in H2O2 production and plasma membrane protein expression in the presence or absence of TNFα, LPS, and MAPK inhibitors were determined by analysis of variance. Where significant differences were detected in the primary analysis, Tukey’s test was used to determine differences between the individual priming agents and control and between the individual inhibitors. All statistical analyses were performed using SPSS for Windows (version 10, SPSS Inc, Chicago, IL). Differences were considered significant at p < 0.05. Data are presented as mean ± S.E. for n observations.

RESULTS

Inhibition of p38 MAPK, but Not ERK, Reduces TNFα and LPS Priming of Phagocytosis-stimulated Respiratory Burst Activity—We have demonstrated previously that preincubation of PMNs for 60 min with 10 μM SB203580 or 50 μM PD98059 significantly inhibits the ability of TNFα and GM-CSF to prime fMLP-stimulated superoxide production in isolated PMNs (28). It is not known if this effect of MAPK inhibition extends to priming of respiratory burst activity stimulated by phagocytosis. Pharmacologic inhibition of p38 MAPK and ERK cascades on TNFα and LPS priming of respiratory burst activity stimulated by the phagoctyosis of S. aureus. Incubation with either 200 units/ml TNFα for 10 min (Fig. 1A) or 100 ng/ml LPS for 60 min (Fig. 1B) significantly increased H2O2 production on subsequent exposure to S. aureus, compared with control. Preincubation with the ERK in-
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Phagocytosis was slightly, but significantly, increased in the presence of TNFα stimulated by pretreatment with TNFα priming. Phagocytosis was significantly increased when PMNs were incubated with 10 μM SB203580 or PD098059 (data not shown).

DeLeo and colleagues (3) have previously shown that LPS increases plasma membrane expression of the KRPB control (*, p < 0.05), but not in the TNFα experiments. Results are presented as mean ± S.E. for 14 experiments with TNFα and 6 experiments with LPS.

Fig. 1. Effect of TNFα (A) and LPS (B) on phagocytosis-stimulated H2O2 production in the presence and absence of inhibitors of p38 MAPK (SB203580) and ERK (PD098059). PMNs (2 × 10⁷/ml) were incubated with 10 μM SB203580, 50 μM PD098059 or MeSO for 60 min at 37°C before the addition of 200 units/ml TNFα for a further 10 min or 100 ng/ml LPS for a further 60 min. S. aureus-stimulated H2O2 production was then measured. Incubation with both TNFα and LPS significantly increased H2O2 production compared with the KRPB control (*, p < 0.05). Preincubation with SB203580 or SB203580 + PD098059 (#, p < 0.05) significantly inhibited the ability of TNFα to increase H2O2 production, whereas preincubation with PD098059, alone, had no effect. Preincubation with PD098059 increased H2O2 production in the absence of the priming agent in the LPS experiments (#, p < 0.05), but not in the TNFα experiments. Results are presented as mean ± S.E. for 14 experiments with TNFα and 6 experiments with LPS.

PMNs store flavocytochrome b₅₅₈ in specific granules, gelatinase granules and secretory vesicles (41). The increased flavocytochrome b₅₅₈ observed in the plasma membrane following exposure to TNFα is likely to derive from exocytosis of one or more of these granules. CD35 and CD66b are specific membrane markers of secretory vesicles and specific granules, respectively (41). We used flow cytometry and specific antibodies to examine the effect of TNFα and LPS on plasma membrane expression of CD35 and CD66b and to determine the role of MAPK modules on this process. The data in Table I show that 200 units/ml TNFα for 10 min and 100 ng/ml LPS for 60 min significantly increased the expression of both CD35 and CD66b. Thus, both priming agents stimulate exocytosis of secretory vesicles and specific granules. The data in Table I also show the effect of pretreatment with 10 μM SB203580 and 50 μM PD098059 on TNFα- and LPS-stimulated increases in CD35 and CD66b expression. Pretreatment with SB203580 significantly inhibited the increase in CD35 and CD66b expression stimulated by TNFα and LPS; although pretreatment with PD098059 had no effect. These data indicate that priming by LPS and TNFα is associated with p38 MAPK-dependent exocytosis of granules containing flavocytochrome b₅₅₈.

SB203580 has been reported to have effects on kinases other than p38 MAPK at concentrations above 3 μM (42–44). Therefore, we examined the concentration-dependent inhibition of TNFα-mediated priming. The effect of SB203580 on priming of respiratory burst activity by TNFα was concentration-dependent, with significant inhibition being achieved at concentrations of 1 μM or higher (Fig. 2A). Furthermore, the effect of SB203580 on the TNFα-induced increase in CD35 expression (Fig. 2B) showed a similar concentration-inhibition profile to that observed for TNFα-induced increase in respiratory burst activity.

If the exocytosis-mediated up-regulation of flavocytochrome b₅₅₈ plays a role in PMN priming, the time course and dose response of TNFα- and LPS-induced exocytosis and priming of the respiratory burst should be the same. Experiments comparing CD35 and CD66b expression and H2O2 production at various concentrations of TNFα and LPS are shown in Fig. 3. Up-regulation of CD35 and CD66b and increased H2O2 production were evident between 1 and 10 units/ml TNFα and were maximal at 100 units/ml (Fig. 3A). Similarly, up-regulation of CD35 and CD66b expression and enhanced H2O2 production were observed between 1 and 10 ng/ml LPS and continued to increase through the highest concentration studied, 1000 ng/ml (Fig. 3B). The time courses of priming and exocytosis by 200 units/ml TNFα and 100 ng/ml LPS are shown in Figs. 4 and 5. TNFα stimulated a demonstrable increase in CD35 and CD66b expression by 5 min that was essentially maximal by 10 min (Fig. 4A). The ability of TNFα to enhance H2O2 production was maximal by 10 min (Fig. 4B). The respiratory burst assay methodology precluded incubation times with TNFα of less than 10 min. LPS stimulated an increase in CD35 and CD66b by 30 min that was maximal by 60 min (Fig. 5A). Priming of the respiratory burst by LPS followed the same time course (Fig. 5B). Thus, both LPS and TNFα stimulate priming of the respiratory burst with the same time course and concentration dependence observed for exocytosis of secretory vesicles and specific granules, resulting in increased expression of the flavocytochrome b₅₅₈.
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PMNs (2 x 10^6/ml) were incubated with 10 μM SB203580, 50 μM PD098059, or MeSO vehicle for 60 min at 37 °C before the addition of 200 units/ml TNFα for an additional 10 min or 100 ng/ml of LPS for an additional 60 min. Plasma membrane expression of flavocytochrome b_{558}, CD35, or CD66b was then determined by flow cytometry. Incubation with TNFα and LPS significantly increased plasma membrane expression of flavocytochrome b_{558}, CD35, and CD66b. The TNFα- and LPS-induced increases in membrane protein expression were significantly attenuated by preincubation with SB203580 but not by preincubation with PD098059. Results are presented as mean ± S.E. for n experiments.

|                  | n     | MeSO         | SB203580 (10 μM) | PD098059 (50 μM) |
|------------------|-------|--------------|------------------|------------------|
| Flavocytochrome b_{558} (Ln(mcf)) | (9)   | 3.2 ± 0.4    | 3.0 ± 0.3        | 4.1 ± 0.4        |
|                  |       | +TNFα        |                  |                  |
|                  | (10)  | 3.2 ± 0.3    | 3.2 ± 0.3        | 3.4 ± 0.4        |
|                  |       | +LPS         |                  |                  |
| CD35 (mcf)       |       |              |                  |                  |
|                  | (9)   | 393 ± 20     | 388 ± 23         | 394 ± 23         |
|                  |       | +TNFα        |                  |                  |
|                  | (6)   | 393 ± 20     | 388 ± 23         | 394 ± 23         |
|                  |       | +LPS         |                  |                  |
| CD66b (mcf)      |       |              |                  |                  |
|                  | (9)   | 351 ± 25     | 322 ± 27         | 386 ± 26         |
|                  |       | +TNFα        |                  |                  |
|                  | (7)   | 329 ± 20     | 297 ± 21         | 365 ± 20         |
|                  |       | +LPS         |                  |                  |

*p < 0.05, +TNFα versus −TNFα or +LPS versus −LPS.

*p < 0.05, SB203580 versus MeSO.

**TABLE I**

*Effect of TNFα and LPS on plasma membrane expression of flavocytochrome b_{558}, CD35, and CD66b in the presence and absence of SB203580 and PD098059.*

**FIG. 2.** Concentration response of SB203580 inhibition of TNFα-induced priming of H₂O₂ production (A) and increase in plasma membrane CD35 expression (B). PMNs (2 x 10^6/ml) were incubated with varying concentrations of SB203580 for 60 min at 37 °C before the addition of 200 units/ml TNFα or KRPB control for a further 10 min. S. aureus-stimulated H₂O₂ production and CD35 expression were then measured. Results are presented as mean ± S.E. for 3 experiments in each panel.

**FIG. 3.** Increase in H₂O₂ production (A) and plasma membrane expression of CD35 (B) and CD66b (C) induced by varying concentrations of TNFα (A) and LPS (B). PMNs (2 x 10^6/ml) were incubated with the indicated concentrations of TNFα for 10 min, or LPS for 60 min, at 37 °C. S. aureus-stimulated H₂O₂ production and CD35 and CD66b expression were then measured. Results are presented as mean ± S.E. for 2–4 experiments.

Time Required to Prime the Respiratory Burst—To determine if granule exocytosis stimulated by TNFα led to measurable release of degradative enzymes, the release of lysozyme, which is contained in specific granules, gelatinase granules, and primary granules, was measured. There was no measurable release of lysozyme from PMNs incubated for 10 min with 200 units/ml TNFα, with only 0.2 ± 0.3% of total cellular lysozyme recovered in the supernatant, compared with 11 ± 4% when the cells were stimulated with 10 ng/ml PMA for 10 min.

TNFα Does Not Prime the Respiratory Burst or Up-regulate CD35 and CD66b Expression in Enucleated PMN Cytoplasts—Enucleated PMN cytoplasts consist of cytoplasm surrounded by plasma membrane. Although some fusion between granules and plasma membrane occurs during cytoplast preparation (45), cytoplasts are essentially devoid of intracellular granules, and they have been used successfully to assess the role of
degranulation in specific cellular responses (46, 47). Therefore, we used enucleated PMN cytoplasts to determine if intracellular granules were necessary for priming of the respiratory burst by TNFα. Incubation with 200 units/ml TNFα for 10 min failed to prime the production of superoxide stimulated by 10⁻⁷ M fMLP (Table II). Similar results were obtained when cytoplasts were stimulated by phagocytosis, although both phagocytosis and H₂O₂ production were less than observed in intact PMNs (data not shown). To confirm that cytoplasts do not contain vesicles, incubation of cytoplasts with TNFα under the same conditions used for intact PMNs failed to increase membrane expression of CD35 (Table II). Taken together, these data suggest that intracellular storage granules are necessary for priming of PMN respiratory burst activity by TNFα.

**DISCUSSION**

The molecular mechanisms through which the respiratory burst of PMNs is primed remain unknown. To produce a respiratory burst, PMNs must assemble NADPH oxidase units in the plasma or phagolysosomal membrane. The NADPH oxidase assembles on flavocytochrome b₅₅₈, which is expressed at low levels in the plasma membranes of resting cells. DeLeo and colleagues (3) reported that exposure of PMNs to LPS leads to an increase in the amount of flavocytochrome b₅₅₈ expressed in the plasma membrane from 25% to about 40% of total cellular content, suggesting that redistribution of this NADPH oxidase component is one mechanism by which the respiratory burst is primed. The results of the present study extend the observations of DeLeo et al. (3) to a second priming agent, TNFα, and provide further evidence that translocation of flavocytochrome b₅₅₈ to the plasma membrane is one mechanism by which PMN priming occurs.

**Intracellular stores of flavocytochrome b₅₅₈** are found in the membranes of secretory vesicles, gelatinase granules, and specific granules. The results of our experiments support a role for secretory vesicles and specific and gelatinase granules in priming of the respiratory burst. First, TNFα and LPS stimulate increased plasma membrane expression of CD35 and CD66b, markers of secretory vesicles and specific granules, at the same times and with the same concentrations that stimulate priming (Figs. 3-5). Second, inhibition of exocytosis by SB203580 results in attenuation of the priming effect of both TNFα and LPS. Finally, a role for exocytosis in priming is supported by observations in cytoplasts, which are devoid of vesicles and granules. Our data demonstrate that TNFα does not stimulate an increase in cytoplast CD35 expression, and cytoplasts cannot be primed by TNFα. These results suggest that priming of the respiratory burst involves recruitment of flavocytochrome b₅₅₈ to the plasma membrane by exocytosis of intracellular granules.

A role for exocytosis in respiratory burst priming is sup-

**FIG. 4.** Time course of TNFα-induced increase in plasma membrane expression of CD35 and CD66b (A) and priming of H₂O₂ production (B). PMNs (2 × 10⁶/ml) were incubated with 200 units/ml TNFα (● CD35, ■ CD66b) or KRPB control (○ CD35, □ CD66b) at 37 °C for varying times up to 30 min. Plasma membrane expression of CD35 and CD66b and S. aureus-stimulated H₂O₂ production were then measured. Results are presented as mean ± S.E. for 2-5 experiments (error bars are omitted from the control data).

**TABLE II**

| Condition          | CD35 Expression (mcf) | Superoxide (nmol/10⁶ cells/10 min) |
|--------------------|-----------------------|-----------------------------------|
| −TNFα              | 272 ± 48              | 0.9 ± 0.5                         |
| +TNFα              | 277 ± 46              | 0.9 ± 0.5                         |

These results suggest that priming of the respiratory burst involves recruitment of flavocytochrome b₅₅₈ to the plasma membrane by exocytosis of intracellular granules.

A role for exocytosis in respiratory burst priming is sup-
ported by the results of several previous studies. Our data for CD35 that was significant after 10 min of incubation. Borregaard et al. (49) found that TNFα increased membrane expression of CD11b, which is contained in specific and gelatinase granules and secretory vesicles, within 15 min. TNFα has also been demonstrated to cause release from PMNs of gelatinase (50) and lysozyme (51, 52), markers of specific and gelatinase granules (41). In the present study, no release of lysozyme was detected after a 10-min incubation with TNFα, an observation also made by Bajaj et al. (53). Those studies that reported significant lysozyme release used incubation times of 30–60 min (51, 52). Thus, sufficient exocytosis to produce measurable release of granular enzymes appears to require a longer time course than that required for priming of the respiratory burst. These data suggest that the initial increase in flavocytochrome b₅₅₈ results primarily from exocytosis of secretory vesicles. Finally, our data demonstrating that cytotoxic proteins cannot be primed with TNFα are consistent with the report of Mege et al. (47) showing that GM-CSF primes fMLP-stimulated superoxide production in intact PMNs but not in cytotoxins.

Both the present study and the report of DeLeo et al. (3) indicate that TNFα and LPS increase the plasma membrane content of flavocytochrome b₅₅₈ by 50–100%. This increase in plasma membrane expression of flavocytochrome b₅₅₈ would be expected to result in a comparable increase in the number of NADPH oxidase units assembled following translocation of the other subunits of the oxidase from the cytosol. However, DeLeo et al. (3) found a 3–5-fold increase in translocation of p47Phox, p67Phox, and Rac2 following fMLP stimulation of LPS-primed PMNs, compared with unprimed cells. Additionally, TNFα and LPS induce a 3–10-fold increase in respiratory burst activity. Thus, it is unlikely that changes in membrane expression of components of flavocytochrome b₅₅₈ fully explain the increase in translocation of cytosolic components or priming of respiratory burst activity.

We have shown previously that inhibition of p38 MAPK by SB203580 markedly attenuates the ability of TNFα to prime respiratory burst activity stimulated by fMLP (28). The results of the present study show a similar effect of SB203580 on the ability of TNFα and LPS to prime the respiratory burst triggered by bacterial phagocytosis. Although TNFα and LPS have been shown previously to activate the p38 MAPK module in human PMNs (18, 24, 28, 31, 32), the mechanism by which p38 MAPK mediates priming is not known. Previous studies showed that inhibition of p38 MAPK attenuated respiratory burst activity by unprimed PMNs stimulated by fMLP or phagocytosis (21, 22, 26). Thus, the effect of SB203580 on priming could result from inhibition of respiratory burst activity independent of the actions of TNFα or LPS. However, our data show that SB203580 had no effect on phagocytosis-stimulated H₂O₂ production in unprimed PMNs (Fig. 1). The finding that similar concentrations of SB203580 inhibit priming of the respiratory burst and the increase in flavocytochrome b₅₅₈, CD35, and CD66b in the plasma membrane suggests that p38 MAPK regulates exocytosis of secretory vesicles and specific and gelatinase granules. That p38 MAPK also plays a crucial role in exocytosis of chemoattractant-stimulated exocytosis. Exocytosis of vesicles and granules proceeds by a series of steps, including release from the cytoskeleton, migration to the plasma membrane, and fusion with the plasma membrane. Many details of this process have not been elucidated in PMNs, and the steps regulated by p38 MAPK remain to be determined.

We have shown previously that inhibition of ERK activity with PD98059 attenuated TNFα- and GM-CSF-mediated priming of respiratory burst activity (28). In the present study, however, inhibition of ERK activity had no effect on TNFα or LPS priming of respiratory burst activity or exocytosis. The reasons for the disparate effects of ERK inhibition are currently unknown. The chemotactic peptide, fMLP, was used to stimulate respiratory burst activity in the previous report, whereas bacterial phagocytosis was used in the present study. Additionally, respiratory burst was measured by extracellular release of superoxide in our previous study, whereas intracellular production of H₂O₂ was used in the present study.

Four isoforms of p38 MAPK have been identified, p38α, p38β, p38γ, and p38δ (27). Of these, only p38α and p38δ are expressed in human PMNs (57, 58). SB203580, in common with other pyridinyl imidazoles, acts as a competitive inhibitor of p38α and p38β activity through binding to the ATP site with an IC₅₀ of about 0.6 µM (59–62). Although reported to have minimal effects on other kinases and phosphatases (59), pyridinyl imidazoles have been shown recently to inhibit JNK activity and phoshoinositide-dependent protein kinase 1 with an IC₅₀ greater than 3 µM (42, 43, 44). Thus, similar to other pharmacologic inhibitors, the specificity of SB203580 could limit interpretation of our data. The demonstration in the present study that SB203580 significantly inhibits priming of respiratory burst activity and up-regulation of CD35 expression at concentrations of 1 µM (Fig. 2), however, suggests that p38 MAPK mediates these activities. Coupled with previous data, our results indicated that p38α mediates TNFα- and LPS-stimulated exocytosis of secretory vesicles and specific granules leading to priming of the respiratory burst. Our data do not exclude other mechanisms by which p38 MAPK regulates priming of respiratory burst activity. Additionally, the observation that inhibition of p38 MAPK does not completely block priming suggests that p38 MAPK-independent mechanisms of priming exist.

Our findings expand the functional consequences of p38 MAPK in human PMNs. Previous studies showed that p38 MAPK participated in adherence, chemotaxis, respiratory burst activity, apoptosis, transcription factor activation, and gene expression (21, 24, 26, 57, 63). The present study indicates that p38 MAPK also plays a crucial role in exocytosis of intracellular granules resulting in increased plasma membrane expression of a number of important molecules. In addition to enhancing respiratory burst capability through increased expression of flavocytochrome b₅₅₈, our results provide a mechanism for the up-regulation of chemoattractant receptors, adhesion molecules, and G protein expression previously reported to accompany priming (6, 11–17).

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