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Role of Stress-Activated Mitogen-Activated Protein Kinase (p38) in \(\beta_2\)-Integrin-Dependent Neutrophil Adhesion and the Adhesion-Dependent Oxidative Burst\(^1\)

Patricia A. Detmers,\(^2\,*\)† Dahua Zhou,* Elizabeth Polizzi,† Rolf Thieringer,† William A. Hanlon,† Sanskruti Vaidya,† and Vinay Bansal‡

Bacterial LPS elicits both rapid activation of the stress-activated MAP kinase p38 in polymorphonuclear leukocytes (PMN) and rapid adhesion of the PMN to ligands for the leukocyte integrin CD11b/CD18. The functional correlation between these two events was examined. The time course for tyrosine phosphorylation of p38 in PMN in response to 10 ng/ml LPS in 1% normal human serum was consistent with participation in signaling for leukocyte integrin-dependent adhesion, with transient phosphorylation peaking at 10 to 20 min. The concentration dependence of p38 phosphorylation also resembled that for PMN adhesion, with <1 ng/ml LPS eliciting a response. Phosphorylation was inhibited by mAb 60b against CD14, but not by mAb 26ic, a nonblocking anti-CD14. The function of p38 in integrin-dependent adhesion and the adhesion-dependent oxidative burst was tested using a specific inhibitor of p38, SB203580. SB203580 inhibited adhesion by diminishing the initial rate of adherence in response to both LPS and TNF, with a half-maximal concentration in the range of 0.1 to 0.6 \(\mu\)M. It did not, however, block adhesion in response to formyl peptide or PMA. The p38 inhibitor also blocked the adhesion-dependent oxidative burst with a half-maximal concentration similar to that for adhesion. Timed delivery of the compound during the lag phase preceding \(\text{H}_2\text{O}_2\) production suggested that p38 kinase activity was required throughout the lag but not after the oxidase was assembled. These results suggest that p38 functions in PMN to signal leukocyte integrin-dependent adhesion and the subsequent massive production of reactive oxygen intermediates. *The Journal of Immunology, 1998, 161: 1921–1929.*

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\(^{3}\)Abbreviations used in this paper: PMN, polymorphonuclear leukocytes; ROI, reactive oxygen intermediates; mCD14, membrane CD14; scCD14, recombinant soluble human CD14; PI3K, phosphatidylinositol 3-kinase; INLLP, formyl-norleucyl-leucyl-phenylalanine; HRP, horseradish peroxidase; PD, cation-deficient Dulbecco’s PBS; HSA, human serum albumin; HAP, Dulbecco’s PBS with 0.5 mg/ml human serum albumin, 0.3 U/ml aprotinin, and 3 mM glucose; KRPG, Krebs-Ringer phosphate buffer with 5.5 mM glucose MAPKAP, mitogen-activated protein kinase-activated protein; hsp27, heat shock protein-27; LSP1, lymphocyte-specific protein-1.

Adhesion and production of ROI are interrelated, since adhesion is a prerequisite for a large oxidative burst in response to cytokines (4). A distinctive feature of this response is a significant lag period (20–60 min) between the addition of agonist and the beginning of ROI production. During this time PMN adhere to the ligand-coated substrate and spread. The adhesion-dependent oxidative burst requires ligation of leukocyte integrins, as demonstrated by blockade of the response by Abs against CD18 and failure of cells from leukocyte adhesion-deficient patients to respond (5). The participation of the microfilamentous cytoskeleton is also necessary, since dihydrocytochalasin B interferes with both cell spreading and ROI production (4).

Low concentrations (<1 ng/ml) of bacterial LPS act through the glycosylphosphatidylinositol anchored protein CD14 on the plasma membrane of PMN (mCD14) to directly elicit leukocyte integrin-dependent adhesion (6). In serum, LPS is delivered to mCD14 through the actions of the transfer protein, LPS binding protein, and the shuttle protein, soluble CD14 (scCD14) (7). The requirement for LPS binding protein to deliver LPS to mCD14 can be circumvented by forming complexes of LPS with scCD14. These complexes have a ratio of 1 to 2 LPS per scCD14 and are capable of stimulating leukocytes in an mCD14-dependent fashion (8). Adhesion in response to LPS exhibits an initial 10- to 15-min lag period before onset, during which time internalization of LPS may be required to initiate signaling for adhesion (9). Phosphatidylinositol 3-kinase (PI3K) is rapidly activated by LPS in PMN, and the PI3K inhibitors, wortmannin and LY294002, block PMN adhesion to fibrinogen in response to LPS, suggesting that PI3K activity is required at an early stage of signaling (9). However, the identity of other signaling components in this pathway remains to be elucidated.

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The stress-activated MAP kinase p38 has been identified as participating in the LPS-mediated production of cytokines by monocytes (10). LPS stimulation leads to phosphorylation and activation of p38 (11, 12), and specific inhibitors of p38 abrogate cytokine production in response to LPS (13, 14).

PMN also phosphorylate and activate p38 in response to LPS (15), suggesting that LPS signaling pathways are similar in PMN and monocytes. Here we present evidence that p38 is phosphorylated in PMN with a time course and concentration dependence similar to those for LPS-stimulated cell adhesion and that a specific inhibitor of p38 kinase activity blocks LPS-stimulated PMN adhesion and the adhesion-dependent oxidative burst.

**Materials and Methods**

**Reagents and mAbs**

Formyl-norleucyl-leucyl-phenylalanine (fNLLP), apotinin, PMA, diisopropyl fluorophosphate, PMSF, scopeoitin, horseradish peroxidase (HRP), Dulbecco’s PBS, and cation-deficient Dulbecco’s PBS (PD) were purchased from Sigma (St. Louis, MO). PBS was obtained from HyClone (Logan, UT). Pyrogen-free human serum albumin (HSA) was purchased from Centent, Armour & Berring (Kankakee, IL). SB203580, antipain, leupeptin, benzamidine, chymostatin, pepstatin A, and human fibrogen were purchased from Calbiochem-Novabiochem (La Jolla, CA). Monoclonal anti-phosphotyrosine, anti-p38, anti-Erk-1, and anti-Erk-2 Abs were obtained from Upstate Biotechnology (Lake Placid, NY). Abs against CD14 (1b9 and 24), (16), were purified from ascites by chromatography on protein A-Sepharose (Sigma). LPS Ra (R60) from *Salmonella minnesota* was obtained from List Biologics (Campbell, CA). TNF-α (recombinant, human) was purchased from Genzyme (Cambridge, MA). Complexes of monomeric LPS and recombinant soluble human CD14 (sCD14), expressed and purified as described below, were made by incubating 5 μg/ml LPS with 500 μg/ml sCD14 for 16 h at 37°C. These were conditions sufficient to assure that all the LPS was bound by sCD14 (7).

**Expression and purification of recombinant human sCD14**

The cDNA encoding the complete open reading frame for human CD14 (17) was cloned into the multiple cloning site adjacent to the metallothionein promoter of expression vector pRmHa3 (18), resulting in the construct pRmHa3-hCD14. Schneider-2 insect cells, 2 × 10^6 in 100 ml T75 tissue culture flasks (19), were cotransfected with 10 μg of salmon sperm DNA, 9 μg of pRmHa3-hCD14, and pcDNA3 (20) using the calcium phosphate transfection method with a commercially available kit as outlined by the manufacturer (Life Technologies, Gaithersburg, MD). After 48-h growth in Schneider medium containing 1 mM CuSO_4, CD14 expression on the cell surface was documented by flow cytometry using FITC-labeled anti-CD14 (clone MY4; Coulter, Hialeah, FL; data not shown). Soluble CD14 secreted into the medium was detected as the dominant band at 45 to 48 kDa in SDS-PAGE and by Western blotting with a polyclonal anti-CD14. Maximal expression was obtained after 3-day growth at 27°C, yielding approximately 5 mg/l of sCD14.

Soluble CD14 was purified from the conditioned medium by a simple three-step procedure. Briefly, protein was precipitated by addition of ammonium sulfate to a final concentration of 60% (w/v). The pellet was dissolved in 0.25% PBS (BioWhittaker, Walkersville, MD) and 1 mM EDTA (buffer A), with 1 mM PMSF and 10 mM HEPES, pH 7.3, and then diazylated against buffer A with 1 mM PMSF. The dialyzed sample was loaded on a MonoQ column (Pharmacia Biotech, Piscataway, NJ) equilibrated in buffer A with 0.42 mM Pefabloc SC (Boehringer Mannheim, Indianapolis, IN). Protein was eluted with a continuous gradient of 38 to 300 mM NaCl in buffer A with Pefabloc SC. Soluble CD14 was identified in the fractions by SDS-PAGE and Western blotting, as described above. Fractions containing scCD14 were pooled, diluted fivefold in buffer B (25 mM 2-[N-morpholino]ethanesulfonic acids (pH 5.8) and 0.42 mM Pefabloc SC, and loaded on a HiTrap SP column (Pharmacia Biotech) equilibrated in buffer B. Protein was eluted from the column by a continuous gradient of 25 to 150 mM NaCl. Fractions containing scCD14 were pooled and dialyzed against PD. All purification steps were performed at 4°C. Soluble CD14 obtained by this protocol was at least 95% pure as judged by SDS-PAGE. Recombinant sCD14 appeared as a mixture of differently glycosylated forms between 45 and 48 kDa, similar to the pattern described by Haziot et al. (21).

All reagents and solutions used for purification of sCD14 were of cell culture quality, if available. Endotoxin contamination of the pre-purification LPS was less than 0.3 ng LPS/mg CD14. The colorimetric *Limulus* amebocyte lysate assay (QCL-1000, BioWhittaker, Walkersville, MD) using RelPS from *Salmonella minnesota* (List Biologics) as the standard. The identity and activity of sCD14 were confirmed by numerous assays, including immunoabs, LPS transfer assays with boron dipyrromethene-labeled LPS as previously described (22), and various cell-based (this manuscript and data not shown).

**Cell preparation**

PMN were prepared from freshly drawn human blood on Neutrophil Isolation Medium (Cardinal Associates, Sante Fe, NM) exactly as previously described (23). Contaminating erythrocytes were removed by hypotonic lysis, and PMN were suspended in Dulbecco’s PBS with 0.5 mg/ml HSA, 0.3 U/ml aprotinin, and 3 mM glucose (HAP buffer) for adhesion assays or in Krebs-Ringer phosphate buffer with 5.5 mM glucose (pH 7.35; KRPG) for assays of the oxidative burst.

**Electrophoresis and Western blotting of PMN lysates**

For each condition, 1 ml of PMN at 5 × 10⁶ cells/ml was used. At the end of the experimental treatment, the cells were washed twice with Dulbecco’s PBS with 0.5 mM Na₃VO₃, 0.5 mM Na₃VO₄, and 3 mM di-isopropyl fluorophosphate on ice. They were lysed by incubation on ice for 20 min in 0.1 ml 100 mM Tris-HCl (pH 8.0), 100 mM NaCl, 2 mM EDTA, 1% Nonidet P-40, 1 mM Na₃VO₄, 50 mM NaF, 0.3 U/ml aprotinin, 2 mM PMSF, 50 μg/ml benzamidine, and 5 μg/ml each of antipain, leupeptin, chymostatin, and pepstatin A. Lysates were centrifuged for 5 min at 12,000 × g, and the supernatants were prepared for SDS-PAGE under reducing conditions.

SDS-PAGE was run on 10% gels (Novex, San Diego, CA) using standard Tris-glycine buffers. Transfer of proteins to nitrocellulose was accomplished in 1.5 h at 300 mA. The filters were blocked overnight with PBS and 1% nonfat dry milk and washed twice with PBS before sequential staining with primary Ab in PBS and 0.1% dry milk (1 μg/ml anti-phosphotyrosine, 0.2 μg/ml anti-p38, anti-Erk-1, and anti-Erk-2) for 2 h, two washes with PBS, and HRP-conjugated goat anti-mouse IgG diluted 1/100 in PBS and 0.1% dry milk for 2 h. Bound Ab was detected with an ECL kit (Amersham, Arlington Heights, IL) according to the manufacturer’s directions.

**Assay for MAPKAP kinase activity**

Lysates of PMN, made as described above, were diluted 10-fold in 50 mM HEPES (pH 7.6), 100 mM NaCl, 1% Triton X-100, 2 mM EDTA, 5 μM pyrophosphate, 0.5 μM okadaic acid, 1 mM Na₃VO₄ and 10× protease Inhibitor Mixture (Calbiochem, 539131). A 21-μl aliquot of diluted cell lysate was mixed with 3 μl of 250 mM HEPES (pH 7.6), 200 mM MgCl₂, 1 mM Na₃VO₄, 20 mM DTT, 200 mM β-glycerol phosphate, and 50 mM NaF for 10 min at ambient temperature. To this, 6 μl of 125 μg/ml recombinant human hsp27 (StressGen Biotechnologies, Victoria, Canada), 100 μM ATP, and 1 μCi/ml [γ-³²P]ATP were added. The reaction mixture was mixed gently and incubated at ambient temperature for 30 min. The reaction was stopped by adding 15 μl of 3× SDS-PAGE gel sample buffer to the reaction and boiling for 3 min. Phosphorylated hsp27 was resolved by SDS-PAGE (12%), and the gel was dried and subjected to phosphorimager analysis for quantitation.

**Assay for inhibition of cellular adhesion**

Adhesion of PMN to fibrinogen-coated Terasaki plates was performed exactly as previously described (25). Briefly, PMN were labeled with the fluorescent dye carboxyfluorescein diacetate succinimidyl ester (Molecular Probes, Eugene, OR), and 10⁶ cells were added to each fibrinogen-coated Terasaki well. After addition of inhibitor, the plates were incubated for 10 min at 37°C before addition of agonists and continued incubation at 37°C. Adhesion was quantitated by measuring the fluorescence in each well before and after washing in a Cytofluor 2300 (PerSeptive Biosystems, Framingham, MA). Percent adhesion was calculated as: (fluorescence after washing/fluorescence before washing) × 100. Samples for each condition were run in triplicate, and the data are presented as the mean ± SD. Experiments representative of at least three repetitions are shown.
Adhesion-dependent oxidative burst

Oxidative burst was measured using the previously described assay of HRP-catalyzed oxidation of scopoletin by H$_2$O$_2$ (24). Polystyrene Primaria 96-well tissue culture plates (Becton Dickinson, Lincoln Park, NJ) were coated with FBS for 30 min at 37°C. Each well was then washed vigorously three times by forceful squirting of 3 ml of 0.9% NaCl to prevent blockade of the reaction by soluble BSA (26). To each well was added 50 μl scopoletin (39 μM in KRPG), 10 μl HRP (0.5 U/ml), 13 μl NaN$_3$ (1 mM), and 10 μl of inhibitor with or without agonist (TNF, fNLLP, LPS/sCD14 complexes, or PMA). The reaction was started by adding 3 × 10$^4$ PMN/well in 20 μl. The plates were maintained at 37°C, and the scopoletin fluorescence was read every 10 min at an excitation of 360 nm and an emission of 460 nm in a Cytofluor 4000 fluorescence plate reader equipped with a temperature control device (PerSeptive Biosystems). Samples were read in quadruplicate, and the data are presented as mean ± SD for a representative experiment of at least three performed with the same results.

Results

Tyrosine phosphorylation of p38 occurs in PMN in response to LPS with a time course and concentration dependence similar to those for PMN adhesion

Dual phosphorylation of p38 on tyrosine and threonine confers enhanced enzymatic activity (27). We confirmed reports that p38 is tyrosine phosphorylated in response to LPS under conditions that stimulate PMN adhesion. Tyrosine phosphorylation of p38 was tested after treatment of PMN with 10 ng/ml LPS with 1% serum in the continued presence of Abs. At the end of the incubation, the cells were washed and lysed, and Western blots of lysates were probed with anti-phosphotyrosine. The m.w. standards are indicated by arrowheads. The data shown are representative of experiments repeated twice with identical results.
phosphorylation and MAPKAP kinase activity (Fig. 2B). Like MAPKAP kinase activity, adhesion could be detected at concentrations of LPS below those that produced a visible band of tyrosine phosphorylation on p38, suggesting that the Western blot assay was less sensitive than the enzymatic and cell-based assays. p38 was also phosphorylated on tyrosine with a time course consistent with its participating in LPS-mediated signaling for PMN adhesion. PMN exposed to 10 ng/ml LPS with 1% serum for increasing times at 37°C showed a clear increase in tyrosine phosphorylation of p38 by 1 to 5 min, that reached a maximum by 20 min and declined by 40 min (Fig. 3). The peak activity of MAPKAP kinase lagged 20 min behind the peak for p38 phosphorylation and declined after 40 min (Fig. 4A). Adhesion in response to LPS exhibits at least a 10-min lag before onset and declines after 40 min (9) (Fig. 6A). Tyrosine phosphorylation of p38 was therefore apparent before adhesion was observed, and both dephosphorylation of p38 and the decline in MAPKAP kinase activity commenced before or contemporaneously with the decline in adhesion. These kinetics suggest that activation of p38 and its downstream effector MAPKAP kinase could participate in signaling for adhesion. Additional studies with an inhibitor of p38 were conducted to test this hypothesis for LPS and other agonists of PMN function.

An inhibitor of p38 blocks adhesion of PMN to fibrinogen in response to LPS and TNF

The compound SB203580 is reported to be a specific inhibitor of p38 kinase activity (30). It blocked LPS-stimulated MAPKAP kinase activity in PMN by about 50% at concentrations of 0.3 μM and above (Fig. 4B), demonstrating its activity on p38 under our experimental conditions. SB203580 was therefore used to test whether p38 functions in β2-integrin-dependent PMN adhesion in response to LPS, TNF, fNLLP, or PMA. Fibrinogen is a ligand for the leukocyte integrin CD11b/CD18 (31) and was therefore chosen as an appropriate substrate for the assay.

PMN were preincubated for 10 min at 37°C with increasing concentrations of SB203580 before addition of agonists for 15 to 20 min at 37°C and subsequent measurement of adhesion. SB203580 blocked PMN adhesion to fibrinogen in response to LPS in a concentration-dependent fashion, with a concentration of about 0.1 μM having a half-maximal effect (Fig. 5A). In the same experiment, SB203580 inhibited adhesion in response to TNF by almost 50% at a concentration of 0.6 μM and above (Fig. 5B). This is consistent with the reported IC₅₀ for SB203580 of about 0.6 μM for the inhibition of p38 activity in vitro and 1 μM for IL-6 production in response to TNF by L929 cells (30, 32). It is also consistent with the concentration dependence observed for SB203580 inhibition of MAPKAP kinase activity in LPS-stimulated PMN (Fig. 4B). For neither MAPKAP kinase activity nor adhesion in response to LPS or TNF was complete inhibition by SB203580 observed. However, MAPKAP kinase activity was inhibited to the same extent as adhesion, suggesting that the effect of SB203580 on adhesion was through its inhibition of p38. The compound inhibited adhesion in response to fNLLP by only about 20% and failed to inhibit adhesion in response to PMA at concentrations up to 2.4
An inhibitor of p38 kinase activity blocked adhesion in response to LPS or TNF but not to fNLLP or PMA. PMN were incubated with the indicated concentrations of SB203580 for 10 min at 37°C (LPS/CD14 complexes (25 ng/ml LPS; A) were added for 20 min, TNF (15 ng/ml; B) was added for 15 min, fNLLP (10^{-7} M; C) was added for 15 min, or PMA (30 ng/ml; D) was added for 20 min at 37°C, and adhesion was measured as described in Materials and Methods. HAP buffer (clear bars) was used for unstimulated controls for each agonist. Data shown are representative of four individual experiments.

FIGURE 5. An inhibitor of p38 kinase activity blocked adhesion in response to LPS or TNF but not to fNLLP or PMA. PMN were incubated with the indicated concentrations of SB203580 for 10 min at 37°C. LPS/CD14 complexes (25 ng/ml LPS; A) were added for 20 min, TNF (15 ng/ml; B) was added for 15 min, fNLLP (10^{-7} M; C) was added for 15 min, or PMA (30 ng/ml; D) was added for 20 min at 37°C, and adhesion was measured as described in Materials and Methods. HAP buffer (clear bars) was used for unstimulated controls for each agonist. Data shown are representative of four individual experiments.

The p38 inhibitor affects the initial rate of adhesion as well as maximal adhesion

The effect of SB203580 on adhesion over time was tested to determine whether the rate of adhesion was slowed by blockade of p38. PMN were preincubated for 10 min at 37°C with 1 μM SB203580, and then agonists were added for different times at 37°C before measurement of adhesion. The results, shown in Figure 6, demonstrate that in the presence of the p38 inhibitor, the initial rate of adhesion was much slower in response to LPS or TNF but was only slightly decreased in response to fNLLP. In addition, the overall extent of adhesion was depressed for both LPS and TNF when p38 was blocked; 1 μM SB203580 inhibited peak adhesion in response to LPS or TNF by 40 to 50%. SB203580 had no effect on either the rate or the extent of adhesion in response to PMA (Fig. 6), which bypasses cell surface receptors to stimulate PKC directly.

The p38 inhibitor also blocks the adhesion-dependent oxidative burst

Since cytokine-stimulated, adherent PMN exhibit a massive oxidative response, we tested whether this secondary response to adhesion was also abrogated by an inhibitor of p38. PMN were preincubated for 10 min at 37°C with increasing concentrations of SB203580 before addition of agonist and further incubation at 37°C. Production of ROI was monitored over time using HRP-catalyzed oxidation of scopoletin by H₂O₂. As was the case for adhesion, SB203580 slowed the initial rate of the adhesion-dependent oxidative burst in response to LPS and TNF in a concentration-dependent manner (Fig. 7, A and B). This led to a large reduction in the amount of H₂O₂ produced by 60 min, with 50% inhibition observed at about 1 μM SB203580. There was also a concentration-dependent decrease in the initial rate of H₂O₂ production in response to fNLLP, but the compound was less potent in blocking the response to fNLLP than it was in blocking that for LPS or TNF (Fig. 7C). Fifty percent inhibition of the fNLLP response was not achieved at concentrations up to 2 μM. As was the case with adhesion, the compound had no effect on the response to PMA (Fig. 7D), indicating that the compound was not toxic to the cells and had no direct effect on the assay. These results suggest that the p38 inhibitor may have its primary effect on the oxidative burst by blocking adhesion. However, we cannot rule out the possibility that p38 participates in additional ways to signal the oxidative burst. Further studies were conducted to determine at what
time after addition of agonist p38 function was required for the adhesion-dependent oxidative burst.

**p38 function is required during the lag period before the adhesion-dependent oxidative burst begins**

To test when p38 function was required for the oxidative burst, 1 μM SB203580 was added either at the same time as the agonist (LPS or TNF) or at intervals after the addition of agonist, and H₂O₂ production was monitored over time at 37°C. The inhibitory effect of SB203580 on the oxidative burst in response to LPS or TNF was gradually lost with the increase in time of addition following stimulation (Fig. 8). The decline in inhibition was observed as early as 10 min for LPS and 5 min for TNF, and by 20 min there was no effect on the oxidative burst (Fig. 8, A and B). Thus, addition of SB203580 after the end of the lag period was not inhibitory. Inhibition during the lag was time dependent, suggesting that p38 kinase activity is important at a very early stage in signaling for the oxidative burst and, further, that its function is required throughout the lag to achieve maximal generation of ROI.

**Discussion**

The role of p38 in signal transduction leading to cytokine production has been explored in several studies (14), but fewer studies have examined the function of p38 in relation to cellular responses that do not require protein synthesis. p38 is present in platelets and is rapidly (1–2 min) activated by thrombin (34), collagen fibers, and the thromboxane analogue U46619 (35). In addition, platelet aggregation in response to collagen and U46619 is blocked by SB203580 (35), suggesting that p38 may participate in signaling for activation of the integrin gpIIb/IIIa. However, aggregation in response to thrombin is nonreproducibly blocked by the inhibitor, and the inhibition of aggregation in response to collagen and U46619 is overcome by increasing the concentration of agonist.

The main focus in PMN has been on the kinase activity of p38 itself and on identifying proteins downstream of the kinase, although some functional correlates have also been made. Phosphorylation and activation of p38 in response to LPS (15, 28), TNF (36), formyl peptide (36, 37), and PMA (15) have been reported. In addition, the immediate downstream effector of p38, MAPKAP kinase-2, is phosphorylated in PMN in response to granulocyte-macrophage CSF (38) and formyl peptide (37, 39), and SB203580 blocks activation of MAPKAP kinase-2 in PMN (37). Functional studies have suggested that p38 may play a role in signaling arachidonic acid release and the production of ROI. SB203580 partially blocks phosphorylation of cPLA₂ and diminishes its activity in PMN stimulated with TNF (40). A competitive inhibitory peptide of hsp27, a protein phosphorylated by MAPKAP kinase-2 (41, 42), introduced into PMN has been reported to inhibit adhesion of PMN to endothelial cells in response to FMLP and the
oxidative burst of cells in suspension in response to the same agonist (43).

Our results both confirm that p38 is phosphorylated by PMN in response to LPS in a mCD14-dependent manner and go beyond previous reports to describe an important functional significance of p38 activation in PMN. Here we demonstrated that blockade of p38 kinase activity with a specific inhibitor diminished two physiologically important functions of PMN. Integrin-dependent adhesion of PMN to fibrinogen and the adhesion-dependent oxidative burst in response to LPS and TNF were both inhibited by SB203580 at concentrations consistent with the IC50 of the compound for p38 inhibition (30, 32). These results define a common component in the signaling pathways for LPS and TNF. LPS and TNF also share PI3K as a common signaling component, since wortmannin effectively blocks adhesion in response to these agonists (9). Preliminary experiments suggest that wortmannin at concentrations that inhibit adhesion does not block phosphorylation of p38 in response to LPS (P. A. Detmers and D. Zhou, unpublished observations). However, published results suggest that wortmannin, but not the alternative PI3K inhibitor LY29002, partially blocks p38 activation in response to formyl peptide (37, 44). Further work will be necessary to determine the order of p38 and PI3K in a cascade or, alternatively, whether they lie on separate pathways.

The inhibitor also partially blocked both adhesion and the oxidative burst in response to fNLLP. It is likely that SB203580 was more effective in blocking ROI production than adhesion in response to fNLLP, because the substrates used for the assays were different. Serum offers fewer ligand binding sites for β2-integrins than does fibrinogen, and adhesion to serum would thus be easier to inhibit. Formyl peptide-stimulated adhesion of PMN to endothelial cells is blocked 50% by 10 μM SKF86002 (43), a p38 inhibitor with an IC50 for the enzyme around 1 μM (45). This suggests that although formyl peptide causes activation of the kinase (36, 37, 43) and MAPKAP kinase 2 (37, 39), adhesion to different substrates in response to formyl peptide may be differentially p38 dependent. SB203580 had no inhibitory effect on PMN responses to PMA, although it was previously reported that PMA can activate p38 (15). Apparently this activity is incidental to the ability of PMA to stimulate adhesion and an oxidative burst.

Several lines of evidence suggest that p38 participates in signaling the production of ROI primarily through its role in signaling integrin-dependent adhesion. SB203580 diminished integrin-dependent adhesion, and it has been previously shown that blockade of leukocyte integrin-ligand interactions by Abs abrogates the production of ROI by PMN in response to TNF (5). Further, p38 kinase activity was required at an early stage in the lag period before the production of ROI, during which adhesion, cell spreading, and assembly of the oxidase components occurs. When SB203580 was added at times longer than 5 or 10 min after addition of LPS or TNF, the inhibitory effectiveness for ROI production declined. Thus, while we cannot rule out the possibility that p38 functions in both signaling for adhesion and in another process necessary for oxidase assembly, blockade of adhesion

FIGURE 7. Inhibition of p38 blocked the adhesion-dependent oxidative burst. PMN were incubated in serum-coated wells for 10 min at 37°C with increasing concentrations of SB203580 (0–2 μM) as indicated before addition of LPS/CD14 (30 ng/ml LPS; A), TNF (100 ng/ml; B), fNLLP (6 μM; C), PMA (30 ng/ml; D), or KRPG (all graphs; △) and continued incubation at 37°C. H2O2 produced was measured at 10-min intervals as described in Materials and Methods. The figure is representative of four separate experiments performed on quadruplicate samples.
controls received SB203580 and KRPG in place of agonist. The cells were

ranged in quadruplicate with similar results.

alone by SB203580 appears sufficient to explain inhibition of the

oxidative burst.

Two observations suggest that p38 has little role in directly reg-

ulating the function of the oxidase complex. When SB203580 was

added to PMN as late as 20 min after addition of the agonist, at the

end of the lag period, ROI production proceeded unimpeded, sug-

gesting that once assembly of the oxidase complex is accom-

plished, p38 function is no longer required. In addition, there was

no inhibition by SB203580 of ROI production in response to PMA,

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