THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 279, No. 26, Issue of June 25, pp. 27059–27068, 2004
Printed in U.S.A.

Distinct Ligand-dependent Roles for p38 MAPK in Priming and Activation of the Neutrophil NADPH Oxidase*

Received for publication, December 29, 2003, and in revised form, April 8, 2004
Published, JBC Papers in Press, April 20, 2004, DOI 10.1074/jbc.M314258200

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In response to certain cytokines and inflammatory mediators, the activity of the neutrophil NADPH oxidase enzyme is primed for enhanced superoxide production when the cells receive a subsequent oxidase-activating stimulus. The relative role of p38 MAPK in the priming and activation processes is incompletely understood. We have developed a 2-step assay that allows the relative contributions of p38 MAPK activity in priming to be distinguished from those involved in oxidase activation. Using this assay, together with *in vitro* kinase assays and immunochemical studies, we report that p38 MAPK plays a critical role in TNFα priming of the human and porcine NADPH oxidase for superoxide production in response to complement-opsonized zymosan (OpZ), but little, if any, role in neutrophil priming by platelet-activating factor (PAF) for OpZ-dependent responses. The OpZ-mediated activation process *per se* is independent of p38 MAPK activity, in contrast to oxidase activation by fMLP, where 70% of the response is eliminated by p38 MAPK inhibitors regardless of the priming agent. We further report that incubation of neutrophils with TNFα results in the p38 MAPK-dependent phosphorylation of a subpopulation of $p47^{phox}$ and $p67^{phox}$ molecules, whereas PAF priming results in phosphorylation only of $p67^{phox}$. Despite these phosphorylations, TNFα priming does not result in significant association of either of these oxidase subunits with neutrophil membranes, demonstrating that the molecular basis for priming does not appear to involve preassembly of the NADPH oxidase holoenzyme/cytochrome complex prior to oxidase activation.

The neutrophil NADPH oxidase is a multiprotein enzyme that catalyzes the production of O_2^- from oxygen using NADPH as an electron donor (1, 2). The O_2^- is subsequently converted to powerful oxidizing agents such as hypohalides, singlet oxygen, peroxides, and chloramines, which together with proteases and ions, are primarily responsible for neutrophil-mediated killing of microorganisms (2–4). These oxidase products, however, are also highly destructive to nearby tissues (see Refs. 5–7). Consequently, tight temporal and spatial regulation of the oxidase is required so that it is activated only under appropriate circumstances.

The NADPH oxidase holoenzyme consists of 6 subunits. In resting cells in which the oxidase is dormant, 4 of the subunits, $p47^{phox}$, $p67^{phox}$, $p40^{phox}$, and the small GTPase Rac2 are localized exclusively in the cytoplasm whereas the remaining two subunits, $p22^{phox}$ and $gp91^{phox}$ form a heterodimeric membrane-bound flavocytochrome known as cytochrome b_{558}. Exposure of neutrophils to a variety of diverse stimuli results in translocation and docking of the cytosolic subunits with the membrane subunits (3, 8–19), allowing electrons to flow from $p67^{phox}$-bound NADPH to molecular oxygen via an FAD moiety and two heme groups in the membrane-bound cytochrome (20). The subunit translocation and oxidase activation process is complex and requires multisite phosphorylation of the cytosolic subunits, followed by a series of SH3 domain- and PX domain-mediated interactions that are only partially understood (3, 20–26). Approximately 50–70% of the reactive oxygen species (ROS)[1] generated by the NADPH oxidase are produced within closed intracellular compartments including phagosomes, endosomes, and intracellular granules (26–30). The remaining 30–50% of the ROS are delivered to the extracellular space either by direct oxidase activation at the plasma membrane or by oxidase recruitment to the developing phagosomes before they are sealed and internalized.

Priming of the neutrophil respiratory burst refers to the 10–20-fold increase in ROS production when solution phase neutrophils are exposed to low levels of cytokines and chemotactic factors such as TNFα, platelet-activating factor (PAF), and LPS prior to administering a stimulus capable of activating the NADPH oxidase directly (31–34). Although high concentrations of these priming agents themselves can lead to the production of O_2^- (35), at typical priming doses these compounds and priming agents do not directly activate the NADPH oxidase in solution phase neutrophils. Rather, priming agents activate various signal transduction cascades that amplify the process, while maintaining an obligatory requirement for a secondary "activating" stimulus to initiate the production of O_2^-. The cytokine-induced priming of neutrophils in suspension is extremely different from that observed in adherent neutrophils in which integrin receptors have been engaged, where cytokine exposure alone is sufficient to activate the NADPH oxidase, presumably through Pyk2, a cytoplasmic tyrosine kinase (36–39).

* This work was supported by National Institutes of Health Grant GM98059 and a Burroughs-Wellcome Career Development Award (to M. B. Y.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: ROS, reactive oxygen species; TNF, tumor necrosis factor; PAF, platelet-activating factor; BSA, bovine serum albumin; PBS, phosphate-buffered saline; CL, chemiluminescence; PMA, phorbol 12-myristate 13-acetate; Erk, extracellular signal-regulated kinase; LPS, lipopolysaccharide; OpZ, opsonized zymosan; MAPK, mitogen-activated protein kinase; AEBSF, 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride.

Priming of neutrophil responses is clinically important in a variety of human diseases, particularly in ischemia-reperfusion injury and in the acute lung injury and multiple organ system failure observed in patients following trauma and/or sepsis (40, 41). In these diseases, excessive production of ROS by primed neutrophils is believed to directly contribute to host-tissue damage through an innocent bystander effect (5, 6, 41–50). In agreement with this, patients with infections have been shown to have primed subpopulations of neutrophils that show increased intracellular ROS products (51). Moore, Silliman, and others (52–54) have shown that PAF is a primary agent responsible for neutrophil priming in human trauma victims. In contrast, LPS and TNFα are the likely priming agents in acute pancreatitis (55, 56), as well as in many patients with sepsis (57–61), and these agents have been directly implicated in causing acute lung injury (62–65) and ischemia-induced acute renal failure (66).

The molecular basis for priming of the neutrophil NADPH oxidase is unknown. A number of signaling pathways have been implicated in the priming process including the phosphatidylinositol 3-kinase pathway (67, 68) and several MAP kinase pathways (69–76). The involvement of p38 MAPK in priming has been particularly contentious with some groups claiming that p38 MAPK plays a critical role (69, 70, 72, 73), whereas others claim that it plays no role in priming whatsoever, and instead is involved only in the oxidase activation step (77). The conclusions from all of these studies have been complicated by the fact that priming cannot be studied in the absence of respiratory burst activity, and therefore it has been difficult to distinguish effects on priming from effects on the activation assay for the priming process. To overcome this limitation and identify specific roles for p38 MAPK in either neutrophil priming or activation, or both, we have developed a novel 2-step assay that separates the priming step from the activation process. Using this assay we report that p38 MAPK plays specific distinct roles in cytokine priming and/or activation in both human and porcine neutrophils, depending on the nature of the priming and/or activating ligand. We find that p38 MAPK plays prominent roles in priming by TNFα and activation by fMLP, but minimal, if any, role for p38 MAPK in priming by PAF or activation by complement-opsonized zymosan. Furthermore, our data suggest a common molecular mechanism for priming that facilitates NADPH oxidase activation prior to the formation of the membrane-bound holoenzyme complex.

EXPERIMENTAL PROCEDURES

Reagents and Materials—Rabbit polyclonal antibodies against $p47^{phox}$ and $p67^{phox}$ were prepared as described previously (26). Antibodies against the Thr-180/Tyr-182-phosphorylated active form of p38 MAPK (78), and the Thr-334-phosphorylated form of MAPKAP kinase-2 (79) were purchased from Cell Signaling Technology (Beverly, MA, products 9211 and 3041). A rabbit polyclonal antibody against p38 MAPK was a kind gift from Drs. Peter Juo and John Blenis (Harvard Medical School). Recombinant GST-ATF2 was expressed in *Escherichia coli* using a plasmid provided by Dr. Roger Davis (University of Massachusetts Medical School) and purified using glutathione-agarose chromatography. SB203580 was purchased from CalBiochem (San Diego, CA). Low-endotoxin bovine serum albumin (BSA) and PAF were purchased from Sigma-Aldrich. Recombinant TNFα was purchased from Roche Applied Science (Indianapolis, IN).

Human and pig complement-opsonized Zymosan (OpZ) was prepared following the procedure of Allen (80). In brief, zymosan (1 g) was rehydrated in 300 ml of 0.9% NaCl (SS, saline solution), incubated at 100 °C with constant stirring for 15 min, pelleted by centrifugation, and washed twice with SS. Plasma was harvested from citrate-anticoagulated blood from human volunteers or from 6-week-old pigs, flash-frozen on liquid nitrogen, and stored at −70 °C. Plasma from at least 8 separate donors was quickly thawed to 4 °C, pooled (~800 ml total volume), supplemented with solid ammonium sulfate to 28.5% saturation, stirred for 1 h at 4 °C and centrifuged at 1200 × *g* for 30 min to precipitate the IgG. The clear supernatant was dialyzed against 20 volumes of Hanks' basic salt solution without divalent cations (HBSS), pH 7.1, for 20 h at 4 °C with 3 changes of buffer. The dialyzed sample was cleared by centrifugation, warmed to 22 °C, and added to pre-warmed zymosan (in HBSS) followed by the addition of $CaCl_2$ to 1.3 mM and $MgCl_2$ to 0.4 mM. Following incubation at 22 °C for 15 min with constant stirring, the mixture was quickly chilled on ice slush. The resulting OpZ was then pelleted, washed five times with SS, and suspended in 200 ml of SS. Aliquots were frozen on liquid nitrogen and stored at −70 °C. Each lot of OpZ was assayed to ensure low primer-independent neutrophil responses but good primer-enhanced (PAF, 12.5 pM) responses (10-fold enhancement) using chemiluminescence assays with diluted whole blood. The amount of OpZ used in a typical experiment was between 1/40 and 1/80 of the final assay volume.

Preparation of Neutrophils and Subcellular Fractions—Heparinized blood was obtained from healthy human volunteers or from healthy Yorkshire swine and used within 5 min of venipuncture. Neutrophils were isolated from whole blood by centrifugation thorough Ficoll-Hypaque (Amersham Biosciences, Piscataway, NJ) followed by dextran (T500, Amersham Biosciences) sedimentation and hypotonic lysis of residual red blood cells as described previously (81). Neutrophils were kept on ice until used, which always occurred within 4 h of venipuncture. All portions of this study were performed in accordance with the relevant directives from the Committee on Clinical Investigations and the Institutional Animal Care and Use Committee at Beth Israel Deaconess Medical Center and the Committee on the Use of Humans as Experimental Subjects at MIT.

Membrane preparations were prepared as described by Quinn and Bokoch (82). PMNs (5×10^7/ml in PBS containing 100 μg/ml BSA, 780 units/ml catalase, 50 units/ml superoxide dismutase (SOD)) were incubated at 37 °C for 15 min with no ligands, 47 pM TNFα, or 1 μg/ml PMA. Reactions were stopped on ice slush, 8 μg/ml 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride was added, and cells were pelleted at 200 × *g* for 10 min at 4 °C. PMNs were resuspended in extraction buffer (RB buffer plus 4 μg/ml each of leupeptin, aprotinin, pepstatin A, 8 μg/ml AEBSF), and the cells disrupted by nitrogen cavitation (450 psi, 15 min, 4 °C). The cavitates were centrifuged at 1,000 × *g* for 5 min, and the supernatants (0.25 ml) layered onto 0.25 ml of 20% (w/v) sucrose containing 10 mM Hepes, pH 7.4. Samples were centrifuged at 200,000 × *g* for 45 min at 4 °C in a TLA 120.1 rotor (Beckman). Supernatants were discarded, 0.5 ml of extraction buffer was added to each tube, and the samples recentrifuged as before. Pellets were resuspended in 150 μl of PBS without divalent cations. SDS-PAGE sample buffer (60 μl of 6× concentrate) was added to 120 μl of membranes, and the samples were incubated at 95 °C for 5 min.

Protein determinations were performed on resuspended membranes prior to addition of PAGE sample buffer using the Bio-Rad protein assay kit. Equal amounts of protein, equivalent to material derived from 1×10^6 cells, were electrophoresed into 12% SDS-polyacrylamide gels. Proteins were transferred to polyvinylidene difluoride membranes and blotted for $p47^{phox}$ and $p67^{phox}$.

One- and Two-dimensional Gel Electrophoresis and Western Blot Analysis—One-dimensional SDS-PAGE was performed using 10% acrylamide-containing SDS gels (83). For two-dimensional electrophoresis, cytoplasmic extracts (100 μg of protein) were suspended in 100 μl of lysis buffer (50 mM Tris-HCl, 1% Nonidet P-40, 150 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 mM Na_3VO_4, 20 nM Microcystin LR, 5 nM okadaic acid, 2 μM cantharidin, 0.00073% *p*-bromotetramisole, 10 μM E64, 1 mM AEBSF) and treated with 5 μl of 2 mg/ml each of DNaseI and RNaseA for 30 min on ice. Samples were precipitated by addition of 10× total volume of ice-cold acetone overnight and resuspended in 100 μl of 9.8 M urea, 2% CHAPs, and 100 mM dithiothreitol, containing 0.5% ampholytes (Pharmalyte pH 6–11, Amersham Biosciences). Samples were applied to 7-cm IPG strips pH 6–11 (all strips from the same lot), which were allowed to rehydrate for 12 h at 50 V, then subjected to isoelectric focusing for 30 min at 200 V, 30 min at 500 V, 1 h at 1000 V, and 5 h at 3500 V using an IPGphor isoelectric focusing system device (Amersham Biosciences). Strips were briefly washed in re-equilibration buffer (50 mM Tris HCl, pH 8.8, 6 M urea, 30% glycerol, 2% SDS) in the presence of 20 mg/ml of dithiothreitol for 10 min, and then in the presence of 25 mg/ml of iodoacetamide for 10 min to irreversibly modify all Cys residues. The strips were then loaded onto 6–18% SDS-PAGE gels and electrophoresed at 100 V for 1 h. Gels were transferred onto polyvinylidene difluoride membranes and used for Western blot analyses using the appropriate antibodies in conjunction with enhanced chemiluminescence reagents (PerkinElmer Life Sciences, Boston, MA).

Determination of sample pI values was performed based on the migration positions of pI standards (Bio-Rad).

Chemiluminescence Assay for ROS Production—For determination of ROS production in whole blood assays, freshly drawn blood was diluted 100-fold in Dulbecco's PBS without divalent cations (PD buffer) and immediately combined with 100 μl of ligand diluted in PD buffer and 600 μl of luminol buffer (Dulbecco's PBS plus 100 μg/ml BSA and 0.15 mM luminol). Chemiluminescence (CL) was assayed at 37 °C with an Autolumat LB953 instrument (PerkinElmer Life Sciences Wallac). Assays involving isolated neutrophils were performed similarly with neutrophils diluted 800-fold after preincubations with inhibitors and priming agents. For studies using dilute whole blood, aliquots were incubated with 9.4 pM TNFα. In studies with isolated neutrophils, comparable priming was observed using 47 pM TNFα. CL results are generally expressed as the integral of the initial 10 min CL signal from which the machine background has been subtracted.

p38 MAPK and Erk1/2 in Vitro Kinase Assays—Freshly prepared neutrophils ($4-8 \times 10^6$ cells/assay in 100 μl PBS) were stimulated with 9.4 pM TNFα, or 12.5 nM PAF at 23 °C for various times, pelleted at 900 rpm in a microcentrifuge for 45 s, and lysed in 200 μl of lysis buffer (10 mM KH_2PO_4, pH 7.2, 1 mM EDTA, 5 mM EGTA, 10 mM $MgCl_2$, 0.5% Nonidet P-40, 0.1% Brij-35, 2 mM dithiothreitol, 50 mM β-glycerophosphate, 1 mM Na_3VO_4, and 1.6 μg/ml each of pepstatin, leupeptin, and aprotinin) at 4 °C for 10 min. Samples were centrifuged at 15,000 rpm in a microcentrifuge at 4 °C for 10 min, and the supernatants incubated with 10 μl of either anti-p38 or anti-Erk1/2 MAPK rabbit polyclonal antiserum at 4 °C for 60 min. Protein A-Sepharose beads (30 μl) were added and the samples incubated with rocking at 4 °C for an additional 60 min. The beads were washed with 1 ml of 10 mM Tris, pH 7.2, 100 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, and 0.5% deoxycholate, then with 1 ml of 10 mM Tris, pH 7.2, 1 M NaCl, 1% Nonidet P-40 and then with 1 ml of 50 mM Tris, pH 7.2, 150 mM NaCl. The beads were resuspended in 30 μl of kinase reaction buffer (20 mM HEPES, pH 7.2, 10 mM $MgCl_2$, 3 mM 2-mercaptoethanol, 100 μg/ml BSA, 50 μM ATP, 10 μCi of [γ-^{32}P]ATP) containing 1 μg of recombinant GST-ATF2 (for p38 assays) or myelin basic protein (for Erk1/2 assays). Kinase reactions were performed at 30 °C for 15 min, and the reactions were terminated by the addition of an equal volume of 2× Laemmli sample buffer followed by heating at 95 °C for 3 min. Samples were analyzed by SDS-PAGE followed by transfer to nitrocellulose for autoradiography and immunoblotting.

RESULTS

Priming of the Neutrophil NADPH Oxidase in Human and Porcine Neutrophils—The phenomenon of priming of the neutrophil respiratory burst is shown in Fig. 1, using the luminol-dependent CL response of human neutrophils to the agonist OpZ and to the priming agents PAF and TNFα (*panels A* and *B*). The luminol-based assay was chosen because it can be used either with whole blood or with isolated neutrophils (80), and it accurately measures NADPH oxidase in both intracellular and extracellular compartments (26, 84). In addition, this assay can be performed using only a few thousand cells, in contrast to measurements of cytochrome *c* reduction, which typically require on the order of 10^6 total cells. This enhanced sensitivity permitted us to develop a dilution-based 2-step assay that separates priming events from agonist-mediated activation events as described below. Fig. 1*A* shows a typical experiment in which diluted whole blood from healthy human donors was initially assayed for CL at 37 °C for 5 min in the absence of agonists or priming agents. Reactions were then initiated by direct addition of the agonist OpZ alone, by addition of OpZ together with the priming agent PAF (12.5 nM final concentration), or by addition of PMA, a direct activator of PKCs. No CL was generated in the absence of agonists, and there was a very low response to OpZ or PAF alone (Fig. 1*A* and *inset*, respectively). Inclusion of PAF with the OpZ dramatically enhanced the kinetics with a net 16–25-fold increase in integrated CL signals over the initial 10 min following addition of ligands, relative to identical reactions containing OpZ alone. Furthermore, preincubation of the samples with PAF prior to addition of activating ligands is not necessary to obtain a primed response for CL production, in contrast priming by to TNFα (see below). In addition to the increased rate of CL production, PAF-primed neutrophils exhibited virtually no lag time in CL generation following the addition of OpZ. In comparison, PMA (30 ng/ml, final concentration) generally induced an intermediate level of CL production with a variable lag time in different experiments that averaged about 2–5 min. The responses to either PMA or OpZ+PAF were prolonged; there was no appreciable decrease in CL generation observed during the 10-min time course following addition of agonists.

Similar experiments were performed using TNFα as the priming agent (Fig. 1*B*). In these experiments, undiluted whole blood samples were preincubated with or without 9.4 pM TNFα for 15 min at 37 °C, diluted into assay buffer containing OpZ and assayed for CL production over 10 min. Exposure to TNFα greatly enhanced CL production in response to OpZ, demonstrating both an increased rate of CL generation and a shortened lag time compared with OpZ alone, similar to the results obtained when PAF was used as the priming agent. No CL was produced when cells were treatment with TNFα alone, in the absence of the agonist OpZ (Fig 1*B*, *inset*). *Panels A* and *B* represent typical examples of neutrophil priming from experiments performed hundreds of times in our laboratory and verify that PAF and TNFα prime human blood neutrophils for CL in response to OpZ.

We next characterized the priming response of porcine neutrophils because pigs are a useful whole animal model for studies of sepsis and acute lung injury with similar physiology to humans (46, 85). As shown in Fig. 1, *panels C* and *D*, both PAF and TNFα strongly primed porcine neutrophils for chemiluminescence production in response to pig complement-opsonized zymosan, with similar kinetics and amplitude as those observed using human neutrophils. Thus, porcine neutrophils exhibit properties of priming *ex vivo* similar to those seen with human neutrophils.

Priming by TNFα, but Not PAF, for CL Responses to OpZ Involves a p38 MAPK-dependent Process—A number of conflicting reports have either implicated, or directly disputed, the role of p38 MAPK in priming of the neutrophil respiratory burst by TNFα, PAF, LPS, and GM-CSF, based largely on studies using the p38 MAPK inhibitor SB203580 (69, 70, 72, 73, 77). The experimental design used in all of those studies, however, could not distinguish between two alternative possibilities: (1) an SB203580-inhibitable process involved specifically in the priming for enhanced CL activity or (2) an SB203580-inhibitable process involved in the general NADPH oxidase activation step required for all CL production, both primed and unprimed. To address this issue, we designed a 2-step procedure to specifically distinguish roles for p38 MAPK in either the priming process and/or the activation step (Fig. 2*A*). In this assay, whole blood or purified neutrophils were preincubated with either an initial agent (buffer, TNFα (9.4 pM), PAF (12.5 nM), or SB203580 (10 μM)) for 15 min at 37 °C followed by a second 15-min incubation with a second set of ligands (buffer, TNFα, PAF, or SB203580). Following these two preincubation steps, the samples were immediately diluted 800-fold into ice-cold assay buffer and were then assayed for CL at 37 °C in response to the oxidase-activating agonist OpZ. Independent experiments verified that the 800-fold dilutions of 9.4 pM TNFα and 12.5 nM PAF reduced their concentrations to below that at which neutrophils exhibit appreciable priming (data not shown). This assay permits inhibition of p38 MAPK either prior to TNFα or PAF priming, or following priming but prior to agonist-mediated activation, thereby separating p38 MAPK-dependent priming events from those involved in the general oxidase activation process.

Fig. 2*B* shows the results obtained using this assay for TNFα

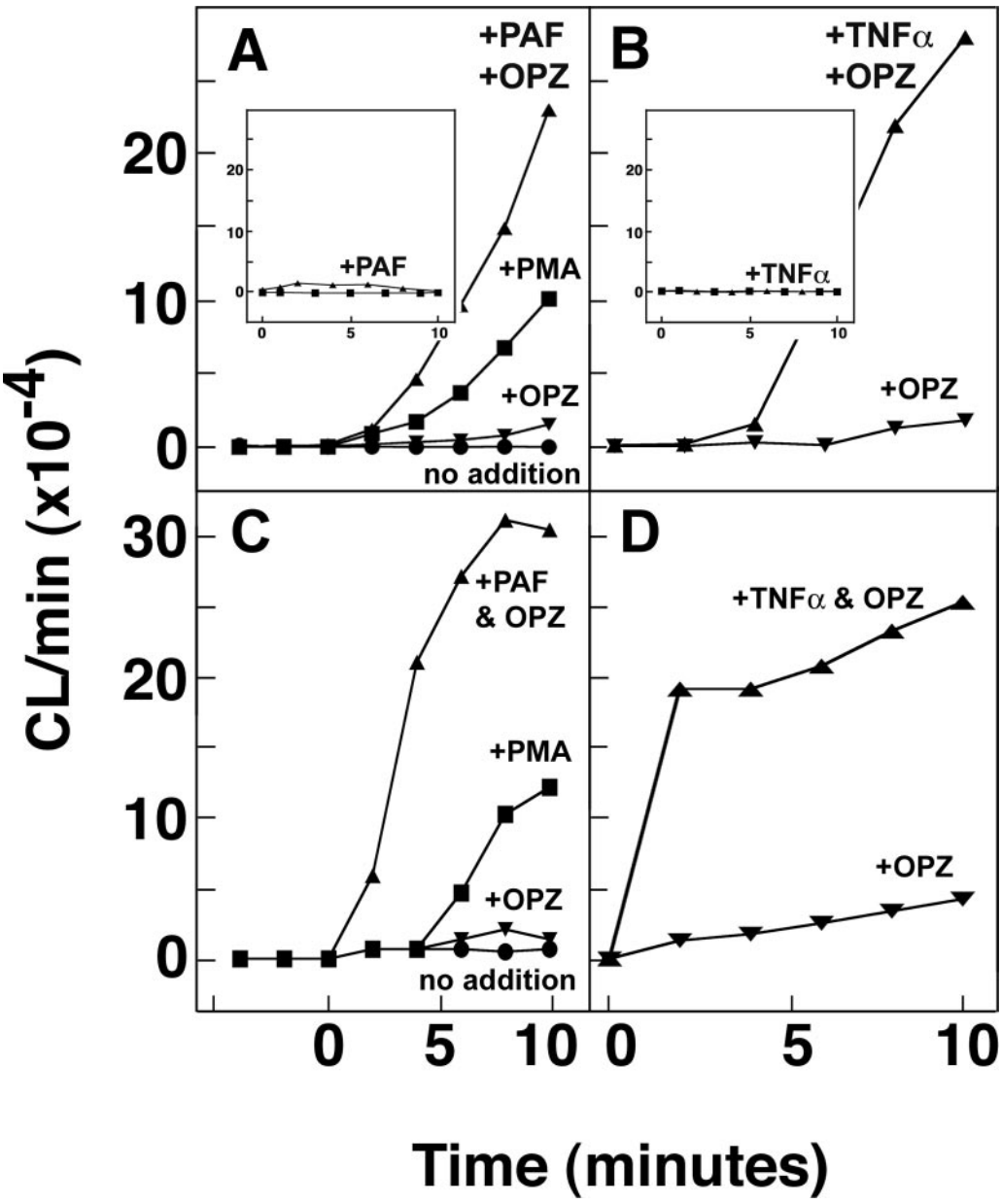

FIG. 1. **Priming of human and porcine neutrophils by PAF and TNFα reduces the lag time and increases the rate of CL production in response to OpZ.** *Panel A*, ROS production by human neutrophils was monitored at 1-min intervals by luminol-dependent CL production in 800-fold diluted whole blood for 5 min at 37 °C prior to addition of ligands (OpZ, PMA) ± priming agents (PAF) at *O min*. (▼), OpZ alone; (■), PMA (30 ng/ml); (▲), PAF (12.5 nM) plus OpZ; (●), control reaction lacking ligands and priming agents. Rate of CL production was monitored in 1-min intervals for 10 min following ligand addition. *Inset*, CL production in the absence (■) or presence (▲) of 12.5 nM PAF in reactions lacking an activating ligand. *Panel B*, CL response of human neutrophils to OpZ following a 15-min preincubation with (▲) or without (▼) TNFα (9.4 pM) at 37 °C. OpZ was added to both sets of reactions at 0 min, and CL production was assayed as in *panel A*. *Inset*, CL production in the absence (■) or presence (▲) of 9.4 pM TNFα in reactions lacking an activating ligand. *Panels C* and *D*, experiments similar to those in *panels A* and *B*, respectively, but performed with porcine blood.

priming and OpZ-mediated activation of human neutrophils. The upper three curves virtually overlap and show identical rates of CL production upon OpZ addition when cells were exposed to TNFα alone for 15 min (*i.e.* agent1/agent2 = buffer/TNFα) or 30 min (TNFα/buffer) or TNFα for 15 min followed by SB203580 for 15 min (TNFα/SB203580). In contrast, the bottom curve shows a significantly decreased rate of CL production when SB203580 is added for 15 min prior to TNFα addition (SB203580/TNFα). The net inhibition of total CL, integrated over the initial 10 min, was 72 ± 7% when the drug was added prior to TNFα but −17 ± 24% when the drug was added after the TNFα (Table I, top 2 lines; ($p < 0.017$; Student's t test, two-tail, type 3; based on ligand-dependent CL). We conclude that a specific SB203580-inhibitable process was involved in TNFα-priming for responses to OpZ, but was relatively unimportant once the primed state had been established.

We next examined whether SB203580 inhibited priming induced by a different type of priming agent, the lipid-derived product PAF, for respiratory burst activity in response to OpZ. PAF was chosen because it binds to a different category of receptor than does TNFα, and because it also has been reported to activate p38 in human neutrophils (69, 73, 86). As seen in Fig. 2*C* and Table II (top line), preincubation of neutrophils in dilute whole blood with SB203580 (10 μM) had no effect on subsequent priming by PAF for CL production in response to OpZ. Thus, in strong contrast to the results obtained with TNFα, PAF-mediated priming for CL in response to OpZ occurs primarily through processes that are p38 MAPK-independent.

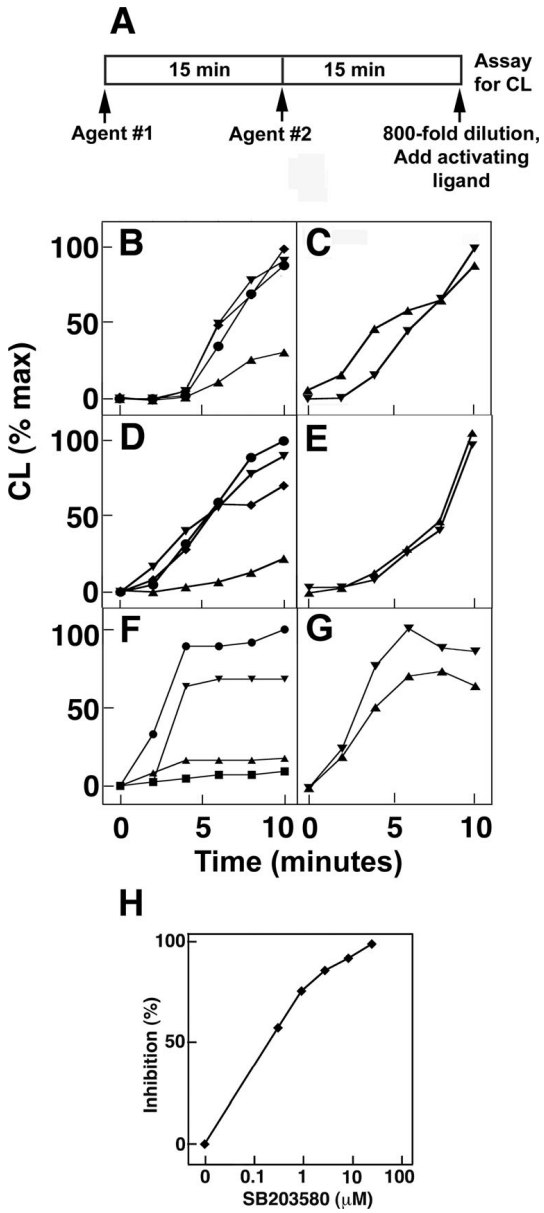

FIG. 2. **The p38 MAPK inhibitor SB203580 inhibits priming by TNFα, but not by PAF, for CL in response to OpZ.** *A*, schematic of the 2-step assay procedure. *B*, 2-step assay reveals a specific role for p38 MAPK in TNFα-mediated priming of NADPH oxidase activity. Aliquots of human blood were preincubated with the indicated ligands in sequential 15-min steps at 37 °C, then assayed for the rate of CL production in response to OpZ as in the legend to Fig. 1. The data were normalized by setting the 10-min time point for the +TNFα/buffer sample equal to 100%. Agent 1 and Agent 2, respectively, were: ●, TNFα/buffer; ▼, buffer/TNFα; ♦, TNFα/SB203580; ▲, SB203580/TNFα. Data are representative of four independent experiments. *C*, 2-step assay reveals no significant role for p38 MAPK in PAF-mediated priming of NADPH oxidase activity. Aliquots of human blood were preincubated with the indicated ligands and then assayed for the rate of CL production in response to OpZ as above. Agents 1 and 2, respectively, were: ▼, buffer/PAF; ▲, SB203580/PAF. Experiment representative of $n = 3$ experiments. *D*, experiment and symbols as in *B*, but performed using purified human neutrophils. Data shown are representative of three independent experiments. *E*, experiment and symbols as in *C*, but performed using purified human neutrophils. Experiment representative of $n = 3$ experiments. *F*, as in panel B, but performed using porcine blood. Agents 1 and 2, respectively, are: ●, buffer/TNFα; ▼, TNFα/SB203580, ▲, SB203580/TNFα; ■, buffer/buffer. Data shown are representative of four independent experiments. *G*, as in *panel C*, but performed with isolated porcine neutrophils. Agents 1 and 2, respectively, are: ▼, buffer/PAF; ▲, SB203580/PAF. Experiment representative of $n = 4$ experiments. *H*, SB203580 log dose-response curve for inhibition of TNFα-mediated priming of CL production in human whole blood using OpZ as the activating ligand as in *B*.

TABLE I
Inhibition of OpZ- and fMLP-dependent ROS production by TNFα-primed human neutrophils in whole blood

Experiments were performed using the 2-step assay procedure in Fig. 2.

Agent 1	Agent 2	Activating ligand	Inhibition ($n = 4$)
			%
SB203580	TNFα	OpZ	72 ± 7
TNFα	SB203580	OpZ	−17 ± 24
SB203580	TNFα	FMLP	73 ± 11
TNFα	SB203580	FMLP	67 ± 7

TABLE II
Inhibition of OpZ- and fMLP-dependent ROS production by PAF-primed isolated human neutrophils

Experiments were performed using the 2-step assay procedure in Fig. 2.

Agent 1	Agent 2	Activating ligand	Inhibition ($n = 3$)
			%
SB203580	PAF	OpZ	1 ± 2
SB203580	PAF	FMLP	65 ± 18
PAF	SB203580	FMLP	69 ± 8

Identical results were obtained for SB203580 effects on TNFα and PAF priming when the experiments were performed using purified isolated neutrophils (Fig. 2, *D* and *E*).

To determine whether these separate p38 MAPK-dependent and -independent signaling pathways for priming of the neutrophil respiratory burst were also present in other species, the same series of experiments were performed using porcine whole blood and isolated neutrophils. Similar to what was observed with human neutrophils, preincubation of porcine blood with SB203580 prior to TNFα treatment dramatically inhibited CL production in response to porcine-complement opsonized zymosan (86 ± 20%), whereas only a small amount of inhibition (27 ± 13%) was observed when cells were treated with SB203580 following 15 min of priming by TNFα (Fig. 2*F*). In addition, as observed with human neutrophils, SB203580 treatment resulted in only minimal inhibition of PAF priming for CL responses to OpZ in porcine neutrophils (Fig. 2*G*). Thus, in both human and porcine neutrophils an SB2035880-inhibitable process is involved in priming by TNFα, at least for a subset of agonists typified by OpZ, whereas p38 MAPK is not required for priming by PAF, at least for a subset of agonists typified by OpZ.

SB203580 has been reported to be specific for p38 when present at low doses, but to also inhibit Erk at doses greater than or equal to 10 μM (87). Because the experiments in Fig. 2 were performed using 10 μM SB203580, the absence of an inhibitory effect on PAF-priming is sufficient to demonstrate that p38 MAPK is not involved. The inhibitory effect of this dose of SB203580 on TNFα priming, however, is not sufficient to exclude the possibility that priming was at least partially because of activation of Erk rather than solely to activation of p38 MAPK. An SB203580 dose-response study for inhibition of priming by TNFα was therefore performed (Fig. 2*H*, representative of $n = 3$ experiments). Aliquots of human blood were preincubated with graded doses of SB203580 for 15 min, followed by the inclusion of 9.4 pM TNFα for a second 15 min. The blood samples were immediately diluted and assayed for CL in response to OpZ for 10 min. By 310 nM SB203580, OpZ-dependent CL production was inhibited by 57 ± 23%, and by 2.8 μM inhibition of OpZ-dependent CL was 85 ± 15%, in excellent agreement with the reported IC_{50} of SB203580 of ~0.6 μM (88), and verifying that doses of SB203580 far below those at which Erk is inhibited efficiently inhibited TNFα-primed CL activity.

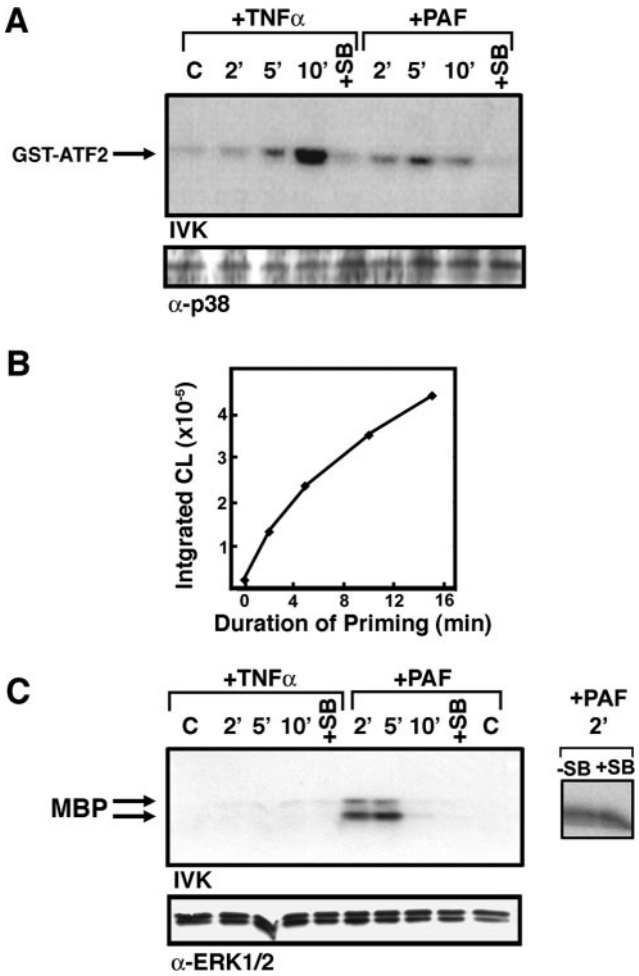

FIG. 3. **TNFα activates p38 MAPK but not Erk1/2 MAPK, with kinetics that parallel priming for NADPH oxidase activity.** *A*, robust induction of p38 MAPK activity by TNFα, compared with PAF. *In vitro* kinase assays for p38 MAPK were performed using purified neutrophils primed with 9.4 pM TNFα or 12.5 nM PAF for the indicated times. The *upper panel* shows p38 MAPK activity against GST-ATF2 detected by autoradiography. The *lower panel* shows equal amounts of immunoprecipitated p38 MAPK in each lane as verified by immunoblotting. The lanes marked *+SB* indicate assays performed on the 10-min samples in the presence of 2.5 μM SB203580. *B*, in a parallel experiment, neutrophils were preincubated with TNFα for the indicated times as in *A* and assayed for NADPH oxidase activity by measuring integrated CL production in response to OpZ. *C*, PAF priming, but not TNFα priming, strongly activates Erk1/2 MAPK. *In vitro* kinase assays for Erk1/2 activity were performed as in *A*. The *upper panel* shows Erk 1/2 activity against myelin basic protein (*MBP*) detected by autoradiography. The *lower panel* shows equal amounts of immunoprecipitated Erk1/2 in each lane as verified by immunoblotting. The small *right panel* demonstrates no inhibitory effect of 2.5 μM SB203580 on the Erk1/2 *in vitro* kinase assays using lysates from cells stimulated with PAF for 2 min.

The kinetics of p38 MAPK activation following treatment of neutrophils with TNFα were investigated using *in vitro* kinase assays. Neutrophils were treated with 9.4 pM TNFα for varying times, then lysed, and p38 MAPK immunoprecipitated and assayed for activity using a GST-ATF2 construct as substrate. As shown in the +TNFα lanes in Fig. 3*A*, we observed detectable p38 MAPK activity within 2 min of TNFα treatment, with extensive activation by 10 min. Addition of 2.5 μM SB203580 to these *in vitro* kinase assays eliminated substrate phosphorylation, verifying the specificity of this assay.

The kinetics of neutrophil priming by TNFα for NADPH oxidase activity were examined as shown in Fig. 3*B*. In these experiments, cells were incubated with 9.4 pM TNFα at 25 °C for varying times, then assayed over 15 min at 25 °C for CL in response to OpZ. These data indicated that TNFα induced a low level of priming within 2 min and that the level of priming increased as exposure to TNFα increased, with near maximal activation at 10–15 min. Thus, the kinetics of priming closely paralleled the induction of p38 activity by TNFα.

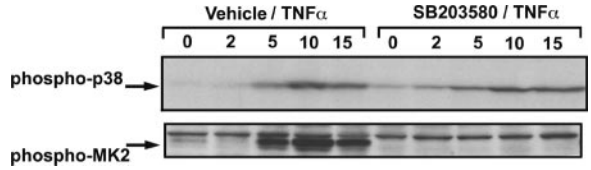

FIG. 4. **SB203580 inhibits TNF-α induced p38 MAPK activity in PMNs *in vivo*.** Neutrophils were treated exactly as in Fig. 2. Total neutrophil lysate from 1.5×10^5 cells was analyzed by SDS-PAGE and immunoblotting using antibodies against the phospho-Thr-180/phospho-Tyr-182-activated form of p38 MAPK (*upper panel*) or against the physiologic p38 MAPK substrate, the phospho-Thr-334 site on MAPKAP kinase-2 (*lower panel*). The *upper band* in the lower panel is a nonspecific cross-reacting band that functions as a loading control.

In contrast to the strong activation of p38 MAPK observed upon TNFα treatment, exposure of neutrophils to PAF resulted in much weaker stimulation of p38 MAPK activity, which appeared to be maximal at 5 min with no further increase by 10 min (Fig. 3*A*, *+PAF lanes*). SB203580 completely eliminated the low levels of p38 MAPK activity detected *in vitro* following PAF treatment, but had no significant effect on PAF-induced priming of CL production *in vivo*.

We performed similar IVK assays to determine whether neutrophil treatment with TNFα or PAF resulted in activation of Erk 1/2 MAPK. As shown in Fig. 3*C*, there was no detectable stimulation of Erk kinase activity by TNFα under these conditions. PAF, however, strongly induced Erk1/2 activity within 2 min, with a return to baseline levels by 10 min. As expected, there was no inhibition of PAF-stimulated Erk activity when 2.5 μM SB203580 was present in the reaction (Fig. 3*C*, *right panel*). Whether this PAF-stimulated increase in Erk1/2 activity is relevant to priming of the NADPH oxidase by PAF, however, was not further investigated.

To verify that the SB203580 effect on TNFα priming involves p38 MAPK inhibition *in vivo*, we performed immunoblotting experiments for the activated form of p38 MAPK, and for one of its phosphorylated substrates, MAPKAP kinase 2, in lysates from cells treated identically as those shown in Fig. 2. As seen in Fig. 4, SB203580 had no effect on the phosphorylation/activation of p38 MAPK by its upstream activating kinases, as expected. However, SB203580 treatment completely eliminated the p38 MAPK-dependent phosphorylation of MAPKAP kinase 2 *in vivo*, verifying that p38 MAPK was efficiently inhibited in these cells *in vivo* under these conditions.

Activation of the Neutrophil CL Response by fMLP, Involves p38 MAPK Independently of Priming Agents—The results presented above directly implicate p38 MAPK in the process of neutrophil priming by TNFα, but not by PAF, for NADPH oxidase responses to OpZ, and clearly distinguish these priming events from the subsequent events involved in the NADPH oxidase activation process. Our results, however, conflict with those of Partrick *et al.* (69), who reported that p38 MAPK was involved in neutrophil priming by PAF on the basis of SB203580 inhibitor studies, and with those of Lal *et al.* (77) who stated that p38 MAPK was not involved in the priming process at all, because these investigators observed similar inhibition of superoxide production by SB203580 using three different priming agents, one of which did not directly activate p38 MAPK. In both the Partick *et al.* (69) and Lal *et al.* (77) studies, however, fMLP, rather than OpZ, was used as the agonist for activating the NADPH oxidase after priming. In contrast to OpZ, which interacts with neutrophils primarily

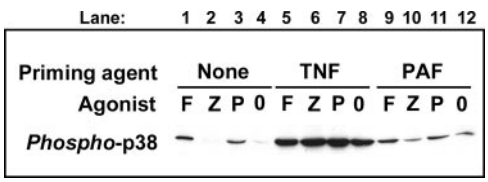

FIG. 5. **Activation loop phosphorylation of p38 MAPK in response to some, but not all activators and priming agents for the NADPH oxidase.** Purified neutrophils in the absence of priming, or following priming by TNFα (47 pM) or PAF (12.5 nM) for 15 min at 37 °C, were then incubated with 1 μM fMLP (*F*), OpZ (Z), 10 ng/ml PMA (*P*), or no agonist (*0*) for 5 min at 37 °C. Cells were lysed and equal amounts corresponding to 1×10^6 cells were immunoblotted for the active phospho-Thr-180/Tyr-182 form of p38 MAPK.

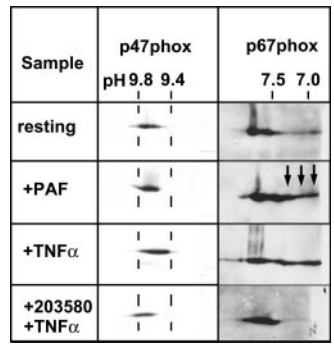

FIG. 6. **Priming-dependent phosphorylation of p47^{phox} and p67^{phox}.** Cytoplasmic extracts from unprimed neutrophils, PAF-primed neutrophils, or TNFα-primed neutrophils in the presence or absence of a prior incubation with SB203580 were analyzed using two-dimensional gel electrophoresis. The isoelectric focusing step was performed simultaneously for all samples using the same lot of Immobiline dry strips, pH 6–11. Following resolution along the second dimension by 10% SDS-PAGE, the gels were transferred to nitrocellulose and immunoblotted for p47^{phox} and p67^{phox}. *Arrows* in the p67^{phox} lanes indicate species enriched in the TNFα- and PAF-primed samples.

through complement receptor 3, fMLP activates respiratory burst activity via binding to a G-protein-coupled receptor. We therefore investigated the effect of SB203580 on the fMLP-mediated activation process, using the two-step procedure. (These experiments were done only with human blood, because in agreement with other studies (89, 90) we were unable to obtain responses from either porcine blood or isolated porcine neutrophils to fMLP.)

In the absence of a priming agent, fMLP stimulation of human neutrophils generates only very low amounts of CL over a prolonged time course. These small amounts of ROS produced in unprimed neutrophils made it impossible to directly determine the effect of SB203580 on fMLP activation in a statistically significant manner. However, both TNFα and PAF can prime neutrophils for a robust CL response to subsequent fMLP stimulation. In contrast to the pattern of SB203580 inhibition observed when OpZ was the agonist, SB203580 was equally effective in inhibiting respiratory burst activity in response to fMLP (1 μM) regardless of whether it was added before (67 $\pm$ 7% inhibition) or after (73 $\pm$ 11% inhibition) the TNFα priming step (Table I, rows 3 and 4). This same ~70% inhibition of CL production by SB203580 was also observed when PAF was used as the priming agent for fMLP-mediated activation, and again, it did not matter whether the cells were treated with the drug before (65 $\pm$ 18% inhibition) or after (69 $\pm$ 8% inhibition) incubation with PAF (Table II, rows 2 and 3, respectively). Taken together, these results implicate p38 MAPK as playing a critical role in the fMLP-mediated activation process, rather than in the PAF-mediated priming step *per se*. Furthermore, these findings rationalize the conflicting findings of Partrick *et al.* (69) and Lal *et al.* (77), because the fMLP-based assays used in those studies could not distinguish between the effects of p38MAPK inhibitors on priming *versus* effects on oxidase activation.

To further verify these conclusions, we directly investigated the activation state of p38 MAPK in response to stimulation of human neutrophils with various activating agents (fMLP, OpZ, PMA, or buffer control), with or without prior priming by TNFα or PAF (Fig. 5). This assay also allowed us to investigate the possibility that neutrophil priming by TNFα and/or PAF somehow facilitated the subsequent activation of p38 MAPK by the activating ligand, regardless of whether the priming event itself involved p38 MAPK. Following activation, with or without priming, total neutrophil lysates were analyzed by SDS-PAGE and immunoblotted with an antibody that specifically recognizes the dual phospho-Thr-180/phospho-Tyr-182 motif on the T-loop of the activated form of p38 MAPK. As shown in Fig. 5, no active p38 MAPK was detected in resting cells (*lane 4*) or in unprimed cells stimulated with OpZ as the agonist (*lane 2*). In contrast, some p38 MAPK activation was observed in these unprimed neutrophils upon activation by either fMLP or PMA (*lanes 1* and *3*, respectively). Priming by TNFα resulted in very strong activation of p38 MAPK, both in the absence or presence of subsequent incubation with an activating agonist (*lanes 5–8*). In contrast, PAF priming resulted in only a small amount of p38 MAPK activation, with levels comparable to or below those observed in unprimed cells activated by fMLP (compare *lanes 12* and *1*). Importantly, neither TNFα- or PAF-priming significantly increased the subsequent p38 MAPK activation response produced by the activating agonists. These findings indicate that (1) both fMLP and PMA, but not OpZ, directly activate p38 MAPK during the oxidase activation step, and (2) TNFα and PAF do not prime for a subsequent enhancement of p38 MAPK activation upon ligand-induced stimulation of the NADPH oxidase.

Incubation of Neutrophils with TNFα Results in SB203580-inhibitable Changes in Phosphorylation of p47^{phox} and p67^{phox}—One role of p38 MAPK and other protein kinase signaling pathways involved in neutrophil priming might involve the direct post-translational modification of p47^{phox} or p67^{phox} proteins, as suggested by others (12, 91–94). To investigate this, p47^{phox} and p67^{phox} from resting neutrophils, TNFα-primed neutrophils, SB203580-treated/TNF-α primed neutrophils, and PAF-primed neutrophils were analyzed by two-dimensional gel electrophoresis and immunoblotting. Both p47^{phox} and p67^{phox} proteins from resting cells exhibited a relatively narrow spread of isoelectric points, consistent with a small amount of basal phosphorylation (Fig. 6, *top row*). (A small fraction of the p67^{phox} from these resting cells migrated at more acidic pI values probably because of inadvertent priming during handling of the cells, because these more acidic spots were eliminated in SB203580-treated cells (*row 4*)). Incubation of the cells with TNFα shifted the isoelectric points of both the p47^{phox} and p67^{phox} species to more acidic pI values, consistent with increased phosphorylation (Fig. 6, *3rd row*). The appearance of these phosphorylated forms was completely prevented by inclusion of SB203580 prior to TNFα treatment (Fig. 6, *4th row*). Treatment of the cells with PAF did not alter the isoelectric profile of p47^{phox}, but did result in the production of multiple acidic p67^{phox} species similar to those seen after TNFα treatment (Fig. 6, *2nd row*). We interpret these data to indicate that neutrophil priming by TNFα induces phosphorylation of p47^{phox} and p67^{phox} via a p38-dependent process, whereas neutrophil priming by PAF, which is p38-independent for OpZ responses, results only in enhanced p67^{phox} phosphorylation.

Neutrophil Priming by TNFα Causes Minimal Increases in the Level of Membrane-associated p47^{phox} or p67^{phox}—During activation of the NADPH oxidase, p47^{phox} and p67^{phox} undergo

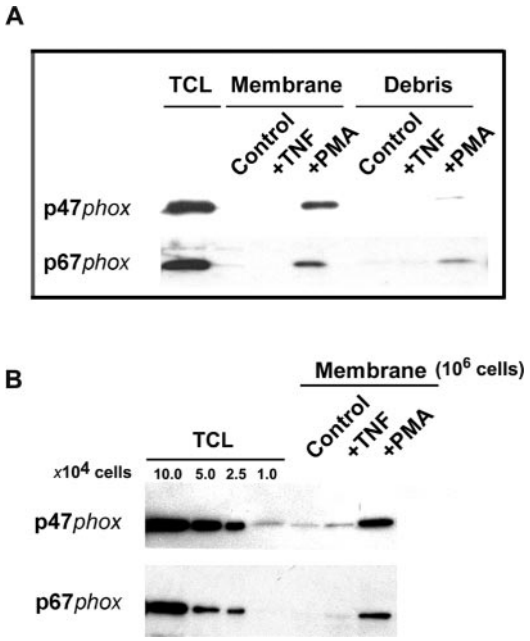

FIG. 7. **Neutrophil priming by TNFα does not induce translocation of p47^{phox} or p67^{phox} to the membrane.** *A*, neutrophils were incubated without ligands (*Control*), with the priming agent TNFα (47 pM) or with the activating agent PMA (1 μg/ml) for 15 min at 37 °C. Cells were disrupted by sonication and a low speed pellet corresponding to debris, and a high speed pellet enriched in neutrophil membranes was prepared as described under "Experimental Procedures." Samples were analyzed by SDS-PAGE followed by immunoblotting for p47^{phox} and p67^{phox}. *TCL* denotes a total cell lysate control for p47^{phox} and p67^{phox} immunoreactivity. *B*, the membrane fraction from 1×10^6 control cells, TNFα-primed cells, or PMA-stimulated cells was quantitated for p47^{phox} and p67^{phox} translocation by immunoblotting. The *left 4 lanes* contain total cell lysate (*TCL*) standards corresponding to 1.0, 2.5, 5.0, and 10.0×10^4 total neutrophils.

multiple phosphorylation events and translocate from the cytoplasm to the membrane where they associate with cytochrome b_{558} to form a catalytically competent enzyme complex capable of transferring electrons from NADPH to molecular oxygen to form superoxide (1, 12, 14, 15). This suggested that the increase in oxidase activity and the decrease in lag time for ROS production seen in primed neutrophils might result from pre-assembly of the oxidase holoenzyme complex at the membrane in a form that could then be rapidly and efficiently activated when the cells are exposed to an additional activating stimulus. To investigate this, we examined the association of p47^{phox} and p67^{phox} with neutrophil membranes before and after priming by 47 pM TNFα for 15 min. As a positive control, a replicate aliquot was incubated with 1 μg/ml PMA. The cells were then sonicated and separated into a low speed, easily sedimentable fraction containing any cellular debris and unbroken cells, a high speed sedimentable membrane fraction and a soluble cytosolic fraction.

As shown in Fig. 7*A*, minimal p47^{phox} and p67^{phox} were found in association with membrane preparations from 10^6 control cells or TNFα-primed cells. In contrast, upon NADPH oxidase activation by PMA, significant amounts of both p47^{phox} and p67^{phox} were easily detectable in the membrane fraction. The material in the debris pellets showed a similar pattern of phox protein association as that observed in the membrane preparations, but to a much lower extent. Analysis of this debris material, presumably the consequence of trapped membrane and unbroken cells, indicates that the lack of p47^{phox} and p67^{phox} seen in the membrane fraction of TNFα-treated cells was not the result of some type of priming-induced formation of insoluble aggregates that would have been missed by analysis of the membrane fraction alone. Identical data were obtained using porcine neutrophils (data not shown). To more rigorously quantitate the priming-induced association of p47^{phox} and p67^{phox} with the membrane, Western blotting was repeated using isolated membrane preparations and graded amounts of whole neutrophil lysate. As shown in Fig. 7*B*, in the TNFα-primed but non-activated samples, the amount of membrane-associated p47^{phox} and p67^{phox} was on the order of 1%. In contrast, treatment of neutrophils with PMA resulted in translocation of 5–10% of the total p47^{phox} and p67^{phox} to the membranes under these conditions, in reasonably good agreement with the 10–18% of p47^{phox} and p67^{phox} reported to become membrane-associated in PMA-treated PMNs by Quinn *et al.* (11). Because activation of the TNFα-primed neutrophils results in significantly more ROS production than that observed following PMA treatment, we conclude that the p38 MAPK-mediated process of neutrophil priming by TNFα does not appear to involve the stable pre-assembly of large amounts of p47^{phox}- or p67^{phox}-containing complexes at the membranes prior to the activation step.

DISCUSSION

Elucidating the signal transduction pathways involved in priming and activation of the NADPH oxidase is important for understanding the pathophysiology of many diseases involving neutrophil-mediated autoinflammatory tissue injury. These diseases include the adult respiratory distress syndrome and multisystem organ failure observed after trauma and sepsis (5, 41, 47–49, 52, 95), and ischemia reperfusion injury to the brain, heart, and kidney (66, 96, 97). The involvement of the p38 MAPK pathway in priming and activation of the neutrophil NADPH oxidase has been a subject of considerable controversy and confusion in the literature. Because p38 MAPK inhibitors are likely to be available for clinical use in the near future, it is important to clarify the exact role of this pathway in neutrophils to determine the rational basis for their use. We therefore, investigated the roles of p38 MAPK in neutrophil NADPH oxidase function by devising a 2-step assay that specifically distinguished p38 MAPK-dependent events in priming from p38 MAPK-dependent events involved in oxidase activation.

Using this assay, we observed that p38 MAPK plays a critical role in neutrophil priming by TNFα, but a much smaller role, if any, in the PAF priming process, for ROS production by the NADPH oxidase in response to OpZ. An essentially identical pattern of p38 MAPK-dependent and -independent signaling responses to TNFα and PAF priming for OpZ responses was observed with human and porcine neutrophils, suggesting that the relevant signaling pathways involved have been evolutionarily conserved. In agreement with the physiological readout of ROS production, we found that p38 MAPK activation in response to TNFα priming is much more pronounced than that observed after PAF priming using both an *in vitro* kinase assay to measure p38 MAPK activity directly (Fig. 3) and by examining the phosophorylation-dependent activation state of p38 MAPK itself (Fig. 5).

We found that p38 MAPK is important in the activation of the NADPH oxidase by fMLP independently of the priming agent, because ~70% of the fMLP-mediated ROS response was eliminated by SB203580 regardless of whether the drug was added before or after the priming process. This 70% inhibition is in excellent agreement with similar values observed by Lal *et al.* (77) and Yan *et al.* (75), and fits with prior observations of fMLP-mediated stimulation of p38 MAPK reported by others (70, 73, 98), The residual ~30% of ROS production that is not SB203580-inhibitable suggests the existence of multiple pathways connecting fMLP to the NADPH oxidase, only one of which involves p38 MAPK. In contrast to fMLP, p38 MAPK

does not be appear to be involved at all in activation of the NADPH oxidase by OpZ. Furthermore, priming of the oxidase response by TNFα or PAF does not result from enhancing the subsequent p38 MAPK response upon activation, above that seen from the sum of the TNFα or PAF response plus the activating ligand response alone (Fig. 5).

We believe that these results resolve much of the conflicting literature regarding the roles of p38 MAPK in neutrophil function, demonstrating roles for p38 MAPK in both priming and activation for some priming and activating agents but not for others. Our results are in best agreement with the studies of McLeish *et al.* (72) and Zu *et al.* (70), both of which showed that treatment of neutrophils in suspension with TNFα resulted in activation of p38 MAPK. The Zu *et al.* (70) study showed that SB203580 blocked ROS production in response to TNFα; however, that study used activating doses of TNFα rather than priming doses, so a specific effect of p38 MAPK on priming could not be determined. McLeish *et al.* (72) reported that, in addition to activation of p38 MAPK, priming doses of TNFα caused a 3–10-fold increase in Erk kinase activity. We were unable to reproduce this observation (Fig. 3), and our finding is in agreement with a similar lack of Erk activation in suspension neutrophils in response to TNFα seen by Zu *et al.* (70), Waterman and Sha'afi (99), and Rafiee *et al.* (100).

Our observation that p38 MAPK plays little if any role in PAF priming for ROS production in response to OpZ using a 2-step assay that separates priming from activation differs from the conclusions reached by Partrick *et al.* (69) who postulated an important role for p38 MAPK in PAF priming for ROS production in response to fMLP. In that study, which employed SB203580 in a 1-step priming and activation assay, the authors clearly appreciated the difficulty in separating the role of p38 MAPKs role in PAF priming from its role in the fMLP activation process, and attempted to overcome this by using PMA as an alternative activating agent. Our data (Fig. 5), and that from others, however, shows that p38 MAPK is also activated in response to PMA (101), and may play a direct role in PMA-mediated activation of the NADPH oxidase (77), although this latter point remains controversial (69). Finally, our results directly contradict the conclusions of Lal *et al.* (77), who claimed that p38 MAPK played no role in neutrophil priming whatsoever. Their experiments, however, relied solely on fMLP-mediated activation of the neutrophil NADPH oxidase to assess the role of p38 MAPK in priming, and therefore, could not distinguish between SB203580 effects on priming *versus* those on fMLP-mediated activation.

Thus in support of our earlier hypothesis (76), p38 plays roles in either/both priming or/and activation of respiratory burst activity, depending on the identity of both the priming agent and the activating ligand. Our data emphasize the importance of separating priming events from activation events using the type of 2-step experiment described here, and strongly argue that studies elucidating the signal transduction pathways involved in priming of the neutrophil NADPH oxidase should be performed in the context of specific activating agents. The wide variety of known priming agents and activating agonists suggests that priming and activation can occur by multiple mechanisms involving p38-MAPK dependent and p38 MAPK-independent processes, and caution against generalizations involving the role of specific kinase pathways in a global neutrophil priming process.

The molecular basis for neutrophil priming of the NADPH oxidase remains obscure. We found that neutrophil priming by TNFα resulted in only a small amount of stable translocation of $p47^{phox}$ or $p67^{phox}$ to intracellular or plasma membranes when the cells were lysed and separated into membrane and cytosol fractions (Fig. 7). Similarly, we previously failed to observe an association of $p47^{phox}$ or $p67^{phox}$ with the membranes when whole cells were primed by TNFα or PAF and then permeabilized with Streptolysin-*O* to deplete them of soluble cytosolic proteins *in situ* (26). Likewise, Dang *et al.* (71) did not observe translocation of $p47^{phox}$ or $p67^{phox}$ to neutrophil membranes in response to GM-CSF priming. Together, these results argue that the increased production of ROS in primed neutrophils does not result from pre-assembly of large amounts of a complete $gp91^{phox}/p22^{phox}/p47^{phox}/p67^{phox}/p40^{phox}$ holoenzyme at the membrane during the priming process.

We found that in response to neutrophil priming by TNFα, which activates p38 MAPK, both $p47^{phox}$ and $p67^{phox}$ displayed more acidic isoelectric points on two-dimensional gels, consistent with priming-induced phosphorylation of both $p47^{phox}$ and $p67^{phox}$ subunits *in vivo*. Furthermore, these TNFα-mediated phosphorylation events were blocked when the cells were pretreated with the p38 MAPK inhibitor SB203580. These results fit well with a detailed series of studies by El-Benna and co-workers that show that p38 MAPK can directly phosphorylate $p47^{phox}$ and $p67^{phox}$ *in vitro* (91, 92), and that TNFα treatment of human neutrophils results in $p47^{phox}$ phosphorylation *in vivo* (102). In contrast to the results obtained with TNFα, we found that only the $p67^{phox}$ subunit appeared to undergo phosphorylation in response to PAF priming (Fig. 6). The significance of these distinct $p47^{phox}$ and $p67^{phox}$ phosphorylation events is currently being explored. However, the most parsimonious explanation for neutrophil priming would appear to involve, at the minimum, the specific phosphorylation of $p67^{phox}$, because this modification was observed by two different priming agents, which appear to utilize distinct signaling pathways. We expect in future work to address this $p67^{phox}$ phosphorylation event in molecular detail, focusing on its mechanistic significance to the processes underlying increased NADPH oxidase activity within primed neutrophils.

Acknowledgments—We thank Kevin Janes for technical assistance and R. C. Allen for helpful discussions. Financial and technical assistance from the Harvard Center for Minimally Invasive Surgery is gratefully acknowledged.

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