Activation of p38 Mitogen-activated Protein Kinase by Lipopolysaccharide in Human Neutrophils Requires Nitric Oxide-dependent cGMP Accumulation*

Darren D. Browning‡, Nancy D. Windes, and Richard D. Ye§

From the Department of Immunology, The Scripps Research Institute, La Jolla, California 92037

This study examined the signal transduction pathway(s) leading to phosphorylation of p38 in human neutrophils stimulated with lipopolysaccharide and formyl peptides. Blockade of the nitric oxide (NO) pathway in neutrophils with the NO synthase inhibitor N-nitro-l-arginine methyl ester or by treatment with the NO scavenger 2-phenyl-tetramethylimidazoline-1-oxyl-3-oxide attenuated phosphorylation of the mitogen-activated protein kinase p38 in response to lipopolysaccharide but not fMet-Leu-Phe. Using the NO releasing agents S-nitroso-N-acetylpenicillamine and sodium nitroprusside it was determined that nitric oxide is sufficient to cause an increase in phosphorylation of p38. Increasing cellular cGMP with phosphodiesterase inhibitors, by stimulation of soluble guanylyl cyclase with YC-1 or with exogenous dibutyl cGMP resulted in mitogen-activated protein kinase/extracellular signal-regulated kinase 3,6 (MEK3,6) activation and phosphorylation of p38. This phenomenon was specific for MEK3,6, because these agents had no effect on the phosphorylation state of MEK1,2. A role for protein kinase G but not protein kinase A downstream of lipopolysaccharide but not formylmethionylleucylphenylalanine was shown using the specific inhibitors KT5823 and H89, respectively. These data indicate that activation of p38 by fMet-Leu-Phe and lipopolysaccharide involve different mechanisms, and that activation of protein kinase G by NO-dependent stimulation of guanylyl cyclase is necessary and sufficient for phosphorylation of p38 downstream of lipopolysaccharide.

Detection of microbial products such as lipopolysaccharide (LPS)1 and N-formyl peptides (fMLF) by neutrophils is an essential event that triggers the microbicidal functions of these cells. Activation of neutrophils by such stimuli results in chemotaxis to inflammatory sites, cytokine production, release of degradative granule enzymes, and generation of toxic oxygen intermediates.

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‡ Established Investigator of the American Heart Association. To whom correspondence should be addressed: Dept. of Pharmacology (MC 868), University of Illinois, 835 S. Wolcott Ave., Chicago, IL 60612. Tel.: 312-996-5087; Fax: 312-996-7857.

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1 The abbreviations used are: LPS, lipopolysaccharide; fMLF, formylmethionylleucylphenylalanine; MAP, mitogen-activate protein; ERK, extracellular signal-regulated kinase; MEK, MAP/ERK kinase; NO, nitric oxide; IBMX, 3-isobutyl-1-methylxanthine; PDE, phosphodiesterase; PKG, protein kinase G.

The MAP kinases are members of discrete signaling cascades that form focal points for diverse extracellular stimuli and function to regulate fundamental cellular processes. Three distinct classes within the MAP kinase family have been described, including ERK, c-jun-NH2 kinase, and p38, each having different physiological roles (1–4). The p38 MAP kinase is associated with immune cell activation, because this kinase is activated by a variety of inflammatory mediators (5, 6). It is well established that p38 has an important role in gene expression in monocytes and macrophages (7–9). A specific function for p38 in neutrophils remains unclear, but it has been suggested to play an important role in the respiratory burst, interleukin 8 production, and apoptosis (10–13). Activation of p38 occurs after phosphorylation of a distinctive TGY motif by the upstream kinases MEK3 or MEK6 (6, 14–16). Stimulation of human neutrophils with classical chemoattractants, including fMLF, results in a rapid and transient phosphorylation and activation of p38 (12, 13, 17–20). It has also been demonstrated that challenge of neutrophils with LPS leads to activation of p38 but with slower kinetics than G-protein-coupled chemoattractant receptors (17, 19).

The molecular mechanism(s) leading to activation of ERK have been largely unraveled, but the regulation of components upstream of p38 remain to be clarified. Although recent reports have examined signaling downstream of fMLF, little attention has focused on the mechanism for LPS-mediated activation of p38 in neutrophils (18, 20). In Jurkat T-cells, long-term exposure to nitric oxide (NO) leads to activation of several MAP kinases, with ERK being the most responsive (21). LPS induces NO production in neutrophils, and this process is thought to mediate many of its inflammatory activities (22–27). Stimulation of neutrophils with fMLF can also induce NO production, which is thought to play an important role in chemotaxis and degranulation elicited by this agent (28–32). The mechanism of action for NO to regulate cell physiology is not clear; however, a key mediator is cGMP, which is generated by direct activation of soluble guanylyl cyclase.

In this study we examined the role of NO in the activation of p38 MAP kinase by LPS and by fMLF in human neutrophils. It was found that NO formation is sufficient to cause phosphorylation of p38 and that its formation is a prerequisite to p38 activation by LPS but not by fMLF. Furthermore, it was found that the mechanism of action involves an increase in intracellular cGMP and possibly activation of protein kinase G.

EXPERIMENTAL PROCEDURES

Reagents—Inhibitors of the nitric oxide system, including N-nitro-l-arginine methyl ester, 2-phenyl-tetramethylimidazoline-1-oxyl-3-oxide, and the NO releasing agents S-nitroso-N-acetylenicillamine, sodium nitroprusside, and N-acetyl-l-cysteine, the phosphodiesterase inhibitors 3-isobutyl-1-methylxanthine (IBMX) and 4-(methyleneedioxy)-benzylamino-6-methoxyquinazoline, RO-20-1724, and the protein kinase G inhibitor KT5823 were purchased from Calbiochem. The guanylyl cy-

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class stimulator YC-1 was from Alexis Biochemicals (San Diego, CA). Dibutyryl cGMP and fMLF were from Sigma; LPS from Salmonella Minnesota was purchased from Calbiochem. Rabbit anti-p38 serum was a generous gift from Dr. J. Han ( Scripps Research Institute). All other antibodies, including those directed against the phosphorylated forms of MEK3,6, MEK1,2, and p38, were purchased from New England Biolabs (Beverly, MA) unless otherwise indicated.

Preparation of Neutrophils from Whole Blood—Neutrophils were prepared from the blood of healthy donors using Percoll gradient (Amersham Pharmacia Biotech) as published elsewhere (33). In brief, coagulation of the blood was prevented using citrate-dextrose during collection, and the erythrocytes were sedimented by adding 0.5 volume of 65% dextran-tetra-starch (Abbott) at room temperature for 45 min. The erythrocyte-depleted supernatants were then layered on top of 50% x g. Neutrophils were then resuspended in serum-free RPMI 1640 medium at a density of 5 x 10^6 cells/ml and maintained at 37 °C.

Assay for Phosphorylation of p38 and MEK—In experiments requiring pretreatment with inhibitors, cells were incubated in microtubes for 30 min (unless otherwise indicated) rotating at 37 °C. In all cases cells were pelleted and resuspended in 0.25 ml of the same medium, (in some cases the incubating agents were added at this time), and the cells were incubated for various periods in a 37 °C water bath. Incubations were terminated by placing tubes on ice and diluting rapidly with 1 ml of ice-cold phosphate-buffered saline. Cells were then pelleted for 5 s in a microcentrifuge at 4 °C, and the supernatant was replaced with SDS-polyacrylamide gel electrophoresis sample buffer.

Gel Electrophoresis and Western Blotting—After resuspension in sample buffer, cell extracts were prepared for electrophoresis by boiling for 6 min followed by clarification by centrifugation. Equal amounts of protein (∼20 μg in 10 μl) of each extract were then loaded onto 10% polyacrylamide minigels and separated using 30 μl/Aggel. Protein profiles were transferred to supported nitrocellulose membranes, which were then blocked by incubating in phosphate-buffered saline containing 0.025% Tween 20 and 4% bovine serum albumin (PBS buffer) for 20 min at room temperature. In all cases the blots were probed by incubating without agitation with 1/1000 primary antibody in PBS at 4 °C overnight. MAP kinases on the blots were then visualized by rotating for 1 h at room temperature in PBS containing 1/2000 peroxidase-conjugated goat anti-rabbit IgG (PharMingen, La Jolla, CA) followed by chemiluminesence using enhanced reagents according to the manufacturer’s instructions (Pierce). Between incubation steps blots were washed twice by centrifugation at 500 g for 3 min at 12 °C. The supernatant was replaced with 0.025% Tween 20 and 4% bovine serum albumin (PBS buffer) for 30 min (unless otherwise indicated) rotating at 37 °C. In all cases cells were pelleted and resuspended in 0.25 ml of the same medium, (in some cases the incubating agents were added at this time), and the cells were incubated for various periods in a 37 °C water bath. Incubations were terminated by placing tubes on ice and diluting rapidly with 1 ml of ice-cold phosphate-buffered saline. Cells were then pelleted for 5 s in a microcentrifuge at 4 °C, and the supernatant was replaced with SDS-polyacrylamide gel electrophoresis sample buffer.

Role of Nitric Oxide in Formyl Peptide- and Lipopolysaccharide-mediated Phosphorylation of p38 in Human Neutrophils—It has been shown previously that LPS and the N-formyl peptide fMLF stimulate the phosphorylation and activation of p38 in human neutrophils (17, 18, 20, 34). MEK3 and MEK6 are upstream kinases that, on activation by phosphorylation, can recognize p38 as a substrate both in vitro and in vivo. The presence or activation of either of these kinases has not previously been demonstrated in neutrophils. As shown here, challenge with 100 nM fMLF caused a rapid and transient phosphorylation of MEK3,6 in neutrophils, with a maximum within 1 min of stimulation and a return to basal levels within 15 min (Fig. 1A). This phosphorylation leads to activation of MEK3,6, as indicated by the equally rapid and transient appearance of phospho-MEK3,6. Stimulation of neutrophils with LPS (10 μg/ml) also resulted in phosphorylation of MEK3,6, with a concomitant appearance of phospho-p38 (Fig. 1B). In agreement with previous findings (17), the LPS-mediated phosphorylation of p38 and MEK3,6 was slower than that of fMLF, increasing after 5 min, and was maximal between 15 and 30 min after stimulation. Because the kinetics of MEK3,6 pho-

RESULTS

The phosphorylation paralleled the appearance of phospho-p38 for both of these stimuli, it is likely that MEK3,6 is upstream of p38 in neutrophils as in other systems. In this study we did not address whether MEK3 or MEK6 (or both) was important in neutrophils, because our antibodies did not distinguish these closely related species. The signal detected on Western blots probed with phospho-MEK3,6 antibodies was very weak when compared with similar blots probed with phospho-MEK1,2 antibodies (see below), which suggests that these proteins are less abundant. This point supports previous findings, which have determined that MEK1 and MEK2 are the major isoforms found in neutrophils (35–37).

Both LPS and fMLF are able to induce NO formation in neutrophils, and this function contributes to the biological activity of these agents. Furthermore, in Jurkat cells NO can activate several MAP kinase pathways (21). To examine the relevance of NO formation in the phosphorylation of p38, neutrophils were treated with either the NO scavenger 2-phenyl-tetramethylimidazoline-1-oxyl-3-oxide or the NO-synthase inhibitor N-nitro-l-arginine methyl ester before stimulation with fMLF and LPS. The increase in phospho-p38 levels measured 20 min after stimulation with LPS was dose-dependently inhibited by both 2-phenyl-tetramethylimidazoline-1-oxyl-3-oxide and N-nitro-l-arginine methyl ester (Fig. 2, A and B). In contrast, neither of these drugs was capable of reducing fMLF-induced phosphorylation of p38 even at doses 100-fold over the published EC50 (Fig. 2, C and D). These data support a physiological role for NO formation in the activation of p38 in neutrophils by LPS but not by fMLF.

Because NO production appeared to have a role in the activation of p38 MAP kinase, it was of interest to determine whether NO was sufficient to stimulate this pathway. Although several NO-releasing compounds are commercially available, some of these are problematic in neutrophils (38). We chose to use agents that have been shown previously to be effective in neutrophils, including S-nitroso-N-acetylpenicillamine, and a combination of sodium nitroprusside (an NO-releasing agent) and N-acetyl-l-cysteine (increases availability of NO) (26, 27). Under the conditions used here, incubation of neutrophils in medium without NO-releasing agents for periods of up to 30 min did not result in significant levels of phospho-p38 (Fig. 3A). However, in the presence of the NO-releasing agents S-nitroso-N-acetylpenicillamine and sodium nitroprusside/N-acetyl-l-cysteine, there was a notable increase.
in the phosphorylation of p38 (Fig. 3, B and C). In both cases the increase in phospho-p38 was detectable within 20 min and increased in amount throughout the period investigated.

**Exogenous Elevation of Intracellular cGMP Levels Results in Activation of the Pathway Leading to Phosphorylation of p38**

The biological effects of NO formation are generally assumed to be attributable to the activation of soluble guanylyl cyclase, leading to cGMP accumulation (39–41). If this mechanism underlies the NO-mediated activation of p38, as measured here, then it follows that cGMP should also play an important role. To examine this question we used several different methods to increase the intracellular concentration of this messenger. Initial studies used phosphodiesterase (PDE) inhibitors, which presumably lead to an increase in basal cGMP levels by preventing cyclic nucleotide degradation. Treatment of neutrophils with the broad-range PDE inhibitor IBMX resulted in an increase in phospho-MEK3,6 and phospho-p38 after 1 min of incubation in the drug with maximal levels at 15 min (Fig. 4A). Although IBMX has been reported to have agonist activity to adenosine receptors, the phosphorylation of p38 by this drug was not affected by other adenosine agonists (data not shown). There are several PDEs known to be expressed in different mammalian tissues. In neutrophils the expression of both the cAMP-specific (PDE-IV) and the cGMP-specific (PDE-V) isoforms have been demonstrated (42–44). Treatment of neutrophils with RO-20-1724, a specific inhibitor of the cAMP-specific PDE-IV, was unable to increase the level of phosphorylated MEK3,6 in similar experiments (Fig. 4B). In contrast, it was found that the specific inhibitor of PDE-V 4-(methylenedioxy)-benzylamino-6-methoxyquinazoline) was equal to IBMX in its ability to increase levels of the phosphorylated forms of both MEK3,6 and p38 in neutrophils, which indicated an important role for cGMP in this phenomenon (Fig. 4C).

To lend support to the idea that cGMP but not cAMP is important to the activation of p38 in neutrophils, neutrophils were treated with activators of soluble guanylyl cyclase or of adenylyl cyclase. Activation of guanylyl cyclase by adding YC-1 to neutrophils resulted in a rapid phosphorylation of both p38 and MEK3,6, and this increased level remained stable within the 15-min period investigated (Fig. 5A). In contrast, increasing the intracellular cAMP concentration by treatment with forskolin did not change the levels of the phosphorylated forms of either p38 or MEK3,6 (Fig. 5B). These studies demonstrate that an elevation in the intracellular concentration specifically of cGMP is sufficient to cause phosphorylation of p38 in neutrophils.

Increasing the intracellular concentration of cGMP by treating cells with increasing doses of the membrane-permeable analog dibutyryl cGMP resulted in phosphorylation of both MEK3,6 and p38 (Fig. 6A). In these studies cGMP was effective at concentrations as low as 250 nm, and at 250 mC the resulting phosphorylation of p38 and MEK3,6 was equal to or greater (respectively) than levels produced by fMLF stimulation of the cells. The similarity in the phosphorylation of MEK3,6 and p38,
both in terms of the effect of PDE inhibition and the dose response for dibutyryl cGMP, further supports the role of MEK3,6 in the regulation of p38 activity in neutrophils. In addition, these data point to cGMP-sensitive regulation of components upstream of MEK3,6 in neutrophils. Treatment of freshly isolated neutrophils with 250 mM dibutyryl cGMP caused a rapid and transient phosphorylation of MEK3 and p38, with maximal levels detected within 1 min after addition of this reagent to the cells. Using antibodies specific to the phosphorylated domain of other MEK isoforms, we found that the effect of cGMP was specific to MEK3,6, because neither MEK1,2 (Fig. 6) nor MEK4 (not shown) was affected by dibutyryl cGMP.

**Discussion**

Stimulation of neutrophils with LPS or fMLF leads to activation of several MAP kinase pathways, although only MEK1,2 has been identified in these cells. In this study it was demonstrated that both LPS and fMLF were able to transiently activate MEK3,6 kinase activity in neutrophils. Both fMLF and LPS cause endogenous nitric oxide formation in neutrophils. Recent work by Lander and colleagues (21) has demonstrated activation of ERK, c-jun-NH₂-kinase, and p38 kinase activities after stimulation of Jurkat T-cells with NO. As shown here in human neutrophils, inhibition of the NO pathway was able to inhibit phosphorylation of p38 downstream of LPS but not fMLF. This result indicates that NO functions specifically downstream of LPS in p38 MAP kinase activation, and that fMLF must use different signaling pathways to achieve the same end. The different kinetics of p38 phosphorylation with fMLF reaching a maximum at 1–2 min, whereas LPS activated p38 only after 10–20 min, might reflect these underlying differences in signaling mechanism. Other work by Lander et al. (45–47) has shown that p21ras is a target for NO-related free radicals, which might explain the activation of ERK in this system. It is not clear from their studies how NO might stimulate p38 MAP kinase activity in their system, or which stimuli might use this pathway to activate MAP kinases. Because activation of soluble guanylyl cyclase by NO is well documented, in this report we examined the role of cGMP in the signaling pathway leading to activation of p38 in neutrophils. It was found that phosphorylation of MEK3,6 was induced by

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**Fig. 4.** Inhibition of cGMP-phosphodiesterase leads to activation of MEK3,6 in neutrophils. Freshly isolated neutrophils were incubated with the nonspecific phosphodiesterase inhibitor IBMX (A), with the cAMP-specific inhibitor RO-20-1724 (RO2; B), or with the cGMP-specific phosphodiesterase inhibitor 4-(methylenedioxy)-benzylamino-6-methoxyquinazoline (MBQ; C) for up to 15 min at 37 °C. At the indicated times cells were harvested, and the phosphorylated forms of MEK3,6 and p38 were measured by Western blotting as detailed under “Experimental Procedures.” The lower panel reflects the protein-loading pattern of the above gels as determined by staining for total p38. Results shown are representative of at least three donors.

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**Fig. 5.** Increased cGMP levels lead to phosphorylation of p38 in neutrophils. The intracellular cyclic nucleotide levels were increased by incubating freshly isolated neutrophils in the activator of soluble guanylyl cyclase YC-1 (A) or the activator of adenyl cyclase forskolin. At the indicated times cells were harvested for analysis of phospho-p38 content using Western blotting as detailed under “Experimental Procedures.” The lower panel shows the same gel reprobed with an antibody directed at the unphosphorylated form of p38. Gels shown were reproduced with similar results in three independent experiments.
increasing intracellular cGMP levels in several different ways: by inhibition of PDEs, by ligand-independent activation of soluble guanylyl cyclase, or by addition of dibutylryl cGMP. The increase in phospho-MEK3,6 levels initiated by each of these treatments corresponded with activation of MEK3,6 kinase activity, as measured by the in vivo phosphorylation of its physiological substrate p38. Because both LPS and fMLF stimulation of neutrophils leads to the phosphorylation of p38, it would follow that signals generated by each ligand must converge at some point upstream of p38. Results shown here suggest that this point of convergence is likely to be at, or upstream of, MEK3,6 activation. In contrast to the previous studies using Jurkat cells, results shown here demonstrate that the MEK3,6/p38 pathway is specifically phosphorylated downstream of the NO system and that activation of the MEK1,2/ERK pathway or the MEK4/c-Jun-NH2-kinase pathway was not affected. This result suggests that NO-related species might signal to the MAP kinases using more than one mechanism and indicates cell type-specific differences in the responsive signaling pathways. To extend these findings, we show that the cGMP-dependent phosphorylation of p38 in neutrophils requires activation of PKG. The lack of effect of cAMP-elevating agents and of the inhibitor of cAMP-dependent protein kinase suggests that this pathway does not play an important role in the activation of p38 by LPS in this system. It is not known from our studies where in the p38 signaling pathway PKG is likely to function, because many proteins have been reported to have a role upstream of MEK3,6. Because the number of substrates for PKG that have been characterized in any system is few, and those that might contribute to activation of MAP kinase pathways have not been explored, this remains a question for future endeavors.

LPS and fMLF use fundamentally different signaling mechanisms; the former is incompletely understood, whereas that latter uses activation of G-proteins. The differential effect of NO inhibitors on fMLF- and LPS-stimulated p38 phosphorylation reveals that there are different biochemical pathways capable of activating p38 phosphorylation in neutrophils. It remains to be determined whether other stimuli such as tumor necrosis factor α or interleukin 1β, which activate p38 with similar kinetics to LPS as measured here, also use NO generation as a mediator of MAP kinase activation. The mechanism used by fMLF to activate p38 in neutrophils requires both phosphatidylinositol 3-kinase and calcium-dependent activation of protein kinase C (18, 20). Other work has shown that fMLF causes PKG to co-localize with intermediate filaments and to phosphorylate vimentin in a calcium-dependent manner, and speculation has suggested that in this context, PKG is involved in cytoskeletal rearrangement associated with the degranulation process (28, 40, 48, 49). However, results shown here would suggest that PKG does not contribute to the phosphorylation of p38 in response to stimulation with fMLF.

Previous work has shown that NO can contribute to cytokine gene expression in neutrophils (26, 50). Nitric oxide has also been associated with the regulation of gene expression in endothelial cells (51–54). Activation of p38 has also been associ-
ated with transcription factor activation (1, 8). Recent work has demonstrated that in LPS-stimulated neutrophils and monocytes, p38 is involved in cytokine production (9, 55). Thus it is likely that the activation of p38 MAP kinase by NO contributes to the inflammatory response in these cells through regulation of cytokine gene expression. It has been shown that p38 activation is important to the up-regulation of iNOS in glial cells and astrocytes (52, 56). Although this has not been demonstrated for myeloid cells, it is an intriguing possibility that longer-term activation of p38 might create an autonomous regulatory loop where the increased NO would help maintain or augment cytokine expression. It has been shown that p38 activation is important to the up-regulation of iNOS in glial cells (41). In myeloid cells, it is an intriguing possibility that a regulatory loop where the increased NO would help maintain or augment downstream of NO formation but upstream of MEK3,6 in this pathway.

In summary, we have investigated the role of NO production on the activation of the p38 MAP kinase pathway in human neutrophils. It was found that exogenous NO caused phosphorylation of p38 in these cells. In addition, using inhibitors of NO synthesis it was shown that LPS but not fMLF used this pathway to activate p38. Preliminary investigations of the synthesis of p38 proteins demonstrated that LPS but not fMLF used this pathway to activate p38. Preliminary investigations of the synthesis of p38 proteins demonstrated that LPS but not fMLF used this pathway to activate p38. Preliminary investigations of the synthesis of p38 proteins demonstrated that LPS but not fMLF used this pathway to activate p38.