Involvement of protein tyrosine kinase in Toll-like receptor 4-mediated NF-κB activation in human peripheral blood monocytes

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Chen, Ling-Yu, Bruce L. Zuraw, Ming Zhao, Fu-Tong Liu, Shuang Huang, and Zhixing K. Pan. Involvement of protein tyrosine kinase in Toll-like receptor 4-mediated NF-κB activation in human peripheral blood monocytes. Am J Physiol Lung Cell Mol Physiol 284: L607–L613, 2003. First published December 20, 2002; 10.1152/ajplung.00116.2002.—Bacterial lipopolysaccharide (LPS) is a powerful activator of the innate immune system. Exposure to LPS induces an inflammatory reaction in the lung mediated primarily by human blood monocytes and alveolar macrophages, which release an array of inflammatory chemokines and cytokines including IL-8, TNF-α, IL-1β, and IL-6. The signaling mechanisms utilized by LPS to stimulate the release of cytokines and chemokines are still incompletely understood. Pretreatment with the protein tyrosine kinase-specific inhibitors genistein and herbimycin A effectively blocked LPS-induced NF-κB activation as well as IL-8 gene expression in human peripheral blood monocytes. However, when genistein was added 2 min after the addition of LPS, no inhibition was observed. Utilizing a communoprecipitation assay, we further showed that LPS-stimulated tyrosine phosphorylation of Toll-like receptor 4 (TLR4) may be involved in downstream signaling events induced by LPS. These findings provide evidence that LPS-induced NF-κB activation and IL-8 gene expression use a signaling pathway requiring protein tyrosine kinase and that such regulation may occur through tyrosine phosphorylation of TLR4.

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LPS, in particular the early intracellular events, that lead to monocyte/macrophage transcription activation, are still not clearly understood. The presence of tyrosine protein kinase activities, first described in several oncogene products of RNA tumor viruses and growth factor receptors (14), has been shown in neutrophils (12, 15), monocytes, and macrophages (36, 40). It is likely that the protein tyrosine kinases play an important role in transducing signals to the cell interior (16, 32).

Our hypothesis is that the protein tyrosine kinases play a key role in the LPS-stimulated signaling events that lead to NF-κB activation and proinflammatory cytokine gene expression in human peripheral blood monocytes. In this report we show that LPS-stimulated NF-κB activation and subsequent IL-8 gene expression are accompanied by tyrosine phosphorylation of TLR4 in peripheral blood monocytes and that inhibition of protein tyrosine kinases blocks LPS-stimulated NF-κB activation and IL-8 gene expression. Utilizing a coimmunoprecipitation assay, we further show that LPS-stimulated tyrosine phosphorylation of TLR4 may be involved in downstream signaling events induced by LPS. These results indicate that protein tyrosine kinase activity is an important signal transducer for LPS-induced NF-κB activation and proinflammatory cytokine gene expression and that such regulation may occur through the tyrosine phosphorylation of TLR4 in activated human peripheral blood monocytes.

EXPERIMENTAL PROCEDURES

Reagents. LPS isolated from Salmonella minnesota Re595 was a gift from R. Ulevitch (Scripps Research Institute). Recombinant murine TNF-α was kindly provided by V. Kravchenko (Scripps Research Institute). Actinomycin D, cycloheximide, and phorbol 12-myristate 13-acetate (PMA) were purchased from Sigma (St. Louis, MO). Recombinant human IL-1β, pyrrolidine dithiocarbamate (PDTC), herbinycin A, and genistein were obtained from Calbiochem (San Diego, CA). An antibody against a COOH-terminal peptide (residues 289–307) of IkB-α was a gift from Dr. W. C. Greene (University of California, San Francisco, CA). A rabbit polyclonal antibody against TLR4 was from Torrey Pines Biolabs (San Diego, CA), and an MAb against phosphotyrosine was purchased from Upstate Biotechnology (Lake Placid, NY).

Oligonucleotides and their complementary strands for electrophoretic mobility shift assays (EMSA) were from Promega (Madison, WI) and Santa Cruz Biotechnology. The sequences are: murine intronic κ-chain κB site (underlined), 5′-AGTTGAGGGGACTTT-CCCAGGC-3′ (NF-κB) (16). Double-stranded oligonucleotide (5 pmol) was 32P-labeled with T4 polynucleotide kinase. [γ-32P]ATP (>5,000 Ci/mmol) was from Amersham (Arlington Heights, IL).

Preparation of monocytes from peripheral blood. Heparinized human peripheral blood from healthy donors was fractionated on Percoll (Pharmacia) density gradients. Mononuclear cells and neutrophils were initially separated by centrifugation through a 55%/74% discontinuous Percoll gradient. Monocytes were further prepared from the mononuclear cell population by adherence to tissue culture flasks (27). The purity of monocytes was >80–90% as determined by staining with the anti-CD14 monoclonal antibody (Coulter Immunology, Miami, FL), and cell viability was >95% as measured by trypan blue exclusion. Monocytes were resuspended in RPMI 1640 medium (Invro Scientific, Santa Ana, CA) with 10% (vol/vol) heat-inactivated fetal bovine serum, penicillin (100 units/ml), streptomycin (100 μg/ml), and L-glutamine (2 mM; Irvine Scientific). All reagents were tested by Limulus amebocyte lysate assay (BioWhittaker) and contained <0.005 ng/ml of endotoxin.

Detection of immunoreactive IL-8. Monocytes were stimulated with LPS at a concentration of 10 ng/ml for various times up to 8 h. The conditioned media were collected, and secreted IL-8 was measured by enzyme-linked immunosorbent assay (ELISA) using a commercial kit (Genzyme) according to the manufacturer’s recommended protocol. The quantities of secreted IL-8 in the test samples were determined by a standard curve generated with purified IL-8.

Preparation of nuclear extracts. Nuclear extracts were prepared by a modified method of Dignam et al. (10). Monocytes were separated and plated at a density of 1 × 106 cells in six-well plates. After stimulation, cells were washed three times with ice-cold PBS, harvested, and resuspended in 0.4 ml of buffer A [10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM sulfo-NHS-SS-biotin (Pierce), 1 μM dithiothreitol, 0.5 mM dithiothreitol, 0.4 mg/ml poly(dI-dC) (Pharmacia), 0.1 mg/ml sonicated double-stranded salmon sperm DNA, and 10% glycerol] for 10 min at 4°C. Lysates were separated by centrifugation at 13,000 g for 10 s and then resuspended in 50 μl of buffer B (20 mM HEPES, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, and 0.1 mM PMSF). After 30 min at 4°C, lysates were separated by centrifugation (13,000 g, 30 s), and the supernatants containing nuclear proteins were transferred to new vials. The protein concentration of extracts was measured with a protein dye reagent (Bio-Rad) with bovine serum albumin as the standard, and the samples were diluted to an equal concentration in buffer B for use directly or storage at −80°C.

EMSA. We performed EMSA by incubating 2.5 μg of the nuclear extract in 12 μl of binding buffer [5 mM HEPES, pH 7.8, 5 mM MgCl2, 50 mM KCl, 0.5 mM dithiothreitol, 0.4 mg/ml poly(dI-dC) (Pharmacia), 0.1 mg/ml sonicated double-stranded salmon sperm DNA, and 10% glycerol] for 10 min at room temperature. The samples were analyzed on 6% acrylamide gels, which were made in 50 mM Tris-borate buffer containing either 1 mM EDTA or 50 mM Tris/380 mM glycine/2 mM EDTA, and were pre-electrophoresed for 2 h at 12 V/cm. Electrophoresis was carried out at the same voltage for 2–2.5 h. Gel contents were transferred to Whatman DE-81 paper, dried, and exposed for 3–5 h at −80°C with an intensifying screen. Using this method, one sometimes sees a nonspecific DNA-protein complex of unknown origin in the autoradiogram.

Immunoprecipitation and immunoblotting. Approximately 10 μg of cytoplasmic extracts, collected after the Nonidet P-40 lysis and centrifugation steps (see Preparation of nuclear extracts above), were incubated with an appropriate amount of antibody for 3 h and then precipitated following absorption onto protein A-Sepharose. Precipitates were washed three times, separated by SDS-PAGE, and transferred to Hybond-ECL nitrocellulose (Amersham). Filter strips were incubated with primary antibody for 30 min at room temperature, followed by addition of peroxidase-conjugated IgG at 1:10,000 for 30 min, and analysis with enhanced chemiluminescence reagents (DuPont-NEB). Luciferase activity assay. The plasmid pIL-8-8cBILUC (WT-IL-8-LUC) contains a κB site from the promoter region of the IL-8 gene, and a separate plasmid pIL-8(Mu)LUC (MU-IL-8-
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Fig. 1. Inhibition of LPS-induced κB binding activity and IL-8 protein production by pyrrolidine dithiocarbamate (PDTC). A: PDTC inhibits LPS-induced κB binding activity. Nuclear protein extracts were prepared from monocytes preincubated for 40 min with PDTC at the indicated concentrations, followed by LPS (10 ng/ml) stimulation for 60 min. The autoradiograph of EMSA results is shown. B: PDTC inhibits LPS-induced IL-8 protein production. Cells pretreated with PDTC, as indicated above, were stimulated with LPS (10 ng/ml) for 4 h and secreted IL-8 was measured by ELISA.

Fig. 2. Specificity of LPS-induced κB binding activity. A: monocytes were either unstimulated (medium, lane 5) or stimulated for 60 min with LPS (10 ng/ml, lanes 1–4). Nuclear extracts prepared from these cells were used for analysis of κB binding activity by EMSA in the presence (lanes 1–3) or absence (lanes 4 and 5) of competitive unlabeled oligonucleotides (Oligo). B: identification of p50 and p65 in the DNA-protein complexes induced by LPS. Nuclear extracts from cells stimulated with LPS (10 ng/ml for 60 min) were incubated in the presence of specific antibodies (Ab, 2 μg/sample) against members of the NF-κB/Rel protein family as noted below: lane 1, control (no antibody); lane 2, IgG from normal rabbit preimmune serum; lanes 3–7, antibodies against c-Rel (lane 3), p52 (lane 4), p50 (lane 5), and p65 (lane 6). Samples were analyzed by EMSA (5% gel in Tris-borate buffer containing Tris/glycine/EDTA) using an oligonucleotide probe containing the consensus κB sequence.

A

B

Control

PDTC (μM)

2

20

200

LPS

1

2

3

4

5

NF-κB

1

2

3

4

5

6

no Ab

Control Ab

c-rel

p52

p50

p65

NF-κB

Control

PDTC

LPS
were subsequently identified that belongs to the NF-κB/Rel family (3). We performed gel supershift assays to determine whether the LPS-induced DNA-protein complexes contain p50 and p65. With nuclear extracts from LPS-stimulated cells, only anti-p50 and anti-p65 antibodies induced a shift of the DNA-protein complexes (Fig. 2B), whereas anti-p52 and anti-c-Rel did not cause a supershift of the DNA-protein complex (Fig. 2B, lanes 3 and 4). The results suggest that LPS stimulate κB binding activity to p50 and p65, but not p52 and c-Rel under the assay conditions.

To further confirm the IL-8 gene expression was the result of LPS-induced NF-κB activation, we transfected the monocyte-like cell line THP1 with a plasmid containing the promoter region of the IL-8 gene fused to the luciferase reporter gene, to assess the effect of LPS-induced NF-κB activation on IL-8 transcription. The plasmid πIL-8(κB)LUC (WT-IL-8-LUC) contains κB site from the promoter region of the IL-8 gene and a separate plasmid πIL-8(mu)LUC (MU-IL-8-LUC) has nonfunctional mutant κB site. Both LPS (10 ng/ml) and PMA (100 nM) induced luciferase activity in cells transfected with the WT-IL-8-LUC (Fig. 3A) compared with the unstimulated control (P < 0.01). In contrast, neither LPS nor PMA caused a significant increase in luciferase activity in cells transfected with MU-IL-8-LUC (from which the κB site was deleted) compared with the cells transfected with WT-IL-8-LUC (Fig. 3B). Together, these results demonstrate that LPS stimulates IL-8 gene expression and that this is a consequence, at least in part, of NF-κB activation.

Protein tyrosine kinase activity is required for LPS-induced NF-κB activation and chemokine gene expression. Because protein tyrosine kinases play an important role in intracellular signal processes linked to diverse receptor types, we examined the role of protein tyrosine kinases in LPS-activated NF-κB and IL-8 gene expression. Genistein and herbimycin A have both been shown to specifically inhibit protein tyrosine kinases in multiple cell types with distinct and different modes of action (1, 37). After pretreatment with genistein, herbimycin A, or medium control, monocytes were stimulated with LPS. NF-κB activation was assessed by EMSA and IL-8 protein production was measured by ELISA. LPS-induced NF-κB activation (Fig. 4) and IL-8 production (not shown) were completely inhibited in monocytes pretreated with genistein (Fig. 4, lane 5) or herbimycin A (Fig. 4, lane 7), and genistein inhibited LPS-stimulated NF-κB activation in a dose-dependent manner (Fig. 5A). These results suggest that protein tyrosine kinase activity is required for LPS-induced NF-κB-mediated cytokine gene transcription. However, the step at which the tyrosine kinase functions and the molecules are tyrosine phosphorylated is not known. We attempted to dissect the transcription activation pathway temporally by intervening pharmacologically at different time points after LPS challenge. Monocytes were stimulated with LPS, and then genistein (100 nM) was added at different times (Fig. 5B). It was observed that genistein blocked NF-κB activation when added within 60 s of the LPS addition, but after this time the addition of genistein failed to inhibit the NF-κB. These results indicate that a tyrosine kinase-phosphorylated intermediate is important for the LPS-induced NF-κB activation and that the tyrosine phosphorylation of this molecule(s) occurs at very early stage to mediate this response.

LPS stimulates tyrosine phosphorylation of TLR4. To determine whether LPS stimulates tyrosine phosphorylation of its receptor, TLR4, we treated cell lysates from control and LPS-stimulated monocytes with antibody against TLR4, and the immunoprecipitates were resolved by SDS-PAGE and then detected using either

![Fig. 3. NF-κB regulation of IL-8 gene transcription induced by LPS. Monocytic cell line THP1 cells were transfected with 2 μg of WT-IL-8-LUC (A) or Mu-IL-8-LUC (B) using DEAE/dextran. After 48 h, cells were divided into 3 equal portions and incubated with or without LPS (100 ng/ml) for 5 h. Luciferase activity was determined using the Luciferase Assay System and Monolight 2010 luminometer.](http://ajplung.physiology.org/)

![Fig. 4. Tyrosine kinase is involved in LPS-induced κB binding activity in human blood monocytes. Cells were pretreated for 10 min with or without inhibitors (Gn, genistein, 50 μM; HA, herbimycin A, 1 μM), followed by LPS (10 ng/ml) stimulation for 60 min for κB binding activity. The EMSA autoradiograph is shown with the DNA-protein complex marked with a bracket and the unbound probe indicated by an arrow.](http://ajplung.physiology.org/)
antiphosphotyrosine antibody or anti-TLR4 antibody (Fig. 6A). As shown in Fig. 6A, LPS stimulated tyrosine phosphorylation of TLR4 in a time-dependent manner. To further confirm the effect of tyrosine phosphorylation, we pretreated monocytes with genistein (100 nM) for 10 min and significantly inhibited LPS-induced TLR4 tyrosine phosphorylation (Fig. 6B, lane 4). When monocytes were stimulated with LPS for 4 min and then genistein (100 nM) was added, the tyrosine phosphorylation of TLR4 was not significantly inhibited by genistein (Fig. 6B, lane 5).

**DISCUSSION**

The major outer membrane component of gram-negative bacteria, LPS, is one of the most important activators of the innate immune system, which involves the host defense against infection (20, 34). Monocytes/macrophages are among the cells that are most sensitive to LPS. Although a great deal has been learned during the past few years about the synthesis and release of proinflammatory cytokines by monocytes/macrophages, relatively little is known about the intracellular events that lead to cytokine gene transcription. To study the signaling mechanisms for LPS-stimulated cytokine gene expression, we have chosen to use NF-κB-driven IL-8 synthesis in monocyte as our primary model system. Using purified peripheral blood monocytes, we showed that PDTC, an antioxidant inhibitor of NF-κB, not only reduced LPS-induced NF-κB activation (Fig. 1A) but also abolished the IL-8 protein secretion (Fig. 1B). These observations suggest that LPS-stimulated IL-8 gene expression could be a consequence of NF-κB activation. To further confirm this hypothesis, we used IL-8 gene promoter-reporter constructs to assess the effect of LPS-induced NF-κB activation on the transcription of IL-8 gene in THP-1 monocyte-like cells. LPS stimulated luciferase activity when the promoter region contained a functional NF-κB site but not when the NF-κB site was mutated and non-functional (Fig. 3). Together with the previous data, these results demonstrate that LPS-stimulated IL-8 gene expression is a consequence, at least in part, of NF-κB activation.

Stimulation of human blood leukocytes with LPS is known to result in an increase of tyrosine phosphorylation (13, 39), which may contribute to the activation of proinflammatory cytokine gene expression (11). We therefore examined the role of protein tyrosine kinase in LPS-induced NF-κB activation IL-8 gene expression. Genistein and herbimycin A have both been shown to specifically inhibit protein tyrosine kinase with different and distinct modes of action. Genistein is a...
competitive inhibitor binding to the ATP-binding site of the protein tyrosine kinase (1); herbimycin A irreversibly inactivates protein tyrosine kinase by binding to the reactive SH domain of the kinase (37). Preincubation of monocytes with either genistein or herbimycin A completely abrogated LPS-induced NF-κB activation as well as IL-8 protein synthesis (both inhibitors alone had no effect on cell activation). These results demonstrate that protein tyrosine kinase is important for LPS-induced NF-κB activation. However, if genistein is added 60 s or more after the LPS challenge, the inhibition is not seen. Inhibition of tyrosine kinase after this time was too late to stop the relay of the signaling. These results suggest that within this time, LPS stimulates a tyrosine kinase involved in NF-κB activation and that early inhibition of the tyrosine kinase abolishes this response. The inability of genistein to block LPS-induced NF-κB activation when added more than 60 s after LPS implies that additional signaling pathways may be involved in LPS induced NF-κB activation. There is increasing evidence that TLR4 mediates LPS-induced signaling events, including activation of MAP kinases and NF-κB (7, 22, 45). On the basis of these results, we hypothesized that tyrosine phosphorylation of TLR4 may be involved in LPS-induced NF-κB activation in monocytes. To address this issue, we immunoprecipitated LPS-stimulated monocytes with antibody against the TLR4 and then detected them using anti-phosphotyrosine antibody. Our results demonstrate that LPS stimulates tyrosine phosphorylation of the TLR4 and that this effect of LPS inhibited by genistein (Fig. 6).

The mechanisms by which LPS induces tyrosine phosphorylation of TLR4 are not clear. Recent evidence from different groups has indicated that several molecules can autophosphorylate on tyrosine residues (8, 31, 44). Therefore, LPS-induced tyrosine phosphorylation of TLR4 may occur by an LPS-stimulated auto-phosphorylation mechanism, independently of other protein tyrosine kinases. Alternatively, increased TLR4 tyrosine phosphorylation could be a consequence of LPS-activated protein tyrosine kinases. The ability of genistein to inhibit both LPS-induced tyrosine phosphorylation and activation of NF-κB suggests that an activated protein tyrosine kinase is necessary for these LPS responses. Genistein inactivates protein tyrosine kinases by binding to the ATP-binding site of the tyrosine kinase. It is known that IκB phosphorylation involves two serine kinases, IKK-α and -β (9, 30, 41). These kinases have been shown to phosphorylate serine residues S32 and S36 of IκB following stimulation with the molecules known to activate NF-κB. These results suggest that genistein does not directly inhibit NF-κB. Thus it seems most likely that genistein inhibits an upstream protein tyrosine kinase that is necessary for the LPS-induced tyrosine phosphorylation of TLR4 and thereby activation of NF-κB.

The results presented above make two important points. First, LPS-stimulated IL-8 gene expression is a consequence of NF-κB activation in human peripheral blood monocytes, and this function of LPS requires protein tyrosine kinase. Second, LPS-stimulated tyrosine phosphorylation of TLR4 may contribute to the downstream signaling events induced by LPS. These results suggest that stimulation of LPS causes a rapid production of signals that induce the activation of NF-κB and that such regulation may occur through the tyrosine phosphorylation of TLR4.

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