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

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Submitted 17 April 2002; accepted in final form 11 December 2002

Bacterial lipopolysaccharide (LPS) is shed from bacteria during normal bacterial growth and during bacteriolysis. Monocytes/macrophages bind LPS and in response rapidly synthesize and secrete biologically active products including cytokines that can mediate inflammation. LPS has been shown to interact with a number of plasma elements including complement proteins, coagulation factors, HDL, and LPS-binding protein (LBP) (35). LBP is a 60-kDa serum glycoprotein synthesized in the liver, the concentration of which can increase 100 times during acute-phase reactions, such as occurs during bacterial infections. LBP binds to LPS on the surface of bacteria or LPS-coated particles and facilitates attachment of these particles to monocytes/macrophages (43). This binding occurs via CD14 (42). CD14 is not a transmembrane protein and lacks the ability to transduce cytoplasmic signal (38). Recently, the signal-transducing receptor for LPS was identified as a member of the Toll-like receptor family. Toll is a transmembrane receptor in Drosophila that is involved in the induction of an antifungal response (17). Activation of the Toll receptor results in the stimulation of several signaling molecules that are homologous to proteins involved in NF-κB in mammalian cells (5). Poltorak et al. (29) found that mutational inactivation of Tlr4 completely abolishes LPS signal transduction. These results document that Toll-like receptor 4 (TLR4) is the cellular LPS receptor. There is increasing evidence that TLR4 mediates LPS-induced signaling events, including activation of MAP kinases and NF-κB (8–10).

LPS is a powerful activator of the innate immune system, stimulating mononuclear phagocytes to synthesize an array of cytokines and chemokines that recruit inflammatory cells to the involved tissue as well as activating immune and inflammatory responses. Repeated exposure to inhaled LPS also occurs as a consequence of its nearly ubiquitous presence in environmental dust (23–25). Stimulation of monocytes/macrophages with LPS induces several cellular functions, including generation of a defined set of gene products, such as interleukin (IL)-1α, IL-1β, and IL-8. IL-8, a chemoattractant for neutrophils and eosinophils, has recently been considered to play a key role in the pathogenesis of lung inflammatory reactions (21, 26). The regulation of IL-8 gene expression in these cells is governed by the activities of transcription factors. NF-κB is an important transcription factor to immune cell function owing to its ability to activate the transcription of many proinflammatory immediate-early genes (4, 33). Numerous stimuli can activate NF-κB, including the bacteria-driven chemoattractant formyl-Met-Leu-Phe, as well as other proinflammatory factors, including IL-1, TNF-α, and LPS (6, 28).

Although the activation of NF-κB has been extensively studied in cultured cell lines of hematopoietic lineage, the signal transduction pathways induced by...
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 communoprecipitation 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), herbimycin A, and genistein were obtained from Calbiochem (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/mmole) 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 (Irvine 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 separately 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 EGTA, 1 mM dithiothreitol, 5 mM MgCl2, 0.5 mM phenylmethylsulfonyl fluoride (PMSF)]. After 10 min, 20 μl of 10% Nonidet P-40 were added and mixed for 2 s. Nuclei were separated from cytosol 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. Then ~20–50 fmol of 32P-labeled oligonucleotide probe (30,000–50,000 counts per min) were added, and the reaction mixture was incubated 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 autoradiograph.

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-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-
LPS-stimulated production of IL-8 involves transcription activation. To assess the relationship between LPS stimulation and synthesis of proinflammatory cytokines, we examined the effects of LPS on IL-8 synthesis in monocytes. IL-8, a prototypic proinflammatory chemokine, induces the expression of a variety of genes whose products are involved in acute and chronic inflammatory conditions. Unstimulated human peripheral blood monocytes produce little IL-8. Addition of LPS (10 ng/ml) resulted in a time-dependent production of IL-8 as measured by ELISA. Pretreatment with either actinomycin D or cycloheximide completely inhibited LPS-induced IL-8 protein synthesis (not shown). These results indicate that LPS stimulates de novo IL-8 protein synthesis in monocytes, which is consistent with what has been previously reported. We previously demonstrated that LPS stimulates NF-κB activity in monocytes (28), suggesting that activation of NF-κB may be involved in LPS-stimulated IL-8 gene expression. To test this hypothesis, we examined the effect of PDTC on both IL-8 gene expression and NF-κB activation. PDTC is an antioxidant that blocks the dissociation of IκB from the cytoplasmic NF-κB, thus preventing the activation and nuclear translocation of NF-κB (2). As shown in Fig. 1A, PDTC treatment of monocytes significantly inhibited LPS-induced expression of IL-8. The same treatment almost completely blocked the activation of NF-κB by LPS (Fig. 1B), suggesting that NF-κB activation is required for LPS-stimulated IL-8 gene expression.

To test the specificity of the observed DNA-protein interaction, we included an excess amount of unlabeled κB oligonucleotide in the EMSA using monocytes stimulated with LPS. Unlabeled probe at 10-fold and 100-fold molar excess successfully competed with the labeled probe (Fig. 2A). The prototypic NF-κB is a heterodimer consisting of the 50-kDa (p50, NF-κB1) and 65-kDa (p65, RelA) proteins. Additional proteins

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.
were subsequently identified that belong 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 a luciferase reporter gene, to assess the effect of LPS-induced NF-κB activation on IL-8 transcription. The plasmid pIL-8(κB)LUC (WT-IL-8-LUC) contains κB site from the promoter region of the IL-8 gene and a separate plasmid pIL-8(mu)LUCA (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.
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 nonfunctional (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 distinct and different modes of action. Genistein is a

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**Fig. 6.** LPS stimulates tyrosine phosphorylation of Toll-like receptor 4 (TLR4) in human blood monocytes. A: time course of TLR4 protein phosphorylation. Cell lysates from control or LPS-treated monocytes were immunoprecipitated with anti-TLR4 Ab and resolved by SDS-PAGE. Blots were probed with an antiphosphotyrosine Ab (top) or anti-TLR4 Ab (bottom). B: effect of tyrosine kinase inhibitor on LPS-stimulated tyrosine phosphorylation of TLR4. Stimulation of media (M), genistein alone (G), or 10 ng/ml LPS (L). Lane 4, monocytes were pretreated for 10 min with genistein (100 μM), followed by LPS (10 ng/ml) stimulation for 4 min. Lane 5, stimulation of LPS (10 ng/ml) for 4 min, and genistein was added (100 μM). Tyrosine phosphorylation of TLR4 was measured as described in 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-kinase 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.

The authors thank Drs. N. Mackman and R. Medzhitov for providing DNA constructs.

This work was supported by United States Public Health Service Grants AI-45324 and HL-69425 to L. K. Pan and National Institutes of Health (NIH) Training Grant T32 AI-07469 to L.-Y. Chen. This work was also supported in part by the Sam and Ross Stein Charitable Trust and NIH Grant M01RR008833 provided to the General Clinical Research Center of the Scripps Research Institute. This is publication 14269-MEM from The Scripps Research Institute.

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