Bacterial Lipopolysaccharide Activates Nuclear Factor-κB through Interleukin-1 Signaling Mediators in Cultured Human Dermal Endothelial Cells and Mononuclear Phagocytes

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Bacterial lipopolysaccharide (LPS)-mediated immune responses, including activation of monocytes, macrophages, and endothelial cells, play an important role in the pathogenesis of Gram-negative bacteria-induced sepsis syndrome. Activation of NF-κB is thought to be required for cytokine release from LPS-responsive cells, a critical step for endotoxic effects. Here we investigated the role and involvement of interleukin-1 (IL-1) and tumor necrosis factor (TNF-α) signal transducer molecules in LPS signaling in human dermal microvascular endothelial cells (HDMEC) and THP-1 monocytic cells. LPS stimulation of HDMEC and THP-1 cells initiated an IL-1 receptor-like NF-κB signaling cascade. In transient cotransfection experiments, dominant negative mutants of the IL-1 signaling pathway, including MyD88, IRAK, IRAK2, and TRAF6 inhibited both IL-1 and LPS-induced NF-κB luciferase activity. LPS-induced NF-κB activation was not inhibited by a dominant negative mutant of TRAF2 that is involved in TNF signaling. LPS-induced activation of NF-κB-responsive reporter gene was not inhibited by IL-1 receptor antagonist. TLR2 and TLR4 were expressed on the cell surface of HDMEC and THP-1 cells. These findings suggest that a signal transduction molecule in the LPS receptor complex may belong to the IL-1 receptor/toll-like receptor (TLR) super family, and the LPS signaling cascade uses an analogous molecular framework for signaling as IL-1 in mononuclear phagocytes and endothelial cells.

Lipopolysaccharide (LPS), or endotoxin, is the major component of the outer surface of Gram-negative bacteria. LPS is a potent activator of cells of the immune and inflammatory systems, including macrophages, monocytes, and endothelial cells, and contributes to systemic changes known as septic shock (1, 2). LPS-induced activation of monocytes/macrophages is mediated through a cell surface receptor glycoprotein, known as membrane CD14 (mCD14). The binding of LPS to mCD14 is enhanced by LPS-binding protein, a plasma protein (3). On the other hand, vascular endothelial cells do not express mCD14 and respond to LPS only in the presence of soluble CD14 (4–6). We (7) and others (8–12) have shown that protein tyrosine phosphorylation and activation of ERK1, ERK2, p38 mitogen-activated protein kinase, and c-Jun N-terminal kinase appear to be important for LPS-induced cellular activation. LPS rapidly induces nuclear factor-κB (NF-κB) in both monocyte (13, 14) and endothelial cells (15). Activation of NF-κB is required for release of proinflammatory cytokines, including interleukin-1 (IL-1), interleukin-6 (IL-6), and tumor necrosis factor (TNF) (16, 17). However, the molecular mechanisms of the signaling cascade induced by LPS to activate NF-κB are unknown. Furthermore, the signaling LPS receptor is still unidentified.

The toll gene controls dorsoventral pattern formation during the early embryonic development of Drosophila melanogaster (18). Toll initiates a signaling pathway homologous to the mammalian NF-κB activation cascade (18). The toll family of receptors is defined by homology to the Drosophila toll protein (19, 20). The mammalian IL-1 receptor is a member of the toll family (18). Other mammalian family members (toll-like receptors 1 through 5, TLR1–5) have been identified, but their function is uncertain. Several TLRs, similar to IL-1R, have been observed to signal through the NF-κB pathway (19–22). LPS signaling also leads to activation of NF-κB, and recent studies suggested that a toll-like receptor (TLR) might be a signaling receptor that is activated by LPS (22, 23). In these reports, expression of TLR2 in LPS-unresponsive human embryonic kidney cells (293 cells) enabled these cells to respond to LPS stimulation (22, 23). These investigators observed that LPS binds to a TLR2 extracellular domain and suggested that TLR2 is a candidate for a long sought LPS receptor, although the data were generated from a transfected and normally LPS-unresponsive cell line (22, 23). A recent study in the LPS-resistant C3H-HeJ mice has implicated another toll homologue (TLR4) as a signal-transducing component in the LPS receptor complex (24). It is known that the IL-1 signaling pathway in mammals is strikingly similar to the toll signaling pathway in Drosophila (19–22). The molecular events linking the IL-1 receptor (IL-1R) signaling complex to the induction of NF-κB have been recently characterized. Upon binding of IL-1 to its receptors (IL-1R), IL-1R associates with the IL-1 receptor accessory protein (IL-1RαcP) (26, 27). The complex then recruits and activates an

The abbreviations used are: LPS, lipopolysaccharide; HDMEC, human dermal microvascular endothelial cells; IL-1, interleukin-1; IL-1R, IL-1 receptor; IL-1RaCP, IL-1R accessory protein; IL-6, interleukin-6; IRAK, IL-1 receptor-associated kinase; MyD88, myeloid differentiation protein; NF-κB, nuclear factor-κB; NIK, NF-κB-inducing kinase; RT-PCR, reverse transcription-polymerase chain reaction; TLR, toll-like receptor; TNF, tumor necrosis factor; TRAF, tumor necrosis factor receptor-associated factor; PAGE, polyacrylamide gel electrophoresis.
Human THP-1 cells were transiently transfected with NF-κB-luciferase and β-galactosidase reporter vectors and dominant negative mutants of MyD88, TRAF6, and TRAF2 for 24 h. The total amount of DNA was kept constant with pcDNA3 vector. The cells were then stimulated with LPS (100 ng/ml) for different periods of time (A) or increasing amounts of LPS for 6 h (B). Some cells were transfected with different amounts of MyD88 mutants and stimulated with 100 ng/ml LPS for 6 h (C). Cells were transfected with TRAF2 and TRAF6 mutants and then stimulated with 100 ng/ml LPS for 6 h (D). Luciferase and β-galactosidase assays were performed as described under “Experimental Procedures.” The time and dose response experiments (A and B) are one representative of two independent experiments with similar qualitative results. C and D were graphed with means and standard deviations of three or more experiments.

In this study, we tested the hypothesis that LPS activates NF-κB through IL-1 signaling molecules, namely MyD88, IRAK, IRAK2, TRAF6, and NIK but not TRAF2 inhibited LPS-induced NF-κB activity in HDMEC. Human dermal endothelial cells were grown on 6-well plates and transfected with dominant negative mutants of MyD88, IRAK, IRAK2, TRAF6, TRAF2, and NIK as well as reporter genes for 24 h. The amount of total DNA was kept constant with pcDNA3 vector. The cells were treated with 100 ng/ml LPS (A), 400 units/ml human IL-1β (B), 200 units/ml human TNF-α (C) for 6 h. Some cells were also treated with 100 ng/ml IL-1 receptor antagonist for 6 h (A and B). NF-κB luciferase activity was obtained with luciferase assay and normalized with β-galactosidase activity. Data shown are one representative experiment of two experiments with similar qualitative results.

with 10% serum. Reporter genes pCMV-β-galactosidase (0.5 µg) and ELAM-NF-κB-luciferase (2 µg) and pcDNA3 empty vector or dominant negative mutants of MyD88, IRAK, TRAF6, TRAF2, and NIK (3 µg each) were used. After a 24-h transfection, cells were stimulated for 6 h with 100 ng/ml LPS. Cells were then lysed, and luciferase activity was measured with a Promega kit (Promega, Madison, WI) and a luminometer. β-Galactosidase activity was determined by the calorimetric method to normalize transfection efficiency as described earlier (30). Data shown are means of two independent experiments.

**Experimental Procedures**

**Cell Culture**—Human THP-1 cells (from ATCC) were cultured in RPMI medium with 10% fetal calf serum. The immortalized human dermal endothelial cells (generous gift of Dr. Candal of the Center for Disease Control, Atlanta) were cultured in MCDB-131 medium with 10% heat-inactivated fetal bovine serum, 2 mM glutamine, and 100 µg/ml penicillin and streptomycin in 6-well plates.

**Expression Vectors and Transfection of THP-1 Cells**—Dominant negative expression vectors of MyD88, IRAK, IRAK2, TRAF2, TRAF6, and NIK have been characterized and described before (30, 32, 33). Cells were used for transfection with FuGene 6 Transfection Reagent (Boehringer Mannheim) following the manufacturer’s instructions in RPMI 1640 medium.

**Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Analysis**—Total RNA was isolated from HDMEC and THP-1 cells using a Qiagen kit (Valencia, CA) and treated with RNase-free DNase I. For RT reaction, the SuperScript™ Preamplification system (Life Technologies, Inc.) was applied. PCR amplification was performed with Taq polymerase (Qiagen, Valencia, CA) for 25 cycles at 95 °C for 40 s, 54 °C for 40 s, and 72 °C for 1 min. PCR primers for TLR2 were 5′-GCC-CAAAAGTCTTGGATGATTTG and 5′-TTGAAAGTTCTCCAGCTCT-G. PCR primers for TLR4 were 5′-TGGATACGTTTCCTTATAAG and 5′-GGAAAGGAGGCACCACTTC. GAPDH primers were obtained from CLONTECH.

**Immunostaining and Immunoblotting**—Smeared THP-1 cell and cultured HDMEC on slides were fixed with acetone for 5 min and then stained with rabbit anti-TLR2 and TLR-4 antibody (1:100) and rabbit anti-TLR4 antibody (1:500) and then probed with FITC-conjugated secondary antibodies.


Fig. 3. Expression of TLR2 and TLR4 in HDMEC and THP-1 cells. Expression of TLR2 mRNA (upper panel, 347 base pairs) and TLR4 mRNA (middle panel, 548 base pairs) in HDMEC (lanes 1 and 2) and THP-1 cells (lanes 2 and 4) was analyzed by PCR following reverse transcription (RT, lanes 1 and 2) or without RT (lanes 3 and 4). RT-PCR analysis of GAPDH expression was used as control (lower panel, 983 base pairs). Labels of base pairs at the right are DNA standard markers.

RESULTS AND DISCUSSION

A sequence of defense mechanisms are triggered in vertebrates and invertebrates in response to Gram-negative bacterial infections by sensing the presence of LPS (1–3). LPS-induced signal relay is thought to be initiated following its binding to specific cellular receptors which then triggers intracellular signaling pathways leading to the activation of NF-κB (4–12) in various LPS-responsive cell types. To date, the identification of functional, signal-transducing component of the putative LPS receptor complex and the signaling pathways involved in LPS-induced activation of NF-κB have remained elusive. Recent findings suggested that LPS might use signaling molecules of the TLR and IL-1R superfamilies to transduce signals (23, 24).

To investigate the potential involvement of IL-1 and TNF signaling transducers in LPS signaling in two LPS-responsive cell types, HDMEC and THP-1 cells, we cotransfected dominant negative constructs of various components of the NF-κB signaling cascades for IL-1 and TNF together with NF-κB-luciferase reporter gene. LPS induced the activation of NF-κB in a time- (Fig. 1A) and dose-dependent (Fig. 1B) manner in THP-1 cells. Activation of NF-κB reached a maximum at a LPS concentration of 100 ng/ml and when cells were stimulated with LPS for 6 h (Fig. 1A and 1B). Similar results were also obtained from endothelial cells.

A mutant version of MyD88 (ΔMyD88), encoding only for the COOH-terminal toll-IL-1R-like domain, which abrogates IL-1R-induced NF-κB activation (30), inhibited both LPS- and IL-1R-mediated NF-κB activation (Figs. 1C and 2A and B) but not TNF-induced NF-κB activation (Fig. 2C). IRAK and IRAK-2 are two additional proximal mediators of the IL-1R signaling complex (30). Dominant negative constructs of IRAK (ΔIRAK) and IRAK2 (ΔIRAK2) inhibited both LPS- (Figs. 1D and 2A) and IL-1R-mediated NF-κB activation (Fig. 2B), but not TNF-induced NF-κB activation (Fig. 2C).

NF-κB activation induced by various cytokine receptors is mediated by members of the TRAF adapter family. While TRAF2 plays a crucial role in NF-κB activation, we next investigated whether dominant negative versions of TRAF6 (ΔTRAF6) or TRAF2 (ΔTRAF2) could act to inhibit LPS-induced NF-κB activity. ΔTRAF6 but not ΔTRAF2 significantly impaired LPS-induced NF-κB activation, suggesting that TRAF6 may act as an additional downstream mediator of LPS-induced NF-κB activation cascade (Figs. 1D and 2A). ΔTRAF2 blocked TNF-induced NF-κB activation in endothelial cells (Fig. 2C), but not LPS-induced NF-κB activation (Figs. 1D and 2A). Because the pathways for IL-1 and TNF-α signaling converge at the level of NIK for NF-κB activation, we next investigated whether dominant negative NIK mutant (ΔNIK) would block LPS-induced, as well as IL-1- and TNF-induced, NF-κB activation. As expected, ΔNIK blocked NF-κB activation induced by LPS, IL-1, and TNF-α (Fig. 2).

IL-1 receptor antagonist had no effect on LPS-induced NF-κB activation (Fig. 2A) but inhibited IL-1-induced NF-κB activation in endothelial cells (Fig. 2B). This observation suggests that NF-κB activation that we measured following 6 h of
LPS stimulation of cells is not due to an autocrine effect such as LPS-induced IL-1 release from endothelial cells. These data suggest that LPS stimulation of endothelial cells and THP-1 cells triggers an IL-1R-like signal relay leading to activation of NF-κB (Fig. 5). Further support for this concept is provided by the observation of a 15-year-old girl with recurrent infections who was found to be resistant to both LPS and IL-1 stimulation in vivo and in vitro (39). The authors suggested that resistance to LPS and IL-1 was due to a defect very early in the common signaling pathway for LPS and IL-1 (39).

The experiments with IL-1R antagonist also suggest that LPS does not use IL-1 receptor to transduce signals in endothelial cells. Although recent findings imply that TLR2 or other members of the TLR family, which use the IL-1R signaling molecules, namely MyD88, IRAK, IRAK2, and TRAF6, but not TNF signaling molecules, TRAF2. We have also shown that TLR2 and TLR4 are expressed on the cell surface of two LPS-responsive cell types, endothelial cells and THP-1 cells. These data strongly suggest that a crucial signaling component in the LPS receptor complex may belong to the IL-1 receptor/TLR superfamily, and the LPS signaling cascade uses an analogous molecular framework for signaling as IL-1. MyD88 appears to represent the most upstream mediator of the LPS-mediated signaling cascade, which ultimately activates NF-κB, thus driving transcriptional activation of several cytokines. Thus, MyD88 may represent a potentially useful therapeutic target to control the molecular switch from innate to the adaptive immune response.

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