Lipopolysaccharide-induced Tumor Necrosis Factor-α Promoter Activity Is Inhibitor of Nuclear Factor-κB Kinase-dependent*

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The adverse effects of lipopolysaccharide (LPS) are primarily mediated by tumor necrosis factor-α (TNF-α). TNF-α production by LPS-stimulated macrophages is regulated both transcriptionally and post-transcriptionally. Transcriptional regulation of the TNF-α gene is dependent on nuclear factor-κB (NF-κB). We examined the signaling pathways involved in the regulation of NF-κB that lead to TNF-α promoter activity. We determined a role for one or both of the recently identified inhibitor of NF-κB kinases, IκB kinase-1 and IκB kinase-2, in LPS induction of an NF-κB reporter and of TNF-α promoter activity. IκB kinase activation is one of the earliest signaling events known to be induced by LPS. Furthermore, our results suggest roles for the IκB kinases NF-κB-inducing kinase and mitogen-activated protein kinase/extracellular signal-regulated kinase kinase kinase in the regulation of IκB kinase-1 in the regulation of IκB kinase-2, as well as in LPS-induced TNF-α transcription.

Lipopolysaccharide (LPS) is a surface component of Gram-negative bacteria that is released following host infection and causes tissue injury and shock (1). LPS mediates such adverse effects by inducing the production of pro-inflammatory cytokines. One of the most important of these LPS-induced pro-inflammatory cytokine mediators is tumor necrosis factor-α (TNF-α). TNF-α production in monocytes and macrophages constitutes between 1 and 2% of secreted proteins in response to LPS (2). Purified TNF-α induces many of the deleterious effects of LPS in vivo (3); passive immunization against TNF-α protects animals from the lethal effects of LPS (4).

The LPS signaling cascade leading to TNF-α production bifurcates to control both transcription of the TNF-α gene and translation of TNF-α mRNA (5). Translational regulation of TNF-α mRNA is mediated by a short AU-rich sequence that is conserved among various cytokines and oncogenes and is present within the 3′-untranslated regions of such genes (6). This element confers a repression of translation that must be overcome in order for translation to proceed (7–11). Recent studies also suggest a role for this element in destabilization of TNF-α mRNA (12). Signaling molecules shown to play a role in translational regulation of TNF-α mRNA include p38 (13) and Jun-N-terminal kinase/stress-activated protein kinase (JNK/SAPK) (14), members of the mitogen-activated protein kinase family, as well as the more proximal signaling molecules Raf and Ras (15). Transcriptional regulation of the TNF-α gene is quite complex; differences exist across species as well as among cell types. Regulation of TNF-α transcription is conferred by transcription factor binding sites present within the TNF-α promoter. The cis-acting elements within the TNF-α promoter that are conserved among species include a Y box motif, an SP-1 binding site, as well as multiple nuclear factor-κB (NF-κB) sites (16, 17). In addition, the human TNF-α promoter contains an AP-1 site (18). LPS induction of both murine and human TNF-α promoter activity is dependent on NF-κB binding sites. Mutation or deletion of such sites results in a loss of LPS responsiveness, whereas multiple copies of the NF-κB sites inserted in front of a reporter gene are LPS-responsive (16, 19, 20). Furthermore, compounds that inhibit NF-κB block TNF-α transcription and TNF-α production in human monocytes (21). Evidence argues against a role for NF-κB in regulation of human TNF-α gene transcription exists (22, 23). However, the discrepancy may be due to the stimulus used to induce TNF-α promoter activity, as well as the cell type.

LPS treatment of macrophages stimulates the nuclear mobilization of NF-κB (24, 25). Under normal conditions, NF-κB is found in a cytoplasmic complex with an inhibitory protein, inhibitor of NF-κB (IκB) (26). Many signals that lead to the nuclear translocation of NF-κB result in the phosphorylation and subsequent degradation of IκB. Phosphorylation of Ser-32 and Ser-36 of IκB targets IκB-α for ubiquitination and degradation by the proteosome, allowing NF-κB to translocate to the nucleus (27–29). Recently, two kinases have been identified that inducibly phosphorylate IκB: IκB kinase-1 (IKK-1), also called IKK-α (30–33), and IκB kinase-2 (IKK-2), also called IKK-β (31, 33, 34). These kinases are activated by TNF-α and interleukin-1β (30, 33, 34) and are required for cytokine-induced activation of NF-κB (30, 32, 33). IKK-1 and IKK-2 show 52% identity at the amino acid level and can exist in a heterodimer that is able to interact with another kinase called NF-κB-inducing kinase (NIK). NIK was originally identified based on its ability to bind to TNF-receptor-associated factor 2 and is a member of the mitogen-activated protein kinase/extracellular signal-regulated kinase kinase kinase (MEKK) family (35). Co-expression of NIK with IKK-1 or IKK-2 enhances IKK activity, whereas overexpression of a kinase mutant NIK blocks cytokine-induced NF-κB activation (30, 34). Another kinase implicated in the regulation of NF-κB activation is MEKK1. MEKK1 activates an IκB-α kinase complex (36, 37) and is also implicated in TNF-α-induced NF-κB activation (38). Furthermore, MEKK1 is required for Tax-induced NF-κB activation (39) and is involved in FceRI-induced activation of the...
human TNF-α promoter (40).

We set out to determine the signaling events initiated by LPS that lead to activation of the murine TNF-α promoter. This promoter lacks elements, such as AP-1, present in the human promoter, thereby allowing a more straightforward analysis of the underlying signaling mechanisms using a physiological promoter. Here, we demonstrate a requirement for one or both IKKs and examine roles of NIK and MEKK1 in LPS-induced TNF-α transcription.

**EXPERIMENTAL PROCEDURES**

**DNA Constructs**—Expression vectors containing the constitutively active version of MEKK1 (MEKK-C) and dominant-negative MEKK1 (D1569A) were described previously (41). The FLAG-tagged wild-type IKK-2, dominant-negative IKK-2 (S177A/S181A), FLAG-tagged wild-type IKK-1, and dominant-negative IKK-1 (S176A/S180A) constructs were described previously (31, 39). Wild-type and dominant-negative NIK (K429A/K430A) were also described previously (39). The TNF-α transcriptional reporter (TNFpro-CAT) was provided by B. Beutler and was described previously (14). The NF-κB reporter (NF-κB-luc) consisted of a triple repeat of the human immunodeficiency virus NF-κB site driving a luciferase cDNA and was provided by A. Thorburn. The dominant-negative IκB construct (IκB-α SS32, 36AA) was provided by R. Gaynor.

**Cells and Transfections**—RAW 264.7 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (Life Technologies, Inc.), 50 units/ml penicillin, 50 μg/ml streptomycin, 2 mM glutamine at 37 °C in 5% CO2. Transfections were performed using the Profection DNA transfection kit (DEAE-dextran) from Promega and following the protocol provided by the manufacturer. DNA amounts transfected were kept constant by the addition of empty DNA amounts transfected were kept constant by the addition of empty DNA amounts transfected were kept constant by the addition of empty DNA amounts transfected were kept constant by the addition of empty

**In Vitro Kinase Assays**—Where indicated, FLAG-IKK-1 and FLAG-IKK-2 were immunoprecipitated from equal amounts of protein (2 mg) from lysates of transfected RAW 264.7 cells using 1 μg of the M-2 anti-FLAG monoclonal antibody (Sigma) for 4 h at 4 °C on a rocking platform. Where indicated, endogenous IKK-1 and IKK-2 were immunoprecipitated from equal amounts of lysates using 1 μg of anti-IKK-1 (M-280; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or anti-IKK-2 (H-470; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) antibodies. CAT assays were performed as described previously (14). CAT activity was quantitated using a PhosphorImager. Data are represented as percent conversion of chloramphenicol to acetylated chloramphenicol. Luciferase assays were performed using the luciferase assay reagent (Promega) and following the manufacturer’s protocol.

**RESULTS**

**LPS Activates the TNF-α Promoter and an NF-κB Reporter in RAW 264.7 Cells; LPS-induced TNF-α Promoter Activity Is NF-κB-dependent**—LPS is a potent inducer of TNF-α bio-activation, activating both transcription of the TNF-α gene and translation of TNF-α mRNA. We previously demonstrated that multiple kinase signaling pathways are stimulated by LPS and that the JNK/SAPK pathway is required for translational induction of TNF-α by LPS (14). In addition, we found no requirement for JNK/SAPK in LPS-induced TNF-α transcription, despite the previously described effects of JNK/SAPK on TNF-α transcription in another system (14, 40). Therefore, we have explored signaling pathways leading to LPS-induced TNF-α promoter activity. It is well documented that LPS activates NF-κB and that NF-κB is crucial for LPS induction of TNF-α gene transcription (20, 21, 24, 25). As a starting point for our experiments, we confirmed that LPS could induce NF-κB activation, as well as TNF-α promoter activity in our system. We transiently transfected RAW 264.7 cells with an NF-κB reporter (NF-κB-luc) or a TNF-α transcriptional reporter consisting of the murine TNF-α promoter driving a CAT cDNA (TNFpro-CAT). After 24 h, cells were stimulated with LPS or diluent for 6 h and were harvested for assessment of CAT activity. LPS enhanced NF-κB and TNF-α promoter activity, as demonstrated by an increase in luciferase (Fig. 1A, left) or CAT activity (Fig. 1A, right) in LPS-treated cells, over that in control cells. The stimulation across experiments was variable, ranging from 2- to 20-fold. To determine the extent to which NF-κB contributes to LPS-induced TNF-α promoter activity, we tested whether overexpression of a dominant-negative IκB could inhibit LPS-induced TNF-α promoter activity. As shown in Fig. 1B, overexpression of the dominant-negative IκB completely abolished LPS-induced TNF promoter activity.

**LPS Activates IKK-1 and IKK-2; IKKs Are Required for LPS-induced TNF-α Promoter Activity**—Because LPS induction of the TNF-α promoter appears strongly linked to NF-κB (20, 21, 24, 25), we set out to examine the signaling molecules involved in NF-κB activation by LPS. Recently, two kinases, IKK-1 and IKK-2, were identified that phosphorylate IκB, releasing NF-κB, leading to its nuclear translocation (30–34). To determine whether LPS is capable of activating IKK-1 or IKK-2, we immunoprecipitated endogenous IKK-1 or IKK-2 from lysates of RAW 264.7 cells that had been treated with LPS for varying...
amounts of time or from unstimulated cells (control). After immunoprecipitation, IKK-1 or IKK-2 activity was assessed in vitro in the presence of [γ-32P]ATP using bacterially expressed glutathione S-transferase-IκB (amino acid, 1-54) as a substrate. As shown in Fig. 2A, LPS activates both IKK-1 and IKK-2. Peak activity occurred 15 min poststimulation and rapidly decreased within 30 min. Because LPS activates an NF-κB reporter and IKK activity, we wanted to determine whether IKK-1 or IKK-2 is involved in LPS induction of TNF-α promoter activity. RAW 264.7 cells were transiently co-transfected with TNFpro-CAT, and either empty vector (control) or inhibitory mutants of IKK-1 (IKK-1 SS/AA) or IKK-2 (IKK-2 SS/AA). These are phosphorylation-defective mutants previously shown to block IκB phosphorylation (39). Cells were treated with diluent or LPS for 6 h and harvested for assessment of CAT activity. As shown in Fig. 2B, both dominant-negative IKK-2 and dominant-negative IKK-1 decreased basal promoter activity and inhibited LPS-induced TNF-α promoter activity. The inhibitory effect was greater with IKK-2. Similar results were obtained with the NF-κB reporter (data not shown). Thus, one or possibly both IKKs are required for LPS-induced regulation of TNF-α transcription. These results further emphasize the predominant role of NF-κB in the LPS-mediated transcription of TNF-α.

**LPS-induced Activation of IKK-2 and TNF-α Promoter Activity Is Blocked by Kinase-defective Mutants of NIK and MEKK1**—NIK and MEKK1 can activate IKK-1 or IKK-2 in HeLa cells and fibroblasts (34, 37, 39, 42). We examined the roles of NIK and MEKK1 in the regulation of IKK-1 or IKK-2 in macrophages. We transiently co-transfected a FLAG-tagged IKK-1 or IKK-2 with increasing amounts of either NIK or a constitutively active version of MEKK1 (MEKKC). Overexpression of either NIK or MEKKC activated IKK-2 by 50-fold or more, whereas neither activated IKK-1, although equal amounts of IKKs were present (Fig. 3A and data not shown). To determine the possible requirement of NIK or MEKK1 for LPS induction of IKK-2 activity, we co-expressed FLAG-tagged IKK-2 with either empty vector (control), a dominant-negative
NIK (NIK KK/AA), or a dominant-negative MEKK1 (MEKK1 DA) in RAW 264.7 cells. After 29 h, the cells were treated with LPS or diluent for the indicated amounts of time. As shown in Fig. 3B, dominant-negative NIK and dominant-negative MEKK1 were each able to block LPS-induced IKK-2 activity. These results suggest functions for both NIK and MEKK-1 in LPS induction of IKK-2 activity. However, because each of these proteins may bind to IKK2, it is possible that they could sequester it, preventing activation by other kinases. Because NIK and MEKK1 stimulated IKK-2 activity (Fig. 3A), we examined their effects on LPS-induced TNF-α promoter activity. RAW 264.7 cells transiently transfected with the TNF-α transcriptional reporter and either a dominant-negative NIK or a dominant-negative MEKK1 were treated with LPS or diluent. As shown in Fig. 3C, expression of either dominant-negative NIK (right) or dominant-negative MEKK1 (left) abolished LPS induction of TNF-α promoter activity. Similar results were obtained with the NF-κB reporter (Fig. 3D). These results further suggest a role for NIK and MEKK-1 in LPS induction of the TNF-α promoter.

Overexpression of NIK or MEKK1 Activates TNF-α Promoter Activity in the Absence of Exogenous Stimuli—Overexpression of MEKK1 activates the human TNF-α promoter (40) and NF-κB (37, 39) in other systems. Because kinase-dead NIK and MEKK1 block LPS-induced TNF-α promoter activity, we tested whether overexpression of NIK or MEKK1 could induce TNF-α promoter activity in the absence of exogenous stimuli. We co-expressed the TNF-α transcriptional reporter with either NIK or MEKK1 (MEKKC) in RAW 264.7 cells. 24 h after transfection, the cells were treated with either diluent or LPS and cells were harvested for assessment of CAT activity. As shown in Fig. 4, in the absence of LPS, overexpression of NIK induced TNF-α promoter activity to a level above that obtained with LPS stimulation alone. LPS did not appear to enhance the ability of NIK to activate the TNF-α promoter. In a similar manner, in the absence of LPS, overexpression of MEKK-1 activated the TNF-α promoter. However, the effects of LPS and MEKK1 were nearly additive in inducing the TNF-α transcriptional reporter. Similar results were obtained using the NF-κB reporter (data not shown). Thus, NIK and MEKK1 are able to induce TNF-α promoter activity in the absence of exogenous stimuli. Because LPS further enhances activation of TNF-α promoter activity by MEKK1, but not by NIK, it may be that MEKK1 is acting by a distinct mechanism.

Overexpression of Dominant-negative IKKs Inhibit NIK- and MEKK1-induced TNF-α Promoter Activity—Because NIK and MEKK1 can stimulate IKK-2, we tested whether IKK-2 was required for NIK- or MEKK1-induced TNF-α promoter activity. We transiently transfected the TNF-α transcriptional reporter with empty expression vector, MEKK1 (MEKKC), or NIK in the presence or absence of dominant-negative IKK-2 (IKK2 SS/AA). As shown in Fig. 5, overexpression of dominant-negative IKK-2 inhibited NIK- and MEKK1-induced TNF-α promoter activity. Similarly, overexpression of a dominant-negative IKK1 (IKK-1 SS/AA) blocked NIK- and MEKK1-induced TNF-α promoter activity, despite the fact that neither NIK nor MEKK1 activated IKK1 in these cells (Fig. 5).

**DISCUSSION**

In this study, we investigated the signaling pathways initiated by LPS that are involved in transcriptional regulation of the TNF-α gene in macrophages. In confirmation of earlier findings concerning regulation of the TNF-α promoter, our work indicates that most or all of the signaling leading to TNF-α transcription is mediated by NF-κB. This conclusion is based, in part, on the finding that dominant-negative IkB completely inhibits LPS-induced TNF-α promoter activity. Dominant-negative IKKs fully block LPS induction of the TNF-α promoter, further implicating NF-κB as the primary mediator of LPS-induced TNF-α promoter activity. In a previous study examining transcriptional regulation of the human TNF-α promoter, it was determined that a dominant-negative JNK/SAPK interfered with FceRI induction of transcription in mast cells (40). We showed that dominant-negative JNK/SAPK had no effect on LPS induction of the murine TNF-α promoter in macrophages (14). Therefore, regulation of TNF-α transcription differs among species and/or cell types. Indeed, the human
NF-κB merely demonstrate a role for IKKs in the regulation of important using these approaches. Because IKK-1 and IKK-2 form heterodimers (3, 33, 34), it is not possible to determine whether one or the other is more important using these approaches.

Because LPS induces TNF-α production in macrophages, one could argue that the effects on the IKKs are caused by an autocrine mechanism. This, however, is not the case because of the rapid kinetics of IKK activation. We determined that LPS induced activation of other signaling molecules, including extracellular signal-regulated kinases, p38, JNK/SAPK, MEK1, 3, 4, and 6, 30–60 min poststimulation in macrophages (14). In contrast, activation of the IKKs peaks within 15 min of LPS stimulation. Therefore, IKK activation is among the earliest signaling events known to be induced by LPS in macrophages.

To test possible links between LPS and the IKKs, we examined two IKK kinases, NIK and MEKK1. Both NIK and MEKK1 stimulate IKK-2 activity in RAW 264.7 cells, and both NIK and MEKK1 are sufficient to activate not only an NF-κB reporter but also the TNF-α promoter in the absence of ligand. Additional findings suggesting that NIK and MEKK1 may both be involved in LPS-mediated transcriptional regulation of the TNF-α gene come from studies with kinase-defective mutants. Either kinase-dead NIK or MEKK1 blocks the induction of IKK-2 activity and induction of the TNF-α promoter by LPS. These results may indicate that both enzymes are required. Alternatively, they may block NF-κB by sequestering IKK-2 or NF-κB activation complexes.

Our results suggest roles for IKK-1, IKK-2, or both, in LPS-induced signaling to the TNF-α promoter. However, we propose that IKK-2 is the more significant mediator of LPS-induced signaling to the TNF-α promoter, since known IKK kinases lead to activation of IKK-2, not IKK-1. LPS-induced IKK activity observed in the IKK-1 immunoprecipitates could be due to the presence of IKK-2. Dominant-negative IKK-1 inhibited LPS-, MEKK1-, and NIK-induced TNF-α promoter, and NF-κB activity could also be attributed to the fact that the IKKs form heterodimers (3, 33, 34). Thus, overexpression of dominant-negative IKK-1 could interfere with IKK-2 function; more extensive studies are necessary to determine such matters definitively.

It is clear that regulation of NF-κB is complex, as multiple kinases and other molecules, such as NF-κB essential modulator (43), appear to be involved. Here, we establish a role for IKKs in LPS-induced NF-κB activation and, more importantly, in LPS induction of TNF-α transcription. Most studies about IKKs merely demonstrate a role for IKKs in the regulation of NF-κB, using NF-κB reporters consisting of multiple copies of a particular site driving the expression of a reporter gene. Our studies establish a potential mechanism for initiation of TNF-α promoter activity, thus providing a better understanding of the regulation of such an important inflammatory mediator.

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