Role of IKK1 and IKK2 in Lipopolysaccharide Signaling in Human Monocytic Cells*

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Maria A. O’Connell, Brydon L. Bennett‡, Frank Mercurio‡, Anthony M. Manning‡, and Nigel Mackman§

From the Departments of Immunology and Vascular Biology, The Scripps Research Institute, La Jolla, California 92037 and ‡Signal Pharmaceuticals Inc., San Diego, California 92121

Mononuclear phagocytes play a major role in immune and inflammatory responses. Bacterial lipopolysaccharide (LPS) induces monocytes to express a variety of genes by activating the NF-κB/Rel transcription factor family. Recently, we have reported that the tumor necrosis factor and interleukin 1 signaling pathways activate two kinases, IKK1 and IKK2. Phosphorylation of the IkB cytoplasmic inhibitors, IkBa, IkBβ, and IkBe, by these kinases triggers proteolytic degradation and the release of NF-κB/Rel proteins into the nucleus. At present, the role of the IKKs in LPS signaling has not been investigated. Here, we report that LPS induces IKK activity in human monocytes and THP-1 monocytic cells. The kinetics of activation of kinase activity in monocytic cells are relatively slow with maximal activity observed at 60 min, which coincides with the degradation of IkBs and the nuclear translocation of NF-κB. In transfection experiments, overexpression of wild type IKK1, a dominant negative mutant IKK1 (K44M), or wild type IKK2 did not affect LPS-induced IkB-dependent transcription in monocytic cells. In contrast, a dominant negative mutant of IKK2 inhibited LPS induction of IkB-dependent transcription in a dose-dependent manner. These results indicate that LPS induction of IkB-dependent gene expression in human monocytic cells requires activation of IKK2.

Monocytes and macrophages play essential roles in inflammation and mobilization of the host defense against bacterial infection. Following exposure to foreign microbes, monocytes develop an increased capacity to kill bacteria and secrete inflammatory mediators that are involved in the pathogenesis of septic shock. Bacterial lipopolysaccharide (LPS), a component of the outer membrane of Gram-negative bacteria, is one of the most potent activators of monocytes (1). LPS stimulation of monocytes activates many second messengers and signal transduction pathways (2–5). These signaling pathways in turn activate various transcription factors, including the NF-κB/Rel family, which coordinate the induction of genes encoding inflammatory mediators, such as tumor necrosis factor α (TNFα) and interleukin 1 (2). The NF-κB/Rel family of proteins comprises RelA (p65), RelB, c-Rel, NF-κB1 (p105/p50), and NF-κB2 (p100/p52), which form various homo- or heterodimers. NF-κB is activated in response to various extracellular stimuli, including cytokines, LPS, phorbol esters, viruses, and oxidative stress (6–8).

In unstimulated monocytes, NF-κB is retained in the cytoplasm by the binding of a family of inhibitors (IkBa, IkBβ, and IkBe). Activation of NF-κB requires sequential phosphorylation, ubiquitination, and degradation of the IkBs, which permits nuclear translocation of NF-κB/Rel proteins. A high molecular mass kinase complex (700 kDa) has been described that contains a kinase activity specific for Ser172 and Ser36 of IkBa and Ser19 and Ser32 of IkBβ (9). More recently, we and others have cloned two IkB kinases, IKK1 (IKKα, CHUK) and IKK2 (IKKβ), that are present in a multisubunit complex, which we termed the IKK signalosome (10–14). Although IKK1 and IKK2 can homo- and heterodimerize, the major form of IKK contains both IKK1 and IKK2. These recent studies have elucidated the mechanism by which the TNFα and interleukin 1 signaling pathways activate NF-κB.

In this study, we used primary human monocyte-derived macrophages (Mo) and human monocytic THP-1 cells to investigate the role of these novel IKKs in the LPS signaling pathway. We demonstrate that LPS activates IKK activity, in particular IKK2 activity, relatively slowly with maximal activity at 60 min. A dominant negative mutant of IKK2 inhibited LPS-induced IkB-dependent transcription in a dose-dependent manner in THP-1 cells. Taken together, these results suggest that LPS-induced nuclear translocation of NF-κB in human monocytic cells is mediated, in part, by the activation of IKK2.

EXPERIMENTAL PROCEDURES

Materials—LPS (Escherichia coli serotype O111:B4) and the proteasome inhibitor, MG132, were purchased from Calbiochem. Recombinant human TNFα was purchased from Collaborative Biomedical Products (Bedford, MA).

Cell Culture—Human monocytic leukemia THP-1 cells (15) were obtained from ATCC (Manassas, VA). THP-1 cells were cultured in RPMI 1640 medium (BioWhittaker, Walkersville, MD) with 8% fetal calf serum (Omega Scientific, Inc., Tarzana, CA), l-glutamine (Irvine Scientific, Santa Ana, CA), and 2-mercaptoethanol (complete media).

Monocyte Isolation—Human peripheral blood mononuclear cells were isolated from heparinized blood from healthy volunteers by buoyant density centrifugation on a low endotoxin Ficoll-Hypaque (16). Peripheral blood mononuclear cells (4 × 10⁶/ml) were seeded in 6-well tissue culture plates in complete media, and monocytes were allowed to adhere for 90 min at 37 °C. Subsequently, cells were washed with phosphate-buffered saline to deplete nonadherent cells. Adherent cells were stimulated with 100 ng/ml LPS for 1 h at 37 °C, and cell lysates were prepared.

NF-κB Kinase Activity—Mo and THP-1 cells were unstimulated or stimulated with LPS for various times. Cell lysates were mixed with 1.5 μg of control rabbit anti-goat IgG antibody, 1.5 μg of a rabbit affinity-purified anti-IKK2 polyclonal antibody, which was raised against a
unique region of the IKK2 C terminus and is specific for IKK2, or 1.5 \mu g of a rabbit affinity-purified anti-IKK no. 1941 polyclonal antibody, which was raised against the C-terminal region of murine IKK1 and cross-reacts with both human IKK1 and IKK2. The immunoprecipitate was prepared as described previously (10), resuspended in kinase buffer containing 10 \mu M adenosine triphosphate and 1-3 \mu Ci of [\gamma-32P]ATP, and incubated at 34 °C for 30 min in the presence of the substrates GST-IκBα-(1–54), GST-IκBβ-(1–44), or mutant substrates. Proteins were separated by SDS-PAGE, and radiolabeled proteins were visualized by autoradiography.

Western Blotting—THP-1 cells were unstimulated or stimulated with 10 \mu g/ml LPS for various times, and cytosolic extracts were prepared (17). Cytosolic proteins were separated by SDS-PAGE and transferred to Nyobond-enhanced chemiluminescence membrane (ECL; Amersham Pharmacia Biotech). IκBα or IκBβ protein was detected according to the ECL protocol (Amersham Pharmacia Biotech) using a 1:1000 dilution of anti-IκBα or anti-IκBβ antibodies (Santa Cruz Biotechnology Inc., Santa Cruz, CA).

Electrophoretic Mobility Shift Assay—Nuclear extracts were prepared from 5 × 10^6 cells as described previously (17). A radiolabeled double-stranded oligonucleotide probe (Operon Technologies Inc., Alameda, CA) containing the murine IκBα binding site (underlined), 5′-CA-GAGGGGACTTTCCGAGA-3′, was used to detect p65-p50 (17). Protein-DNA complexes were separated from a free DNA probe by electrophoresis through 6% nondenaturing acrylamide gels (Novex, San Diego, CA) in 0.5× Tris borate EDTA buffer. Gels were dried, and protein-DNA complexes were visualized by autoradiography.

Plasmids and Transfections—In THP-1 cells, NF-κB-dependent transcription was measured using a firefly luciferase reporter plasmid, p(B−a)4LUC, which contained four NF-κB sites cloned upstream of the minimal (SV40) promoter in pGL2 promoter (Promega Corp.); pGL2 basic (Promega Corp., Madison, WI) (17). pTNF-LUC contains the minimal (SV40) promoter in pGL2 promoter (18). Eukaryotic expression plasmids containing cDNAs encoding wild type IKK1, mutant IKK1 (K44M), wild type IKK2, and mutant IKK2 (K44M) have been described (10). pcDNA3 (Invitrogen Corp., San Diego, CA) was used as a control. THP-1 cells were transfected using the DEAE-dextran protocol (19). After transfection, cells were incubated in complete media for 46 h at 37 °C before stimulating cells stimulated with pRLTK that expresses Renilla luciferase (Promega Corp.). Renilla luciferase was measured according to the manufacturer’s protocol (Promega Corp.) and used to normalize the activity of the firefly luciferase. Results were expressed as mean ± S.D. Statistical analysis was performed using the unpaired Student’s t test. Expression levels of wild type and mutant IKK1 in transfected cells were determined by Western blotting using an anti-hemagglutinin (1:1000) antibody.

RESULTS

LPS Induces IKK Activity in Human Monocytes and THP-1 Cells—IKK activity was determined in human Mo and human THP-1 monocytes stimulated with LPS. Following immunoprecipitation of the IKK1-IKK2 complex from cell extracts using an anti-IKK2 antibody, IKK activity was determined by phosphorylation of the substrate GST-IκBα-(1–54) (Fig. 1). LPS stimulation dramatically increased IKK activity in both Mo and THP-1 cells. No phosphorylation of IκBα-(1–54) was observed when either the anti-IKK2 antibody was replaced with a control rabbit anti-goat IgG antibody or when GST-IκBα-(1–54) was replaced with a mutant GST-IκBα-(1–54, S92T, S36T) demonstrating specificity for the kinase and for the serines required for target degradation of IκBα. Similar results were observed using an antibody that recognizes both IKK1 and IKK2 (data not shown).

These results indicated that THP-1 cells were a valid model for studying LPS-induced IKK activation in monocytes. Our previous studies have shown that LPS activation of NF-κB and induction of κB-dependent gene expression in THP-1 cells are similar to that of human monocytes (17, 20). The activation of IKK activity was then determined in greater detail in THP-1 cells stimulated with LPS for various times (0–60 min). Following immunoprecipitation of the IKK1-IKK2 complex from cell extracts using an antibody that recognizes both IKK1 and IKK2, IKK activity was determined by phosphorylation of the substrates GST-IκBα-(1–54) and GST-IκBβ-(1–44) (Fig. 2). In contrast to the rapid activation of IKK activity in TNF-stimulated HeLa cells (10, 11), LPS induced a slow increase in IKK activity that continued to increase up to 1 h. A longer time course (0–180 min) revealed that LPS-induced IKK activity peaks at 60 min and decreases thereafter but is still present at 180 min (Fig. 3, top panels).

The kinetics of IKK activity in response to LPS were compared with the kinetics of degradation of IκBα and IκBβ (Fig. 3, middle panels). IκBα was rapidly degraded within 15 min of LPS stimulation with maximal degradation between 30 and 90 min. Resynthesis of IκBα was observed at 180 min. In contrast, degradation of IκBβ occurred more slowly with maximal degradation at 60 min and no resynthesis at 180 min. Nuclear translocation of NF-κB was examined by electrophoretic mobility shift assay (Fig. 3, bottom panel). In unstimulated THP-1 cells, no nuclear NF-κB was detected. LPS stimulation induced a rapid nuclear translocation of NF-κB, which was detectable within 15 min, reached a maximum at 30 min, and was still present at 180 min.

A Dominant Negative IKK2 (K44M) Inhibits LPS-stimulated κB-dependent Transcription—LPS stimulation of THP-1 cells strongly induced κB-dependent transcription (21 ± 2, mean-fold induction ± S.D., n = 7). This induction was strongly inhibited (>95%) by the proteasome inhibitor MG132 (5 μM), suggesting that it was mediated by NF-κB/Rel proteins. To determine whether IKK1 and IKK2 were required for LPS
signaling in monocytic cells, THP-1 cells were co-transfected with a κB-dependent reporter gene and plasmids expressing either wild type or mutant versions of the IKKs or a vector control. Fig. 4 demonstrated that wild type IKK1 or IKK2 had no effect on LPS-induced κB-dependent transcription. An IKK1 mutant, IKK1 (K44M), also did not affect LPS-induced κB-dependent transcription. Western blotting for hemagglutinin-tagged IKK1 and IKK1 (K44M) confirmed that these proteins were expressed in transfected cells (data not shown). In contrast to the results with IKK1 (K44M), a dominant negative IKK2 mutant significantly inhibited LPS-induced κB-dependent reporter activity (Fig. 4). Mean inhibition of κB-dependent transcription IKK2 (K44M) was 44 ± 2.5% (mean ± S.E., n = 7, p = 0.0008).

A detailed dose experiment performed with IKK2 (K44M) demonstrated a dose-dependent inhibition of κB-dependent transcription (Fig. 5). Maximal inhibition (44%) was observed with 4 μg of IKK2 (K44M), and no further inhibition was observed with 5 or 6 μg of plasmid DNA. For comparison, we determined the degree of inhibition of TNF-stimulated κB-dependent transcription by IKK2 (K44M). IKK2 (K44M) inhibited TNF and LPS-stimulated κB-dependent transcription to a similar extent (Fig. 6).

To determine whether IKK2 (K44M) inhibited an authentic κB-dependent promoter in a similar manner to the multimerized κB reporter, we examined the effect of IKK2 (K44M) on the human TNF promoter. Fig. 7 shows that IKK2 (K44M) significantly inhibited LPS induction of the cloned human TNF promoter in THP-1 cells. These data indicate that IKK2 plays a biological role in LPS induction of κB-dependent gene expression in human monocytic cells.

**DISCUSSION**

LPS stimulates multiple signaling pathways in monocytes, some of which lead to proteolytic degradation of IκBα and IκBβ and activation of NF-κB. Signaling molecules activated by LPS include G proteins, phospholipids, ceramide, and protein kinases, such as cAMP-dependent protein kinase, protein kinase C, tyrosine kinases, phosphatidylinositol 3-kinase, and the
ERK, p38, and JNK mitogen-activated protein kinase pathways (2–5). The search for the IkB kinase led to many potential candidates that phosphorylate IkB in vitro, including protein kinase C (21) and casein kinase II (22). However, these kinases lacked the ability to target the serines of IkBα that are phosphorylated by phorbol ester (25). These studies suggested that there may be multiple IkB kinases that are differentially activated by various stimuli (26). We and others have shown that the TNFα and interleukin-1 signaling pathways activate a novel IKK1-IKK2 complex in HeLa cells (10–14).

In this study, we examined the role of IKK1-IKK2 in LPS activation of NF-κB in human monocytic cells. LPS stimulated IKK activity in human Mo and human THP-1 monocytic cells. IkBα was rapidly phosphorylated and degraded with resynthesis by 3 h after LPS stimulation. In contrast, IkBβ was phosphorylated and degraded more slowly, and no resynthesis was noted at 3 h. LPS-stimulated monocytic cells exhibit prolonged NF-κB activity in the nucleus (3–4 h), which may be because of the prolonged degradation of IkBβ. Similarly, in murine 70Z/3 pre-B cells, LPS induces nuclear translocation of NF-κB by 15 min and remains for >4 h, which coincides with a slow persistent degradation of IkBβ (27). In contrast, TNFα induces a rapid IkBα degradation without affecting IkBβ in 70Z/3 cells and EL-4 T cells (27, 28). Our results suggest that the persistence of NF-κB that is observed in LPS-stimulated monocytic cells may be because of a slower IKK-dependent phosphorylation of IkBβ.

Overexpression of the wild type IKK1 and IKK2 had no effect on LPS-induced κB-driven gene transcription (Fig. 4). A dominant negative IKK1 (K44M) did not inhibit κB-dependent transcription in THP-1 cells, which is consistent with the inability of IKK1 (K44M) to inhibit κB-dependent transcription or nuclear translocation of RelA in TNFα-stimulated HeLa cells (10, 11). In an independent study using 293 cells, expression of a dominant negative IKK1 (K44A) inhibited TNFα-induced κB-dependent transcription of the E-selectin promoter (13). Differences in the cell type, reporter plasmids, or different substitutions of the lysine residue may be responsible for this anomaly. Antisense to IKK1 inhibited TNFα and interleukin-1-induced κB-dependent transcription in HeLa cells, suggesting that IKK1 functions in cytokine-stimulated activation of NF-κB in HeLa cells (29).

Expression of a dominant negative IKK2 (K44M) inhibited LPS-induced κB-dependent transcription in a dose-dependent manner (Fig. 4). IKK2 (K44M) and other IKK2 mutants strongly inhibit cytokine-induced κB-dependent transcription and RelA nuclear translocation in HeLa cells and 293 cells (10, 12, 14). A dominant negative mutant of IKK2 appears to have less of an effect on LPS-induced κB-dependent transcription in monocytic cells (maximal inhibition of 44%) than on cytokine-induced κB-dependent transcription in HeLa cells. We com-
pared LPS and TNFα-mediated κB-dependent transcription in THP-1 cells (Fig. 6). IKK2 (K44M) inhibition of LPS-mediated κB-driven transcription (44%) was similar to that of TNF (32%), suggesting that there are differences in the relative efficiency of the dominant negative mutants to inhibit IKK2 in different cell types. IKK2 (K44M) also inhibited LPS-induced TNF promoter activity (25%). The lesser effect of IKK2 (K44M) on LPS-induced TNF promoter activity may be because of compensation by other transcription factors that are involved in the induction of this complex promoter (18). Thus, monocytic cells appear to be more resistant than other cell types to the effects of dominant negative kinases.

NF-κB-inducing kinase, a member of the TNF signaling pathway, has been reported to phosphorylate IKK1 on Ser176 (31). However, overexpression of MEK kinase 1, a member of the 900-kDa signalingome and an activator of NF-κB, preferentially stimulates IKK2 activity (31). These results suggest that NF-κB-inducing kinase and MEK kinase 1 independently activate the IKK complex, and the kinase activities of IKK1 and IKK2 are differentially regulated by NF-κB-inducing kinase and MEK kinase 1, which respond to different stimuli. The upstream members of the LPS signaling pathway leading to IKK2 activation have not yet been elucidated.

In summary, this study demonstrates that LPS stimulation of human monocytes induces nuclear translocation of NF-κB by activation of IKK2. NF-κB is a key regulator of LPS-induced gene expression in monocytes, and therefore targeting key components of the LPS-mediated NF-κB signaling pathway, such as IKK2, may prove beneficial in the treatment of sepsis and inflammatory diseases.

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