IMMUNOBIOLOGY

IκB Kinase Complex Is an Intracellular Target for Endotoxic Lipopolysaccharide in Human Monocytic Cells

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Endotoxic lipopolysaccharide (LPS) is a proinflammatory agonist produced by gram-negative bacteria and a contributor to the majority of the 400,000 septic shock cases recorded annually in US hospitals. The primary target cells for LPS are monocytes and macrophages. Their response consists of massive production of proinflammatory cytokines, reactive oxygen- and nitrogen-intermediates, procoagulants, and cell adhesion molecules. In turn, expression of these LPS-responsive factors contributes to collapse of the circulatory system, to disseminated intravascular coagulation, and to a 30% mortality rate. A common intracellular mechanism responsible for the expression of septic shock genes in monocytes and macrophages involves the activation of NF-κB. This transcription factor is regulated by a family of structurally related inhibitors including IκBα, IκBβ, and IκBe.

Proinflammatory stimuli signal to the nucleus via transcription factor NF-κB, which activates a set of genes responsive to inflammatory, immune, and oxidant stress. These stimuli encompass biologic agents such as endotoxic lipopolysaccharide (LPS) and bacterial superantigens (eg, toxic shock syndrome toxin and streptococcal pyrogenic exotoxin), which are responsible for the majority of septic shock cases. In addition, intracellular bacteria (eg, Mycobacterium tuberculosis), viruses (eg, human immunodeficiency virus, human T-cell lymphotropic virus, and cytomegalovirus), cytokines (tumor necrosis factor-α [TNF-α], interleukin-1 [IL-1]), and lipid peroxides induce nuclear import of NF-κB.1,3 In response to these agents, the cytoplasmic ankyrin motif-rich inhibitors of NF-κB, IκBα, IκBβ, and IκBe are phosphorylated, ubiquitinated, and then degraded by adenosine triphosphate (ATP)-dependent 26S proteasomes.1-3 Thus, phosphorylation-dependent proteolysis of IκB proteins releases NF-κB for subsequent import to the nucleus. In turn, NF-κB stimulates transcription of a number of genes containing cognate κB sites in their enhancer/promoter regions. These NF-κB-responsive genes encode cytokines (TNF-α, IL-1, IL-6, IL-8, and IL-12), procoagulant molecules (tissue factor and plasminogen activator inhibitor 1), signal transducers (inducible nitric oxide synthase and cyclooxygenase 2), cell adhesion molecules (endothelial selectin, intracellular cell adhesion molecule-1, and vascular cell adhesion molecule-1), and growth factors (granulocyte-colony-stimulating factor).3,6,10 The products of these NF-κB-regulated genes expressed in monocytes, macrophages, and vascular endothelial cells contribute to the development of septic shock and disseminated intravascular coagulation, leading to multiple organ failure and death.1,3 These findings highlight NF-κB as a major intracellular mediator of the systemic inflammatory response syndrome known as sepsis and septic shock.11

The fact that multiple proinflammatory stimuli, LPS, TNF-α, and IL-1, involved in induction and mediation of septic shock syndrome, evoke activation of NF-κB indicates that the signals generated by these stimuli and their cognate receptors converge at the common step of NF-κB activation.6 This step appears to be specific phosphorylation of inhibitory proteins, such as IκB, by their kinase(s). A primary target for IκB kinases is IκBe. This inhibitor is characterized by a signal response domain in the amino-terminal segment, ankyrin repeats in the middle section, and a PEST domain in the carboxyl terminal segment.12 The signal response domain contains two serines at positions 32 and 36, which are targeted for specific phosphorylation by IκB kinases.5,13 The PEST domain bears serines phosphorylated by casein kinase II involved in constitutive turnover of IκBe.14

The IκBe kinase that catalyzes inducible phosphorylation of serines 32 and 36 in response to cytokines was initially reported by Chen et al15 as part of a 700-kD complex requiring ubiquitin for full activity. Subsequently, two serine/threonine kinases termed IκB kinase α (IKKα or IKK1) and IκB kinase β (IKKβ or IKK2) of molecular weight 85 kD and 87 kD, respectively, were identified.16-18 Both of these kinases are homologous to a previously reported conserved helix-loop-helix ubiquitous kinase with a unique structure containing a kinase domain linked to a leucine zipper domain and a helix-loop-helix domain. Both IKKα and IKKβ phosphorylate serines 32 and 36 in IκBe, suggesting functional homology as well.19,20 Although it is well known that primary target cells for LPS are monocytes and macrophages,2,20 the mechanism by which...
LPS induces NF-κB in this cellular setting remained unknown. Therefore, we hypothesized that the recently discovered IκB kinases constitute an intracellular target for LPS. Our results provide evidence that IκB kinase in human monocytic THP-1 cells is targeted by LPS, thereby assigning this multiunit kinase complex a primary role as the signal transducer responsible for NF-κB mobilization in response to septic-shock inducers.

**MATERIALS AND METHODS**

**Reagents.** Rabbit polyclonal antibodies were generated by immunizing rabbits with synthetic peptides corresponding to amino acids 1-28 (NH2-terminal peptide) and 731-744 (COOH-terminal peptide) of human IκKa and a synthetic peptide corresponding to amino acids 743-756 of human IκKβ. The peptides were covalently coupled to keyhole limpet hemocyanin before injection together with complete Freund adjuvant. The antibody IgG fraction of each antiserum was purified by absorption to and elution from Protein A Sepharose (Zymed, San Francisco, CA). IκBa-specific (amino acids 289-317) antipeptide rabbit antibodies were prepared as described previously.9 Glutathione S-transferase (GST)-IκBo (1-54), IκBβ (1-44), and IκBe (1-61) fusion proteins (wild type and IκBo mutant S32A and S36A) were prepared as described.17,19,21

LPS preparations were derived from: (1) Escherichia coli 0127:B8 (extracted by Boivin method; DIFCO, Detroit, MI), (2) E coli 0127:B8 (prepared by phenol extraction and gel filtration chromatography), (3) Pseudomonas aeruginosa serotype 10 (chromatographically purified by gel filtration), and (4) Salmonella minnesota (prepared by phenol extraction and gel filtration chromatography). The latter three LPS preparations were obtained from Sigma Chemical Company (St. Louis, MO).

**Cell culture.** Human monocytic THP-1 cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum containing no detectable LPS (<0.006 ng/mL in Limulus Amebocyte Gel Clot as determined by the manufacturer, Atlanta Biologicals, Norcross, GA). 2 mmol/L L-glutamine, and antibiotics as described.4 Where indicated, 5 µL of THP-1 cells (106/mL) were stimulated with E coli LPS O127:B8 (DIFCO) or TNF-α with the potency of 32,000 U per µg (Mallinkrodt, St Louis, MO) at concentrations and times specified in the text.

**Immunoprecipitation and immunoblotting.** Cytoplasmic extracts were prepared from THP-1 cells by detergent lysis (0.1% Nonidet P40 in 10 mmol/L HEPES, pH 7.9, 10 mmol/L KCl, 1.5 mmol/L MgCl2, 300 mmol/L sucrose) in the presence of phosphate inhibitors (12.5 mmol/L β-glycerophosphate, 2 mmol/L NaF, 100 µmol/L Na3VO4) and protease inhibitors (0.1 mmol/L EDTA, 0.1 mmol/L EGTA, 1 mg/mL pefabloc SC, 50 µg/mL antipain, 1 mg/mL aprotinin, 20 µg/mL chymostatin, 5 µg/mL E64, 1 µg/mL leupeptin, 1 µg/mL pepstatin, and 20 µg/mL phosphoramidon). Lysates were adjusted to a final concentration of 50 µmol/L HEPES (pH 7), 250 µmol/L NaCl, and 5 mmol/L EDTA before addition of specific IκKα or IκKβ antibody IgG (20 µg added to reaction mixture). Specific IκBa antipeptide antibody was used as described previously.4

Immunoprecipitations were typically performed in 400 µL reaction mixtures containing 100 to 500 µg of total cytoplasmic protein and 10 µL of protein A-Sepharose beads (Zymed). In some immunoprecipitation experiments, protein A-Sepharose beads were precoated with specific antibodies and washed once with ELB buffer (50 mmol/L HEPES, 250 mmol/L NaCl, 5 mmol/L EDTA, and 0.1% Nonidet P-40).4 Immunoprecipitates were washed three times with 0.1 ELB buffer in the presence of phosphate inhibitors, resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to polyvinylidene difluoride membranes. Immunoreactive polypeptides were detected with anti-IκK antibodies and an enhanced chemiluminescence system (Amersham Pharmacia Biotech, Piscataway, NJ).

**RESULTS**

**Expression of endogenous IκKα and IκKβ in human monocytic THP-1 cells.** At least 2 kinases specific for IκBo and IκBβ were recently identified in Hela and 293 EBNA cells and named IκKα and IκKβ.16,19 To monitor their expression in human monocytic cells, we prepared rabbit polyclonal antibodies directed against peptides derived from the NH2-terminal segment (residues 1-28) and the COOH-terminal segment (residues 731-744) of IκKα and the COOH-terminal segment of IκKβ (residues 743-756). The antibodies were non-specific for homologous peptides in an enzyme-linked immunosorbent assay using peptide-coated wells (results not shown). When cytoplasmic extracts from monocytic THP-1 cells were immunoprecipitated and then probed with these antibodies by immunoblotting, 2 bands of 85 kD and 87 kD, apparent molecular weights, were discerned with anti-IκKα antibody (Fig 1A, lane 1). In contrast, anti-IκKβ antibody precipitated and reacted in immunoblotting with the 87-kD band representing IκKβ (Fig 1B, lane 2). Thus, the anti-IκKβ antibody was monospecific for IκKβ and did not cross-react with IκKα (Fig 1B, lane 1). These bands were not visualized when antibodies were preincubated with homologous peptides before the immunoblotting (results not shown).

**Stimulus-dependent activation of IκKα and IκKβ in human monocytic THP-1 cells.** Having established the presence of IκKα and IκKβ in the cytoplasmic fraction of human monocytic THP-1 cells, we examined the kinase activity in immunoprecipitates obtained from LPS-stimulated cells as compared with those from TNF-α-stimulated cells (Fig 2A). Stimulated and nonstimulated THP-1 cells were lysed, and the cytoplasmic fraction was prepared by differential centrifugation. Immunoprecipitated IκKα and IκKβ were assayed for kinase activity with 32P-γ-ATP and a fusion protein made of IκBo residues 1-54 attached to GST.17,22 Nonstimulated THP-1 cells had no detectable kinase activity in immunoprecipitates obtained with anti-IκKα antibodies (Fig 2A, lane 1). Low-level basal kinase activity was detectable in anti-IκKβ antibody immunoprecipi-
Fig 1. Detection of IKKα and IKKβ in human monocytic THP-1 cells by immunoprecipitation and immunoblotting. Cytoplasmic lysates from unstimulated cells were immunoprecipitated with anti-IKKα NH2-terminal peptide antibody (lane 1) and anti-IKKβ COOH-terminal peptide antibody (lane 2). Immunoprecipitated proteins were resolved by (A) SDS-PAGE and immunoblotted with anti-IKKα NH2-terminal peptide antibody, with (B) anti-IKKβ COOH-terminal peptide antibody (NS represents nonspecific band).

IKKα and IKKβ are differentially activated by LPS and TNF-α. Stimulation of human monocytic THP-1 cells by LPS or TNF-α was followed by determination of enzymatic activity of IKKα and IKKβ in cytoplasmic extracts of stimulated cells prepared at different time intervals. Three NF-κB inhibitors, IκBα, IκBβ, and IκBe, presented as GST fusion proteins were tested as phosphorylation substrates in standard in vitro kinase assays with immune complex containing IKK. LPS stimulation of THP-1 cells for 10 minutes was sufficient to induce measurable kinase activity, which peaked at 30 minutes (Fig 5). The significant increase in IκBα kinase activity at 10 minutes is consistent with phosphorylation and degradation of IκBα in LPS-treated THP-1 cells after 10 minutes stimulation as documented by immunoblotting with specific IκBα antipeptide antibody. Concurrently, the nuclear import of NF-κB monitored by the electrophoretic mobility shift assay shows progressive increase reaching maximum at 30 minutes (Fig 6A and B). Significantly, all three inhibitory proteins, IκBα, IκBβ, and IκBe, used as GST fusion proteins at equivalent concentrations were phosphorylated with similar kinetics in response to LPS. In contrast, TNF-α stimulation of THP-1 cells resulted in much faster kinetics of phosphorylation of IκBα-GST fusion protein reaching peak at 3 to 5 minutes and declining after 20 minutes (Fig 5). This pattern of rapid activation by TNF-α is consistent with much faster degradation of endogenous IκBα and nuclear import of NF-κB reaching maximum at 10 minutes in TNF-α-stimulated THP1 cells (Fig 6A and B). The 5 times lower concentration of TNF-α (20 U/mL) did not change the rapid course of IKKα/IKKβ activation (results not shown). Thus, the differences in activation kinetics of IκB kinase complex induced by LPS and TNF-α suggest that these proinflammatory agonists evoke different signaling pathways resulting in activation of IKKα-containing and IKKβ-containing complex in human monocytic cells.
The results presented here have several important implications for the cellular and molecular pathogenesis of septic shock induced by LPS. First, our studies provide evidence that IκB kinase complex containing IκKα and IκKβ is an intracellular target for LPS produced by different gram-negative bacteria (enterobacteriaceae and nonenterobacteriaceae).

Second, IκKβ appears to be a more LPS-responsive signal transducer than IκKα based on observed kinase activity (Fig 2). This is consistent with recent demonstration that IκKα is not required for signaling induced by proinflammatory cytokines, IL-1, or TNF-α.23–25 The lowest concentration of LPS that induces detectable activation of IκKα and IκKβ in human monocytic THP-1 cells was 10 ng/mL. Treatment of human peripheral blood mononuclear cells (“adherent monocytes”) with 100 ng/mL of E. coli 0111:B4 lipopolysaccharide also induces IKK activity, thus validating THP-1 cells as a model for studying LPS-induced IKK activation in human cells of the myelomonocytic lineage.26 Moreover, LPS-stimulated and TNF-α-stimulated IKK complex can phosphorylate not only inhibitory proteins IκBα and IκBβ, but also IκBe. The IκB kinases provide a pivotal checkpoint in NF-κB-mediated signaling to the nucleus. Thus, activation of IκB kinases by LPS in human monocytic cells constitutes an important link in understanding the NF-κB recruitment in molecular pathogenesis of septic shock. Whether continuing activation of IκB kinases in vivo by LPS derived from different gram-negative bacteria results in persistent nuclear import of NF-κB demonstrated in nonsurvivors of septic shock11 remains to be established with in vivo models. Importantly, kinetics of phosphorylation of all three inhibitory proteins, IκBα, IκBβ, and IκBe, as demonstrated herein by IKK complex activated by LPS can contribute to progressive activation of the nuclear import of NF-κB.

Third, the distinct activation kinetics of IκKα-containing and IκKβ-containing complex by LPS and TNF-α in human monocytic cells is consistent with different rate of the nuclear import of NF-κB in these cells (Fig 6).4,9,27 These agonists signal through distinct receptors (toll-like receptor 2 and 4 or TNF-α receptor 1 and 2).28–31 Receptor-proximal steps in intracellular signaling induced by TNF-α that interacts with its cognate receptor, TNFR1, and by LPS interacting with its cognate receptor TLR2 differ.29 Whereas TNF-α–induced signaling requires TRAF2 in mammalian cell transfectants, LPS-induced signaling via TLR2 seems to require TRAF6.29,32 The latter is required for IL-1–induced signaling, which also uses an adaptor protein MyD88.33–35 Dominant-negative mutants of MyD88 and TRAF6 expressed in TLR2- and IL-1R–transfected cells selectively blocked signaling evoked by LPS and IL-1 but remained ineffective toward TNF-α–induced signaling.29,32 These differences in receptor-proximal signaling may explain the different kinetics of IKK complex activation by LPS and TNF-α in human monocytic THP-1 cells used in our experiments. Further dissection of LPS-stimulated and TNF-α-stimulated specific pathways of IKK complex activation will help in the development of new inhibitors designed to interrupt potentially lethal lipopolysaccharide-induced intracellular mechanism of septic shock.

**DISCUSSION**

**A. LPS**

![Figure 3.](image)

**B. TNFα**

![Figure 4.](image)

![Figure 5.](image)
ACKNOWLEDGMENT

The authors thank Traci Tidwell, Susan Rowlinson, and Erica Holleran for assistance in preparation of the manuscript, Zhi-Liang Chu and Min Dai for preparation of GST fusion proteins, and Hiroyasu Nakano, Juntendo University School of Medicine, Tokyo, Japan, for plasmid pGEX-4T-IκBε (1-61).

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