Protein-tyrosine Kinase Activation Is Required for Lipopolysaccharide Induction of Interleukin 1β and NFκB Activation, but Not NFκB Nuclear Translocation

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In human monocytes, interleukin 1β (IL-1β) protein production and steady state mRNA levels are increased in response to lipopolysaccharide, predominantly as a result of increased transcription of the interleukin 1β gene. Expression of interleukin 1β and other cytokines, such as interleukin 6 and tumor necrosis factor α, has been shown to be dependent on the activation of the transcription factor, NFκB. Since recent studies have shown that lipopolysaccharide-induced tyrosine kinase activation is not required for NFκB nuclear translocation, we sought to determine whether NFκB translocated in the absence of tyrosine kinase activity was active in stimulating transcription.

We have found that, in the human pro-monocytic cell line, THP-1, the lipopolysaccharide-induced expression of interleukin 1β is dependent on tyrosine kinase activation. Tyrosine kinases are not required for lipopolysaccharide-mediated nuclear translocation of NFκB. However, in the absence of tyrosine kinase activity, the ability of NFκB to stimulate transcription is impaired. This inhibition of transcription is specific for NFκB; in the absence of tyrosine kinase activity, the NFκB translocated to the nucleus in the absence of PTK activity. These data indicate that PTK activity is not obligatory for NFκB nuclear translocation, while lipopolysaccharide-induced expression of inflammatory mediators requires tyrosine kinase activity, tyrosine kinase activity is not obligatory for lipopolysaccharide signal transduction.

Septic shock is a lethal syndrome and the principle cause of death in patients in intensive care units (1). A number of laboratories have demonstrated a role for protein-tyrosine phosphorylation in intracellular signals initiated by endotoxin. Endotoxin stimulation has been shown to rapidly increase tyrosine phosphorylation of a number of proteins (9, 10), and protein-tyrosine kinases (PTKs) appear to be linked to endotoxin signaling at a number of levels, including through the activation of receptor PTKs, p56lck, p58fgr, and p59fgr (11), as well as through a PTK-dependent transient activation of the mitogen-activated protein kinase (MAPK or ERK) family (12, 13). In addition, PTK inhibitors have been shown to be effective in preventing endotoxin-induced cytokine production, both in vivo (14) and in vitro (10, 15).

Recent data have indicated that PTK activity is not involved in the endotoxin-mediated nuclear localization of NFκB (16). Although specific NFκB family members may mediate transcription depending on the inducer, cell type, and gene context (for review, see Ref. 17), nuclear localization of NFκB is generally paralleled by increases in NFκB-dependent transcription. While it is likely that endotoxin signaling pathways other than those regulating NFκB activity are involved in the production of inflammatory mediators, NFκB is thought to play a central role in the transcription of IL-1β gene (5, 6).

Using the human pro-monocytic cell line, THP-1, we sought to determine the role of PTKs in endotoxin-stimulated transcription activation. Specifically, we tested the transcription competency of NFκB translocated to the nucleus in the absence of PTK activity. We found that, similar to peripheral blood monocytes, endotoxin-induced IL-1β expression by THP-1 cells was dependent on PTK activity. In the absence of PTK activity, endotoxin was unable to induce IL-1β mRNA or activate NFκB-dependent transcription, despite nuclear translocation of NFκB. These results suggest that NFκB can be regulated at two separate and distinct levels, nuclear translocation and transcription activation. PTKs appear to play a critical role at the level of transcription activation mediated by NFκB. We further show that, in the absence of PTK activity, endotoxin-mediated activation of AP-1 is enhanced. These data indicate that PTK activity is not obligatory for endotoxin signal transduction and suggest a complex mechanism for signal transduction in the activation of transcription factors by endotoxin. These studies provide further evidence suggesting a pivotal role for NFκB in mediating the expression of inflammatory mediators.

EXPERIMENTAL PROCEDURES

Cell Culture—THP-1 cells were maintained in RPMI 1640 (Life Technologies, Inc.) with 10% fetal bovine serum (HyClone) as described previously (3). Low passage number and log-phase cells were used for all experiments. RNA isolation and Northern Analysis—THP-1 cells were pretreated with 20 μg/ml of actinomycin D and 10 μg/ml of cycloheximide for 2 h prior to treatment with LPS or lipopolysaccharide.
for 30 min with 30 μg/ml genistein (LC Laboratories) before addition of endotoxin (1 μg/ml, or as indicated in the legends to the figures, Escherichia coli lipopolysaccharide 0111: B4, Sigma) for 1 to 3 h. Total RNA was isolated from 2 × 10^7 cells/condition using RNA STAT-60™ (TEL-TEST B, Inc.) according to the manufacturer’s instructions. After transfection to nylon membranes, IL-1β, tumor necrosis factor α, junB, and glyceraldehyde-3-phosphate dehydrogenase mRNAs were visualized by autoradiography as described previously (3).

Transfection and Assay for CAT Activity—THP-1 cells (1 × 10^7 cells/ transfection) were transfected with plasmids containing 6 repeats of the NF-κB consensus binding site of the kappa light chain immunoglobulin enhancer (pNH.dAN.6XKB.gfib.CAT, kindly provided by Dr. E. O’Neill) or 3 repeats of the AP-1 consensus binding site of the human collagenase gene (Cd-TRE × 3/TK CAT, kindly provided by Dr. P. Angel) linked to the CAT (chloramphenicol acetyltransferase) reporter gene using DEAE-dextran as described by Shirakawa and co-workers (18). After 18–20 h, cells were pretreated with genistein (30 μg/ml) and then stimulated with TNF-α and endotoxin (1 μg/ml) (1 μg/ml genistein (LC Laboratories) or genistein and stimulated with endotoxin (1 μg/ml) as described under “Experimental Procedures.” The results (expressed as -fold increase over -LPS) of a single representative experiment are shown in A. The averaged result of three separate experiments is shown in B, where the endotoxin-stimulated CAT activity in genistein-pretreated samples is expressed as a percentage of the CAT activity after pretreatment with vehicle alone (100%).

Nuclear Extraction Preparation and Assay for DNA Binding of Transcription Factors—THP-1 cells (2 × 10^6 cells/condition) were pretreated with genistein (30 μg/ml) for 30 min before stimulation with endotoxin (1 μg/ml) or PMA (10 ng/ml) for 30 min as indicated in the figures. Nuclear extracts were prepared and assayed for DNA binding of transcription factors as described previously (20). Oligonucleotide probes used in DNA binding assays were prepared using an Applied Biosystems model 380B automated DNA synthesizer and contained the following sequences: a consensus NF-κB binding site derived from the kappa light chain immunoglobulin enhancer, 5′-AATTTCCAACAGAGGGGACTTCGCAG-3′ and the complementary strand; a consensus AP-1 binding site derived from the human collagenase gene, 5′-TAATTCCCTAGATTGATGGCAGCGATCCGCCCGAG-3′ and the complementary strand; Antibodies used in the supershift assays were obtained from Santa Cruz Biotechnology.

RESULTS

Protein-tyrosine Kinases Mediate Endotoxin Induction of IL-1β—Bacterial endotoxin activates monocytes through mechanisms that are incompletely characterized; however, recent
studies have implicated PTKs in endotoxin signal transduction (9–15). Endotoxin is a potent inducer of IL-1β expression in human monocytes and in the pro-monocytic cell line, THP-1. To determine the role of PTKs in the induction of IL-1β expression, THP-1 cells were stimulated with endotoxin in the presence of inhibitors of PTKs. As shown in Fig. 1, preincubation with the PTK inhibitor, genistein, inhibited endotoxin-stimulated IL-1β mRNA accumulation. Dose-response studies confirmed the inhibitory effects of genistein at a variety of endotoxin concentrations. Similar results were seen in human blood monocytes and with another PTK inhibitor, herbimycin A (data not shown). Endotoxin-mediated tumor necrosis factor α mRNA accumulation is similarly inhibited by preincubation with genistein. In contrast, endotoxin-stimulated JunB mRNA accumulation is unaffected by preincubation with protein-tyrosine kinase inhibitors. These results indicated that PTKs can specifically mediate endotoxin induction of IL-1β and allowed us to further investigate the role of PTKs in the activation of transcription factors by endotoxin in THP-1 cells.

Protein-tyrosine Kinases Mediate Endotoxin Induction of NFκB Activation—Recent studies have shown that endotoxin-induced NFκB nuclear localization is not dependent on PTK activation (16). We directly tested the transcription competency of NFκB translocated to the nucleus in the absence of PTK activity. Using a CAT reporter gene that is dependent only on NFκB activity, we found that in the absence of PTK activity, endotoxin was unable to activate NFκB-dependent transcription (Fig. 2, A and B). Nuclear localization of NFκB induced by endotoxin in THP-1 cells was unaffected by the presence of PTK inhibitors, both with respect to DNA binding (Fig. 2C) and NFκB subunit composition (Fig. 2D). These results suggest that NFκB can be regulated at two separate and distinct levels, nuclear translocation and transcription activation. PTKs are apparently involved in the regulation of NFκB transcription activity.

Protein-tyrosine Kinases Mediate Endotoxin Induction of AP-1 Activation—We further investigated the specificity of PTK-mediated transcription in response to endotoxin using a CAT reporter gene that is dependent on the transcription factor, AP-1. Treatment of THP-1 cells with PTK inhibitors prior to endotoxin stimulation did not affect DNA binding or inhibit CAT expression mediated by AP-1. Endotoxin-mediated activation of AP-1 appears to be enhanced in the absence of PTK activity (Fig. 3, A and B). Furthermore, AP-1 transcription activity in THP-1 cells appears to be negatively regulated by PTKs, as protein kinase C-induced AP-1 transcription activity is also enhanced in the absence of PTK activity (Fig. 3C). DNA binding by AP-1 is not significantly affected by any of these treatments (Fig. 3D). These results demonstrate that PTK activity is not required for the activation of transcription factors by endotoxin and suggest a complex mechanism for activation of gene expression in response to endotoxin. These studies further emphasize a potentially critical role for NFκB in mediating the expression of inflammatory genes.

DISCUSSION

It has been well established that many transcription factors are modified in response to a variety of stimuli to achieve gene activation/repression (for review, see Ref. 21). While the pattern of transcription factor modification, most commonly protein phosphorylation, has been relatively easily elucidated, the physiologic mechanisms involved in transcription activation have only now begun emerging. Transcription factor nuclear localization, DNA binding, and interactions with the basic transcription machinery as well as with other transcription factors are all potential regulatory targets in the activation of gene expression by extracellular signals.
Non-receptor PTKs (11) and the MAPK/ERK family (12, 13) have been implicated in the signal transduction pathway utilized by endotoxin. Significantly, in vitro studies have shown that at least one transcription factor, ATF-2, is directly phosphorylated by the p38 member of the MAPK/ERK family in response to endotoxin (22). Phosphorylation of ATF-2 by p38 was mapped to the amino-terminal activation domain at sites known to increase the transcriptional activity of ATF-2. Subcellular localization of p38 showed cytoplasmic and nuclear pools, establishing a spatial link between this kinase and potential transcription factor targets within the nucleus. Specific inhibitors of p38 MAPK/ERK have implicated this kinase in the signal transduction pathway to increased expression of inflammatory cytokines (23), and recent co-transfection studies have established a direct link between p38 activation and tumor necrosis factor promoter activation (24).

In the absence of genetic analyses, like gene “knock-out” or “dominant negative” mutants, signal transduction pathways are often dissected through the use of protein kinase inhibitors. Many classes of inhibitors have been utilized in numerous studies, yet the complex and compensatory nature of intracellular signaling has made exact mapping of these pathways elusive. In the studies we have presented, the activation of single transcription factors were used as a measure of intracellular signaling by endotoxin. We have been able to demonstrate that NF-κB can be regulated not only at the level of nuclear translocation (which has already been well characterized and reviewed in Ref. 25), but more importantly, at the level of transcription activation. Our results indicate that PTKs play an important role in the regulation of the latter process. Recent studies have shown that the transactivating domain of the p65 subunit of NF-κB interacts with the TATA-binding protein component of the basic transcription machinery (26). What role PTKs may play in this subsequent step in NF-κB transactivation is not yet known.

We also observed enhanced transactivation by AP-1 in the absence of PTK activity. Activation of AP-1 is known to involve JNK or SAPK, which are functionally distinct members of the MAPK/ERK family (27). The endotoxin signaling pathway can diverge at MKK4, the MAPK kinase thought to be immediately upstream of JNK/SAPK and p38 kinases. Our results show that endotoxin induction of JunB mRNA accumulation is unaffected by PTKs and suggest that endotoxin signals through MKK4 may affect gene expression, in part by differential activation/repression of transcription factors. The enhanced AP-1 transcription activation that we have observed may be due, in part, to increased accumulation of the JunB component of the AP-1 transcription complex. Alternatively, a number of parallel intracellular signals may be integrated at the nucleus, ultimately determining the potential for gene expression. Our assay system allows us to measure the transactivation potential of individual transcription factors, thus simplifying the integration of intracellular signals, and may allow more precise mapping of signaling pathways to the nucleus.

The salient feature of the MAPK/ERK family is the requirement for dual phosphorylation on threonine and tyrosine residues for kinase activity. The molecular targets for MAPK/ERK activity include the microtubule-associated protein 2, the S6 ribosomal kinase, and transcription factors, such as c-jun and ATF-2. The precise function of MAP kinases in monocytes is not known, and many, if not all, of these molecular targets may respond to endotoxin. We have shown that endotoxin mediates gene expression, at least in part, through the activation of transcription factors and that the transactivation potential of a given transcription factor may be determined by protein-tyrosine kinases activated by endotoxin. Whether this response is altered by endotoxin as a direct result of MAPK/ERK activity is not clear at this time. Tyrosine kinases are not required for endotoxin-mediated signaling to the nucleus, as is evidenced by endotoxin-mediated nuclear translocation of NF-κB and enhanced AP-1 activation in the presence of PTK inhibitors. These results suggest a complex mechanism for signal transduction in the activation of gene expression by endotoxin. Our finding that NF-κB is inactive and that IL-1β (and tumor necrosis factor α) is not expressed in the absence of PTK activity provides further support for a model in which NF-κB plays a critical role in mediating the expression of inflammatory mediators.

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