Lipopolysaccharide stimulation of ERK1/2 increases TNF-α production via Egr-1

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Shi, Liang, Raj Kishore, Megan R. McMullen, and Laura E. Nagy. Lipopolysaccharide stimulation of ERK1/2 increases TNF-α production via Egr-1. Am J Physiol Cell Physiol 282: C1205–C1211, 2002. First published February 6, 2002; 10.1152/ajpcell.00511.2001.—Lipopolysaccharide (LPS) is a potent activator of tumor necrosis factor-α (TNF-α) production by macrophages. LPS stimulates the phosphorylation of extracellular signal-regulated kinase (ERK) 1/2 and increases TNF-α mRNA and protein accumulation in RAW 264.7 murine macrophages. However, the role of ERK1/2 activation in mediating LPS-stimulated TNF-α production is not well understood. Inhibition of ERK1/2 activity with PD-98059 or overexpression of dominant-negative ERK1/2 decreased LPS-induced TNF-α mRNA quantity. LPS rapidly increased early growth response factor (Egr)-1 binding to the TNF-α promoter; this response was blunted in cells treated with PD-98059 or transfected with dominant-negative ERK1/2. Using a chloramphenicol acetyltransferase reporter gene linked to the Egr-1 promoter, we show that LPS increased Egr-1 promoter activity via an ERK1/2-dependent mechanism. These results delineate the role of ERK1/2 activation of Egr-1 activity in mediating LPS-induced increases in TNF-α mRNA expression in macrophages.

MACROPHAGES PLAY AN IMPORTANT role in regulating immune and inflammatory activities. Upon activation by lipopolysaccharide (LPS), cell wall components of gram-negative bacteria, macrophages secrete an array of proinflammatory cytokines and oxidants, including tumor necrosis factor-α (TNF-α). TNF-α serves as an important mediator for activation of the host immune response against infection and tumor formation, as well as in tissue remodeling. However, in addition to its beneficial effects, TNF-α also mediates septic shock during chronic infection, cachexia, some autoimmune diseases, and activation of human immunodeficiency virus (18).

LPS-induced TNF-α production is controlled at transcriptional (3, 19), posttranscriptional (8, 12), and posttranslational levels (13). The following seven transcription factor binding sites identified within the promoter region of the TNF-α gene are required for full transcriptional activation of the TNF-α gene after LPS treatment: an early growth response factor (Egr)-1 site, three E26 transformation-specific (Ets) sites, a cAMP response element (CRE)/activator protein (AP)-1 site, a nuclear factor (NF)-κB site, and a promoter specificity protein 1 (Sp1) site (19, 23). LPS stimulates a complex array of signal transduction pathways, leading to the activation of the transcription factors regulating TNF-α expression. Extracellular/signal-regulated kinase (ERK) 1/2 is a member of the mitogen-activated protein kinase family, which are activated by protein phosphorylation at tyrosine and threonine residues (20). Although it is well documented that treatment of macrophages with LPS activates ERK1/2 (5, 16, 21), the role of ERK1/2 in regulation of LPS-induced TNF-α mRNA expression is controversial. One report indicates that ERK1/2 activation is required for TNF-α mRNA expression in murine J774 macrophages (19). In contrast, another investigation found that inhibition of ERK1/2 activation had no effect on TNF-α mRNA expression in RAW 264.7 macrophages (11). Because of the potentially key role of LPS activation of ERK1/2 in regulating TNF-α expression, here we have made use of a specific inhibitor of mitogen/extracellular signal-regulated kinase kinase, PD-98059, and dominant-negative forms of ERK1/2, to address the role of ERK1/2 in mediating LPS-stimulated TNF-α accumulation. We show that LPS-induced ERK1/2 activation increases TNF-α mRNA expression in RAW 264.7 cells via regulation of Egr-1 production and binding to the TNF-α promoter.

EXPERIMENTAL PROCEDURES

Reagents. LPS from Escherichia coli serotype 026:B6 was purchased from Sigma (St. Louis, MO). PD-98059 was obtained from Calbiochem (La Jolla, CA). Antibodies were from the following sources: anti-active ERK1/2 polyclonal antibody (Promega, Madison, WI), anti-ERK1/2 (Upstate Biotechnology, Lake Placid, NY), anti-Egr-1, and anti-SP1 (Santa Cruz Biotechnology, Santa Cruz, CA). oligonucleotide probes were synthesized by IDT Technologies (Coralville, IA). The ribonuclease protection assay system was from Ambion (Austin, TX). The template for transcribing the anti-sense chloramphenicol acetyltransferase (CAT) riboprobe was purchased from Promega. Kinase dead dominant-negative constructs

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for ERK1/2 were a gift from Dr. R. L. Eckert and have been described previously (14). An Egr-1 promoter linked to a CAT reporter construct (pEgr-1B950 CAT) and control vector (pCAT) were a gift from Dr. R. P. Huang (7).

Culture and transfection of RAW 264.7 macrophages. The mouse macrophage cell line RAW 264.7 was cultured as previously described (10). For transfections, RAW 264.7 macrophages were grown in 100-mm dishes to 60% confluence and were transiently transfected with either empty vectors or dominant-negative ERK1/2 and/or Egr-1 promoter-CAT reporter expression vectors using TransFast transfection reagent (Promega) following the manufacturer’s instructions. A single plate of transfected cells was then used to set up the experimental cultures required for each assay, ensuring equal transfection efficiencies between different treatments, and cells were cultured for an additional 48 h before treatment with LPS.

ERK1/2 activation. After 48 h, the cells were washed one time with 2 ml of DMEM with 10% FBS and were treated with or without LPS in DMEM with 10% FBS for the indicated times. In some cases, cells were preincubated with PD-98059 for 2 h before addition of LPS. Activation of ERK1/2 was measured by Western blot analysis (9). Membranes were first probed with anti-ERK1/2 antibody and then stripped and reprobed with antibody to total ERK1/2.

TNF-α mRNA. Cells were treated with or without LPS as described above and then were washed one time with PBS and lysed in 1 ml of Trizol reagent (Life Technologies, Gaithersburg, MD) for 5 min. Total RNA was isolated with TRIzol reagent according to the manufacturer’s instructions, and TNF-α mRNA was quantified by Northern blot analysis or ribonuclease protection assays.

Electrophoretic mobility shift assays. RAW 264.7 cells were treated or not with LPS as described above, and then nuclear extracts were prepared for electrophoretic mobility shift assay as described (9). The binding of nuclear proteins to an oligonucleotide corresponding to the Egr-1 binding site in the promoter region of the murine TNF-α gene (5′-AACCTCTTGGCCCGCGATGGAG-3′) was measured as described previously (9). In competition and antibody supershift assays, competing oligonucleotides (50-fold excess) or 2 μg of antibodies were included in the binding reaction mixtures 10 min before the addition of labeled oligonucleotides.

TNF-α bioassay. RAW 264.7 cells were treated or not with LPS for 2–4 h, and cell culture media were removed. Accumulated TNF-α was measured by bioassay (1).

Statistical analysis. All values are expressed as means ± SE. Student’s t-test was used to compare between groups.

RESULTS

ERK1/2 are involved in LPS-induced TNF-α mRNA and protein accumulation. LPS treatment activated ERK1/2 in RAW 264.7 cells. Phosphorylation peaked 40 min after LPS treatment (Fig. 1A) and returned to basal levels by 3 h after LPS treatment (data not shown). LPS treatment did not change the total amount of ERK1/2 protein (Fig. 1A). Pretreatment of RAW 264.7 cells with PD-98059 for 2 h dose dependently decreased LPS-induced ERK1/2 phosphorylation (Fig. 1B). Phosphorylation of ERK1/2 was decreased by ~70% of control in the presence of 50 μM PD-98059 (Fig. 1C). LPS treatment also increased TNF-α mRNA accumulation (Fig. 1D) and peptide secretion from RAW 264.7 cells (Fig. 1F). Pretreatment of RAW 264.7 cells with 50 μM PD-98059 reduced LPS-induced TNF-α mRNA accumulation by 65% at 30 min and 37% at 45 min after LPS treatment (Fig. 1, D and E). Similarly, pretreatment with 50 μM PD-98059 decreased LPS-induced secretion of TNF-α by 65% at 2–4 h after LPS treatment (Fig. 1F).

To further substantiate the role of ERK1/2 in LPS-induced TNF-α production, we transfected RAW 264.7 macrophages with empty vector or dominant-negative ERK1/2 constructs. Activation of ERK1/2 by LPS was decreased in cells transfected with dominant-negative ERK1/2 constructs (Fig. 2A), whereas transfection with empty vector had no effect. Similarly, although LPS increased TNF-α mRNA accumulation over 30–60 min in cells transfected with empty vectors, overexpression of dominant-negative ERK1/2 decreased LPS-mediated TNF-α mRNA accumulation by 60% (Fig. 2B). Overexpression of dominant-negative ERK1/2 also suppressed the release of TNF-α peptide (Fig. 2C).

ERK1/2 activation is required for LPS-induced Egr-1 binding to the TNF-α promoter. Seven transcription factor binding sites have been identified in the promoter region of the TNF-α gene; the sites contribute to upregulation of the TNF-α gene after LPS treatment in macrophages and T cells (3, 23). One of these transcription factors, Egr-1, is regulated by an ERK1/2-dependent mechanism in other cell types (4, 22). Therefore, we asked whether LPS-stimulated Egr-1 binding to the murine TNF-α was mediated via ERK1/2. Although multiple proteins bound to the probe containing the Egr-1 site, stimulation with LPS increased the binding activity of only one protein (Fig. 3A). Supershift assays utilizing antibodies against Sp1 and Egr-1, two transcription factors reported to bind to Egr-1 consensus sites, identified this protein as Egr-1 (Fig. 1–Fig. 3A). Pretreatment of RAW 264.7 cells with 50 μM PD-98059 for 2 h decreased LPS-induced Egr-1 binding by 40 ± 11% (P < 0.05 compared with control, n = 3; Fig. 3A). Increased Egr-1 DNA binding activity was associated with a greater accumulation of Egr-1 protein in the nucleus after LPS treatment (Fig. 3B). Pretreatment of PD-98059 for 2 h decreased LPS-induced Egr-1 quantity in the nucleus by 42 ± 5% (P < 0.05 compared with controls not treated with PD-98059, n = 6; Fig. 3B). Overexpression of dominant-negative ERK1/2 also decreased LPS stimulation of Egr-1 binding to the TNF-α promoter, with binding reduced to 21 ± 5% (P < 0.05 compared with cells transfected with empty vector, n = 3; Fig. 3C).

To further investigate the role of Egr-1 in LPS-dependent TNF-α accumulation, we compared the time course of LPS-stimulated Egr-1 binding with the time course for TNF-α mRNA accumulation. LPS rapidly increased Egr-1 binding to the TNF-α promoter; maximal binding activity was observed after 30 min (Fig. 4A). This peak of Egr-1 binding precedes the 45- to 60-min peak for LPS-stimulated TNF-α mRNA accumulation (Fig. 4B), consistent with the hypothesis that Egr-1 binding to the TNF-α promoter is required for maximal activation in response to LPS.
LPS stimulates Egr-1 promoter activation. Using a CAT reporter construct linked to the Egr-1 promoter, we tested whether treatment of RAW 264.7 macrophages with LPS increased Egr-1 promoter activity. RAW 264.7 macrophages were transfected with pCAT control vector or an Egr-1 promoter-CAT reporter construct and then were stimulated or not with 100 ng/ml LPS for 60 min. LPS had no effect on CAT mRNA expression in cells transfected with pCAT control vector (Fig. 5) but increased CAT mRNA expression by 2.2-fold in cells expressing the Egr-1 promoter-CAT construct (Fig. 5). Consistent with a role for ERK1/2 activation in mediating LPS-induced increases in Egr-1 expression, cotransfection with dominant-negative ERK1/2 completely abrogated the ability of LPS to increase Egr-1 promoter-driven CAT mRNA expression (Fig. 5).

DISCUSSION

Activation of macrophages with LPS initiates a complex set of signal transduction cascades, ultimately resulting in increased secretion of TNF-α. Increased TNF-α expression in response to LPS requires the activation of a distinct set of transcription factors binding to the TNF-α promoter (19, 23). Although the exact array of transcription factors interacting with the TNF-α promoter is to some extent cell and species specific (11), recruitment of NF-kB, Egr-1, and c-Jun appears to be required for full activation of TNF-α expression in most types of macrophages (19, 23). Despite advances in our understanding of the transcriptional regulation of TNF-α expression, the signal transduction cascades linking the binding of LPS to cell surface receptors with increased TNF-α expression are only partially understood. Here we report that LPS-induced activation of ERK1/2 is required for LPS-stimulated TNF-α mRNA and peptide accumulation in RAW 264.7 macrophages. In other cell types, activation of ERK1/2 activation leads to an increase in the activity of the transcription factor Egr-1 (4, 22). Therefore, we asked whether ERK1/2-dependent activation of Egr-1 contributed to LPS-stimulated TNF-α produc-
Fig. 2. Dominant-negative ERK1/2 decreases LPS-stimulated TNF-α mRNA and peptide accumulation. RAW 264.7 macrophages were transiently transfected with empty vector or dominant-negative (DN) ERK1/2 constructs, replated, and cultured for 48 h and then stimulated or not with 100 ng/ml LPS. A: after 30 min of stimulation with LPS, phosphorylation of ERK1/2 was measured by Western blot. B: after 30–60 min of stimulation with LPS, total RNA was isolated, and TNF-α mRNA and β-actin mRNA were detected by RNase protection assays (bottom). Values represent means ± SE; n = 3. *P < 0.05. A representative experiment is shown at top. C: cell supernatants were then harvested and assayed for TNF-α by bioassay. Values represent means ± SE; n = 3–4.

Fig. 3. ERK1/2 activation is required for LPS-induced Egr-1 binding to the murine TNF-α promoter. RAW 264.7 cells were pretreated with 0 or 50 μM PD-98059 for 2 h and then stimulated or not with 100 ng/ml LPS for 60 min. A: binding of nuclear extracts to the Egr-1 site on the TNF-α promoter was determined by electrophoretic mobility shift assay (EMSA). Identification of the proteins binding to the Egr-1 site was assessed using an Egr-1 and Sp1 specific antibody to supershift the Egr-1-bound oligonucleotide. B: quantity of Egr-1 in lysates of isolated nuclei was measured by Western blot analysis using Egr-1-specific antibodies. The same blot was stripped and reprobed with PU.1 specific antibodies to ensure equal loading of nuclear proteins. C: RAW 264.7 macrophages were transiently transfected with empty vector or dominant-negative ERK1/2 constructs, subcultured for 48 h, and then stimulated with 0 or 100 ng/ml LPS for 30 min. Binding of nuclear extracts to the Egr-1 site on the TNF-α promoter was determined by EMSA. Autoradiograms are representative of 3 experiments.
We report that LPS-stimulated activation of ERK1/2 increased the binding of Egr-1 to the murine TNF-α promoter. Pretreatment of RAW 264.7 macrophages with PD-98059 or overexpression of dominant-negative ERK1/2 decreased LPS-stimulated Egr-1 binding to the Egr-1 site from the TNF-α promoter. Inhibition of ERK1/2 activation also decreased LPS-induced Egr-1 promoter activity and protein accumulation.

Fig. 4. LPS induction of Egr-1 DNA binding activity precedes maximal stimulation of TNF-α mRNA. RAW 264.7 macrophages were stimulated with 100 ng/ml LPS for 0–180 min. A: binding of nuclear extracts to the Egr-1 site on the TNF-α promoter was measured by EMSA. B: total RNA was isolated, and TNF-α mRNA was measured by Northern blot. Values represent means ± SE, n = 3 for EMSA and 5 for Northern blots. Representative experiments are shown at right.

Fig. 5. LPS increases Egr-1 promoter activity by an ERK1/2-dependent mechanism. RAW 264.7 macrophages were transfected with pCAT control vector or cotransfected with Egr-1 promoter-CAT reporter construct with dominant-negative ERK1/2 expression vector or its empty vector control, subcultured for 48 h, and then stimulated with 100 ng/ml LPS for 0–60 min. Expression of CAT and β-actin mRNA was measured by RNase protection assay. Values represent means ± SE, n = 3, *P < 0.05.
loration inside nuclei of RAW 264.7 macrophages (Figs. 3B and 5), suggesting that LPS-induced Egr-1 binding to the TNF-α promoter is dependent on stimulation of Egr-1 synthesis via ERK1/2 activation. Indeed, the time course for activation of Egr-1 DNA binding activity is consistent with a maximal increase in Egr-1 activity before maximal increases in TNF-α mRNA accumulation (see Fig. 4). Egr-1 is an immediate early gene and a member of the zinc finger transcription factor family. Its synthesis is rapidly and transiently upregulated in response to a variety of stimuli, including LPS in peritoneal macrophages (2) and primary cultures of rat Kupffer cells (9). This response is similar to the rapid increase in Egr-1 DNA binding activity in response to LPS reported here (Fig. 4). Activation of Egr-1 promoter activity, and induction of Egr-1 expression, was dependent on activation of ERK1/2 in response to LPS in RAW 264.7 macrophages (Fig. 5). A recent report demonstrated a similar involvement of ERK1/2 and Egr-1 in LPS-stimulated TNF-α production in the human macrophage cell line THP-1 (6). Similarly, Egr-1 induction in a number of other cell types, including astroglial cells (17), endothelial cells (22), and hepatocytes (15), requires ERK1/2 activation. The essential role of LPS-stimulated ERK1/2 activation in TNF-α mRNA accumulation reported here was based on data collected using two different methods of inhibition of ERK1/2: pretreatment with PD-98059, a specific inhibitor of ERK1/2 phosphorylation, and overexpression of dominant-negative ERK1/2 construct. The use of these two distinct methods of inhibiting ERK1/2 makes it unlikely that the results are because of nonspecific effects of either inhibitor. Although pretreatment of RAW 264.7 macrophages with 50 μM PD-98059 did not completely inhibit ERK1/2 activation, significant reductions in nuclear Egr-1 protein and DNA binding activity were observed (see Fig. 3). Instead of using higher concentrations of PD-98059 to attempt to completely inhibit ERK1/2 activation, which would have a higher risk for potential nonspecific effects of the inhibitor, we made use of a dominant-negative ERK1/2 construct. Overexpression of dominant-negative ERK1/2 resulted in undetectable levels of ERK1/2 phosphorylation. With complete inhibition of ERK1/2, Egr-1 binding activity was reduced to baseline (Fig. 3C), and activation of the Egr-1 promoter was completely blocked (Fig. 5). Taken together, the strong correlation between the extent of inhibition of ERK1/2 with PD-98059 or overexpression of dominant-negative ERK1/2 with decreased Egr-1 DNA binding activity provides strong support for the role of ERK1/2 in LPS-induced activation of Egr-1 DNA binding activity.

The data presented here demonstrating a role for ERK1/2 and Egr-1 in mediating LPS-stimulated TNF-α production differ from a previous report indicating that LPS-induced TNF-α mRNA expression in RAW 264.7 cells was independent of ERK1/2 activation (11). Here we have used a slightly higher concentration of the ERK1/2 inhibitor (50 μM) compared with 20 μM PD-98059 in the previous report (14) and overexpression of a dominant-negative ERK1/2 construct, which may have resulted in a greater degree of inhibition of ERK1/2 activation. Moreover, it is likely that inhibition of ERK1/2 has a rapid effect on TNF-α mRNA. TNF-α mRNA accumulation peaked at 45–60 min after stimulation by LPS (Fig. 4). At 30–60 min after LPS stimulation, activity of ERK1/2 contributed up to 50% of the stimulus toward TNF-α mRNA accumulation (see Figs. 1, D and E, and 2B). In the study by Means et al. (11), which reported no effect of ERK1/2 inhibition on LPS-dependent responses, TNF-α mRNA was not measured until 4 h after LPS stimulation, well past the peak of LPS-stimulated TNF-α mRNA observed here (Fig. 4B). Despite this potentially transient inhibition of mRNA accumulation, inhibition of ERK1/2 activation, either with PD-98059 or overexpression of dominant-negative ERK1/2, inhibited TNF-α protein production at 2 and 4 h after LPS stimulation (Figs. 1F and 2C). Thus LPS stimulation of ERK1/2 contributes to maximal TNF-α production in RAW 264.7 macrophages by increasing Egr-1 quantity and DNA binding activity to the TNF-α promoter.

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