Transcriptional Activation of the Cyclooxygenase-2 Gene in Endotoxin-treated RAW 264.7 Macrophages*

(Received for publication, October 12, 1999, and in revised form, December 9, 1999)

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Cyclooxygenase-2 (COX-2), the enzyme primarily responsible for induced prostaglandin synthesis, is an immediate early gene induced by endotoxin in macrophages. We investigated the cis-acting elements of the COX-2 5'-flanking sequence, the transcription factors and signaling pathways responsible for transcriptional activation of the COX-2 gene in endotoxin-treated murine RAW 264.7 macrophages. Luciferase reporter constructs with alterations in presumptive cis-acting transcriptional regulatory elements demonstrate that the cyclic AMP-response element and two nuclear factor interleukin-6 (CCAAT/enhancer-binding protein (C/EBP)) sites of the COX-2 promoter are required for optimal endotoxin-dependent induction. In contrast, the E-box and NF-kB sites are not required for endotoxin-dependent induction. Inhibition of endotoxin-induced NF-kB activation by expression of an inhibitor-κB α mutant does not block endotoxin-dependent COX-2 reporter activity. Overexpression of c-Jun, C/EBPβ, and C/EBPβ enhances induction of the COX-2 reporter, while overexpression of cyclic AMP-response element-binding protein or "dominant negative" C/EBPβ represses COX-2 induction. In addition, endotoxin rapidly and transiently elicits c-Jun phosphorylation in RAW 264.7 macrophages. Cotransfection of the COX-2 reporter with dominant negative expression vectors shows that endotoxin-induced COX-2 gene expression requires signaling through a Ras-independent pathway involving the adapter protein ECSIT and the signaling kinases MEKK1 and JNK. In contrast, endotoxin-induced COX-2 reporter activity is not blocked by overexpression of dominant-negative forms of Raf-1, ERK1, or ERK2.

Macrophages play an important role in the regulation of inflammation and the immune response. When activated, macrophages release growth factors, cytokines, and lipid mediators such as prostaglandins and leukotrienes. Secreted prostaglandins promote inflammation by increasing vascular permeability (1) and vasodilation (2) and by directing cellular migration into the site of inflammation through the production and release of proinflammatory cytokines such as interleukin-6 (3). Induced prostaglandin synthesis is associated with the onset of symptoms resulting from acute immune system activation. For example, a knockout mouse strain unable to induce prostaglandin production does not develop fever in response to normally pyrogenic doses of bacterial endotoxin (4). Elevated prostaglandin levels are also associated with conditions of both chronic inflammation and cancer (5, 6). Because of the many potent effects of prostaglandins, control of prostaglandin synthesis is a critical element in the regulation of many physiological processes and the abatement of a number of pathophysiological conditions.

The synthesis of prostaglandins is dependent on the activity of the cyclooxygenase (COX)1 enzyme. COX converts arachidonic acid released from membrane stores by phospholipase to prostaglandin H2, the common precursor to all prostaglandins, thromboxanes, and prostacyclins (5, 6). There are two isozforms of COX enzyme, encoded by distinct genes. The COX-1 protein is expressed constitutively in most cell types and is involved in normal kidney, gastrointestinal, and reproductive function (2, 7, 8). The COX-2 protein has low basal expression in most tissues but can be rapidly and transiently induced by a wide variety of mitogens, hormones, and other ligands (8). Induction of COX-2 transcription can occur independent of de novo protein synthesis and can be inhibited by glucocorticoids (8). Since treatment with glucocorticoids, as well as antisense COX-2 oligonucleotides (9, 10) and COX-2-specific enzyme inhibitors (11), is frequently able to block prostaglandin production, induced prostaglandin synthesis is attributed primarily to the COX-2 enzyme.

Macrophages secrete prostaglandins upon activation by the bacterial endotoxin lipopolysaccharide (LPS), due primarily to induced transcription of the COX-2 gene and production of the COX-2 enzyme (9, 12). Experiments with pharmacological protein tyrosine kinase inhibitors have demonstrated the necessity of signaling kinases in LPS-dependent COX-2 transcription in the murine RAW 264.7 macrophage cell line (13, 14). Synthetic peptide inhibitors of nuclear factor κB (NF-κB) trans-
location to the nucleus suggest that NF-κB activity is also required for COX-2 production in RAW 264.7 cells treated with LPS (14). However, the cis-acting elements in the COX-2 promoter responsible for LPS-dependent transcription in macrophages, the transcription factors modulating COX-2 expression following macrophage activation, and the signaling pathways from the activated endotoxin/LPS receptor to the COX-2 gene have not been well elucidated. In this study, we show that induction of a murine COX-2 reporter by LPS in RAW 264.7 macrophages requires the cyclic AMP-response element (CRE) site and nuclear factor interleukin-6 (NF-IL6) sites of the COX-2 promoter but not the presence of the E-box or the putative NF-κB site. We find that NF-κB activity is not required for efficient COX-2 reporter transcription. We also demonstrate a requirement for the MAPK/ERK kinase kinase (MEKK1) and c-Jun N-terminal kinase (JNK) kinases in this induction. LPS-dependent activation of the COX-2 reporter through these signaling kinases is independent of Ras function and involves a recently discovered adapter protein, ECSIT (evolutionarily conserved signaling intermediate in toll pathways) (15). Finally, we provide evidence suggesting a role for c-Jun and the CCAAT/enhancer-binding protein (C/EBP) family of transcription factors in the LPS-dependent activation of the COX-2 promoter in RAW 264.7 macrophages.

**EXPERIMENTAL PROCEDURES**

**Ligands**—Fetal bovine serum was from Sigma. Omega Scientific. LPS was from Sigma and was resuspended in sterile double-distilled H₂O to make a 250 μg/ml stock, which was stored at −70 °C.

**Plasmids**—The wild-type COX-2 promoter fragment was made by polymerase chain reaction (PCR) using pT10L (16) as template, with −724U and +7L primers. Mutant COX-2 promoter fragments were constructed by two-stage Bridge polymerase chain reaction (17), using pT10L as original template for the generation of the 5′ and 3′ mutant mCRE, mNF-IL6(1), mNF-IL6(2), and mNF-κB fragments and for the generation of the 5′ mE-box fragment. For construction of the 3′ E-box fragment, we used pT1S10−37mE-box (18) as template. The PCR generating the 5′ mNF-IL6(1 + 2) fragment used mNF-IL6(2) fragment as template. Resulting 5′ and 3′ mutant paired fragments were gel-purified from a nondenaturing polyacrylamide gel and used together as a double-stranded template pair. The PCR amplification was carried out for 30 cycles with annealing at 72°C and +7L oligonucleotides as primers. All resulting full-length wild-type and mutant COX-2 promoter fragments were digested with HindIII and XhoI, polyacrylamide gel-purified, and ligated into the HindIII-XhoI sites of the polycloning site of the firefly luciferase reporter plasmid (pXP2). Wild-type E-box sequence CATGCT was changed to CACGCT. Wild-type CRE sequence CTCATGCA was changed to CTGATTCGCA. Wild-type NF-IL6(1) sequence TGGGGAAAG was changed to TGAATGGCG. Wild-type NF-IL6(2) sequence TGCCGAC was changed to AAGCTCGAC. Wild-type NF-κB sequence TGGTGTATC was changed to GGTGTGTATC. Wild-type E-box sequence GGGATTCCC was changed to GGTGTGTATC. Wild-type NF-IL6(1) sequence CTACGTCA was changed to CTGATTCA. Wild-type NF-IL6(2) sequence CTACGTCA was changed to CTGATTCA. Wild-type NF-IL6(1) sequence CTACGTCA was changed to CTGATTCA. Wild-type NF-IL6(2) sequence CTACGTCA was changed to CTGATTCA. Wild-type NF-κB site, Are Essential for Optimal COX-2 Reporter Induction in LPS-stimulated Macrophages—To investigate the cis-acting elements of the COX-2 gene necessary for LPS-induced COX-2 transcription, we generated by PCR both wild-type and mutant murine COX-2 promoter fragments spanning nucleotides −724 to +7. These fragments were then cloned into the polycloning site of the firefly luciferase reporter plasmid pXP2. Sites in the COX-2 promoter targeted for mutation included a proposed NF-IL6(2) fragment used mNF-IL6(2) fragment as template. The PCR generating the 5′ mNF-IL6(1 + 2) fragment used mNF-IL6(2) fragment as template. Resulting 5′ and 3′ mutant paired fragments were gel-purified from a nondenaturing polyacrylamide gel and used together as a double-stranded template pair. The PCR amplification was carried out for 30 cycles with annealing at 72°C and +7L oligonucleotides as primers. All resulting full-length wild-type and mutant COX-2 promoter fragments were digested with HindIII and XhoI, polyacrylamide gel-purified, and ligated into the HindIII-XhoI sites of the polycloning site of the firefly luciferase reporter plasmid (pXP2). Wild-type E-box sequence CATGCT was changed to CACGCT. Wild-type CRE sequence CTCATGCA was changed to CTGATTCGCA. Wild-type NF-IL6(1) sequence TGGGGAAAG was changed to TGAATGGCG. Wild-type NF-IL6(2) sequence TGCCGAC was changed to AAGCTCGAC. Wild-type NF-κB sequence TGGTGTATC was changed to GGTGTGTATC. Wild-type E-box sequence GGGATTCCC was changed to GGTGTGTATC. Wild-type NF-IL6(1) sequence CTACGTCA was changed to CTGATTCA. Wild-type NF-IL6(2) sequence CTACGTCA was changed to CTGATTCA. Wild-type NF-κB site, Are Essential for Optimal COX-2 Reporter Induction in LPS-stimulated Macrophages—To investigate the cis-acting elements of the COX-2 gene necessary for LPS-induced COX-2 transcription, we generated by PCR both wild-type and mutant murine COX-2 promoter fragments spanning nucleotides −724 to +7. These fragments were then cloned into the polycloning site of the firefly luciferase reporter plasmid pXP2. Sites in the COX-2 promoter targeted for mutation included a proposed NF-κB site, two presumptive NF-IL6 (C/EBP) sites, the CRE site (18), and an E-box (18) (Fig. 1).

The COX-2 promoter constructs were transiently transfected into subconfluent RAW 264.7 macrophages maintained in endotoxin-free medium supplemented with 10% fetal bovine serum and antibiotics. Following transfection, cells were allowed to recover for 18 h. Cells were subsequently induced with LPS (10 ng/ml) for 5 h and then harvested and lysed for assay of their luciferase activity. Mutation of the E-box, the NF-κB site, or the 3′ NF-IL6 site (NF-IL6(1)) does not affect LPS-induced reporter activity (Fig. 1). Mutation of the 5′ NF-IL6 site (NF-IL6(2)) has a moderate effect on COX-2 reporter induction, while mutation of both NF-IL6 sites strongly represses LPS induction of reporter activity. As previously observed in NIH3T3 fibroblasts induced by are, serum, and platelet-de-
rived growth factor (18, 19), mutation of the CRE site severely represses both basal and induced COX-2 reporter activity in RAW 264.7 macrophages.

**Dominant Negative Inhibition of NF-kB Activity Does Not Repress LPS-induced COX-2 Reporter Activity in Macrophages**—We were surprised to find no requirement for the putative NF-kB site in endotoxin-induced expression from the murine COX-2 promoter in macrophages. To further investigate the role of NF-kB activation in COX-2 induction, we compared luciferase activity of RAW 264.7 macrophage cells transfected with the wild-type COX-2 reporter with the luciferase activity of cells transfected with [NF-kB][luc], a plasmid that drives the luciferase reporter gene from multimerized NF-kB response elements. We cotransfected RAW 264.7 macrophages either with the wild-type COX-2 reporter or with [NF-kB][luc], along with either empty vector (as a control) or a plasmid expressing mutant I-kBα protein. This mutant I-kBα protein is neither phosphorylated nor degraded following cellular activation and therefore remains irreversibly bound to NF-kB in the cytoplasm (20). This restriction prevents free, active NF-kB transcription factor from translocating to the nucleus, binding to NF-kB binding sites on DNA, and activating transcription. Mutant I-kBα protein therefore acts as a dominant negative for NF-kB activity. LPS treatment strongly induces NF-kB activity in RAW 264.7 macrophages, as reflected by substantial induction of the luciferase activity from the [NF-kB][luc] reporter (Fig. 3, *right panel*). Expression of the mutant I-kBα protein completely inhibits LPS-dependent activation of the [NF-kB][luc] reporter, indicating that the mutant I-kBα protein is a very effective dominant negative repressor of NF-kB activity. In contrast, when the mutant I-kBα expression vector is co-
of the COX-2 reporter in macrophages during the first 5 h following treatment with LPS.

Expression of C/EBPβ (LAP) and C/EBPδ Enhances, and Dominant Negative C/EBPβ (LIP) Represses, LPS-induced COX-2 Reporter Activity in Macrophages—Since mutation of both NF-IL6 sites severely represses LPS-dependent COX-2 promoter activity, we investigated the roles of transcription factors that bind to these elements. The wild-type COX-2 reporter was cotransfected into RAW 264.7 macrophages, along with one of three different expression vectors encoding various members of the C/EBP family of transcription factors. Expression of C/EBPβ wild type, also known as LAP (21), is able to enhance COX-2 reporter activity (Fig. 4). This stimulatory effect is LPS-dependent. In contrast, expression of another C/EBP family member, C/EBPδ, enhances both basal and LPS-induced COX-2 reporter activity. A naturally occurring alternate C/EBPβ translation product, known as LIP, lacks an “activation domain” yet retains the ability to bind to NF-IL6 sites and to C/EBP family members (21). LIP therefore acts as a dominant negative for C/EBP activity (21). LIP expression strongly represses LPS-dependent COX-2 reporter activity (Fig. 4), suggesting that C/EBP activity is important for induction of the COX-2 gene by LPS in macrophages.

Expression of Wild-type CREB Represses, and c-Jun Enhances, LPS-induced COX-2 Reporter Activity in Macrophages—Mutation of the COX-2 CRE site completely abrogates COX-2 reporter activity (Fig. 2). We therefore examined activation at this site in more detail. As seen activation at CRE sites in many promoters is often associated with transcriptional activation by CREB, we examined the effect of a cotransfected CREB expression vector on the LPS induction of the wild-type COX-2 reporter. Expression of wild-type CREB substantially represses LPS-dependent COX-2 reporter activity (Fig. 5).

The c-Jun transcription factor can also bind at consensus CRE sites. Moreover, c-Jun, and not CREB, is the transcription factor that modulates growth factor and oncogene induction of the COX-2 gene at the CRE in both fibroblasts (18, 19) and mammary epithelial cells (22, 23). Overexpression of wild-type c-Jun enhances LPS-dependent COX-2 reporter activation (Fig. 5), suggesting that c-Jun also plays a role in the activation of the COX-2 promoter by LPS treatment in macrophages.

In untreated RAW 264.7 cells, c-Jun predominate exists in a transcriptionally inactive, unphosphorylated state (Fig. 6). Following stimulation by LPS, c-Jun is rapidly and transiently phosphorylated. c-Jun activation is maximal by 40 min after LPS treatment in RAW 264.7 macrophages. By 120 min following LPS treatment, most c-Jun protein has returned to the unphosphorylated state. The rapid and transient activation of c-Jun is consistent with a role for this transcription factor in the LPS-dependent initiation of COX-2 transcription in macrophages.

Optimal COX-2 Reporter Activation by LPS in Macrophages Requires a Ras-independent JNK/MEKK1 Signaling Pathway—Since c-Jun overexpression enhances LPS-dependent COX-2 reporter activity, and the c-Jun protein is rapidly phosphorylated upon LPS induction, we anticipated that the MEKK/JNK kinase cascade that leads to phosphorylation of c-Jun would be required for LPS-dependent activation of the COX-2 promoter in RAW 264.7 macrophages. Activation by JNK and MEKK1 is, indeed, required for activation of the COX-2 reporter; expression of dominant negative JNK or dominant negative MEKK1 significantly represses LPS-dependent activation of the COX-2 reporter in RAW 264.7 macrophages (Fig. 7).

In NIH3T3 fibroblasts, signaling to the COX-2 promoter through JNK and MEKK requires Ras activity (18, 19). However, when a vector overexpressing a dominant negative Ras protein is cotransfected with the COX-2 reporter in RAW 264.7 macrophages, there is no repression of basal or LPS-induced luciferase activity (Fig. 8). Thus, activation of the JNK/MEKK1 signaling pathway and LPS-induced COX-2 transcription does not require Ras activity in LPS-treated RAW 264.7 macrophages.

The Raf-1/MAPKK/ERK Signaling Pathway Does Not Mediate LPS-dependent Activation of the COX-2 Reporter in
Macrophages—Another Ras-dependent signaling pathway required for optimal induction of the COX-2 reporter in fibroblasts (18) involves Raf1 and the ERK1 and ERK2 MAP kinases. However, overexpression of dominant negative Raf-1, dominant negative ERK1, or dominant negative ERK2 proteins fails to repress induction of COX-2 reporter activity in LPS-treated RAW 264.7 macrophages (Fig. 9). Activation of the Ras/Raf-1/ERK signaling pathway does not modulate COX-2 transcription in LPS-treated RAW 264.7 macrophages.

ECSIT Links LPS Receptor Signaling to Induced COX-2 Reporter Expression in Macrophages—The toll gene product has been identified as the major LPS receptor (reviewed in Ref. 24). Using a dominant negative toll-2 expression plasmid, we have demonstrated that COX-2 induction by LPS in RAW 264.7 macrophages is mediated by toll-2. Kopp et al. (15) recently identified ECSIT as an adapter protein that bridges toll/tumor necrosis factor-associated factor 6 activation to MEKK1 and facilitates MEKK1 activation of c-Jun. In contrast to results in fibroblasts (18, 19), activation of MEKK1 and JNK in LPS-treated RAW 264.7 cells is not Ras-dependent (Fig. 8). We thought it likely that ECSIT might couple LPS activation to MEKK1/JNK/c-Jun-dependent induction of COX-2 gene expression in RAW 264.7 macrophages. Cotransfection of the wild-type COX-2 luciferase reporter with a plasmid expressing a dominant negative ECSIT protein significantly represses LPS-induction of COX-2 reporter activity in RAW 264.7 macrophages. These data suggest that LPS-activated macrophages signal to the MEKK1/JNK pathway from ligand-bound LPS receptors through ECSIT rather than Ras.

COX-2 Induction in Macrophages by Endotoxin

S. Krutzik, D. Wadleigh, P. Godowski, R. Modlin, H. Herschman, unpublished observation.
factor and is independent of Ras activation. Signaling at the NF-IL6 sites appears to be modulated by members of the C/EBP transcription factor family.

**DISCUSSION**

*Fig. 10. Expression of dominant negative (dn) ECSIT represses LPS-induced COX-2 reporter activity in RAW 264.7 macrophages.* RAW 264.7 macrophages were transiently transfected for 2 h with 4.75 μg of COX2 reporter, 0.5 μg of pRL-TK, and 4.75 μg of either a plasmid expressing dominant negative Ras or a plasmid expressing dominant negative ECSIT. Cells were incubated for 18 h and then were induced with LPS (10 ng/ml) for 5 h and lysed. Firefly and Renilla luciferase activities were determined. Values shown are means ± S.D.

Transcription Factors That Mediate LPS Induction of the COX-2 Gene in Murine Macrophages—As observed in murine fibroblasts (18), mast cells (38) and osteoblasts (39), CREB does not play a positive regulatory role at the CRE in RAW 264.7 macrophages. Several proteins, including CREB and c-Jun, bind to the murine COX-2 CRE in electrophoretic gel shift mobility experiments (43). In contrast to CREB, c-Jun overexpression enhances COX-2 reporter activation in LPS-treated RAW 264.7 macrophages. Moreover, c-Jun plays a role in at least the early stages of LPS-induced COX-2 induction in macrophages. LPS-induced c-Jun phosphorylation is transient, with phospho-c-Jun levels returning to basal levels after 120 min. It is possible that another transcription factor(s) may subsequently be activated, possibly also at the CRE site, during paradigms of prolonged COX-2 transcription in macrophages.

Activation at NF-IL6 sites is most often associated with C/EBP transcription factors (36). Overexpression of a truncated alternate C/EBPβ translation product, LIP, which acts as a dominant negative inhibitor of C/EBP activity (21), severely represses COX-2 reporter activity in LPS-stimulated macrophages. Enhancement of COX-2 reporter activity in macrophages by C/EBPβ (LAP) overexpression is dependent on LPS stimulation. This LPS-dependent enhancement of COX-2 transcription by LAP overexpression reflects LPS-stimulated phosphorylation and consequent activation of C/EBPβ as a transcription factor (33, 44). C/EBPβ has relatively high basal expression in many tissues and cell lines, but the transcriptional activity of C/EBPβ requires phosphorylation by signaling kinases (33, 40, 45). C/EBP activity may also be regulated by the relative expression of C/EBPβ alternate translation products: the activating LAP protein and the repressing LIP protein (21). We find (i) that untreated RAW 264.7 macrophages have modest C/EBPβ (LAP) basal levels, with undetectable levels of LIP, and (ii) that treatment of RAW 264.7 cells for 5 hours with though we do not see a requirement for this putative NF-κB sequence in the early induction of the COX-2 reporter by LPS in RAW 264.7 macrophages, it is possible that there may be additional NF-κB sites in the murine COX-2 promoter upstream of the 724 base pairs examined in this study.

Mutation of three bases in the COX-2 CRE site completely represses both basal and LPS-induced COX-2 reporter activity in RAW 264.7 macrophages. Mutation of the CRE site in the COX-2 promoter significantly represses COX-2 reporter induction by v-Src, PDGF, and serum in murine fibroblasts (18, 19). The COX-2 CRE has been identified as a cis-acting regulatory element of the COX-2 gene in several other studies as well (22, 23, 35–37). More recently, we have demonstrated that this same CRE site plays a critical role in COX-2 induction in activated murine mast cells (38) and ligand-stimulated murine osteoblasts (39).

LPS-dependent COX-2 activation requires the presence of at least one NF-IL6 (C/EBPβ) site. Mutation of either NF-IL6 site alone results in only moderate repression of COX-2 reporter activity. Presumably, activation at these sites involves the C/EBP family of transcription factors, which are able to bind to and promote activation of transcription at consensus IL6 sites (40). Five deletion and site-directed mutagenesis studies in M3T3-E1 osteoblasts have suggested the involvement of a NF-IL6 site in the induction of the COX-2 promoter by tumor necrosis factor-α (41, 42). The NF-IL6 site is also observed in the aberrant COX-2 overexpression seen in mouse skin carcinoma cells (25) and in LPS and TPA-directed COX-2 induction in vascular endothelial cells (35).

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**Transcription Factors That Mediate LPS Induction of the COX-2 Gene in Murine Macrophages**—We expected an E-box would have no effect on COX-2 reporter induction in LPS-treated RAW 264.7 macrophages. The E-box does not play a role in oncogene or growth factor induction of COX-2 in murine fibroblasts (18). In contrast, Kim and Fisher report that the E-box of the murine COX-2 gene mediates COX-2 transcriptional activation in mouse skin tumor cells (25). However, the E-box and the CRE of the murine and human COX-2 promoters overlap. The E-box mutation used by Kim and Fisher (25) contains mutations in two 3′ base pairs of the critical COX-2 CRE CGTCA sequence. An E-box in the rat COX-2 promoter, which shares identical sequence and location with the murine COX-2 E-box, is required for COX-2 induction in rat ovarian granulosa cells (26). The rat COX-2 promoter, however, lacks the CRE that is present in the murine and human COX-2 promoters. Since the rat E-box and the murine CRE are roughly at the same place in their respective promoters, these sites may play similar roles in promoting proper DNA folding and protein-protein contacts with the polymerase complex to facilitate ligand-induced COX-2 gene transcription.

We were surprised to find that mutation of the putative NF-κB site did not affect COX-2 reporter activity in LPS-treated RAW 264.7 macrophages. NF-κB activity has been implicated in COX-2 induction in many cell types, including LPS-treated macrophages (14, 27). Most of these experiments, however, involve the use of chemical (27, 28) or synthetic peptide (14, 29) inhibitors, or oligonucleotide decoys (30), which may affect NF-κB activity (as well as the activity of other transcription factors) at other sites on the COX-2 promoter. Using similar techniques, other laboratories report that NF-κB activity is not required for COX-2 induction in some cases, e.g. in rat vascular smooth muscle cells (31, 32). The putative NF-κB site targeted for mutation in this study matches exactly the NF-κB consensus 5′-GGGRNNYYCC-3′ (33) (although it lacks an internal, degenerate nucleotide seen in the 5′-GGGRNNNYCC-3′ TRANSFAC NF-κB consensus (34)).
LPS results in only a slight enhancement of C/EBPβ (LAP) expression (data not shown). Overexpression of C/EBPβ enhances COX-2 reporter activity in both basal and LPS-induced macrophages. Unlike C/EBPα, C/EBPβ activity does not appear to be controlled by LPS-dependent post-translational modifications (33, 40). Our results with C/EBPβ (LAP), C/EBPβ (LIP), and C/EBPγ overexpression are consistent with a role for the C/EBP transcription factors in LPS-induced COX-2 gene expression in murine macrophages. The relative involvement of C/EBP family members for induced COX-2 expression most likely varies for alternate cell types and stimulatory ligands.

Activation of the NF-κB transcription factor system has been implicated in induction of COX-2 gene expression in several contexts (14, 27–30). Although mutation of the putative NF-κB sites within the COX-2 promoter is not required for LPS induction of luciferase expression in RAW 264.7 macrophages (Fig. 2), it is possible that NF-κB transcriptional activation in response to LPS plays a role in COX-2 induction in these cells, either at another site on the COX-2 promoter or by an indirect mechanism. However, inhibition of the NF-κB activation mechanism, which completely blocks transcriptional stimulation of a conventional NF-κB reporter, has no repressive effect on transcriptional activation at the COX-2 promoter in LPS-stimulated RAW 264.7 macrophages (Fig. 3). Similar studies in catalase, interleukin-1β, and tumor necrosis factor-α-induced rat vascular smooth muscle cells also showed a lack of a requirement for NF-κB activity in COX-2 induction (31, 32). Unlike activation of c-Jun, which appears to be very wide, if not ubiquitous, required for transcriptional activation of the murine and human COX-2 genes, NF-κB transcriptional activation of the COX-2 gene appears to be context-sensitive with respect to species, cell type, and inducer.

Signal Transduction Pathways That Modulate LPS Induction of the COX-2 Gene in Murine Macrophages—In murine fibroblasts, oncogene- and growth factor-induced COX-2 transcription requires Ras-dependent MEKK1/JNK activation, leading to c-Jun phosphorylation (18, 19). Since c-Jun appears to be a Jun-NH2-terminal kinase substrate of MEK1/2, it is possible that MEKK1 and JNK are required for LPS-dependent COX-2 induction in macrophages. However, a recently identified adapter protein, ECSIT, has recently been shown to link signaling from ligand-bound Toll-domain receptors, such as the LPS receptor, to MEKK1 (15). Expression of a truncated, dominant negative ECSIT protein represses induction of the COX-2 reporter. Activation at the NF-IL6 sites most likely occurs through some combination of C/EBP family members.

Acknowledgments—We thank Victor Grijalva, Art Catapang, and Raymond Basconcillo for technical assistance and Drs. M. Cobb, M. Motminny, C. Sawyers, M. Green, M. Karin, S. Macdonald, S. Smale, R. Davis, G. Cheng, and R. Modlin for gifts of plasmids and reagents.

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J. Biol. Chem. 2000, 275:6259-6266.
doi: 10.1074/jbc.275.9.6259

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