Macrophage expression of cyclooxygenase-2 (COX-2), the inducible isoform of COX, is up-regulated by pro-inflammatory stimuli both in vivo and in vitro. Here we investigated the mechanisms regulating COX-2 gene expression in macrophage/ monocytic cells. Lipopolysaccharide (LPS) is known to induce de novo COX-2 mRNA expression in these cells. Transient cotransfections with a COX-2 promoter-luciferase construct and different expression vectors showed that LPS up-regulates COX-2 transcription through both mitogen-activated protein kinase (MAPK) and protein kinase C (PKC) pathways. Cotransfections with expression vectors for dominant negative mutants of MAPK and PKC isoforms did not suppress the effects of LPS on COX-2. Electrophoretic mobility shift assays and transient transfection experiments with deleted and mutated variants of a COX-2 promoter-luciferase construct showed that NF-kB, NF-IL6, and CRE promoter sites mediate gene transcription independently in response to LPS treatment. In these experiments, isolated NF-kB, NF-IL6, and CRE promoter sites were less effective than the intact promoter in mediating COX-2 transcription. Cotransfections with mutated COX-2 promoter-luciferase constructs and expression vectors showed that each one of these promoter elements can be activated by LPS through both MAPK and PKC pathways to induce gene expression. In summary, there is redundancy in the signaling pathways and promoter elements regulating COX-2 transcription in endotoxin-treated cells of macrophage/monocytic lineage.

Cyclooxygenase (COX)\(^1\) can be a rate-limiting step in the synthesis of prostaglandins (PGs), lipid mediators that contribute to the development of inflammatory responses (1). In this process, phospholipase A\(_2\) catalyzes the release of arachidonic acid from membrane phospholipids, while COX catalyzes the conversion of arachidonic acid into PGs (2, 3). There are two isoforms of COX, COX-1 and COX-2, the product of two different genes. COX-1 is expressed constitutively in most tissues and may be responsible for housekeeping functions (4). In contrast, COX-2 is not detectable in most normal tissues or resting immune cells, but its expression can be induced by endotoxin, cytokines, growth factors, and carcinogens (5–7).

Macrophage activation is accompanied by a significant increase in COX-2 expression, whereas levels of COX-1 remain unchanged (7, 8). \textit{In vivo} macrophage COX-2 immunoreactivity is a characteristic finding in the synovium of patients with osteoarthritis as well as in other forms of inflammation, whereas COX-1 expression typically remains unchanged (9, 10). Nonsteroidal anti-inflammatory drugs inhibit PG synthesis through the inhibition of COX activity, which confers on them anti-inflammatory and analgesic properties (11). Studies with newly developed selective COX-2 inhibitors also have shown suppression of PG production and acute tissue inflammation (12). Moreover, homozygous deletion of the COX-2 gene in mice led to a striking mitigation of endotoxin-induced hepatocellular cytotoxicity (13). Therefore, COX-2 may play a critical role in the development of local and systemic inflammatory responses.

The different responses of the genes encoding COX-1 and COX-2 reflect, at least in part, differences in the regulatory elements in the 5' flanking regions of these two genes. In the COX-2 gene (Fig. 1), promoter elements for nuclear factor \(\kappa\)B (NF\(\kappa\)B, \(-223/-214\)) and nuclear factor interleukin-6 (NF-IL6, \(-132/-124\)) and a cAMP-responsive element (CRE, \(-59/-53\)) have been found to be important in regulating transcription (14–16). The CRE appears to be the crucial site in epithelial cells (17, 18), whereas other promoter elements, such as those for NF\(\kappa\)B and NF-IL6, seem to have a role in regulating COX-2 gene transcription in macrophage-like cells (19–21). However, the relative contribution of the different promoter elements in mediating COX-2 transcription in macrophages has not been completely elucidated.

In the present work, we have investigated the regulation of COX-2 gene expression in endotoxin-treated RAW 264.7 macrophages and THP-1 monocytic cells. Our data show that there is transcriptional redundancy among the NF\(\kappa\)B, NF-IL6, and CRE promoter sites although maximal transcriptional activity requires cooperation among these elements. Also in a redundant manner, mitogen-activated protein kinase (MAPK) and protein kinase C (PKC) signaling pathways are capable of inducing COX-2 transcription through NF\(\kappa\)B, NF-IL6, or CRE.
promoter sites. These results are important for understanding why COX-2 expression is up-regulated during macrophage activation.

EXPERIMENTAL PROCEDURES

Materials—RPMI and fetal bovine serum were from Life Technologies, Inc. Escherichia coli (strain 055:B5) lipopolysaccharide, DEAE-dextran, and α-nitrophenyl-β-D-galactopyranoside were from Sigma. [32P]ATP was from PerkinElmer Life Sciences. Endotoxin-free plasmid DNA was prepared using Qiagen DNA purification kits (Chatsworth, CA). Reagents for the luciferase assay were from Analytical Luminescence (San Diego, CA). Mutagenesis kits were from Stratagene (La Jolla, CA). Oligonucleotides were synthesized by Genosys Biotechnologies Inc. (The Woodlands, TX). T4 polyadenylate kinase was from New England Biolabs, Inc. (Beverly, MA).

Cell Lines—Murmur macrophage-like RAW 264.7 cells and human THP-1 monocytic cells were maintained in RPMI supplemented with 10% fetal bovine serum and antibiotics (100 units/ml penicillin G, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B).

Plasmids—Expression vectors for Ras, MAPK kinase-1, mitogen-activated protein kinases (ERK-1 and -2, p38, and JNK), PKC isoforms (α, ζ, and δ), and their dominant negative mutants were provided by Dr. Andrew J. Dannenberg (Weill Medical College of Cornell University, New York, NY). Human COX-2 promoter-luciferase deletion constructs (−327/+59, −220/+59, −124/+59, and −52/+59) and mutant constructs (NFκB mutant (designated KBM), NF-IL6 mutant (designated ILM), CRE mutant (designated CRM), the double mutant ILM/CRM, and the triple mutant KBM/ILM/CRM) have been described previously (14, 15, 20). The double mutants KBM/ILM and KBM/CRM were created using site-directed mutagenesis kits. Briefly, primers that incorporate mutations (lowercase letters) for NFκB (sense: 5′-CCCTGGCCGAGGCTTTAAGGGGAGGAG-3′) or CRE (sense: 5′-GCGGAAGGGACATCCCGCGGAG-3′) elements found in the COX-2 promoter were amplified using T4 polynucleotide kinase and used to amplify the KBM construct. This was achieved with the use of phi29 Turbo-DNA polymerase and sequential cycling in a 480 PerkinElmer Life Sciences thermocycler as per the protocol of the mutagenesis kit. The parental DNA was then digested with DpnI, and the new constructs harboring the KBM/ILM or KBM/CRM mutations were used to transform E. coli competent cells. Incorporation of the desired mutations was confirmed by DNA sequencing (Fig. 1).

Transient Transfection Assays—RAW 264.7 or THP-1 cells (5 × 10⁶ per treatment group) were washed twice in serum-free RPMI and then suspended in 0.5 ml of transfection solution containing 50 μg Tris and 500 mg/ml DEAE-dextran. Subsequently, 2 μg of a COX-2 promoter-luciferase construct, 2 μg of either an expression vector or empty plasmid, and 0.5 μg of the control plasmid pSV-β-galactosidase were added, and the mixture was incubated at 37 °C and 5% CO₂ for 30 min. Me_SO (100 μM of transfection mixture) was added for 1 min at room temperature, and the reaction was stopped by adding 10 volumes of RPMI. After pelleting by centrifugation, transfected cells were plated in 100-mm dishes and incubated in 10% fetal calf serum RPMI for 24 h. Subsequently, cells were treated with fresh 3% fetal bovine serum RPMI with or without LPS (50 ng/ml). Luciferase and β-galactosidase activities were measured in cellular extracts 6 h later as described previously (22).

Electrophoretic Mobility Shift Assays—Cells were plated in 100-mm dishes at a density of 3 × 10⁶ cells/dish and allowed to attach for 24 h prior to experiments. Cells were then treated with fresh 3% fetal bovine serum RPMI with or without LPS (50 ng/ml). Nuclear extracts were obtained 30 min later as described previously (23). Briefly, cells were lysed by incubation in buffer A (10 mM HEPES, pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM dithiothreitol, and 0.2 mM phenylmethylsulfonyl fluoride) on ice for 15 min, followed by vortexing for 10 s. Nuclei were pelleted, and nuclear extracts were obtained by high salt extraction after incubating nuclei in buffer C (20 mM HEPES, pH 7.9, 2.5% glycerol, 420 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM dithiothreitol, and 0.2 mM phenylmethylsulfonyl fluoride) for 20 min. Nuclei were pelleted again by centrifugation for 5 min, and the supernatant fraction (nuclear extract) was stored at −70 °C. Double-stranded DNA oligonucleotides containing the consensus binding sites for the NFκB (sense: 5′-GGAG-AGTGGGACTACCCCTCTG-3′), NF-IL6 (sense: 5′-CACCAGGGCTTACCAATTTTAAAA-3′), or CRE (sense: 5′-AACAGTGATTTGACG-3′) elements found in the COX-2 promoter were labeled with [32P]ATP using T4 kinase. 4 μg of nuclear extract were incubated with 1 μl of DNA probe in a total of 10 μl containing 4% glycerol, 50 mM NaCl, 10 mM Tris, pH 7.5, 1.5 mM MgCl₂, 0.5 mM EDTA, 0.5 mM dithiothreitol, and 1 μg of poly(dI-dC). Cold chase was carried out with a 50 molar excess of the same, unlabeled probe or with a probe that contained mutated binding sequences (lowercase letters) for NFκB (cgggACC), NF-IL6 (TAgtagttC), or CRE (TTTAgtagtC). The nuclear extract-DNA complexes were resolved in 4% polyacrylamide gels using 0.5 × TBE at 150 V. The gels were then dried and autoradiographed.

Statistics—Comparisons among groups were made by Student’s t test. A difference among groups of p < 0.05 was considered significant.

RESULTS

LPS Induces COX-2 Transcription through MAPK and PKC Signaling Pathways—RAW 264.7 and THP-1 cells were transiently cotransfected with a −327/+59 COX-2 promoter-luciferase reporter construct and different MAPK (ERK-1 and -2, p38, and JNK) and PKC (PKC-ε, PKC-ζ, and PKC-δ) expression
Transfection experiments with deletion constructs harboring deleted and mutated variants of the promoter elements were essential to induce luciferase activity when the KBM/ILM/CRM construct was used; therefore, the combination of at least two promoter elements appears to be necessary to activate transcription. The ability of the NFκB, NF-IL6, and CRE sites to individually support transcription was assessed in transfection experiments with COX-2 promoter constructs containing only one functional promoter element (i.e. double mutant constructs). As seen in Fig. 3, C and F, there were differences in the basal luciferase activity level sustained by each of these promoter sites, but all of them mediated induction of luciferase activity after LPS treatment. Remarkably, none of these individual promoter elements, not even the sum of their effects, were as effective in inducing luciferase activity after LPS treatment as a promoter with three functional sites. Therefore, the coexistence of NFκB, NF-IL6, and CRE sites in the intact promoter has synergistic rather than additive effects.

**LPS Induces Binding of Nuclear Proteins to the NFκB, NF-IL6, and CRE Promoter Sites of the COX-2 Gene**—For LPS to induce COX-2 gene transcription through NFκB, NF-IL6, and CRE promoter sites, it should induce binding of nuclear proteins to all three promoter sites (Fig. 4). In combination, the data from electrophoretic mobility shift assay and transfection experiments indicate that maximal LPS induction of COX-2 transcription is achieved through the activation and binding of transcription factors to any combination of two promoter sites. Because the presence of all three promoter elements is not necessary to mediate this effect, but only any combination of two, it appears that there

![Fig. 2. LPS induces COX-2 transcription through MAPK and PKC signaling pathways.](http://www.jbc.org/)

**Fig. 2.** LPS induces COX-2 transcription through MAPK and PKC signaling pathways. RAW 264.7 and THP-1 cells were transfected with a −327/+59 COX-2 promoter-luciferase reporter construct and either an empty vector (EV) or expression vectors for ERK-1 and -2, p38, and JNK (A and D) or for PKC-ε, PKC-ζ, and PKC-δ (B and E). Cells were transfected also with dominant negative (DN) mutants of MAPK and PKC expression vectors (C and F). Subsequently, cells were treated with control media or media containing LPS (50 ng/ml). Reporter activities were measured in cell extracts 6 h later. Luciferase activity represents data that have been normalized with β-galactosidase activity. Columns, means; bars, S.D., n = 6; *, p < 0.05; **, p < 0.01; and ***, p < 0.005 for MAPK, or PKC-transfected control and LPS-treated cells versus vector-transfected control and LPS-treated cells, respectively.
is redundancy also at the promoter level in LPS-mediated COX-2 transcription.

LPS Activates MAPK and PKC Signaling Pathways to Induce COX-2 Gene Transcription through NFκB, NF-IL6, or CRE Promoter Sites—We further investigated the signaling pathways by which LPS induces NFκB, NF-IL6, and CRE-mediated COX-2 transcription. We performed transient co-transfections with those MAPK and PKC expression vectors that induced COX-2 gene transcription (Fig. 2) and double mutant reporter constructs that contained only a functional NFκB site (Fig. 5A), NF-IL6 (Fig. 5B), or CRE (Fig. 5C) promoter site. As shown in Fig. 5A, ERK-2, p38, JNK, and PKC-ζ overexpression had a synergistic effect with LPS to induce luciferase activity, indicating that these pathways participate in LPS-mediated gene transcription through the NFκB site. Overexpression of upstream signaling intermediates, such as Ras and MEKK-1, had the same effect (data not shown). Fig. 5B shows that p38 and PKC-ζ overexpression synergize with LPS to induce transcription through the NF-IL6 promoter site. In Fig. 5C, overexpression of ERK-2, JNK, and PKC-ζ are synergistic with LPS in inducing transcription through the CRE site. Overexpression of dominant negative mutants for Ras, MEKK, MAPKs, or PKC isoforms does not inhibit the stimulatory effect of LPS on gene transcription through the NFκB, NF-IL6, or CRE sites (data not shown). Therefore, it appears that COX-2 gene transcription through each promoter element (i.e. NFκB, NF-IL6, or CRE-mediated transcription) is supported by at least two different signaling pathways.

DISCUSSION

COX-2 expression and PG synthesis in macrophages may be important in eliciting local and systemic inflammatory responses. When exposed to LPS and other pro-inflammatory stimuli, macrophages secrete PGs as a result of increased arachidonic acid release and COX-2 enzyme activity. Increased COX-2 activity may result from increased enzymatic activity or mRNA stability (26–28), but in endotoxin-treated macrophages it results mainly from augmented rates of transcription of the COX-2 gene. Therefore, the signaling mechanisms governing COX-2 gene transcription could be a potential target of strategies designed to suppress local or systemic inflammatory responses. However, the inter-relation between signaling pathways and promoter sites in the COX-2 gene and the individual role of these cis-acting elements in mediating transcription are not well understood.

Our data show that in both RAW 264.7 macrophages and THP-1 monocytic cells there is redundancy in the transcriptional activation of the COX-2 gene both at the extranuclear signaling level and at the promoter level. Overexpression of dominant negative mutants for different MAPKs and PKC isoforms does not abrogate the effects of LPS on COX-2 transcription indicating that there is redundancy at the signaling level. Mutation of a single NFκB, NF-IL6, or CRE site also does not abrogate the LPS effect indicating that there is redundancy at the promoter level. Therefore, transcriptional repression of COX-2 could not be accomplished by targeting a single signaling pathway or transcription factor/promoter element.
JNK pathways. Moreover, PKC-NF-IL6 via a p38 pathway, and through CRE via ERK-2 and NF-κB transcription factors to at least two promoter sites. Therefore, individual signaling pathways, such as the MAPK signaling seems to mediate transcription after LPS treatment through all three promoter sites. LPS can activate different MAPK pathways to induce COX-2 gene transcription: through NFκB via ERK-2, p38, and JNK pathways, through NF-IL6 via a p38 pathway, and through CRE via ERK-2 and JNK pathways. Moreover, PKC-ζ signaling seems to mediate transcription through NFκB, NF-IL6, and CRE promoter sites.

Previous studies have shown that pharmacological inhibition of NFκB activation decreases COX-2 expression in macrophage-like cells (19), and COX-2 transcription through the NFκB promoter site also has been demonstrated (20). The role of other cis-acting elements and their cooperation in promoting transcription was not evaluated in those studies. Other investigators have attempted to characterize the signaling pathways leading to COX-2 transcriptional activation. In one study, MAPK signaling was found to be necessary in LPS-mediated activation of the COX-2 NFκB promoter site (19), but other studies could not confirm this finding (29). A recent report on the transcriptional regulation of the murine COX-2 gene in LPS-treated RAW 246.7 cells showed that two NF-IL6 sites are necessary for maximal induction of transcription and that without a functional CRE site transcription cannot occur (21).

Our results indicate that LPS induces COX-2 expression in cells of macrophage/monocytic cell lineage through the binding of transcription factors to the NFκB, NF-IL6, and CRE promoter elements situated within the first 327 base pairs in the 5′ flanking region. The participation of at least two of these cis-acting elements is necessary to achieve maximal induction of transcription. We also show that there is lack of specificity in the signaling pathways that mediate COX-2 gene expression through each of these promoter sites. LPS can activate different MAPK pathways to induce COX-2 gene transcription: through NFκB via ERK-2, p38, and JNK pathways, through NF-IL6 via a p38 pathway, and through CRE via ERK-2 and JNK pathways. Moreover, PKC-ζ signaling seems to mediate transcription after LPS treatment through all three promoter sites. Therefore, individual signaling pathways, such as ERK-2, p38, JNK, or PKC-ζ, appear to be sufficient to mediate COX-2 gene transcription by virtue of their ability to recruit transcription factors to at least two promoter sites.

In addition, activation of a MEKK-1/JNK pathway was necessary for induction of murine COX-2 transcription. This study differs from ours in that specificity rather than redundancy, both at the signaling pathway and promoter levels, characterizes LPS-mediated induction of COX-2 transcription. The differences in results may reflect differences in transcriptional regulation between the murine promoter, studied by these investigators, and the human promoter, utilized in our work.

In summary, we have presented experiments with mutant COX-2 promoter reporter constructs and with expression vectors for wild type or dominant negative mutants for MAPK and PKC isoforms. These experiments demonstrate the redundant
rather than specific character of the cell signaling leading to COX-2 gene transcription in endotoxin-treated macrophage/monocytic cells. Redundancy in the signaling pathways and promoter elements regulating COX-2 gene transcription may constitute an important mechanism ensuring increased levels of COX-2 in macrophage/monocytic cells during inflammation.

REFERENCES

1. Davies, P., Bailey, P. J., Goldenberg, M. M., and Ford-Hutchinson, A. W. (1984) Annu. Rev. Immunol. 2, 335–357
2. Marnett, L. J., Rowlinson, S. W., Goodwin, D. C., Kalugtar, A. S., and Lanzo, C. A. (1999) J. Biol. Chem. 274, 22903–22906
3. Smith, W. L. (1992) Am. J. Physiol. 263, F181-F191
4. Funk, C. D., Funk, L. B., Kennedy, M. E., Pong, A. S., and Fitzgerald, G. A. (1991) J. Biol. Chem. 266, 2304–2312
5. Hempel, S. L., Monick, M. M., and Hunninghake, G. W. (1994) J. Clin. Invest. 93, 391–396
6. Kelley, D. J., Mestre, J. R., Subbaramaiah, K., Sacks, P. G., Schantz, S. P., Tanabe, T., Inoue, H., Ramonetti, J. T., and Dannenberg, A. J. (1997) J. Biol. Chem. 270, 24965–24971
7. Riese, J., Hoff, T., Nordhoff, A., De Witt, D. L., Resch, K., and Kaever, V. (1994) J. Leukocyte Biol. 55, 476–482
8. Nanayama, T., Hara, S., Yokoyama, C., and Tanabe, T. (1995) Prostaglandins 49, 371–382
9. Sano, H., Hla, T., Maier, J. A., Crofford, L. J., Case, J. P., Maciag, T., and Wilder, R. L. (1992) J. Clin. Invest. 89, 57–108
10. Vane, J. R., Mitchell, J. A., Appleton, I., Tomlinson, A., Bishop-Bailey, D., Crockstall, J., and Willoughby, D. A. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 2046–2050
11. Meade, E. A., Smith, W. L., and De Witt, D. L. (1993) J. Biol. Chem. 268, 6610–6614
12. Anderson, G. D., Hauser, S. D., McGarity, K. L., Bremer, M. E., Isakson, P. C., and Gregory, S. A. (1996) J. Clin. Invest. 97, 2672–2679
13. Dinchuk, J. E., Car, B. D., Focht, R. J., Johnston, J. J., Jaffe, B. D., Cervero, M. B., Contel, N. R., Eng, V. M., Collins, R. J., Czerniak, P. M., et al. (1995) Nature 378, 406–409
14. Inoue, H., Nanayama, T., Hara, S., Yokoyama, C., and Tanabe, T. (1994) FEBS Lett. 350, 51–54
15. Inoue, H., Yokoyama, C., Hara, S., Tone, Y., and Tanabe, T. (1995) J. Biol. Chem. 270, 24965–24971
16. Kosaka, T., Miyata, A., Ibara, H., Hara, S., Sugimoto, T., Takeda, O., Takahashi, K., and Tanabe, T. (1994) Eur. J. Biochem. 221, 889–897
17. Subbaramaiah, K., Telang, N., Ramonetti, J. T., Araki, R., De Vito, B., Wessler, B. B., and Dannenberg, A. J. (1996) Cancer Res. 56, 4424–4429
18. Xie, W., and Herschman, H. R. (1995) J. Biol. Chem. 270, 27622–27628
19. Hwang, D., Jang, B. C., Yu, G., and Boudreau, M. (1996) Biochem. Pharmacol. 51, 87–96
20. Inoue, H., and Tanabe, T. (1998) Biochem. Biophys. Res. Commun. 244, 143–148
21. Wadleigh, D. J., Reddy, S. T., Kopp, E., Ghosh, S., and Herschman, H. R. (2000) J. Biol. Chem. 275, 6253–6266
22. Mestre, J. R., Subbaramaiah, K., Sacks, P. G., Schantz, S. P., Tanabe, T., Inoue, H., and Dannenberg, A. J. (1997) Cancer Res. 57, 1081–1085
23. Zhang, F., Altorki, N. K., Mestre, J. R., Subbaramaiah, K., and Dannenberg, A. J. (1999) Carcinogenesis 20, 445–451
24. Deleted in proof.
25. Deleted in proof.
26. Dixon, D. A., Kaplan, C. D., McIntyre, T. M., Zimmerman, G. A., and Prescott, S. M. (2000) J. Biol. Chem. 275, 11750–11757
27. Sheng, H., Shao, J., Dixon, D. A., Williams, C. S., Prescott, S. M., Du Bois, R. N., and Beauchamp, R. D. (2000) J. Biol. Chem. 275, 6628–6635
28. Simmons, D. L., Botting, R. M., Robertson, P. M., Madsen, M. L., and Vane, J. R. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 3275–3280
29. von Kriehn, A., Calissen, D., and Brune, B. (1999) Mol. Biol. Cell 10, 361–372
