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Expression of Mitogen-Inducible Cyclooxygenase Induced by Lipopolysaccharide

MEDIATION THROUGH BOTH MITOGEN-ACTIVATED PROTEIN KINASE AND NF-κB SIGNALING PATHWAYS IN MACROPHAGES

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ABSTRACT. The mitogen-inducible cyclooxygenase (COX-2) is selectively expressed in lipopolysaccharide (LPS)-stimulated macrophages. However, the signaling pathways that lead to the expression of COX-2 in LPS-stimulated macrophages are not well understood. LPS activates members of mitogen-activated protein kinases (MAPKs) and NF-κB transcription factor in macrophages. We have shown that protein tyrosine kinase (PTK) inhibitors suppress the LPS-induced expression of COX-2 in macrophages (Chanmugam *et al.*, *J Biol Chem* **270:** 5418–5426, 1995). These PTK inhibitors also inhibit LPS-induced activation of MAPKs. Thus, in the present study, we determined whether the activation of MAPKs and NF-κB is necessary for the signaling pathway for the LPS-induced expression of COX-2 in the murine macrophage cell line RAW 264.7. The findings demonstrated that inhibition of extracellular signal-regulated protein kinases 1 and 2 (ERK-1 and -2) by the selective inhibitor PD98059 or inhibition of P38 by the specific inhibitor SB203580 results in partial suppression of COX-2 expression. However, activation of MAPKs by phorbol 12-myristate 13-acetate, H_2O_2, sorbitol, sodium vanadate, or a combination of these agents failed to induce the expression of COX-2. Inhibitors of NF-κB suppressed COX-2 expression without affecting tyrosine phosphorylation of MAPKs. The PTK inhibitors that suppressed the activation of MAPKs and COX-2 expression also inhibited the degradation of IκB-α. Together, these results indicate that the activation of NF-κB is required to induce the expression of COX-2 in LPS-stimulated RAW 264.7 cells. Inhibition of ERK-1 and 2 or P38 results in partial suppression of COX-2 expression. However, the activation of MAPKs alone is not sufficient to induce the expression of COX-2 in these cells. BIOCHEM PHARMACOL **54**;1:87–96, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. cyclooxygenase; mitogen-activated protein kinases; lipopolysaccharide; protein tyrosine kinase inhibitors; macrophages; NF-κB

COX† (PG endoperoxide synthase, EC 1.14.99.1) is the rate-limiting enzyme that catalyzes the conversion of arachidonic acid to PG endoperoxide (PGH_2). The endoperoxide, in turn, is converted to various PGs and thromboxane, which exert diverse pathophysiological actions. Two isoforms of COX have been identified: constitutively expressed COX-1 [1–5] and mitogen-inducible COX-2 [6–11]. COX-2 is expressed in response to various mitogenic stimuli [6, 9, 11, 12]. In our previous studies, it was shown that COX-2 but not COX-1 is selectively expressed in macrophages stimulated by LPS [13]. However, the signal transduction pathway that leads to the expression of COX-2 in LPS-stimulated macrophages is not well understood.

The stimulation of macrophages by LPS results in activation of members of MAPKs [14–16]. Currently, there are three distinct MAPK cascades found in vertebrates: ERK-1 and ERK-2, c-Jun N-terminal kinases/stress-activated protein kinases (JNK/SAPKs), and P38 [17–23]. ERK-1 and ERK-2 require phosphorylation of both Thr-183 and Tyr-185 for activation [24]. The immediate upstream protein kinases that phosphorylate these residues in ERK-1 and ERK-2 are MAPK kinases (or MEK1 and MEK2). MEKs possess dual specificity for the tyrosine and threonine residues in ERK-1 and ERK-2 [25–27]. MKK3 and MKK4 are the MAPK kinases activating P38 and JNK, respectively [28–30]. The MAPK cascade is one of the major signaling pathways transmitting signals from growth factors, hormones, and cytokines to the immediate early genes (genes induced by a stimulus in the absence of *de novo* synthesis of proteins). Activated MAPKs, in turn, can activate transcription factors that bind *cis*-acting elements of immediate early genes [31]. The 5′-flanking regions of COX-2 genes

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† *Abbreviations:* COX, cyclooxygenase; DMEM, Dulbecco's modified Eagle's medium; ERK, extracellular signal-regulated protein kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; JNK, c-Jun N-terminal kinase; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; MBP, myelin basic protein; PG, prostaglandin; PMA, phorbol 12-myristate 13-acetate; and Z-LLF-CHO, benzyloxycarbonyl-Leu-Leu-phenylalaninal.

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from different species contain *cis*-acting elements such as CRE, AP2, SP1, and NF-IL6 [32–36] that can be transactivated by MAPK-activated transcription factors [17, 31]. Participation of these *cis*-acting elements in transcriptional regulation of the COX-2 gene has been demonstrated by using the promoter constructs coupled to the reporter gene [34, 35, 37].

Our previous studies have indicated that the expression of COX-2 in LPS-stimulated macrophages is suppressed by the tyrosine kinase inhibitors [38]. Our preliminary studies have shown that tyrosine kinase inhibitors also suppress the LPS-induced activation of members of MAPK. Therefore, in this study we determined whether the activation of MAPKs is the major signaling pathway necessary to induce the expression of COX-2 in LPS-stimulated macrophages.

In macrophages, LPS also activates NF-κB transcription factor, which leads to the induction of the expression of many immediate early genes [39]. The presence of the *cis*-acting NF-κB element has been demonstrated in the 5′-flanking regions of COX-2 genes [32–36]. In resting cells, NF-κB is bound by the inhibitory protein, IκB-α, thereby preventing its nuclear translocation. Appropriate cellular stimuli cause degradation of IκB-α, resulting in the release of active NF-κB, which, in turn, translocates to the nucleus and induces gene transcription through *cis*-acting κB elements [40]. It has been well documented that activation of NF-κB correlates with rapid proteolytic degradation of IκB-α regardless of signal or cell type [41, 42]. Thus, we investigated whether the activation of NF-κB is also required for the LPS-induced expression of COX-2 protein by monitoring the degradation of IκB-α as an indication of NF-κB activation.

MATERIALS AND METHODS

Reagents

4G10 antiphosphotyrosine monoclonal antibody was purchased from Upstate Biotechnology, Inc. (UBI, Lake Placid, NY). Rabbit polyclonal anti-ERK-2 antibody and polyclonal ERK-1 antibody cross-reacting with ERK-2 also were purchased from UBI. Rabbit polyclonal anti-P38 antibody was a gift from R. J. Ulevitch (The Scripps Research Institute, La Jolla, CA). Polyclonal JNK-1 antibody was purchased from Santa Cruz Biotech (Santa Cruz, CA). MBP was purchased from GIBCO (Grand Island, NY). Goat anti-mouse and anti-rabbit IgG conjugated to horseradish peroxidase were purchased from the Amersham Corp. (Arlington Heights, IL). Enhanced chemiluminescence (ECL) western blotting detection reagents were purchased from Amersham. Polyvinylidene difluoride (PDVF) transfer membrane was purchased from Millipore (Bedford, MA). Arachidonic acid was purchased from Nu-Chek (Eslyan, MN). The inhibitor of IκB-α degradation, Z-LLF-CHO, was obtained from Frank Mercurio (Signal Pharmaceuticals). The inhibitor of NF-κB translocation, a synthetic peptide containing a cell membrane-permeable motif and nuclear localization sequence (SN50, AAVALLPAVLLALLAPVQRKRQKLMP), was obtained from Yao-Zhong Lin (Vanderbilt University School of Medicine). The selective inhibitors of MEK1 and MEK2 (PD98059) and of P38 (SB203580) were obtained from Alan Saltiel (Parke–Davis Pharmaceutical) and Peter Young (SmithKline Beecham Pharmaceuticals), respectively.

Cell Culture and Stimulation

The murine macrophage cell line RAW 264.7 (ATCC, T1B71) was cultured in DMEM containing 10% heat-inactivated fetal bovine serum (Intergen) at 37° with a 5% CO_2/air mixture. About 10×10^6 cells were seeded in a 100-mm plate (Costar), and then cultured for about 18 hr to allow the cell number to approximately double. Cells were treated with agonists, inhibitors, or vehicle for the indicated time periods. For the COX activity assay, cells were seeded in 24-well plates, and after near confluency cells were treated with aspirin (250 μM) for 2.5 hr to inactivate endogenous COX. After being washed three times with DMEM, cells were treated with agonist for 8 hr.

Preparation of Cell Lysates

After stimulation, cells were washed with ice-cold PBS containing 1 mM Na_3VO_4, and then were lysed in 750 μL of lysis buffer [50 mM Tris–HCl (pH 7.4), 1 mM diethyldithiocarbamic acid (DDTC)-sodium, 10 mM EDTA, 1% Tween-20, 1% Triton X-100, 10 μM leupeptin, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM Na_3VO_4] for 20 min on ice. In cells used for COX and GAPDH immunoblots Na_3VO_4 was not included in either the washing or the lysis buffers. Cells were scraped off dishes and solubilized by sonication. The detergent-insoluble material was removed by centrifugation at 10,000*g* for 20 min at 4°. Protein concentrations were determined by the Bradford method [43].

Antiphosphotyrosine Immunoblotting

This was carried our essentially the same as previously described [38]. Briefly, solubilized proteins were separated in a 12% SDS-polyacrylamide gel, and transblotted onto PDVF membrane. The membrane was blocked with 2% BSA in Tris-buffered saline containing 0.05% Tween-20. Tyrosine-phosphorylated proteins in the membrane were treated with 4G10 monoclonal antiphosphotyrosine antibody, and then with goat anti-mouse IgG coupled to horseradish peroxidase. The membrane was analyzed by the ECL detection system (Amersham).

Immunoprecipitation

Cell lysates prepared using the lysis buffer were incubated with polyclonal P38, ERK-2, or JNK-1 antibodies at 4° overnight. The immune complexes were captured by incubating with protein A-Sepharose beads for 3 hr at 4°. After centrifugation, the supernatant fraction was collected and concentrated using Centricon-10 (Amicon, Beverly, MA).

The Sepharose beads were washed three times with ice-cold wash buffer [1% Triton X-100, 500 mM Nacl, 10 mM Tris (pH 7.5), 1 mM EGTA, 1 mM Na_3VO_4], and then were resuspended in 2× Laemmli sample buffer and boiled for 5 min. Depleted supernatant and immunoprecipitates were resolved on a 12% SDS-polyacrylamide gel and subjected to antiphosphotyrosine immunoblot analyses for ERK-2 and P38. For JNK-1, samples were resolved on an 8% SDS-polyacrylamide gel and probed with JNK-1 antibody.

Immunoblotting for JNK-1, COX-2, IκB-α, and GAPDH

Immunoblotting for these proteins was carried out as described above for antiphosphotyrosine immunoblotting. However, the PDVF membrane was blocked with 5% nonfat dry milk in PBS containing 0.1% Tween-20 overnight. Polyclonal antibodies for COX-2 and GAPDH were prepared as described previously [38]. Polyclonal antibodies for JNK-1 and IκB-α were purchased (Santa Cruz Biotech).

In-gel Kinase Assay

This was performed according to the method described by Kameshita and Fugisawa [44]. Solubilized proteins were separated on a 12% SDS-polyacrylamide gel containing 5 mg MBP as a substrate for ERK-1 and -2 in the separation gel. After electrophoresis overnight, SDS was removed by washing the gel with two changes of 20% 2-propanol in 50 mM Tris–HCl (pH 8.0) for 1 hr at room temperature with gentle agitation, and then with 50 mM Tris–HCl (pH 8.0) containing 5 mM β-mercaptoethanol (Buffer A). MAP kinases were denatured by treating the gel first with two changes of 6 M guanidine–HCl in Buffer A at room temperature for 30 min and then were renatured with five changes of Buffer A containing 0.04% Tween 40 (pH 8.0) at 4° for a total of at least 16 hr with gentle agitation. After renaturation, the gel was preincubated at 25° for 30 min with 40 mM HEPES–NaOH (pH 8.0) containing 2 mM dithiothreitol, 0.1 mM EGTA, and 5 mM $MgCl_2$. The kinase assay was carried out by incubating the gel at 25° for 1 hr with 40 mM HEPES–NaOH (pH 8.0) containing 2 mM dithiothreitol, 0.1 mM EGTA, 5 mM $MgCl_2$, 50 μM ATP, and 50 μCi [γ-^{32}P]ATP (Amersham; specific activity 3000 Ci/mmol). After incubation, the gel was washed with 5% (w/v) trichloroacetic acid solution containing 1% sodium pyrophosphate at room temperature until the radioactivity of the solution became negligible (usually more than five changes of 250 mL each were necessary). The gel was then dried on Whatman 3 MM filter paper and exposed to Kodak X-ray film at −80°.

Assay for COX Activity of Newly Synthesized COX

Cells were pretreated with aspirin (250 μM) for 2.5 hr to inactivate endogenous COX [13]. After washing the cells three times with DMEM, cells were incubated with agonists for 8 hr. The medium was removed, and cells were incubated for 10 min in fresh DMEM containing arachidonic acid (30 μM). Levels of PGE_2 were determined by radioimmunoassay as described previously [13].

RNase Protection Assay

Total cellular RNA was isolated by TRIZOL reagent (GIBCO, BRL) and quantitated by its absorbance at 260 nm. Two micrograms of total RNA was hybridized with 1×10^6 cpm of ^{32}P-labeled antisense riboprobes. The RNase protection assay was performed as described previously [38].

RESULTS AND DISCUSSION

Stimulation of Tyrosine Phosphorylation of MAPKs and the Expression of COX-2 by LPS in RAW 264.7 Cells

It has been demonstrated that LPS stimulates the tyrosine phosphorylation of three MAPK subfamilies (ERK-1,

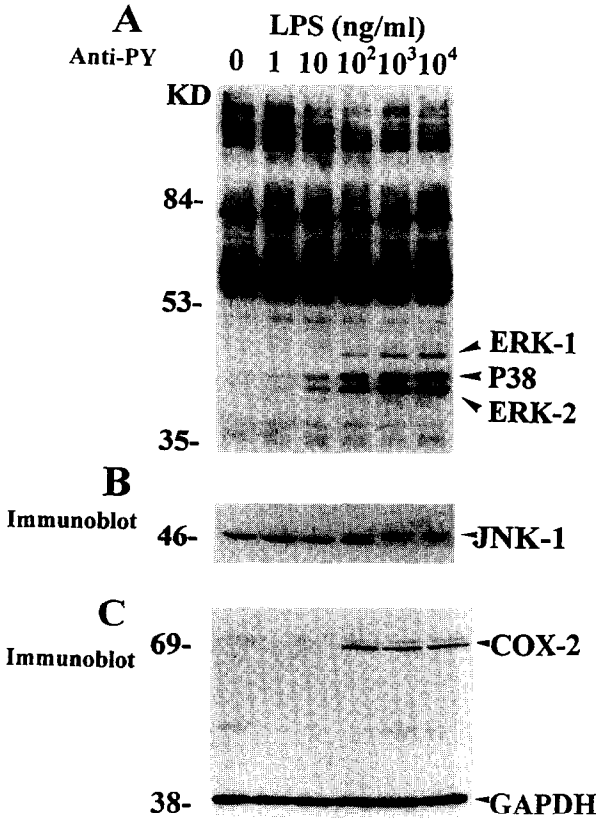

FIG. 1. Concentration-dependent stimulation of protein tyrosine phosphorylation of ERK-1, ERK-2, and P38, and concomitant stimulation of the expression of COX-2 protein by LPS in RAW 264.7 cells. (A) Cells were stimulated with various concentrations of LPS for 30 min. Solubilized proteins were analyzed by antiphosphotyrosine immunoblot, as described under Materials and Methods. (B) For JNK-1 immunoblots, the same cell lysates as used in panel A were analyzed. (C) For COX-2 and GAPDH immunoblots, cells were stimulated with various concentrations of LPS for 8 hr. Solubilized proteins were analyzed by COX-2 and GAPDH immunoblotting as described under Materials and Methods. Shown are representative immunoblots from more than three different analyses.

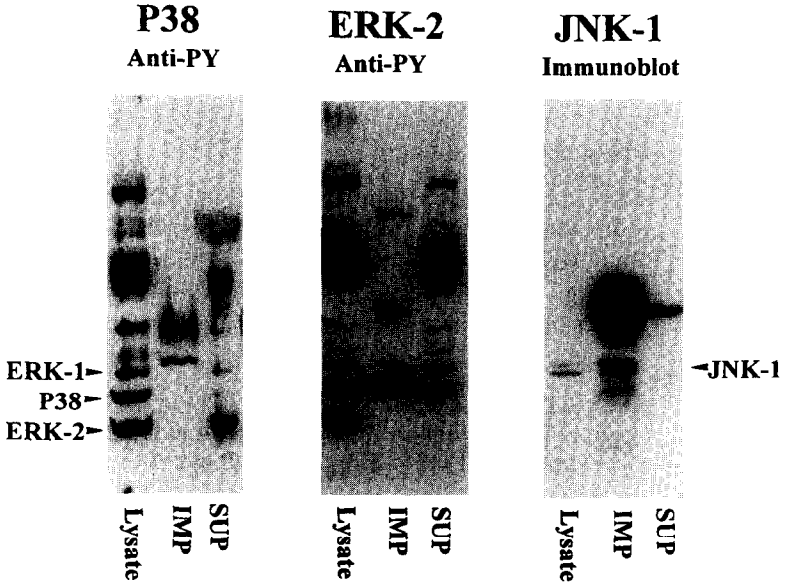

FIG. 2. Antiphosphotyrosine immunoblot analysis of immunoprecipitated P38 and ERK-2, and immunoblot analysis of immunoprecipitated JNK-1. Cells were incubated with LPS (1 μg/mL) for 30 min, and solubilized proteins were immunoprecipitated with P38, ERK-2, or JNK-1 antibody. P38 and ERK-2 immunoprecipitate, and respective depleted supernatants were analyzed by antiphosphotyrosine immunoblot. JNK-1 immunoprecipitate was analyzed by JNK-1 immunoblot. The mobility shift of phosphorylated JNK-1 (upper band) is shown in doublet bands. IMP = immunoprecipitate; and SUP = supernatant.

ERK-2, and P38) and JNK in macrophage cell lines [14–16]. Among the proteins that were tyrosine phosphorylated in response to LPS, MAPKs exhibited the most dramatic change in the extent of tyrosine phosphorylation (Fig. 1, A and B), and this change was correlated with the increased expression of COX-2 (Fig. 1C). The stimulation of protein tyrosine phosphorylation by LPS occurred in a concentration- and time-dependent manner. The maximum stimulation was reached with 1 μg/mL of LPS (Fig. 1A). The time–course indicated that the maximum stimulation occurred within 30–45 min of incubation, and tyrosine phosphorylation of MAPKs was reduced rapidly after 60 min of incubation (data not shown). Thus, all subsequent incubations to study the tyrosine phosphorylation of MAPKs were carried out using 1 μg/mL of LPS for 30 min. The catalytic activity of ERK-2 requires phosphorylation of both tyrosine and threonine residues [24]. Thus, the extent of tyrosine phosphorylation of MAPKs assessed by anti-phosphotyrosine immunoblot can be used to compare the catalytic activities of MAPKs.

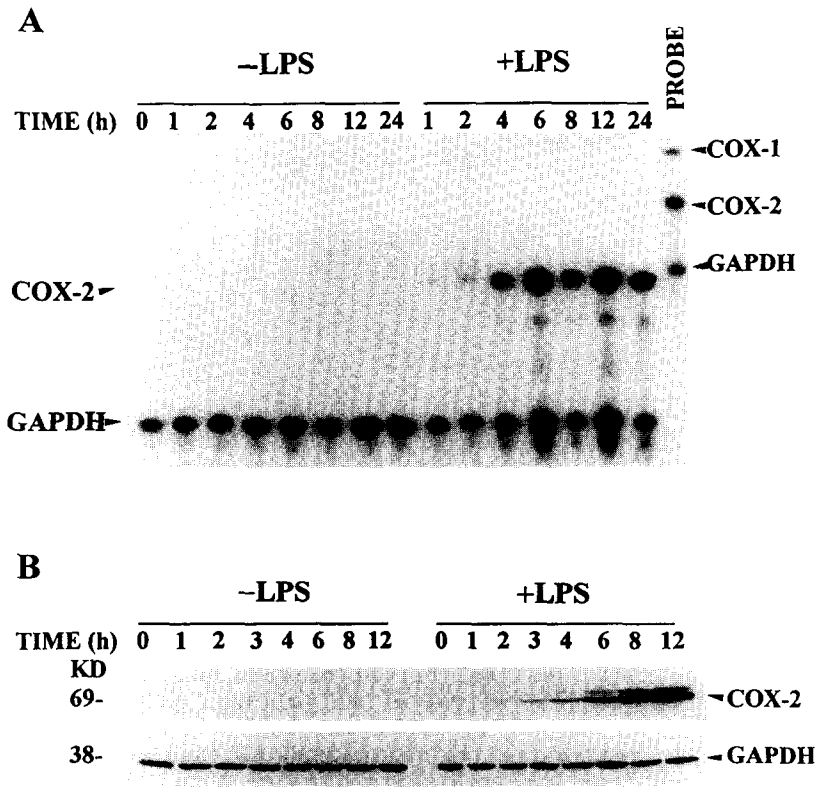

FIG. 3. Time-course of the expression of COX-2 mRNA and protein induced by LPS. (A) Cells were incubated with LPS (1 μg/mL) for specified time periods. Total RNA was isolated by TRIZOL reagent. Abundance of mRNA was determined by RNase protection assay. (B) For COX-2 and GAPDH proteins, solubilized proteins of cells stimulated with LPS (1 μg/mL) for specified time periods were analyzed by immunoblot using polyclonal COX-2 and GAPDH antibodies as described under Materials and Methods.

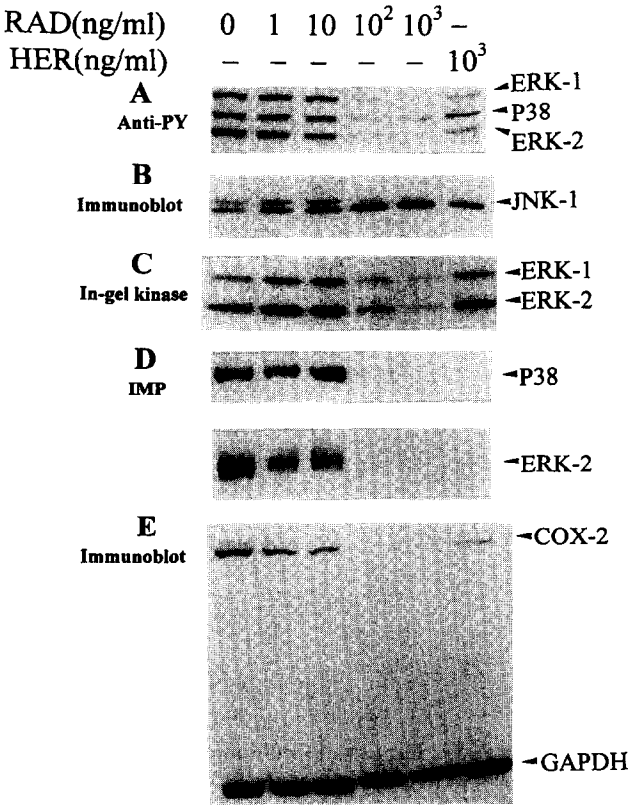

FIG. 4. Inhibition of protein tyrosine phosphorylation and kinase activity of MAPKs and the expression of COX-2 protein by the tyrosine kinase inhibitors radicicol and herbimycin A. (A) Cells were pretreated with the inhibitors for 4 hr and then stimulated with LPS (1 μg/mL) in the presence of inhibitors for 30 min. Solubilized proteins were analyzed by antiphosphotyrosine immunoblot as described under Materials and Methods. (B) Immunoblot of the same samples used in panel A, using polyclonal JNK-1 antibody. (C) In-gel kinase assays of the solubilized proteins from cells treated the same way as those in panel A, using MBP as a substrate for ERK-1 and ERK-2. (D) Immunoprecipitated P38 or ERK-2 from the solubilized samples used in panel A was analyzed by antiphosphotyrosine immunoblot. (E) COX-2 and GAPDH immunoblot of solubilized proteins from cells stimulated with LPS (1 μg/mL) in the presence of the tyrosine kinase inhibitors. IMP = immunoprecipitation. Representative results of more than five different analyses are shown.

Identities of ERK-2 and P38 were determined by antiphosphotyrosine immunoblot analyses of immunoprecipitated ERK-2 and P38, as shown in Fig. 2. The disappearance of tyrosine-phosphorylated ERK-2 or P38 in depleted supernatant after immunoprecipitation (Fig. 2) indicates that ERK-2 or P38 bands shown in antiphosphotyrosine immunoblots of cell lysates (Fig. 1) indeed represent tyrosine-phosphorylated ERK-2 or P38. Mobility retardation of immunoprecipitated ERK-2 or P38 in SDS–PAGE is due to the heavy chain of IgG derived from the rabbit polyclonal antibodies (Fig. 2). Mobility retardation did not occur for immunoprecipitated JNK-1 because only 0.2 μg of IgG (that is, one-tenth of the amount of ERK-2 or P38 antibody) was used to deplete JNK-1 completely from 100 μg of solubilized protein. The monoclonal antiphosphotyrosine antibody (4G10) does not recognize phosphorylated JNKs. Therefore, the extent of tyrosine phosphorylation of JNK was assessed by the electrophoretic mobility shift of phosphorylated JNK-1, as shown in Fig. 1B.

Time–Course of the Expression of COX-2 mRNA and Protein Induced by LPS in RAW 264.7 Cells

The expression of COX-2 mRNA began within 1 hr of the LPS stimulation, and the maximum induction was reached within 4–6 hr. COX-1 mRNA was not detected in these assays (Fig. 3A). The maximum expression of COX-2 protein was reached 8–12 hr after stimulation with LPS (Fig. 3B). COX-1 protein as assessed by polyclonal anti-COX-1 antibodies was not detected in these assays (data not shown). Similar time–courses for the expression of COX-2 mRNA and protein were demonstrated in rat alveolar macrophages stimulated with LPS in our previous studies [38]. To determine the levels of expression of COX-2 protein in response to various inhibitors used in this study, cells were incubated with LPS in the presence or absence of each inhibitor for 8 hr.

Inhibition of Tyrosine Phosphorylation and Kinase Activity of MAPKs, and COX-2 Expression by Tyrosine Kinase Inhibitors in RAW 264.7 Cells

The tyrosine phosphorylation and kinase activity of MAPKs stimulated by LPS was inhibited by radicicol, the novel tyrosine kinase inhibitor, in a concentration-dependent manner (Fig. 4, A–D). Another protein tyrosine kinase inhibitor, herbimycin A, also inhibited tyrosine phosphorylation of MAPK subfamilies. Radicicol at concentrations that suppressed tyrosine phosphorylation of MAPK subfamilies also inhibited the expression of COX-2 (Fig. 4E). These results raised an important question as to whether the activation of MAPKs is required for the expression of COX-2 protein in RAW 264.7 cells stimulated by LPS. To answer this question, first, we pretreated cells with a selective inhibitor of ERK-1 and ERK-2 or P38 and determined whether this leads to the suppression of COX-2 expression. Second, cells were stimulated with agents other than LPS to activate MAPKs, and whether or not the activation of MAPKs by these agents also leads to the expression of COX-2 was determined.

Partial Suppression of COX-2 Expression by Inhibitors of MEK and P38 in LPS-Stimulated RAW 264.7 Cells

PD98059 was shown to be a selective inhibitor of phosphorylation and kinase activity of MEK1 and MEK2 [45]. MEK1 and MEK2 are the upstream kinases dedicated to phosphorylation of both tyrosine and threonine residues of ERK-1 and ERK-2. Pretreatment of cells with PD98059 resulted in partial inhibition of the kinase activities and tyrosine phosphorylation of ERK-1 and ERK-2 stimulated by LPS (Fig. 5, A, C and D). However, this inhibitor

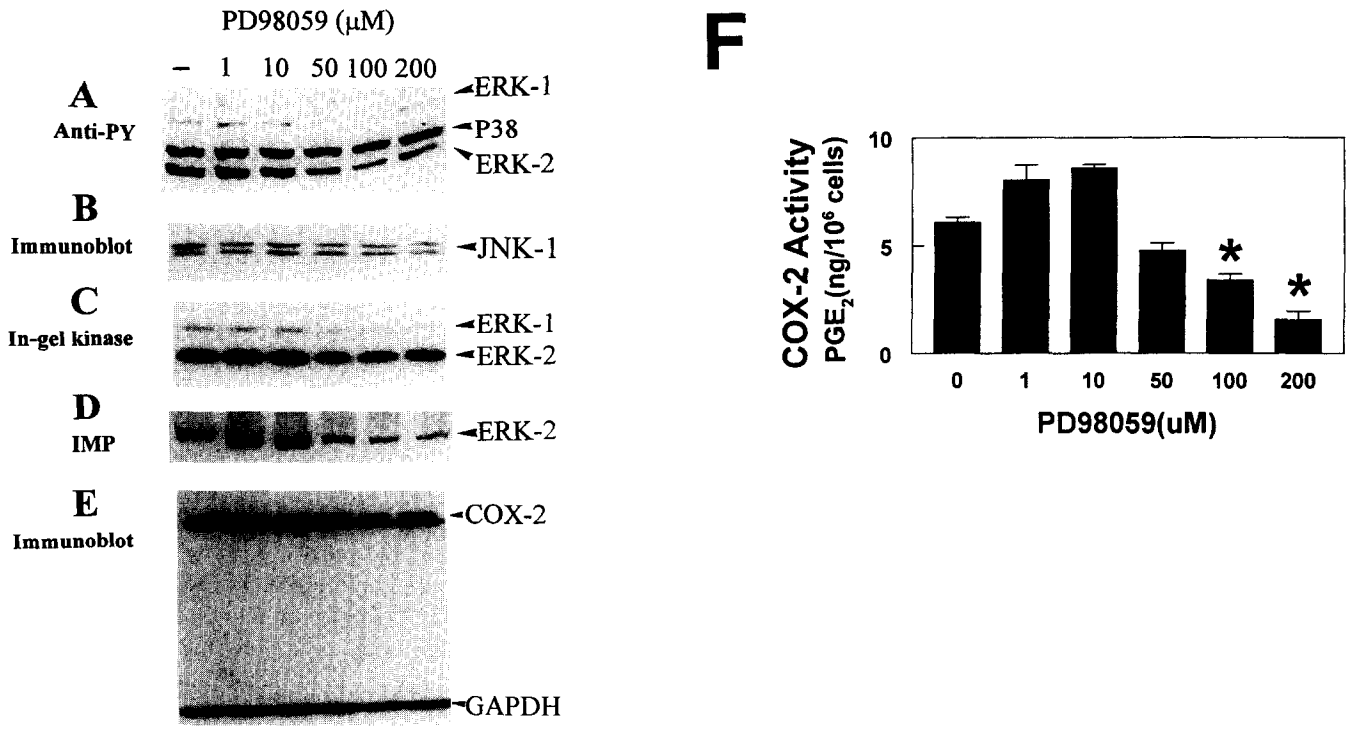

FIG. 5. Inhibition of the tyrosine phosphorylation of ERK-1 and ERK-2 (but not P38), and partial inhibition of the expression of COX-2 by PD98059 (inhibitor of MEK1 and MEK2) in RAW 264.7 cells. (A) Cells were pretreated with various concentrations of PD98059 for 1 hr and then were stimulated with LPS (1 μg/mL) in the presence or absence of the inhibitor for 30 min. Solubilized proteins were analyzed by antiphosphotyrosine immunoblot as described under Materials and Methods. (B) Immunoblot of the same samples used in panel A using polyclonal JNK-1 antibody. (C) In-gel kinase assays of the same samples used in panel A. (D) Immunoprecipitated ERK-2 from the same samples used in panel A was analyzed by antiphosphotyrosine immunoblot. (E) COX-2 and GAPDH immunoblot of solubilized proteins from cells treated the same way as in panel A but incubated for 8 hr. IMP = immunoprecipitation. (F) COX activity in cells pretreated with aspirin (250 μM) to inactivate the endogenous COX for 2.5 hr and then treated with the inhibitor for 8 hr. COX activity was determined by measuring PGE_2 after incubating cells with exogenous arachidonic acid (30 μM) for 10 min. Representative results of more than three different analyses are shown. Values are means ± SEM (N = 3). Key: (*) significantly different from the control ($P < 0.05$).

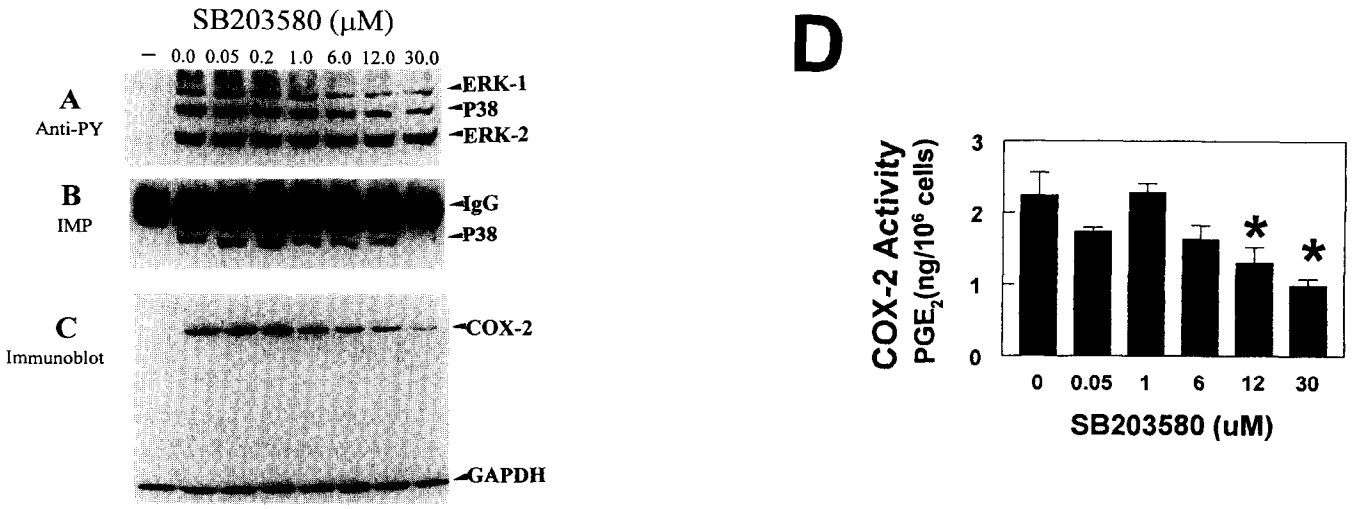

FIG. 6. Inhibition of tyrosine phosphorylation of P38 and partial inhibition of the expression of COX-2 by SB203580 (inhibitor of P38) in RAW 264.7 cells. (A) Cells were pretreated with various concentrations of SB203580 for 1 hr and then stimulated with LPS (1 μg/mL) in the presence or absence of the inhibitor for 30 min. Solubilized proteins were analyzed by antiphosphotyrosine immunoblot as described under Materials and Methods. (B) Immunoprecipitated P38 from the solubilized samples used in panel A was analyzed by antiphosphotyrosine immunoblot. (C) COX-2 and GAPDH immunoblot of solubilized proteins from cells treated the same way as in panel A but incubated for 8 hr. (D) COX activity in cells treated the same way as in panel C. Values are means ± SEM (N = 3). IMP = immunoprecipitation. Representative results of more than two different analyses are shown. Key: (*) significantly different from the control ($P < 0.05$).

affected neither the activity of P38 nor tyrosine phosphorylation of JNK (Fig. 5B). The partial inhibition of the activities and tyrosine phosphorylation of ERK-1 and ERK-2 by PD98059 caused partial suppression of the LPS-induced COX-2 expression (Fig. 5, E and F). It has been demonstrated that production of interleukin-1 and tumor necrosis factor from stimulated human monocytes is inhibited by a new series of pyridinyl-imidazole compounds [46]. The molecular target of these compounds was found by binding assays to be P38. The inhibition of P38 phosphorylation by the selective inhibitor SB203580 (Fig. 6, A and B) also led to the partial suppression of LPS-induced COX-2 expression in RAW 264.7 cells (Fig. 6, C and D).

Failure to Induce the Expression of COX-2 Protein by the Activation of MAPK Subfamily by Agents Other Than LPS in RAW 264.7 Cells

As shown in Fig. 7A–C, PMA stimulated tyrosine phosphorylation and kinase activity of ERK-1 and ERK-2, whereas sorbitol (0.4 M) stimulated tyrosine phosphorylation and kinase activity mainly of P38 and JNK-1. Hydrogen peroxide (1 mM) activated all three subfamilies of MAPKs. Hydrogen peroxide at lower concentrations (<1 mM) mainly activated P38 (data not shown). Increased tyrosine phosphorylation and kinase activities of ERK-1, ERK-2, and P38 by these agents failed to induce COX-2 expression (Fig. 7, D and E). MKP-1 (3CH134), an immediate early gene product, is a dual specificity phosphatase that dephosphorylates both T183 and Y185 in ERK-2, and thus inactivates the kinase [47]. Therefore, we determined whether the protein tyrosine phosphatase (PTPase) inhibitor sodium orthovanadate stimulates tyrosine phosphorylation of MAPKs and leads to the induction of COX-2 expression in macrophages. Results show that sodium orthovanadate caused increased tyrosine phosphorylation and kinase activity of P38, ERK-1, ERK-2, and JNK-1 (Fig. 7), but failed to induce COX-2 expression (Fig. 7, D and E).

Taken together, these results indicate that the activation of MAPK subfamilies (ERK-1, ERK-2, JNK-1, and P38) alone is not sufficient to induce the expression of COX-2 protein in LPS-stimulated RAW 264.7 cells. Furthermore, these results suggest that an additional signaling pathway(s) independent from the MAPK cascade is required to induce the expression of COX-2 protein in LPS-stimulated RAW 264.7 cells. Recently, Xie and Herschman [37] showed that enhanced promoter activity of a COX-2 promoter/luciferase reporter construct induced by overexpression of $pp60^{v\text{-}src}$ is mediated by activation of JNK, ERK-1, and ERK-2 in NIH 3T3 cells. This implies that the role of MAPKs in the induction of COX-2 expression is agonist and cell type specific.

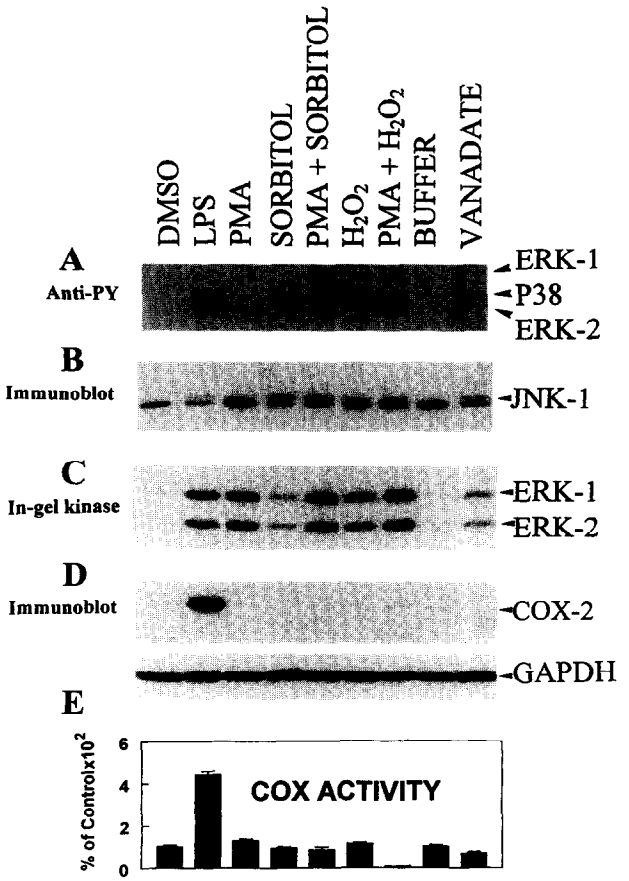

FIG. 7. Effects of LPS, PMA, sorbitol, H_2O_2, a combination of these agents, or sodium orthovanadate, on protein tyrosine phosphorylation and kinase activity of MAPKs, and the expression of COX-2. (A) Cells were treated with the indicated agents for 30 min, and solubilized proteins were analyzed by antiphosphotyrosine immunoblot as described under Materials and Methods. (B) Immunoblot using polyclonal JNK-1 antibody with the samples used in panel A. (C) In-gel kinase assays of the solubilized proteins from cells treated the same way as those in panel A using MBP as a substrate for ERK-1 and ERK-2. (D) COX-2 and GAPDH immunoblot of solubilized proteins from cells treated with the indicated agents for 8 hr. (E) COX activity in cells pretreated with aspirin (250 μM) for 2.5 hr to inactivate the endogenous COX and then treated with the indicated agents for 8 hr. COX activity was determined by measuring PGE_2 after incubating cells with exogenous arachidonic acid (30 μM) for 10 min. Values are means ± SEM (N = 3). The control (DMSO) value was 0.573 ± 0.02 ng/10^6 cells. Representative results of more than three different analyses are shown.

Suppression of the Expression of COX-2 Protein by Inhibitors of NF-κB in LPS-Stimulated RAW 264.7 Cells

It has been demonstrated that LPS also activates NF-κB transcription factor that leads to the induction of the expression of many immediate early genes [39]. The *cis*-acting NF-κB element is known to be present in the 5′-flanking regions of COX-2 genes of different species [32–36]. Thus, we determined whether the activation of NF-κB is also required for the LPS-induced expression of COX-2 protein in RAW 264.7 cells. Since it has been well

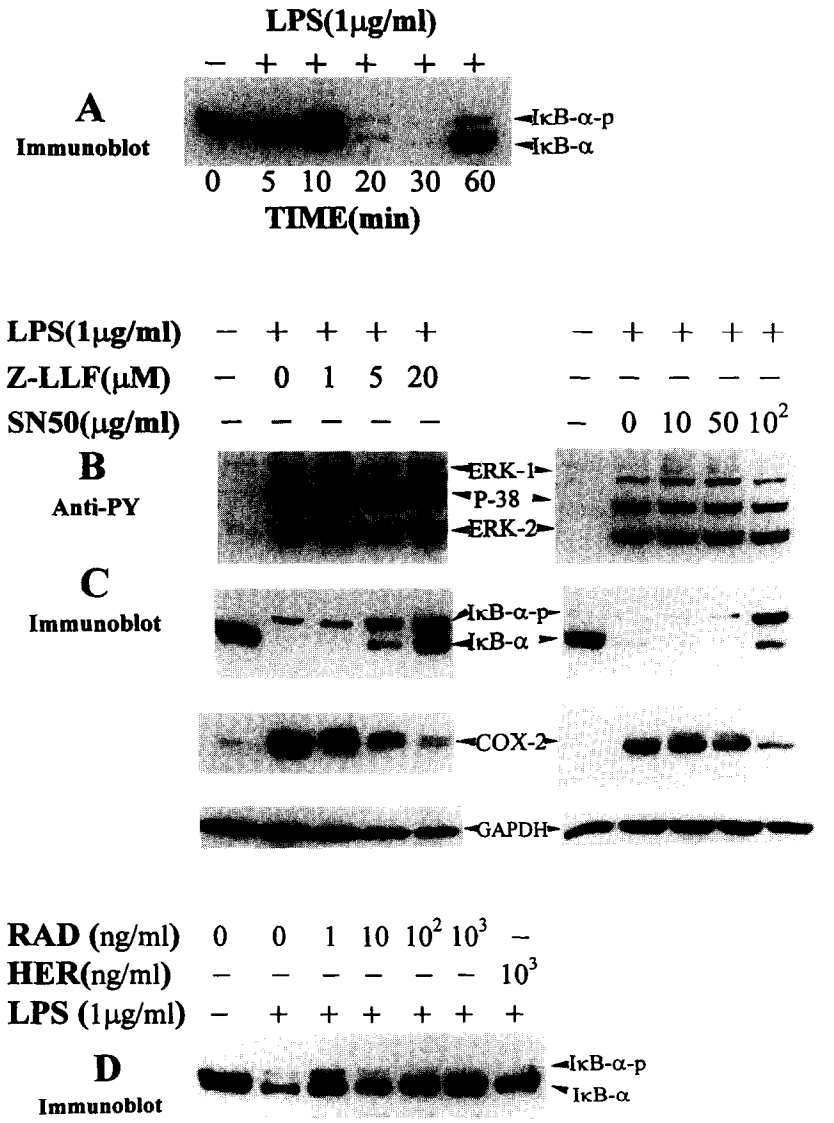

FIG. 8. Degradation of IκB-α induced by LPS (A); and suppression of the COX-2 expression, without affecting tyrosine phosphorylation of MAPKs, by inhibition of the LPS-induced degradation of IκB-α by SN50 or Z-LLF-CHO (B and C); and protein kinase inhibitors inhibit the degradation of IκB-α (D). (A) Cells were stimulated with LPS for specified time periods, and solubilized proteins were analyzed by IκB-α immunoblot. (B) Cells were pretreated for 1 hr with various concentrations of SN50 or Z-LLF-CHO and then stimulated with LPS (1 μg/mL) in the presence or absence of the inhibitors for 30 min. Solubilized proteins were analyzed by antiphosphotyrosine immunoblot. (C) The same samples used in panel B were analyzed by anti-IκB-α. Anti-COX-2 or anti-GAPDH immunoblot was carried out with cell lysates from cells treated the same way as for panel B but incubated for 8 hr. (D) Cells were pretreated with the protein tyrosine kinase inhibitors radicicol or herbimycin A for 4 hr and then stimulated with LPS (1 μg/mL) in the presence of the inhibitors for 30 min. Cell lysates were analyzed by IκB-α immunoblot. Representative results of more than three different analyses are shown. IκB-α-P = phosphorylated IκB-α.

documented that the activation of NF-κB correlates with rapid proteolytic degradation of IκB-α, the degradation of IκB-α was used as an indication for NF-κB activation in this study. We used the multicatalytic protease inhibitor Z-LLF-CHO [48] and a synthetic peptide containing a cell membrane-permeable motif and nuclear localization sequence (SN50) [49] as inhibitors of NF-κB. It has been demonstrated that SN50 inhibits nuclear translocation of NF-κB in monocytic cells stimulated by LPS in a concentration-dependent manner [49]. Z-LLF-CHO blocks IκB-α degradation, and the inhibition of NF-κB induction was directly proportional to the extent of inhibition of IκB-α degradation [49]. LPS induced the transient degradation of IκB-α in RAW 264.7 cells (Fig. 8A). Z-LLF-CHO inhibited the degradation of IκB-α in a concentration-dependent manner (Fig. 8C). SN50 at a high concentration also slightly inhibited the degradation of IκB-α. These compounds did not inhibit tyrosine phosphorylation of MAPKs at the concentrations used in this study (Fig. 8B). However, the expression of COX-2 was inhibited by these compounds in a concentration-dependent manner (Fig. 8C). These results suggest that the expression of COX-2 induced by LPS requires the activation of NF-κB in RAW 264.7 cells.

Radicicol and herbimycin A, tyrosine kinase inhibitors that suppressed the activation of MAPKs and COX-2 expression as shown in Fig. 4, also inhibited the degradation of IκB-α (Fig. 8D). These results suggest that tyrosine kinase(s) may mediate the activation of both MAPKs and NF-κB induced by LPS in RAW 264.7 cells. This may be the reason why radicicol exerts a much more pronounced inhibition of COX-2 expression than either PD98059 or SB203580. PMA, sorbitol, sodium vanadate, and hydrogen peroxide did not induce the degradation of IκB-α (data not shown). It has been shown that hydrogen peroxide induces the activation of NF-κB in some cells, and antioxidants inhibit the activation [50]. However, hydrogen peroxide does not induce the degradation of IκB-α in RAW 264.7 cells. The selective inhibitors of MEK1 and P38 (PD98059 and SB203580, respectively) also did not inhibit the LPS-induced degradation of IκB-α (data not shown).

In summary, LPS activates both the MAPK cascade and NF-κB in RAW 264.7 cells. We determined whether the expression of COX-2 protein induced by LPS was mediated through the MAPK cascade and/or NF-κB signaling pathways. Tyrosine kinase inhibitors (radicicol and herbimycin A) blocked activation of ERK-1, ERK-2, P38, and JNK-1 in a concentration-dependent manner in LPS-stimulated RAW 264.7 cells. This inhibition resulted in the concomitant suppression of the expression of COX-2 protein. The selective inhibitors of MEK1 and MEK2 and of P38 resulted in partial inhibition of the expression of COX-2 protein induced by LPS. It remains to be determined whether the activation of ERK-1 and ERK-2 or of P38 is required for the expression of COX-2 protein induced by LPS. However, the activation of ERK-1 and ERK-2, P38, and JNK-1 by PMA, H_2O_2, sorbitol, or sodium vanadate did not lead to the induction of COX-2 expression. Inhibition of the LPS-induced degradation of IκB-α by a protease inhibitor (Z-LLF-CHO) or the compound inhibiting the nuclear translocation of NF-κB resulted in suppression of COX-2 expression. Taken together, these results suggest that the activation of NF-κB is required to induce the expression of COX-2 protein in LPS-stimulated RAW 264.7 cells. However, the activation of the MAPKs alone is not sufficient for the expression of COX-2 protein in these cells.

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