0026-895X/03/6303-512–523$7.00
MOLECULAR PHARMACOLOGY
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Mol Pharmacol 63:512–523, 2003

Vol. 63, No. 3
2135/1047484
Printed in U.S.A.

Potentiation of Lipopolysaccharide-Inducible Cyclooxygenase 2 Expression by C2-Ceramide via c-Jun N-Terminal Kinase-Mediated Activation of CCAAT/Enhancer Binding Protein β in Macrophages

YANG HEE CHO, CHANG HO LEE, and SANG GEON KIM

National Research Laboratory (MDT) (Y.H.C., S.G.K.), College of Pharmacy and Research Institute of Pharmaceutical Sciences, Seoul National University, Seoul, Korea; and Department of Pharmacology and Institute of Biomedical Science (C.H.L.), College of Medicine, Hanyang University, Seoul, Korea

Received September 9, 2002; accepted December 9, 2002

This article is available online at http://molpharm.aspetjournals.org

ABSTRACT

Ceramide, formed by sphingomyelinase, is involved in the expression of cyclooxygenase-2 (COX-2). This study examines the effect of C2-ceramide (C2), a cell-permeable ceramide analog, on the lipopolysaccharide (LPS)-inducible COX-2 expression and signaling pathways. C2 did not induce COX-2 but potentiated LPS-inducible COX-2 expression in Raw264.7 cells, whereas dihydro-C2 was inactive. Treatment of cells with C2 notably increased LPS-inducible CCAAT/enhancer binding protein (C/EBP) DNA binding. Antibody supershift experiments revealed that LPS-induced C/EBP DNA binding activity depended on C/EBPβ and C/EBPδ but not C/EBPα, C/EBPε or CBP/p300. C/EBPβ contributed to C2-enhanced DNA binding activity. 4-(4-Fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl) 1*H*-imidazole (SB203580), a p38 kinase inhibitor, completely inhibited LPS-inducible and C2-potentiated LPS-inducible COX-2 expression. Enhancement of LPS-inducible COX-2 expression and C/EBP DNA binding by C2 was abrogated in dominant-negative mutant of JNK1 [JNK1(−)] cells. 2′-Amino-3′-methoxyflavone (PD98059) or stable transfection with dominant-negative mutant of MKK1 decreased COX-2 induction by LPS but failed to inhibit C2-enhanced LPS induction of COX-2. Transfection with dominant-negative mutant of C/EBP inhibited the ability of C2 to potentiate the induction of COX-2 by LPS. In LPS-treated cells, C2 enhanced both the nuclear translocation and the expression of LPS-inducible C/EBPβ with an increase in AP-1 DNA binding activity. These enhancements were abolished by JNK1(−) transfection. AP-1 decoy oligonucleotide suppressed C2-potentiated C/EBPβ expression, indicating that AP-1 was responsible for C2-mediated C/EBPβ expression. These results demonstrate that C2 increases C/EBPβ-mediated COX-2 induction by LPS and that the pathway of JNK1 but not ERK1/2 is responsible for C/EBPβ activation involving activator protein-1-mediated enhanced C/EBPβ expression.

Cyclooxygenase-2 (COX-2) plays roles in pathophysiological processes, including inflammation, angiogenesis, and tumorigenesis. Lipopolysaccharide (LPS) and many growth factors induce COX-2 (Wadleigh et al., 2000). LPS is an endotoxin that induces septic shock syndrome and stimulates the production of inflammatory mediators such as COX-2, nitric oxide, tumor necrosis factor-α (TNF-α), interleukins, prostanoids, and leukotrienes (Lee et al., 1992; Kubes and McCafferty, 2000; Hewett and Roth, 1993). Development of COX-2 inhibitors represents a major advance in the therapy of inflammatory processes, and their use includes prevention or treatment of disorders associated with the induction of this enzyme (e.g., colon cancer). Despite the importance of COX-2 as a target for the treatment of human inflammatory disorders, the functional role of the signaling pathways for the induction of COX-2 is largely unsolved or contradictory.

A variety of stimuli increase intracellular ceramide through sphingomyelinase or de novo synthesis (Hannun, 1994). Ceramide is implicated in cell growth, differentiation, apoptosis, inflammation, and immune responses. Several laboratories studied the ceramide signaling pathways. Cer-

This work was supported by National Research Laboratory Program (2001), KISTEP, The Ministry of Science and Technology, Republic of Korea.

ABBREVIATIONS: COX-2, cyclooxygenase 2; LPS, lipopolysaccharide; TNF-α, tumor necrosis factor-α; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; MAP, mitogen-activated protein; NF-κB, nuclear factor-κB; C2, C2-ceramide; AP-1, activator protein 1; C/EBP, CCAAT/enhancer binding protein; CREB, cAMP response element binding protein; PD98059, 2′-amino-3′-methoxyflavone; SB203580, 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1*H*-imidazole; AC/EBP, C/EBP-specific dominant-negative; RT, reverse transcription; PCR, polymerase chain reaction; PI, propidium iodide; MEM, minimal essential medium; ODN, oligodeoxynucleotide; JNK1(−), JNK1 dominant-negative mutant; MKK1(−), MKK1 dominant-negative mutant; SP-1, specific protein-1.

amide induced COX-2 in mammary epithelial cells (Subbaramaiah et al., 1998). The signaling pathway of TNF-α–induced COX-2 expression was mediated with the formation of ceramide and sequential activation of extracellular signal-regulated kinase 1/2 (ERK1/2), p38 kinase, c-Jun N-terminal kinase (JNK), I-κB kinase complex 1/2, and nuclear factor-κB (NF-κB) in the COX-2 promoter (Chen et al., 2001). Also, ceramide mediates age-associated increase in COX-2 expression (Claycombe et al., 2002). The ceramide-induced up-regulation in prostaglandin E_2 production was mediated through transcriptional up-regulation of COX-2. By contrast, a study from another laboratory showed that C2-ceramide (C2), a cell-permeable ceramide analog, inhibited LPS-elicited COX-2 induction and prostaglandin E_2 formation with the inhibition of NF-κB and activator protein-1 (AP-1) activation (Hsu et al., 2001). They proposed the hypothesis that the inhibition of COX-2 induction by C2 resulted from the inhibition of LPS-stimulated I-κB kinase, p38 kinase, and protein kinase C. Given these controversial reports on the role of ceramide in the induction of COX-2, we were tempted to study the effect of C2 on LPS-inducible expression of COX-2 and to explore the cell signaling.

Studies have shown that CCAAT/enhancer binding protein (C/EBP), cyclic-AMP response element-binding protein (CREB) and NF-κB were commonly or individually involved in the regulation of the *COX-2* gene. Evidence has accumulated to show that the C/EBP element plays an important role in the induction of COX-2. In particular, activation of C/EBPβ leads to the induction of COX-2 (Thomas et al., 2000; Wadleigh et al., 2000). The regulatory region for the *COX-2* gene includes cAMP response element/E-box elements. NF-κB, which is activated by the inflammatory responses during viral and bacterial infections (Grilli and Memo, 1999; Kim et al., 2000), is involved in the expression of inflammatory genes (e.g., TNF-α) (Muller et al., 1993). Nevertheless, the role of NF-κB in COX-2 induction is controversial. In the present study, we examined alterations in the activation of these transcription factors by C2 in LPS-treated macrophages.

Nuclear translocation of C/EBP and C/EBP DNA binding were monitored by immunoblotting, immunocytochemical, and gel mobility shift assays in association with the induction of COX-2 by LPS + C2. Now, we report that C2 potentiates LPS-inducible COX-2 expression in macrophages as a result of the increase in C/EBP activation. C2 increases the expression of C/EBPβ and induces nuclear translocation and DNA binding of C/EBPβ via the pathway involving JNK1, which leads to enhancement of LPS-stimulated COX-2 induction. Toward the end of this study, we studied the effect of C2 on AP-1 activation and the role of JNK pathway in the enhanced AP-1 activation, which we expect to contribute to the increase in C/EBPβ expression.

Materials and Methods

Materials. C2 was obtained from Merck Co. (Darmstadt, Germany). Anti–COX-2 antibody was obtained from Cayman (Ann Arbor, MI). Anti-C/EBP-α, -β, -δ, and -ϵ form antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Horseradish peroxidase- or fluorescein isothiocyanate-conjugated goat anti-rabbit IgGs were purchased from Zymed Laboratories Inc. (San Francisco, CA). PD98059 was obtained from Calbiochem (San Diego, CA). [γ-^{32}P]ATP (3000 mCi/mmol) was obtained from PerkinElmer Life Sciences (Boston, MA). The consensus oligonucleotides of C/EBP, CREB, NF-κB, AP-1, and random prime/5′-end labeling kits were supplied from Promega Corporation (Madison, WI). JNK1 dominant-negative mutant (KmJNK1) was kindly provided from Dr. N. Dhanasekaran (Fels Institute for Cancer Research and Molecular Biology, Department of Biochemistry, Temple University, Philadelphia, PA). MKK1 dominant-negative mutant was gifted from Dr. N.G. Ahn (Howard Hughes Medical Institute, University of Colorado, Boulder, CO). C/EBP-specific dominant-negative expression plasmid (AC/EBP) was a gift from Dr. C. Vinson (National Institutes of Health, Besthesda, MD) (Ahn et al., 1998). Most reagents for the molecular studies were obtained from Sigma Chemical (St. Louis, MO).

Cell Culture. Raw264.7 cells, a murine macrophage cell line (American Type Culture Collection, Manassas, VA), were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. Raw264.7 cells were plated at a density of 2 to 3 $\times$ 10^6/ml and preincubated for 24 h at 37°C. Cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2. For all experiments, cells were grown to 80 to 90% confluence and were subjected to no more than 20 cell passages. Cells were incubated with 0.1 μg/ml LPS (*Escherichia coli* 026:B6; Difco, Detroit, MI) for the time periods indicated under *Results* and in the figure legends. C2 (as dissolved in dimethyl sulfoxide) was simultaneously added with LPS to the incubation medium.

Immunoblot Analysis. The expression of COX-2 was immunochemically monitored in lysates of Raw264.7 cells using anti-mouse COX-2 antibody. The secondary antibodies were horseradish peroxidase-conjugated anti-rabbit antibodies. The band of COX-2 protein was developed using enhanced chemiluminescence immunoblot detection system according to the manufacturer's instructions (Amersham Biosciences, Buckinghamshire, UK). C/EBP-α, -β, -δ, and -ϵ forms in the nuclear or lysate fractions were immunoblotted with the respective form-specific antibodies.

Phosphorylated and unphosphorylated p38 kinase, JNK, and ERK were immunochemically assessed in cell lysates using the specific antibodies (Cho et al., 2002) and developed using the ECL chemiluminescence system (Amersham Biosciences).

RT-PCR Analysis. Total RNA (2 μg) obtained from the cells was reverse-transcribed using an oligo(dT) adaptor as a primer to produce cDNAs. The specific cDNA probe for the *COX-2* gene was amplified by reverse transcription (RT)-polymerase chain reaction (PCR) using the selective primers and cloned in a TA vector (Promega). The primers used were COX-2: sense primer, 5′-TACAAGCAGTGGCAAAGGC-3′; antisense primer, 5′-CAGTATTGAGGAGAACAGATGGG-3′ (287 bp). Expression of *glyceraldehyde-3-phosphate dehydrogenase* gene was assessed by PCR using the sense primer 5′-TCGTGGAGTCTACTGGCGT-3′ and the antisense primer 5′-GCCTGCTTCACCACCTTCT-3′ (510 bp). PCRs were conducted using the following conditions for 38 cycles: denaturation at 94°C for 0.5 min, annealing at 49°C for 1 min, and elongation at 68°C for 1.5 min. Band intensities of the amplified DNAs were compared after visualization on an UV transilluminator.

Preparation of Nuclear Extracts. Nuclear extracts were prepared essentially according to the previously published method (Schreiber et al., 1990). Briefly, the cells in dishes were washed with ice-cold PBS. Cells were then scraped, transferred to microtubes, and allowed to swell after the addition of 100 μl of hypotonic buffer containing 10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.5% Nonidet P-40, 1 mM dithiothreitol, and 0.5 mM phenylmethylsulfonyl fluoride. The lysates were incubated for 10 min in ice and centrifuged at 7,600*g* for 6 min at 4°C. Pellets containing crude nuclei were resuspended in 50 μl of extraction buffer containing 20 mM HEPES, pH 7.9, 400 mM NaCl, 1 mM EDTA, 10 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride and then incubated for 1 h in ice. The samples were centrifuged at 15,000*g* for 10 min to obtain supernatants containing nuclear fractions. Nuclear fractions were stored at −70°C until use.

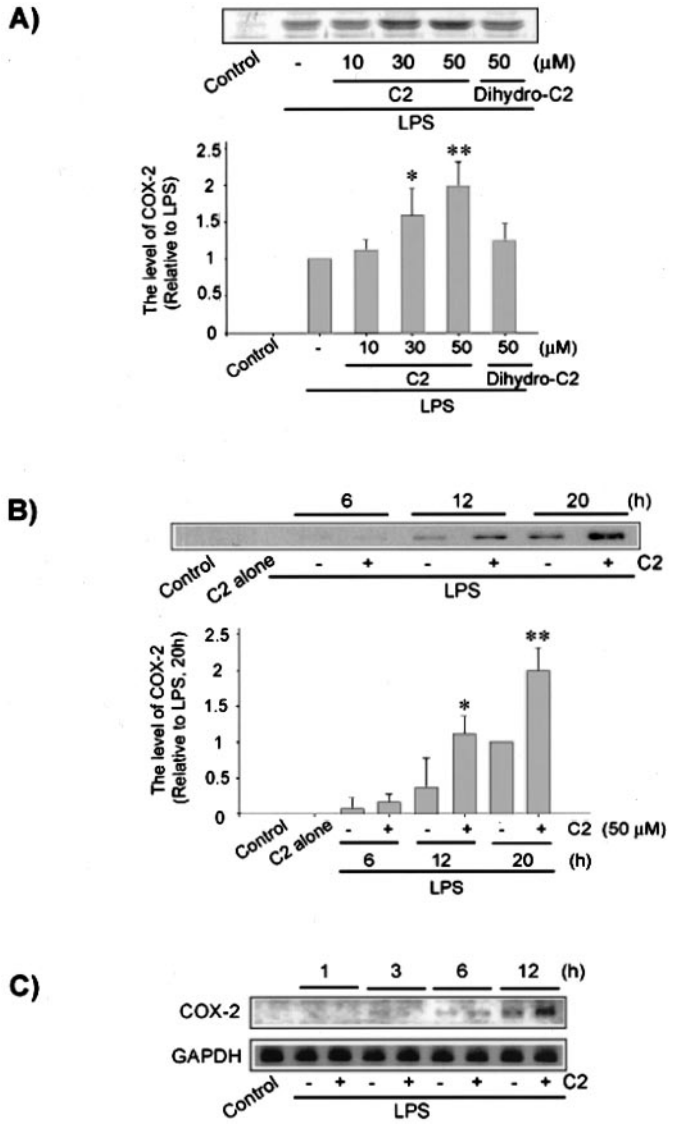

Fig. 1. The effect of C2 on LPS-inducible COX-2 expression. A, enhancement of COX-2 induction by C2 in Raw264.7 cells. Cells were treated with varying concentrations of C2 dissolved in dimethyl sulfoxide for 20 h in combination with LPS (0.1 μg/ml). Control cells were incubated with vehicle alone. Dihydro-C2 was used for comparison. The level of COX-2 was monitored as described under *Materials and Methods*. Data represent the mean ± S.D. with at least three separate experiments (*, $p < 0.05$; **, $p < 0.01$, significant compared with LPS alone) (LPS alone = 1). B, time course of COX-2 expression in cells treated with LPS in the presence or absence of C2 (50 μM). The relative COX-2 protein levels were measured by scanning densitometry of the immunoblot bands. Data represent the mean ± S.D. with at least three separate experiments *, $p < 0.05$; **, $p < 0.01$, significant compared with LPS alone at 20 h (i.e., 1). C, the effects of C2 on the COX-2 mRNA level. Raw264.7 cells were exposed to LPS (0.1 μg/ml) with or without C2 (50 μM). RT-PCR analysis was performed to determine COX-2 mRNA in total RNA fractions (2 μg each) isolated from cells treated with LPS or LPS + C2 for 1 to 12 h. Equal RNA loading was confirmed by RT-PCR of glyceraldehyde-3-phosphate dehydrogenase mRNA.

Gel Retardation Assay. Double stranded DNA probes for the consensus sequences of C/EBP (5′-TGCAGATTGCGCAATCTGCA-3′), CREB (5′-AGAGATTGCCTGACGTCAGAGAGCTAG-3′), NF-κB (5′-AGTTGAGGGGACTTTCCCAGGC-3′), and AP-1 (5′-CGCTTGATGAGTCAGCCGGAA-3′) were used for gel-shift analyses after end-labeling of each probe with [γ-^{32}P]ATP and T_4 polynucleotide kinase. Nuclear extracts were prepared by modification of the procedure published previously (Schreiber et al., 1990). The reaction mixtures contained 2 μl of 5× binding buffer containing 20% glycerol, 5 mM $MgCl_2$, 250 mM NaCl, 2.5 mM EDTA, 2.5 mM dithiothreitol, 0.25 mg/ml poly(dI-dC), and 50 mM Tris-Cl, pH 7.5, 5 μg of nuclear extracts, and sterile water in a total volume of 10 μl. Reactions were initiated by addition of 1 μl probe (10^6 cpm) after 10 min of preincubation and continued for 30 min at room temperature. The specificity of the DNA/protein binding was confirmed by competition reactions, in which a 20-fold molar excess of unlabeled C/EBP, CREB, NF-κB, or AP-1 oligonucleotide was added to each reaction mixture before the addition of radiolabeled probe. For supershift assay, the antibodies (2 μg each) were added to the reaction mixture and additionally incubated for 1 h at 25°C. Samples were loaded onto 4% polyacrylamide gels at 100 V. The gels were removed, fixed, and dried, followed by autoradiography.

Immunocytochemistry. Raw264.7 cells were grown on Lab-TEK chamber slides (Nalge Nunc International, Naperville, IL) and incubated in medium for 24 h. Standard immunocytochemical methods were used as described previously by Cho et al. (2002). For immunostaining, cells were fixed in 100% methanol for 30 min and washed three times with PBS. After blocking in 5% bovine serum albumin in PBS for 1 h at room temperature or overnight at 4°C, cells were incubated for 2 h with polyclonal rabbit anti-C/EBPβ antibody in PBS containing 1% bovine serum albumin. Cells were incubated with 1:100 dilution of fluorescein isothiocyanate-conjugated goat anti-rabbit IgG after serial washing with PBS. Counter-staining with propidium iodide (PI) verified the location and integrity of nuclei. Stained cells were washed and examined using a laser scanning confocal microscope (Leica TCS NT; Leica Microsystems, Wetzlar, Germany).

Transient Transfection with Dominant-Negative Mutant of C/EBP. Cells were plated at a density of 0.5×10^6 cells/well in six-well dishes and transfected the following day. Briefly, cells were incubated with 1 μg of C/EBP dominant-negative mutant (A/CEBP) plasmid or pCMV500 plasmid (an empty vector that was used as a control) DNA and 3 μl of LipofectAMINE reagent (Invitrogen, Carlsbad, CA) in 1 ml of antibiotic-free minimal essential medium (MEM) for 3 h. The cells were incubated in Dulbecco's modified Eagles medium for 3 h and then exposed to LPS or LPS + C2 for 20 h.

Decoy Oligodeoxynucleotide Technique. Double-stranded oligodeoxynucleotide (ODN) was prepared from complementary single-stranded phosphorothioate-bonded ODN (Bioneer, Chungbuk, Korea) by melting at 95°C for 5 min followed by a cool-down phase at ambient temperature (Cho et al., 2002). The sequences of the single-stranded ODN were as follows. Underlined letters denote phosphorothioate-bonded bases: AP-1, 5′-*CGCT*TGATGACTCAGCC*GGAA*-3′ and mutant AP-1 (mAP-1), 5′-*CGCT*TGATTACTTAGCC*GGAA*-3′ (Cho et al., 2002). Cells were preincubated with 10 μM decoy ODN for 1 h and then further incubated with LPS (0.1 μg/ml) or LPS + C2 (50 μM) for 12 h. Transfection of the decoy ODN was achieved without using a cationic lipid or liposomal complex.

Stable Plasmid Transfection. Cells were transfected using Transfectam according to the manufacturer's instructions (Promega). Cells were replated 24 h before transfection at a density of 2×10^6 cells in a 10-cm^2 plastic dish. For use in JNK1 dominant-negative mutant [JNK1(−)] or MKK1 dominant-negative mutant [MKK1(−)] stable transfection, 20 μl of Transfectam was mixed with 10 μg of a JNK1(−) or MKK1(−) plasmid in 2.5 ml of MEM. Cells were transfected by addition of MEM containing each plasmid and Transfectam and then incubated at 37°C in a humidified atmosphere of 5% CO_2 for 6 h. After addition of 6.25 ml MEM with 10% fetal bovine serum, cells were incubated for additional 48 h at 37°C and 50 μg/ml of G-418 was added to select the resistant colonies.

Scanning Densitometry. Scanning densitometry of the immunoblots was performed with Image Scan and Analysis System (α-Innotech Corporation, San Leandro, CA). The area of each lane was integrated using the software AlphaEase version 5.5, followed by background subtraction. One-way analysis of variance procedures were used to assess significant differences among treatment groups.

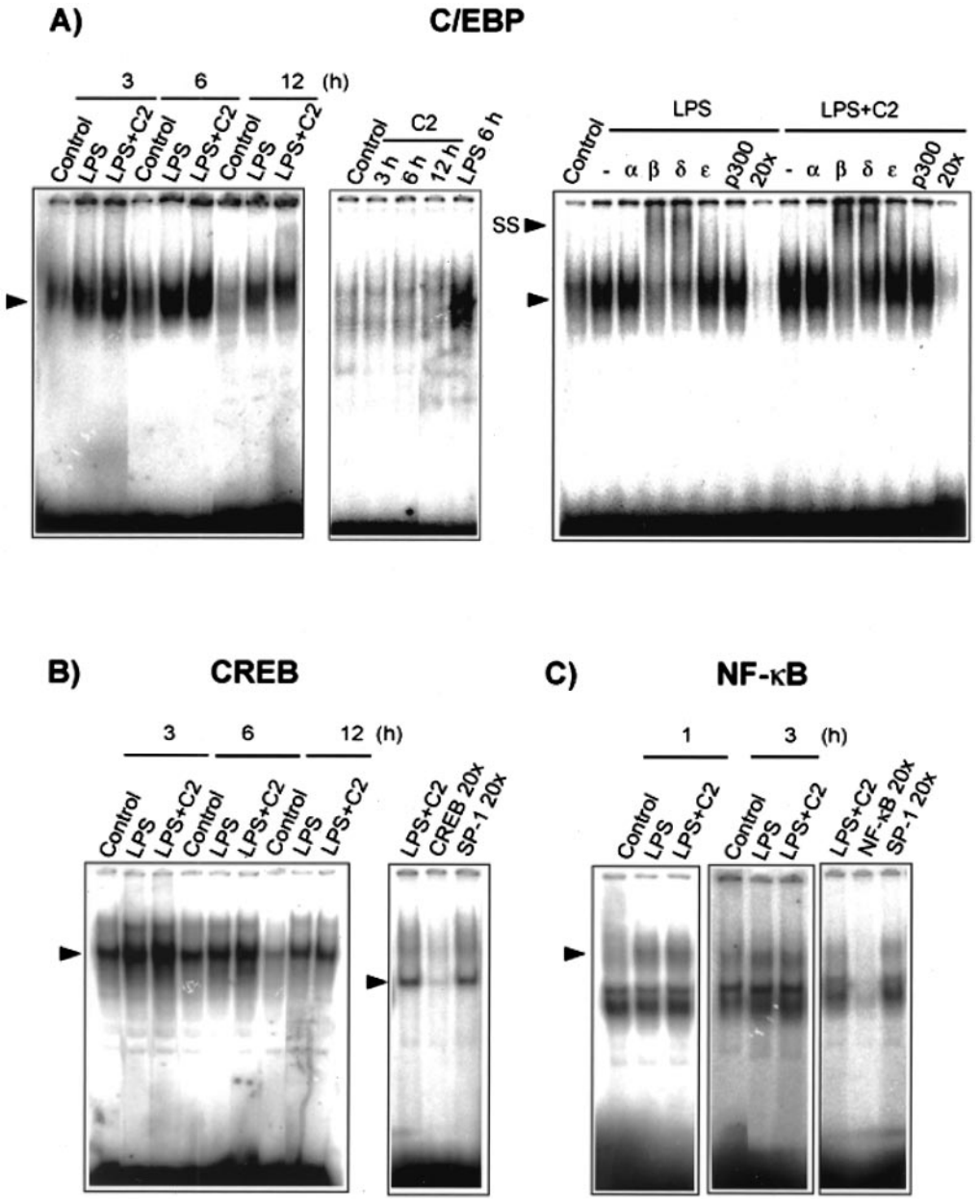

Fig. 2. Gel-shift analyses of C/EBP, CREB, and NF-κB transcription complexes. Nuclear extracts were prepared from Raw264.7 cells cultured with LPS (0.1 μg/ml) or LPS + C2 (50 μM). A, gel-shift analyses of C/EBP. All lanes contained 5 μg of nuclear extracts and 5 ng of labeled C/EBP consensus oligonucleotide. Supershift analyses were carried out by incubating the nuclear extracts (LPS or LPS + C2, 6 h) with the specific polyclonal antibodies directed against C/EBP forms (α, β, δ, and ε) and CBP/p300 (p300) for 1 h. SS, supershift of the retarded C/EBP complex. Competition studies were carried out by adding a 20-fold excess of an unlabeled C/EBP oligonucleotide (20×) to the nuclear extracts from cells treated with LPS or LPS + C2, and the DNA-binding reactions were performed by gel-shift analysis. B, gel-shift analysis of CREB. Gel-shift analysis was performed as described in A. C, gel-shift analysis of nuclear extracts using the consensus oligonucleotide of NF-κB. The specificity of CREB or NF-κB binding was confirmed by addition of an excess amount of free probe (20 ×). Results were confirmed by repeated experiments.

For each significant effect of treatment, the Newman-Keuls test was used for comparisons of multiple group means. The criterion for statistical significance was set at $p < 0.05$ or 0.01.

Results

C2 Potentiation of COX-2 Induction by LPS. First, we determined whether C2 altered the level of COX-2 protein in macrophages incubated with LPS. Whereas COX-2 was not detected in control cells, LPS (0.1 μg/ml, 20 h) notably increased the COX-2 protein level. The induction of COX-2 by LPS was enhanced by C2 at the concentrations of 30 to 50 μM (20 h), whereas dihydro-C2, an inactive analog of C2, had no effect (Fig. 1A). Cell viability was not affected by C2 at the concentrations employed. The expression of COX-2 was assessed at 50 μM of C2 in subsequent experiments. To determine the time points for C2 enhancement of COX-2 induction by LPS, cells were treated with LPS (0.1 μg/ml) in the presence or absence of C2 for 6, 12, or 20 h (Fig. 1B). C2 enhanced COX-2 induction by LPS at 12 or 20 h. C2 alone did not induce COX-2 in macrophages at 20 h, which was in agreement with the previous report (Hsu et al., 2001). Studies were extended to determine whether the expression of COX-2 protein paralleled that of its mRNA. LPS increased the COX-2 mRNA at 6 to 12 h. RT-PCR analysis showed that C2 notably enhanced LPS-inducible increase in the COX-2 mRNA at 12 h (Fig. 1C).

Effect of C2 on LPS-Inducible C/EBP, CREB, and NF-κB Activation. Expression of the *COX-2* gene depends on the C/EBP element present in the upstream region of the gene (Thomas et al., 2000). To test whether C2 potentiation of the *COX-2* gene induction was mediated by C/EBP activation, electrophoretic mobility shift for C/EBP binding activity was performed with the nuclear extracts of cells exposed to LPS in the presence or absence of C2 using a radiolabeled C/EBP consensus oligonucleotide. Treatment of cells with LPS for 3 to 12 h resulted in an increase in C/EBP DNA

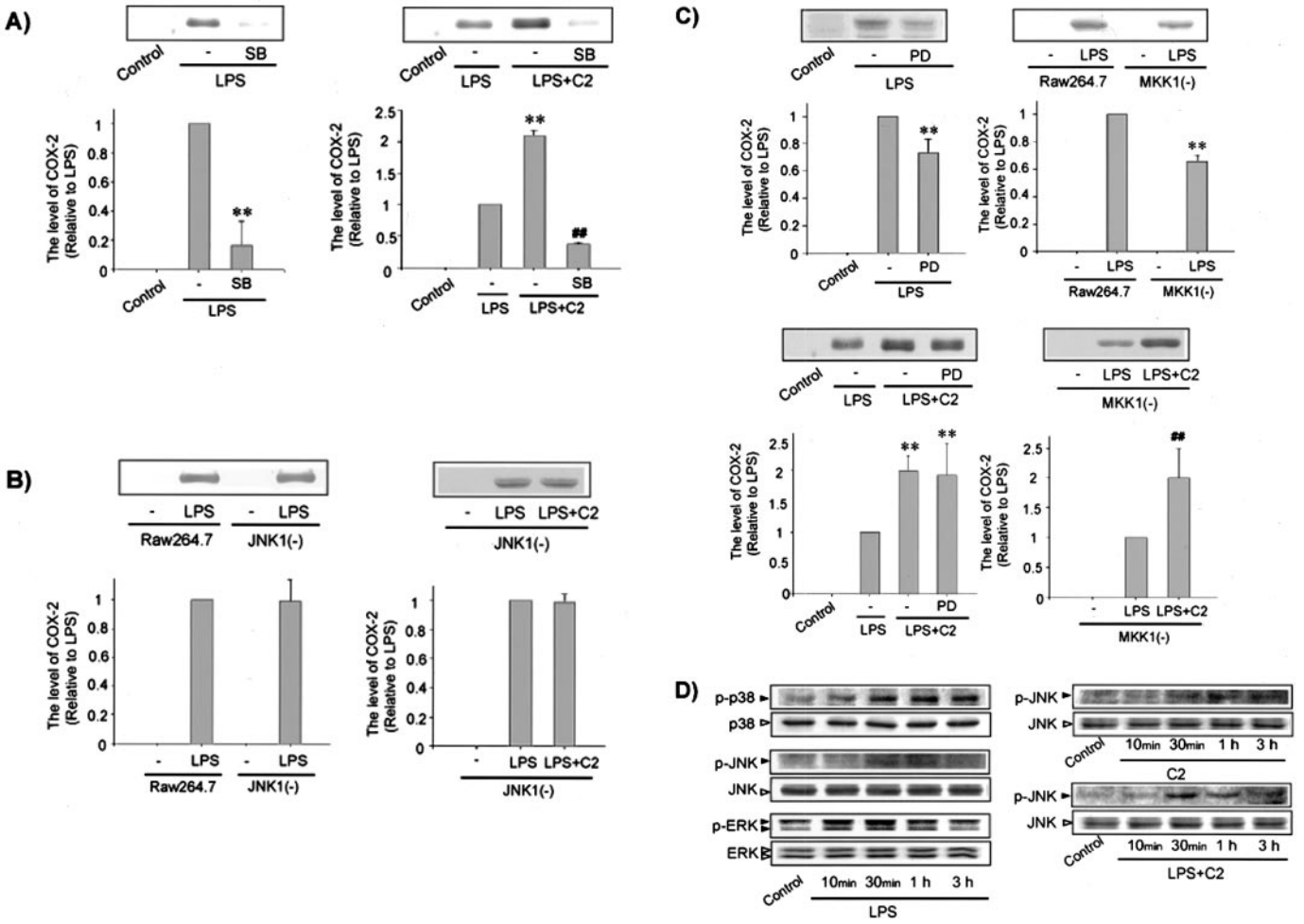

Fig. 3. The role of MAP kinases in C2-enhanced LPS induction of COX-2. A, the role of p38 kinase pathway. Cells were treated with SB203580 (SB, 10 μM) for 1 h and further incubated with LPS (0.1 μg/ml) or LPS + C2 (50 μM) for 20 h. Data represent the mean ± S.D. with at least three separate experiments (**, $p < 0.01$, significant compared with LPS alone; ##, $p < 0.01$, significant compared with LPS + C2). B, the role of JNK pathway. Raw264.7 cells or cells stably expressing JNK1(−) were used to assess LPS- or LPS + C2-inducible COX-2. C, the role of MKK1/ERK1/2 pathway. Cells were treated with PD98059 (PD, 30 μM) and subsequently exposed to LPS or LPS + C2. Cells stably expressing MKK1(−) were used to assess the role of MKK1 in C2 enhancement of LPS-inducible COX-2 expression. Data represent the mean ± S.D. of at least three separate experiments (**, $p < 0.01$, significant compared with LPS alone in Raw264.7 cells; ##, $p < 0.01$, significant compared with LPS alone in MKK1(−) cells). D, MAP kinase activation. Phosphorylation of p38 kinase (p38), JNK, or ERK in cell lysates was assessed by immunoblotting with the specific antibodies. Solid and open arrowheads indicate phosphorylated and unphosphorylated forms of MAP kinases, respectively. Results were confirmed by repeated experiments.

binding compared with control, whereas concomitant treatment of cells with C2 notably enhanced LPS-inducible C/EBP binding (Fig. 2A, left). The band intensity of C/EBP DNA binding maximally increased at 6 h and returned toward that of LPS alone at 12 h. C2 alone (3–12 h) had no effect or minimally increased C/EBP binding to the C/EBP binding site (Fig. 2A, middle).

To identify the factor(s) that makes up the inducible C/EBP activity, highly specific antibodies directed against individual C/EBP proteins were evaluated for the ability to inhibit the DNA binding activity. Competition experiments indicated that C/EBP DNA binding activity in cells treated with either LPS or LPS + C2 depended specifically on C/EBPβ and C/EBPδ but not C/EBPϵ or CBP/p300 (Fig. 2A, right). In cells treated with LPS + C2, anti-C/EBPβ antibody immunochemically competed with the band of C/EBP DNA binding, inducing supershift with complete reduction in the band shift. However, anti-C/EBPδ antibody partially reduced the band intensity with weak supershift. Addition of a 20-fold excess of unlabeled C/EBP to the nuclear extract abolished the C/EBP binding complex (Fig. 2A, right). These data indicated that C/EBPβ contributed to the enhanced C/EBP DNA binding in cells treated with LPS + C2.

It has been shown that mutation of the cAMP-response element site in the *COX-2* gene abrogated COX-2 reporter activity and that the expression of CREB repressed LPS-dependent COX-2 reporter activity, presumably through cAMP response element site(s) (Wadleigh et al., 2000). We monitored the effect of C2 on LPS-inducible CREB binding activity. In cells exposed to LPS for 3 to 12 h, C2 (50 μM) enhanced CREB DNA binding, most notably at 3 h (Fig. 2B, left). Addition of a 20-fold excess of unlabeled CREB, but not specific protein-1 (SP-1), abolished the CREB binding complex (Fig. 2B, right).

NF-κB is activated in cells challenged with LPS and other inflammatory insults and is involved in the transcriptional activation of the responsive genes (Baldwin, 1996). Previous studies have shown that NF-κB was activated at early times

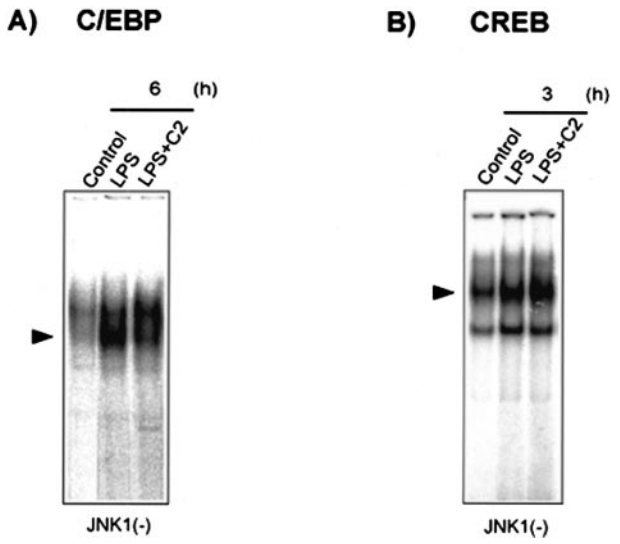

Fig. 4. Gel-shift analyses of C/EBP and CREB in JNK1(−) cells. Raw264.7 cells stably transfected with JNK1(−) were cultured with LPS (0.1 μg/ml) or LPS + C2 (50 μM). Nuclear extracts prepared at the indicated times were subjected to gel-shift analysis of C/EBP (A) or CREB (B). All lanes contained 5 μg of nuclear extracts and 5 ng of labeled C/EBP or CREB consensus oligonucleotide. Arrowhead indicates C/EBP or CREB DNA binding complex.

after LPS treatment (Kim et al., 2000). Gel-shift analysis was conducted to determine whether C2 affected NF-κB DNA binding activity in macrophages. LPS (0.1 μg/ml, 1–3 h) increased the binding activity of nuclear extracts to the NF-κB DNA consensus sequence, whereas C2 did not change LPS-inducible increase in the band intensity of NF-κB complex (Fig. 2C). Addition of excess unlabeled NF-κB, but not SP-1, abolished the NF-κB DNA binding complex (Fig. 2C).

Cell Signaling for C2 Enhancement of COX-2 Induction. Major signaling pathways for the induction of COX-2 transmit through MAP kinase pathways (Paul et al., 1999; Wadleigh et al., 2000). The role of the MAP kinase signaling pathways in the potentiation of COX-2 induction by C2 was investigated using specific inhibitors and/or stable transfection with dominant-negative mutant vectors. First, the pathway responsible for the induction of COX-2 by LPS was assessed. It has been shown that the p38 kinase pathway was involved in COX-2 induction (Chen et al., 1999). SB203580 (10 μM) completely inhibited the enzyme induction by LPS. In cells treated with LPS + C2, the enhanced COX-2 induction was also completely blocked by treatment with SB203580 (Fig. 3A). Cells transfected with JNK1(−) were used to test whether blockade of JNK cascade led to change in the COX-2 induction by LPS or LPS + C2. The stable JNK1(−) transfection experiment revealed that the JNK1 pathway was responsible for C2-potentiated induction of COX-2 but not the induction of COX-2 by LPS alone (Fig. 3B). PD98059, a chemical inhibitor of MKK1, decreased COX-2 induction by LPS (Fig. 3C, top). COX-2 expression was also decreased in cells stably transfected with MKK1(−), which was consistent with the result of chemical inhibition. Interestingly, however, C2-potentiated COX-2 induction by LPS was not affected by PD90859 or stable transfection with MKK1(−) (Fig. 3C, bottom). These data provided evidence that p38 kinase was required for the inducible and potentiated COX-2 expression, whereas JNK1, but not ERK1/2, played a role in the enhanced COX-2 induction by C2.

We next confirmed the activation of MAP kinases by LPS (Fig. 3D, left). The level of phosphorylated p38 kinase was enhanced in cells stimulated by LPS from 30 min through 3 h. JNK was also phosphorylated in cells treated with LPS. JNK activation was increased at 30 min to 1 h. The level of phosphorylated ERK1/2 was also increased 15 to 30 min after LPS treatment. Given the role of JNK in the enhanced induction of COX-2 by C2 in LPS-treated cells, we measured the extents of JNK phosphorylation as a function of time in cells treated with C2 or LPS + C2 (Fig. 3D, right). Either C2 or LPS + C2 was capable of increasing JNK phosphorylation.

Effects of C2 on DNA Binding Transcription Factors in JNK1(−) Cells. Given the importance of the JNK pathway for C2-potentiated COX-2 induction, studies were extended to determine whether C2 changed activation of the transcription factors C/EBP, CREB, and NF-κB in JNK1(−) cells. Gel-shift retardation analysis revealed that LPS-inducible band intensity of C/EBP transcription complex was not further increased by C2 in JNK1(−) cells (6 h) (Fig. 4A). By contrast, LPS-inducible CREB transcription complex (3 h) was potentiated by C2 in JNK1(−) cells (Fig. 4B). In JNK1(−) cells, C2 did not change LPS-inducible increase in NF-κB DNA binding (data not shown). These data indicate that the JNK1 pathway controlled C2 enhancement in C/EBP DNA binding but not that of CREB or NF-κB.

Changes in the Nuclear C/EBPβ and δ Levels by LPS + C2. In view of the importance of C/EBPs as the transcriptional factors responsible for COX-2 induction, we sought to determine the levels of nuclear C/EBPs in macrophages exposed to LPS with or without C2. LPS increased nuclear C/EBPβ isoforms (38, 35, and 19 kDa). In cells treated with LPS + C2, the level of nuclear 38-kDa C/EBPβ was increased to a greater extent (Fig. 5A). Conversely, the C/EBPδ isoform, which was increased by LPS at 3 to 12 h (a maximal increase at 6 h), was rather suppressed by concomitant C2 treatment compared with that of LPS alone at the respective time point. The levels of nuclear C/EBPα and -ϵ forms were not changed (Fig. 5A).

Because C/EBPβ was the component of C/EBP DNA binding complex in cells treated with LPS + C2, we determined translocation of C/EBPβ into the nucleus by immunocytochemistry (Fig. 5B, left). Raw264.7 cells were incubated with LPS in the presence or absence of 50 μM C2 for 3 to 12 h, fixed, and permeabilized. C/EBPβ protein was located predominantly in the cytoplasm of control cells. C/EBPβ protein began to move into the nucleus 3 h after LPS treatment. C/EBPβ nuclear translocation was accelerated in cells exposed to LPS + C2 (3 h) (Fig. 5B, left). The intensities of nuclear C/EBPβ in cells exposed to LPS + C2 could not be immunocytochemically differentiated from those in LPS-treated cells at 6 to 12 h because of intense staining of nuclear C/EBPβ. Cellular localization of C/EBPα, which was assessed as a control, was not affected by LPS or LPS + C2 (Fig. 5B, right). This was in agreement with the result of the immunoblot analysis. Nuclear integrity was confirmed by PI staining of the identical cells.

To determine whether the expression of C/EBPβ was increased by LPS + C2, the expression of C/EBPβ was determined in cell lysates. Immunoblot analysis revealed that the level of C/EBPβ was increased by LPS + C2, compared with LPS alone (Fig. 5C), indicating that C2 further stimulates the expression of C/EBPβ in cells treated with LPS.

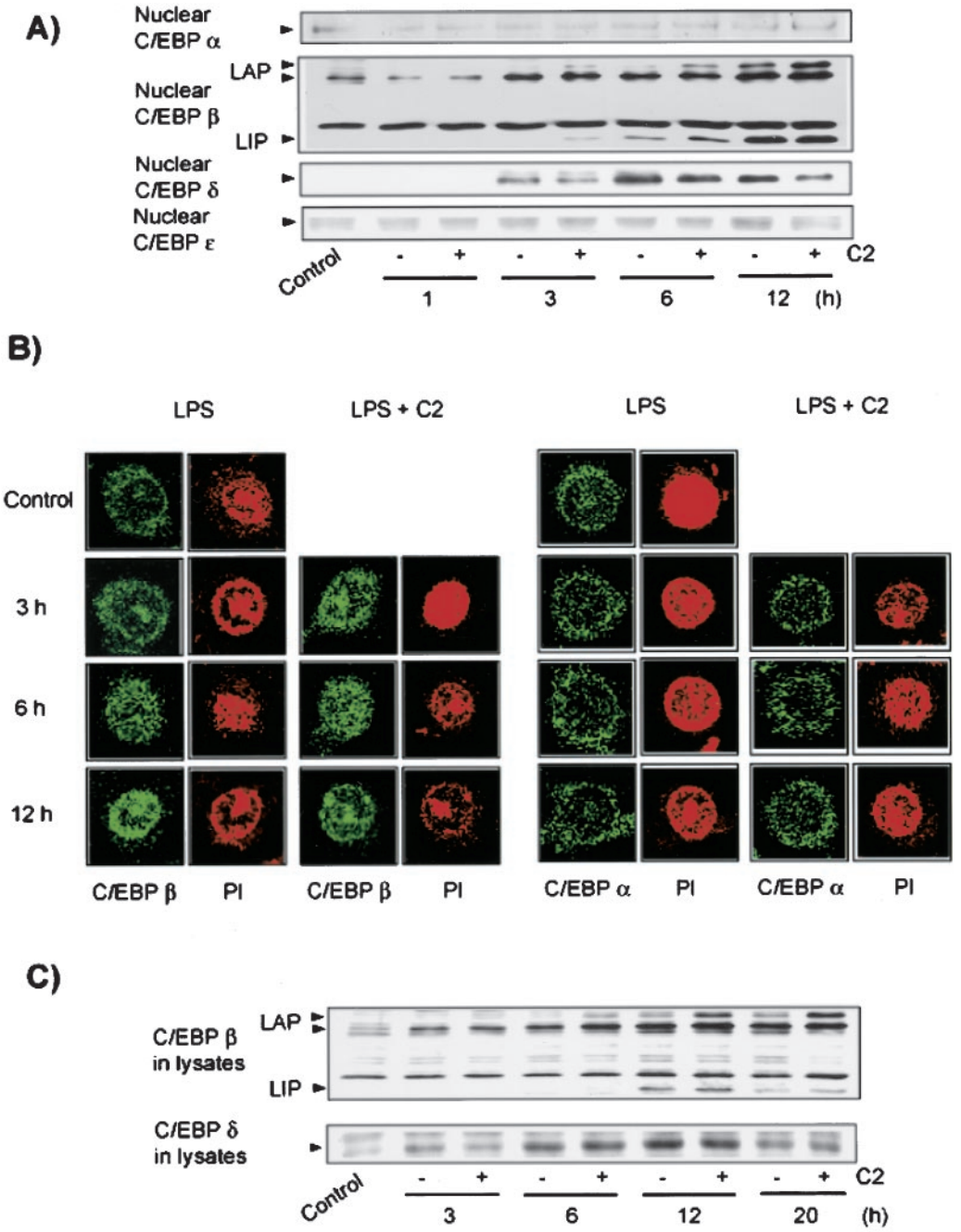

Fig. 5. The effects of C2 on the activation and expression of C/EBPs. A, Western blot analyses of nuclear C/EBP isoforms in Raw264.7 cells. The levels of C/EBPα, C/EBPβ (LAP and LIP), C/EBPδ, and C/EBPϵ were determined in the nuclear fractions of cells treated with LPS (0.1 μg/ml) or LPS + C2 (50 μM) for 1 to 12 h. B, immunocytochemistry of C/EBPβ or C/EBPα in cells treated with LPS or LPS + C2. Distribution of C/EBPβ or C/EBPα was also monitored in cells incubated with vehicle (dimethyl sulfoxide) or 50 μM C2 for 3 to 12 h. The same fields were counter-stained with PI. C, expression of C/EBPβ and C/EBPδ. The levels of C/EBPβ [LAP (liver activating protein) and LIP (liver inhibitory protein)] and C/EBPδ isoforms were determined in the lysates of cells treated with LPS (0.1 μg/ml) or LPS + C2 (50 μM) for 3 to 20 h. Each lane was loaded with 30 μg of cell lysates. The results in A, B, and C were confirmed by repeated experiments.

Abrogation of C2-Mediated C/EBPβ Activation by JNK1(−) Transfection. Next, we determined whether JNK1(−) transfection abolished C2-enhanced nuclear translocation of C/EBPβ. Immunoblot analysis showed that C2 (6–12 h) failed to increase LPS-inducible nuclear translocation of 38-kDa C/EBPβ in JNK1(−) cells (Fig. 6A). The decrease in nuclear translocation of C/EBPδ isoform by C2 was not affected by JNK1(−) transfection. Immunocytochemistry showed that LPS induced nuclear translocation of C/EBPβ in JNK1(−) cells that was slower, however, than in control cells (Fig. 6B). Consistent with the result of immunoblot analysis, C2 did not increase LPS-inducible nuclear translocation of C/EBPβ in JNK1(−) cells. These data provide evidence that the JNK1 pathway controls C2-mediated activation of C/EBPβ in LPS-treated cells.

Inhibition of C2-Potentiated COX-2 Induction by AC/EBP. Given the activation of C/EBPβ by LPS + C2, we assessed the role of C/EBP in the *COX-2* gene expression. Constitutively active AC/EBP was expressed in cells before treatment with LPS + C2. Expression of AC/EBP completely inhibited the ability of C2 to enhance LPS induction of COX-2 (Fig. 7, top). Transfection with pCMV500, a control vector, allowed C2 to potentiate the induction of COX2 by LPS. Overexpression of AC/EBP reduced the extent of COX-2 induction in cells exposed to LPS alone (Fig. 7, bottom), confirming the fact that C/EBP was involved in LPS-inducible COX-2 expression.

Effect of C2 on LPS-Inducible AP-1 Activation. AP-1 is a heterodimer of Fos and Jun and is activated in cells challenged with LPS and other inflammatory insults

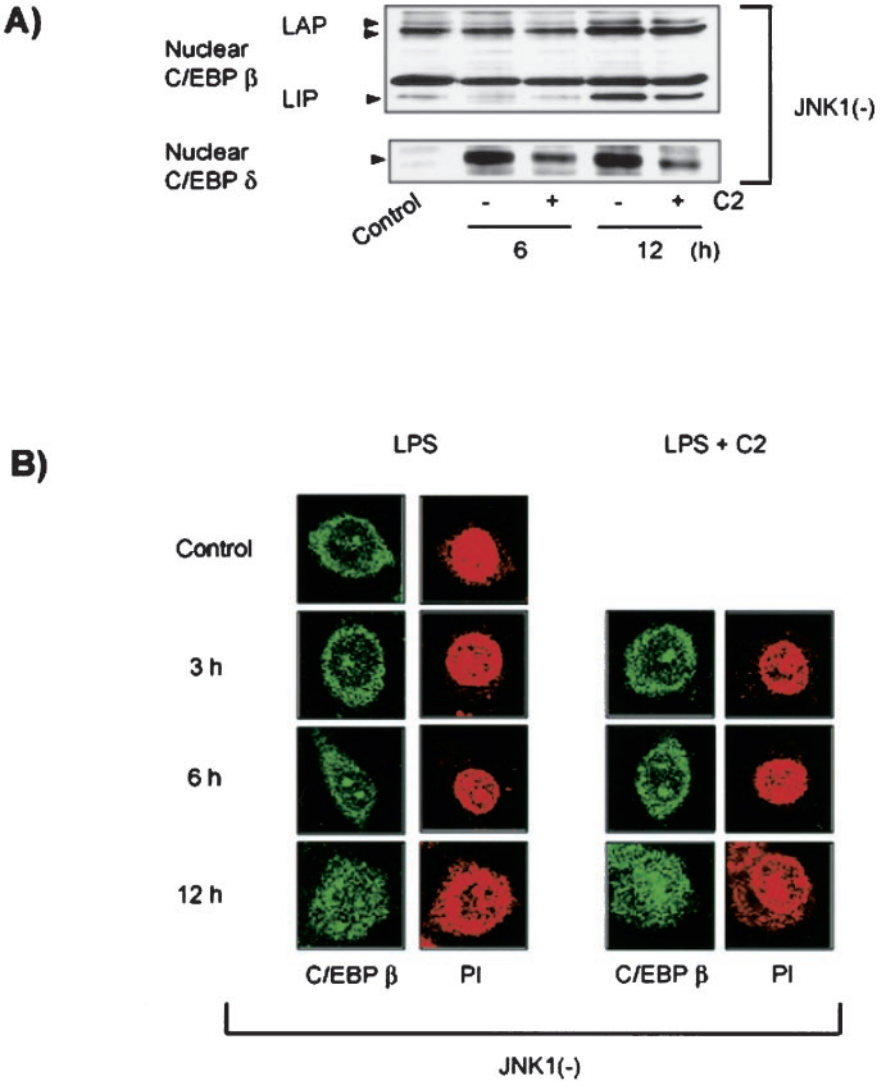

Fig. 6. The effects of C2 on the levels of nuclear C/EBPβ [LAP (liver activating protein) and LIP (liver inhibitory protein)] and C/EBPδ in JNK1(−) cells. A, the levels of nuclear C/EBP isoforms in JNK1(−) cells. Western blot analyses were performed with the nuclear fractions prepared from JNK1(−) cells treated with LPS (0.1 μg/ml) or LPS + C2 (50 μM) for 6 or 12 h. B, immunocytochemistry of C/EBPβ in JNK1(−) cells treated with LPS or LPS + C2. Nuclear translocation of cytosolic C/EBPβ was monitored in JNK1(−) cells, as described in Fig. 5B. The results were confirmed by repeated experiments. The same fields were counter-stained with PI.

(Tengku-Muhammad et al., 2000). The JNK pathway regulates AP-1 activation (Karin, 1995). The promoter region of the *C/EBPβ* gene contains the putative binding sites for the transcription factors AP-1, C/EBP, and CREB (Foka et al., 2001). As part of the efforts to determine how the JNK pathway controls C2-mediated enhanced activation of C/EBPβ in LPS-treated cells, we next assessed AP-1 activation. Gel-shift analyses revealed that LPS or C2 alone increased AP-1 DNA binding activity (6–12 h) (Fig. 8A). AP-1 was activated to a greater extent in cells exposed to LPS + C2 compared with LPS or C2 alone. Addition of a 20-fold excess of an unlabeled AP-1 binding oligonucleotide to the nuclear extract completely abolished the binding activity, whereas excess unlabeled SP-1 oligonucleotide failed to inhibit binding, suggesting that the binding protein is AP-1. We next monitored whether C2 activated AP-1 in JNK1(−) cells. AP-1 DNA binding activity was not potentiated by C2 in LPS-treated JNK1(−) cells (Fig. 8B). Hence, AP-1 activation was controlled by JNK1.

To determine whether increase in C/EBPβ expression by C2 resulted from the activation of AP-1, cells were preincubated with AP-1 specific decoy ODN for 1 h, and further incubated with LPS or LPS + C2 for 12 h. AP-1, but not mutant AP-1, decoy ODN reduced LPS + C2-inducible C/EBPβ expression (Fig. 8C). LPS-inducible C/EBPβ expression was also decreased by AP-1, compared with that by mutant AP-1. These data demonstrated that AP-1 was responsible for C/EBPβ expression.

C2 Potentiation of COX-2 Induction by TNF-α or Type I Collagen. Studies were extended to assess whether C2 increased the expression of COX-2 by other inducers including TNF-α and type I collagen. We found that type I collagen induces nitric-oxide synthase and COX-2 in macrophages (Cho et al., 2002; Y. H. Cho and S. G. Kim, unpublished observations). Western blot analysis revealed that the extent of COX-2 induction by TNF-α or type I collagen was potentiated by concomitant C2 treatment (Fig. 9). C2 potentiation of COX-2 induction was also abolished in JNK1(−) cells (data not shown).

Discussion

Macrophages secrete inflammatory mediators, including lipid metabolites (e.g., prostaglandins) and cytokines. COX-2 catalyzes the inducible production of prostaglandins, which represents an important step in the inflammatory process (Wadleigh et al., 2000). The production of prostaglandins by LPS in macrophages is primarily caused by the transcrip-

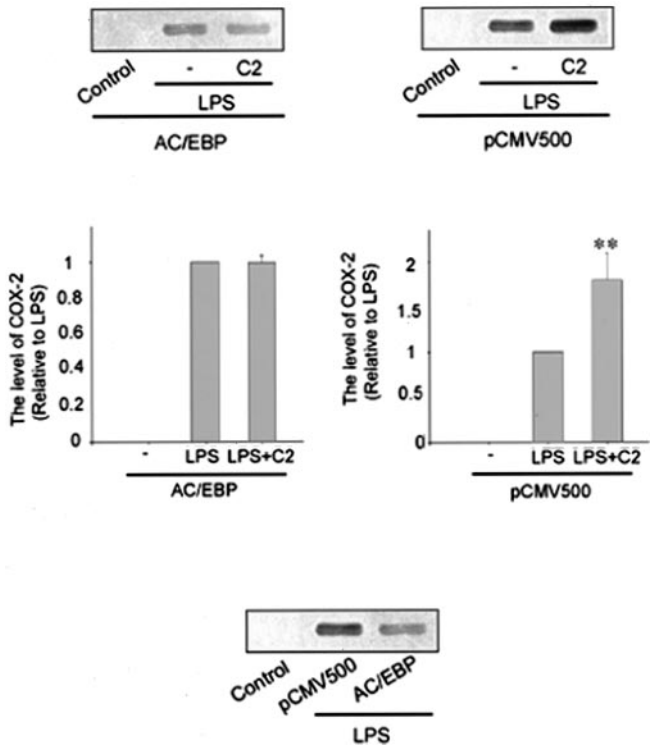

Fig. 7. Inhibition of LPS + C2-inducible COX-2 expression by AC/EBP. Raw264.7 cells transfected with the plasmid encoding for AC/EBP were exposed to LPS (0.1 μg/ml) or LPS + C2 (50 μM) for 20 h. Control cells were transfected with pCMV500. The relative COX-2 protein levels were measured by scanning densitometry of the immunoblots. Data represent the mean ± S.D. of at least three separate experiments [**, $p < 0.01$, significant compared with LPS alone (i.e., 1).

tional activation of the *COX-2* gene (Lee et al., 1992; Reddy and Herschman, 1994). The *cis*-acting elements identified on the promoter region of the murine *COX-2* gene include C/EBP, CREB, and NF-κB (Caivano and Cohen, 2000; Caivano et al., 2001). The complex consisting of C/EBPβ homodimers is involved in the activation of C/EBP response element in macrophages exposed to LPS (Granger et al., 2000). If C/EBPβ is inactivated, the expression of COX-2 by LPS is impaired (Gorgoni et al., 2001). Although the initial phase of COX-2 expression by LPS involved CREB (Caivano et al., 2001), the activation of CREB was not sufficient to *trans*-activate the gene (Caivano and Cohen, 2000). In contrast, Eliopoulos et al. (2002) recently showed that Tpl2-dependent CREB activation signals regulate the induction of COX-2 by LPS in macrophages and that p38 kinase and ERK contribute to cell signaling. Despite the presence of the NF-κB binding site in the regulatory region of the *COX-2* gene, the putative NF-κB was not required for the induction of COX-2 by LPS, as shown by dominant-negative inhibition of NF-κB and COX-2 reporter gene activity (Wadleigh et al., 2000).

Ceramide signaling has been linked to inflammation and tumorigenesis. Because C2 is a cell-permeable analog, the agent has been widely used to study the pathophysiological effects of ceramide. C2 induced COX-2 in human epithelial carcinoma cells (Chen et al., 2001). We observed that C2 enhanced the extent of COX-2 induction by LPS in macrophages, although C2 alone did not induce the enzyme. C/EBP, which was activated by LPS + C2, was apparently involved in the induction of COX-2, whereas CREB might indirectly affect the enzyme expression. The present study showed that the expression of C/EBPβ in total cell lysates was increased by LPS and to a greater extent by LPS + C2. The expression of C/EBPβ is regulated by CREB (Belmonte et al., 2001). The increase in CREB DNA binding by LPS + C2 at earlier times may stimulate the C/EBPβ expression. We found in additional that C2 potentiated LPS-inducible AP-1 DNA binding activity. Because the promoter region of C/EBPβ contains putative AP-1 binding sites, the enhanced AP-1 activation by C2 is highly likely to be responsible for the increase in C/EBPβ expression and thus for C/EBPβ-mediated induction of COX-2. This is supported by blockage of LPS + C2-inducible C/EBPβ expression in cells treated with AP-1 decoy ODN. In addition to the increase in the C/EBPβ expression, C2 elicited its nuclear translocation in LPS-treated cells. Competition experiments using the specific antibodies revealed that LPS induced C/EBP DNA binding activity and that the binding complex comprised C/EBPβ and δ forms. The nuclear translocation of C/EBPβ was increased by C2 in LPS-treated cells, whereas that of C/EBPδ was rather decreased. This raised the notion that C/EBPβ form plays an important role in the transcriptional activation of the *COX-2* gene. Increase in the nuclear translocation of C/EBPβ and the altered ratio of nuclear C/EBPβ to C/EBPδ by C2 would contribute to C2-potentiated COX-2 induction. This is consistent with the observation that the increase in C/EBPβ homodimer complex *trans*-activated the *COX-2* gene expression (Granger et al., 2000). In the present study, the crucial role of C/EBP in the enhancement of COX-2 induction was further supported by the experiment using dominant-negative mutant of C/EBP.

NF-κB is activated by oxidative stress and/or inflammation. Activation of the NF-κB complex is related with the cellular redox state (Hirota et al., 1999). The intracellular thiol level changes the expression of several genes after early activation of NF-κB (Parmentier et al., 2000). In a previous study from another laboratory (Hsu et al., 2001), C2, when present with LPS, inhibited LPS-induced NF-κB activation. Hsu et al. (2001) proposed the hypothesis that the inhibition of LPS-mediated IKK, p38 kinase, PKC, NF-κB, and AP-1 activation by C2 may lead to COX-2 inhibition (2001). However, another study showed that activation of NF-κB was not responsible for the induction of COX-2 (Wadleigh et al., 2000). We found that NF-κB was not further activated by C2, which was consistent with the notion that the putative NF-κB was not required for COX-2 induction by LPS in macrophages (Wadleigh et al., 2000). Because all of the experimental conditions in the present study were identical to those of Chen et al. (2001), the discrepancy implicating the role of ceramide in the induction of COX-2 is mystery.

Studies have shown that ceramide activates MAP kinases, including p38 kinase, JNK, and ERK1/2 (Chen et al., 2001). The pathways of the MAP kinases, in particular p38 kinase and ERK1/2, regulate the expression of COX-2 (Nagano et al., 2002). Hence, the signaling pathway of ceramide is likely to produce cross-talk with that for COX-2 expression. In the present study, we confirmed that the MAP kinases controlled the expression of COX-2 by LPS. p38 kinase played a critical role in LPS-inducible or C2-potentiated COX-2 induction, as evidenced by complete blockage of COX-2 induction by p38 kinase inhibition. The ERK1/2 pathway was involved in the

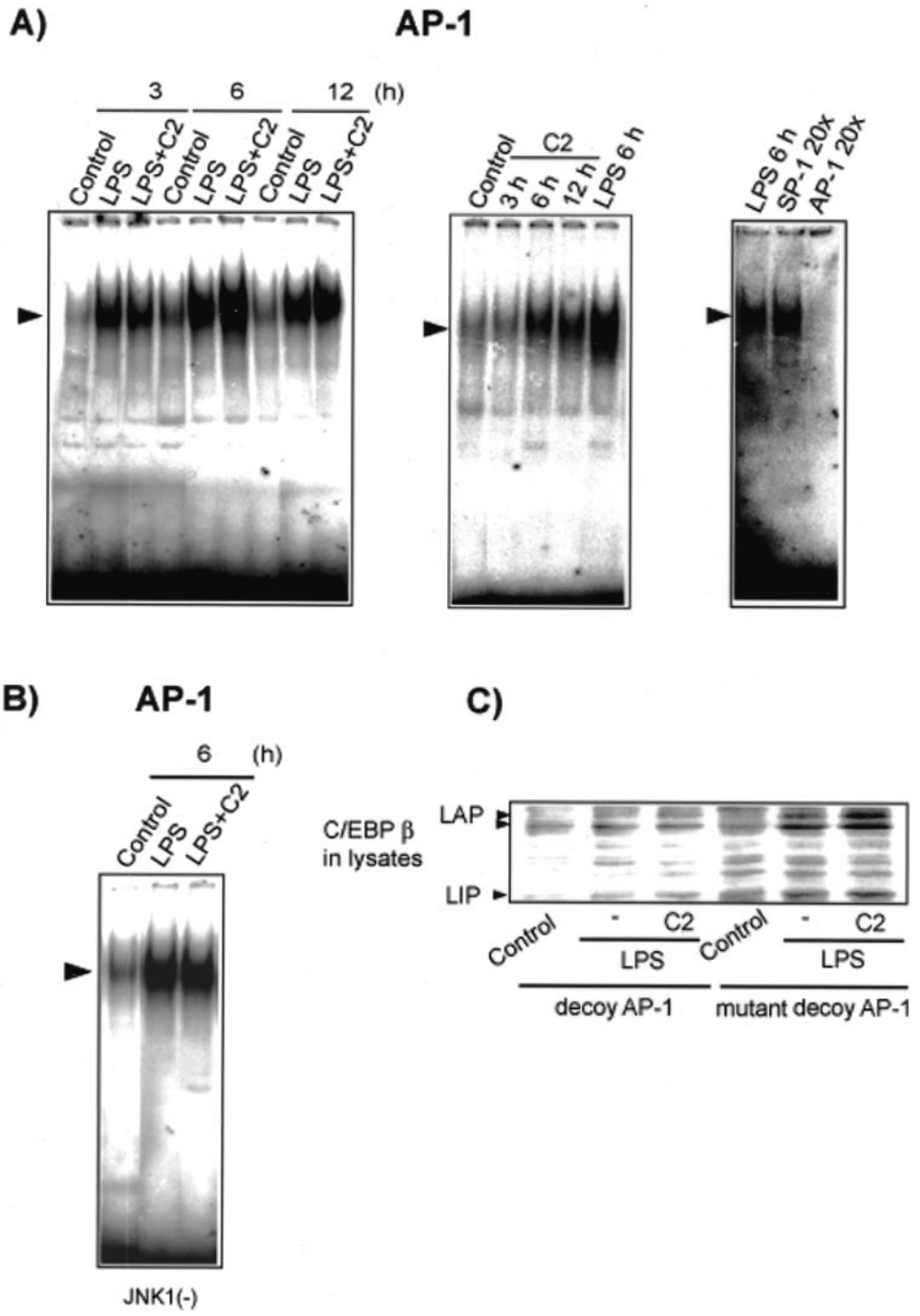

Fig. 8. Gel-shift analysis of AP-1. A, gel-shift analysis of AP-1 with the nuclear extracts prepared from cells treated with LPS (0.1 μg/ml) or LPS + C2 (50 μM) for 3 to 12 h. Each lane contained 5 μg of nuclear extracts. The specificity of AP-1 binding was assessed by competition assays with 20-fold molar excess of unlabeled AP-1 or SP-1 oligonucleotide. Arrowhead, the retarded AP-1 band. B, gel-shift analysis of AP-1 with the nuclear extract prepared from JNK1(−) cells that were treated with LPS or LPS + C2 for 6 h. Results were confirmed by repeated experiments. C, the effect of AP-1 consensus decoy ODN on the expression of C/EBPβ by LPS or LPS + C2. Cells were incubated with 10 μM of AP-1 decoy ODN or mutant AP-1 decoy ODN for 1 h and further incubated with LPS or LPS + C2 for 12 h. C/EBPβ in cell lysates was immunoblotted.

induction of COX-2 by LPS. However, the inhibition of the MKK/ERK1/2 failed to suppress C2 enhancement in COX-2 induction by LPS, as evidenced by the experiments using a specific chemical inhibitor of MKK1 or cells stably expressing MKK1(−). Hence, the ERK1/2 pathway was not responsible for the C2 potentiation of COX-2 induction by LPS. It is highly likely that the MAP kinase signaling pathways responsible for COX-2 induction switch in cells exposed to C2 in combination with LPS. In the present study, we showed for the first time that the JNK pathway, which is not involved in the COX-2 induction by LPS alone, contributes to C2 potentiation of COX-2 induction instead of the ERK pathway (Fig. 11).

We found that JNK1(−) transfection abrogated both enhanced C/EBP DNA binding and C/EBPβ nuclear translocation by C2. In contrast, the decrease in the translocation of C/EBPδ by C2 was not restored by JNK1(−) transfection, which indicated that the JNK1 pathway was not responsible for the reduced translocation of C/EBPδ. We conclude that

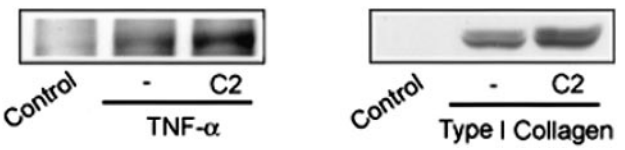

Fig. 9. Enhancement of TNF-α- or type I collagen-inducible COX-2 expression by C2 in Raw264.7 cells. Cells were treated with C2 (50 μM) in combination with TNF-α (10 ng/ml) or type I collagen (10 μg/ml) for 20 h.

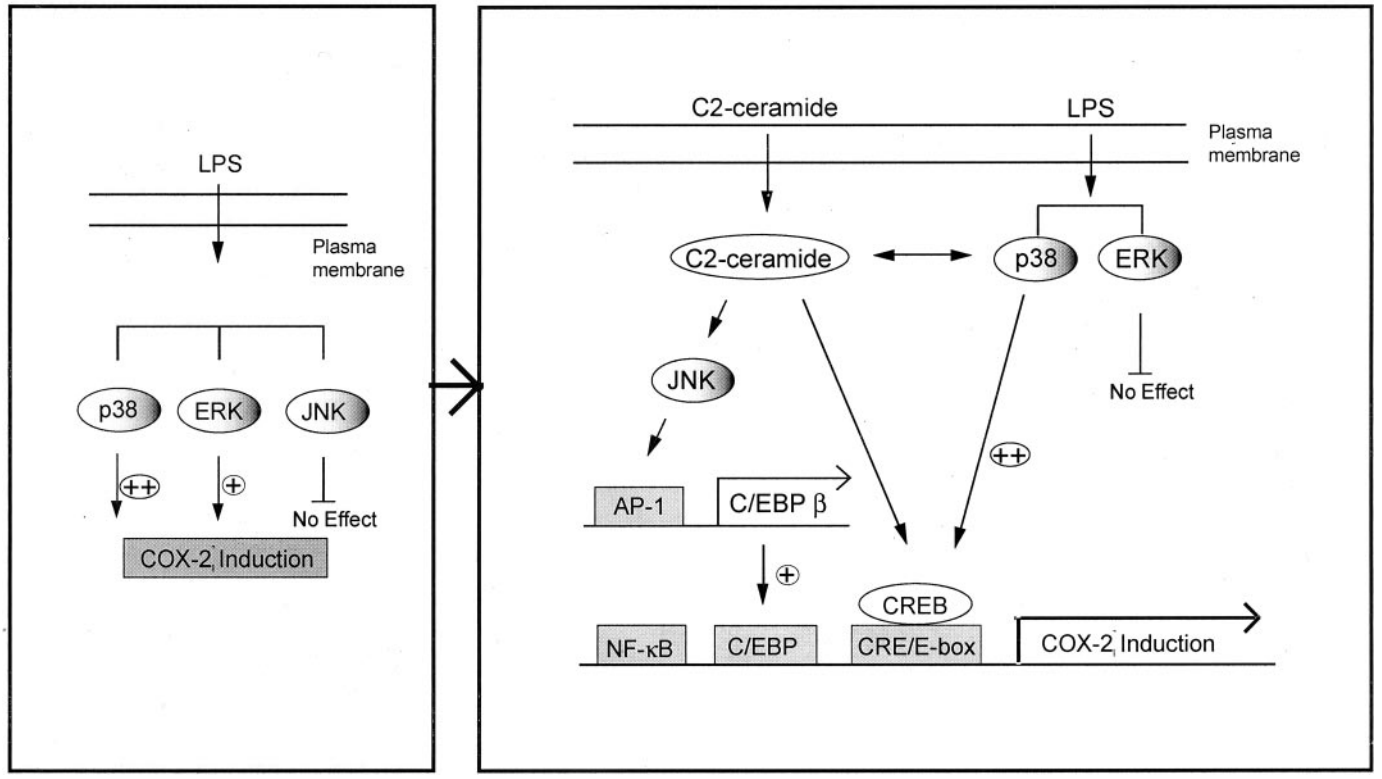

Fig. 10. Schematic diagram illustrating the proposed mechanisms by which C2 enhances LPS-inducible COX-2 expression. JNK/AP-1–mediated C/EBPβ activation by C2 potentiates COX-2 induction by LPS. p38 kinase plays a role in the inducible and potentiated COX-2 expression.

activation of C/EBPβ, but not C/EBPδ, is dependent on JNK1. It has been reported that c-Jun mediates the induction of COX-2 by ceramide or sphingomyelinase (Subbaramaiah et al., 1998), and enhanced phosphorylation of c-Jun in combination with c-Fos potentiates its ability to activate transcription of the responsible genes (Karin, 1995). C/EBPβ and other transcription factors (e.g., c-Jun) may be involved in the formation of enhanceosome protein complex for *trans*-activation of the *COX-2* gene.

We observed that the expression of C/EBPβ was enhanced by LPS + C2, compared with LPS or C2 alone, and that the enhanced C/EBPβ expression was controlled by JNK. JNK has been proposed to play a role in AP-1–mediated transcriptional activation of the target genes (Karin, 1995). The promoter region of the *C/EBP*β gene contains the AP-1 binding sites (Foka et al., 2001). Thus, JNK-mediated AP-1 activation by C2 in combination with LPS may contribute to the transcriptional activation of the gene. Our observation has an implication for the finding of AP-1 as a putative transcriptional factor responsible for enhanced C/EBPβ expression (Fig. 11). Hence, the pathway involving JNK may be responsible for the greater induction and activation of C/EBPβ by C2 in LPS-treated cells and may consequently lead to C2-potentiated induction of COX-2.

Activation of JNK is an early cellular response after exposure to a variety of stressors such as heat, UV irradiation, DNA damaging agents, and osmotic shock (Adler et al., 1995; Rosette and Karin, 1995), which represents separate stress-activated apoptotic pathway (Leppa and Bohmann, 1999). Ceramide signaling regulates the expression of the genes implicated in inflammation. We found that C2 also enhanced TNF-α–inducible COX-2 expression. We speculate that the induction of COX-2 by inflammatory mediators would be potentiated in cells whose JNK and ceramide pathways are both activated by stressors (e.g., apoptotic cells). A previous study showed that bovine type I collagen induced nitric-oxide synthase (Cho et al., 2002) and COX-2 (Y. H. Cho and S. G. Kim, unpublished observations). We observed that C2 enhanced COX-2 induction by bovine type I collagen. Hence, C2 was capable of enhancing COX-2 induction by other inducers. Thus, the ceramide-signaling pathway significantly contributes to the induction of COX-2 by LPS and other inducer(s) in macrophages.

In summary, the present study demonstrates that C2 potentiates C/EBPβ activation and COX-2 induction by LPS, that the pathway of JNK1, but not ERK1/2, is responsible for C/EBPβ-mediated potentiation of COX-2 induction (which involves AP-1–mediated C/EBPβ expression), and that p38 kinase plays an essential role in the inducible and potentiated COX-2 expression.

Acknowledgments

We gratefully acknowledge the kind donation of pCMV500 and AC/EBP plasmids by Dr. C. Vinson.

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Address correspondence to: Sang Geon Kim, Ph.D., College of Pharmacy, Seoul National University, Sillim-dong, Kwanak-gu, Seoul 151-742, South Korea. E-mail: sgk@snu.ac.kr