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Tumor Necrosis Factor-α-Induced Cyclooxygenase-2 Expression via Sequential Activation of Ceramide-Dependent Mitogen-Activated Protein Kinases, and IκB Kinase 1/2 in Human Alveolar Epithelial Cells

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ABSTRACT

The role of p44/42 mitogen-activated protein kinase (MAPK), p38, and c-Jun NH_2-terminal kinase (JNK) in tumor necrosis factor (TNF)-α-induced cyclooxygenase (COX)-2 expression was studied in NCI-H292 epithelial cells. TNF-α-mediated COX-2 expression and COX-2 promoter activity were inhibited by the MAPK kinase inhibitor PD98059 or the p38 inhibitor SB203580. Treatment of cells for 10 min with TNF-α resulted in activation of p44/42 MAPK, p38, and JNK. C2-ceramide (a cell-permeable ceramide analog), bacterial neutral sphingomyelinase (Smase; an enzyme that degrades sphingomyelin to ceramide), and *N*-oleoylethanolamine (a ceramidase inhibitor) all induced activation of MAPKs, COX-2 expression, nuclear factor (NF)-κB DNA-protein binding, and COX-2 promoter activity. The inactive analog, dihydro-C2-ceramide, had no effect. SMase- or C2-ceramide-induced COX-2 expression and COX-2 promoter activity were also inhibited by PD98059 or SB203580. Glutathione, a neutral SMase inhibitor, attenuated TNF-α- or SMase-induced activation of MAPKs, COX-2 expression, and COX-2 promoter activity. TNF-α- or C2-ceramide-induced COX-2 promoter activity was inhibited by the dominant negative mutant of extracellular signal-regulated kinase 2, p38, JNK, IκB kinase (IKK)1, or IKK2. IKK activity was stimulated by either TNF-α or C2-ceramide, and these effects were inhibited by PD98059 or SB203580. All these results suggest that, in NCI-H292 epithelial cells, activation of MAPKs by ceramide contributes to the TNF-α signaling that occurs downstream of neutral SMase activation and results in the stimulation of IKK1/2, and NF-κB in the COX-2 promoter, followed by initiation of COX-2 expression.

The enzyme cyclooxygenase (COX) is a rate-limiting step in the synthesis of prostaglandins. To date, two isoforms of this enzyme have been described: COX-1, which is constitutively expressed in most human tissues (O'Neill and Ford-Hutchinson, 1993), and COX-2, expression of which is readily induced in a variety of cells by inflammatory stimuli, such as lipopolysaccharide and cytokines (O'Banion et al., 1992; Habib et al., 1993). Much evidence suggests that COX-2 is an important therapeutic target for preventing or treating arthritis and cancer. Reducing the levels of COX-2 will be an effective strategy for inhibiting inflammation and carcinogenesis (Anderson et al., 1996; Lipsky and Isakson, 1997; Kawamori et al., 1998); to develop an effective approach, however, it is important to define the signaling mechanisms that govern COX-2 expression. Studies on the transcriptional regulation of *COX-2* genes have led to the identification of a number of transcriptional factors that are mediated through specific *cis*-acting elements. In the human *COX-2* gene, the nucleotide sequence of the 5′-flanking region contains a canonical TATA box and consensus sequences of the NF-κB, NF-IL6 (C/EBPβ), and CRE sites in the 275-bp region upstream from the transcriptional start site (Kosaka et al., 1994). Sequences homologous to these consensus sites are also found in the corresponding regions of the mouse and rat *COX-2* genes (Fletcher et al., 1992; Sirosis et al., 1993). NF-κB consensus sites in the COX-2 promoter region are important in the induction of COX-2 mRNA by TNF-α (Yamamoto et al., 1995). CRE and C/EBPβ (NF-IL6) act as positive regulatory

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ABBREVIATIONS: COX, cyclooxygenase; PG, prostaglandin; NF, nuclear factor; C/EBP, CCAAT/enhancer-binding protein; CRE, cAMP-responsive element; TNF, tumor necrosis factor; PKC, protein kinase C; MAPK, mitogen-activated protein kinase; JNK, c-Jun NH_2-terminal kinase; SMase, sphingomyelinase; GST, glutathione *S*-transferase; ERK, extracellular signal-regulated kinase; IκB, inhibitory protein of NF-κB; IKK, IκB kinase; FCS, fetal calf serum; MBP, myelin basic protein; OE, *N*-oleoylethanolamine; ECL, enhanced chemiluminescence; PAGE, polyacrylamide gel electrophoresis; MEK, mitogen-activated protein kinase kinase; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; SM, sphingomyelin; MEKK, mitogen activated protein kinase kinase kinase; EMSA, electrophoretic mobility shift assay.

elements for COX-2 transcription (Sirosis and Richards, 1993; Inoue et al., 1994; Xie et al., 1994).

Epithelial cells play an active role in inflammation by producing various cytokines and eicosanoids (Devalia and Davies, 1993). Airway epithelial cells respond to proinflammatory cytokines, such as IL-1β, by COX-2 induction and PGE2 release (Mitchell et al., 1994). We have also demonstrated that TNF-α induced a dose- and time-dependent increase in COX-2 expression and PGE2 release in airway epithelial cells (Chen et al., 2000b). The intracellular signaling pathways by which TNF-α induces COX-2 expression are largely unresolved, but involve a series of events resulting in the transmission of the signal from the plasma membrane through the cytoplasm to the nucleus, where *COX-2* gene expression is up-regulated. Previous studies have shown that TNF-α activates phosphatidylinositol-phospholipase Cγ^2 by tyrosine phosphorylation to induce PKCα activation, which then results in the stimulation of NF-κB in the COX-2 promoter, initiating COX-2 expression and, finally, PGE2 release (Chen et al., 2000b). In mammalian cells, three distinct and parallel MAPK cascades, p44/42 MAPK, p38, and JNK/stress-activated protein kinases, have been identified (Boulton et al., 1991; Derijard et al., 1994; Han et al., 1994). Although TNF-α is reported to activate all three of these MAPKs (Kyriakis and Avruch, 1996), the activation-signaling pathway and their functional roles are largely unresolved. In the present study, we used MAPK pathway specific inhibitors and dominant negative mutants, and found that p44/42 MAPK, p38, and JNK must all be activated for TNF-α-induced transactivation of NF-κB in the COX-2 promoter, followed by COX-2 expression. Activation of neutral sphingomyelinase (SMase) by TNF-α and the subsequent formation of ceramide contribute to the activation of these MAPKs.

Experimental Procedures

Materials. GST c-jun, the NF-κB probe, and goat polyclonal antibodies specific for COX-1 and COX-2 or rabbit polyclonal antibodies specific for p42 MAPK (ERK2), p38, JNK1, or IKKβ were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Recombinant human TNF-α were purchased from R&D Systems Inc. (Minneapolis, MN). RPMI 1640 medium, fetal calf serum (FCS), penicillin, and streptomycin were from Life Technologies (Gaithersburg, MD). Myelin basic protein (MBP), bacterial neutral SMase, and glutathione were from Sigma (St. Louis, MO). PD98059, SB203580, *N*-acetyl D-erythrosphingosine (C2 ceramide), dihydro-C2 ceramide, and *N*-oleoylethanolamine (OE) were from Calbiochem (San Diego, CA). T4 polynucleotide kinase and rabbit polyclonal antibodies specific for the phosphorylated form of p44/42 MAPK, p38, or JNK were from New England Biolab (Beverly, MA). Poly(dI/dC), horseradish peroxidase-labeled donkey anti-rabbit second antibody and the ECL detecting reagent were from Amersham Pharmacia Biotech (Piscataway, NJ). [γ-^{32}P]ATP (3000 Ci/mmol) was from DuPont-New England Nuclear (Boston, MA). Tfx-50 and the luciferase assay kit were from Promega (Madison, WI).

Plasmids. The COX-2 promoter construct (−459/+9) was a generous gift from Dr. L.H. Wang (University of Texas–Houston, Houston, TX). The dominant negative mutant for ERK2 was provided by Dr. M. Cobb (South-Western Medical Center, Dallas, TX), that for p38 (T180A/Y182F) by Dr. J. Han (The Scripps Research Institute, San Diego, CA), and that for JNK (T183A/Y185F) by Dr. M. Karin (University of California, San Diego, CA). The IKK1 (KM) and IKK2 (KM) dominant negative mutants were from Signal Pharmaceutical (San Diego, CA). pGEX-IκBα (1–100) was a gift from Dr. H. Nakano (University of Juntendo, Japan).

Cell Culture. NCI-H292 cells, a human alveolar epithelial cell carcinoma, were obtained from the American Type Culture Collection (Manassas, VA) and cultured in RPMI 1640 medium supplemented with 10% FCS, 100 U/ml of penicillin, and 100 μg/ml of streptomycin in 6-well plates (COX-2 expression and transfection) or in 10-cm dishes (activation of MAPKs, NF-κB gel shift assay, and IKK activation).

Preparation of Cell Extracts and Western Blot Analysis of COX-2 or COX-1. After 16 h treatment with TNF-α, C2 ceramide, SMase, or OE, the cells were harvested and collected and cell lysates prepared and subjected to SDS-PAGE using 7.5% running gels as described previously (Chen et al., 1998). The proteins were transferred to nitrocellulose paper and the membrane incubated successively at room temperature with 0.1% milk in TTBS for 1 h, with goat antibody specific for COX-2 or COX-1 for 1 h, and with horseradish peroxidase-labeled anti-goat antibody for 30 min. After each incubation, the membrane was washed extensively with Tris-buffered saline/Tween 20. The immunoreactive band was detected using ECL detection reagent and visualized using Hyperfilm-ECL. The quantitative data were obtained using a computing densitometer and ImageQuant software (Molecular Dynamics, Sunnyvale, CA). In pretreatment experiments, cells were incubated for 30 min with the MEK inhibitor PD98059, the p38 inhibitor SB203580, or the neutral SMase inhibitor glutathione (Liu and Hannun, 1997; Liu et al., 1998) before addition of TNF-α. These inhibitors had no cytotoxic effect on NCI-H292 cells and 0.001% DMSO (vehicle) used through this study had no effect on TNF-α- or TPA-induced COX-2 expression.

Preparation of Cell Extracts and Western Blot Analysis of Phosphorylated p44/42 MAPK, Phosphorylated p38, Phosphorylated JNK, p42 MAPK, p38, and JNK1. After 10 min treatment with TNF-α, C2 ceramide, SMase, or OE, or 30 min pretreatment with PD98059, SB203580, or glutathione before challenge with TNF-α, the cells were rapidly washed with PBS, then lysed with ice-cold lysis buffer (50 mM Tris-HCl, pH 7.4, 1 mM EGTA, 150 mM NaCl, 1% Triton X-100, 1 mM PMSF, 5 μg/ml of leupeptin, 20 μg/ml of aprotinin, 1 mM NaF, and 1 mM Na_3VO_4) as described previously (Chen and Chen, 1998). The lysates were then subjected to SDS-PAGE using a 7.5% running gel. The proteins were transferred to nitrocellulose paper and immunoblot analysis performed as described above, except that rabbit antibodies specific for phosphorylated MAPKs or nonphosphorylated MAPKs were used.

Immunoprecipitation and Assay of p38 and JNK1 Activity. The immunoprecipitation experiment was performed as described previously (Chen and Chen, 1998). Briefly, 50 μg of total cell lysate was incubated with 1 μg of anti-p38 antibody or 0.5 μg of anti-JNK1 antibody for 1 h at 4°C and the antibody-bound material was collected using protein A-Sepharose CL-4B beads (Sigma). The beads were then washed three times with lysis buffer and incubated for 30 min at 30°C with 30 μl of kinase reaction mixture containing 20 mM HEPES, pH 7.4, 10 mM $MgCl_2$, 100 μM Na_3VO_4, and 50 μM [γ-^{32}P]ATP (2000 cpm/pmol), together with 1 μg MBP for the p38 activity assay (Chen and Wang, 1999) or 1 μg of GST c-jun for the JNK1 activity assay. The reaction was stopped by the addition of Laemmli buffer and subjected to SDS-PAGE, phosphorylated MBP or GST c-jun being visualized by autoradiography.

Preparation of Nuclear Extracts and the Electrophoretic Mobility Shift Assay (EMSA). Cells were treated for 1 h with TNF-α, C2 ceramide, SMase, or OE (Chen et al., 2000b), then nuclear extracts were isolated as described previously (Chen et al., 1998). Briefly, cells were washed with ice-cold PBS and pelleted, then the cell pellet was resuspended in hypotonic buffer (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF, 1 mM NaF, and 1 mM Na_3VO_4), incubated for 15 min on ice, then lysed by the addition of 0.5% Nonidet P-40, followed by vigorous vortexing for 10 s. The nuclei were pelleted and resuspended in extraction buffer (20 mM HEPES, pH 7.9, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, 1 mM NaF, and 1 mM Na_3VO_4), and the tube vigorously shaken for 15 min at 4°C on a

shaking platform. The nuclear extracts were then centrifuged and the supernatants aliquoted and stored at −80°C.

Oligonucleotides corresponding to the downstream NF-κB consensus sequences in the human COX-2 promoter (5′-AGAGTGGGGACTACCCCCTCT-3′) were synthesized, annealed, and end-labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase. The nuclear extract (6–10 μg) was incubated at 30°C for 20 min with 1 ng of ^{32}P-labeled NF-κB probe (40,000–60,000 cpm) in 10 μl of binding buffer containing 1 μg of poly (dI/dC), 15 mM HEPES, pH 7.6, 80 mM NaCl, 1 mM EGTA, 1 mM DTT, and 10% glycerol, as described previously (Chen et al., 1998). DNA-nuclear protein complexes were separated from the DNA probe by electrophoresis on a native 6% polyacrylamide gel, then the gel was vacuum dried and subjected to autoradiography using an intensifying screen at −80°C.

Transient Transfection and Luciferase Assay. NCI-H292 cells, grown in 6-well plates, were transfected with the human COX-2 firefly luciferase (LUC) plasmid, pGS459 (−459/+9) using Tfx-50 (Promega) according to the manufacturer's recommendations. Briefly, reporter DNA (0.4 μg) and β-galactosidase DNA (0.1 μg) were mixed with 2.25 μl of Tfx-50 in 1 ml of serum-free RPMI 1640 medium. The plasmid pRK containing the β-galactosidase gene driven by the constitutively active SV40 promoter was used to normalize transfection efficiency. After 10 to 15 min of incubation at room temperature, the mixture was applied to the cells. One hour later, 1 ml of RPMI 1640 medium containing 20% FCS was added, then the cells were grown in medium containing 10% FCS. On the following day, they were exposed for 6 h to 30 ng/ml of TNF-α, 100 mU/ml of SMase, or 50 μM C2 ceramide, then cell extracts were prepared. The luciferase (Promega) and β-galactosidase activity was measured, and the luciferase activity of each well normalized to the β-galactosidase activity. In dominant negative mutant experiments, cells were cotransfected with reporter and β-galactosidase and either the dominant negative mutant for ERK2, p38, JNK, IKK1, or IKK2 (0.4 μg of DNA) or the empty vector.

In Vitro IKK Activity Assay. After a 10-min treatment with TNF-α or C2 ceramide or a 30-min pretreatment with PD98059 or SB203580 before addition of TNF-α or C2 ceramide, cells were rapidly washed with PBS, then lysed with ice-cold lysis buffer as described above, and the IKK proteins were immunoprecipitated. Fifty micrograms of total cell extract was incubated for 1 h at 4°C with 0.5 μg of anti-IKKβ antibody and the antibody-bound protein collected using protein A-Sepharose CL-4B beads (Sigma). The beads were then washed three times with lysis buffer without Triton X-100 and incubated for 30 min at 30°C in 20 μl of kinase reaction mixture containing 20 mM HEPES, pH 7.4, 5 mM $MgCl_2$, 5 mM $MnCl_2$, 0.1 mM Na_3VO_4, 1 mM DTT, 1 μg of bacterially expressed GST-IκBα (1–100), and 10 μM [γ-^{32}P]ATP. The reaction was stopped by the addition of Laemmli buffer and the material subjected to 10% SDS-PAGE, phosphorylated-GST-IκBα (1–100) being visualized by autoradiography.

Statistical Analyses. All data are expressed as mean ± S.E.M. Statistical analyses were done with Student's *t* test.

Results

TNF-α-Induced Activation of p44/42 MAPK, p38, and JNK and Inhibition of TNF-α-Induced COX-2 Expression by PD98059 and SB203580. In NCI-H292 cells, TNF-α activated p44/42 MAPK, p38, and JNK. When cells were treated with 30 ng/ml of TNF-α for 10, 30, or 60 min, maximal activation of these three MAPKs was seen after 10-min treatment, with lower or no activation being seen after 30- or 60-min treatment (Fig. 1A). To determine whether activation of p44/42 MAPK and p38 was involved in the regulation of TNF-α-induced COX-2 expression, a MEK inhibitor and a p38 inhibitor were used. As shown in Fig. 2, 30 μM PD98059 or SB203580 resulted, respectively, in 77 or 85% inhibition of the TNF-α-induced COX-2 expression. PD98059 (30 μM) almost totally blocked TNF-induced activation of p44/42 MAPKs without any effect on p38 and JNK1 (Fig. 1B), whereas 30 μM SB203580 almost completely blocked p38 activation by TNF-α but had no effect on p44/42 MAPK and JNK1 (Fig. 1B). Neither of these treatments had any effect on the expression of p42 MAPK or p38.

Ceramide-Induced COX-2 Expression and p44/42 MAPK, p38, and JNK1 Activation. Ceramide, a novel lipid second messenger, is formed when the membrane phospholipid sphingomyelin (SM) undergoes hydrolysis (Heller and

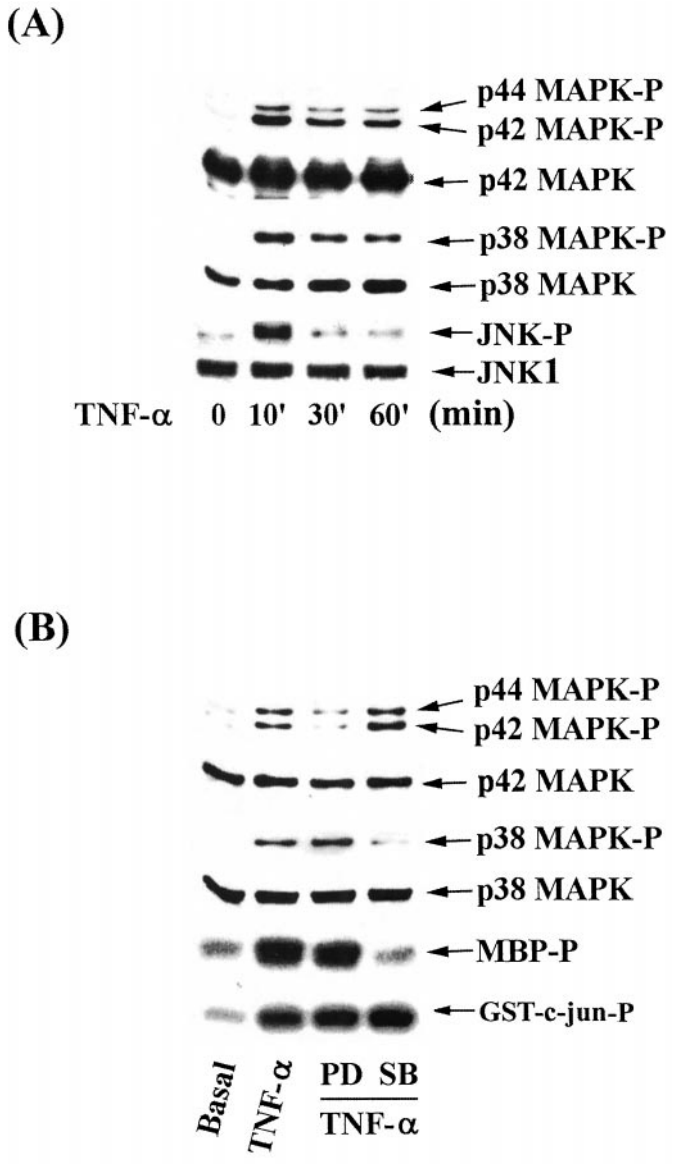

Fig. 1. Time-dependent activation of p44/42 MAPK, p38, and JNK by TNF-α, and effects of PD98059 or SB203580 on p44/42 MAPK and p38 activation in NCI-H292 epithelial cells. Cells were treated with 30 ng/ml of TNF-α for the indicated time intervals (A), or pretreated for 30 min with 30 μM PD98059 or SB203580 before incubation for 10 min with 30 ng/ml of TNF-α (B). Whole-cell lysates were prepared and subjected to Western blotting using antibody specific for the phosphorylated form of p44/42 MAPK, p38, or JNK or for p42 MAPK, p38, or JNK1, or immunoprecipitated with anti-p38 or anti-JNK1 antibody, followed by autoradiography for phosphorylated MBP or GST-c-jun as described under *Experimental Procedures*. Similar results were seen in three independent experiments.

Kronke, 1994; Spiegel et al., 1996). A cell-permeable ceramide analog, C2 ceramide, is reported to activate MAPKs (Reunanen et al., 1998; Subbaramaiah et al., 1998). When cells were treated for 16 h with 50 μM C2-ceramide, induction of COX-2 expression was seen, whereas dihydro C2-ceramide, the inactive analog of C2-ceramide, had no effect (Fig. 3A). Bacterial neutral SMase, an enzyme that degrades SM to ceramide (100 mU/ml), also induced expression of COX-2. OE, an inhibitor of ceramidase, the enzyme responsible for catabolism of ceramide to sphingosine and fatty acid, was also used to examine the role of increased intracellular ceramide in the induction of COX-2 expression. When cells were treated for 16 h with 100 μM OE, induction of COX-2 expression was seen. When C2-ceramide, SMase, or OE was added together with TNF-α, greater induction of COX-2 expression was seen than with either agent alone. None of these treatments affected COX-1 expression (Fig. 3A).

Because TNF-α-induced activation of p44/42 MAPK and p38 had been shown to be involved in TNF-α-induced COX-2 expression in NCI-H292 cells, the ability of C2-ceramide, SMase, and OE to activate MAPKs was examined. As shown in Fig. 3B, 10-min treatment of cells with all three agents induced activation of p44/42 MAPK, p38, and JNK1. PD98059 or SB203580 (30 μM) inhibited SMase-induced COX-2 expression by 75 and 85%, respectively, and C2-ceramide-induced COX-2 expression by 71 and 81%, respectively (Fig. 4).

Because ceramide-dependent activation of MAPKs was responsible for induction of COX-2 expression, the question of whether TNF-α acted through neutral SMase to induce COX-2 expression was examined using the neutral SMase inhibitor glutathione (Liu and Hannun, 1997; Liu et al., 1998). When cells were pretreated with 5, 10, or 20 mM glutathione, TNF-α-induced COX-2 expression was inhibited in a dose-dependent manner (33, 62, or 96% inhibition, respectively) (Fig. 5). TNF-α−, or SMase-induced p44/42 MAPK, p38 and JNK1 activation was also inhibited by glutathione. However, C2-ceramide-induced p44/42 MAPK and JNK1, but not p38 activation, were inhibited by glutathione (Fig. 6).

TNF-α, C2-ceramide, or SMase Induction of NF-κB in the Nucleus and COX-2 Promoter Activity, and Effects of Various Inhibitors or Dominant Negative Mutants. Nuclear extracts prepared from NCI-H292 cells were assayed for activated NF-κB in an EMSA. This transcriptional factor has been demonstrated to be involved in COX-2 expression in NCI-H292 cells (Chen et al., 2000b). In nonstimulated cells, no NF-κB-specific DNA-protein complex formation was identified. TNF-α, C2 ceramide, SMase, and OE all activated NF-κB (Fig. 7). A supershift assay has demonstrated the p65/p50 heterodimer of NF-κB in NCI-H292 cells (Chen et al., 2000b).

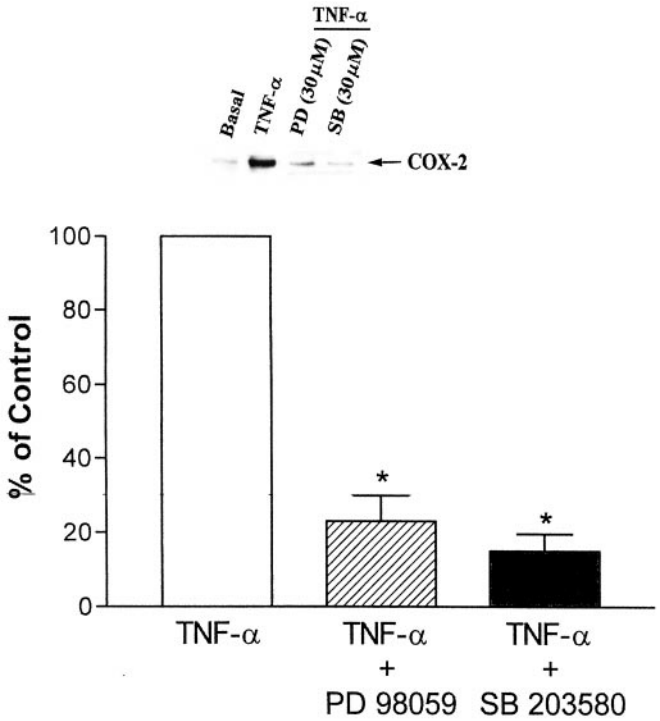

Fig. 2. Effect of PD98509 or SB203580 on TNF-α-induced COX-2 expression in NCI-H292 epithelial cells. Cells were pretreated for 30 min with 30 μM PD98059 or SB203580 before incubation for 16 h with 30 ng/ml of TNF-α. Whole-cell lysates were prepared and subjected to Western blotting using antibody specific for COX-2 as described under *Experimental Procedures*. COX-2 expression was quantified using a densitometer with ImageQuant software. Results are expressed as the mean ± S.E.M. of three independent experiments. *$p < 0.05$ compared with TNF-α alone.

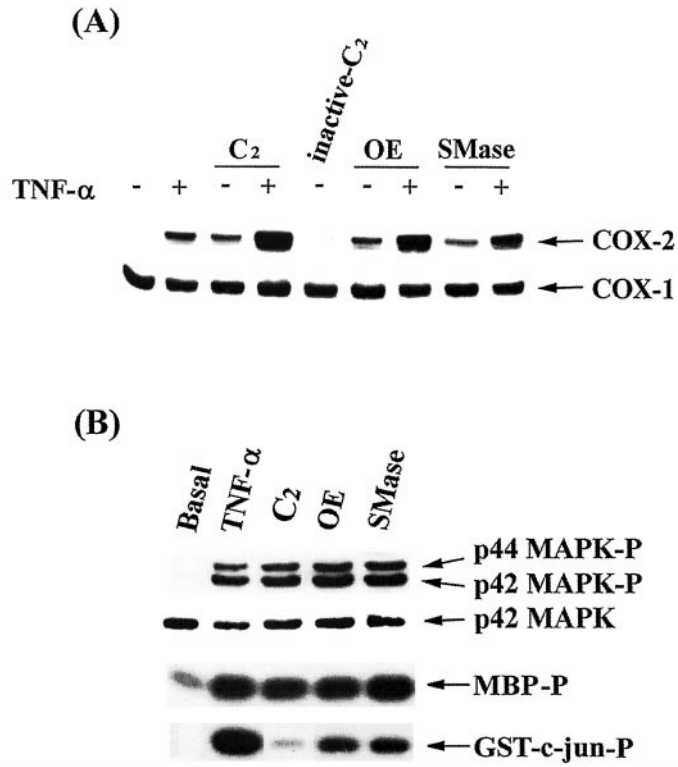

Fig. 3. Effect of C2-ceramide, SMase, or OE on COX-2 expression, and on p44/42 MAPK, p38, and JNK1 activation in NCI-H292 epithelial cells. In A, cells were treated for 16 h with 50 μM C2-ceramide (C2) or dihydro-C2-ceramide (inactive-C2), 100 mU/ml of SMase, or 100 μM OE in the presence or absence of 30 ng/ml of TNF-α. Whole-cell lysates were prepared and subjected to Western blotting using antibody specific for COX-1 and COX-2 as described in *Experimental Procedures*. In B, cells were treated for 10 min with 30 ng/ml of TNF-α, 50 μM C2-ceramide (C2), 100 mU/ml of SMase, or 100 μM OE. Whole-cell lysates were prepared and subjected to Western blotting using antibodies specific for the phosphorylated form of p44/42 MAPK or for p42 MAPK, or immunoprecipitated with anti-p38 or anti-JNK1 antibody, followed by autoradiography for phosphorylated MBP or GST-c-jun as described under *Experimental Procedures*. Similar results were seen in three independent experiments.

To further investigate the involvement of neutral SMase-dependent ceramide formation leading to activation of MAPKs in TNF-α-induced COX-2 expression, transient transfections were performed using the human COX-2 promoter-luciferase construct, pGS459 (−459/+9) (Tazawa et al., 1994). PGS459 contains both upstream (−447/−438) and downstream (−223/−214) NF-κB sites in the COX-2 promoter. Treatment with TNF-α, SMase, or C2-ceramide led to increases of 2.7-, 2.4-, or 2.1-fold, respectively, in COX-2 promoter activity, whereas dihydro-C2-ceramide had no effect (Fig. 8A). The TNF-α-, SMase-, or C2-ceramide-induced COX-2 promoter activity was inhibited by PD98059 or SB203580 (Fig. 8A) or glutathione (Fig. 8B). None of these inhibitors affected the basal luciferase activity (data not shown).

In cotransfection experiments, induction of COX-2 promoter activity by TNF-α or C2-ceramide was inhibited by the

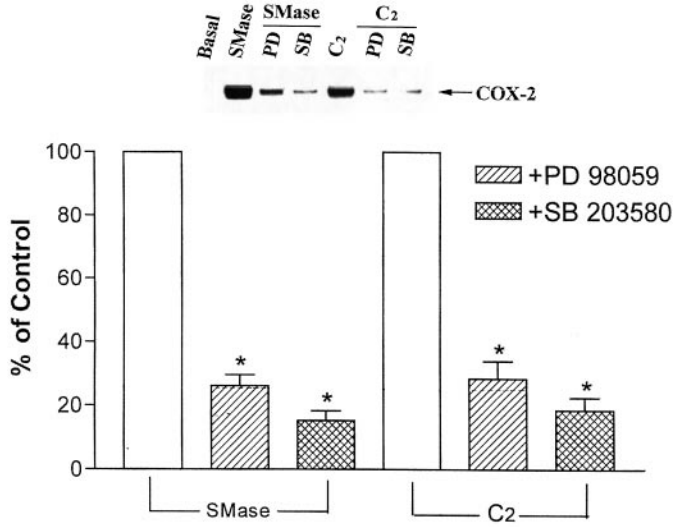

Fig. 4. Effect of PD98059 or SB203580 on C2-ceramide- or SMase-induced COX-2 expression in NCI-H292 epithelial cells. Cells were pretreated for 30 min with 30 μM PD98059 (PD) or SB203580 (SB) before incubation for 16 h with 50 μM C2-ceramide (C2) or 100 mU/ml of SMase. Whole cell lysates were prepared and subjected to Western blotting using antibody specific for COX-2 as described under *Experimental Procedures*. COX-2 expression was quantified using a densitometer with ImageQuant software. Results are expressed as the mean ± S.E.M. of three independent experiments. $^*p < 0.05$ compared with C2-ceramide or SMase alone.

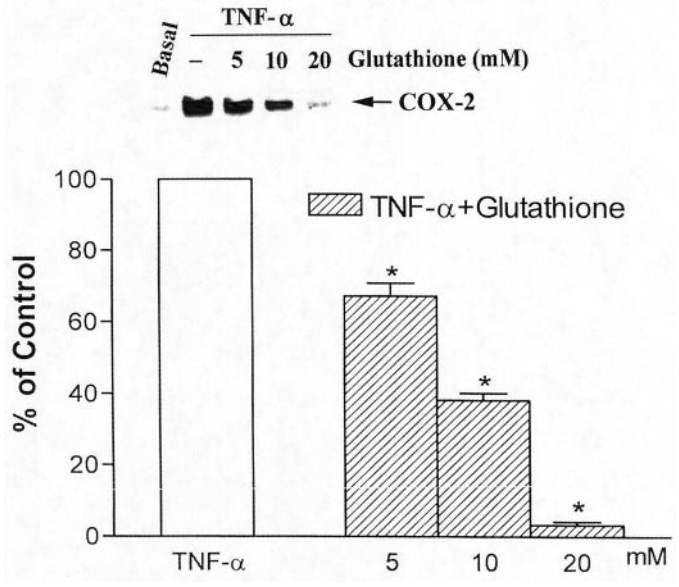

Fig. 5. Concentration-dependent inhibitory effect of glutathione on TNF-α-induced COX-2 expression in NCI-H292 epithelial cells. Cells were pretreated for 1 h with the indicated concentrations of glutathione before incubation for 16 h with 30 ng/ml of TNF-α. Whole-cell lysates were prepared and subjected to Western blotting using antibody specific for COX-2 as described under *Experimental Procedures*. COX-2 expression was quantified using a densitometer with ImageQuant software. Results are expressed as the mean ± S.E.M. of three independent experiments. $^*p < 0.05$ compared with TNF-α alone.

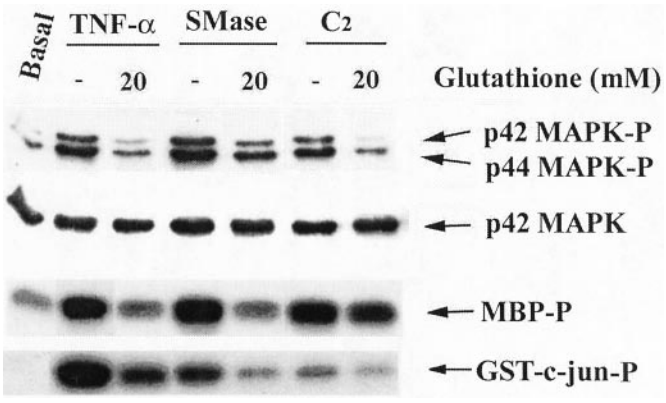

Fig. 6. Effect of glutathione on TNF-α-, C2-ceramide-, or SMase-induced p44/42 MAPK, p38, and JNK1 activation in NCI-H292 epithelial cells. Cells were pretreated for 30 min with 20 mM glutathione before incubation for 20 min with 30 ng/ml of TNF-α, 50 μM C2-ceramide (C2), or 100 mU/ml of SMase. Whole-cell lysates were prepared and subjected to Western blotting using antibodies specific for the phosphorylated form of p44/42 MAPK or for p42 MAPK, or immunoprecipitated with anti-p38 or anti-JNK1 antibody, followed by autoradiography for phosphorylated MBP or GST-c-jun as described under *Experimental Procedures*. Similar results were seen in three independent experiments.

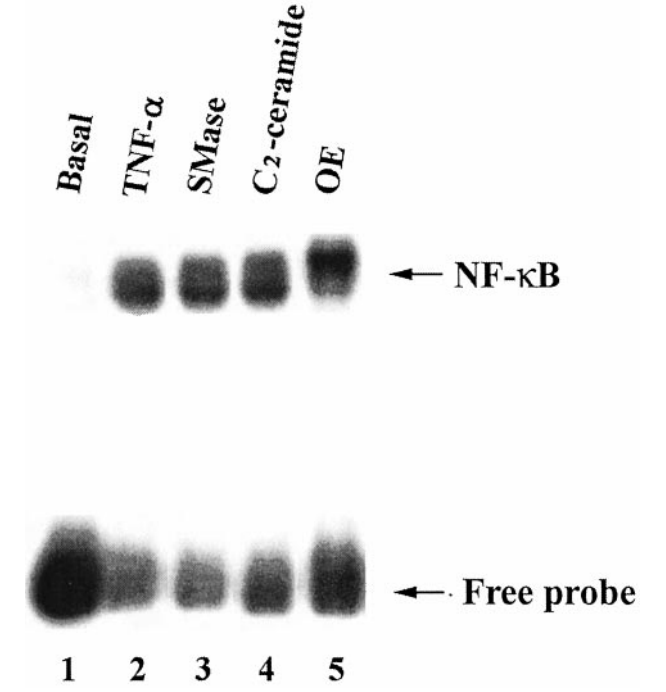

Fig. 7. Induction of NF-κB-specific DNA-protein complex formation by C2-ceramide, SMase, or OE. Cells were treated for 1 h with 30 ng/ml of TNF-α, 50 μM C2-ceramide, 100 mU/ml of SMase, or 100 μM OE, then nuclear extracts were prepared and NF-κB DNA-protein binding activity was determined by EMSA as described under *Experimental Procedures*. Similar results were seen in three independent experiments.

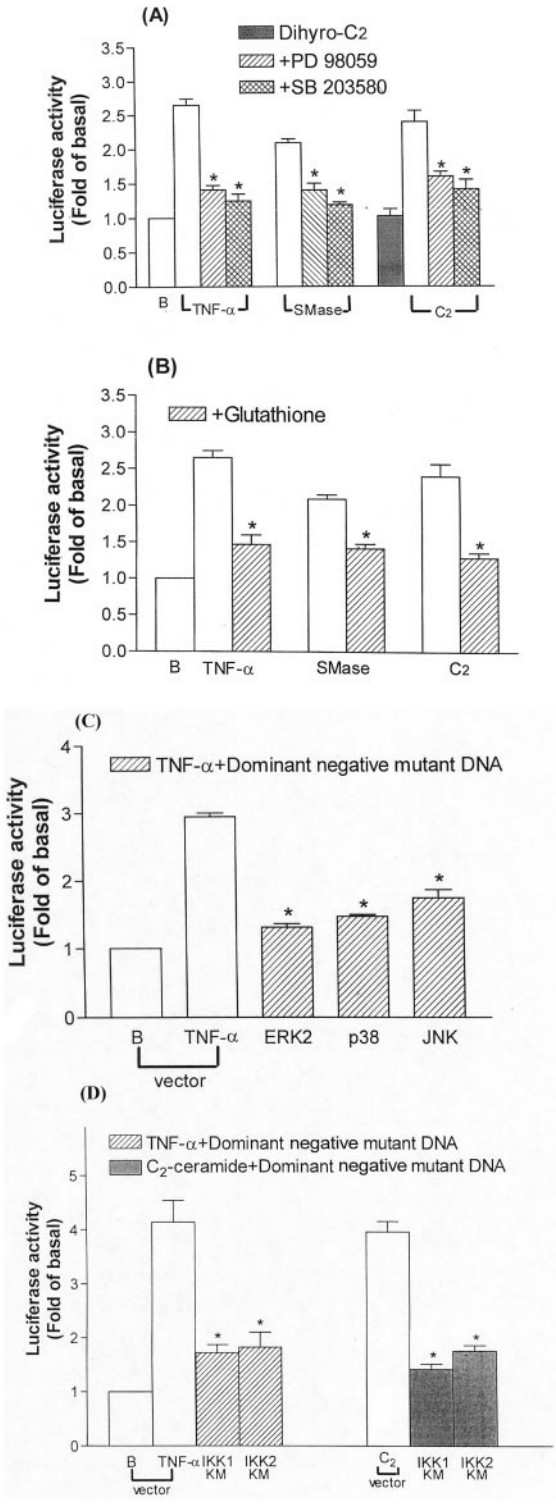

Fig. 8. Effects of PD98059, SB203580, glutathione, or various dominant negative mutants on TNF-α-induced COX-2 promoter activity. Cells were transfected with the pGS459 luciferase expression vector, then treated for 6 h with 30 ng/ml of TNF-α, 100 mU/ml of SMase, or 50 μM C2-ceramide (C2) or dihydro-C2-ceramide or pretreated for 30 min with 30 μM PD98059 or SB203580 (A) or 10 mM glutathione (B) before incubation with TNF-α, SMase, or C2-ceramide. Dominant negative mutants for ERK2, p38, JNK (C), IKK1, or IKK2 (D) or the empty vector were cotransfected with pGS459. Luciferase activity was assayed, and the results normalized to the β-galactosidase activity. Results are expressed as the mean ± S.E.M. of three independent experiments performed in triplicate. *: $p < 0.05$ compared with TNF-α, SMase, or C2-ceramide alone.

dominant negative mutants of ERK2, p38, JNK, IKK1 (KM), or IKK2 (KM) (Fig. 8, C and D).

Induction of IKK Activation by TNF-α or C2-ceramide, and Inhibitory Effect of PD98059 or SB203580. The endogenous IKK complex was isolated by immunoprecipitation with anti-IKKβ antibody and tested for in vitro kinase activity. When cells were treated with 30 ng/ml of TNF-α for 10, 30, or 60 min, maximal activation of IKK activity was seen after 10-min treatment, with lower or no activation being seen after 30- or 60-min treatment, respectively (Fig. 9A). The TNF-α- and C2-ceramide-induced IKK activity after 10-min treatment were inhibited by PD98059 or SB203580 (Fig. 9B).

Discussion

In NCI-H292 cells, TNF-α induced activation of p44/42 MAPK, p38, and JNK1, activation being maximal after 10 min treatment and declining after 30 or 60 min of treatment. We used the specific MEK inhibitor PD98059 or the p38 inhibitor SB203580 to study the relationship between TNF-α-elicited p44/42 MAPK and p38 activation and COX-2 expression in epithelial cells. PD98059 almost completely blocked TNF-α-induced activation of p44/42 MAPKs, had no effect on p38 or JNK1 activation, and abrogated TNF-α-induced COX-2 expression and COX-2 promotor activity. SB203580 had a similar inhibitory effect on TNF-α-induced p38 activation, COX-2 expression, and COX-2 promoter activity. These results emphasize the importance of both p44/42 MAPK and p38 activation in mediating TNF-α-induced COX-2 expression in epithelial cells. Cotransfection with the dominant negative mutant for ERK2 or p38 and the COX-2 promoter-luciferase construct further demonstrated the involvement of these two MAPKs. Using a dominant negative JNK mutant, JNK was also demonstrated to be involved, despite the current lack of a specific inhibitor. It seems that either MAPK was required for mediating TNF-α-induced COX-2 expression, because dominant negative ERK2, p38 or JNK mutant induced respective inhibition of

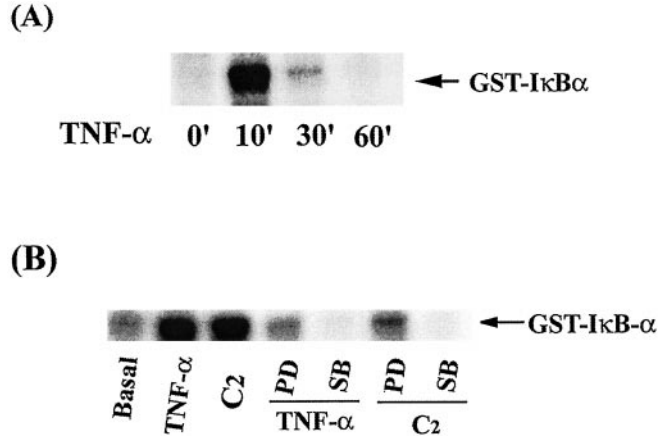

Fig. 9. Time-dependent activation of IKK activity by TNF-α, and effect of PD98059 or SB203580 on IKK activity in NCI-H292 epithelial cells. Cells were treated with 30 ng/ml of TNF-α for the indicated intervals (A), or pretreated for 30 min with 30 μM PD98059 (PD) or SB203580 (SB) before incubation for 10 min with 30 ng/ml of TNF-α or 50 μM C2-ceramide (C2) (B). Whole-cell lysates were immunoprecipitated with anti-IKKβ antibody, followed by autoradiography for phosphorylated GST-IκBα (1–100) as described under *Experimental Procedures*. Similar results were seen in three independent experiments.

82, 77 or 62% in COX-2 promoter activity (Fig. 8C). Activation of p38 and JNK by IL-1β in renal mesangial cells (Guan et al., 1998a), of ERK, JNK, and p38 by ceramide in human mammary epithelial cells (Subbaramaiah et al., 1998), of p38 by MEKK1 in NIH3T3 cells (Guan et al., 1998b), and of p44/42 MAPK by Ras in Rat-1 cells (Sheng et al., 1998) have also been reported to be involved in COX-2 expression. However, activation of MAPKs pathways are not involved in IL-1β-induced ICAM-1 expression in epithelial cells (Chen et al., 2000a), and p38 but not p44/42 MAPK is involved in LPS-induced iNOS expression in macrophages (Chen and Wang, 1999).

Because the activation of MAPKs pathway was involved in TNF-α-induced COX-2 expression, the signaling mediating activation of MAPKs was further examined. TNF-α-induced activation of MAPKs was not affected by either tyrosine kinase or PKC inhibitors (data not shown), indicating that upstream tyrosine kinase or PKC was not involved in activation of MAPKs. Several kinds of lipid messengers are known to mediate agonist-induced cellular responses (Liscovitch and Cantley, 1994). Ceramide, generated from SM by hydrolysis, has been shown to be a novel lipid second messenger in various cell systems (Hannun, 1994). TNF-α is reported to stimulate ceramide production (Wiegman et al., 1994), and ceramide is reported to induce activation of MAPKs (Reunanen et al., 1998; Subbaramaiah et al., 1998). Several lines of evidence suggest that, in NCI-H292 cells, TNF-α acts via ceramide-dependent activation of MAPKs to induce COX-2 expression. First, C2-ceramide, bacterial neutral SMase, or OE, but not dihydro-C2-ceramide, induced COX-2 expression, COX-2 promoter activity, and NF-κB-DNA protein binding. Second, the COX-2 expression and COX-2 promoter activity induced by these three agents were inhibited by PD98059 or SB203580. Third, all three agents induced activation of MAPKs in NCI-H292 cells. Fourth, glutathione, a neutral SMase inhibitor, inhibited TNF-α- or SMase-induced COX-2 expression, COX-2 promoter activity, and activation of MAPKs. Therefore, activation of MAPKs by ceramide formation because of downstream neutral SMase activation is responsible for TNF-α-induced COX-2 expression in NCI-H292 cells. Although glutathione was reported to be a neutral SMase inhibitor (Liu and Hannun, 1997; Liu et al., 1998), the reason for its inhibition on C2-ceramide-induced activation of MAPKs and promoter activity was unknown (Figs. 6 and 8). It is probable that it exerts other action, such as antioxidant, in addition to neutral SMase inhibition. TNF-α triggering of the ceramide signaling pathway initiated by neutral SMase resulting in activation of MAPKs has also been reported in human skin fibroblast cultures (Reunanen et al., 1998). Although ceramide-dependent activation of MAPKs has been reported to be involved in COX-2 expression (Subbaramaiah et al., 1998), the agonist-inducing ceramide formation was not defined. The p38 and p44/42 MAPK pathways have been reported to be required for TNF-α-induced NF-κB (p65) *trans*-activation (Berghe et al., 1998).

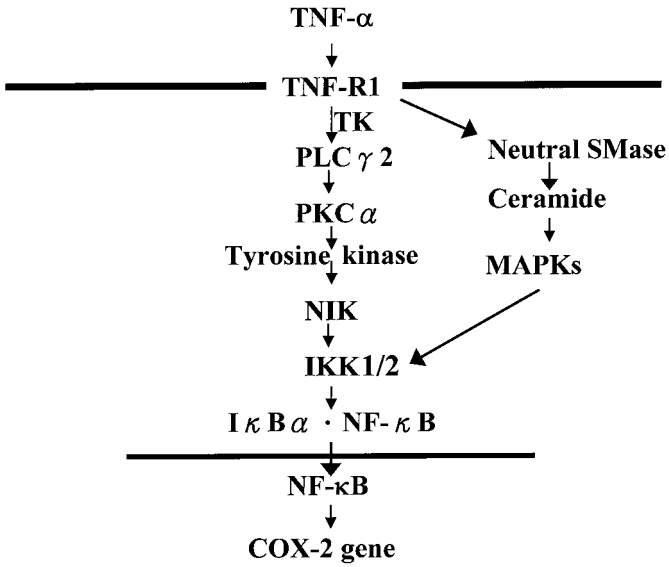

Fig. 10. Schematic representation of the signaling pathway of TNF-α-induced COX-2 expression in NCI-H292 epithelial cells. TNF-α binds to TNF RI, and activates PLCγ2 via tyrosine phosphorylation to induce PKCα and tyrosine kinase activation. TNF-α also activates MAPKs through ceramide that occurs downstream of neutral SMase activation. These two pathways result in stimulation of IKK1/2, and NF-κB in the COX-2 promoter, initiating COX-2 expression and PGE2 release.

In nonstimulated cells, NF-κB dimers are present as cytoplasmic latent complexes as a result of the binding of specific inhibitors, the IκBs, which mask their nuclear localization signal. After stimulation by proinflammatory cytokines, the IκBs are rapidly phosphorylated at two conserved NH_2-terminal serine residues; this post-translational modification is rapidly followed by their polyubiquitination and proteasomal degradation (Thanos and Maniatis, 1995; Chen et al., 1996). This results in the unmasking of the nuclear localization signal in the NF-κB dimers, which is followed by their translocation to the nucleus, binding to specific DNA sites (κB sites), and targeting of gene activation. The protein kinase that phosphorylates IκBs in response to proinflammatory stimuli has been identified biochemically and molecularly (DiDonato et al., 1997; Mercurio et al., 1997; Regnier et al., 1997; Woronicz et al., 1997; Zandi et al., 1997). Named IKK, it exists as a complex, termed the IKK signalsome, which is composed of at least three subunits, IKK1 (IKKα), IKK2 (IKKβ), and IKKγ (Zandi and Karin, 1999). IKK1 and IKK2 are very similar protein kinases that act as the catalytic subunits of the complex (DiDonato et al., 1997; Mercurio et al., 1997; Regnier et al., 1997; Woronicz et al., 1997; Zandi et al., 1997). In mammalian cells, IKK1 and IKK2 form a stable heterodimer that is tightly associated with IKKγ, a regulatory subunit (Rothwarf et al., 1998). The physiological function of the two catalytic subunits is still unclear. Initially, overexpression of catalytically inactive forms of IKK1 and IKK2 that block IKK and NF-κB activation suggested that both subunits play similar, and possibly redundant, roles in IκB phosphorylation and NF-κB activation (Zandi et al., 1997). Recent studies have shown that IKK2, not IKK1, is the target for proinflammatory stimuli and plays the major role in IKK activation and induction of NF-κB activity (Delhase et al., 1999; Li et al., 1999). However, our results show that TNF-α-induced COX-2 promoter activity in NCI-H292 cells is inhibited by the dominant negative mutants for both IKK1 (KM) and IKK2 (KM). This is consistent with the findings that the IKK1 (KM, AA, or KA) mutant and the IKK2 (KM, AA, or KA) mutant inhibit TNF-α-induced κB-dependent transcription in HeLa and 293 cells (Mercurio et al., 1997; Woronicz et al., 1997). C2-ceramide-induced COX-2 promoter activity was also inhibited by the dominant negative mutants

for both IKK1 and IKK2, indicating that IKK1/2 is involved in the downstream of activation of MAPKs in COX-2 expression induction. IKK activity was stimulated by both TNF-α and C2-ceramide and inhibited by PD98059 and SB203580, confirming that activation of MAPKs occurs downstream of ceramide in IKK activation. Thus, TNF-α acts via sequential activation of MAPKs, IKK1/2, and NF-κB in the COX-2 promoter to induce COX-2 expression.

In summary, the signaling pathway involved in TNF-α-induced COX-2 expression in NCI-H292 cells has been explored. In addition to activating the phosphatidylinositol-phospholipase Cγ2 pathway (Chen et al., 2000b), TNF-α also activates neutral SMase to induce ceramide formation, which is followed by sequential activation of p44/42 MAPK, p38, JNK, IKK1/2, and NF-κB in the COX-2 promoter, then initiation of COX-2 expression. A schematic representation of the signaling pathway for the TNF-α-induced COX-2 expression in NCI-H292 epithelial cells is shown in Fig. 10.

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