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TNF-α-Induced Cyclooxygenase-2 Expression in Human Lung Epithelial Cells: Involvement of the Phospholipase C-γ2, Protein Kinase C-α, Tyrosine Kinase, NF-κB-Inducing Kinase, and I-κB Kinase 1/2 Pathway[1] FREE

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TNF-α-Induced Cyclooxygenase-2 Expression in Human Lung Epithelial Cells: Involvement of the Phospholipase C-γ2, Protein Kinase C-α, Tyrosine Kinase, NF-κB-Inducing Kinase, and I-κB Kinase 1/2 Pathway[1]

Ching-Chow Chen,[2] Yi-Tao Sun, Jun-Jie Chen, and Kuo-Tung Chiu

TNF-α induced a dose- and time-dependent increase in cyclooxygenase-2 (COX-2) expression and PGE_2 formation in human NCI-H292 epithelial cells. Immunofluorescence staining demonstrated that COX-2 was expressed in cytosol and nuclear envelope. Tyrosine kinase inhibitors (genistein or herbimycin) or phosphoinositide-specific phospholipase C inhibitor (U73122) blocked TNF-α-induced COX-2 expression. TNF-α also stimulated phosphatidylinositol hydrolysis and protein kinase C (PKC) activity, and both were abolished by genistein or U73122. The PKC inhibitor, staurosporine, also inhibited TNF-α-induced response. The 12-*O*-tetradecanoylphorbol 13-acetate (TPA), a PKC activator, also stimulated COX-2 expression, this effect being inhibited by genistein or herbimycin. NF-κB DNA-protein binding and COX-2 promoter activity were enhanced by TNF-α, and these effects were inhibited by genistein, U73122, staurosporine, or pyrolidine dithiocarbamate. TPA stimulated both NF-κB DNA-protein binding and COX-2 promoter activity, these effects being inhibited by genistein, herbimycin, or pyrolidine dithiocarbamate. The TNF-α-induced, but not the TPA-induced, COX-2 promoter activity was inhibited by phospholipase C-γ2 mutants, and the COX-2 promoter activity induced by either agent was attenuated by dominant-negative mutants of PKC-α, NF-κB-inducing kinase, or I-κB (inhibitory protein that dissociates from NF-κB) kinase (IKK)1 or 2. IKK activity was stimulated by both TNF-α and TPA, and these effects were inhibited by staurosporine or herbimycin. These results suggest that, in NCI-H292 epithelial cells, TNF-α might activate phospholipase C-γ2 via an upstream tyrosine kinase to induce activation of PKC-α and protein tyrosine kinase, resulting in the activation of NF-κB-inducing kinase and IKK1/2, and NF-κB in the COX-2 promoter, then initiation of COX-2 expression and PGE_2 release. ***The Journal of Immunology*****, 2000, 165: 2719–2728.**

The cyclooxygenases (COX),[3] also referred to as PG endoperoxide synthases, catalyze the rate-limiting step in the synthesis of PGs, a potent group of autocrine and paracrine lipid mediators (1, 2) that are implicated in many normal cellular and pathophysiological processes, such as inflammation, edema, bronchoconstriction, platelet aggregation, fever, and hyperalgesia (1–3). Two forms of COX have been identified, these being the constitutively expressed form, COX-1, and the inducible form, COX-2. Both isoforms perform two enzymatic functions; as COX, they convert arachidonic acid into PGG_2, and, as peroxidases, they convert PGG_2 into PGH_2, which serves as a common precursor for PGs, prostacyclin, and thromboxanes (4). The two COX isoforms are encoded by distinct genes. That coding for COX-1 is a housekeeping gene and appears to be responsible for the production of PGs that mediate normal physiological functions, such as maintenance of the integrity of the gastric mucosa and regulation of renal blood flow (1, 5). In contrast, COX-2 is thought to mediate inflammatory events and shows low basal expression, but is rapidly induced by proinflammatory mediators (6, 7). Epithelial cells play an active role in inflammation by producing various cytokines and eicosanoids (8). Airway epithelial cells respond to proinflammatory cytokines, such as IL-1β, by induction of COX-2 and release of PGE_2 (6).

Induction of COX-2 expression requires de novo mRNA and protein synthesis (7), indicating regulation at the transcriptional level. 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. Transcriptional activation in response to extracellular signals involves the regulated assembly of multiprotein complexes on enhancers and promoters (9). In the human COX-2 gene, the nucleotide sequence of the 5′-flanking region contains a canonical TATA box and the consensus sequences of the NF-κB, NF-IL-6 (CCAAT/enhancer-binding protein β; C/EBPβ), and cAMP response element (CRE) sites in the 275-bp region upstream of the transcriptional start site (10). Sequences homologous to the consensus sequences of the NF-κB, NF-IL-6, and CRE sites are also found in the corresponding regions of the mouse (11, 12) and rat (13) COX-2 gene. The NF-κB consensus sites are found in the mouse COX-2 promoter region and are important in COX-2 mRNA induction by TNF-α (12). CRE and C/EBPβ act as positive regulatory elements for COX-2 transcription (14–16).

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[3] Abbreviations used in this paper: COX, cyclooxygenase; C/EBPβ, CCAAT/enhancer-binding protein β; CRE, cAMP response element; DAG, diacylglycerol; I-κB, inhibitory protein that dissociates from NF-κB; IKK, I-κB kinase; IP, inositol phosphate; NIK, NF-κB-inducing kinase; PC-PLC, phosphatidylcholine-specific phospholipase; PDTC, pyrolidine dithiocarbamate; PI, phosphatidylinositol; PI-PLC, phosphoinositide-specific phospholipase C; PKC, protein kinase C; PLC, phospholipase C; TPA, 12-*O*-tetradecanoylphorbol 13-acetate.

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TNF-α is a pleiotropic cytokine, primarily produced by activated macrophages and some T lymphocytes, that is involved in a wide range of biological effects, including inflammation, mitogenesis, differentiation, immune modulation, and antitumor immunity (17). These activities result from interaction with specific cell surface receptors expressed in most cell types. Two types of TNF receptor, TNFR55 and TNFR75, with respective molecular masses of 55 and 75 kDa, have been characterized (18). TNF-α is synthesized as a 26-kDa type II transmembrane proform, which is then processed by a metalloprotease, resulting in the release of mature TNF-α, consisting of three 17-kDa subunits (19, 20); this trimeric ligand can bind to TNFR55 or TNFR75, thereby engaging three receptor chains. Although TNF-α has a higher affinity for TNFR75 than for TNFR55, most of the biological responses of TNF-α are thought to be mediated through TNFR55 (18). Ligand-bound trimeric TNFR55 recruits TNFR-associated proteins (e.g., TNFR-associated death domain protein, Fas-associated death domain protein, and TNFR-associated factor 2) to its intracytoplasmic domain to generate intracellular signaling cascades that act via second messengers, including ceramide, diacylglycerol (DAG), and arachidonic acid metabolites (21). Although, in the main, not well understood, certain intracellular signaling pathways by which TNF-α induces COX-2 expression have been proposed; these include the activation of tyrosine kinase (22) and protein kinase C (PKC) (23). However, the relationship between these pathways is unknown. In the present study, we explored the intracellular signaling pathway involved in TNF-α-induced COX-2 expression in a human alveolar epithelial cell line, NCI-H292. The results show that TNF-α might activate phospholipase C-γ2 (PLC-γ2) via an upstream tyrosine kinase, resulting in activation of PKC-α, protein tyrosine kinase, NF-κB-inducing kinase (NIK), I-κB kinase (IKK) 1/2, and NF-κB in the COX-2 promoter, which is followed by COX-2 expression and PGE_2 release.

Materials and Methods

Materials

Goat polyclonal Abs specific for COX-1 or COX-2 or rabbit polyclonal Abs specific for the p65, p50, or p52 subunit of NF-κB or for I-κB-α or IKKβ were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Human rTNF-α was purchased from R&D Systems (Minneapolis, MN). RPMI 1640 medium, FCS, penicillin, and streptomycin were obtained from Life Technologies (Gaithersburg, MD). Staurosporine, pyrolidine dithiocarbamate (PDTC), and histone III-S were obtained from Sigma (St. Louis, MO). D609, U73122, U73343, genistein, and herbimycin were obtained from Calbiochem (San Diego, CA). T4 polynucleotide kinase was obtained from New England Biolabs (Beverly, MA). Poly(dI-dC), the PGE_2 enzyme immunoassay kit, HRP-labeled donkey anti-rabbit second Ab, and the enhanced chemiluminescence (ECL) detecting reagent were obtained from Pharmacia Biotech (Uppsala, Sweden). Myo-[^{3}H]inositol (23.5 Ci/mmol) and [γ-^{32}P]ATP (3000 Ci/mmol) were obtained from DuPont-New England Nuclear (Boston, MA). FITC-conjugated and HRP-conjugated rabbit anti-goat IgG Abs were obtained from Cappel (Aurora, OH). Tfx-50 and the luciferase assay kit were obtained from Promega (Madison, WI).

Cell culture

NCI-H292 cells, a human alveolar epithelial cell carcinoma, were obtained from American Type Culture Collection (Manassas, VA) and cultured in RPMI 1640 supplemented with 10% FCS, 100 U/ml of penicillin, and 100 μg/ml of streptomycin in six-well plates for experiments involving COX-2 expression, phosphatidylinositol (PI) hydrolysis assay, and transfection on 24-mm glass coverslips in 35-mm dishes for COX-2 immunofluorescence studies, in 6-cm dishes for PKC activity measurements, or in 10-cm dishes for the NF-κB gel-shift assay.

Plasmids

The COX-2 promoter construct (−459/+9) was a generous gift from Dr. L. H. Wang (University of Texas, Houston, TX). The PLC-γ2 mutants were gifts from Dr. T. Kurosaki (Kansai Medical University, Moriguchi, Japan) (the mutants SH2(N) and SH2(C) in which Arg^{564} or Arg^{672} are replaced, respectively, by Ala). The dominant-negative mutants were provided by Dr. A. Altman (La Jolla Institute for Allergy and Immunology, San Diego, CA) (the PKC-α mutant, PKC-α/KR), Dr. M. Karin (University of California, San Diego, CA) (the NIK mutant KKAA), and Signal Pharmaceutical (San Diego, CA) (the IKK1 mutant KM and the IKK2 mutant KM). pGEX-I-κBα(1–100) was a gift from Dr. Nakano (University of Junteudo, Tokyo, Japan).

Preparation of cell extracts and Western blot analysis of COX-2 and COX-1

Following treatment with TNF-α or 12-*O*-tetradecanoylphorbol 13-acetate (TPA), the cells were harvested and cell lysates were prepared and subjected to SDS-PAGE using 7.5% running gels, as described previously (24). The proteins were transferred to a nitrocellulose membrane, which was then incubated successively at room temperature with 0.1% milk in TTBS (50 mM Tris-HCl, pH 7.5, 0.15 M NaCl, and 0.05% Tween-20) for 1 h, with goat Ab specific for COX-2 or COX-1 for 1 h, and with HRP-labeled anti-goat Ab for 30 min. After each incubation, the membrane was washed extensively with TTBS. The immunoreactive band was detected using ECL detection reagent and developed with Hyperfilm-ECL (Pharmacia Biotech). Quantitative data were obtained using a computing densitometer and ImageQuant software (Molecular Dynamics, Sunnyvale, CA). In pretreatment experiments, cells were incubated for 30 min at 37°C with the tyrosine kinase inhibitors, genistein and herbimycin, the phosphoinositide-specific phospholipase C (PI-PLC) inhibitor, U73122, or the PKC inhibitor, staurosporine, before addition of TNF-α for 16 h. At the concentrations used, these inhibitors had no cytotoxic effect on NCI-H292 cells.

Immunofluorescence staining

NCI-H292 cells grown on coverslips were treated for 16 h with TNF-α or TPA in growth medium, rapidly washed with PBS, then fixed at room temperature for 10 min with 3.7% paraformaldehyde. After washing with PBS, the cells were blocked for 15 min with 1% BSA in TTBS containing 0.1% Triton X-100, then incubated with anti-COX-2 Ab (1:100) for 1 h, washed extensively, and stained for 30 min with anti-goat IgG-fluorescein (1:200). After further washes, the coverslips were mounted on glass slides using mounting medium (2% *n*-propyl gallate in 60% glycerol, 0.1 M PBS, pH 8). Optical sections of the immunostained cells were observed and photographed using a Zeiss Axiovert inverted microscope equipped with a photoMicroGraph Digitized Integration System (Zeiss, Oberkochen, Germany).

Measurement of PI hydrolysis

PI hydrolysis was assessed by measuring the accumulation of [^{3}H]inositol phosphates (IP) in cells labeled by 24-h incubation in growth medium containing myo-[^{3}H]inositol (2.5 μCi/ml), as previously described (25). After incubation, the cells were washed with physiologic salt solution (PSS: 118 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgCl_2$, 1.2 mM KH2PO4, 11 mM glucose, and 20 mM HEPES, pH 7.4) containing 10 mM LiCl and incubated at 37°C for 20 min. Pretreatment with 10 μM U73122 or U73343 or 30 μM genistein was performed by adding the reagent to the PSS 30 min before stimulation with 30 ng/ml of TNF-α for 10 min.

PKC activity assay

Cells treated with TNF-α for 10 min, 1 h, or 2 h were harvested, and membrane fractions were prepared and assayed for PKC activity, as previously described (26); the assay was performed at 30°C for 5 min in 25 μl of 30 mM Tris-HCl buffer, pH 7.5, containing 6 mM magnesium acetate, 0.12 mM [γ-^{32}P]ATP, 0.4 mM $CaCl_2$, 40 μg/ml of phosphatidylserine, 8 μg/ml of 1,2-dioleoylglycerol, 1 mg/ml of histone III-S, and the membrane preparation (2.5–5 μg protein). The Ca^{2+}- and phospholipid-independent activity was measured under the same conditions in the absence of Ca^{2+} and phospholipid and in the presence of 2 mM EGTA.

Preparation of nuclear extracts and the EMSA

Control cells, or cells pretreated with various inhibitors, were treated with TNF-α or TPA for 1 h, then nuclear extracts were isolated as described previously (24). Briefly, cells were washed with ice-cold PBS and pelleted. 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 NaVO4) and incubated for 15 min on ice before being 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),

then the tube was vigorously shaken at 4°C for 15 min on a shaking platform. The nuclear extracts were then centrifuged, and the supernatants were aliquoted and stored at −80°C.

Oligonucleotides corresponding to the NF-κB consensus sequences in the human COX-2 promoter (underlined: 5′-AGAGTGGGGACTAC CCCCTCT-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 the ^{32}P-labeled NF-κB probe (40,000–60,000 cpm) in 10 μl of binding buffer (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 (24). 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. When supershift assays were performed, polyclonal Abs specific for p65, p50, or p52 were added to the nuclear extracts 30 min before the binding reaction, and the DNA/nuclear protein complexes were separated on a 4.5% polyacrylamide gel.

In NF-κB (p65) translocation studies, both cytosolic and nuclear extracts were used, while only cytosolic extracts were used in I-κB-α degradation studies. The extracts were subjected to SDS-PAGE using a 10% running gel, and immunoblot analysis was performed as described above.

Determination of PGE_2 concentrations

Following treatment of cells with TNF-α for 4, 8, 16, or 24 h, PGE_2 levels in the culture medium were measured using an enzyme immunoassay kit from Amersham Pharmacia Biotech (Piscataway, NJ).

Transient transfection and luciferase assay

NCI-H292 cells, grown in six-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. The plasmid pRK containing the β-galactosidase gene driven by the constitutively active SV40 promoter was used to normalize the transfection efficiency. After 10–15-min incubation at room temperature, the mixture was applied to the cells. One hour later, 1 ml of RPMI 1640 containing 20% FCS was added; from this point, the cells were grown in medium containing 10% FCS. The following day, cells were exposed to 30 ng/ml of TNF-α or 1 μM TPA for 6 h, then cell extracts were prepared and luciferase (Promega), and β-galactosidase activity were measured. The luciferase activity of each well was normalized to the β-galactosidase activity. In dominant-negative mutant experiments, cells were cotransfected with reporter and β-galactosidase and either the PLC-γ2 mutant or dominant-negative PKC-α, NIK, IKK1, or IKK2 mutant or the empty vector.

In vitro IKK activity assay

Following 10-min treatment with TNF-α or TPA or 30-min pretreatment with staurosporine or herbimycin before addition of TNF-α or TPA, cells were rapidly washed with PBS, then lysed with ice-cold lysis buffer, as described above, and the IKK proteins immunoprecipitated. Fifty micrograms of total cell extract were incubated for 1 h at 4°C with 0.5 μg of anti-IKKβ Ab, and the Ab-bound protein was 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 Na3VO4, 1 mM DTT, 1 μg of bacterially expressed GST-I-κBα(1–100), and 10 μM [γ-^{32}P]ATP. The reaction was stopped by the addition of Laemmli buffer and subjected to 10% SDS-PAGE, phosphorylated-GST-I-κBα(1–100) being visualized by autoradiography.

Results

TNF-α induced COX-2 expression in NCI-H292 cells

When the cells were exposed to 10 ng/ml of IL-1β, TNF-α, or IFN-γ, 1 μg/ml of LPS, or 1 μM TPA, only TNF-α and TPA stimulated COX-2 expression (data not shown). TNF-α induced COX-2 expression in a concentration- and time-dependent manner. For an exposure period of 16 h, maximum COX-2 expression was obtained using 30 ng/ml of TNF-α (Fig. 1), while, when cells were treated with 30 ng/ml of TNF-α for various times, COX-2 expression was significant at 4 h and maximal at 16 h, remaining at this level for up to at least 24 h. COX-1 expression was not affected by

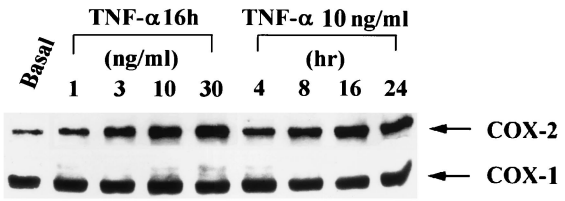

FIGURE 1. Concentration- and time-dependent TNF-α-induced COX-2 expression in NCI-H292 epithelial cells. Cells were incubated at 37°C with various concentrations of TNF-α for 16 h or with 30 ng/ml of TNF-α for various time intervals. Whole cell lysates were prepared and subjected to Western blotting using Ab specific for COX-1 or COX-2, as described in *Materials and Methods*.

these treatments (Fig. 1). Fig. 2 shows the concentration- and time-dependent production of PGE_2 in response to TNF-α. The basal release of PGE_2 was 61.6 pg/μg of total protein. After 16-h treatment with 1, 3, 10, or 30 ng/ml of TNF-α, this rose to 140, 194, 274, or 323 pg/μg protein, respectively (Fig. 2*A*), while, after treatment with 30 ng/ml of TNF-α for 4, 8, 12, or 24 h, PGE_2 release was 81, 102, 279, or 475 pg/μg protein, respectively (Fig. 2*B*). NS-398 (COX-2 inhibitor) (10 μM) (27) attenuated the PGE_2 release induced by 16-h treatment with 30 ng/ml of TNF-α (323 pg/μg protein) to 102 pg/μg protein. Induction of COX-2 by TNF-α was further demonstrated by immunofluorescence staining. As shown in Fig. 3, no COX-2 expression was seen in the basal state (Fig. 3*B*), but was apparent in the cytosol and nuclear envelope following TNF-α treatment (Fig. 3*D*). In the following COX-2 expression experiments, the cells were treated with 30

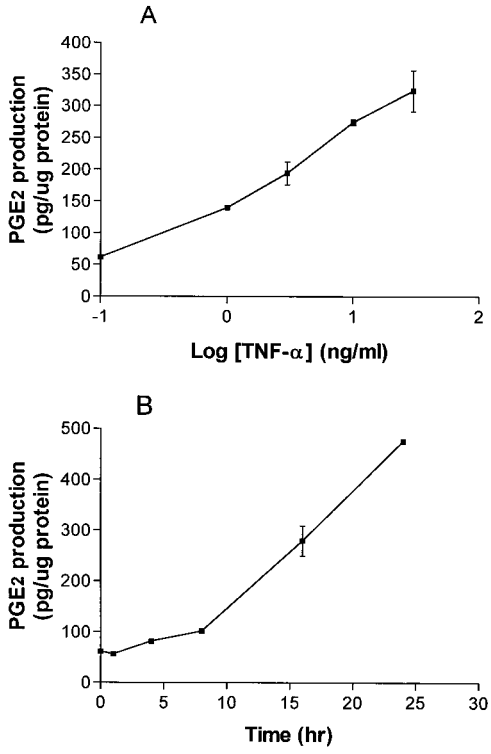

FIGURE 2. Concentration- and time-dependent TNF-α-induced PGE_2 production in NCI-H292 epithelial cells. Cells were incubated at 37°C with various concentrations of TNF-α for 16 h or with 30 ng/ml of TNF-α for various time intervals, then the medium was removed and analyzed for PGE_2 production. Results are expressed as the mean ± SEM of three independent experiments performed in triplicate.

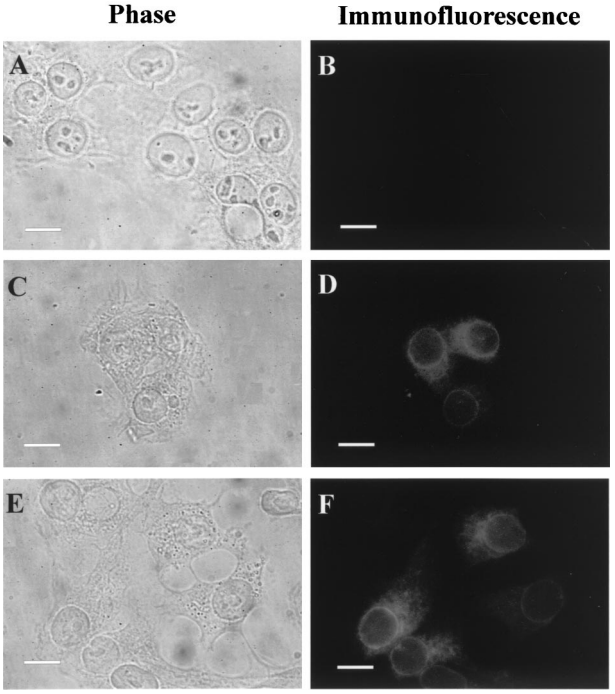

FIGURE 3. COX-2 is located around the nuclear envelope. Immunofluorescent staining of NCI-H292 epithelial cells with affinity-purified anti-COX-2 Ab (1:100). Cells were fixed and stained as described in *Materials and Methods*. Control cells (*A*, *B*), cells after 16-h treatment with 30 ng/ml of TNF-α (*C*, *D*), and cells after 16-h treatment with 1 μM TPA (*E*, *F*). Bar = 200 μm.

ng/ml of TNF-α for 16 h; under these conditions, both transcriptional (actinomycin D) and translational (cycloheximide) inhibitors blocked the TNF-α-induced COX-2 expression (data not shown).

Inhibitory effect of tyrosine kinase, PI-PLC, or PKC inhibitors on TNF-α-induced COX-2 expression and activation of PKC by TNF-α

To study the intracellular signaling pathway involved in TNF-α-induced COX-2 expression, the tyrosine kinase inhibitors, genistein and herbimycin (28, 29), were used. When cells were pretreated for 30 min with 10–100 μM genistein or 1 μM herbimycin, TNF-α-induced COX-2 expression was inhibited. The extent of inhibition seen using 10, 50, or 100 μM genistein was 49%, 93%, or 97%, respectively, while that using 1 μM herbimycin was 87% (Fig. 4*A*). When cells were pretreated with 10 μM U73122 (a PI-PLC inhibitor) (30), TNF-α-induced COX-2 expression was inhibited by 86%, while 10 μM U73343 (an inactive analogue of U73122), 100 μM D609 (a PC-PLC inhibitor) (31), or 100 μM propranolol (a phosphatidate phosphohydrolase inhibitor) (32) had no effect (Fig. 4*B*).

Because TNF-α-induced COX-2 expression was inhibited by U73122, indicating involvement of the PI-PLC pathway, TNF-α-induced PI hydrolysis was measured. Following 10-min treatment of cells with 30 ng/ml of TNF-α, a 5.5-fold increase in IP formation was seen; this effect was less marked after 1 h, although the values were still higher than basal (Fig. 5*A*). The TNF-α-induced IP formation seen after 10-min treatment was inhibited by 96% or 94% by 10 μM U73122 or 30 μM genistein, respectively, while 10 μM U73343 had no effect (Fig. 5*B*), indicating that, in NCI-H292 cells, TNF-α stimulates the PI-PLC pathway by activating tyrosine kinase.

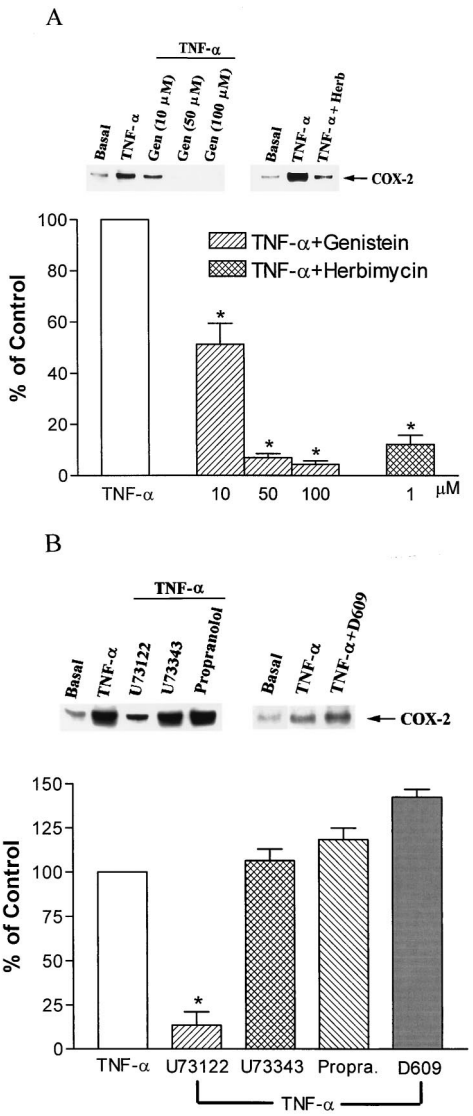

FIGURE 4. Inhibitory effect of genistein, herbimycin, or U73122 on TNF-α-induced COX-2 expression in NCI-H292 epithelial cells. Cells were pretreated with the indicated concentrations of genistein or with 1 μM herbimycin (*A*) or with 10 μM U73122 or U73343 or 100 μM propranolol or D609 (*B*) for 30 min before incubation with 30 ng/ml of TNF-α for 16 h. Whole cell lysates were prepared and subjected to Western blotting using Ab specific for COX-2, as described in *Materials and Methods*. COX-2 expression was quantified using a densitometer with ImageQuant software. Results are expressed as the mean ± SEM of three independent experiments. *, $p < 0.05$ as compared with TNF-α alone.

In addition to causing increased IP formation, the PI-PLC pathway also increases DAG levels, then activates PKC. PKC activity was measured after treatment with 30 ng/ml of TNF-α for various times. As shown in Fig. 6*A*, membrane PKC activity increased 3-fold after treatment with TNF-α for 10 min; after 2 h, the effect was slightly less, although still higher than basal (Fig. 6*A*). U73122 (10 μM) or genistein (30 μM) inhibited the TNF-α-induced increase in PKC activity by 90% and 83%, respectively, while 10 μM U73343 had no effect (Fig. 6*B*).

To determine whether PKC activation by TNF-α was involved in the regulation of TNF-α-induced COX-2 expression, staurosporine, a PKC inhibitor (33), was used. Following pretreatment of cells with 30 or 100 nM staurosporine, TNF-α-induced COX-2 expression was inhibited by 64% or 82%, respectively (Fig. 7).

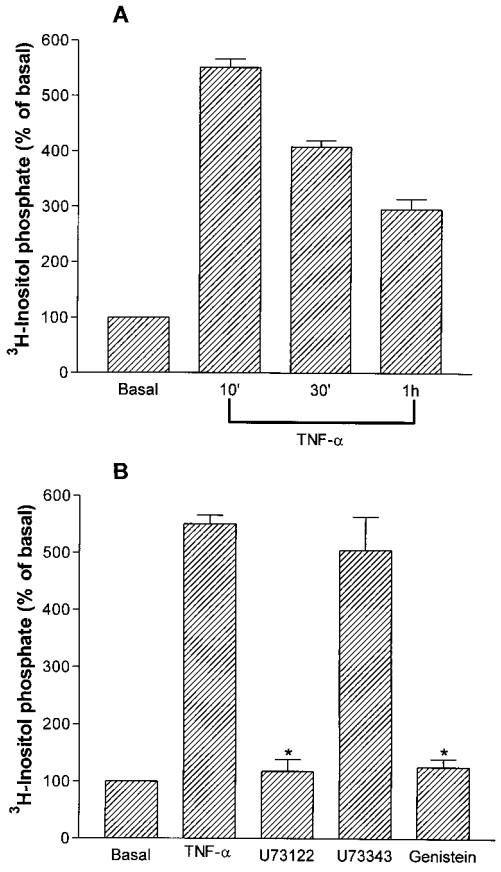

FIGURE 5. [^{3}H]Inositol phosphate formation in response to TNF-α in NCI-H292 cells and the effect of U73122, U73343, or genistein. Prelabeled cells were treated with 30 ng/ml of TNF-α for various time intervals (*A*) or pretreated with 10 μM U73122 or U73343 or 30 μM genistein for 30 min before incubation with 30 ng/ml of TNF-α for 10 min (*B*). Results are expressed as the mean $\pm$ SEM of three independent experiments performed in triplicate. $*$, $p < 0.05$ as compared with the basal level (*A*) or TNF-α alone (*B*).

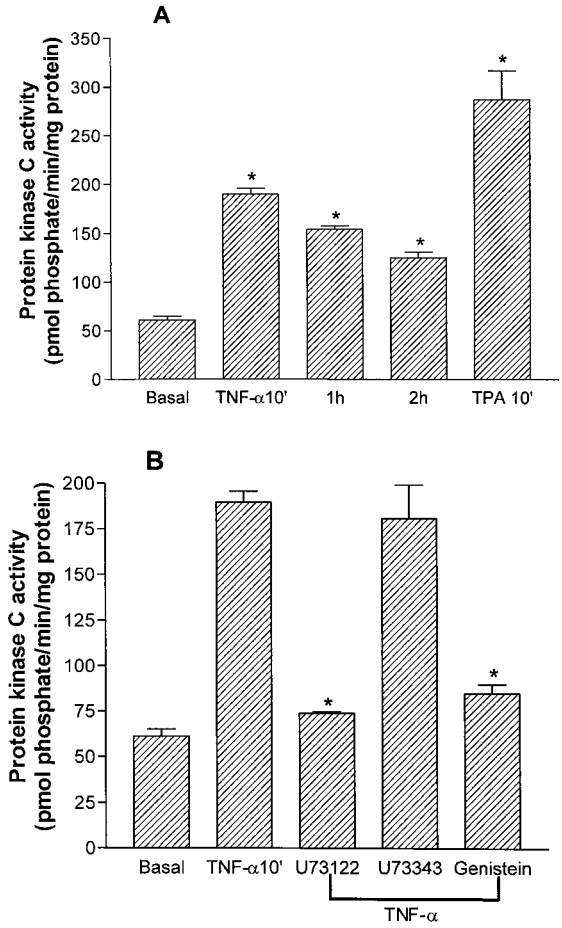

FIGURE 6. PKC activity in response to TNF-α in NCI-H292 epithelial cell membranes and the effect of U73122, U73343, or genistein. Cells were incubated with 30 ng/ml of TNF-α for the indicated time or with 1 μM TPA for 10 min (*A*) or pretreated with 30 μM U73122 or U73343 or 30 μM genistein for 30 min before incubation with 30 ng/ml of TNF-α for 10 min (*B*), then fractionated into cytosol and membranes. PKC activity in the membrane was measured as described in *Materials and Methods*. The results are expressed as the mean $\pm$ SEM of three independent experiments performed in duplicate. $*$, $p < 0.05$ as compared with the basal level (*A*) or TNF-α alone (*B*).

Other PKC inhibitors, such as Ro 31-8220 or calphostin C, were not tested in this study because of cytotoxicity seen after 16 h of incubation. Because PKC had been shown to be involved, the effect of direct TPA-mediated PKC activation on COX-2 expression was examined. TPA (1 μM) also induced a time-dependent increase in COX-2 expression, which was significant at 4 h, maximal at 16 h, then declined after 24 h (Fig. 8*A*). COX-1 expression was not affected by TPA treatment. Induction of COX-2 expression by TPA was also demonstrated by immunofluorescence staining (Fig. 3*F*). When cells were pretreated with 10–100 μM genistein or 1 μM herbimycin, TPA-induced COX-2 expression was inhibited, the extent of inhibition being 49%, 79%, or 90% using 10, 30, or 100 μM genistein, respectively, and 81% using 1 μM herbimycin (Fig. 8*B*). TPA-induced COX-2 expression was inhibited by staurosporine (data not shown).

NF-κB induction in the nuclei of TNF-α-stimulated NCI-H292 cells, and inhibition by PDTC, genistein, U73122, or staurosporine

PDTC, an antioxidant, caused dose-dependent inhibition of TNF-α- or TPA-induced COX-2 expression and induction of NF-κB DNA-protein-binding activity (Fig. 9; see Fig. 11, *A* and *E*), 100 and 200 μM PDTC, respectively, inhibiting the TNF-α-induced COX-2 expression by 26% or 55% and the TPA response by 69% and 77% (Fig. 9).

The time course of NF-κB activation after treatment with TNF-α was examined. Nuclear extracts prepared from NCI-H292 cells were assayed by EMSA for activated NF-κB. In nonstimulated NCI-H292 cells, one faint NF-κB-specific DNA-protein complex was identified. TNF-α rapidly (10 min) activated NF-κB, similar activation being seen after 1 and 24 h (Fig. 10*A*). For subsequent EMSA experiments, cells were treated with TNF-α for 1 h. To identify the specific subunits involved in the formation of the banding pattern of the NF-κB dimer after TNF-α stimulation, supershift assays were performed in the presence of Abs specific for the p65, p50, or p52 subunit. As shown in Fig. 10*B*, incubation with anti-p65 or anti-p50 Abs induced a supershift (arrows a and b, respectively), but no shift occurred in the presence of anti-p52 Ab. Thus, our data demonstrate the presence of the p65/p50 NF-κB heterodimer in NCI-H292 cells. To characterize the proteins involved in NF-κB activation, the amount of p65 in cytosolic and nuclear extracts was assayed by Western blotting. As shown in Fig. 10*C*, p65 was rapidly (10 min) translocated from the cytosol

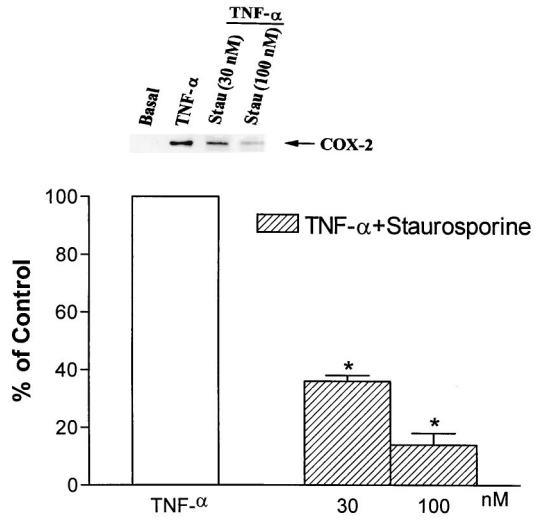

FIGURE 7. Effect of staurosporine on TNF-α-induced COX-2 expression in NCI-H292 epithelial cells. Cells were pretreated with 30 or 100 nM staurosporine for 30 min before incubation with 30 ng/ml of TNF-α for 16 h. Whole cell lysates were prepared and subjected to Western blotting using Ab specific for COX-2, as described in *Materials and Methods*. COX-2 expression was quantified using a densitometer with ImageQuant software. Results are expressed as the mean ± SEM of three independent experiments. *, $p < 0.05$ as compared with TNF-α alone.

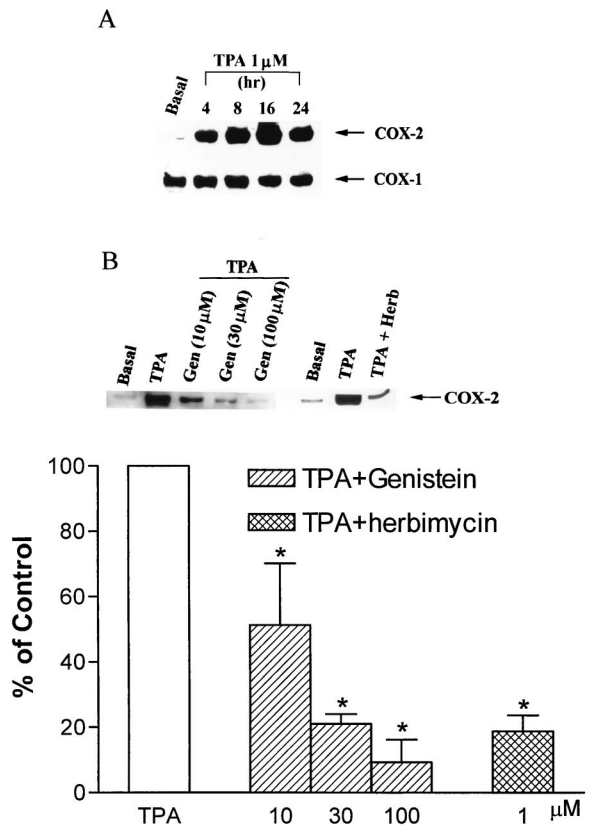

FIGURE 8. Time-dependent TPA-induced COX-2 expression in NCI-H292 epithelial cells and the inhibitory effect of genistein or herbimycin. In *A*, cells were incubated at 37°C with 1 μM TPA for various time intervals. In *B*, cells were pretreated with the indicated concentrations of genistein or with 1 μM herbimycin for 30 min before incubation with 1 μM TPA for 16 h. Whole cell lysates were prepared and subjected to Western blotting using Ab specific for COX-2 or COX-1, as described in *Materials and Methods*. COX-2 expression was quantified using a densitometer with ImageQuant software. Results are expressed as the mean ± SEM of three independent experiments performed in triplicate. *, $p < 0.05$ as compared with TPA alone.

to the nuclear compartment in stimulated cells. Because the amount of NF-κB protein released to migrate to the nucleus is thought to be proportional to the degradation of I-κB, the I-κB-α protein level in the cytosol was also measured. As shown in Fig. 10*C*, TNF-α rapidly induced complete degradation of I-κB-α, but the level was restored after 1 h of TNF-α treatment.

After treatment of cells for 30 min with 10 μM U73122, 30 μM genistein, or 100 nM staurosporine, the TNF-α-elicited activation of NF-κB-specific DNA-protein complex formation was inhibited (Fig. 11, *A* and *B*). Following exposure of cells to 1 μM TPA, rapid activation (10 min) of NF-κB was seen; this was sustained for 24 h (Fig. 11*C*) and inhibited by 30 μM genistein (Fig. 11*D*).

Induction of COX-2 promoter activity by TNF-α and the inhibitory effect of PDTC, genistein, herbimycin, U73122, staurosporine, PLC-γ2 mutants, or dominant-negative mutants of PKC-α, NIK, or IKK1/2

To further study the involvement of the PI-PLC-dependent PKC pathway in TNF-α-induced COX-2 expression, transient transfections were performed using the human COX-2 promoter-luciferase construct, pGS459 (−459/+9) (34). This construct contains both upstream (−447/−438) and downstream (−223/−214) NF-κB sites in the COX-2 promoter. Treatment with 30 ng/ml TNF-α or 1 μM TPA led to a 3.2- or 5.2-fold increase, respectively, in COX-2 promoter activity, these effects being inhibited by 64% or 54%, respectively, by 100 μM PDTC (Fig. 12*A*). These data further indicate that NF-κB is responsible for mediating TNF-α- or TPA-induced COX-2 expression in NCI-H292 cells. The TNF-α-induced COX-2 promoter activity was blocked by genistein, herbimycin, U73122, or staurosporine, while the TPA-induced activity was inhibited by genistein or herbimycin (Fig. 12*A*). In cotransfection experiments, the induction of COX-2 promoter activity by TNF-α was inhibited by the mutant PLC-γ2 SH2(N) or PLC-γ2 SH2(C) (Fig. 12*B*), or by the dominant-negative mutants PLC-α/KR, NIK (KKAA), or IKK1/2 (KM) (Fig. 12*C*), while that induced by TPA was inhibited by the dominant-negative mutants PKC-α/KR, NIK (KKAA), or IKK1 or 2 (KM), but not by the mutant PLC-γ2 SH2(N) or PLC-γ2 SH2(C) (Fig. 12, *B* and *C*).

Induction of IKK activation by TNF-α or TPA, and inhibitory effect of herbimycin

The endogenous IKK complex was isolated by immunoprecipitation with anti-IKKβ Ab and tested for in vitro kinase activity. As shown in Fig. 13, both TNF-α and TPA induced IKK activation, and these effects were inhibited by staurosporine and herbimycin.

Discussion

In the present study, we have shown that TNF-α induced COX-2 expression in NCI-H292 cells and that this occurred in the cytosol and nuclear envelope, as demonstrated by immunofluorescence staining. The immunofluorescence results are consistent with previous findings that COX is detected in the nuclear envelope and endoplasmic reticulum (35), and with the observation of cytosolic PLA_2 (phosholipase A_2 from honey bee venom) translocation to the nuclear envelope and endoplasmic reticulum (36, 37). Two NF-κB consensus sites are found in the promoter region of the human COX-2 gene (34), these being the NF-κB-5′ site (−447/−438) and the NF-κB-3′ site (−223/−214). The NF-κB-5′ site has been shown to be involved in TNF-α-mediated COX-2 induction in a

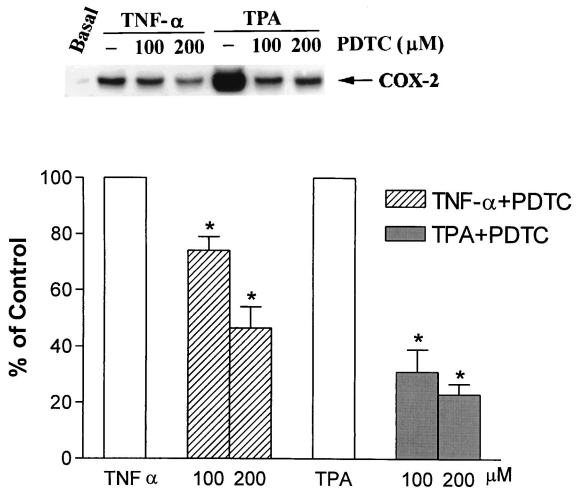

FIGURE 9. Inhibitory effect of PDTC on TNF-α-induced or TPA-induced COX-2 expression in NCI-H292 epithelial cells. Cells were pretreated with the indicated concentrations of PDTC for 30 min before incubation with 30 ng/ml of TNF-α or 1 μM TPA for 16 h. Whole cell lysates were prepared and subjected to Western blotting using Ab specific for COX-2, as described in *Materials and Methods*. COX-2 expression was quantified using a densitometer with ImageQuant software. Results are expressed as the mean $\pm$ SEM of three independent experiments. *, $p < 0.05$ as compared with TNF-α or TPA alone.

mouse osteoblast cell line (12). The NF-κB-3′ site, in concert with the NF-IL-6 and CRE sites, may play a role in facilitating the induction of COX-2 by LPS and phorbol ester in bovine aortic endothelial cells (38). In NCI-H292 cells, TNF-α increased the levels of the NF-κB-specific DNA-protein complex in nuclear extracts (Fig. 10*A*). The NF-κB blocker, PDTC, partially inhibited TNF-α-induced COX-2 expression and NF-κB-specific DNA-protein binding (Figs. 9 and 11*A*). Induction of COX-2 promoter activity by TNF-α or TPA was also partially blocked by PDTC (Fig. 12). These results indicate that NF-κB is indeed responsible for the TNF-α-mediated induction of COX-2 expression in NCI-H292 cells. However, another transcriptional factor, NF-IL-6, might also be involved, as reported in MC3T3-E1 cells (12). TNF-α did increase C/EBP-specific DNA-protein binding in NCI-H292 cells (data not shown). EMSA studies showed rapid activation (10 min) of NF-κB in response to TNF-α stimulation (Fig. 10*A*) and the parallel translocation of p65 into the nucleus. Complete degradation of I-κB-α was also seen (Fig. 10*C*), as reported in RAW 264.7 cells exposed to LPS (39), and seen in A549 cells exposed to TNF-α or IL-1β (40). The supershift assay demonstrated the presence of the p65/p50 NF-κB heterodimer in NCI-H292 cells (Fig. 10*B*), in contrast to the p50/p50 homodimer and p65/p50 heterodimer found in human hepatic stellate cells (41).

We demonstrated that the PKC inhibitor, staurosporine, inhibited the TNF-α-mediated induction of COX-2 expression in a dose-dependent manner, indicating that PKC activation is an obligatory event in TNF-α-mediated COX-2 expression in these cells. This was further confirmed by the result that the dominant-negative PKC-α mutant, PKC-α/KR, inhibited the TNF-α-induced COX-2 promoter activity (Fig. 12*B*). TNF-α caused PKC activation, this phenomenon occurring after 10-min treatment and lasting for 1 h (Fig. 4*A*). PKC is activated by the physiological activator, DAG, which can be generated either directly, by the action of PLC, or indirectly, by a pathway involving the production of phosphatidic acid by phospholipase D, followed by a dephosphorylation reaction catalyzed by phosphatidate phosphohydrolase. Normally, the PLC involved in the production of DAG is PI-PLC, but PC-PLC can also be involved (42, 43). The PI-PLC inhibitor, U73122, inhibited TNF-α-induced COX-2 expression, whereas the PC-PLC inhibitor, D609, or the phosphatidate phosphohydrolase inhibitor, propranolol, did not. The inactive analogue, U73343, had no effect. TNF-α also stimulated PI hydrolysis, this effect being blocked by U73122, but not by U73343. Genistein also blocked TNF-α-induced PI hydrolysis, indicating that the PI-PLC involved might be PLC-γ, because PLC-γ is a SH2 domain-containing protein that utilizes this module to link phosphotyrosine-containing sequences in a receptor protein or cytoplasmic protein tyrosine kinase to PI hydrolysis (44). Structurally, PLC-γ has a putative pleckstrin homology domain at its amino terminus, which is followed by the two conserved parts of the catalytic domain, separated by two tandem SH2 domains and an SH3 domain (45). Both the N-terminal SH2 (SH2(N)) and the C-terminal SH2 (SH2(C)) domain mutants of PLC-γ2 were not functional in surface IgM-induced apoptosis in B cells lacking wild-type PLC-γ2 (46). In the present study, despite the existence of endogenous PLC-γ2, either mutant inhibited TNF-α-, but not TPA-induced COX-2 promoter activity (Fig.

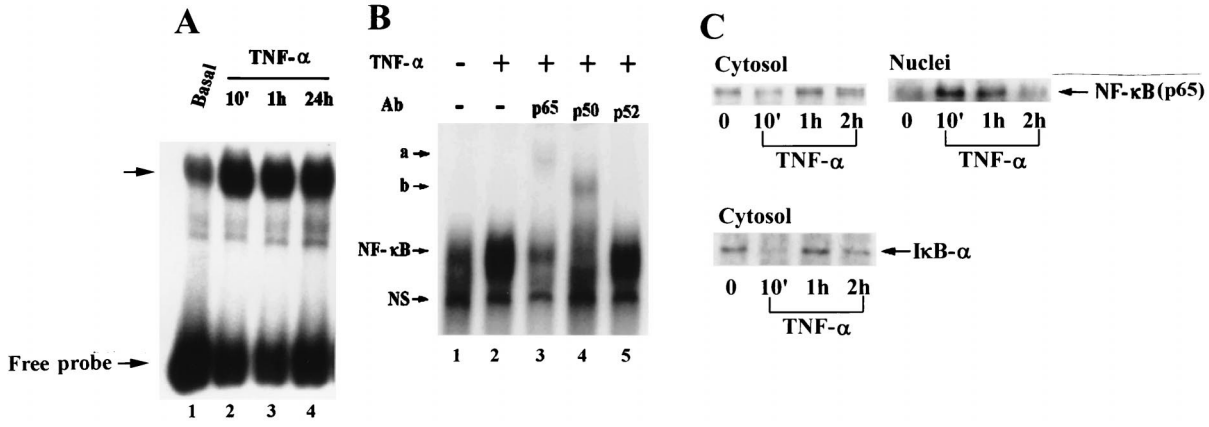

FIGURE 10. Kinetics of TNF-α-induced NF-κB-specific DNA-protein complex formation, NF-κB translocation, and I-κB-α degradation in NCI-H292 epithelial cells. Cells were treated with 30 ng/ml of TNF-α for 10 min, 1 h, or 24 h (*A*), then cytosolic and nuclear extracts were prepared. In *A*, NF-κB-specific DNA-protein-binding activity in nuclear extracts was determined by EMSA, as described in *Materials and Methods*. In *B*, supershift assays were performed using 2 μg of the indicated Abs, as described in *Materials and Methods*. In *C*, cytosolic and nuclear levels of NF-κB (p65) proteins and cytosolic levels of I-κB-α were immunodetected using specific Ab, as described in *Materials and Methods*.

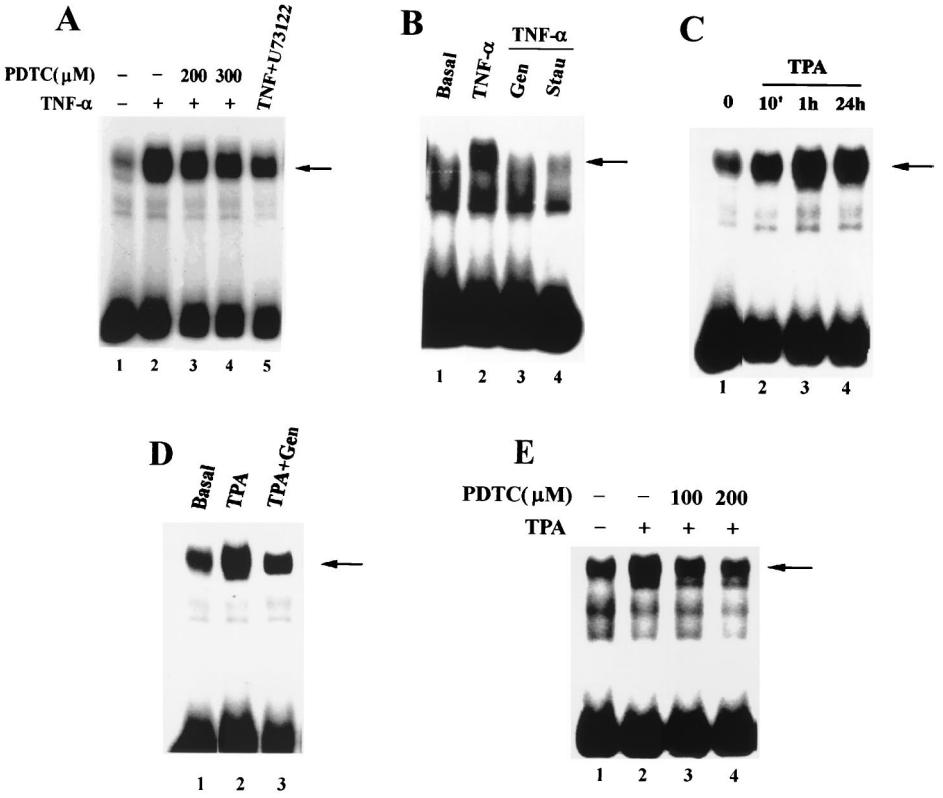

FIGURE 11. Kinetics of TPA-induced NF-κB-specific DNA-protein complex formation and the effect of various inhibitors on TNF-α-induced or TPA-induced NF-κB-specific DNA-protein complex formation in nuclear extracts of NCI-H292 epithelial cells. Cells were pretreated with 200 or 300 μM PDTC or 10 μM U73122 (*A*) or with 30 μM genistein or 30 nM staurosporine (*B*) for 30 min before incubation with 30 ng/ml of TNF-α for 1 h, or cells were treated with 1 μM TPA for 10 min, 1 h, or 24 h (*C*), or pretreated with 30 μM genistein (*D*), or 100 or 200 μM PDTC (*E*) for 30 min before incubation with 1 μM TPA for 1 h, then nuclear extracts were prepared and NF-κB DNA-protein-binding activity determined by EMSA, as described in *Materials and Methods*.

12*B*), indicating the possible involvement of PLC-γ2 in TNF-α-induced COX-2 expression in NCI-H292 cells. Thus, TNF-α might act through the PI-PLC-γ2 pathway, but not through the PC-PLC or PC-phospholipase D pathway, to induce PKC activation in NCI-H292 cells. Genistein blocked TNF-α-induced PKC activation (Fig. 6*B*), again indicating the requirement for an initial protein tyrosine phosphorylation event in this PKC activation process.

Because PKC had been shown to be involved in the TNF-α effect, direct activation of PKC by TPA was tested and found to induce COX-2 expression, as shown both by Western blotting (Fig. 8*A*) and immunofluorescence staining (Fig. 3*F*). This TPA-induced COX-2 expression was inhibited in a dose-dependent manner by genistein or herbimycin (Fig. 8*B*), as was the TNF-α-induced COX-2 expression (Fig. 4*A*). TPA also stimulated NF-κB DNA-protein binding and COX-2 promoter activity, and these effects were also inhibited by genistein or herbimycin (Figs. 11*D* and 12*A*), as was the TNF-α-induced activation of NF-κB and COX-2 promoter activity (Figs. 11*B* and 12*A*). These results suggest that protein tyrosine phosphorylation might also occur downstream of PKC in the induction of NF-κB activation. Further demonstration was discussed below.

In nonstimulated cells, NF-κB dimers are present as cytoplasmic latent complexes due to the binding of specific inhibitors, the I-κBs, which mask their nuclear localization signal. Upon stimulation by proinflammatory cytokines, the I-κBs are rapidly phosphorylated at two conserved NH2-terminal serines, this posttranslational modification being rapidly followed by their polyubiquitination and proteasomal degradation (47–49). This results in the unmasking of the nuclear localization signal in NF-κB dimers, which is followed by their translocation to the nucleus, binding to specific DNA sites (κB sites), and targeting gene activation. The protein kinase that phosphorylates I-κBs in response to proinflammatory stimuli has been identified biochemically and molecularly (50–54). 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 (55). IKK1 and IKK2 are very similar protein kinases that act as the catalytic subunits of the complex (50–54). In mammalian cells, IKK1 and IKK2 form a stable heterodimer that is tightly associated with IKKγ, a regulatory subunit (56). The IKKs bind NIK (52, 54), a member of the mitogen-activated protein kinase kinase kinase family that interacts with TNFR-associated factor 2, thus linking I-κB degradation and NF-κB activation to the TNFR complex (57). NIK activates and phosphorylates IKKα in cotransfection experiments, but is unable to phosphorylate IKKβ (58). However, both IKKα and IKKβ activity are reported to be regulated by NIK (59). The physiological function of IKK1 and IKK2 is still unclear. Initially, overexpression of catalytically inactive forms of IKK1 and IKK2 that blocked IKK and NF-κB activation suggested that both subunits play similar, and possibly redundant, roles in I-κB phosphorylation and NF-κB activation (50). Recent studies have shown that IKK2, and not IKK1, is the target for proinflammatory stimuli and plays the major role in IKK activation and induction of NF-κB activity (60, 61). However, our results show that TNF-α-induced COX-2 promoter activity in NCI-H292 epithelial cells was inhibited by the dominant-negative mutants NIK (KKAA), IKK1 (KM), or IKK2 (KM). This is consistent with the findings that IKK1 (KM, AA, or KA) and the IKK2 (KM, AA, or KA) mutant inhibit TNF-α-induced κB-dependent transcription in HeLa and 293 cells (53, 54). TPA-induced COX-2 promoter activity was also inhibited by the dominant-negative mutant for NIK, IKK1, or IKK2, indicating that NIK and IKK1/2 are involved in the downstream of PKC activation in COX-2 expression induction. IKK activity was stimulated by both TNF-α and TPA and inhibited by staurosporine and herbimycin (Fig. 13), indicating that tyrosine kinase activation occurs downstream of PKC in IKK activation. This tyrosine kinase has been demonstrated to be Src family member, c-Src, or Lyn. Both

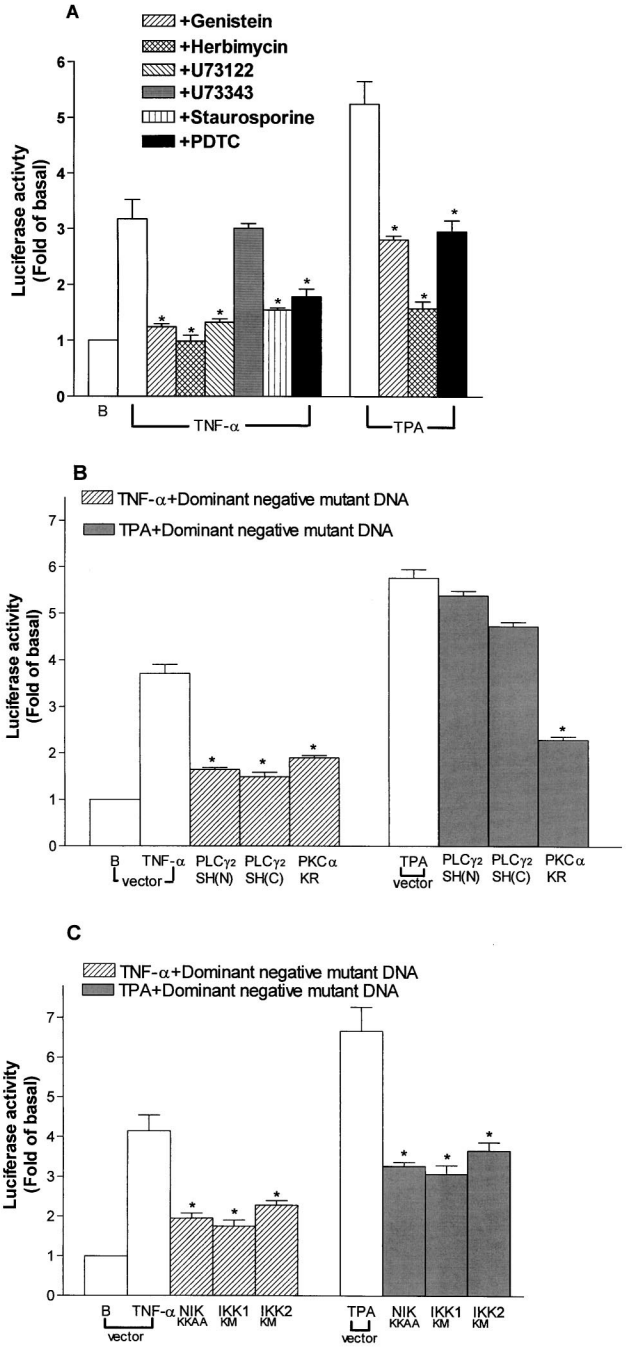

FIGURE 12. Effect of various inhibitors, mutants, or dominant-negative mutants on TNF-α-induced or TPA-induced COX-2 promoter activity. In *A*, cells were transfected with the pGS459 luciferase expression vector, as described in *Materials and Methods*, then pretreated with 30 μM genistein, 1 μM herbimycin, 10 μM U73122 or U73343, 30 nM staurosporine, or 100 μM PDTC for 30 min before incubation with 30 ng/ml of TNF-α or 1 μM TPA for 6 h. In *B* and *C*, cells were cotransfected with pGS459 and the PLC-γ2 mutants or the dominant-negative mutant for PKC-α (*B*), NIK, IKK1 or IKK2 (*C*), or empty vector. Luciferase activity was assayed as described in *Materials and Methods*. The results were normalized using the β-galactosidase activity and expressed as the mean $\pm$ SEM of three independent experiments performed in triplicate. $*$, $p < 0.05$ as compared with TNF-α or TPA alone.

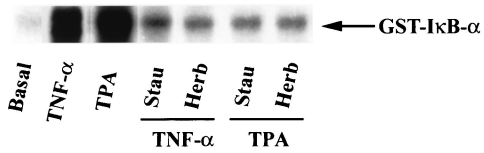

FIGURE 13. TNF-α- and TPA-induced IKK activation in NCI-H292 epithelial cells. Cells were treated for 10 min with 30 ng/ml of TNF-α or 1 μM TPA, or pretreated for 30 min with 30 nM staurosporine or 1 μM herbimycin before addition of TNF-α or TPA. Whole cell lysates were immunoprecipitated with anti-IKKβ Ab, followed by autoradiography for phosphorylated GST-I-κBα (1–100), as described in *Materials and Methods*.

TNF-α and TPA induced c-Src or Lyn activation, these effects being inhibited by staurosporine and herbimycin (unpublished data). Wild-type NIK induced COX-2 promoter activity in NCI-H292 cells, and this effect was not affected by either staurosporine or herbimycin (data not shown), confirming that NIK was involved in the downstream of TNF-α-induced PKC and tyrosine kinase activation. The present finding of the involvement of IKK1/2 in PKC-induced COX-2 expression contrasts with the results that PKC activates IKKβ, but not IKKα, in 293 cells, and only IKKβ is the target of PKC in T lymphocytes (62, 63).

In summary, the signaling pathway involved in TNF-α-induced COX-2 expression in human NCI-H292 epithelial cells has been explored. TNF-α might activate PLC-γ2 via an upstream protein tyrosine kinase to induce activation of PKC-α, protein tyrosine kinase, NIK, IKK1/2, and NF-κB in the COX-2 promoter, followed by initiation of COX-2 expression and PGE_2 release.

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