0026-895X/01/6006-1439–1448$3.00
MOLECULAR PHARMACOLOGY
Copyright © 2001 The American Society for Pharmacology and Experimental Therapeutics
Mol Pharmacol 60:1439–1448, 2001

Vol. 60, No. 6
972/951291
Printed in U.S.A.

Conjugated Polyhydroxybenzene Derivatives Block Tumor Necrosis Factor-α–Mediated Nuclear Factor-κB Activation and Cyclooxygenase-2 Gene Transcription by Targeting IκB Kinase Activity

CHING-CHOW CHEN, KUO-TUNG CHIU, SHU-TING CHAN, and JI-WANG CHERN

Department of Pharmacology (C.-C.C., K.-T.C., S.-T.C.) and School of Pharmacy (J.-W.C.), College of Medicine, National Taiwan University, Taipei, Taiwan

Received March 20, 2001; accepted August 21, 2001

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

ABSTRACT

Because the transcription factor, nuclear factor (NF)-κB, plays a key role in cellular inflammatory and immune responses, components of the NF-κB–activating signaling pathways are frequently used as targets for anti-inflammatory agents. This study shows that 2-(3′4′-dihydroxyphenyl)-5-hydroxybenzo[*b*-]furan (GF-015) and 2,3-di(3′4′-dihydroxy-transstyryl) pyridine (GF-90), two conjugated polyhydroxybenzene derivatives, inhibited a common step in NF-κB activation in human NCI-H292 epithelial cells by preventing tumor necrosis factor (TNF)-α– and 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced IκB kinase (IKK) complex activation. Both agents inhibited the TNF-α- or TPA-induced expression of cyclooxygenase (COX)-2 mRNA and protein, COX-2 promoter activity, and prostaglandin E_2 (PGE_2) production. Overexpression of wild-type NF-κB–inducing kinase, IKKα, and IKKβ led, respectively, to 3.5-, 2.6-, and 2.6-fold increases in COX-2 promoter activity, and these effects were inhibited by both compounds. GF-015 and GF-90 also prevented the TNF-α- and TPA-induced activation of IKK and NF-κB–specific DNA-protein binding activity. These results suggest that the inhibitory effect of GF-015 and GF-90 on TNF-α–induced COX-2 protein expression was caused by suppression of IKK activity and NF-κB activation in the COX-2 promoter, resulting in attenuation of COX-2 gene expression and PGE_2 production.

Cyclooxygenase (COX) is the rate-limiting enzyme in the conversion of arachidonic acid into prostaglandin H_2 (PGH_2), the precursor of a large group of biologically active mediators, such as PGE_2, prostacyclin, and thromboxane A_2, which are involved in several biological processes, including inflammation, immune responses, cell growth, ovulation, and regulation of vascular tone (Williams and DuBois, 1996). The two COX isoforms, COX-1 and COX-2, are encoded by separate genes (Fletcher et al., 1992; Hla and Neilson, 1992; Kraemer et al., 1992). Although their enzymatic function is similar, regulation of their cellular expression differs. COX-1 gene expression is largely constitutive, whereas COX-2 gene expression is negligible under basal conditions but can increase dramatically in many cell types in response to mitogenic and inflammatory stimuli (Smith and Dewitt, 1996). COX-2 is expressed in activated macrophages, monocytes, and several other cell types and has been identified in chronic inflammatory conditions in vivo (Vane et al., 1994). It is implicated in physiological processes, such as ovulation and delivery (Lim et al., 1997), and in pathological states, such as colorectal cancer, Alzheimer's disease, heart failure, and even hypertension (Levy, 1997; Oka and Takashima, 1997; Hartner et al., 1998; Wong et al., 1998). Much evidence suggests that COX-2 is an important therapeutic target for the prevention and treatment of arthritis and cancer. Reducing the levels of COX-2 will be an effective strategy for inhibiting inflammation and carcinogenesis (Anderson et al., 1996; Kawamori et al., 1998). Because of this, there has been great interest in the role(s) of COX-2 and the usefulness of drugs that can selectively block this isozyme (Frolich, 1997).

COX-2 is an early gene expressed in response to many cytokines, and its transcriptional regulation is, at least in

This work was supported by a research grant from the National Science Council of Taiwan.

ABBREVIATIONS: COX, cyclooxygenase; PG, prostaglandin; NF, nuclear factor; IκB, inhibitory protein of nuclear factor-κB; NIK, nuclear factor-κB–inducing kinase; IKK, IκB kinase; TNF, tumor necrosis factor; PKC, protein kinase C; GF-015, 2-(3′4′-dihydroxyphenyl)-5-hydroxybenzo[*b*]furan; GF-90, 2,3-di(3′,4′-dihydroxy-transstyryl) pyridine; FCS, fetal calf serum; ECL, enhanced chemiluminescence; SSC, standard saline citrate; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; PAGE, polyacrylamide gel electrophoresis; TTBS, 0.1% nonfat dry milk in Tris-buffered saline containing Tween 20; PMSF, phenylmethylsulfonyl fluoride; DTT, dithiothreitol; GST, glutathione *S*-transferase; MG132, N-CBZ-Leu-Leu-Leu-al; PBS, phosphate-buffered saline; NSAIDs, nonsteroidal anti-inflammatory drugs; NS-398, *N*-(2-cyclohexyloxy-4-nitrophenyl)methane sulfonamide.

part, under the control of NF-κB (Newton et al., 1997). NF-κB activation is tightly regulated by its endogenous inhibitor IκB, which complexes with and sequesters NF-κB in the cytoplasm. After cytokine stimulation, IκBα is phosphorylated at serines 32 and 36, initiating the selective ubiquitination and rapid degradation of this inhibitor by the nonlysosomal, ATP-dependent 26S proteolytic complex composed of a 700-kDa proteasome (Stancovski and Baltimore, 1997). IκBα phosphorylation involves the successive participation of various kinases linked to cytokine-specific membrane receptor complexes and adapter proteins, which converge on NF-κB–inducing kinase (NIK) (Malinin et al., 1997). Activated NIK then phosphorylates and activates the IκB kinase (IKK) complex (Ling et al., 1998; Lin et al., 1998). IKK is part of a multiprotein complex that contains IKKα and IKKβ subunits (Woronicz et al., 1997). Activation of the IKK complex leads to specific IκBα phosphorylation/degradation and the subsequent release of NF-κB, which then translocates to the nucleus and activates the transcription of multiple κB-dependent genes, including COX-2 (Barnes and Karin, 1997). Because NF-κB plays a central role in regulating the genes involved in the initiation of immune, acute phase, and inflammatory responses, there is growing interest in modulating its activity. The pathways leading to NF-κB activation are therefore frequent targets for a variety of anti-inflammatory drugs. The anti-inflammatory drugs aspirin and salicylate suppress inducible COX-2 gene transcription (Xu et al., 1999) and inhibit IκB kinase-β (Yin et al., 1998). We have demonstrated that, in NCI-H292 human alveolar epithelial cells, TNF-α induces COX-2 expression via the PKC- and mitogen-activated protein kinase-dependent IKK1/2 and NF-κB activation pathway (Chen et al., 2000, 2001). In this study, we have identified two conjugated polyhydroxybenzene derivatives, 2-(3′,4′-dihydroxyphenyl)5-hydroxybenzo-[*b*]furan (GF-015) and 2,3-di(3′,4′-dihydroxy-transstyryl) pyridine (GF-90) (Fig. 1) that block TNF-α–induced COX-2 expression through NF-κB inhibition by targeting the IKK complex.

Experimental Procedures

Materials. The NF-κB probe, goat polyclonal antibodies specific for COX-2, and rabbit polyclonal antibodies specific for IKKβ and IκBα were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). MG132 was from Sigma (St. Louis, MO). Recombinant human TNF-α was purchased from R & D Systems, Inc. (Minneapolis, MN). RPMI 1640 medium, fetal calf serum (FCS), penicillin, and streptomycin were from Invitrogen (Gaithersburg, MD). T4 polynucleotide kinase and rabbit polyclonal antibody specific for the phosphorylated form of IκBα (Ser 32) were from New England Biolabs (Beverly, MA). Poly (dI/dC), horseradish peroxidase-labeled donkey anti-goat or anti-rabbit second antibody and the ECL detection reagent were from Amersham Pharmacia Biotech (Piscataway, NJ). [γ-^{32}P]ATP (3000 Ci/mmol) and [α-^{32}P]dCTP (3000 Ci/mmol) were from PerkinElmer Life Sciences (Boston, MA). Tfx-50 and the luciferase assay kit were from Promega (Madison, WI). GF-015 and GF-90 were synthesized (J.-W. Chern, unpublished observations) and dissolved as stock solutions (50 mM) in dimethyl sulfoxide.

Plasmids. The COX-2 promoter construct (−459/+9) was a generous gift from Dr. L. H. Wang (University of Texas-Houston, Houston, TX). Plasmids containing wild-type NIK, IKKα, and IKKβ were provided by Dr. M. Karin (University of California, San Diego, CA). pGEX-IκBα (1–100) and pGEX-IκBα (1–100) (S32A, S36A) were gifts from Dr. H. Nakano (Juntendo University, Tokyo, 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 penicillin, and 100 μg/ml streptomycin in either six-well plates (COX-2 protein expression, PGE_2 production, and transfection) or 10-cm dishes (COX-2 mRNA expression, NF-κB gel shift assay, and IKK activation).

Northern Blot Analysis. Total cellular RNA was isolated from cultured cells using TRIzol (Invitrogen). A sample of the RNA (20–30 μg/lane) was fractionated on a formaldehyde-containing 1% agarose gel and transferred to a positively charged Immobilon-N (Millipore Corporation, Bedford, MA) membrane. After UV cross-linking, the membranes were prehybridized for 1 h at 60°C and then probed for 12 h at 60°C with the human COX-2 cDNA probe (1.9 kilobases) labeled with [α-^{32}P]dCTP by random primer using Rediprime (Amersham Pharmacia Biotech). Hybridization reactions were performed in Church buffer consisting of 7% SDS, 1% bovine serum

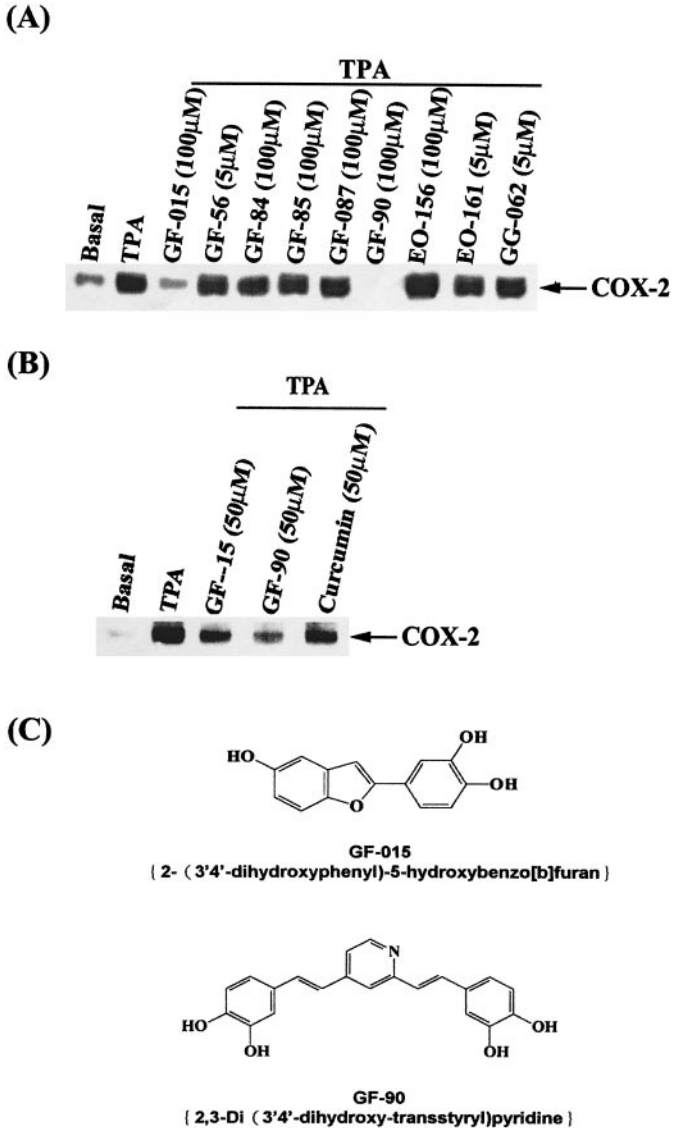

Fig. 1. Effect of nine conjugated polyhydroxybenzene derivatives and curcumin on TPA-induced COX-2 expression in NCI-H292 cells (A and B) and chemical structure of GF-015 and GF-90 (C). A, cells were pretreated with 100 or 5 μM different derivatives; B, cells were pretreated with 50 μM GF-015, GF-90, or curcumin. After pretreatment for 30 min, 1 μM TPA was added for 16 h. Whole-cell lysates were prepared and subjected to Western blotting using antibody specific for COX-2, as described under *Materials and Methods*.

albumin, 10 mM EDTA, and 0.4 M NaH_2PO_4, pH 7.2. The membranes were washed at room temperature for 60 min once in 2× SSC containing 1% SDS and again in 0.2× SSC containing 1% SDS before exposure to film. The membranes were then stripped in 0.2× SSC containing 1% SDS at room temperature for 60 min and rehybridized with human glyceraldehyde-3-phosphate dehydrogenase cDNA (1 kilobase). Blots were quantitated using a computer densitometer and ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

Preparation of Cell Extracts and Western Blot Analysis of COX-2. After 16-h treatment with TNF-α or TPA, the cells were harvested and collected, and cell lysates were prepared and subjected to SDS-PAGE using 7.5% running gels as described previously (Chen et al., 2000). The proteins were then transferred to nitrocellulose membranes, which were incubated successively at room temperature with 0.1% nonfat dry milk in Tris-buffered saline containing Tween 20 (TTBS) for 1 h, with goat antibody specific for COX-2 for 1 h, and with horseradish peroxidase-labeled anti-goat antibody for 30 min. After each incubation, the membrane was washed extensively with TTBS. The immunoreactive band was detected using ECL detection reagent and visualized using Hyperfilm ECL (Amersham Pharmacia Biotech). Quantitative data were obtained using a computing densitometer and ImageQuant software (Molecular Dynamics). In pretreatment experiments, cells were incubated for 30 min with various concentrations of GF-015, GF-90, or curcumin before addition of TNF-α or TPA. These inhibitors (except 100 μM curcumin) had no cytotoxic effect on NCI-H292 cells, and the 0.001% dimethyl sulfoxide (vehicle) used as a control had no effect on TNF-α- or TPA-induced COX-2 expression.

Determination of PGE_2 Concentration. Cells were pretreated with 10 or 50 μM GF-015 or GF-90 for 30 min; then, TNF-α or TPA was added for 16 h. PGE_2 levels in the culture medium were measured using an enzyme immunoassay kit from Amersham Pharmacia Biotech.

Transient Transfection and Luciferase Assay. NCI-H292 cells grown in six-well plates were transfected with the human COX-2 firefly luciferase plasmid, pGS459 (−459/+9), using Tfx-50 (Promega) according to the manufacturer's recommendations. Briefly, reporter DNA (0.3 μg) and β-galactosidase DNA (0.1 μg) were mixed with 1.8 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 a medium containing 10% FCS. On the following day, they were pretreated with various concentrations of GF-015 or GF-90 before exposure to 30 ng/ml TNF-α or 1 μM TPA for 6 h; then cell extracts were prepared, luciferase (Promega) and β-galactosidase activity measured, and the luciferase activity of each well normalized to the β-galactosidase activity. In experiments involving overexpression of wild-type plasmids, cells were cotransfected with reporter, β-galactosidase, and either wild-type NIK, IKKα, IKKβ (0.3 μg of DNA), or the empty vector.

In Vitro IKK Activity Assay. After 10 min of treatment with TNF-α or TPA or 30 min of pretreatment with GF-015 or GF-90 before addition of TNF-α or TPA, cells were washed rapidly with PBS and 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 leupeptin, 20 μg/ml aprotinin, 1 mM NaF, and 1 mM Na_3VO_4), 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 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 consisting of 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 an equal volume of Laemmli buffer and the material subjected to 10% SDS-PAGE, and phosphorylated GST-IκBα (1–100) was visualized by autoradiography.

Preparation of Cell Extracts and Western Blot Analysis of Phosphorylated IκBα and IκBα. After 5, 10, 30, and 60 min of treatment with TNF-α or 60 min of pretreatment with MG132, GF-015, GF-90, or curcumin before challenge with TNF-α for 5 or 10 min, the cells were washed rapidly with PBS and then lysed with fresh ice-cold lysis buffer as described previously (Chen et al., 2001). The lysates were then subjected to SDS-PAGE using a 10% running gel. The proteins were transferred to nitrocellulose paper, and immunoblot analysis was performed as described above, except that rabbit antibodies specific for phosphorylated IκBα or nonphosphorylated IκBα were used.

Preparation of Nuclear Extracts and the Electrophoretic Mobility Shift Assay. Cells were pretreated with 10 or 50 μM GF-015 or GF-90 for 30 min before addition of TNF-α or TPA for 1 h; then nuclear extracts were prepared as described previously (Chen et al., 2000). 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, and the cells 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 for 15 min at 4°C 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 (5′-AGAGTG**GGGACTAC-CC**CCTCT-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., 2000). 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 at −80°C using an intensifying screen. Quantitative data were obtained using a computing densitometer and ImageQuant software (Molecular Dynamics).

Statistical Analyses. All data are expressed as the mean ± S.E.M. Statistical analyses were performed using Student's t test.

Results

Testing of Nine Conjugated Polyhydroxybenzene Derivatives. Nine conjugated polyhydroxybenzene derivatives are tested for their inhibitory effect on TPA-induced COX-2 expression in NCI-H292 cells. As shown in Fig. 1A, six of them at 100 μM had no cytotoxic effect on the cells and only two (GF-015 and GF-90) of these six showed inhibitory effect on TPA-induced COX-2 expression. Because three (GF-56, EO-161, and GG-062) of the nine derivatives had cytotoxicity on cells at 100 μM, the concentration was reduced to 5 μM. They had no inhibitory effect on TPA-induced COX-2 expression at this concentration (Fig. 1A). Therefore, the mechanism of action of GF-015 and GF-90 in inhibiting COX-2 induction was further studied. The synthesis of GF-015 was based on stilbene, whereas that of GF-90 was based on curcumin. The purity and structure of these two compounds were proved by NMR spectrum and elemental analysis. Potency of curcumin and these two compounds was

compared. As shown in Fig. 1B, GF-90 at 50 μM was more potent than curcumin in inhibiting COX-2 induction. Concentration of 100 μM was not applied, because curcumin at this concentration showed serious cytotoxicity to NCI-H292 cells.

Effects of GF-015 and GF-90 on TNF-α- or TPA-Induced Expression of COX-2 mRNA and Protein and PGE_2 Release. The effects of the conjugated polyhydroxybenzene derivatives GF-015 and GF-90 on COX-2 gene expression and the IKK/NF-κB pathway were investigated using NCI-H292 epithelial cells, in which TNF-α has been shown to induce a dose- and time-dependent increase in COX-2 protein expression and PGE_2 formation, the maximal effect being obtained by treatment with 30 ng/ml TNF-α for 16 h. This COX-2 expression has been demonstrated to involve the PKC-dependent activation pathway; therefore, direct activation of PKC by TPA also induced COX-2 protein expression (Chen et al., 2000). TNF-α (30 ng/ml) also induced the expression of COX-2 mRNA in a time-dependent manner; this effect was significant and maximal at 1 h, declined slightly after 3 and 6 h, and was no longer seen after 9 h of treatment (Fig. 2A). To determine the effects of GF-015 and GF-90 on TNF-α–induced COX-2 gene expression, cells were pretreated for 30 min with each of the compounds at concentrations of 10 or 50 μM before stimulation with TNF-α for 3 h. When COX-2 mRNA expression was analyzed by Northern blotting, GF-015 and GF-90 both inhibited TNF-α–induced COX-2 mRNA expression in a dose-dependent manner (Fig. 2B).

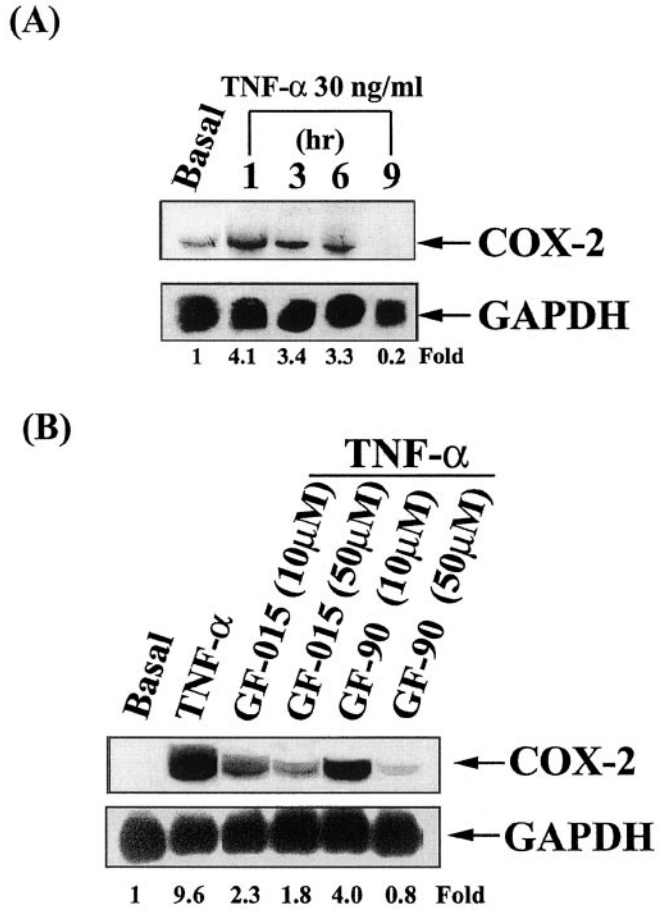

Fig. 2. Time-dependent TNF-α–induced COX-2 mRNA expression in NCI-H292 epithelial cells and inhibitory effect of GF-015 and GF-90. Cells were incubated at 37°C with 30 ng/ml TNF-α for various time intervals (A) or pretreated with 10 or 50 μM GF-015 or GF-90 for 30 min before incubation with 30 ng/ml TNF-α for 3 h (B). Total RNA was analyzed by Northern blotting, as described under *Materials and Methods*.

Inhibition of COX-2 gene expression was confirmed by measuring COX-2 protein expression. To determine the effect of GF-015 and GF-90 on TNF-α- or TPA-induced COX-2 protein expression, cells were incubated for 30 min with or without the test compounds at concentrations varying from 3 to 30 μM before induction for 16 h with either TNF-α (30 ng/ml) or TPA (1 μM). Both compounds led to a significant concentration-dependent reduction in TNF-α- or TPA-induced COX-2 expression (Fig. 3). The IC_{50} values for GF-015 for the inhibition of TNF-α- and TPA-induced COX-2 expression were 13.3 and 12.1 μM, respectively (Fig. 3A), whereas the corresponding values for GF-90 were 5.2 and 7.1 μM (Fig. 3B).

Because the major prostaglandin synthesized by COX in alveolar epithelial cells is PGE_2, we next examined whether TNF-α- and TPA-induced PGE_2 release was inhibited by these two compounds. Both TNF-α (30 ng/ml) and TPA (1 μM) cause PGE_2 release in NCI-H292 cells, and these effects are blocked by 10 μM NS-398 (a COX-2 inhibitor) (Chen et al., 2000; data not shown). After pretreatment of cells with 10 or 50 μM GF-015, TNF-α–induced PGE_2 production was inhibited by 24 or 71%, respectively, whereas TPA-induced PGE_2 production was inhibited by 47 or 75% (Fig. 4A). Similarly, using 10 or 50 μM GF-90, TNF-α–induced PGE_2 production was inhibited by 63 or 88%, respectively, whereas TPA-induced PGE_2 production was inhibited by 54 or 92% (Fig. 4B).

GF-015 and GF-90 Inhibit TNF-α- and TPA-Induced COX-2 Promoter Activity. To elucidate the mechanism involved in the GF-015– and GF-90–mediated inhibition of COX-2 expression, transient transfections were performed using a human COX-2 promoter-luciferase construct, pGS459. As reported previously (Chen et al., 2000), treatment with TNF-α or TPA led to a 4.1- or 5-fold increase, respectively, in COX-2 promoter activity, and these effects were inhibited by GF-015 and GF-90 in a dose-dependent manner (Fig. 5). The IC_{50} values for GF-015 for inhibition of TNF-α- and TPA-induced COX-2 promoter activity were 12.5 and 11 μM, respectively, whereas the corresponding values for GF-90 were 5.9 and 6.4 μM (Fig. 5).

Cytokine-mediated IκB phosphorylation/degradation and NF-κB activation involve the activation of at least two sequential proximal kinases, NIK and IKK (Maniatis, 1997). IKKα/β binds NIK, a member of the mitogen-activated protein kinase kinase kinase family, to link IκB degradation and NF-κB activation to the TNF-α receptor complex (Malinin et al., 1997). TNF-α–induced COX-2 expression involves the PKC-dependent NIK, IKK1/2, and NF-κB activation pathway (Chen et al., 2000). To investigate the effect of both compounds on NF-κB activation-induced COX-2 expression, cotransfection with wild-type NIK, IKKα, or IKKβ and the COX-2 promoter was performed. Overexpression of wild-type NIK, IKKα, or IKKβ DNAs resulted in a 3.5-, 2.6-, or 2.6-fold increase, respectively, in COX-2 promoter activity, and these effects were suppressed by 10 or 50 μM GF-015 or GF-90 (Fig. 6). Pretreatment with 10 or 50 μM GF-015 resulted in 34 or 65% inhibition, respectively, of COX-2 promoter activ-

ity induced by NIK, 64 or 87% inhibition of that induced by IKKα, and 36 or 77% of that induced by IKKβ (Fig. 6A); the corresponding values for 10 and 50 μM GF-90 were 48 and 100% for NIK, 78 and 100% for IKKα, and 58 and 100% for IKKβ (Fig. 6B).

GF-015 and GF-90 Inhibit TNF-α- or TPA-Induced IKK Activation, IκBα Phosphorylation, and NF-κB DNA-Protein Binding Activity. Because COX-2 promoter activity induced by TNF-α, TPA, or wild-type NIK, IKKα, or IKKβ was inhibited by GF-015 and GF-90, indicating that both compounds targeted IKKα/β to inhibit TNF-α- and TPA-induced COX-2 gene expression; the effect of the test compounds on TNF-α- and TPA-induced IKK activity was examined. As reported previously (Chen et al., 2000), both TNF-α and TPA induced IKK activation after 10 min of treatment. The IKK complex kinase activity was specific for Ser 32/Ser 36, because it was not observed when both serines in IκBα substrate were substituted with alanines (Fig. 7A, lanes 4–6). Equal amount of IκBα substrate present in the in vitro kinase assay or the amount of IKK complex immunoprecipitated (Fig. 7A, lanes 1–6). GF-015 and GF-90 (10 or 50 μM) inhibited the TNF-α and TPA-induced IKK activations in a dose-dependent manner (Fig. 7, B and C).

Phosphorylation of IκBα on serines 32 and 36 by IKK is necessary for its degradation and subsequent NF-κB activation (Chen et al., 1995). Because TNF-α- or TPA-induced IKK activity was inhibited by GF-015 and GF-90, and NF-κB activation is essential for TNF-α–induced COX-2 expression in NCI-H292 cells (Chen et al., 2000), we next examined the effect of GF-015 and GF-90 on TNF-α- induced IκBα phosphorylation and degradation. Endogenous IκB phosphorylation was assessed using Western blot with a specific IκBα phospho-serine 32 antibody. As shown in Fig. 8A, when cells were stimulated with TNF-α for 5, 10, 30, or 60 min, phosphorylation of IκBα was seen at 5 min of treatment, whereas it disappeared at 10 or 30 min and reappeared at 60 min. Almost complete degradation of IκBα was seen after 10 min of treatment with TNF-α, and full restoration was seen at 60 min, as reported previously (Chen et al., 2000). The lack of phosphorylated IκBα at 10 min was caused by degradation of IκBα protein (Fig. 8A, lane 3), because phosphorylated IκBα was seen at 10 min in the presence of proteasome inhibitor MG132, which allowed accumulation of the unstable phosphorylated IκBα (compare Fig. 8C, lane 8, with 8B, lane 2) (Chen et al., 1995). GF-015, GF90, and curcumin but not MG132 inhibited the accumulation of phosphorylated IκBα protein seen at 5 min of treatment with TNF-α (compare Fig. 8B, lanes 3–5 and 8 with lane 2). The inhibitory effect of GF-015 and GF90 on IκBα phosphorylation was further demonstrated by combination of these two compounds with MG132. As shown in Fig. 8, B and C, the phosphorylated IκBα seen in the presence of MG132 at 5 or 10 min of treatment with TNF-α was decreased by either GF-015 or GF-90 (compare Fig. 8B, lanes 6–7 with lane 8, and Fig. 8C, lanes 6–7 with lane 8). These results indicated that these two compounds inhibited the phosphorylation of IκBα protein but not the activity of proteasome complex. However, the TNF-α–induced IκBα degradation seen at 10 min of treatment was not prevented by GF-015 and GF-90 but prevented by curcumin and MG132 (Fig. 8C, lanes 3–5 and 8).

To test whether the inhibitory effect of GF-015 and GF-90 on IKK activity led to NF-κB inhibition, the effect of both compounds on TNF-α- and TPA-stimulated NF-κB–specific DNA-protein binding activity was examined. Both compounds at concentrations of 10 or 50 μM inhibited TNF-α- or TPA-induced NF-κB–specific DNA-protein binding in a dose-dependent manner (Fig. 9).

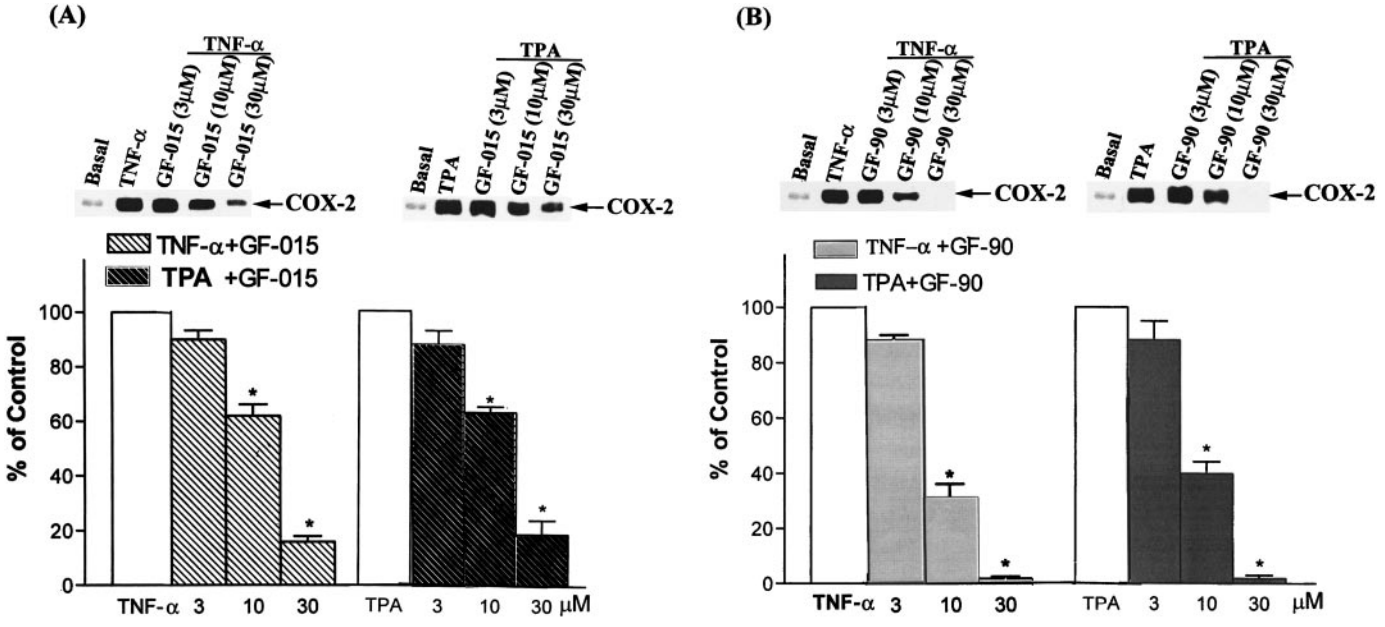

Fig. 3. Inhibitory effect of GF-015 and GF-90 on TNF-α- or TPA-induced COX-2 protein expression in NCI-H292 epithelial cells. Cells were pretreated with 3, 10, or 30 μM GF-015 (A) or GF-90 (B) for 30 min before incubation with 30 ng/ml TNF-α or 1 μM TPA for 16 h. Whole-cell lysates were prepared and subjected to Western blotting using antibody specific for COX-2, as described under *Materials and Methods*. COX-2 expression was quantified using a densitometer with ImageQuant software. The results are expressed as the mean ± S.E.M. of three independent experiments. *, $P < 0.05$ compared with TNF-α or TPA alone.

Discussion

An expanding body of evidence indicates that COX-2 inhibitors are useful for treating inflammation and preventing cancer (Anderson et al., 1996; Oshima et al., 1996; Kawamori et al., 1998). The search for selective COX-2 inhibitors started after the identification of this cytokine-inducible isoform of COX (Fu et al., 1990). So far, more than a dozen such compounds have been described (Frolich, 1997; DeWitt, 1999), all of which act as competitive inhibitors of the enzyme. An alternative approach chosen by some research groups is to develop irreversible COX-2–selective inhibitors (Kalgutkar et al., 1998) that could prevent the resumption of prostaglandin production once drug plasma levels fall, similar to the effect of aspirin on COX-1 and, to a lesser extent, on COX-2. Moreover, because of the rapid induction of COX-2 by proinflammatory stimuli, another interesting approach would be to identify drugs that selectively block the expression of the enzyme (Pennisi, 1998). In this study, we analyzed the effect of nine conjugated polyhydroxybenzene derivatives on TPA-induced COX-2 expression and found that two, GF-015 and GF-90, had inhibitory effects. We then studied the effect of GF-015 and GF-90 on the IKK/NF-κB pathway and their mechanisms of action. We report here that pretreatment with either compound resulted in inhibition of TNF-α–mediated NF-κB activation with concomitant down-regulation of COX-2 gene expression and that NF-κB blockade by both compounds involved inhibition of TNF-α–mediated IKK activation.

It is rational to study COX-2 gene expression and the accompanying signaling pathways in alveolar epithelial cells. Epithelial cells play an active role in inflammation by producing various cytokines that are involved in the late asthmatic response (Barnes, 1994). The respiratory epithelium contributes to normal pulmonary function by removing inhaled particulates by its mucociliary action, ensures alveolar patency through surfactant secretion, and facilitates bacterial opsonization by the production of secretory immunoglobulin. There is now considerable evidence in support of an additional role for airway epithelial cells in amplifying cytokine signals, including the activation of alveolar macrophages and the secretion of chemokines, arachidonic acid metabolites, and phospholipids, which recruit additional inflammatory cells into the airway mucosa (Standiford et al., 1990; Stadnyk, 1994). Of the potent alveolar macrophage-derived cytokines studied, TNF-α in particular has been implicated in the pathophysiology of neutrophilic infiltrating

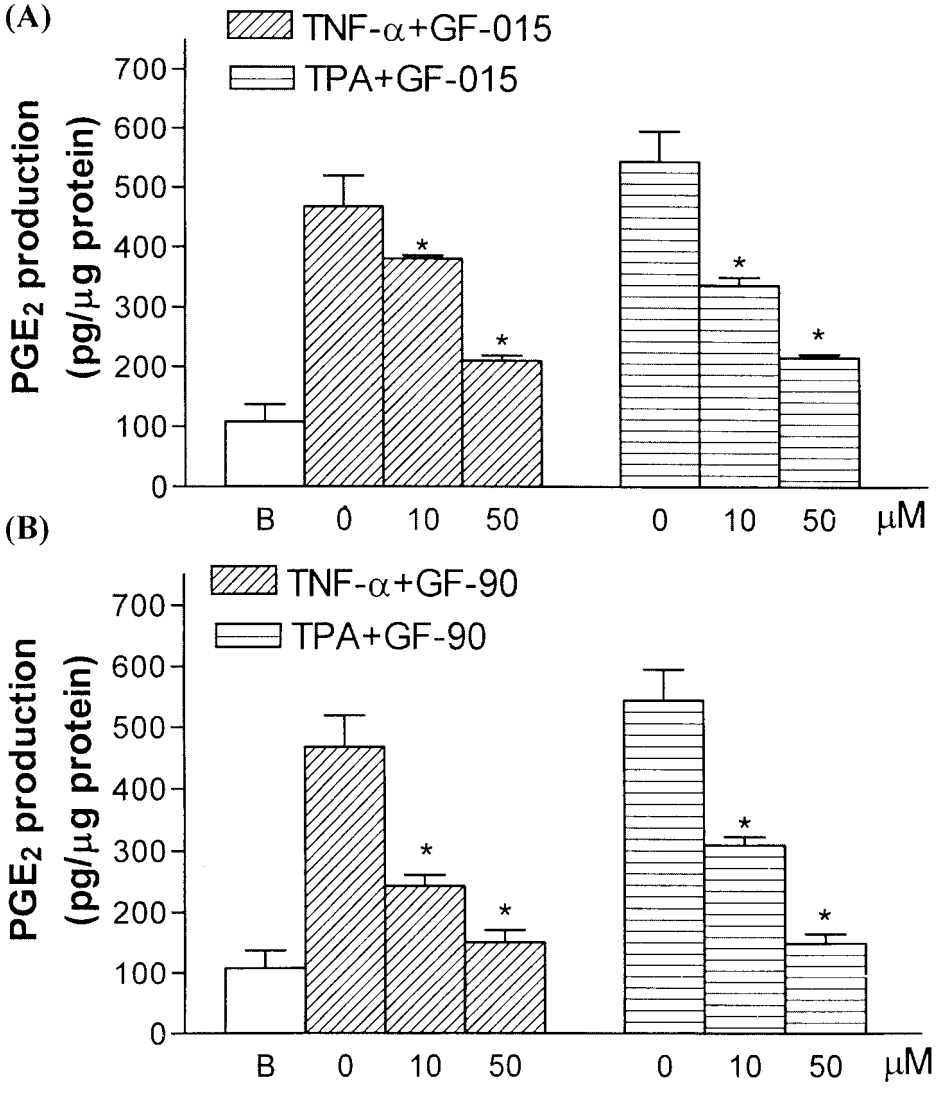

Fig. 4. Inhibitory effect of GF-015 and GF-90 on TNF-α- or TPA-induced PGE_2 production in NCI-H292 epithelial cells. Cells were pretreated with 10 or 50 μM GF-015 (A) or GF-90 (B) for 30 min before incubation with 30 ng/ml TNF-α or 1 μM TPA for 16 h; then the medium was removed and analyzed for PGE_2 production. The results are expressed as mean ± S.E.M. of three independent experiments performed in triplicate. *, $P < 0.05$ compared with TNF-α or TPA alone.

disorders, including acute lung injury from sepsis, silica-induced pulmonary fibrosis, allograft rejection, and acute respiratory tract infection (Piguet et al., 1990; Suter et al., 1992; Beutler, 1995). Upon binding to its cell surface receptors, TNF activates the cytoplasmic form of NF-κB (Baeuerle and Henkel, 1994; Baldwin, 1996).

Some of the conjugated polyhydroxybenzene derivatives tested in this study were based on the structure of stilbene, one of the major structural classes of COX-2 inhibitors (DeWitt, 1999), whereas others were based on curcumin, which has anti-inflammatory and chemopreventive activity (Ammon and Wahl, 1991; Rao et al., 1993). On the nine tested, only GF-015 and GF-90 had an inhibitory effect on TPA-induced COX-2 expression (Fig. 1A). The potency of GF-90 is greater than that of curcumin (Fig. 1B). Further experiments demonstrated that TNF-α- or TPA-induced expression of COX-2 mRNA and protein, PGE_2 production, and COX-2 promoter activity were attenuated by pretreatment with GF-015 and GF-90. Using molecular biological approaches, ectopic expression of NIK or IKKα/β allowed us to bypass the TNF-α receptor and specifically address the effect of these two compounds on more proximal NF-κB–inducing signals. Using this approach, we found that GF-015 and GF-90 interfere directly with NIK- or IKK-induced COX-2 promoter activity, indicating that these two compounds act at the level of IKK in TNF-α–mediated NF-κB activation, leading to COX-2 expression. This was further confirmed by the finding that TNF-α–induced IKK activation was inhibited by GF-015 and GF-90 (Fig. 7). Thus, these two conjugated polyhydroxybenzene derivatives inhibit the signal going to the IKK complex by directly interfering with this complex and then inhibit NF-κB–specific DNA-protein binding activity. Because phosphorylated IκBα protein was seen at 5 min of treatment with TNF-α and disappeared at 10 min due to IκBα degradation (Fig. 8A), cells were treated with TNF-α for 5 min to examine the phosphorylation of IκBα (Fig. 8B) or 10 min to examine the degradation of IκBα (Fig. 8C). Accumulation of phosphorylated IκBα protein was inhibited by GF-015 and GF-90, but not by MG132 (Fig. 8B), indicating that these two compounds inhibited IKK activity but not the proteasome complex. This finding was further demonstrated by the result that the phosphorylated IκBα protein seen in the presence of MG132 at 10 min of treatment with TNF-α was inhibited by GF-015 and GF-90 (Fig. 8C). TNF-α–induced degradation of IκBα protein was prevented by curcumin, which also inhibited accumulation of phosphorylated IκBα protein (Fig. 8B) and

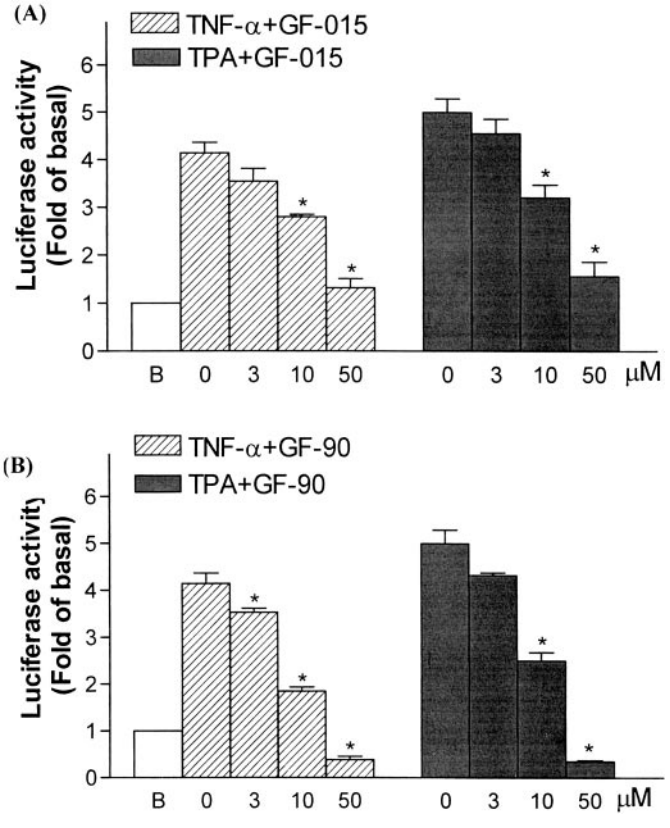

Fig. 5. Inhibitory effect of GF-015 and GF-90 on TNF-α- or TPA-induced COX-2 promoter activity in NCI-H292 epithelial cells. Cells were transfected with the pGS459 luciferase expression vector and then pretreated with 3, 10, or 50 μM GF-015 (A) or GF-90 (B) for 30 min before incubation with 30 ng/ml TNF-α or 1 μM TPA for 6 h. Luciferase activity was assayed as described under *Materials and Methods*. The results were normalized to the β-galactosidase activity and expressed as the mean ± S.E.M. of three independent experiments performed in triplicate. *, $P < 0.05$ compared with TNF-α or TPA alone.

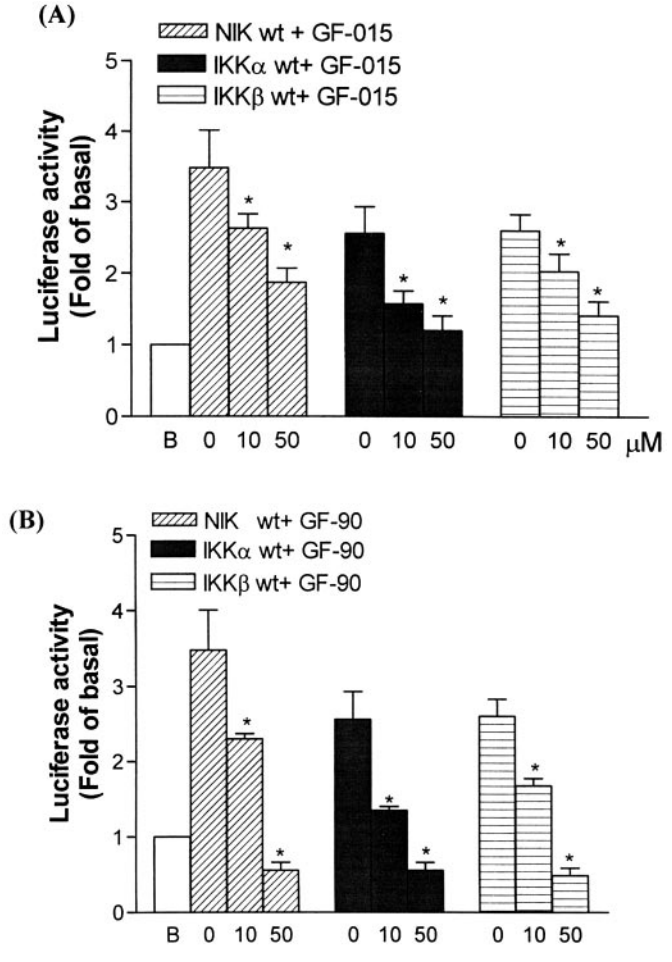

Fig. 6. Inhibitory effect of GF-015 and GF-90 on wild-type NIK-, IKKα-, or IKKβ-induced COX-2 promoter activity. Cells were cotransfected with plasmids coded for wild-type NIK, IKKα, IKKβ, or empty vector and the pGS459 construct and then treated with 10 or 50 μM GF-015 (A) or GF-90 (B) for 6 h. Luciferase activity was assayed as described under *Materials and Methods*. The results were normalized to β-galactosidase activity and expressed as the mean ± S.E.M. of three independent experiments performed in triplicate. *, $P < 0.05$ compared with wild-type NIK, IKKα, or IKKβ alone.

MG132, but not by GF-015 and GF-90 (Fig. 8C). The discrepancy that GF-015 and GF-90 inhibit IKK activity but do not prevent IκBα degradation is unknown at present and requires further investigation. The ability to inhibit the NIK-IKK signaling complex may be common to other anti-inflammatory chemopreventive agents. Aspirin and salicylate, shown previously to inhibit IκB phosphorylation and degradation (Kopp and Ghosh, 1994), inhibit IκB phosphorylation by specifically reducing the binding of ATP to IKKβ (Yin et al., 1998). Salicylate also inhibits COX-2 induction by lipopolysaccharide in macrophages (Tordjman et al., 1995) and by interleukin-1β and TPA in endothelial cells (Xu et al., 1999). Similar interference at the IKKα/β level, leading to the inhibition of NF-κB activation and COX-2 expression, is seen with curcumin (Plummer et al., 1999). Overexpression of COX-2 in colon epithelial cells may promote tumor development (Tsujii and Dubois, 1995), and nonsteroidal anti-inflammatory drugs (NSAIDs), which directly inhibit COX-2 activity (Vane and Botting, 1995), cause regression of adenomatous polyps (Giardiello et al., 1995). COX-2 has therefore been suggested as an important target for the chemopreventive effects of these agents (Giardiello et al., 1997). However, chronic administration of NSAIDs results in serious side effects because of concomitant inhibition of COX-1, making the development of selective COX-2 inhibitors highly desirable. Such agents could act either by direct inhibition of COX-2 and/or inhibition of COX-2 gene expression (Subbaramaiah et al., 1997). COX-1 enzyme activity is inhibited only 30% by 10 μM GF-015 or GF-90, whereas 10 nM indomethacin produces 58% inhibition (our unpublished observations). In addition, COX-2 enzyme activity is not inhibited but increased 18 or 42%, respectively, by 10 μM GF-015 or GF-90 compared with the 51% inhibition seen using the specific COX-2 inhibitor celecoxib at 0.3 μM. Thus, GF-015 and GF-90 inhibit COX-2 gene expression and also have the advantage of having less of an inhibitory effect on COX-1 enzyme activity compared with NSAIDs.

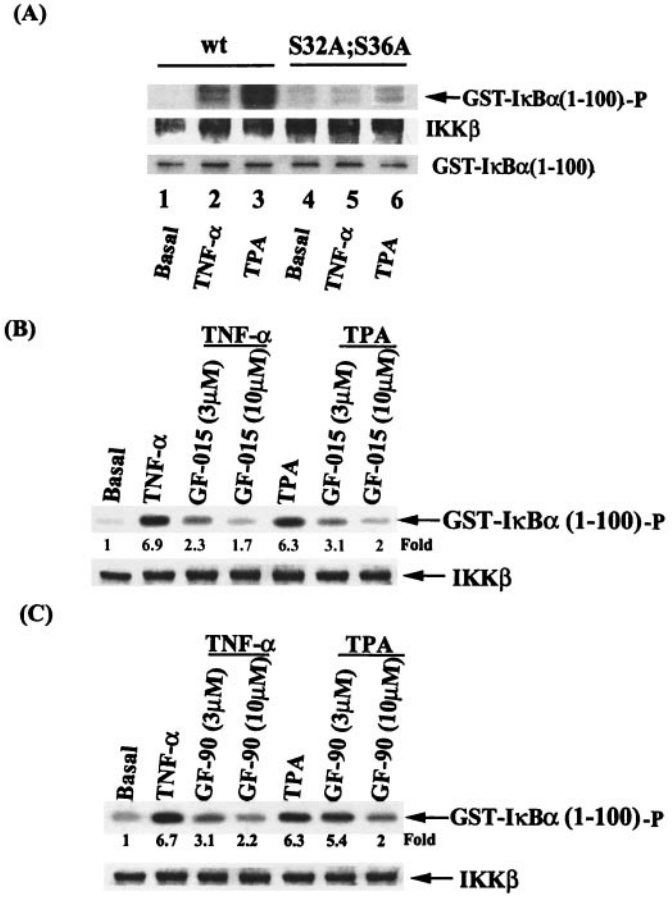

Fig. 7. Activation of IKK activity by TNF-α and TPA in NCI-H292 epithelial cells and inhibitory effects of GF-015 and GF-90. A, cells were treated with 30 ng/ml TNF-α or 1 μM TPA for 10 min; then whole cell lysates were immunoprecipitated with anti-IKKβ antibody followed by antoradiography for phosphorylated GST-IκBα (1–100) or GST-IκBα (1–100) mutant (S32A; S36A). The level of immunoprecipitated IKKβ were detected using IKKβ specific antibody and levels of GST-IκBα were stained by Coomassie Brilliant blue. B and C, cell were pretreated with 10 or 50 μM GF-015 or GF-90, respectively, for 30 min before incubation with 30 ng/ml TNF-α or 1 μM TPA for 10 min; then whole cell lysates were immunoprecipitated with anti-IKKβ antibody followed by electrophoresis and autoradiography of phosphorylated GST-IκBα (1–100) as described under *Materials and Methods*.

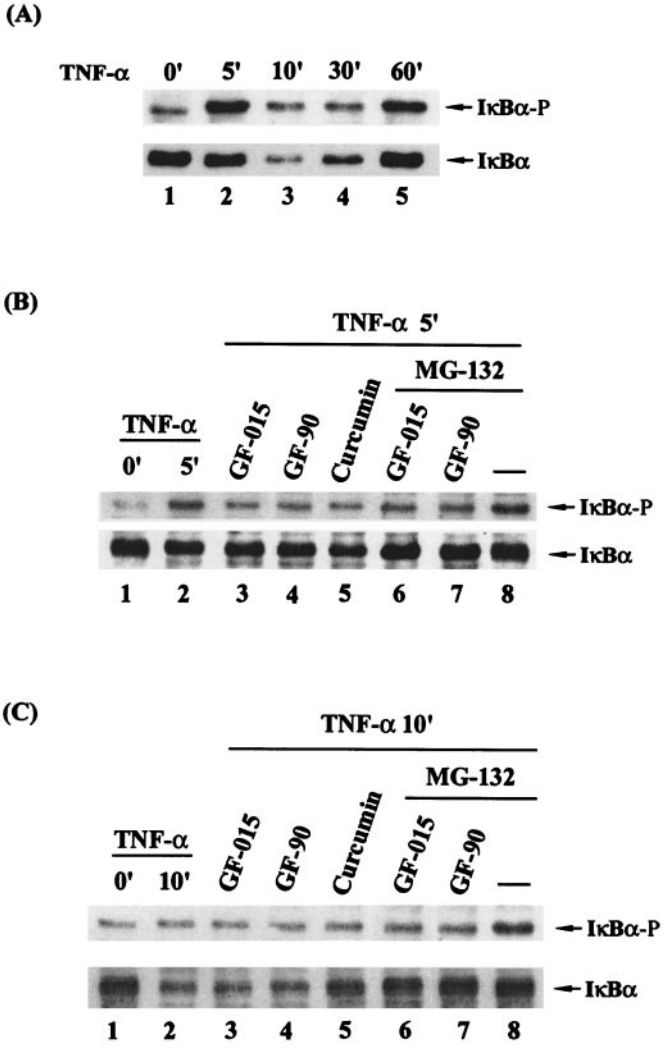

Fig. 8. Time-dependent phosphorylation of IκBα serine 32 and IκBα degradation by TNF-α in NCI-H292 cells and the effects of GF-15, GF-90, curcumin, and MG132. A, cells were stimulated with 30 ng/ml TNF-α for 5, 10, 30, or 60 min. B and C, cells were pretreated with 50 μM GF-015, GF-90, curcumin or 30 μM MG132 or MG132 plus GF-015 or GF-90 for 60 min before stimulation with TNF-α for 5 min (B) or 10 min (B). Cell lysates were prepared and subjected to Western blotting using antibody specific for phosphorylated form of IκBα; then membranes were striped and reprobed with anti-IκBα antibody as described under *Materials and Methods*.

The development of new drugs that inhibit the NF-κB activation pathway will require pharmacokinetic and toxicity studies in conjunction with clinical verification of in vivo activity. In this study, the inhibitory effect of GF-015 and GF-90 on TNF-α- and TPA-induced COX-2 protein expression was caused by suppression of IKK activity and NF-κB activation in the COX-2 promoter, resulting in attenuation of COX-2 gene expression and PGE_2 production. Because these compounds have less of an effect on COX-1 enzyme activity than NSAIDs, these two conjugated polyhydroxybenzene derivatives could serve as lead compounds for the development of new drugs in preventing or treating inflammation and cancer.

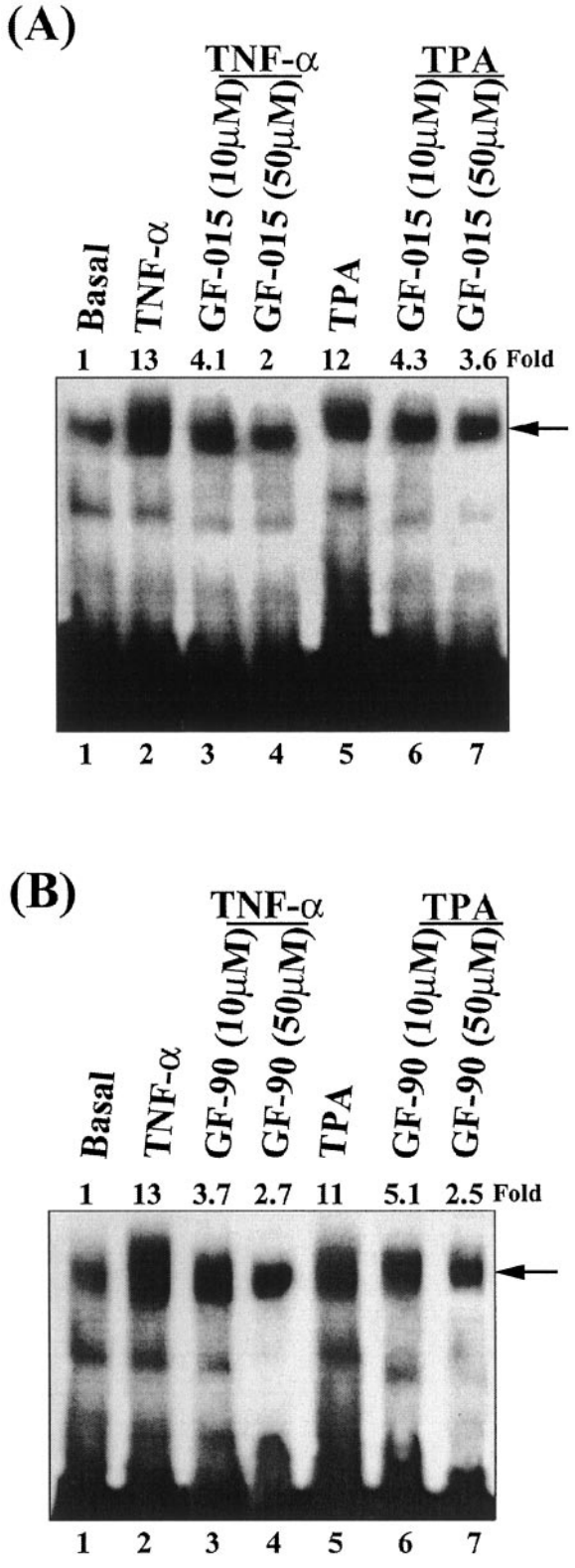

Fig. 9. Inhibitory effect of GF-015 and GF-90 on TNF-α- or TPA-induced NF-κB–specific DNA-protein complex formation in nuclear extracts of NCI-H292 epithelial cells. Cells were pretreated with 10 or 50 μM GF-015 (A) or GF-90 (B) for 30 min before incubation with 30 ng/ml TNF-α or 1 μM TPA for 1 h; then nuclear extracts were prepared and NF-κB DNA-protein binding activity was determined by electrophoretic mobility shift assay as described under *Materials and Methods*.

References

Ammon HP and Wahl MA (1991) Pharmacology of *Curcuma longa*. *Planta Med* **57:**1–7.

Anderson GD, Hauser SD, McGarity KL, Bremer ME, Isakson PC, and Gregory SA (1996) Selective inhibition of cyclooxygenase reverses inflammation and expression of COX-2 and interleukin 6 in rat adjuvant arthritis. *J Clin Invest* **97:**2672–2679.

Baeuerle PA and Henkel T (1994) Function and activation of NF-κB in the immune system. *Annu Rev Immunol* **12:**141–179.

Baldwin AS (1996) The NF-κB and IκB proteins: new discoveries and insights. *Annu Rev Immunol* **14:**649–681.

Barnes PJ (1994) Cytokines as mediators of chronic asthma. *Am J Respir Crit Care Med* **150:**S42–S49.

Barnes PJ and Karin M (1997) Nuclear factor-κB, a pivotal transcription factor in chronic inflammatory diseases. *N Engl J Med* **336:**1066–1071.

Beutler B (1995) TNF, immunity and inflammatory disease: lessons of the past decade. *J Clin Med* **43:**227–235.

Chen CC, Sun YT, Chen JJ, and Chang YJ (2001) TNF-α-induced COX-2 expression via sequential activation of ceramide-dependent mitogen-activated protein kinases and IKK1/2 in human alveolar epithelial cells. *Mol Pharmacol* **59:**493–500.

Chen CC, Sun YT, Chen JJ, and Chiu KT (2000) TNF-α-induced COX-2 expression in human lung epithelial cells: involvement of the PLC-γ2, PKCα, tyrosine kinase, NIK and IKK1/2 pathway. *J Immunol* **165:**2719–2728.

Chen Z, Hagler J, Palombella VJ, Melandri F, Scherer D, Ballard D, and Maniatis T (1995) Signal-induced site-specific phosphorylation targets IκBα to the ubiquitin-proteasome pathway. *Genes Dev* **9:**1586–1597.

DeWitt DL (1999) COX-2 selective inhibitors: the new super aspirins. *Mol Pharmacol* **54:**625–631.

Fletcher BS, Kujubu DA, Perrin DM, and Herschman HR (1992) Structure of the mitogen-inducible TIS10 gene and demonstration that the TIS10-encoded protein is a functional prostaglandin G/H synthase. *J Biol Chem* **267:**4338–4344.

Frolich JC (1997) A classification of NSAIDs according to the relative inhibition of cyclooxygenase isoenzyme. *Trends Pharmacol Sci* **18:**30–34.

Fu JY, Masferrer JL, Seibert K, Raz A, and Needelman P (1990) The induction and suppression of prostaglandin H2 synthase (cyclooxygenase) in human monocytes. *J Biol Chem* **265:**16737–16740.

Giardiello FM, Hamilton SR, Hylind LM, Yang VW, Tamez P, and Casero RA (1997) Ornithine decarboxylases and polyamines in familial adenomatous polyposis. *Cancer Res* **57:**199–201.

Giardiello FM, Offerhaus GJA, and DuBois RN (1995) The role of nonsteroidal anti-inflammatory drugs in colorectal cancer prevention. *Eur J Cancer* **31A:**1071–1076.

Hartner A, Goppelt-Struebe M, and Hilgers KF (1998) Coordinate expression of cyclooxygenase-2 and renin in the rat kidney in renovascular hypertension. *Hypertension* **31(Suppl 2):**201–205.

Hla T and Neilson K (1992) Human cyclooxygenase-2 cDNA. *Proc Natl Acad Sci USA* **89:**7384–7388.

Kalgutkar AS, Crews BC, Rowlinson SW, Garner C, Seibert K, and Marnett LJ (1998) Aspirin-like molecules that covalently inactivate cyclooxygenase-2. *Science (Wash DC)* **280:**1268–1270.

Kawamori T, Rao CV, Seibert K, and Reddy BS (1998) Chemopreventive activity of celecoxib, a specific cyclooxygenase-2 inhibitor, against colon carcinogenesis. *Cancer Res* **58:**409–412.

Kopp E and Ghosh S (1994) Inhibition of NF-κB by sodium salicylate and aspirin. *Science (Wash DC)* **265:**956–959.

Kraemer SA, Meade EA, and DeWitt DL (1992) Prostaglandin endoperoxide synthase gene structure: identification of the transcriptional start site and 5′-flanking regulatory sequences. *Arch Biochem Biophys* **293:**391–400.

Levy GN (1997) Prostaglandin H synthases, non-steroidal antiinflammatory drugs and colon cancer. *FASEB J* **11:**234–247.

Lim H, Paria BC, Das SK, Diachuck JE, Langenbach R, Trazskos JM, and Dey SK (1997) Multiple female reproductive failures in cyclooxygenase-2-deficient mice. *Cell* **91:**197–208.

Lin X, Mu Y, Cunningham ET, Marcu KB, Geleziunas R, and Greene WC (1998) Molecular determination of NF-κB-inducing kinase action. *Mol Cell Biol* **18:**5899–5907.

Ling L, Cao Z, and Geddel DV (1998) NF-κB-inducing kinase activates IKKα by phosphorylation of Ser-176. *Proc Natl Acad Sci USA* **95:**3792–3797.

Malinin NL, Boldin MP, Kovalenko AV, and Wallach D (1997) MAP3K-related kinase involved in NF-κB induction by TNF, CD95 and IL-1. *Nature (Lond)* **385:**540–544.

Maniatis T (1997) Catalysis by multiprotein IκB kinase complex. *Science (Wash DC)* **278:**818–819.
Newton R, Kuitert LM, Bergmann M, Adock IM, and Barnes PJ (1997) Evidence for involvement of NF-κB in the transcriptional control of COX-2 gene expression by IL-1β. *Biochem Biophys Res Commun* **237:**28–32.
Oka A and Takashima S (1997) Induction of cyclooxygenase 2 in brain of patients with Down's syndrome and dementia of Alzheimer type: specific localization in affected neurones and axons. *Neuroreport* **8:**1161–1164.
Oshima M, Dinchuk JE, Kargman SL, Oshima H, Hancock B, Kwong E, Traskos JM, Evans JF, and Taketo MM (1996) Suppression of intestinal polyposis in Apc delta 716 knockout mice by inhibition of cyclooxygenase (COX-2). *Cell* **87:**803–809.
Pennisi E (1998) Building a better aspirin. *Science (Wash DC)* **280:**1191–1192.
Piguet PF, Collart MA, Grau GE, Sappino AP, and Vassalli P (1990) Requirement of tumor necrosis factor for development of silica-induced pulmonary fibrosis. *Nature (Lond)* **344:**245–247.
Plummer SM, Holloway KA, Manson MM, Munks RJL, Kaptein A, Farrow S, and Howells L (1999) Inhibition of cyclooxygenase-2 expression in colon cells by the chemopreventive agent curcumin involves inhibition of NF-κB activation via the NIK/IKK signaling complex. *Oncogene* **18:**6013–6020.
Rao CV, Simi B, and Reddy BS (1993) Inhibition by dietary curcumin of azoxymethane-induced ornithine decarboxylase, tyrosine protein kinase, arachidonic acid metabolism and aberrant crypt foci formation in the rat colon. *Carcinogenesis* **14:**2219–2225.
Smith WL and Dewitt DL (1996) Prostaglandin endoperoxide H synthases-1 and -2. *Adv Immunol* **62:**167–215.
Stadnyk AW (1994) Cytokine production by epithelial cells. *FASEB J* **8:**1041–1047.
Stancovski I and Baltimore D (1997) NF-κB activation: the IκB kinase revealed? *Cell* **91:**299–302.
Standiford TJ, Kankel SL, Basha MA, Chensue SW, Lynch JP, Toews GB, Westwick J, and Strieter RM (1990) Interleukin-8 gene expression by a pulmonary epithelial cell line. A model for cytokine networks in the lung. *J Clin Invest* **86:**1945–1953.
Subbaramaiah K, Zakim D, Weksler BB, and Dannenberg AJ (1997) Inhibition of cyclooxygenase: a novel approach to cancer prevention. *Proc Soc Exp Biol Med* **216:**201–210.
Suter PM, Suter S, Girardin E, Roux-Lombard P, Grau GE, and Dayer JM (1992) High bronchoalveolar levels of tumor necrosis factor and its inhibitors, interleukin-1, interferon and elastase, in patients with adult respiratory distress syndrome after trauma, shock or sepsis. *Am Rev Respir Dis* **145:**1016–1022.
Tordjman C, Coge F, Andre N, Rique H, Spedding M, and Bonnet J (1995) Characterisation of cyclooxygenase 1 and 2 expression in mouse resident peritoneal macrophages in vitro; interactions of nonsteroidal anti-inflammatory drugs with COX-2. *Biochim Biophys Acta* **1256:**249–256.
Tsujii M and Dubois RN (1995) Alterations in cellular adhesion and apoptosis in epithelial cells overexpressing prostaglandin endoperoxide synthase 2. *Cell* **83:**493–501.
Vane JR and Botting RM (1995) New insights into the mode of action of anti-inflammatory drugs. *Inflamm Res* **44:**1–10.
Vane JR, Mitchell JA, Appleton I, Tomlinson A, Bishop-Bailey D, Croxtall J, and Willoughby DA (1994) Inducible isoforms of cyclooxygenase and nitric oxide synthase in inflammation. *Proc Natl Acad Sci USA* **91:**2046–2050.
Williams CS and DuBois RN (1996) Prostaglandin endoperoxide synthase: why two isoform? *Am J Physiol* **270:**G393–G400.
Wong SCY, Fukuchi M, Melnyk P, Rodger I, and Giaid A (1998) Induction of cyclooxygenase-2 and activation of nuclear factor-κB in myocardium of patients with congestive heart failure. *Circulation* **98:**100–103.
Woronicz JD, Gao X, Gao Z, Rothe M, and Goeddel DV (1997) IκB kinase-β: NF-κB activation and complex formation with IκB kinase α and NIK. *Science (Wash DC)* **278:**866–869.
Xu XM, Sansores-Garcia L, Chen XM, Matijevic-Aleksic N, Du M, and Wu KK (1999) Suppression of inducible cyclooxygenase 2 gene transcription by aspirin and sodium salicylate. *Proc Natl Acad Sci USA* **96:**5292–5297.
Yin MJ, Yamamoto Y, and Gaynor RB (1998) The anti-inflammatory agents aspirin and salicylate inhibit the activity of IκB kinase-β. *Nature (Lond)* **396:**77–80.

Address correspondence to: Dr. Ching-Chow Chen, Department of Pharmacology, College of Medicine, National Taiwan University, No.1, Jen-Ai Road, 1st Section, Taipei, 10018, Taiwan. E-mail: ccchen@ha.mc.ntu.edu.tw