

Curcumin (diferuloylmethane) down-regulates cigarette smoke-induced NF- κ B activation through inhibition of I κ B α kinase in human lung epithelial cells: correlation with suppression of COX-2, MMP-9 and cyclin D1

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Cigarette smoke (CS) is a major cause of a variety of malignancies including cancers of the larynx, oral cavity and pharynx, esophagus, pancreas, kidney, bladder and lung. The signal transduction pathway that mediates the effects of CS is not well understood but nuclear factor-kappa B (NF- κ B) is probably involved. The gas phase of CS contains free radicals such as superoxide radicals, hydroxyl radicals and hydrogen peroxide, which potentially can activate NF- κ B. Benzo[*a*]pyrene, another potent carcinogen of CS, can also activate NF- κ B, but by an as yet unknown mechanism. Various other agents that activate NF- κ B are either tumor initiators or tumor promoters, and NF- κ B activation can block apoptosis, promote proliferation and mediate tumorigenesis. Therefore, NF- κ B is an ideal target for preventing CS-induced lung carcinogenesis. Thus, agents that abrogate NF- κ B activation have the potential to suppress lung carcinogenesis. Because curcumin, a diferuloylmethane, is anticarcinogenic, we investigated the effect of this phytochemical on CS-induced NF- κ B activation and NF- κ B-regulated gene expression in human non-small cell lung carcinoma cells. Exposure of cells to CS induced persistent activation of NF- κ B, and pre-treatment with curcumin abolished the CS-induced DNA-binding of NF- κ B, I κ B α kinase activation, I κ B α phosphorylation and degradation, p65 nuclear translocation and CS-induced NF- κ B-dependent reporter gene expression. The inhibition of NF- κ B activation correlated with suppression of CS-induced NF- κ B-dependent cyclin D1, cyclooxygenase-2 and matrix metalloproteinase-9 expression. Overall our results indicate that CS-induced NF- κ B activation and NF- κ B-regulated gene expression in human non-small cell lung carcinoma cells is suppressed by curcumin through suppression of I κ B α kinase.

Introduction

Cigarette smoke (CS) is a major risk factor in cancer development. Smokers have a high incidence of lung cancer which is

Abbreviations: B[*a*]P, benzo[*a*]pyrene; COX-2, cyclooxygenase-2; CS, cigarette smoke; CSC, cigarette smoke condensate; EMSA, electrophoretic mobility shift assay; I κ B, inhibitory subunit of NF- κ B; IKK, I κ B α kinase; NF- κ B, nuclear factor-kappa B; MMP-9, matrix metalloproteinase-9; SDS, sodium dodecyl sulfate; SEAP, secretory alkaline phosphatase; TNF, tumor necrosis factor.

the most common cause of cancer in western countries, accounting for more deaths than those caused by prostate, breast and colorectal cancers combined (1). In addition to this, CS is also implicated in cancers of the larynx, oral cavity, pharynx, esophagus, pancreas, kidney and bladder. Recent estimates indicate that CS causes ~80–90% of lung cancer in the US and in Canada an estimated 20% of all deaths and ~30% of deaths from cancer are caused by it (2).

CS is a complex chemical mixture containing thousands of different compounds, of which 100 are known carcinogens, co-carcinogens, mutagens and/or tumor promoters (3). Metabolites of tobacco-specific *N*-nitrosamines, such as 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone and polycyclic aromatic hydrocarbons, such as diol epoxides of benzo[*a*]pyrene (B[*a*]P), are believed to be the primary tobacco carcinogens (4), although high levels of free radicals may also play an important role. Each puff of smoke contains over 10 trillion free radicals in both mainstream and side stream smoke, which also may contribute to both tumor initiation and promotion. Active smokers have >25% lower circulating concentrations of ascorbic acid, alpha-carotene, beta-carotene and cryptoxanthin (5). Also, CS is known to induce morphological changes in lungs, placenta, liver and kidneys of pregnant rats (6). Additionally, exposure of cells to B[*a*]P mutates the p53 gene at the same site as is found in 60% of lung cancer patients (7). A tobacco-specific *N*-nitrosamine or cigarette smoke condensate (CSC) causes neoplastic transformation of xenotransplanted human bronchial epithelial cells (8).

Among the potential mediators of CS-induced alterations is nuclear factor-kappa B (NF- κ B), whose activation has been implicated in chemical carcinogenesis and tumorigenesis (9,10). Expression of several genes, such as cyclooxygenase (COX)-2, matrix metalloproteinase (MMP)-9, inducible nitric oxide synthase, tumor necrosis factor (TNF), interleukin (IL)-8, eotaxin, cell surface adhesion molecules and anti-apoptotic proteins, involved in tumor initiation, tumor promotion and metastasis are regulated by NF- κ B (11). This ubiquitous nuclear transcription factor plays a major regulatory role in carcinogenesis. It resides in the inactive state in cytoplasm as a heterotrimer consisting of p50, p65 and I κ B α subunits. As a transcription factor, it assumes a dimeric form composed of different members of the Rel/NF- κ B family of polypeptides (12). The p50–p65 heterodimer is retained in the cytoplasm by the inhibitory subunit I κ B α . On activation of the complex, I κ B α sequentially undergoes phosphorylation, ubiquitination and degradation, thus releasing the p50–p65 heterodimer for translocation to the nucleus. An I κ B α kinase, IKK, has been identified that phosphorylates serine residues in I κ B α at positions 32 and 36 (13). Treatment of cells with various inflammatory and oxidative stress stimuli activates the IKK, thus leading to the degradation of I κ B α and activation of the transcription factor.

We have demonstrated that CS activates NF- κ B in a wide variety of different cell types and that this activation is

mediated through induction of IKK, leading to phosphorylation and degradation of I κ B α (14). Therefore, agents that can suppress NF- κ B activation have a potential to suppress CS-induced carcinogenesis. One such agent, curcumin (diferuloylmethane), has been shown to be pharmacologically safe, to be involved in the suppression of carcinogenesis and to suppress effects of various inflammatory stimuli (15–18). In the present report we investigated whether curcumin could block CS-induced NF- κ B activation in human non-small cell lung carcinoma cells and whether it abrogates the expression of genes implicated in carcinogenesis.

Materials and methods

Materials

Curcumin, with a purity >98%, was purchased from LKT laboratories (Minneapolis, MN), dissolved in dimethylsulfoxide (DMSO) as a 100 mM stock solution and stored at -20°C . Bacteria-derived human TNF, purified to homogeneity with a specific activity of 5×10^7 U/mg, was kindly provided by Genentech, (South San Francisco, CA). Penicillin, streptomycin, Iscove's modified Dulbecco's medium, RPMI 1640 medium, keratinocyte serum-free medium and fetal bovine serum (FBS) were obtained from Invitrogen (Grand Island, NY). Tris, glycine, NaCl, SDS and bovine serum albumin, were obtained from Sigma Chemical Co. (St Louis, MO). The following polyclonal antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA): anti-p65, against the epitope corresponding to amino acids mapping within the N-terminal domain of human NF- κ B p65; anti-p50, against a peptide 15 amino acids long mapping at the nuclear localization sequence region of NF- κ B p50; anti-I κ B α , against amino acids 297–317 mapping at the C-terminus of I κ B α /MAD-3 and anti-c-Rel and anti-cyclin D1 against amino acids 1–295, which represents full-length cyclin D1 of human origin. Phospho-I κ B α (Ser32) antibody was purchased from New England Biolabs (Beverly, MA). Anti-IKK α and anti-IKK β antibodies were kindly provided by Imgenex (San Diego, CA). Anti COX-2 antibody was purchased from Transduction Labs (now Invitrogen, Carlsbad, CA) and anti-MMP-9 antibody was purchased from Cell Sciences (Norwood, MA).

Cell lines

The cells used in our studies included immortalized human bronchial epithelial cells (BEAS-2B), human non-small cell lung carcinoma (H1299), and human lung epithelial cell carcinoma (A549). All lung cells were kindly provided by Dr Reuben Lotan (from our Institute). Dominant-negative I κ B α transfected Jurkat cells were kindly provided by Dr Dean W. Ballard (University of Pennsylvania School of Medicine, Philadelphia). BEAS-2B cells were maintained in keratinocyte serum-free medium, and other cell lines were cultured in RPMI 1640 medium supplemented with 10% FBS, 100 U/ml penicillin and 100 $\mu\text{g}/\text{ml}$ streptomycin.

Preparation of CSC

The CSC was prepared from the University of Kentucky Reference Cigarette 1R4F (9 mg tar and 0.8 mg nicotine/cigarette). The 'tar' or particulate phase of smoke was collected on a Cambridge filter pad from cigarettes using a smoking machine set to a 35 ml puff volume of 2 s duration under standard conditions prescribed by the Federal Trade Commission (19). The smoke particulate matter was dissolved in DMSO at 40 mg/ml, aliquoted into small vials and stored frozen at -80°C . On the day of the experiment, each vial of CSC solution was opened and diluted in the serum-free cell culture medium to the desired concentration, vortexed vigorously and used for treatment of cells. Control cells were treated with medium containing an equivalent amount of DMSO. The number of freeze-thaw cycles for the CSC solution was minimized.

NF- κ B activation

To determine NF- κ B activation, we carried out electrophoretic mobility shift assay (EMSA) essentially as described previously (20). Briefly, nuclear extracts prepared from cells ($2 \times 10^6/\text{ml}$) treated with carcinogens were incubated with ^{32}P -end-labeled 45mer double-stranded NF- κ B oligonucleotide (4 μg of protein with 16 fmol of DNA) from the human immunodeficiency virus long terminal repeat, 5'-TTGTTACAAGGGACTTTC CGCTG **GGGACTTTC** CAGGGA GGCGT GG-3' (text bold indicates NF- κ B-binding sites), for 15 min at 37°C , and the DNA-protein complex formed was separated from free oligonucleotide on 6.6% native polyacrylamide gels. A double-stranded mutated oligonucleotide, 5'-TTGTTACA**ACTCACTTTC** CGCTGCTCA**CTTTC** CAGGGAGG CGTGG-3', was used to examine the

specificity of binding of NF- κ B to the DNA. The specificity of binding was also examined by competition with the unlabeled oligonucleotide. For super-shift assays, nuclear extracts prepared from CS-treated cells were incubated with antibodies against either p50 or p65 of NF- κ B for 30 min at room temperature before the complex was analyzed by EMSA. Antibodies against cyclin D1 and pre-immune serum were included as negative controls. The dried gels were visualized, and radioactive bands were quantitated using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA) using Imagequant software.

I κ B α degradation

To determine the effect of curcumin on CS-dependent I κ B α degradation, cytoplasmic extracts were prepared as described previously (21) from H1299 cells ($2 \times 10^6/\text{ml}$) pre-treated with curcumin for 2 h and then exposed to 10 $\mu\text{g}/\text{ml}$ CS for various times. The extracts were then resolved on 10% SDS-polyacrylamide gels. After electrophoresis, the proteins were electrotransferred to nitrocellulose filters, probed with rabbit polyclonal antibodies against I κ B α and detected by chemiluminescence (ECL, Amersham). The bands obtained were quantified using a Personal Densitometer Scan v1.30 using Imagequant software version 3.3 (Molecular Dynamics).

I κ B α phosphorylation

To determine the effect of curcumin on CS-dependent I κ B α phosphorylation, cytoplasmic extracts were prepared from H1299 cells ($2 \times 10^6/\text{ml}$) treated with 50 μM curcumin for 2 h and then treated with 10 $\mu\text{g}/\text{ml}$ CS for various times. The extracts were then resolved on 10% SDS-polyacrylamide gels and analyzed by western blotting using antibody against phosphorylated I κ B α as described previously (21).

IKK assay

To determine the effect of curcumin on CS-induced IKK activation, we assayed IKK by a method described previously (22). Briefly, the IKK complex was precipitated from whole-cell extracts with antibody to IKK α and IKK β and then treated with 20 μl of protein A/G-Sepharose (Pierce, Rockford, IL). After 2 h, the beads were washed with lysis buffer and then assayed in kinase assay mixture containing 50 mM HEPES (pH 7.4), 20 mM MgCl_2 , 2 mM DTT, 20 μCi [γ - ^{32}P]ATP, 10 μM unlabeled ATP, and 2 μg of substrate GST-I κ B α (1–54). After incubation at 30°C for 30 min, the reaction was terminated by boiling with 5 μl of 5 \times SDS sample buffer for 5 min. Finally, the protein was resolved on 10% polyacrylamide gel under reducing conditions, the gel was dried, and the radioactive bands were visualized using a PhosphorImager. To determine the total amounts of IKK α and IKK β in each sample, 30 μg of the whole-cell extract protein was resolved on a 7.5% acrylamide gel and then electrotransferred to a nitrocellulose membrane. The membrane was blocked with 5% non-fat milk protein for 1 h and then incubated with either anti-IKK α or anti-IKK β (1:1000 dilution) for 1 h. The membrane was then washed and treated with horseradish peroxidase-conjugated secondary anti-mouse IgG, antibody and proteins were detected by chemiluminescence (Amersham).

NF- κ B-dependent reporter gene transcription

The effect of curcumin on CS-induced NF- κ B dependent reporter gene transcription was measured as described previously (23). Briefly, H1299 cells were seeded at 1.5×10^5 cells per well in six-well plates. After overnight culture, the cells were transfected with pNF- κ B-secretory alkaline phosphatase (SEAP) by using 6 μl of Lipofectamine 2000 (Invitrogen Life Technologies, Carlsbad, CA) using the manufacturer's protocol. The transfection efficiency of this method was 35% as determined by X-gal (5-bromo-4-chloro-3-indolyl- β -D-galactoside) staining. After 6 h, the cells were incubated in medium containing curcumin (25 μM) for 12 h. The cells were then exposed to CSC (10 $\mu\text{g}/\text{ml}$) or TNF (0.1 nM) for 24 h. The cell culture medium was then harvested and analyzed for SEAP activity essentially according to the protocol described by the manufacturer (Clontech, Palo Alto, CA) using a 96-well fluorescence plate reader (Fluoroscan II, Labsystems, Chicago, IL) with excitation set at 360 nm and emission set at 460 nm.

Transient transfection and luciferase assay

H1299 cells were seeded at a concentration of 1.5×10^5 cells per well in six-well plates. After overnight culture, the cells in each well were transfected with 2 μg DNA consisting of COX-2 promoter-luciferase reporter plasmid, along with 6 μl of Lipofectamine (Life Technologies) by following the manufacturer's protocol. The COX-2 promoter (–375 to +59) amplified from human genomic DNA by using the primers 5'-GAGTCTCTTATTTATTTT-3' (sense) and 5'-GCTGCTGAGGAGTTCCTGGACGTGC-3' (antisense) was kindly provided by Dr Xiao-Chun Xu (M.D. Anderson Cancer Center). After a 6 h exposure to the transfection mixture, the cells were incubated in medium containing curcumin (25 μM) for 12 h. The cells were then exposed to CSC (10 $\mu\text{g}/\text{ml}$) or TNF (0.1 nM) for 24 h and then harvested. Luciferase activity was measured by using the Promega luciferase assay system according to the

manufacturer's protocol and detected by using Monolight 2010 (Analytical Luminescence Laboratory, San Diego, CA). All experiments were performed in triplicates, and repeated at least twice to prove their reproducibility.

NF- κ B p65 localization

The effect of curcumin on CS-induced nuclear translocation of p65 was examined using an immunocytochemical method as described previously (24). Briefly, cells were grown in chamber slides and fixed with cold acetone. After a brief washing in PBS, slides were blocked with 5% normal goat serum for 1 h and then incubated with rabbit polyclonal anti-human p65 antibody (dilution, 1:100). After overnight incubation, the slides were washed and then incubated with goat anti-rabbit IgG-Alexa 594 (1:100) for 1 h and counterstained for nuclei with Hoechst stain (50 ng/ml) for 5 min. Stained slides were mounted with mounting medium (Sigma Chemical) and analyzed under an epifluorescence microscope (Labophot-2; Nikon, Tokyo, Japan). Images were captured using a Photometrics Coolsnap CF color camera (Nikon, Lewisville, TX) and MetaMorph version 4.6.5 software (Universal Imaging, Downingtown, PA).

Western blot analysis

Thirty to sixty micrograms of whole-cell protein was resolved on 10% SDS-PAGE gel. The protein was transferred to a nitrocellulose membrane, blocked with 5% non-fat milk, and probed with specific antibodies against I κ B α (1:3000), phospho-I κ B α (1:1000), cyclin D1, MMP-9 and COX-2 (1:1000) separately. The blots were washed, exposed to HRP-conjugated secondary antibodies for 1 h, and finally detected by ECL reagent (Amersham Pharmacia Biotech.). For COX-2 and MMP-9 assays, whole-cell extracts were prepared from treated cells (2×10^6 cells in 2 ml medium) and resolved on 7.5% SDS-polyacrylamide gels.

RNA analysis and RT-PCR

H1299 cells were cultured at a density of 2×10^6 cells/ml and kept overnight in serum-free medium. Cells were washed and then treated with either different concentrations of CSC or pre-treated with curcumin before treatment with CSC. The growth medium was removed, cells were suspended in Trizol reagent and total RNA was extracted according to the manufacturer's instructions (Invitrogen, Life Technologies, Grand Island, NY). Two micrograms of total RNA was converted to cDNA by Superscript reverse transcriptase and then amplified by Platinum *Taq* polymerase using Superscript One Step RT-PCR kit (Invitrogen, Life Technologies). The relative expression of COX-2 was analyzed using quantitative RT-PCR with β actin as an internal control. The RT-PCR reaction mixture contained 25 μ l of $2 \times$ reaction buffer, 2 μ l each of RNA and forward and reverse COX-2 or β actin primers and 1 μ l of RT-Platinum *Taq* in a final volume of 50 μ l. The primer sequences for COX-2 were as follows: sense 5' TTCAAATGAGATTGTGGGAAAATTGCT 3' and anti-sense 5' AGATCATCTCTGCCTGAGTATCTT 3'. For β actin the primer sequences were as follows: sense 5'GGGTCAGAAGGATTCCTATG3' and antisense 5'GGTCTCAAACATGATCTGGG3'. The reaction was performed at 50°C for 30 min, 94°C for 2 min, 35 cycles at 94°C for 15 s, 60°C for 30 s and 72°C for 1 min with extension at 72°C for 10 min. PCR products were run on 2% agarose gel and then stained with ethidium bromide. Stained bands were visualized under UV light and photographed.

Results

Several genes implicated in CS-induced carcinogenesis are regulated by NF- κ B. Because of the critical role of NF- κ B in CSC-induced carcinogenesis, the present study was designed to examine the effect of curcumin on induction of NF- κ B activation by CSC. Because the mechanism of NF- κ B activation by TNF is better understood, we used this cytokine as a control for comparison with CSC. Because they are more convenient to culture, human non-small cell lung carcinoma (H1299) cells were used for most experiments. Results were independently confirmed in immortalized human bronchial epithelial cells (BEAS-2B) and in human lung epithelial cell carcinoma (A549) cells.

CSC activates NF- κ B in a dose- and time-dependent manner in lung cells

We investigated the ability of CSC to activate NF- κ B in lung cells. To examine the effect of CSC on NF- κ B activation, we

treated H1299 cells with various concentrations of CSC for 30 min or with 0.1 μ g/ml CSC for various times, prepared the nuclear extracts and examined NF- κ B activation by EMSA. As shown in Figure 1, CSC activated NF- κ B maximally at 0.1 μ g/ml (panel A), and the activation reached optimum at 30 min. Interestingly, however, the activation of NF- κ B continued without any significant decline even up to 4 h.

Various combinations of Rel/NF- κ B proteins can constitute an active NF- κ B heterodimer that binds to a specific sequence in DNA (12). To show that the retarded band visualized by EMSA in CSC-treated cells was indeed NF- κ B, we incubated nuclear extracts from CSC-activated cells with antibody to either the p50 (NF- κ B1) or the p65 (RelA) subunit of NF- κ B. Both shifted the band to a higher molecular mass (data not shown), thus suggesting that the CSC-activated complex consisted of p50 and p65 subunits. Neither pre-immune serum (PIS) nor the irrelevant antibody anti-cyclin D1 had any effect. Excess unlabeled NF- κ B (100-fold) caused the band to completely disappear.

CSC induces the phosphorylation and degradation of I κ B α in lung cells

TNF-induced NF- κ B activation requires degradation of I κ B α (25), but not that induced by H₂O₂, X-rays, γ -radiation or pervanadate treatment (26–29). To determine whether CSC activates NF- κ B through I κ B α degradation in lung cells, we treated H1299 cells with CSC for various times, prepared cytoplasmic extracts and assayed them for I κ B α degradation by western blot analysis. As shown in Figure 1C (right top panel), evidence of I κ B α degradation appeared at 30 and 60 min of CSC treatment. In comparison, TNF-induced degradation of I κ B α could be seen as early as 10 min and was complete by 30 min. Re-synthesis of I κ B α , which is NF- κ B dependent, could be seen at 60 min in TNF treated cells but not CSC-treated cells. These results indicate that like TNF, CSC-induced NF- κ B activation is accompanied by I κ B α degradation, however, the kinetics differ. To determine whether I κ B α degradation was preceded by I κ B α phosphorylation, we examined the CSC-induced phosphorylated form of I κ B α by western blot analysis, using antibody that detects only the serine-phosphorylated form of I κ B α . Like TNF, CSC induced I κ B α phosphorylation as early as 5 min in lung cells (Figure 1D).

Curcumin inhibits CSC-dependent NF- κ B activation

Curcumin has been shown to suppress TNF-induced NF- κ B activation, but whether it suppresses CSC-induced NF- κ B activation in lung cells has not been reported. H1299 cells were pre-incubated with different concentrations of curcumin for 2 h and then treated with either TNF or CSC. As shown in Figure 2A, curcumin inhibited CSC-mediated NF- κ B activation in a dose-dependent manner (right panel), with maximum inhibition occurring at 50 mM. The results were comparable with those of TNF (left panel).

The activation of NF- κ B by CSC and its suppression by curcumin was also confirmed independently by immunocytochemistry. Curcumin-pre-treated cells were exposed to CSC and then cytospun on a glass slide, immunostained with antibody p65, and then visualized by the Alexa-594-conjugated second antibody as described in the Materials and methods. The results in Figure 2B clearly demonstrate that CSC induced nuclear translocation of p65 and curcumin prevented the translocation in H1299 cells. These cytological findings were consistent with the NF- κ B inhibition observed by EMSA.

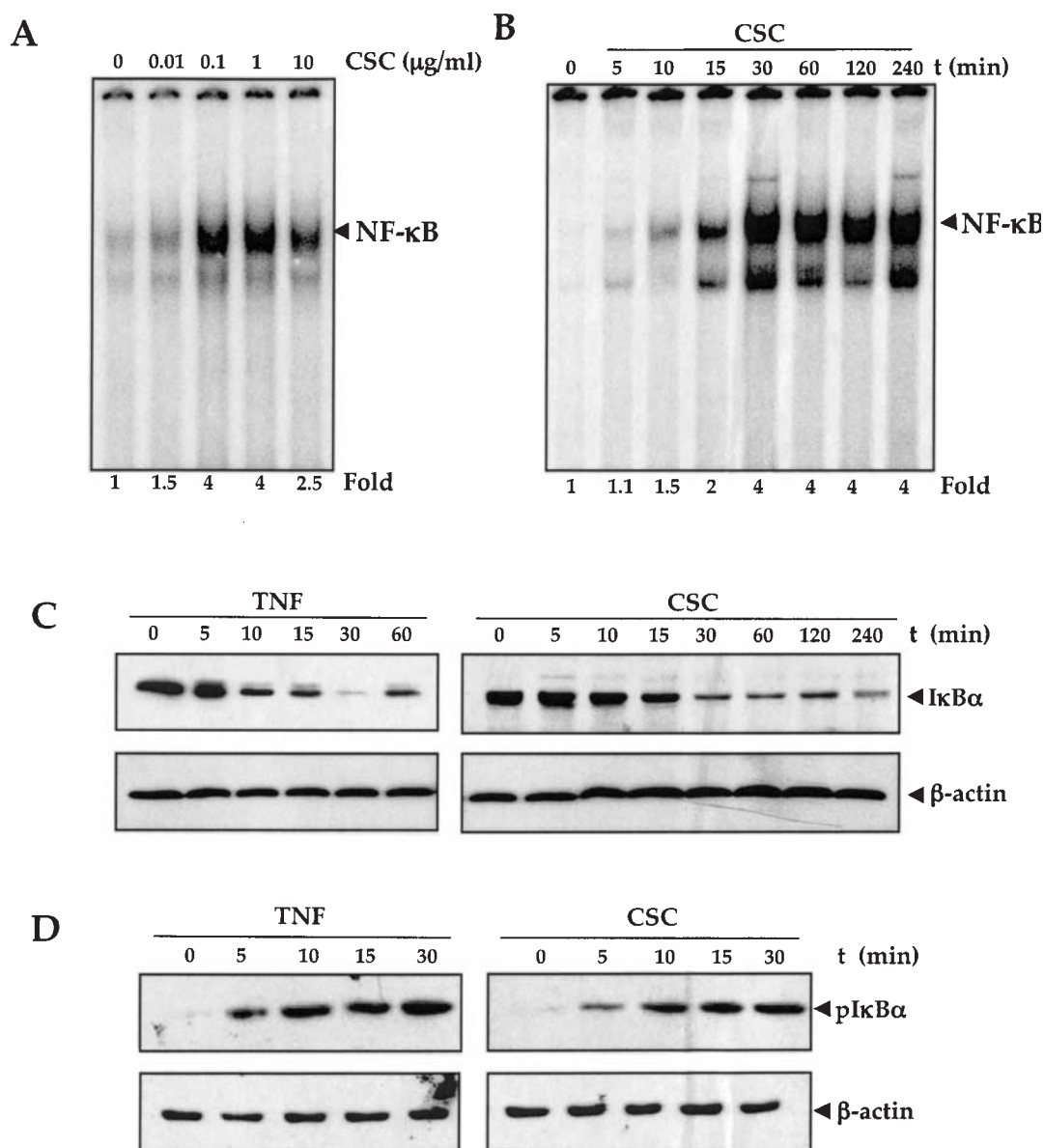


Fig. 1. CSC activates NF- κ B. **(A)** Dose-dependent activation of NF- κ B. Two million H1299 cells in 1 ml were treated with different concentrations of CSC for 30 min, and the nuclear extracts were prepared and assayed for NF- κ B by EMSA as described in the Materials and methods. **(B)** Time course for activation of NF- κ B. Two million H1299 cells in 1 ml were treated with 0.1 μ g/ml CSC for different times. The nuclear extracts were prepared and assayed for NF- κ B by EMSA as described in the Materials and methods. **(C)** CSC induces degradation of I κ B α . Two million H1299 cells per milliliter were treated with either 0.1 nM TNF or 10 μ g/ml CSC for different times. The cytoplasmic extracts were prepared and assayed for I κ B α by western blot using I κ B α -specific antibodies. **(D)** CSC induces phosphorylation of I κ B α . Two million H1299 cells per milliliter were pre-treated with 100 μ g/ml ALLN for 1 h and then treated with either 0.1 nM TNF or 10 μ g/ml CSC for different times. The cytoplasmic extracts were prepared and assayed for phosphorylated I κ B α by western blot using phosphorylated I κ B α -specific antibodies.

Inhibition of NF- κ B activation by curcumin is not cell type-specific

Distinct signal transduction pathways mediate NF- κ B induction in epithelial and lymphoid cells (30). We investigated whether CSC could activate NF- κ B in immortalized human bronchial epithelial cells (BEAS-2B) and in other human lung epithelial cell carcinoma (A549) and whether curcumin could inhibit this activation. Cells were pre-treated with curcumin and then exposed to CSC. CSC activated NF- κ B in both cell types and curcumin completely inhibited this activation (Figure 3), indicating a lack of cell type-specificity.

Curcumin inhibits CSC-dependent I κ B α degradation

Whether inhibition of CSC-induced NF- κ B activation was due to inhibition of I κ B α degradation was examined next. We pre-treated cells with curcumin before exposing them to CSC for different times and then examined them for NF- κ B in the nucleus and for I κ B α in the cytoplasm by western blot analysis. As shown in Figure 4A, CSC activated NF- κ B, but pre-treatment with curcumin abolished the CSC-induced NF- κ B activation. Similarly, CSC induced I κ B α degradation in untreated cells, but in curcumin-pre-treated cells CSC had no effect on I κ B α degradation (Figure 4B). These results clearly

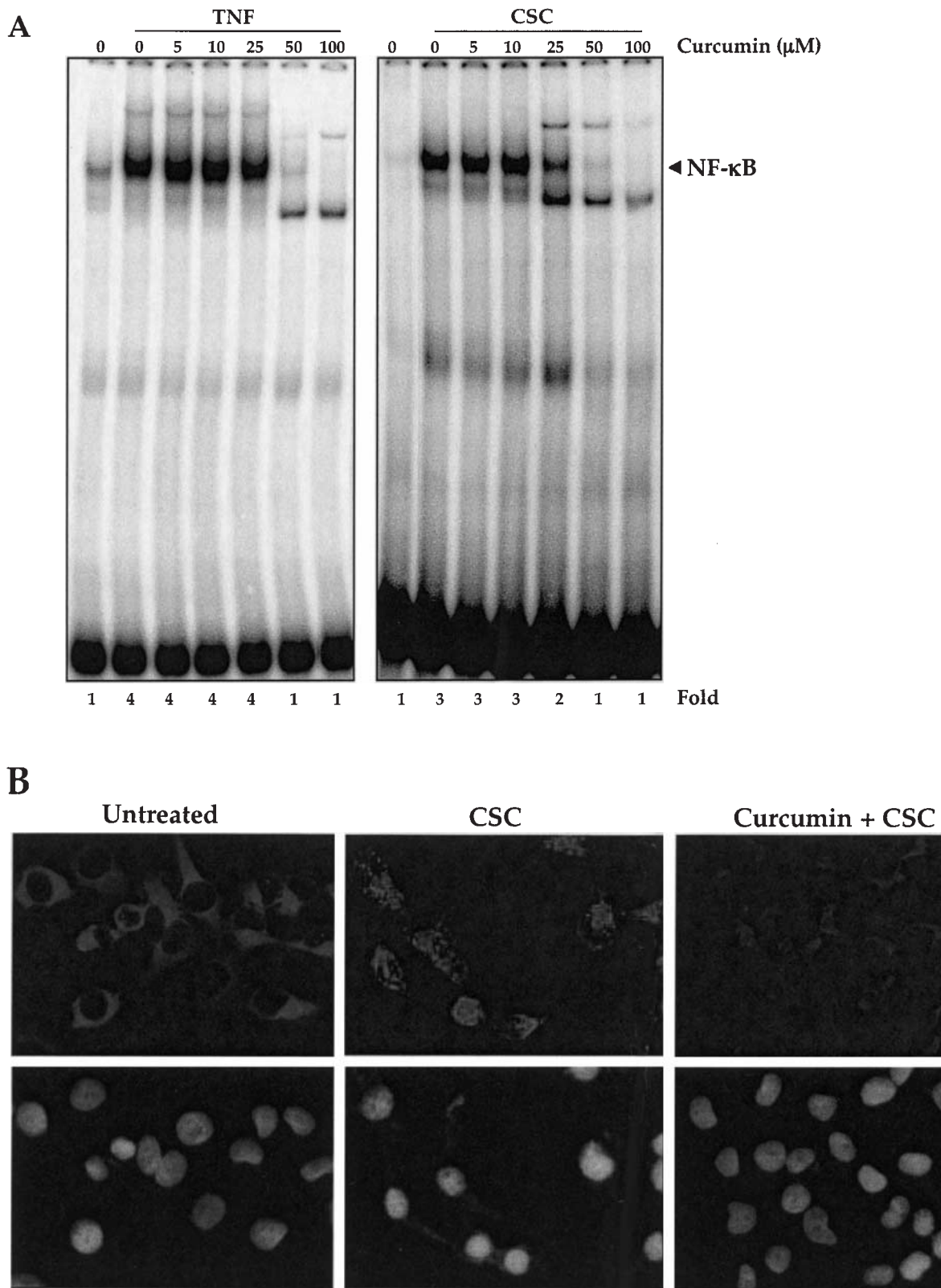


Fig. 2. (A) Curcumin-inhibited TNF- and CSC-dependent NF- κ B activation. H1299 cells (2×10^6 /ml) were pre-incubated with different concentrations of curcumin for 2 h at 37°C and then treated with 0.1 nM TNF or 0.1 μ g/ml CSC for 30 min. Nuclear extracts were prepared and tested for NF- κ B activation, as described in Materials and methods. (B) Curcumin-inhibited CSC-induced nuclear translocation of p65. H1299 cells (1×10^6 /ml) were first treated with 50 μ M curcumin for 2 h at 37°C and then exposed to 10 μ g/ml CSC. After cytopspin, immunocytochemical analysis was performed as described in Materials and methods. The upper three panels show cells stained first with anti p65 antibody and then with alexa-conjugated second antibody. The lower three panels show nuclei as detected with Hoechst stain.

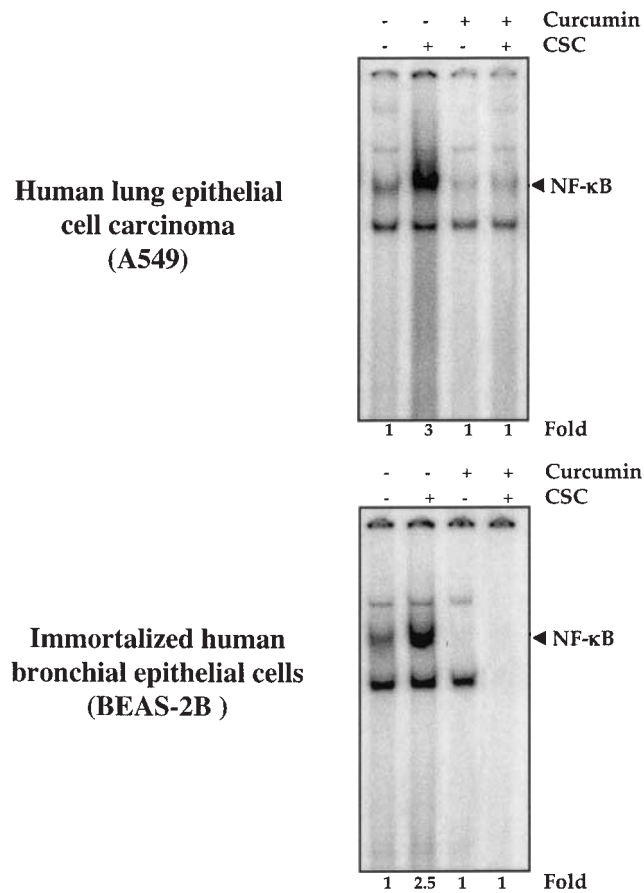


Fig. 3. Cell-type specificity of curcumin-induced inhibition of CSC-induced NF-κB activation. Two million A549 or BEAS-2B cells were pre-treated with 50 μM curcumin for 2 h and then treated with 0.1 μg/ml CSC for 30 min. The nuclear extracts were then prepared and assayed for NF-κB by EMSA as described in the Materials and methods.

indicate that curcumin inhibited both CSC-induced NF-κB activation and IκBα degradation.

Curcumin blocks CSC-induced activation of IκBα kinase

TNF-induced IκBα degradation requires phosphorylation of IκBα at serine 32 and 36 (31). NF-κB activation by pervanadate and H₂O₂, however, requires phosphorylation of IκBα at tyrosine 42 (32–34). The phosphorylation of IκBα at serine 32 requires the activation of IKK (35). Exposure of cells to CSC induced the phosphorylation of IκBα at serine 32 (Figure 5A, left panel), and pre-treatment with curcumin abolished the CSC-induced phosphorylation.

As phosphorylation of IκBα requires the activation of IKK, we directly examined the activation of IKK by immunoprecipitating it using specific antibodies and immunocomplex kinase assays with GST-IκBα as a substrate. CSC activated IKK within 20 min (Figure 5B, upper left panel) and pre-treatment of lung cells with curcumin completely suppressed the CSC-induced activation of IKK. CSC or curcumin had no direct effect on the expression of either IKKα (middle panel) or IKKβ proteins (lower panel), as their levels, as revealed by western blot analysis, were unaffected (Figure 5B, lower two panels).

Curcumin inhibits CSC-induced NF-κB-dependent reporter gene expression

DNA binding alone does not always correlate with NF-κB-dependent gene transcription, suggesting additional regulatory steps are required for NF-κB activation (36). To determine the effect of curcumin on CSC-induced NF-κB-dependent reporter gene expression, we transiently transfected the H1299 cells with the SEAP reporter construct and then treated them with 25 μM of curcumin for 2 h before treatment with CSC for 24 h. SEAP activity reached nearly 10-fold that of the untreated vector control noted upon stimulation with CSC (Figure 6).

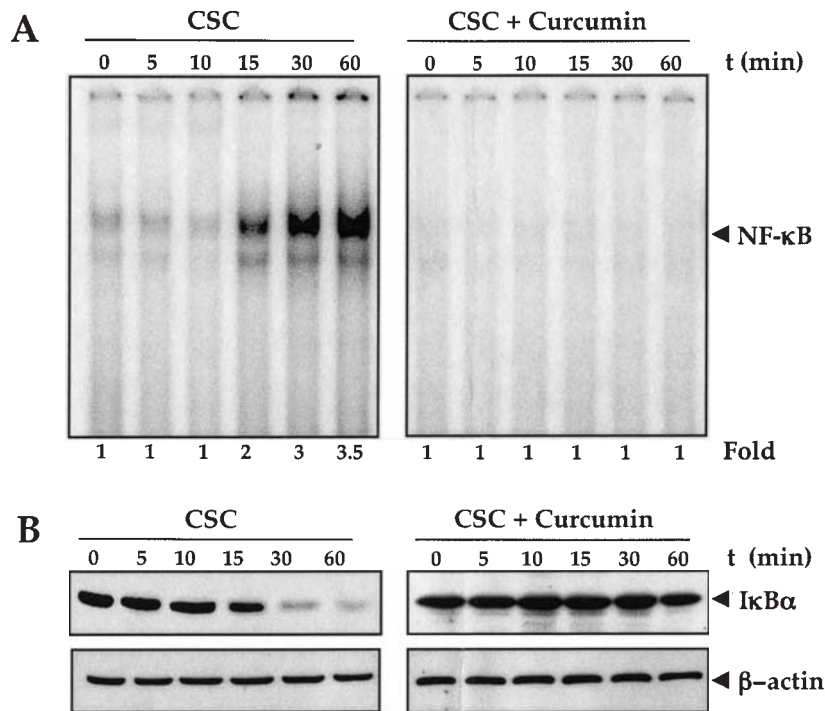


Fig. 4. Curcumin inhibited CSC-induced NF-κB activation and IκBα degradation. H1299 cells (2×10^6 /ml) were incubated with 50 μM curcumin for 2 h at 37°C, treated with 0.1 μg/ml CSC for indicated times at 37°C, and tested for NF-κB activation by EMSA (A), and for IκBα in cytosolic fractions by western blot analysis (B) with antibodies against IκBα. Equal protein loading was evaluated by β-actin.

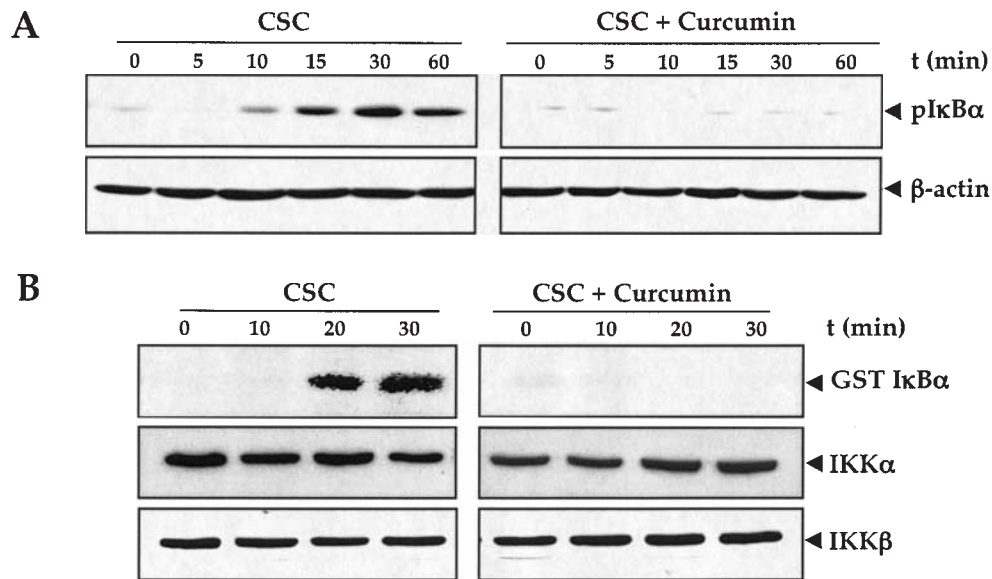


Fig. 5. (A) Curcumin inhibited CSC-induced I κ B α phosphorylation and I κ B α kinase activity. H1299 cells (2×10^6 /ml) were incubated with 50 μ M curcumin for 2 h at 37°C, treated with 0.1 μ g/ml CSC for indicated times at 37°C, and tested for phosphorylated I κ B α in cytosolic fractions by western blot analysis with antibodies against phosphorylated I κ B α . Equal protein loading was evaluated by β -actin. (B) Curcumin inhibited CSC-induced I κ B α kinase activity. H1299 cells (2×10^6 /ml) were treated with 50 μ M curcumin for 2 h and then treated with 10 μ g/ml CSC for different time intervals. Whole-cell extracts were prepared, and 200 μ g of extract was immunoprecipitated with antibodies against IKK α and IKK β . Thereafter immune complex kinase assay was performed as described in Materials and methods. To examine the effect of curcumin on the level of expression of IKK proteins, 30 μ g cytoplasmic extracts was run on 7.5% SDS-PAGE, electrotransferred and immunoblotted with indicated antibodies as described in Materials and methods.

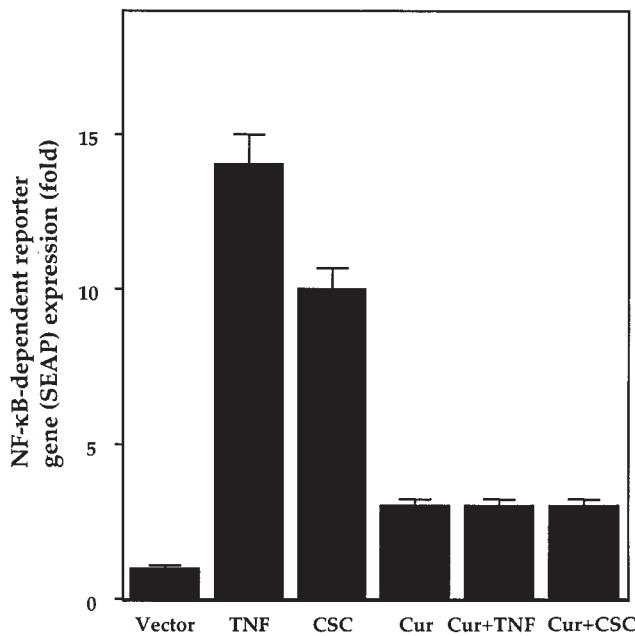


Fig. 6. Curcumin inhibited CSC-induced NF- κ B-dependent reporter gene (SEAP) expression. H1299 cells were transiently transfected with an NF- κ B-containing plasmid linked to the SEAP gene, then treated with 25 μ M curcumin for 12 h. Thereafter, cells were activated for NF- κ B by either 10 μ g/ml CSC or 1 nM TNF as indicated. After 24 h the cell supernatants were collected and assayed for SEAP activity as described in Materials and methods. Results are expressed as fold activity of the vector control activity.

The CS-induced SEAP activity was abolished by dominant-negative I κ B α , indicating specificity (data not shown). These results demonstrate that curcumin inhibits NF- κ B-dependent reporter gene expression induced by CS.

Curcumin inhibited CSC-induced COX-2, MMP-9 and cyclin D1 activation

Because cyclin D1, COX-2 and MMP-9 are NF- κ B-regulated genes (11,37–39), we investigated whether suppression of CSC-induced NF- κ B activation by curcumin abrogates induction of these three genes. H1299 cells, either untreated or pre-treated with curcumin, were exposed to CSC for different times. Whole-cell extracts were prepared and analyzed by western blotting. CSC induced COX-2 (Figure 7A), cyclin D1 (Figure 7B) and MMP-9 (Figure 7C) expression in a time-dependent manner, and curcumin abolished the CSC-induced expression of these gene products.

NF- κ B activation is required for induction of COX-2, cyclin D1 and MMP-9 by CSC

Whether MMP-9, COX-2 and cyclin D1 expression was activated through the activation of NF- κ B was further investigated. Dominant-negative I κ B α -transfected and control Jurkat (2×10^6 cells/ml) cells were treated with either TNF or CSC and tested for NF- κ B activation by EMSA. As shown in Figure 8A, both CSC and TNF activated NF- κ B in control cells but not in dominant-negative I κ B α -transfected cells. We then examined the ability of CSC to induce the expression of MMP-9, COX-2 and cyclin D1 in whole-cell extracts prepared from CSC-treated control and dominant-negative I κ B α -transfected cells. Western blotting showed that CSC induced COX-2, cyclin D1 and MMP-9 expression in a time-dependent manner in control cells but not in DN-I κ B α -transfected cells (Figure 8B). These results further strengthen our postulate that NF- κ B plays a role in CSC-induced activation of the above-mentioned NF- κ B-regulated genes.

Curcumin inhibited CSC-induced COX-2 mRNA transcription

As down-regulation of NF- κ B by curcumin suppresses the expression of NF- κ B-regulated gene products, we also

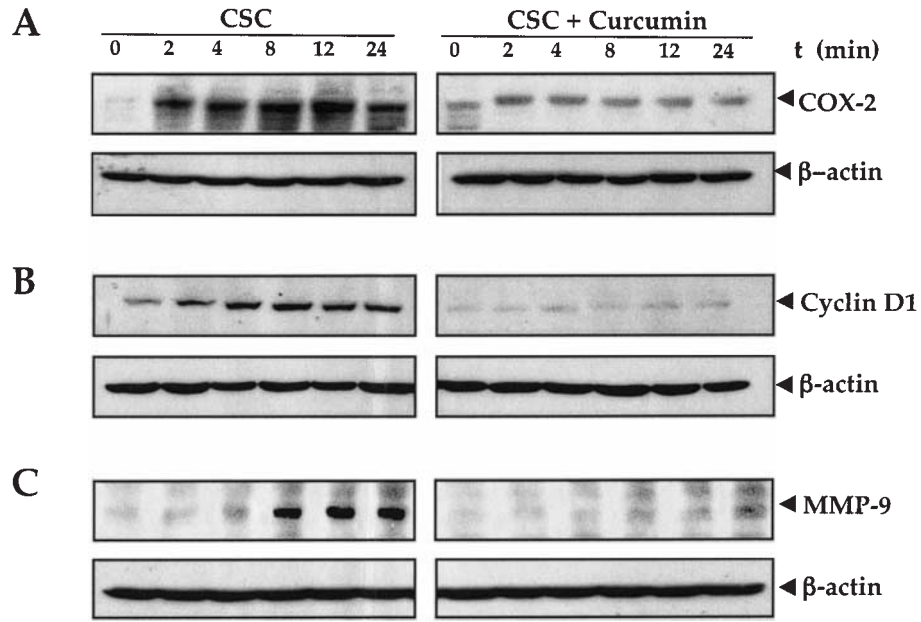


Fig. 7. Curcumin inhibited induction of COX-2, cyclin D1 and MMP-9 by CSC. H1299 cells (2×10^6 /ml) were left untreated or incubated with $50 \mu\text{M}$ curcumin for 2 h and then treated with $10 \mu\text{g/ml}$ CSC for different times. Whole-cell extracts were prepared and analyzed by western blotting using antibodies against COX-2 (A), cyclin D1 (B) and MMP-9 (C).

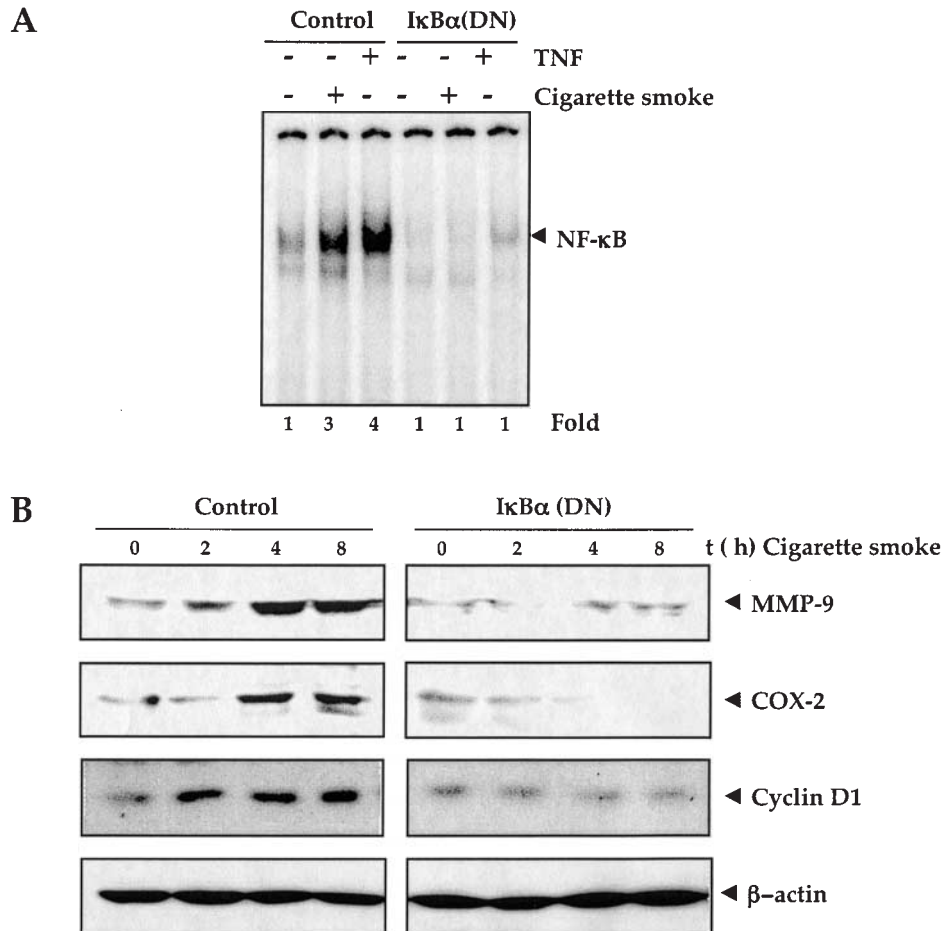


Fig. 8. Role of NF-κB in CSC induction of MMP-9, COX-2 and Cyclin D1. Two million Jurkat or dominant-negative IκBα transfected Jurkat cells per milliliter were treated with either 0.1 nM TNF or $0.1 \mu\text{g/ml}$ CSC for 30 min and the nuclear extracts were prepared and assayed for NF-κB by EMSA as described in the Materials and methods (A). To determine the induction of COX-2, cyclin D1 and MMP-9 by CSC, Jurkat or dominant-negative IκBα transfected Jurkat cells (2×10^6 /ml) were left untreated or incubated with $10 \mu\text{g/ml}$ CSC for different times. Whole-cell extracts were prepared and analyzed by western blotting using antibodies against COX-2, cyclin D1 and MMP-9 (B).

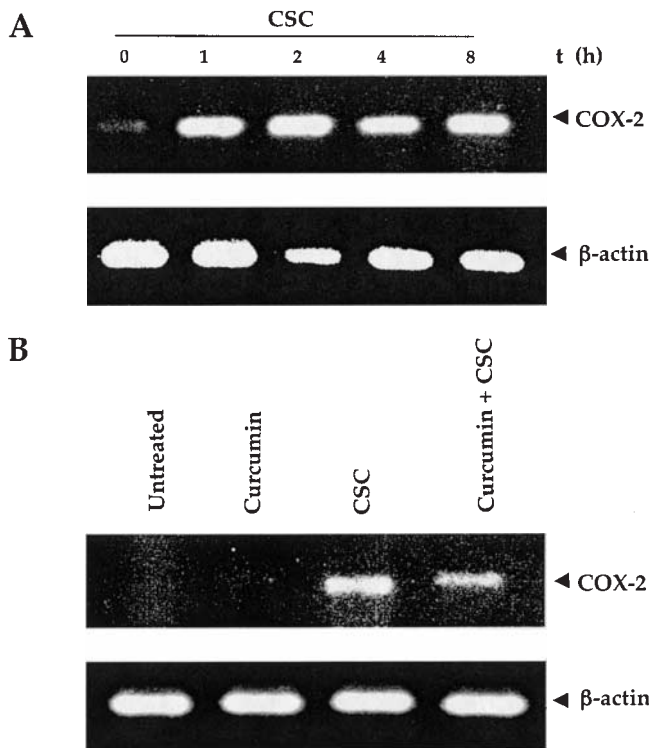


Fig. 9. Curcumin inhibition of CSC-induced COX-2 expression in H1299 cells. RT-PCR analysis of COX-2 expression in H1299 cells, pretreated with 50 μ M curcumin for 2 h followed by 10 μ M CSC for 1 h shows inhibition of CSC induced COX-2 expression by curcumin.

examined the effect of CSC on induction of COX-2 mRNA and its suppression by curcumin. As shown in Figure 9, CSC induced the mRNA for COX-2 in a time-dependent manner (Figure 9A), and treatment with curcumin down-regulated COX-2 mRNA expression (Figure 9B). The up-regulation of COX-2 gene activity by CSC and its suppression by curcumin was also confirmed independently by transient transfection of cells with COX-2 promoter-luciferase reporter plasmid and luciferase assay (Figure 10). These results also show that curcumin down-regulates the COX-2 promoter activity.

Discussion

In the present report we demonstrate that exposure of human non-small cell lung carcinoma cells to CSC activates NF- κ B as indicated by the formation of a NF- κ B-DNA complex, phosphorylation and degradation of I κ B α and the subsequent translocation of p65. Pre-treatment of cells with curcumin abolished these responses to CSC as well as the activation of I κ B α kinase and induction of NF- κ B-dependent reporter gene transcription. We also show that exposure of lung cells to CSC induced cyclin D1, COX-2 and MMP-9; pre-treatment with curcumin abrogated the expression of these CSC-induced gene products.

That CSC can activate NF- κ B in human non-small cell lung carcinoma cells as shown here is in agreement with a previous report in which we showed CSC activates NF- κ B in myeloid cells, lymphoid cells, and head and neck squamous cell carcinoma cells (14). Like TNF, CSC activated NF- κ B through sequential activation of IKK, I κ B α phosphorylation and degradation. Unlike TNF, however, CSC-induced NF- κ B

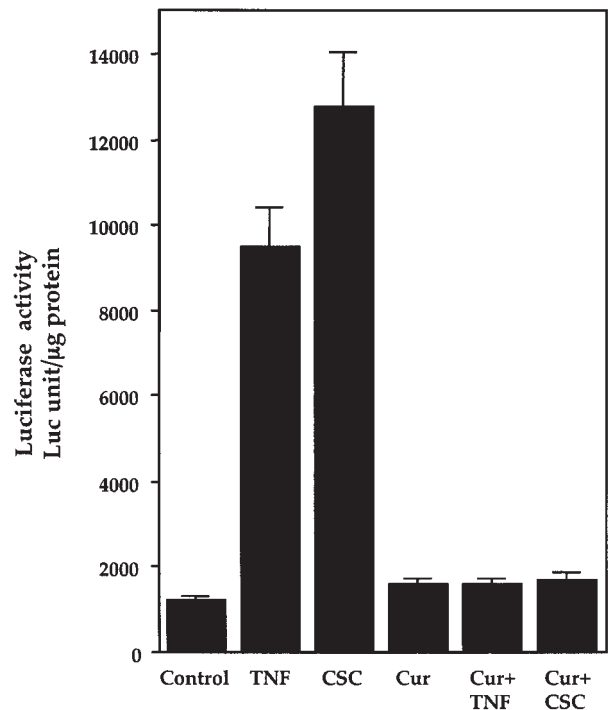


Fig. 10. Curcumin inhibited CSC-induced COX-2-dependent luciferase activity. H1299 cells were transiently transfected with COX-2-luciferase plasmid, then treated with 25 μ M curcumin for 12 h. The cells were then exposed to 10 μ g/ml CSC or 1 nM TNF as indicated. After 24 h, the cells were harvested and assayed for luciferase activity as described in Materials and methods.

activation was persistent; even 4 h later NF- κ B remained. Because persistent activation of NF- κ B by such agents as lipopolysaccharide is mediated through I κ B β (40), it is possible that I κ B β also plays a role in CSC-induced NF- κ B activation. TNF-induced NF- κ B activation leads to resynthesis of I κ B α (see Figure 1C). No re-synthesis of I κ B α was observed on treatment of cells with CSC, which may explain the persistence of CSC-induced NF- κ B activation.

Our results further indicate that pre-treatment of cells with curcumin abrogated CSC-induced IKK activation, I κ B α phosphorylation and NF- κ B activation. These results are in agreement with previous reports from our laboratory and others that curcumin is a potent inhibitor of NF- κ B activation (15,39,41–46). We found that curcumin inhibited CS-induced NF- κ B activation by blocking the activation of IKK, as have previous reports on colon cancer cells and macrophages (39,43). Because curcumin inhibited IKK activity both *in vivo* and *in vitro* (24), we suggest that curcumin may be a direct inhibitor of IKK. A recent report showed that PS-1145, a rationally designed IKK inhibitor, blocked TNF-induced NF- κ B activation in MM.1 cells (47). The concentration of curcumin required for blocking IKK activity in the cells was comparable with that reported for PS-1145.

Our studies indicate that activation of NF- κ B by CSC induces NF- κ B-dependent reporter gene expression and expression of NF- κ B-regulated COX-2, MMP-9 and cyclin D1. We found that induction of COX-2, MMP-9 and cyclin D1 by CSC was a direct result of NF- κ B activation, as CSC did not induce the expression of these genes in dominant-negative I κ B α transfected cells (Figure 8B). We found that suppression of NF- κ B by curcumin down-regulated the expression of these genes.

COX-2 has been implicated in carcinogenic processes (48), and its over-expression by malignant cells has been shown to enhance cellular invasion, induce angiogenesis, regulate anti-apoptotic cellular defences and augment immunologic resistance through production of PGE-2 (49). Additionally, it has been demonstrated that COX-2 is over-expressed in patients with lung cancer, head and neck cancer or breast cancer (50–53). MMP-9 plays a crucial role in tumor invasion and angiogenesis by mediating degradation of extracellular matrix in breast cancer cells (54) and destruction of lung elastin in chronic obstructive pulmonary disease (55). Inhibition of MMP activity has been shown to suppress lung metastasis (56). The over-expression of cyclin D1 has been noted in a wide variety of tumors (57). Altered expression of cyclin D1 is an early event in non-small cell lung carcinoma development and cyclin D1 is known to be required for cells to advance from G₁ to S phase of the cell cycle. Curcumin has been shown to induce G₁/S arrest (24), most likely through the down-regulation of cyclin D1 as shown here.

Because of their therapeutic potential, several NF- κ B blockers are being investigated. These include PS341 (a proteasome inhibitor), PS 1145 (an IKK inhibitor) and thalidomide (a TNF inhibitor) (47,58–60). Non-specific toxicity is one of the major problems in the development of these inhibitors. Curcumin, however, has been found to be pharmacologically safe in centuries of dietary consumption and in several recent phase I clinical trials (18,61). One of the studies showed no dose limiting toxicity in humans even when consuming up to 8 g of curcumin/day (18). These studies, however, indicated that curcumin has a poor bioavailability when consumed orally. In an attempt to improve the bioavailability, liposomal formulation of curcumin has been used (62). Curcumin has also been delivered in combination with piperine, a known inhibitor of intestinal and hepatic glucuronidation (63). The latter was shown to increase the bioavailability of curcumin by 2000% in humans with no adverse effects. The pharmacological safety, combined with its ability to suppress CS-induced NF- κ B activation and down-regulation of COX-2, MMP-9 and cyclin D1, provide sufficient rationale for a clinical trial of curcumin as a chemopreventive agent in former and current cigarette smokers.

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