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Cigarette smoke condensate activates nuclear transcription factor-κB through phosphorylation and degradation of IκBα: correlation with induction of cyclooxygenase-2

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Cigarette smoke (CS) contains several carcinogens known to initiate and promote tumorigenesis and metastasis. Because various genes that mediate carcinogenesis and tumorigenesis are regulated by nuclear factor-κB (NF-κB), we postulated that the effects of CS must be mediated through activation of this transcription factor. Therefore, in the present report we investigated whether cigarette smoke condensate (CSC) activates NF-κB, and whether the pathway employed for activation is similar to that of TNF, one of the potent activators of NF-κB. Our results show that the treatment of human histiocytic lymphoma U-937 cells with CSC activated NF-κB in a dose- and time-dependent manner. The kinetics of NF-κB activation by CSC was comparable with that of TNF. CSC-induced NF-κB activation was not cell type-specific, as it also activated NF-κB in T cells (Jurkat), lung cells (H1299), and head and neck squamous cell lines (1483 and 14B). Activation of NF-κB by CSC correlated with time-dependent degradation of IκBα, an inhibitor of NF-κB. Further studies revealed that CSC induced phosphorylation of the serine residue at position 32 in IκBα. *In vitro* immunocomplex kinase assays showed that CSC activated IκBα kinase (IKK). The suppression of CSC-activated NF-κB-dependent reporter gene expression by dominant negative form of IκBα, TRAF2, NIK and IKK suggests a similarity to the TNF-induced pathway for NF-κB. CSC also induced the expression of cyclooxygenase-2, an NF-κB regulated gene product. Overall, our results indicate that through phosphorylation and degradation of IκBα, CSC can activate NF-κB in a wide a variety of cells, and this may play a role in CS-induced carcinogenesis.

Introduction

Cigarette smoke (CS) is a major risk factor for a number of diseases including cancer, chronic obstructive pulmonary disease (COPD) and cardiovascular disease. Smokers exhibit high incidence of lung cancer, which is the leading cause of cancer mortality in men and women (1). Both active and passive smoking have been implicated in lung cancer. Additionally, cancers of the other sites, e.g. larynx, oral cavity, pharynx, esophagus, pancreas, kidney and bladder are also associated with smoking. Worldwide, 15% of all cancer cases are attributed to CS. Recent estimates indicates that CS causes ~80–90% of lung cancer in the US (2).

CS is a complex chemical mixture containing ~4800 different compounds, of which ~100 are known carcinogens, co-carcinogens, mutagens and/or tumor promoters (3). Metabolites of tobacco-specific *N*-nitrosamines, like 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and polycyclic aromatic hydrocarbons, like diolepoxides of benzo[*a*]pyrene (B[*a*]P) are believed to be the primary tobacco carcinogens (4), although other smoke constituents like high levels of free radicals may also play a role. For example each puff of smoke contains over 10 trillion free radicals present in both mainstream and sidestream smoke, which also may contribute to both tumor initiation as well as promotion. One common feature in the pathogenesis of various smoking-associated diseases is inflammation. Yet, the mechanisms underlying the role of smoking in these diseases is presently unknown or poorly understood. Additionally, exposure of cells to B[*a*]P also mutates p53 gene at the same location as found in 60% of lung cancer patients (5). A tobacco-specific *N*-nitrosamine or cigarette smoke condensate (CSC) causes neoplastic transformation of xenotransplanted human bronchial epithelial cells (6).

Nuclear transcription factor-κB (NF-κB) is a ubiquitous nuclear transcription factor that plays a major regulatory role in inflammation. It resides in the inactive state in cytoplasm as a heterotrimer consisting of p50, p65,and IκBα subunits. This transcription factor is a dimeric complex composed of different members of the Rel/NF-κB family of polypeptides (for refs, see 7). The p50–p65 heterodimer is retained in the cytoplasm by the inhibitory subunit IκBα. On activation of the complex, IκBα sequentially undergoes phosphorylation, ubiquitination and degradation, thus releasing the p50–p65 heterodimer for translocation to the nucleus. An IκBα kinase, IKK, has been identified that phosphorylates serine residues in IκBα at position 32 and 36 (8). Treatment of cells with various inflammatory and oxidative stress stimuli activates the IKK, thus leading to the degradation of IκBα and activation of the transcription factor.

Whether CS or its components cause tumor development through the NF-κB activation pathway is not understood. We postulate that the effects of CS in tumorigenesis are mediated through NF-κB activation for several reasons: (i) CS is known to increase oxidative stress and the latter is known to activate NF-κB; (ii) NF-κB activation has been implicated in chemical carcinogenesis and tumorigenesis (9,10); (iii) expression of several genes, such as cyclooxygenase (COX)-2, MMP-9, iNOS, TNF, interleukin (IL)-8, cell surface adhesion molecules, and antiapoptotic proteins involved in tumor initiation, tumor promotion, and metastasis are regulated by NF-κB (for refs,

Abbreviations: B[*a*]P, benzo[*a*]pyrene; COPD, chronic obstructive pulmonary disease; COX-2, cyclooxygenase; CS, cigarette smoke; CSC, cigarette smoke condensate; EMSA, electrophoretic mobility shift assay; IL, interleukin; NF-κB, nuclear transcription factor-κB.

see 11). Therefore, in the present report we investigated whether CSC can activate NF-κB, and whether the pathway employed for activation is similar to that of TNF, an inducer of NF-κB with a well-defined pathway. We demonstrate that CSC activates NF-κB in a wide variety of different cell types and that this activation is mediated through induction of IKK leading to phosphorylation and degradation of IκBα.

Materials and methods

Materials

Bacteria-derived human rTNF with a specific activity of 5×10^7 U/mg, was a kind gift from Genentech (South San Francisco, CA). Penicillin, streptomycin, RPMI 1640 medium and FBS were obtained from Life Technologies (Grand Island, NY). Tris, glycine, NaCl, SDS, PMA and BSA were obtained from Sigma Chemical (St Louis, MO). Rabbit polyclonal antibodies to IκBα, p50 and p65 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). IκBα-Ser^{32}-phospho-specific polyclonal antibodies were obtained from New England BioLab (Beverly, MA). Monoclonal antibodies to IKKα and IKKβ were kindly provided by Imgenex (San Diego, CA) and antibodies against COX-2 were obtained from Transduction Laboratories (Lexington, KY). Expression plasmids encoding FLAG-tagged NIK (12) were kindly provided by Dr David Wallach (Weizmann Institute of Science, Rehovot, Israel). The expression plasmids encoding myc-tagged TRAF2, NIK, p65 and dominant negative (DN)-IκBα have been described previously (13).

Cell culture

Human histiocytic lymphoma (U937), human epithelial (HeLa) and human T (Jurkat) cells were obtained from American Type Culture Collection (IL). Human lung epithelial cells (H1299), human head and neck cancer cell lines (14B and 1483) were kindly supplied by Dr Reuben Lotan (University of Texas M. D. Anderson Cancer Center, Houston, TX). U937 and Jurkat cells were grown in RPMI-1640 containing 10% FBS, A293 and HeLa cells were grown in MEM containing 10% FBS, and the remaining three cell lines were cultured in DMEM/F12 containing 10% FBS. Cells were subcultured every 3 days. For most studies human monocytic cell line U937 was used because a significant amount of information is available about the mechanism of NF-κB activation in these cells and this cell line is routinely employed in our laboratory to study NF-κB.

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 vol of 2 s duration under standard conditions prescribed by Federal Trade Commission (14). 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 desired concentration, vortexed vigorously and used for treatment of cells. Control cells were treated with medium containing an equivalent amount of DMSO. Repeated freezing and thawing of the CSC solution was avoided as much as possible.

Electrophoretic mobility shift assay (EMSA)

U937 cells (2×10^6/ml) were treated with different concentrations of either CSC (0, 0.01, 0.1, 1 or 10 μg/ml) or TNF (0, 0.01, 0.1 or 1 nM) in serum-free medium for 30 min at 37°C for dose–response and 10 ug/ml CSC or 0.1 nM TNF at 0, 5, 15, 30, 60 and 120 min. NF-κB activation was analyzed by EMSA as described (15). In brief, 8-μg nuclear extracts prepared from CSC-treated or untreated cells were incubated with ^{32}P-end-labeled 45mer double-stranded NF-κB oligonucleotide from human immunodeficiency virus-1 long terminal repeat (5′-TTGTTACAA GGGACTTTCCGCTGGGGAC-TTTCCAG GGAGGCGTGG-3′; underline sequence indicates the binding site) for 15 min at 37°C, and the DNA–protein complex resolved in a 6.6% native polyacrylamide gel. The specificity of binding was examined by competition with unlabeled 100-fold excess oligonucleotide and with mutant oligonucleotide. The composition and specificity of binding was also determined by supershift of the DNA–protein complex using specific and irrelevant antibodies. For the supershift experiment, the antibody-treated samples of NF-κB were resolved on a 5% native gel. The radioactive bands from the dried gels were visualized and quantified by PhosphorImager (Molecular Dynamics, Sunnyvale, CA) using ImageQuant software (Molecular Dynamics).

IKK assay

The IKK assay was performed by a method described earlier (16). Briefly, IKK signalosomes were precipitated by treating 300 μg of cytoplasmic extracts with 1 μg IKKα antibody, followed by treatment with 20 μl protein A/G Sepharose (Pierce). The beads were washed three times with lysis buffer and three times with kinase assay buffer. Beads were then resuspended in 20 μl kinase assay mixture containing 50 mM HEPES (pH 7.4), 20 mM $MgCl_2$, 2 mM DTT, 20 μCi γ-ATP, 10 μM unlabeled ATP and 2 μg substrate [GST-IκBα (1–54)]. The mixture was incubated at 30°C for 30 min, and the reaction was terminated by adding 5 μl of 5× SDS sample buffer and boiling for 5 min. Finally, the protein was resolved on 10% acrylamide gel under reducing conditions. The gel was dried, and the radioactive bands were visualized by PhosphorImager as described previously. To determine the total amounts of IKKα and IKKβ in each sample, 60 μg of cytoplasmic proteins were resolved on 7.5% acrylamide gels. Resolved samples were electrotransferred to nitrocellulose membranes, and the membranes were blocked with 5% non-fat milk protein for 1 h and incubated separately with IKKα and IKKβ antibodies (1:500) for 1 h. The membrane was washed and treated with HRP-conjugated secondary antibodies and finally detected by chemiluminescence (ECL, Amersham Pharmacia Biotech., Arlington Heights, IL).

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 IκBα (1:3000), phospho-IκBα (1:1000) 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 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.

NF-κB SEAP reporter assay

The effect of TNF and CSC on NF-κB-SEAP reporter gene expression assay was based on our earlier report (13). Briefly, 293 and Hela cells (0.6×10^6 cells/well) were plated in 6 well plates, and cells were transfected by the calcium phosphate method (Gibco BRL) with various plasmids. At 12 h post-transfection, CSC or TNF was added to the wells in fresh serum-free medium and incubated for an additional 12 h. The supernatants were collected and assayed for SEAP activity. For this, the supernatants were centrifuged for 2 min at 13 000 *g*, and 25 μl of each sample was incubated with 75 μl of SEAP buffer (500 mM Tris pH 9.0 containing 0.5% BSA) at 65°C for 30 min. The plate was chilled on ice for 2 min. Then 50 μl of 1 mM 4-methylumbelliferyl phosphate was added to each well and incubated at 37°C for 2 h. The activity of SEAP was assayed on a 96 well fluorescence plate reader (Fluoroscan II labsystems, Needham, Heights, MA) with excitation set at 360 nm and emission at 460 nm.

Results

Several genes that are implicated in chemical carcinogenesis and tumorigenesis are regulated by NF-κB. Because of the critical role of NF-κB in these processes, we investigated the effect of CSC on NF-κB activation. Because the mechanism of NF-κB activation by TNF is better understood, throughout we used this cytokine as a control for comparison with CSC.

CSC activates NF-κB in dose- and time-dependent manner

To investigate the effect of CSC on NF-κB activation, U937 cells were treated with variable concentrations of either TNF for 30 min or CSC for 60 min, nuclear extracts prepared and NF-κB activation examined by EMSA. As shown in Figure 1A, TNF activated NF-κB at 0.1 nM and produced a slight additional increase at 1 nM. CSC activated NF-κB at 0.1 μg/ml and reached optimum activation at 1–10 μg/ml. To examine the kinetics of NF-κB activation, cells were treated with either 0.1 nM TNF or with 10 μg/ml CSC for different times. The result shown in Figure 1B indicates that an optimum NF-κB activation by CSC could be seen within 15 min, which was comparable with the kinetics of TNF.

CSC-activated NF-κB consists of p50 and p65 subunits

Various combinations of Rel/NF-κB proteins can constitute an active NF-κB heterodimer that binds to specific sequences in DNA (17). To show that the retarded band visualized by EMSA in CSC-treated cells was indeed NF-κB, we incubated nuclear extracts from CSC/TNF-activated cells with antibody

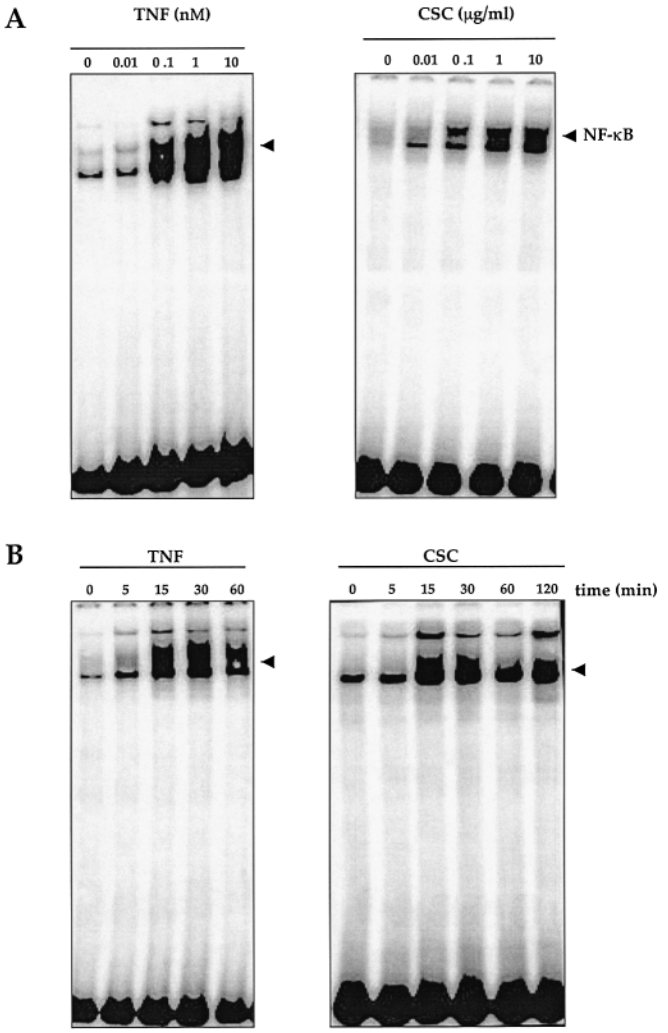

Fig. 1. CSC activates NF-κB. (**A**) Dose-dependent activation of NF-κB. Two million U937 cells in 1 ml were treated with different concentrations of either CSC or TNF for 30 min, and the nuclear extracts were prepared, and assayed for NF-κB by EMSA as described in the Materials and methods. (**B**) Time course for activation of NF-κB. Two million U937 cells in 1 ml were treated with either 0.1 nM TNF or 10 μg/ml CSC for different times. The nuclear extracts were prepared and assayed for NF-κB by EMSA as described in the Materials and methods.

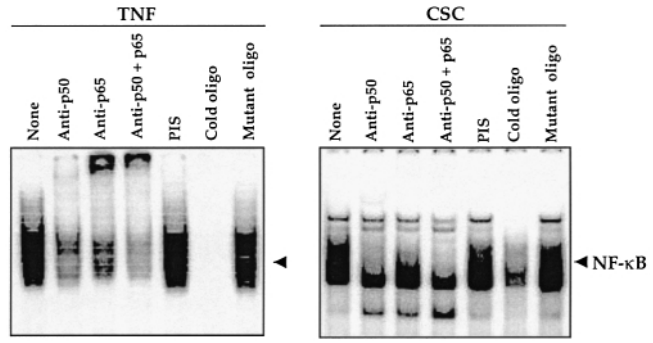

Fig. 2. CSC-induced NF-κB is composed of p50 and p65 subunits. Nuclear extracts prepared from CSC (10 μg/ml)-treated cells were incubated at 37°C for 30 min with either no antibodies, or anti-p50 antibodies, or anti-p65 antibodies, or a mixture of anti-p50 and anti-p65 antibodies, or pre-immune sera, or unlabeled oligo, or mutant oligo, and then assayed for NF-κB as described in the Materials and methods.

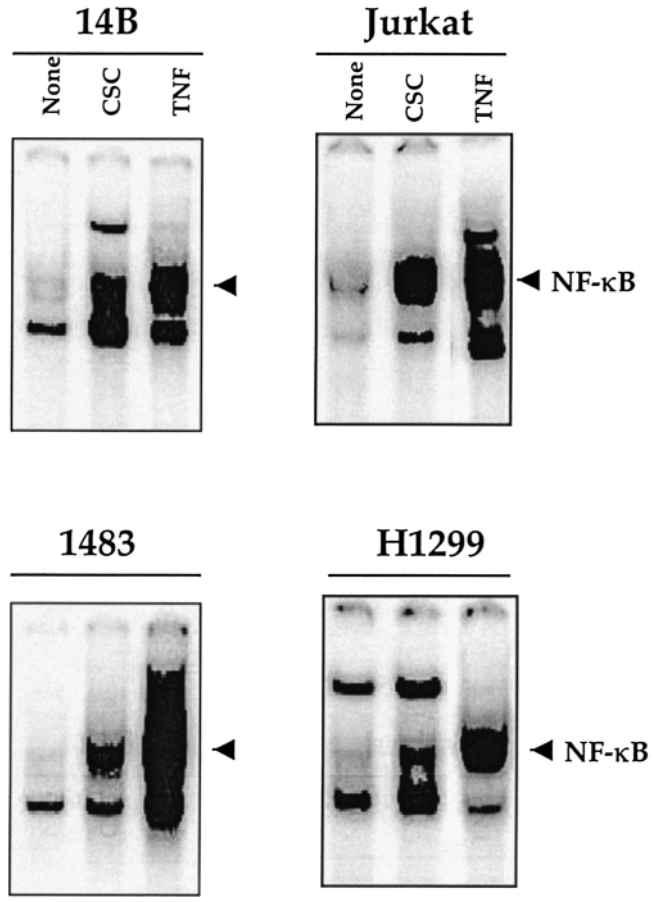

Fig. 3. CSC-induced NF-κB activation is not specific to cell type: two million cells in 1 ml were treated with either CSC (10 μg/ml) or TNF (0.1 nM) for 30 min. The nuclear extracts were then prepared and assayed for NF-κB by EMSA as described in the Materials and methods.

to either the p50 (NF-κBI) or p65 (Rel A) subunits and then conducted EMSA. Antibodies to either subunit of NF-κB decreased the DNA binding (Figure 2), thus suggesting that the TNF-activated complex consisted of p50 and p65 subunits. Pre-immune serum had no affect on the mobility of NF-κB. Excess unlabeled NF-κB (100-fold) caused complete disappearance of the band but mutant oligo did not, indicating the specificity of NF-κB. These EMSA results thus suggest that CSC induced NF-κB activation in monocytic U937 cells.

Activation of NF-κB by CSC is not cell type-specific

That distinct signal transduction pathways could mediate NF-κB induction in epithelial and lymphoid cells has been demonstrated (18–20). All the effects of CSC described thus far were with U937 cells. Whether CSC could activate NF-κB in cell types other than myeloid cells, was investigated. To probe whether CSC also activates NF-κB in other cells, T-cell (Jurkat), lung cancer cell (H1299) and squamous cell carcinoma (1483 and 14B) were treated with CSC and nuclear extracts were analyzed by EMSA. CSC activated NF-κB in all four cell types tested, although the activation was low compared with that by TNF (Figure 3). The results indicate that CSC can activate NF-κB in a wide variety of different cell types.

CSC induces degradation of IκBα

In the previous sections, we have seen that CSC activates NF-κB in different cell lines, but it was not clear how NF-κB is activated. Although TNF-induced NF-κB activation requires

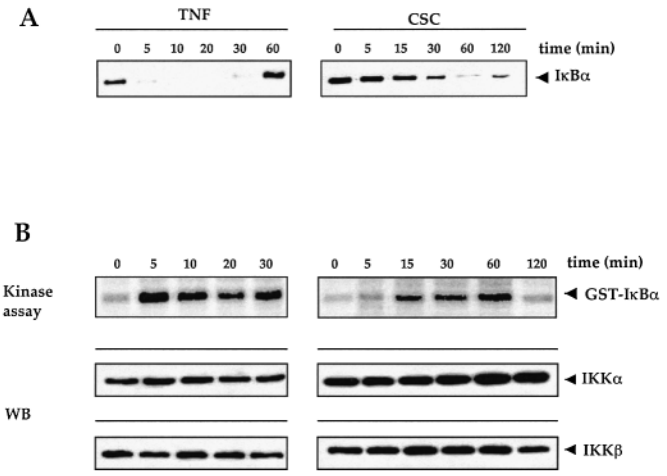

Fig. 4. (**A**) CSC induces degradation of IκBα. Two million U937 cells per ml were treated with either 0.1 nM TNF or 10 μg/ml CSC for different times. The cytoplasmic extracts were prepared and assayed for IκBα by western blot using IκBα-specific antibodies. (**B**) CSC activates IκBα kinase. Two million U937 cells per milliliter were treated with either 0.1 nM TNF or with 25 μg/ml CSC for different times. Thereafter the cytoplasmic extracts were prepared and assayed for IKK by the immunocomplex kinase assay (upper panel) and for IKKα (middle panel) and IKKβ (lower panel) protein by western blot analysis as described in Materials and methods.

degradation of IκBα (21), no IκBα degradation occurs for NF-κB activated by H_2O_2, X-rays, γ-radiation and pervanadate treatment (22–26). Thus, whether CSC activates NF-κB through IκBα degradation was an open question. To determine this, we treated U937 cells with CSC for 0–120 min, prepared the cytoplasmic extracts, and assayed for the IκBα degradation by western blot analysis. As shown in Figure 4A (right top panel), IκBα degradation began to occur at 15 min of CSC treatment and was almost complete at 60 min. For comparison, TNF-induced the degradation of IκBα could be seen as early as 5 min and was complete by 10 min. These results indicate that CSC-induced NF-κB activation, like TNF-induced activation, is accompanied by IκBα degradation.

CSC induces the activation of IκBα kinase

TNF-induced IκBα degradation requires phosphorylation of IκBα at serine 32 and 36 (27). NF-κB activation by pervanadate and H_2O_2, however, require phosphorylation of IκBα at tyrosine 42 (28–30). The phosphorylation of IκBα at serine 32 requires the activation of IKK (31). As CSC phosphorylated IκBα at serine 32, we directly examined the activation of IKK by immunoprecipitation of IKK using specific antibodies followed by immunocomplex kinase assays using GST–IκBα as a substrate. CSC activated IKK within 15 min of treatment (Figure 4B, upper right panel) whereas TNF activated IKK within 5 min. Kinase activation was not due to the synthesis of IKKα and IKKβ proteins, as their levels as revealed by western blot analysis were unaffected (Figure 4B, lower two panels). These results suggest that the mechanism of activation of NF-κB by CSC was similar to that by TNF.

CSC activates NF-κB-dependent reporter gene expression

Although we had shown by EMSA that CSC induced the NF-κB activation, IκBα phosphorylation and degradation, and IKK activation, DNA binding alone does not always correlate with NF-κB-dependent gene transcription, suggesting there are additional regulatory steps (32). To determine the effect of CSC on NF-κB-dependent reporter gene expression, we transiently transfected the A-293 cells with the SEAP reporter construct

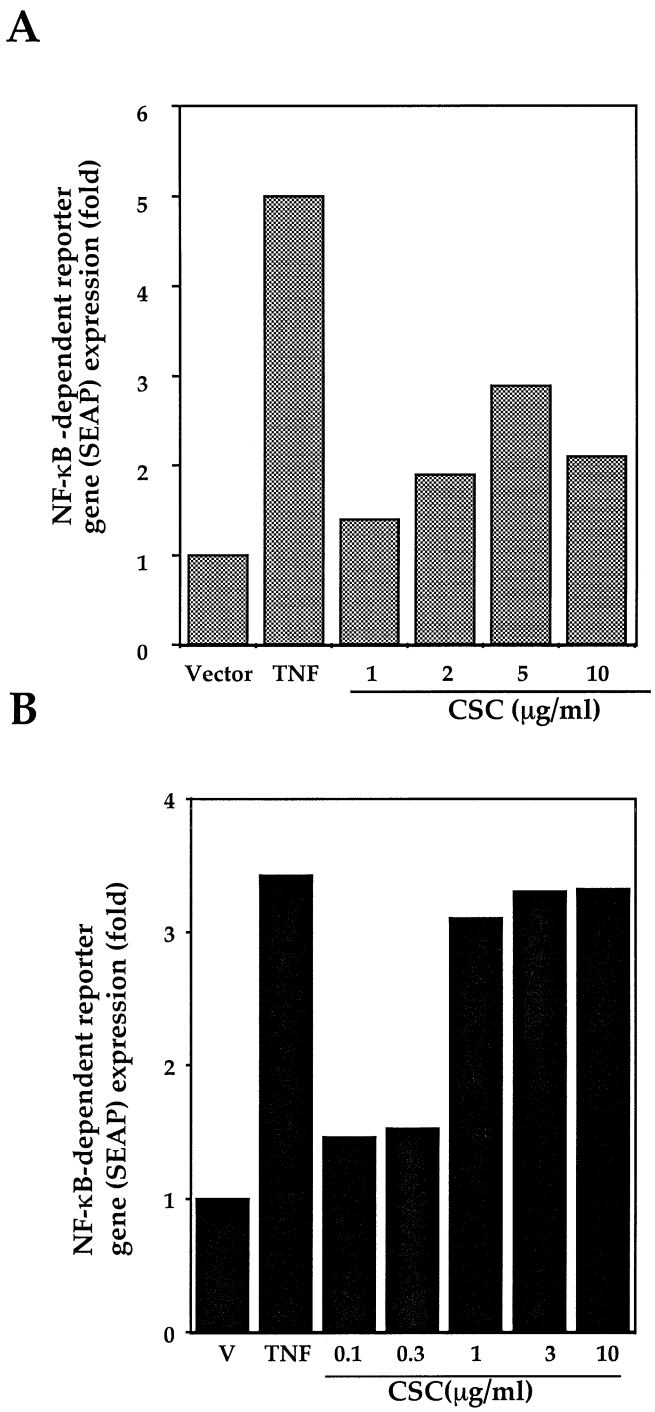

Fig. 5. CSC activates NF-κB-dependent reporter gene expression. (**A**) Cell line 293 cells (0.6×10^6 cells/well) were seeded in 6 well plates and transfected with 0.5 μg SEAP and 2 μg PCMV Flag plasmids. After 24 h incubation, fresh serum-free medium containing different concentrations of CSC (1–10 μg/ml) or TNF (1 nM) were added and incubated for 12 h. The supernatant was collected and assayed for SEAP activity as described in Materials and methods. (**B**) HeLa cells (0.5×10^6) were seeded in 6 well plates and transfected with 0.5 μg SEAP and 2 μg PCMV plasmids. After 24 h incubation, fresh serum-free medium containing different concentrations of CSC (0.1–10 μg /ml) or TNF (1 nM) were added and incubated for 12 h. The supernatant was collected and assayed for SEAP activity as described in Materials and methods.

and then treated them with different concentrations of CSC. An almost 2-fold increase in SEAP activity over the untreated vector control was noted upon stimulation with CSC

(Figure 5A). In contrast, TNF induced a 4-fold activation of the NF-κB SEAP gene reporter construct. To ascertain that CSC can activate NF-κB reporter gene expression in other cell types, we transfected human epithelial HeLa cells with the reporter construct and activated with either TNF or CSC. Again both TNF and CSC induced a significant NF-κB-reporter activity (Figure 5B). These results demonstrate that CSC-induced NF-κB was transcriptionally active and the effects were not cell type-specific.

DN-IκBα, DN-TRAF2, DN-NIK and DN-IKK supress CSC-induced NF-κB-dependent reporter gene expression

TNF-induced NF-κB activation is mediated through sequential interaction of the TNF receptor (TNFR1) with TRADD, TRAF2, NIK and IKK-β, resulting in phosphorylation of IκBα (12,31,33,34). To delineate the CSC-signaling pathway leading to NF-κB activation, cells were transiently transfected with DN-TRAF2, DN-NIK and DN-IKK plasmids, along with NF-κB-SEAP plasmid, and the NF-κB-dependent SEAP expression was then monitored in CSC-treated cells. TNF-induced NF-κB activation served as a control. Suppression of TNF- or CSC-induced NF-κB reporter activity by the DN-IκBα plasmid indicates that this assay is quite specific (Figure 6). NF-κB reporter expression induced by both TNF (upper panel) and by CSC was inhibited by transfection of cells with the plasmids expressing DN-TRAF2, DN-NIK and DN-IKK. These results suggest that the pathway by which CSC activates NF-κB is similar to that of TNF.

CSC induces expression of COX-2

So far our results indicate that CSC activates NF-κB through activation of IKK leading to phosphorylation and degradation of IκBα and that this NF-κB is transcriptionally active. Whether CSC-induced NF-κB activation correlates with induction of COX-2, a gene regulated by NF-κB (35), was examined. U937 cells were treated with CSC for different times, the whole-cell extracts prepared and analyzed by western blot for the expression of COX-2 (Figure 7A). CSC induced COX-2 expression at 4 h and expression reached maximum at 12 h. Like CSC, TNF also induced COX-2 expression, which reached maximum at 8 h. These results indicate that CSC also induces COX-2, an NF-κB regulated gene product. We have shown above that CSC induces NF-κB-dependent reporter gene expression. Whether CSC also induces COX-2 expression in A293 cells was examined. As shown in Figure 7B, CSC induced COX-2 in a time-dependent manner. These results indicate that induction of COX-2 by CSC is not cell type-specific.

Discussion

In the present report we investigated the molecular basis by which CS could mediate carcinogenesis and atherogenesis. Because of the critical role of NF-κB in carcinogenesis and atherogenesis, we investigated the ability of CSC to activate NF-κB. Our results clearly demonstrate that CSC can activate NF-κB as indicated by the formation of NF-κB–DNA complex, phosphorylation and degradation of IκBα, activation of IκBα kinase, activation of NF-κB-dependent reporter gene transcription, and expression of COX-2 a gene product regulated by NF-κB. Our results also indicate that the activation of NF-κB by CSC is not cell type-specific and occurs in lymphoid, myeloid and epithelial cells. Our results also show that the pathway through which CSC activates NF-κB is very similar to that of TNF.

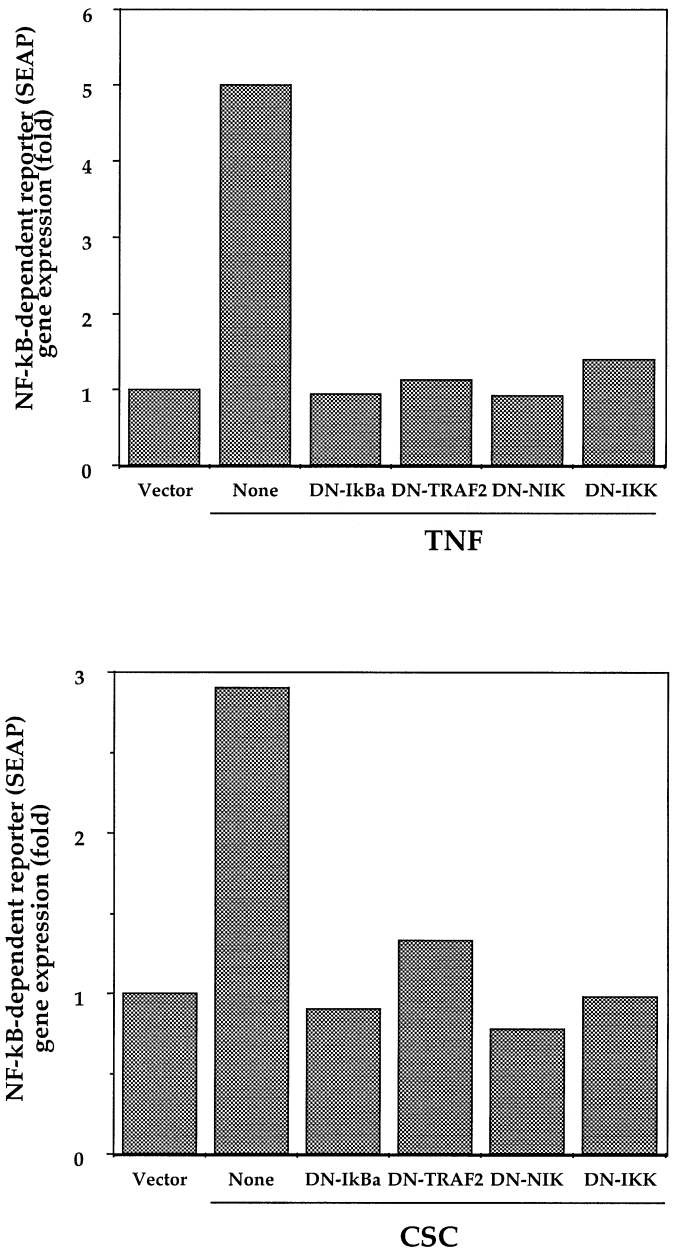

Fig. 6. CSC-induced NF-κB-dependent reporter gene expression is suppressed by DN-IκBα, DN-TRAF2, DN-NIK and DN-IKK. Line 293 cells (0.6×10^6 cells/well) were seeded in 6 well plates and transfected with 0.5 μg SEAP, along with 1 μg of any of the indicated DN-plasmids and 1 μg PCMV flag. At 12 h after transfection, 5 μg/ml CSC or 0.1 nM TNF was added to the wells in fresh serum-free medium and incubated for 12 h. The supernatants were collected and assayed for secreted alkaline phosphatase (SEAP) activity as described in Materials and methods. Results are expressed as fold activity over the non-transfected control.

The chemical moiety in the CSC that activates NF-κB is not, however, clear. CS is an aerosol of complex chemical composition containing both organic and inorganic compounds, of which 4800 have been identified so far (36). Both vapor phase and particulate phase of smoke are known to possess free radicals (37,38). While the gas phase radicals are generally short-lived, the radicals in the particulate phase are relatively stable and consist of a hydroquinone–semiquinone–quinone complex. (39). This complex is an active redox system capable of reducing molecular oxygen to produce superoxide, eventually leading to hydrogen peroxide and hydroxyl radicals.

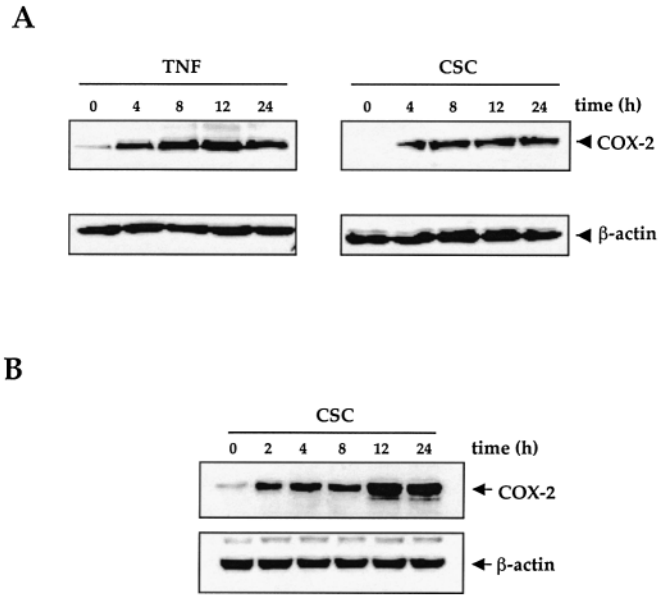

Fig. 7. CSC induces COX-2 gene product. (**A**) Two million U937 cells were incubated with 0.1 nM TNF or 10 μg/ml CSC in serum-free medium for the indicated time intervals. Whole-cell lysates were prepared and 60 μg protein was loaded on gels and analyzed for COX-2 by western blot using COX-2-specific antibodies as described in Materials and methods. (**B**) Two million A293 cells were incubated with 10 μg/ml CSC in serum-free medium for the indicated time period. Whole-cell lysates were prepared and 80 μg protein was loaded on gels and analyzed for COX-2 by western blot using COX-2-specific antibodies as described in Materials and methods.

Another important constituent of CS are metals like cadmium and nickel, which have a long half-life. In addition, at least 40 different CS carcinogens have been implicated in tumor initiation and promotion (40). These cacinogens include nitrosamine, polynuclear aromatic hydrocarbons, aromatic amines, unsaturated aldehydyes (e.g. crotonaldehyde) and some phenolic compounds (acrolein). The most potent carcinogenic agent contained in CS is the NNK, formed by nitrosation of nicotine; it is thought to be an important etiological factor in tobacco smoke-related human cancers (41). NNK is a site-specific carcinogen in that irrespective of the route of admininstration, NNK has remarkable specificity for the lung (42).

H_2O_2, a constituent of the CS, is known to activate NF-κB (30). It is unlikely, however, that NF-κB activation observed in our studies was due to H_2O_2 present in the CSC. Because first, particulate matter of CSC contains very little H_2O_2 (43); secondly, H_2O_2 activates NF-κB not through serine but tyrosine phosphorylation of IκBα (30). NO_2, another constituent of CSC (44), also is unlikely because it is not known to activate NF-κB. The CSC also contains B[*a*]P, a well-known potent carcinogen. Two recent reports indicate that B[*a*]P can activate NF-κB (45,46). It is unlikely, however, that in our studies the activation of NF-κB is due to the B[*a*]P present in CSC. First, the concentration of B[*a*]P in CSC is not sufficient to activate NF-κB; secondly, the level of NF-κB binding activity observed following treatment with B[*a*]P is very minimal as compared with that noted in our studies (45,46). Whether NNK, a lung carcinogen, can activate NF-κB, however, is not clear. Some of our preliminary studies indicate that NNK alone is not sufficient to activate NF-κB. Thus, it is quite probable that NF-κB activation observed in our studies is due to a mixture of inducers present in the CSC.

Our results indicate that CSC can activate NF-κB in a wide variety of cells including myeloid cells, lymphoid cells, lung epithelial cells and in head and neck squamous cell carcinoma cells. Whether CSC activates NF-κB in all these cell types through identical mechanism is, however, not clear. Published reports indicate that the mechanism of NF-κB activation may vary from one cell type to another (19,20). For instance, protein kinase C inhibitor calphostin C, blocked NF-κB activation by TNF in Jurkat cells but not in MCF-7 A/Z cells (19). Similarily, PTEN, a dual specificity lipid phosphatase, inhibited TNF-induced NF-κB activation in PC-3 cells but not in DU145 cells, both prostate cancer cell lines (20).

Our results demonstrate that CSC activates NF-κB through IKK activation leading to IκBα phosphorylation and degradation. Recently Gebel and Muller (47) showed that treatment of 3T3 murine fibroblast cells with CS-bubbled PBS causes a decrease in NF-κB activation during the first 2 h followed by an increase at 4 and 6 h. Although not clear from the data, the results suggest that 3T3 cells must have constitutive NF-κB to be downregulated by the smoke-bubbled PBS. Besides no supershift by the antibodies against the NF-κB protein or competition by excess unlabeled oligo probe was shown, to suggest it is indeed NF-κB that was being modulated by the smoke-bubbled PBS. Therefore, it is not too surprising that authors found no phosphorylation or degradation of IκBα in their experiments (47). Furthermore, it should be pointed out that the CSC preparation used in our study contained both water-soluble and insoluble constituents of smoke as compared with smoke-bubbled PBS used in the above study, which probably contained largely water-soluble fraction of smoke. Our results also indicate that CSC-induced NF-κB activation is blocked by DN TRAF-2, NIK and IKK. This suggests that CSC activates NF-κB through a pathway very similar to that of TNF. Although TNF-induced NF-κB activation is also blocked by DN forms of TRAF-2 and NIK, the deletion of genes for TRAF2 or NIK have no effect on TNF-induced NF-κB activation (33,34), suggesting an alternate pathway. Gene deletion for receptor-interacting protein or MEKK3, however, has been shown to abrogate the TNF-induced NF-κB activation (48–50). Whether RIP or MEKK3 also mediate CSC-induced NF-κB activation is not known at present. Like TNF, CSC-induction of NF-κB does require the activation of IKK.

Tumorigenesis of the lung, larynx, oral cavity and pharynx, esophagus, pancreas, kidney and bladder has been linked to CS (1,2,51). Our results show that CSC can activate NF-κB in myeloid, lymphoid, and head and neck squamous cells and in lung cells. These observations suggest that CS, through NF-κB activation, has the potential to initiate carcinogenic cascade in most cell types.

How activation of NF-κB by CSC contributes to carcinogenesis and tumorigenesis is not clear. Several genes that are regulated by NF-κB can contribute to carcinogenesis and atherogenesis including gene products which block apoptosis (e.g. TRAF1, TRAF2, cIAP-1, cIAP-2, XIAP, bcl-2 and bcl-xl), gene products which promote angiogenesis (e.g. cell surface adhesion molecules on endothelial cells, vascular endothelial cell growth factor, COX-2, matrix metalloprotease-9 and urokinase plasminogen activator), and inflammatory cytokines (e.g. TNF, IL-1, IL-6 and IL-8) (for refs, see 11). Indeed the expression of adhesion molecules in cultured endothelial cells by CSC has been reported (52,53). Our studies indicate that CSC can also induce the expression of COX-2 protein. Whether the induction of COX-2 by CSC is a direct result of NF-κB activation, however, is less clear from our

studies. COX-2 has been implicated in carcinogenic process (54). Additionally, the levels of COX-2 have been shown to be over expressed in patients with lung cancer, head and neck cancer or breast cancer (55–58).

Our results indicate that exposure of cells for 15–30 min to 1–10 μg/ml CSC is sufficient to activate NF-κB. Whether the concentration of CSC used in our studies is achieved by cigarette smokers is not clear. One standard research cigarette can provide up to 10 mg of the CSC (3). Based on 20 cigarettes consumed per day by human (with a body weight of 70 kg), he/she receives ~6.8 mg smoke tar/kg body wt (59), resulting in exposure of lung cells to microgram quantities of tar. Thus, the cumulative dose of CS consumed by the smokers may be sufficient to activate NF-κB and induce gene expression.

The delineation of targets of CS, such as NF-κB as described here, suggests that suppression of NF-κB activation induced by CSC should diminish the CS-induced damage. CS is known to play a role not only in cancer but also in cardiovascular diseases and in COPD (60). NF-κB activation has been implicated in both cardiovascular diseases as well as in COPD (61–66). Therefore, it is possible that suppression of NF-κB induced by CSC may prove useful in those diseases as well.

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