Cigarette smoke (CS), a major risk factor for developing lung cancer, is known to activate transcriptional activator nuclear factor kappa B (NF-κB). However, the underlying mechanism of this activation remains unclear because of conflicting reports. As NF-κB has a pivotal role in the generation and maintenance of malignancies, efforts were targeted towards understanding its activation mechanism using both ex vivo and in vivo studies. The results show that CS-induced NF-κB activation mechanism is different from that of other pro-inflammatory signals such as lipopolysaccharide (LPS). The NF-κB dimer that translocates to the nucleus upon stimulation with CS is predominantly composed of c-Rel/p50 and this translocation involves degradation of IκBε and not IκBα. This degradation of IκBε depends on IKKβ activity, which preferentially targets IκBε. Consistently, CS-activated form of IKKβ was found to be different from that involved in LPS activation as neither Ser177 nor Ser181 of IKKβ is crucial for CS-induced NF-κB activation. Thus, unlike other pro-inflammatory stimulations where p65 and IκBα have a central role, the predominantly active signaling cascade in CS-induced NF-κB activation in the lung epithelial cells comprises of IKKβ–IκBε–c-Rel. Thus, this study uncovers a new axis of NF-κB activation wherein IκBε and c-Rel have the central role.

INTRODUCTION
Cigarette smoke (CS), a known etiological agent for inflammatory response in the lung, may significantly contribute to the development of various inflammatory diseases including lung cancer. CS contains high levels of reactive oxygen species (ROS) ranging from short-lived oxidants to long-lived organic radicals such as semiquinones. In addition, exposure to CS causes activation of enzymes such as NADPH oxidase that are involved in intracellular ROS generation. As a result, ROS levels increase within the cell and this triggers redox-sensitive pathways. Nuclear factor kappa B (NF-κB) is one such redox-sensitive transcription factor. It controls several important cellular processes including cell survival and inflammation and has been shown to be activated by CS. Active NF-κB is a hetero/homo dimeric complex consisting of members of the Rel family (p65 (RelA), RelB, c-Rel, p50 and p52), all of which contain the Rel homology domain; however, only p65, c-Rel and RelB possess transcriptional activation domain. In resting cells, NF-κB is sequestered in the cytosol as a result of physical association with a member of a family of inhibitory proteins called IκB, which mask the nuclear localization signal of NF-κB. The principal members of the IκB family are IκBα, IκBβ, IκBε and Bcl-3. Depending on the cell type and the nature of stimulus, different IκB forms complex with different NF-κB proteins. These different combinations greatly contribute to the NF-κB functional diversity observed in different cells under varied conditions. Stimulation of cells with external stimuli, such as lipopolysaccharide (LPS), elicits signal across the plasma membrane through specific receptors that causes activation of IκB kinase (IKK) complex. The activated IKK in turn phosphorylates IκB. IKK complex contains two catalytic subunits, IKKα and IKKβ, and one regulatory subunit, IKKγ. Previous reports show that IKKβ has a vital role in pro-inflammatory stimuli-mediated NF-κB activation, wherein it phosphorylates IκBα.

The modified IκB undergoes proteasomal degradation thereby freeing NF-κB to translocate into the nucleus and transactivate its target genes.

Although CS has been known to cause NF-κB activation for long time, the mechanism of this activation remains unclear as available reports are conflicting in nature. There are reports that showed the NF-κB activation by CS extract (CSE) in cultured cell lines, including alveolar epithelial H1299 cells, is mediated by p65 and p50 nuclear translocation resulting from IκBα degradation. Consistent with this, Rajendrasozhan et al. showed the degradation of IκBα and nuclear entry of p65 in CS-exposed rat lung extract. In contrast, Marwick et al. have demonstrated CS-induced NF-κB activation in the rat lungs, which is independent of IκBα degradation.

As cigarette smoking is a major etiological agent for several pulmonary diseases, including lung cancer wherein NF-κB has an important role, it is vital to understand the underlying mechanism of CS-induced NF-κB activation. With the aim of elucidating the mechanism of CS-induced NF-κB activation, we have performed both the ex vivo experiments using alveolar epithelial A549 cells and the in vivo experiments in guinea pig. On the basis of these experiments we report that c-Rel/p50 dimer is predominantly involved in CS-induced NF-κB activation in lung epithelial cells as a result of IκBα degradation by IKKβ. Thus, the present study provides a new axis of NF-κB activation comprising IKKβ–IκBε–c-Rel/p50 in lung epithelial cells.

RESULTS
CS-induced NF-κB activation predominantly involves nuclear translocation of c-Rel and p50 in lung epithelia

To study the mechanism of CSE-induced NF-κB activation in A549 alveolar epithelial cells, the optimum condition of NF-κB activation...
was standardized by electrophoretic mobility shift assay (EMSA). It was observed that the treatment of cells with 2% of CSE for 30 min resulted in considerable NF-κB activation (Supplementary Figure S1a, left panel) and this activation is mediated by ROS as pretreatment with 20 mM N-acetyl cystiene, a known anti-oxidant, before CSE treatment reduces this activation substantially (Supplementary Figure S1a, right panel). The functional transactivation property of the nuclear-translocated NF-κB complex, following CSE treatment, was confirmed by luciferase assay and induction of known NF-κB target genes (Supplementary Figure S1b and S1c).

To understand the specific components that constitute active NF-κB in the nucleus in response to CSE treatment, nuclear-cytosolic fractionation of CSE-treated and -untreated A549 cells was performed. The results showed the nuclear accumulation of c-Rel and p50 with time in CSE-treated cells (Figure 1a; upper panel). Immunolocalization of c-Rel, p65 and p50 was demonstrated by Western blot analysis of nuclear and cytosolic fractions of CSE-treated and -untreated A549 cells (Figure 1b). c-Rel downregulation inhibits CSE-induced NF-κB activation. A549 cells were transfected with pSuper (empty vector), anti-c-Rel (si-c-Rel) and anti-p65 (si-p65) si-RNA constructs. After 24 h of transfection, cells were harvested and cell extracts were analyzed by western blotting (left panel). Tubulin serves as loading control. These transfectants harboring pSuper, si-c-Rel and si-p65 constructs were treated with 2% CSE for 30 min and harvested. Nuclear extracts were analyzed by EMSA using radiolabeled NF-κB probe (right panel). Chromatin immunoprecipitation (ChIP) analysis of p65, c-Rel and p50 recruitment at IL-8 and cyclin D1 upstream promoter sequences was performed. A549 cells were treated with 2% CSE for 30 min and cross-linked with paraformaldehyde. Immunoprecipitations were carried out using anti-p65, c-Rel and p50 antibodies. Immunoprecipitated DNA was amplified by PCR primers corresponding to the NF-κB-binding site(s) at IL-8 and cyclin D1 upstream promoter sequences as indicated in the upper panel and analyzed by agarose gel electrophoresis.

**Figure 1.** CSE-induced NF-κB activation in alveolar epithelial A549 cells predominantly involves nuclear translocation of c-Rel and p50. (a) CSE treatment predominantly induces nuclear translocation of c-Rel and p50. A549 cells were treated with either 2% CSE (upper panels) or 1 μg/ml LPS (lower panels) for different time periods as indicated. Nuclear and cytosolic fractions were prepared and separated by SDS-polyacrylamide gel electrophoresis. Western blot analysis was performed with anti-c-Rel, anti-RelA, anti-p50 and anti-tubulin antibodies. C and N indicate cytosolic and nuclear fractions, respectively. (b) Immunolocalization of c-Rel, p65 and p50. A549 cells were treated with 2% CSE for 30 min, fixed and probed with anti-c-Rel, anti-p65 and anti-p50 primary antibodies. (c) c-Rel downregulation inhibits CSE-induced NF-κB activation. A549 cells were transfected with pSuper (empty vector), anti-c-Rel (si-c-Rel) and anti-p65 (si-p65) si-RNA constructs. After 24 h of transfection, cells were harvested and cell extracts were analyzed by western blotting. Tubulin serves as loading control. These transfectants harboring pSuper, si-c-Rel and si-p65 constructs were treated with 2% CSE for 30 min and harvested. Nuclear extracts were analyzed by EMSA using radiolabeled NF-κB probe. (d) Chromatin immunoprecipitation analysis of p65, c-Rel and p50 recruitment at IL-8 and cyclin D1 upstream promoter sequences.
exposed to CS for 4 days. Consistent with the exposure (Figure 2, left panel). As considerable NF-κB-binding activity in lung nuclear extracts was examined by EMSA. Guinea pigs were exposed to CS for 3 - 6 days and NF-κB activation in response to CS treatment, whereas little nuclear accumulation of p65 was observed under the same conditions (Figure 1b).

To further confirm, si-RNA-mediated gene knockdown experiments were performed. Cells, which were transfected with si-c-Rel construct before CSE treatment, showed downregulation of c-Rel and also exhibited marked reduction in CSE-induced NF-κB activation (Figure 1c). In contrast, cells that were transfected with si-p65 construct exhibited little effect on CSE-induced NF-κB activation, although p65 was downregulated. To gain more confidence, the binding of c-Rel, p50 and p65 to the upstream promoter sequence of the two NF-κB target genes IL-8 and cyclin D1, which were found to be upregulated by CSE (Supplementary Figure S1c), were examined in CSE-treated cells by chromatin immunoprecipitation assay. Consistent with the cell fractionation and immunofluorescence results, better DNA binding was observed for c-Rel and p50 compared with p65 (Figure 1d). Taken together, these results indicate that consistent with previously published results, although some p65 does enter the nucleus in response to CSE induction, c-Rel appears to be primarily responsible for NF-κB activation under these conditions. Thus, NF-κB activation in response to CSE treatment differs from the activation observed after LPS treatment and is predominantly mediated by c-Rel/p50 complex in A549 cells.

These ex vivo results were further bolstered by in vivo experiments in guinea pig lung. To standardize NF-κB activation, guinea pigs were exposed to CS for 3 - 6 days and NF-κB DNA-binding activity in lung nuclear extracts was examined by EMSA. The results showed NF-κB activation in lung tissue by 3 days of CS exposure (Figure 2, left panel). As considerable NF-κB activation was observed by 4 days of CS-exposure, the subcellular distribution of c-Rel and p65 was examined immunohistochemically using lung tissue sections obtained from the guinea pigs exposed to CS for 4 days. Consistent with the ex vivo results, while there was substantial nuclear accumulation of c-Rel, little nuclear accumulation of p65 was observed (Figure 2, right panel). As expected, the p50 distribution pattern was similar to c-Rel (Supplementary Figure S3). These results indicate that c-Rel and p50 form active NF-κB nuclear complex in guinea pig lung in response to CS exposure and thus lends support to the ex vivo studies.

CSE-induced NF-κB activation is predominantly mediated through the degradation of IκBα.

As NF-κB dimer is retained in the cytosol by associating with IκBα, the degradation of latter is required for nuclear translocation of NF-κB components. Anto et al.7 had reported the involvement of IκBα in this process. Therefore, the change in the level of IκBα was monitored in A549 cells following CSE treatment by western blotting. Although the result showed a reduction in IκBα level with time, the rate was found to be very slow (Figure 3a) and did not correspond with the substantial translocation of c-Rel and p50 that had been observed within the indicated time period (Figure 1a). In contrast substantial IκBα degradation was observed in control experiments where the cells were treated with LPS (Supplementary Figure S4). Consistent with these results, transient transfection of A549 cells with IκBα super repressor resulted in a substantial reduction of LPS-induced NF-κB activity (Figure 3b). In contrast, the same IκBα super repressor exhibited little effect on IκBα activity (Figure 3b). These results indicate that IκBα is unlikely to be the primary IκBα in CSE-induced NF-κB signaling cascade in A549 cells, which is consistent with the report of Marwick et al.10

In order to identify the IκBα involved in this signaling cascade, the change in the levels of IκBα was investigated as this IκBα isoform is expressed highly in lung and has been reported to have interaction with c-Rel.11 The results showed a substantial reduction of IκBα with the progression of CSE treatment (Figure 3a), which is consistent with the nuclear translocation of c-Rel.

As CSE treatment induces nuclear translocation of CSE-induced NF-κB activation and this association would be disrupted following treatment with CSE. This hypothesis was tested by performing co-immunoprecipitation experiment using anti-c-Rel antibody followed by blotting for both IκBα and p50, in CSE-untreated and -treated cells. Figure 3c shows co-immunoprecipitation of IκBα and p50 with c-Rel in CSE-untreated A549 cells. As expected, IκBα was not detected in this experiment. These results indicate an association between IκBα and c-Rel/p50 in resting A549 cells. Following CSE treatment, although p50 remained associated with c-Rel, a reduced association between c-Rel and IκBα was observed (Figure 3c). Consistent with the time-dependent degradation of IκBα in CSE-treated cells (Figure 3a), a time-dependent loss of IκBα from the c-Rel complex was also observed in CSE-treated cells (Figure 3d). Thus, these results show that IκBα prevents nuclear translocation of c-Rel/p50 in resting A549 cells and following CSE treatment, IκBα degradation results in nuclear entry of c-Rel/p50 complex.

To further validate these ex vivo results in an in vivo system, the levels of IκBα and IκBβ were monitored by western blotting in the lung extracts obtained from guinea pigs that were either exposed or not exposed to CS. The results showed that while IκBα was almost completely degraded following CS exposure, a small decrease in the levels of IκBβ was observed (Figure 3e). Therefore,
IKK activity is required for CSE-induced I-κB degradation and NF-κB activation

The degradation of I-κB proteins requires active IKK as IKK-mediated phosphorylation of I-κB is a prerequisite for its degradation. Therefore, the kinase activity of IKK complex, following CSE treatment for different time periods, was examined. IKK complex was immunoprecipitated with anti-IKKγ antibody from CSE-untreated and -treated A549 cells and assayed for its ability to phosphorylate GST-I-κB (1-27 aa), which was expressed and purified from bacteria. This region of I-κBα was chosen as it contains two serine residues (S18 and S22) that appear to be similar to classical IKK target site (S32 and S36) present on I-κBα.11

A time-dependent increase in kinase activity of IKK, following CSE treatment, was observed as there was an increase in phosphorylation of GST-I-κBα with time (Figure 4a, upper panel). This time-dependent increase corresponds to the degradation pattern of I-κBα (Figure 3a) and thus establishes a linear relation between IKK activation and I-κBα degradation. In contrast, consistent with previous results, a similar experiment with GST-I-κBα substrate exhibited little phosphorylation in response to CSE-treatment (Figure 4a; lower panel). Taken together these results demonstrate that treatment of A549 cells with CSE causes the activation of IKK that preferentially phosphorylates and causes degradation of I-κBα.

Figure 3. I-κBα undergoes degradation upon exposure to CS. (a) Effect of CSE on I-κBα in A549 cells. Cells were treated with 2% CSE for different time periods (as indicated) and harvested. Whole-cell extracts were prepared and examined for I-κBα and I-κBζ by western blotting. Tubulin serves as loading control. (b) Effect of I-κBα super repressor on CS-induced NF-κB activity. A549 cells were transiently transfected with a plasmid construct that expresses I-κBα super repressor (I-κBαSR) along with a NF-κB reporter construct and a lacZ construct. After 24 h of transfection, cells were treated either with 2% CSE or with LPS (1 μg/ml) for 60 min or left untreated (control). Cell extracts were prepared and tested for luciferase activity. Results were normalized for transfection efficiencies with respect to beta galactosidase activity. Result represents the mean ± s.d. of three independent experiments. (c) Interaction of c-Rel with p50 and I-κBα in A549 cells. Cells were treated with either 2% CSE for 60 min or left untreated. Cell extracts were prepared and immunoprecipitated with anti-c-Rel antibody. Immunoprecipitates were analyzed for I-κBα and p50 by western blotting. The panel marked with star (*) shows the immunoglobulin heavy-chain band. Bottom panel shows the input. (d) Time-dependent loss of I-κBα from c-Rel upon CSE-treatment. A549 cells were treated with 2% CSE for different lengths of time as indicated. Whole-cell extracts were prepared and immunoprecipitations were performed with mouse monoclonal anti-c-Rel antibody. Immunoprecipitates were analyzed by immunoblotting with rabbit polyclonal antibodies against I-κBα and c-Rel. (e) CS exposure induces I-κBα degradation in guinea pig lung. Guinea pig eggs were either exposed to CS for 4 days or left unexposed. Lung tissue extracts were analyzed for I-κBα and I-κBζ by western blotting. Tubulin serves as loading control.

both the ex vivo and in vivo results demonstrate that exposure to CS primarily causes degradation of I-κBα resulting in the release and subsequent nuclear translocation of c-Rel/p50. As in two cases the ex vivo results mirror those obtained in vivo (Figures 2 and 3e), subsequent experiments for further elucidation of NF-κB activation mechanism have been carried out in A549 cells only.

Figure 4. CSE-induced NF-κB activation requires IKK activity. (a) CSE activates IKK. A549 cells were treated with 2% CSE and harvested at different time points as indicated. Cell extracts were prepared and subjected to immunoprecipitation (IP) using anti-IKKγ antibody. Kinase assays were performed with the immunoprecipitates and purified recombinant substrates, either GST-I-κBα (1-27 aa) or GST-I-κBζ (1-32 aa) in presence [γ-32P]-ATP. The mixtures were separated by SDS-polyacrylamide gel electrophoresis. The gels were stained with Coomassie blue and autoradiograms were captured. I and III are autoradiograms, whereas II and IV are corresponding Coomassie-stained gels. (b) Downregulation of IKKβ inhibits CSE-induced I-κBα degradation. A549 cells were either transfected with pSuper (empty vector) or anti-IKKβ siRNA construct (si-IKKβ). Twenty-four hours after transfection, cells were treated with 2% CSE for different time periods as indicated. Cell extracts were analyzed by western blotting with antibodies against I-κBα, IKKβ and tubulin. (c) IKKβ downregulation impairs CSE-induced NF-κB activation. A549 cells transfected with either pSuper or si-IKKβ were treated with 2% CSE for 30 min and harvested. Nuclear extracts were analyzed by EMSA using radiolabeled NF-κB probe.

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demonstrates that nuclear activation of NF-κB is predominantly mediated by c-Rel/p50 heterodimer. Consistently, IκBκ was shown to be in a complex with c-Rel/p50 in resting A549 cells and treatment of cells with CSE leads to loss of IκBκ from this complex. As the results show that CSE causes the degradation of IκBκ, the observed loss of IκBκ from the complex can be attributed to its degradation. Thus, the current study reveals that CSE-induced NF-κB activation in lung epithelial cells involves the degradation of IκBκ followed by nuclear translocation of c-Rel/p50.

The current study shows that the signaling cascade involving IκBκ-c-Rel/p50 is predominantly operated in CSE-induced NF-κB activation in contrast to IκBκ-p65/p50 as previously reported. Although IκBκ-c-Rel/p50 axis is predominant, we have observed reduction of CSE-induced NF-κB activation by both si-p65 and IκBκ super repressor and binding of p65 at upstream promoter sequences of NF-κB target genes that are upregulated by CSE. Taking these observations into account, it can be said that the classical axis, comprising IκBκ-p65/p50, is also active with minor contribution. In the previous studies, it is possible that the researchers overlooked the axis that is operating predominantly in this event. In addition, in the cell culture-based studies, the difference in preparation of CSE and applied dose may also contribute towards the observed discrepancy. Anto et al. and Shishodia et al. used the particulate phase of CS extracted with DMSO, whereas in the current study aqueous extract has been used. Therefore, the outcomes present on these two preparations are likely to differ considerably. Aqueous extract is more relevant in the physiological context as smoke is absorbed in aqueous respiratory tract lung fluid. Moreover, Anto et al. and Shishodia et al. stored the DMSO-dissolved tar phase at −80 °C till the start of the experiment. In contrast, experiments described in this study were performed with freshly prepared CSE. Storage at −80 °C may result in the destruction of potent unstable components from CS condensate and may also generate different stable new ones having different activities.

The current study shows the activation of c-Rel/p50 heterodimer by CS in lung epithelial cells. Although all the members of Rel/NF-κB family have been implicated in human cancer except RelB, c-Rel is the most oncogenic member among them.15–17 The underlying mechanism of this tumorigenic potential is not clear. However, a bias towards expression of genes involved in survival oncogenic activity of CS might be controlled by persistent activity of IκBκ-the degradation of IκBκ follows by nuclear translocation of c-Rel/p50.

DISCUSSION
In the present study efforts were targeted towards understanding the CS-induced NF-κB activation in alveolar epithelial cells using cultured epithelial A549 cell line. In vivo experiments were also performed in guinea pig to lend credence to the results obtained from ex vivo studies. Guinea pig has been chosen as experimental animal as like human, guinea pig cannot synthesize vitamin C, an antioxidant known to counter the effect of CS.14 A previous report by Marwick et al. demonstrated that CS-induced NF-κB activation does not require the degradation of IκBκ and proposed an IκBκ-independent mechanism for the same. In agreement with Marwick et al., the present study found that IκBκ degradation is not the prime event in CS-induced NF-κB activation. Instead IκBκ degradation has the central role. The current study also

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Although the mechanism of this selectivity is unknown, functionally it can contribute to a great extent towards the tumorigenic effects of CS in lung. It is known that the different I-CB proteins exhibit different pattern of degradation as well as resynthesis. I-CB is degraded and re-synthesized rapidly (in 1 h) and is therefore involved in functions associated with transient NF-κB activity. In this study it was found that I-CB is 4 h to reappear after the first round of degradation in CSE-treated A549 cells (Supplementary Figure S5). The delayed reappearance of I-CB significantly contributes towards sustained NF-κB activation by prolonging c-Rel/p50 nuclear activity. Thus, the involvement of c-Rel activity coupled with the dynamics of I-CB levels in CS-induced NF-κB activation makes CS a potentially strong tumorigenic agent and may explain the strong correlation between cigarette smoking and lung carcinogenesis.

Till date several NF-κB complexes, in combination with different I-CB, have been identified for stimulus-specific activation of NF-κB in different cell lines. This study has identified an NF-κB complex consisting of I-CB, c-Rel and p50. Previous studies document the interaction between c-Rel and I-CB, and also an active NF-κB complex formation between c-Rel and p50. However, the signaling cascade comprising of stimulus (in this case CS), IKK/1-I-CB/c-Rel/p50, is neither previously documented nor functionally implicated elsewhere. In conclusion, a new axis of NF-κB activation in lung epithelial cells is proposed wherein I-CB and c-Rel, instead of I-CB and p65, have the central role.

MATERIALS AND METHODS

Cell culture and transfection

All in vitro experiments were performed on Human lung alveolar type II cell line A549 that were grown in Ham’s F12-nutrient mixture.20 Transient transfections were performed using PolyFect reagent (Qiagen, Hilden, Germany).

Plasmids and protein expression

siRNA sequences targeted against p65, c-Rel and IKKβ were designed using siDESIGN software (Thermo Scientific, Asheville, NC, USA) and cloned into pSuper Retro Puro vector (Oligo-Engine, Seattle, WA, USA). For the construction of GST-I-CB (1-27 aa), expression plasmid, oligonucleotides corresponding to 1-27 aa of I-CB: cDNA were annealed and cloned into EcoRI and Xhol sites of pGEX-4T1 (Amersham Biosciences, Little Chalfont, UK). GST fusion proteins, GST-I-CB (1-54 aa) and GST-I-CB (1-27 aa), were expressed in Escherichia coli (BL21 DE3) and purified by glutathione-sepharose beads. The sequences of the oligonucleotide primers that have been used for making different constructs are given in Supplementary Table S1.

Preparation of CSE

CSE were prepared from filter tipped 69-mm cigarettes from Indian Tobacco Company as described by Maity et al.20

Exposure of guinea pigs to CS

Three- to four- month-old male guinea pigs (350-400 g) were used. Animal care procedures were as per NIH (National Institutes of Health) guidelines and approved by the Institutional Animal Ethics Committee. The guinea pigs were fed a vitamin C-free diet for 7 days to minimize the vitamin C level in the plasma and tissues as vitamin C is a potential inhibitor of CS-induced oxidative stress.14 The composition of the diet was as per Banerjee et al.21 After feeding vitamin C-free diet for 7 days, each guinea pig was given oral supplement of 1 mg vitamin C/day as maintenance dose and subjected to CSE exposure (three cigarettes/animal/day with two puffs/cigarette) in a smoke chamber.14 Guineas were exposed to smoke environment for 1 min during each puff and exposed to fresh air for the next 1 min.

Nuclear - cytosolic fractionation

Nuclear - cytosolic fractions of differentially treated A549 cells were carried out as described by Chaturvedi et al.22 For lung tissue, single cell was prepared by passing tissue homogenate through a micro-sieve (Sigma-Aldrich, St Louis, MO, USA). Thereafter, the procedure, used for A549 cells, was followed to obtain nuclear -cytosolic fractions of these cells.

Electrophoretic mobility shift assay

EMSA were performed using 32P-labeled oligonucleotide probe containing the consensus sequences for NF-κB according to previously described method by Chaturvedi et al.22 DNA protein complexes were resolved on a non-denaturing 5% polyacrylamide gel and subsequently exposed to either X-ray film (Kodak, Rochester, NY, USA) or phosphor imaging system (Amersham Biosciences).

Immunoprecipitation

Lysates were prepared from differentially treated A549 cells and protein concentrations were determined. Hundred micrograms of each lysate was incubated overnight at 4°C with specific antibody (as per the requirement). Protein A Sepharose beads were added and kept at 4°C on a rotating platform for 2 h. Thereafter immune complexes were isolated, separated by SDS-polyacrylamide gel electrophoresis and analyzed by immunoblotting.

Immunohistochemistry and immunofluorescence

Lung tissue from guinea pigs was fixed in formaldehyde. Fixed tissue was paraffin-embedded, and serially sectioned at 5 μm. These sections were then deparaffinized and made permeable by treating with 0.1% Triton X-100. Thereafter, antigens were unmasked by heating the sections at 90°C for 10 min in 10 mM Na-citrate buffer, pH 6.0. These sections were then incubated overnight at 4°C with specific antibodies as per the requirement. Sections were then incubated with fluorescein isothiocyanate conjugated secondary antibody at room temperature for 2 h, washed and stained with 46-diamidino-2-phenyl indole. Fluorescent signals were viewed under fluorescence microscope (Olympus IX71, Tokyo, Japan). Immunofluorescence was performed as described by Bernard et al.23

Kinase assay

The IKK assay was performed as described by Delhase et al.15 Briefly, IKK complex was immunoprecipitated from 300 μg of cell extracts obtained from CSE-treated or -untreated A549 cells using IKKα antibody with Protein A Sepharose beads. The beads were then washed and resuspended in 25 μl of kinase assay mixture containing 3 μg of substrate (GST-I-CB or GST-I-CB) and 8 μM of ATP in addition to the components of assay buffer and incubated at 37°C for 30 min. Finally, the proteins were separated by SDS-polyacrylamide gel electrophoresis, stained with Coomassie blue and exposed to either X-ray film (Kodak) or phosphor imaging system (Amersham Biosciences) to obtain autoradiogram of the gel.

Chromatin immunoprecipitation assay

A549 cells were treated with 2% CSE for 30 min and thereafter chromatin immunoprecipitation was performed as described previously by Majumder et al.24 The DNA-protein complex was immunoprecipitated using anti-p65, p50 or c-Rel antibody in separate reactions. In a separate reaction, a non specific anti-rabbit IgG was also added as control. The DNA obtained was analyzed by PCR using specific primers (Supplementary Table S1) designed to target the NF-κB-binding sites at IL-8 and cyclin D1 promoters as indicated in the figure.

CONFLICT OF INTEREST

The authors declare no conflict of interests.
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