

ORIGINAL ARTICLE

# IKK $\beta$ -I- $\kappa$ B $\epsilon$ -c-Rel/p50: a new axis of NF- $\kappa$ B activation in lung epithelial cells

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Cigarette smoke (CS), a major risk factor for developing lung cancer, is known to activate transcriptional activator nuclear factor kappa B (NF- $\kappa$ B). However, the underlying mechanism of this activation remains unclear because of conflicting reports. As NF- $\kappa$ B 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- $\kappa$ B activation mechanism is different from that of other pro-inflammatory signals such as lipopolysaccharide (LPS). The NF- $\kappa$ B dimer that translocates to the nucleus upon stimulation with CS is predominantly composed of c-Rel/p50 and this translocation involves degradation of I- $\kappa$ B $\epsilon$  and not I- $\kappa$ B $\alpha$ . This degradation of I- $\kappa$ B $\epsilon$  depends on IKK $\beta$  activity, which preferentially targets I- $\kappa$ B $\epsilon$ . Consistently, CS-activated form of IKK $\beta$  was found to be different from that involved in LPS activation as neither Ser177 nor Ser181 of IKK $\beta$  is crucial for CS-induced NF- $\kappa$ B activation. Thus, unlike other pro-inflammatory stimulations where p65 and I- $\kappa$ B $\alpha$  have a central role, the predominantly active signaling cascade in CS-induced NF- $\kappa$ B activation in the lung epithelial cells comprises of IKK $\beta$ -I- $\kappa$ B $\epsilon$ -c-Rel/p50. Thus, this study uncovers a new axis of NF- $\kappa$ B activation wherein I- $\kappa$ B $\epsilon$  and c-Rel have the central role.

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## 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)<sup>1</sup> 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.<sup>2</sup> As a result, ROS levels increase within the cell and this triggers redox-sensitive pathways. Nuclear factor kappa B (NF- $\kappa$ B) is one such redox-sensitive transcription factor. It controls several important cellular processes including cell survival and inflammation<sup>3</sup> and has been shown to be activated by CS.<sup>4,5</sup> Active NF- $\kappa$ B 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.<sup>6</sup> In resting cells, NF- $\kappa$ B is sequestered in the cytosol as a result of physical association with a member of a family of inhibitory proteins called I- $\kappa$ Bs, which mask the nuclear localization signal of NF- $\kappa$ B. The principal members of the I- $\kappa$ B family are I- $\kappa$ B $\alpha$ , I- $\kappa$ B $\beta$ , I- $\kappa$ B $\epsilon$  and Bcl-3. Depending on the cell type and the nature of stimulus, different I- $\kappa$ B forms complex with different NF- $\kappa$ B proteins. These different combinations greatly contribute to the NF- $\kappa$ B functional diversity observed in different cells under varied conditions.<sup>7</sup> Stimulation of cells with external stimuli, such as lipopolysaccharide (LPS), elicit signal across the plasma membrane through specific receptors that causes activation of I- $\kappa$ B kinase (IKK) complex.<sup>3</sup> The activated IKK in turn phosphorylates I- $\kappa$ B. IKK complex contains two catalytic subunits, IKK $\alpha$  and IKK $\beta$ , and one regulatory subunit, IKK $\gamma$ . Previous reports show that IKK $\beta$  has a vital role in pro-inflammatory stimulus-mediated NF- $\kappa$ B activation, wherein it phosphorylates I- $\kappa$ B $\alpha$ .<sup>6</sup>

The modified I- $\kappa$ B undergoes proteasomal degradation thereby freeing NF- $\kappa$ B to translocate into the nucleus and transactivate its target genes.

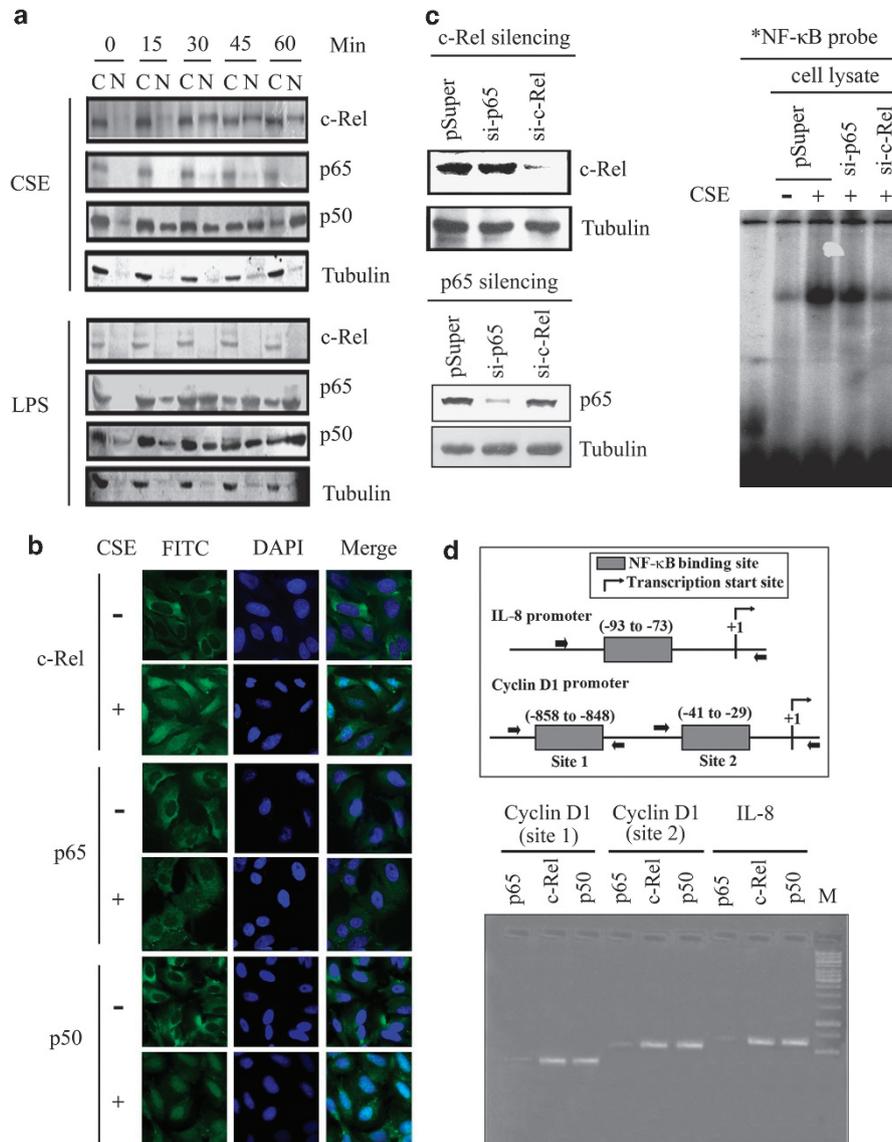
Although CS has been known to cause NF- $\kappa$ B activation for long time,<sup>4</sup> the mechanism of this activation remains unclear as available reports are conflicting in nature. There are reports that showed the NF- $\kappa$ B activation by CS extract (CSE) in cultured cell lines, including alveolar epithelial H1299 cells, is mediated by p65 and p50 nuclear translocation resulting from I- $\kappa$ B $\alpha$  degradation.<sup>5,8</sup> Consistent with this, Rajendrasozhan *et al.*<sup>9</sup> showed the degradation of I- $\kappa$ B $\alpha$  and nuclear entry of p65 in CS-exposed rat lung extract. In contrast, Marwick *et al.*<sup>10</sup> have demonstrated CS-induced NF- $\kappa$ B activation in the rat lungs, which is independent of I- $\kappa$ B $\alpha$  degradation.

As cigarette smoking is a major etiological agent for several pulmonary diseases, including lung cancer wherein NF- $\kappa$ B has an important role, it is vital to understand the underlying mechanism of CS-induced NF- $\kappa$ B activation. With the aim of elucidating the mechanism of CS-induced NF- $\kappa$ B 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- $\kappa$ B activation in lung epithelial cells as a result of I- $\kappa$ B $\epsilon$  degradation by IKK $\beta$ . Thus, the present study provides a new axis of NF- $\kappa$ B activation comprising IKK $\beta$ -I- $\kappa$ B $\epsilon$ -c-Rel/p50 in lung epithelial cells.

## RESULTS

CS-induced NF- $\kappa$ B activation predominantly involves nuclear translocation of c-Rel and p50 in lung epithelia

To study the mechanism of CSE-induced NF- $\kappa$ B activation in A549 alveolar epithelial cells, the optimum condition of NF- $\kappa$ B activation



**Figure 1.** CSE-induced NF- $\kappa$ B 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  $\mu$ g/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- $\kappa$ B 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- $\kappa$ B probe (right panel). The first lane of the gel was loaded with the free probe. **(d)** Chromatin immunoprecipitation (ChIP) analysis of p65, c-Rel and p50 recruitment at IL-8 and cyclin D1 upstream promoter sequences. 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- $\kappa$ B-binding site(s) at IL-8 and cyclin D1 upstream promoter sequences as indicated in the upper panel and analyzed by agarose gel electrophoresis. DAPI, 4, 6-diamidino-2-phenyl indole; FITC, fluorescein isothiocyanate.

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- $\kappa$ B activation (Supplementary Figure S1a, left panel) and this activation is mediated by ROS as pretreatment with 20 mM *N*-acetyl cysteine, 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- $\kappa$ B complex,

following CSE treatment, was confirmed by luciferase assay and induction of known NF- $\kappa$ B target genes (Supplementary Figure S1b and S1c).

To understand the specific components that constitute active NF- $\kappa$ B 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

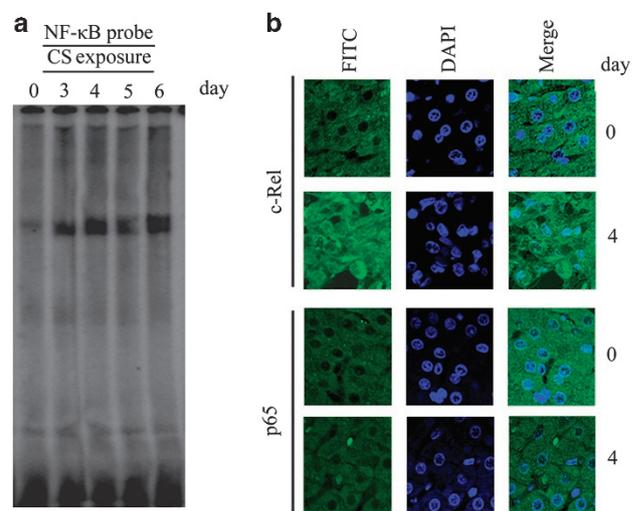
panel). While no detectable nuclear accumulation was observed for RelB and p52, a little p65 was detected in the nucleus (Supplementary Figure S2 and Figure 1a). In contrast, the nuclear accumulation of p65 and p50 was observed with time in response to LPS treatment as expected (Figure 1a; lower panel). As an independent verification, immunolocalization of c-Rel, p65 and p50 was performed in CSE-treated A549 cells. Congruent with cell fractionation results, immunofluorescence experiment demonstrated a substantial nuclear translocation of c-Rel and p50 in response to CSE treatment, whereas little nuclear accumulation of p65 was observed under the same conditions (Figure 1b).

To further confirm, si-RNA-mediated gene knockdown experiments for c-Rel and p65 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- $\kappa$ B activation (Figure 1c). In contrast, cells that were transfected with si-p65 construct exhibited little effect on CSE-induced NF- $\kappa$ B 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- $\kappa$ B 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- $\kappa$ B activation under these conditions. Thus, NF- $\kappa$ B 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- $\kappa$ B activation, guinea pigs were exposed to CS for 3–6 days and NF- $\kappa$ B DNA-binding activity in lung nuclear extracts was examined by EMSA. The results showed NF- $\kappa$ B activation in lung tissue by 3 days of CS exposure (Figure 2, left panel). As considerable NF- $\kappa$ B 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- $\kappa$ B nuclear complex in guinea pig lung in response to CS exposure and thus lends support to the *ex vivo* studies.

CSE-induced NF- $\kappa$ B activation is predominantly mediated through the degradation of I- $\kappa$ B $\epsilon$

As NF- $\kappa$ B dimer is retained in the cytosol by associating with I- $\kappa$ B, the degradation of latter is required for nuclear translocation of NF- $\kappa$ B components. Anto *et al.*<sup>5</sup> had reported the involvement of I- $\kappa$ B $\alpha$  in this process. Therefore, the change in the level of I- $\kappa$ B $\alpha$  was monitored in A549 cells following CSE treatment by western blotting. Although the result showed a reduction in I- $\kappa$ B $\alpha$  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 I- $\kappa$ B $\alpha$  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 I- $\kappa$ B $\alpha$  super repressor resulted in a substantial reduction of LPS-induced NF- $\kappa$ B activity (Figure 3b). In contrast, the same I- $\kappa$ B $\alpha$  super repressor exhibited little effect on



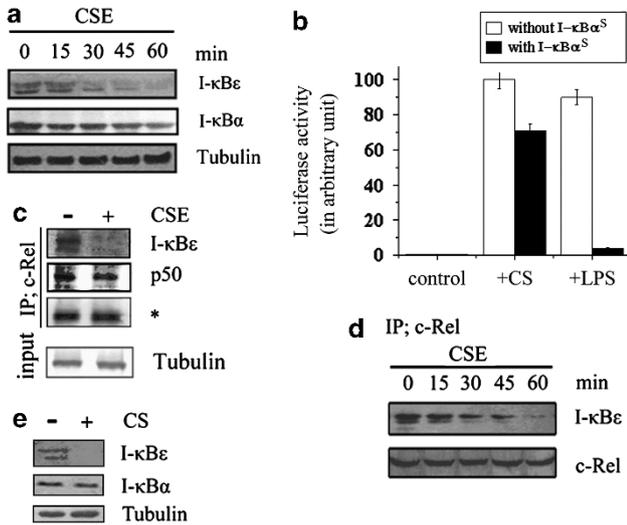
**Figure 2.** CS-induced NF- $\kappa$ B activation in guinea pig lung. (a) Exposure of guinea pigs to CS induces alveolar NF- $\kappa$ B activation. Guinea pigs were exposed to CS for 0, 3, 4, 5 and 6 days. Nuclear extracts were prepared from lung tissues, and NF- $\kappa$ B activation was assayed by EMSA using radiolabeled wild-type NF- $\kappa$ B probe. (b) Immunohistochemistry of c-Rel and p65. Lung tissue from guinea pigs that were either exposed to CS for 4 days or left unexposed (0 day) were fixed and paraffin sections were prepared. Thereafter sections were immunostained with anti-c-Rel and anti-p65 antibodies. Nuclei were stained with DAPI. FITC, fluorescein isothiocyanate.

CS-induced NF- $\kappa$ B activity (Figure 3b). These results indicate that I- $\kappa$ B $\alpha$  is unlikely to be the primary I- $\kappa$ B in CSE-induced NF- $\kappa$ B signaling cascade in A549 cells, which is consistent with the report of Marwick *et al.*<sup>10</sup>

In order to identify the I- $\kappa$ B involved in this signaling cascade, the change in the levels of I- $\kappa$ B $\epsilon$  was investigated as this I- $\kappa$ B isoform is expressed highly in lung and has been reported to have interaction with c-Rel.<sup>11</sup> The results showed a substantial reduction of I- $\kappa$ B $\epsilon$  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 c-Rel and p50, these two NF- $\kappa$ B components must be in a complex with I- $\kappa$ B $\epsilon$  in resting A549 cells 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 I- $\kappa$ B $\epsilon$  and p50, in CSE-untreated and -treated cells. Figure 3c shows co-immunoprecipitation of I- $\kappa$ B $\epsilon$  and p50 with c-Rel in CSE-untreated A549 cells. As expected, I- $\kappa$ B $\alpha$  was not detected in this experiment. These results indicate an association between I- $\kappa$ B $\epsilon$  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 I- $\kappa$ B $\epsilon$  was observed (Figure 3c). Consistent with the time-dependent degradation of I- $\kappa$ B $\epsilon$  in CSE-treated cells (Figure 3a), a time-dependent loss of I- $\kappa$ B $\epsilon$  from the c-Rel complex was also observed in CSE-treated cells (Figure 3d). Thus, these results show that I- $\kappa$ B $\epsilon$  prevents nuclear translocation of c-Rel/p50 in resting A549 cells and following CSE treatment, I- $\kappa$ B $\epsilon$  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 I- $\kappa$ B $\epsilon$  and I- $\kappa$ B $\alpha$  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 I- $\kappa$ B $\epsilon$  was almost completely degraded following CS exposure, a small decrease in the levels of I- $\kappa$ B $\alpha$  was observed (Figure 3e). Therefore,

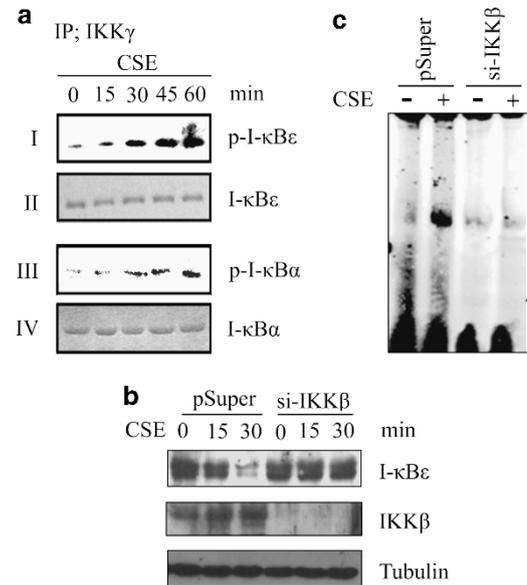


**Figure 3.** I- $\kappa$ B $\epsilon$  undergoes degradation upon exposure to CS. (a) Effect of CSE on I- $\kappa$ B in A549 cells. Cells were treated with 2% CSE for different time periods (as indicated) and harvested. Whole-cell extracts were prepared and examined for I- $\kappa$ B $\epsilon$  and I- $\kappa$ B $\alpha$  by western blotting. Tubulin serves as loading control. (b) Effect of I- $\kappa$ B $\alpha$  super repressor on CS-induced NF- $\kappa$ B activity. A549 cells were transiently transfected with a plasmid construct that expresses I- $\kappa$ B $\alpha$  super repressor (I- $\kappa$ B $\alpha$ <sup>S</sup>) along with a NF- $\kappa$ B reporter construct and a *lacZ* construct. After 24 h of transfection, cells were treated either with 2% CSE or with LPS (1  $\mu$ g/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  $\pm$  s.d. of three independent experiments. (c) Interaction of c-Rel with p50 and I- $\kappa$ B $\epsilon$  in A549 cells. Cells were treated either with 2% CSE for 60 min or left untreated. Cell extracts were prepared and immunoprecipitations were performed using anti-c-Rel antibody. Immunoprecipitates were analyzed for I- $\kappa$ B $\epsilon$  and p50 by western blotting. The panel marked with star (\*) sign shows the immunoglobulin heavy-chain band. Bottom panel shows the input. (d) Time-dependent loss of I- $\kappa$ B $\epsilon$  from c-Rel upon CS-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- $\kappa$ B $\epsilon$  and c-Rel. (e) CS exposure induces I- $\kappa$ B $\epsilon$  degradation in guinea pig lung. Guinea pigs were either exposed to CS for 4 days or left unexposed. Lung tissue extracts were analyzed for I- $\kappa$ B $\epsilon$  and I- $\kappa$ B $\alpha$  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- $\kappa$ B $\epsilon$  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- $\kappa$ B activation mechanism have been carried out in A549 cells only.

#### IKK activity is required for CSE-induced I- $\kappa$ B $\epsilon$ degradation and NF- $\kappa$ B activation

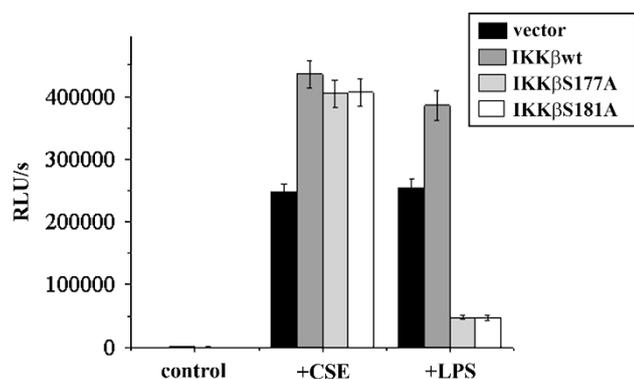
The degradation of I- $\kappa$ B proteins requires active IKK as IKK-mediated phosphorylation of I- $\kappa$ B is a prerequisite for its degradation. Therefore, the kinase activity of IKK complex, following CSE treatment for different time periods, was examined. IKK complex was immunoprecipitated with anti- $\text{IKK}\gamma$  antibody from CSE-untreated and -treated A549 cells and assayed for its ability to phosphorylate GST-I- $\kappa$ B $\epsilon$  (1-27 aa), which was expressed and purified from bacteria. This region of I- $\kappa$ B $\epsilon$  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- $\kappa$ B $\alpha$ .<sup>11</sup>



**Figure 4.** CSE-induced NF- $\kappa$ B 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- $\text{IKK}\gamma$  antibody. Kinase assays were performed with the immunoprecipitates and purified recombinant substrates, either GST-I- $\kappa$ B $\epsilon$  (1-27 aa) or GST-I- $\kappa$ B $\alpha$  (1-32 aa) in presence [ $\gamma$ -<sup>32</sup>P]-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 IKK $\beta$  inhibits CSE-induced I- $\kappa$ B $\epsilon$  degradation. A549 cells were either transfected with pSuper (empty vector) or anti- $\text{IKK}\beta$  siRNA construct (si- $\text{IKK}\beta$ ). 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- $\kappa$ B $\epsilon$ , IKK $\beta$  and tubulin. (c) IKK $\beta$  downregulation impairs CSE-induced NF- $\kappa$ B activation. A549 cells transfected with either pSuper or si- $\text{IKK}\beta$  were treated with 2% CSE for 30 min and harvested. Nuclear extracts were analyzed by EMSA using radiolabeled NF- $\kappa$ B probe.

A time-dependent increase in kinase activity of IKK, following CSE treatment, was observed as there was an increase in phosphorylation of GST-I- $\kappa$ B $\epsilon$  with time (Figure 4a, upper panel). This time-dependent increase corresponds to the degradation pattern of I- $\kappa$ B $\epsilon$  (Figure 3a) and thus establishes a linear relation between IKK activation and I- $\kappa$ B $\epsilon$  degradation. In contrast, consistent with previous results, a similar experiment with GST-I- $\kappa$ B $\alpha$  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- $\kappa$ B $\epsilon$  (Figures 3a and 4a).

IKK consists of two catalytic subunits, IKK $\alpha$  and IKK $\beta$ , of which IKK $\beta$  has been shown to be indispensable for I- $\kappa$ B phosphorylation and subsequent NF- $\kappa$ B activation in inflammatory responses.<sup>3</sup> CS is an inflammatory agent and IKK $\beta$  has already been reported to be involved in CSE-induced NF- $\kappa$ B activation.<sup>5,9,12</sup> Therefore, it is likely that IKK $\beta$  is responsible for CSE-induced I- $\kappa$ B $\epsilon$  degradation and subsequent NF- $\kappa$ B activation. This hypothesis was tested by determining the effect of decreased IKK $\beta$  expression on I- $\kappa$ B $\epsilon$  degradation in CSE-treated A549 cells. IKK $\beta$  expression was downregulated with IKK $\beta$ -targeted si-RNA (Figure 4b, middle panel). It was observed that CSE treatment did not result in I- $\kappa$ B $\epsilon$  degradation in cells that were transfected with IKK $\beta$ -targeted siRNA (Figure 4b, top panel). However, I- $\kappa$ B $\epsilon$  degradation was



**Figure 5.** IKK $\beta$  activated by CS is different from LPS-activated IKK $\beta$ . A549 cells were transiently transfected with a NF- $\kappa$ B reporter construct, a *lacZ* construct and any one of the following: (i) wild-type IKK $\beta$ , (ii) mutant IKK $\beta$  (IKK $\beta$  S177A), (iii) mutant IKK $\beta$  (IKK $\beta$  S181A) and (iv) empty vector. After 24 h of transfection, cells were treated either with 2% CSE or with 1  $\mu$ g/ml LPS for 60 min or left untreated (control). Cell extracts were prepared and tested for luciferase activity. Results were normalized for transfection efficiencies by beta galactosidase activity assay. Result represents the mean  $\pm$  s.d. of three independent experiments. RLU, relative luminescence unit.

evident in control cells transfected with empty vector. Consistently, EMSA showed suppression of CSE-induced NF- $\kappa$ B activity in cells transfected with si-RNA construct targeted against IKK $\beta$  (Figure 4c). Thus, these results establish the role of IKK $\beta$  in I- $\kappa$ B $\epsilon$  degradation and the concomitant NF- $\kappa$ B activation.

IKK $\beta$  is involved in both LPS and CS-induced NF- $\kappa$ B activation and therefore exhibits differential substrate specificity, I- $\kappa$ B $\alpha$  in case of LPS and I- $\kappa$ B $\epsilon$  in case of CSE. This raises the possibility that the activated forms of IKK $\beta$  in case of these two different stimuli (LPS and CS) may differ and the CS-induced activated IKK $\beta$  may not involve the classical phosphorylation at Ser177 and Ser181 residues that has been observed for LPS-induced IKK $\beta$  activation.<sup>13</sup> To test this hypothesis, A549 cells were transiently transfected individually with wild-type IKK $\beta$  as well as two different mutants of IKK $\beta$  (IKK $\beta$  S177A and IKK $\beta$  S181A). These transfectants were treated either with CSE or with LPS, and NF- $\kappa$ B activity was measured in each case. Congruent with the previously published result, it was observed that cells transfected with either of the mutants exhibited reduced NF- $\kappa$ B activity upon LPS stimulation compared with the cells transfected either with vector alone or with wt IKK $\beta$  (Figure 5). In contrast, when the cells were induced with CSE, all transfectants carrying different forms of IKK $\beta$  exhibit similar levels of NF- $\kappa$ B activity (Figure 5). These results suggest that the induced form of IKK $\beta$  is different in case of LPS and CSE induction.

## DISCUSSION

In the present study efforts were targeted towards understanding the CS-induced NF- $\kappa$ B 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.<sup>14</sup> A previous report by Marwick *et al.*<sup>10</sup> demonstrated that CS-induced NF- $\kappa$ B activation does not require the degradation of I- $\kappa$ B $\alpha$  and proposed an I- $\kappa$ B-independent mechanism for the same. In agreement with Marwick *et al.*, the present study found that I- $\kappa$ B $\alpha$  degradation is not the prime event in CS-induced NF- $\kappa$ B activation. Instead I- $\kappa$ B $\epsilon$  degradation has the central role. The current study also

demonstrates that nuclear activation of NF- $\kappa$ B is predominantly mediated by c-Rel/p50 heterodimer. Consistently, I- $\kappa$ B $\epsilon$  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 I- $\kappa$ B $\epsilon$  from this complex. As the results show that CSE causes the degradation of I- $\kappa$ B $\epsilon$ , the observed loss of I- $\kappa$ B $\epsilon$  from the complex can be attributed to its degradation. Thus, the current study reveals that CS-induced NF- $\kappa$ B activation in lung epithelial cells involves the degradation of I- $\kappa$ B $\epsilon$  followed by nuclear translocation of c-Rel/p50.

The current study shows that the signaling cascade involving I- $\kappa$ B $\epsilon$ -c-Rel/p50 is predominantly operated in CSE-induced NF- $\kappa$ B activation in contrast to I- $\kappa$ B $\alpha$ -p65/p50 as previously reported. Although I- $\kappa$ B $\epsilon$ -c-Rel/p50 axis is predominant, we have observed reduction of CSE-induced NF- $\kappa$ B activation by both si-p65 and I- $\kappa$ B $\alpha$  super repressor and binding of p65 at upstream promoter sequences of NF- $\kappa$ B target genes that are upregulated by CSE. Taking these observations into account, it can be said that the classical axis, comprising I- $\kappa$ B $\alpha$ -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 constituents present in 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  $-80^{\circ}\text{C}$  till the start of the experiment. In contrast, experiments described in this study were performed with freshly prepared CSE. Storage at  $-80^{\circ}\text{C}$  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- $\kappa$ B family have been implicated in human cancer except RelB, c-Rel is the most oncogenic member among them.<sup>15-17</sup> The underlying mechanism of this tumorigenic potential is not clear. However, a bias towards expression of genes involved in survival and proliferation such as IL-2 and Bcl-x<sub>L</sub><sup>18,19</sup> may explain its oncogenicity. The tumorigenic potential can also be attributed to the increased and sustained nuclear accumulation of NF- $\kappa$ B proteins. The absence of NES in c-Rel<sup>17</sup> clearly supports its longer retention in the nucleus. Therefore, it is possible that pro-oncogenic activity of CS might be controlled by persistent activity of nuclear c-Rel through constitutive activation of proliferative and anti-death-inducing factors.

c-Rel/p50 is activated through degradation of a specific member of I- $\kappa$ B family, I- $\kappa$ B $\epsilon$ , by the action of IKK $\beta$ . Although I- $\kappa$ B $\alpha$  is present in the lung epithelial cells and undergoes phosphorylation and subsequent degradation by other stimuli such as LPS, CS-induced IKK $\beta$  preferentially targets I- $\kappa$ B $\epsilon$ . It is evident from our experiment that mutations at Ser177 and Ser181, which are involved in NF- $\kappa$ B activation by known pro-inflammatory agents such as LPS, failed to block CSE-induced NF- $\kappa$ B activation. Therefore, the observed preferential choice of substrate may arise from differential phosphorylation of active IKK $\beta$ . All these results indicate that the precise mechanism of IKK $\beta$  activation differs between CS and other stimuli such as LPS and TNF $\alpha$ . Understanding this critical regulatory point may help in designing better IKK inhibitors that will selectively block a specific function of IKK without affecting other functions. Presently, the role of other two subunits of IKK, IKK $\alpha$  and IKK $\gamma$ , in CS-induced NF- $\kappa$ B activation is not clear. Further investigations are required to elucidate their roles in this event.

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

Till date several NF- $\kappa$ B complexes, in combination with different I- $\kappa$ B, have been identified for stimulus-specific activation of NF- $\kappa$ B in different cell lines. This study has identified an NF- $\kappa$ B complex consisting of I- $\kappa$ B $\epsilon$ , c-Rel and p50. Previous studies document the interaction between c-Rel and I- $\kappa$ B $\epsilon$ , and also an active NF- $\kappa$ B complex formation between c-Rel and p50. However, the signaling cascade comprising of stimulus (in this case CS), IKK $\beta$ -I- $\kappa$ B $\epsilon$ -c-Rel/p50, is neither previously documented nor functionally implicated elsewhere. In conclusion, a new axis of NF- $\kappa$ B activation in lung epithelial cells is proposed wherein I- $\kappa$ B $\epsilon$  and c-Rel, instead of I- $\kappa$ B $\alpha$  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.<sup>20</sup> Transient transfections were performed using PolyFect reagent (Qiagen, Hilden, Germany).

### Plasmids and protein expression

siRNA sequences targeted against p65, c-Rel and IKK $\beta$  were designed using siDESIGN<sup>+</sup> software (Thermo Scientific, Asheville, NC, USA) and cloned into pSuper Retro Puro vector (Oligo-Engine, Seattle, WA, USA). For the construction of GST-I- $\kappa$ B $\epsilon$  (1-27 aa) expression plasmid, oligonucleotides corresponding to 1-27 aa of I- $\kappa$ B $\epsilon$  cDNA were annealed and cloned into *Eco*RI and *Xho*I sites of pGEX-4T1 (Amersham Biosciences, Little Chalfont, UK). GST fusion proteins, GST-I- $\kappa$ B $\alpha$  (1-54 aa) and GST-I- $\kappa$ B $\epsilon$  (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.*<sup>20</sup>

### 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.<sup>14</sup> The composition of the diet was as per Banerjee *et al.*<sup>21</sup> 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 CS exposure (three cigarettes/animal/day with two puffs/cigarette) in a smoke chamber.<sup>14</sup> Guinea pigs 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 fractionations of differentially treated A549 cells were carried out as described by Chaturvedi *et al.*<sup>22</sup> 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 fractionations of these cells.

### Electrophoretic mobility shift assay

EMSA were performed using <sup>32</sup>P-labeled oligonucleotide probe containing the consensus sequences for NF- $\kappa$ B according to previously described method by Chaturvedi *et al.*<sup>22</sup> 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 lysates were 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  $\mu$ m. 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. The 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.*<sup>23</sup>

### Kinase assay

The IKK assay was performed as described by Delhase *et al.*<sup>13</sup> Briefly, IKK complex was immunoprecipitated from 300  $\mu$ g of cell extracts obtained from CSE-treated or -untreated A549 cells using IKK $\gamma$  antibody with Protein A Sepharose beads. The beads were then washed and resuspended in 25  $\mu$ l of kinase assay mixture containing 3  $\mu$ g of substrate (GST-I- $\kappa$ B $\alpha$  or GST-I- $\kappa$ B $\epsilon$ ) and 8  $\mu$ moles of [ $\gamma$ -<sup>32</sup>P] 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.*<sup>24</sup> 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- $\kappa$ B-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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