PUBLICATIONS

Vitamin C forestalls cigarette smoke induced NF-κB activation in alveolar epithelial cells

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**HIGHLIGHTS**

- Vitamin C prevents CS-induced nuclear translocation of c-Rel.
- Vitamin C prevents CS-induced degradation of IκBα.
- Vitamin C prevents CS-induced NF-κB activation in alveolar epithelial cells.
- This activity of vitamin C is conferred by its ROS neutralization ability.

**ARTICLE INFO**

**ABSTRACT**

Cigarette smoking causes cellular oxidative stress resulting in inflammatory diseases of lung wherein transcription factor NF-κB plays an important role. It is possible that vitamin C, an antioxidant, may prevent cigarette smoke (CS)-induced NF-κB activation that involves degradation of IκBα and nuclear translocation of c-Rel/p50 in alveolar epithelial cells. Therefore, to examine the hypothesis, we verified the effect of vitamin C on CS-induced expression of NF-κB driven luciferase reporter and NF-κB binding at its target DNA by EMSA in alveolar epithelial A549 cells. We also examined the level of IκBα and sub-cellular distribution of c-Rel by western blotting and immunofluorescence respectively in CSE-treated A549 cells with or without vitamin C pretreatment. We observed a significant reduction in CSE induced luciferase expression, NF-κB DNA binding, IκBα degradation and c-Rel nuclear translocation in cells pretreated with vitamin C. To further validate the result, we examined sub-cellular distribution of c-Rel in lungs of CS-exposed guinea pigs treated or untreated with vitamin C. Result showed that vitamin C treatment resulted in markedly reduced c-Rel nuclear translocation. All these results demonstrate that vitamin C prevents CS(E)-induced NF-κB activation and thus it could be used for the prevention of CS-induced inflammatory diseases.

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1. Introduction

Cigarette smoke (CS) has been implicated in the initiation and progression of various inflammatory diseases in human lung such as emphysema and cancer (Bhalla et al., 2009; Chiu et al., 2001; Vassallo and Ryu, 2008). The signal transduction pathways triggered by cigarette smoke involves various cellular redox sensitive transcription factor including nuclear factor kappa B (NF-κB) (Maity et al., 2012; Tharappel et al., 2010). Transcriptional activator NF-κB regulates the expression of genes that plays very important roles in cellular inflammation and inflammatory diseases (Karin et al., 2004; Rahman and Fazal, 2011; Tak and Firestein, 2001). Active NF-κB is a hetero/homo dimeric complex consisting of members of the Rel family (p65 (RelA), RelB, c-Rel, p50 and p52). In a resting cell NF-κB resides in the cytoplasm in association with inhibitory factor IκB (Hayden and Ghosh, 2004). When cells are activated by various extracellular signals, like tumor necrosis factor alpha (TNF-α), interleukin-1 α, lipopolysaccharide (LPS), IκB is phosphorylated and degraded rapidly. Subsequently, free NF-κB dimer enters the nucleus and activates the transcription of many different target genes (Pahl, 1999). The conventional inflammatory stimuli mediated NF-κB activation involves degradation of IκBα and nuclear translocation of p65/p50 dimer (Ghosh and Karin, 2002). In contrast, in our previous report we have shown a new axis of CS-induced NF-κB activation in alveolar epithelial cells wherein the NF-κB dimer that translocates to the nucleus is predominantly composed of c-Rel/p50 and this translocation involves degradation...
of I–κBε (Maity et al., 2012). Since NF-κB activation plays an important role in inflammation, agents that can block its activation are potentially important for better management of inflammatory diseases caused by CS.

CS is a complex mixture of over 4700 chemical compounds, and high concentrations of free radicals and other oxidants. In addition to its contents, CS can also activate intracellular enzymes responsible for generating reactive oxygen species (ROS) thereby leading to cellular oxidative stress. In our previous study we have shown that CS-induced oxidative stress plays an important role in NF-κB activation. Therefore, it is likely that an antioxidant like vitamin C, which can effectively scavenge a wide array of ROS and free radicals, can be used to inhibit CS-induced NF-κB activation. Vitamin C is a strong antioxidant that maintains a balance of ROS within the cell. Though cells have other antioxidant machinery (e.g., catalase, peroxidase, superoxide dismutase, etc.) to combat the impact of oxidative stress, their level cannot be manipulated by simple means (Frei et al., 1989). However, the importance of vitamin C lies with the fact that this antioxidant is water soluble and it can be easily manipulated by dietary supplementation. In a previous report, Silva Bezerra et al. (2006) demonstrated the effect of vitamin C on the level of alveolar macrophage (AM) and polymorphonuclear cells (PMN) in the lung of CS-exposed mice and in this context they also examined the level of nuclear p65 in lung tissue extract in CS-exposed mice that were either treated or not with vitamin C. They observed that vitamin C treatment resulted in a significant reduction in both the levels of AM and PMN as well as nuclear p65 in lung tissue extracts. However, they did not examine the level of c-Rel, which we observed to be the predominant NF-κB component translocated to the nucleus in alveolar epithelial cells. Besides, they have looked at nuclear p65 level in whole lung extract that includes considerable amount of inflammatory cells. Thus the observed nuclear p65 in their experiment may be a major contribution from these inflammatory cells. Therefore we have targeted our efforts to investigate the effect of vitamin C on CS-induced NF-κB activation in alveolar epithelial cells.

2. Materials and methods

2.1. Cell culture and media supplements

All in vitro experiments were carried out in Human lung alveolar type II cell line A549 grown in (Ham) F12-Nutrient Mixture (Gibco, Invitrogen) supplemented with 10% Fetal Bovine Serum (Gibco, NZ origin), and 100 U/ml penicillin and 100 μg/ml streptomycin. A549 cells were transiently transfected with luciferase reporter plasmid construct containing a luciferase gene driven from NF-κB promoter (Promega Inc., E894A) using Polyfect reagent (Bangalore Genei, India) according to manufacturer’s instruction.

2.2. Preparation of cigarette smoke extract

Cigarette smoke extract (CSE) were prepared from filter tipped 60 mm cigarette as described previously (Maity et al., 2009).

2.3. Electrophoretic mobility shift assay (EMSA)

EMSA were performed using 32P-labeled oligonucleotide probe containing the consensus sequences for NF-κB, according to previously described method (Chaturvedi et al., 2000). 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, USA).

2.4. Immunofluorescence

Immunofluorescence was performed as described previously (Bernard et al., 2002). A549 cells plated on cover slips in 30 mm tissue culture plates were used to study the sub-cellular localization of c-Rel. The cells were differentially treated with CSE and vitamin C (Sigma–Aldrich) as per the requirement of the experiment and then stained with specific antibody against c-Rel (Santa cruz biotechnology). FITC-tagged secondary antibody (Bangalore Genei, India) was used to observe the sub-cellular localization of c-Rel. Images were taken using a Fluorescent Microscope (Olympus IX 71, Japan).

2.5. Exposure of guinea pigs to CS

Three- to four-month-old male guinea pigs (350–400 g) were purchased from University of Calcutta authorized animal supplier. Animal care procedures were as per NIH (National Institutes of Health) guidelines and approved by the Institutional Animal Ethics Committee (University of Calcutta, India). The guinea pigs were fed a vitamin C-free diet for 7 days to minimize the vitamin C level in the plasma and tissues. The composition of the diet was as previously mentioned (Banerjee et al., 2007). 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. For experimental purpose one set of animals were given an additional dose of 5 mg vitamin C/day, whereas the control set was supplemented with the normal dose of 1 mg vitamin C/day. All the animals were subjected to CS exposure (three cigarettes/animal/day with two puffs/cigarette) in a smoke chamber (Ray et al., 2010) for 5 days. Guinea pigs were exposed to smoke environment for 1 min during each puff and exposed to fresh air for the next 1 min. For each group, a minimum of 3 animals were used.

2.6. Immunohistochemistry

Lung tissue from guinea pigs was fixed in formaldehyde and immunohistochemistry was performed as described previously (Maity et al., 2012). Briefly fixed tissue sections were incubated overnight at 4°C with specific antibodies as per the requirement. Then the sections were incubated with FITC conjugated secondary antibody (Bangalore Genei, India) at room temperature for 2 h, washed and stained with 4,6-diamino-2-phenylindole (DAP). Fluorescent signals were viewed under fluorescence microscope (Olympus IX71, Japan).

2.7. In situ ROS measurement in A549 cells

ROS generation in A549 cells was detected by flow cytometric analysis (FACS). A549 cells that were either pretreated or not with vitamin C were treated with CSE for 30 min. After the CSE treatment cells were incubated with 10 μM 2′,7′-dichlorofluorescin diacetate (DCFHDA) for 20 min, washed with ice cold PBS and harvested. Next, harvested cells were analyzed for the presence of oxidized 2′,7′-dichlorofluorescin (DCF), by flow cytometry (BD Pharmingen). A total of 10,000 cells were acquired for each sample and the cells were gated out based on their fluorescent property.

2.8. Statistics

Results are expressed as mean ± standard deviation (SD). Statistical significance between groups was determined using one-way ANOVA and P < 0.01 was considered significant. Statistical analysis was performed using Minitab 16.

3. Results

3.1. Effect of vitamin C on CSE-induced transcriptional response mediated by NF-κB

To investigate the effect of vitamin C on the activation of NF-κB transcription induced by CSE, a luciferase reporter driven from a promoter containing NF-κB binding site was used. A549 transfectants harboring this reporter construct were pretreated with different concentrations of vitamin C for 1 h followed by treatment with 2% CSE for 30 min. A gradual reduction of luciferase activity was observed with the increasing concentration of vitamin C and the maximum reduction was observed for cells pretreated with 100 μM vitamin C (Fig. 1A). Henceforth all the subsequent experiments were done with 100 μM vitamin C. In order to further confirm the result observed in the luciferase reporter assay EMSA was performed. Nuclear extracts from CSE-treated A549 cells that were either pretreated or untreated with vitamin C were prepared and EMSA was performed as described previously. Congruent with the luciferase assay we observed a marked reduction in the band intensity of NF-κB bound DNA complex in the nuclear extract obtained from vitamin C pretreated cells compared to nuclear extract obtained from cells treated with only CSE (Fig. 1B; compare lanes 1 and 3). This result thereby confirms reduced activation of NF-κB in vitamin C pretreated cells. Thus both luciferase assay and EMSA demonstrate that pretreatment of alveolar epithelial cells with vitamin C prevents CSE induced N F-κB activation.
Fig. 1. Effect of vitamin C on NF-κB activation. (A) Effect of vitamin C pretreatment on CSE-induced NF-κB-driven luciferase reporter activity. A549 cells were transiently transfected with a NF-κB reporter construct along with a lacZ construct. After 24 h of transfection, cells were pretreated for 1 h with different concentration of vitamin C as indicated in the figure and then treated either with 2% CSE for 30 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 ± SD of three independent experiments. Mean value with different letters indicate significant difference among different treatments, whereas mean values with same letter indicates no significant difference. P < 0.01 was considered significant. (B) Vitamin C pretreatment reduces DNA binding of NF-κB. A549 cells were pre-treated with vitamin C and then treated with 2% CSE for 30 min. Nuclear extracts were prepared from the treated cells, and NF-κB activation was assayed by EMSA using radiolabeled wild-type NF-κB probe. Arrow head indicates DNA bound NF-κB complex.

3.2. Effect of vitamin C on CSE-induced nuclear translocation of c-Rel in A549 cells

In our previous report we have shown that CSE causes nuclear translocation of c-Rel for NF-κB activation (Maity et al., 2012). Since vitamin C prevents CSE-induced NF-κB activation we investigated whether vitamin C pretreatment can affect the nuclear translocation of c-Rel. To verify this nuclear-cytosolic fractionation was performed with CSE-treated A549 cells that were either pretreated or left untreated with 100 μM vitamin C. As expected, western blot analysis showed that vitamin C prevents nuclear translocation of c-Rel (Fig. 2A, compare lanes 2 and 6). Congruent with the cell fractionation study, the immunofluorescence study showed reduced nuclear accumulation of c-Rel in vitamin C pretreated cells (Fig. 2B). Thus our results demonstrate that vitamin C prevents the nuclear translocation of c-Rel thereby leading to reduced NF-κB activity in CSE-treated A549 cells.

3.3. Effect of vitamin C on CSE-induced degradation of IκBα in A549 cells

CSE-induced NF-κB activation in A549 cells requires degradation of IκBα (Maity et al., 2012). To get an insight into vitamin C mediated prevention of NF-κB activation in CSE treated cells, we investigated the status of IκBα in CSE-treated A549 cells that were either pretreated or untreated with 100 μM vitamin C. The result showed that pretreatment with vitamin C resulted in marked reduction in CSE-induced degradation of IκBα (Fig. 3). Taken together, these observations lead to the conclusion that vitamin C pretreatment leads to decreased degradation of IκBα, thereby
blocking the nuclear entry of c-Rel ultimately leading to reduced NF-κB activation in CSE treated A549 cells.

3.4. Effect of pretreatment of cells with vitamin C on CSE-induced intracellular level of ROS

The CS-induced oxidative stress is known to be the major causative agent for the inflammatory effect of cigarette smoke. Vitamin C being an antioxidant is most likely to cause a reduction in the level of cellular ROS and thereby preventing NF-κB activation. To verify this we have examined the level of intracellular ROS in CSE-treated A549 cells that were either pretreated or untreated with vitamin C. To determine intracellular ROS, differentially treated A549 cells were stained with the redox sensitive dye DCFH-DA. DCFH-DA is a non-polar compound that readily diffuses into the cells, where it is hydrolyzed to the non-fluorescent polar derivative DCFH and is thereby trapped within the cells. In the presence of ROS, DCFH is oxidized to the highly fluorescent DCF. The presence of oxidized DCF was analyzed by FACS analysis. Consistent with our expectation, the result showed that the treatment of cells with vitamin C resulted in a marked reduction of CSE-induced level of intracellular ROS (Fig. 4). Therefore, the observed impairment of CS-induced activation of NF-κB by vitamin C can be attributed partly if not exclusively to its ability to neutralize cellular ROS.

3.5. Effect of dietary supplement of vitamin C on CS-induced nuclear translocation of c-Rel in guinea pig lung

To validate the in vitro results we performed similar experiments in in vivo animal model using guinea pig. For this purpose, guinea pigs were exposed to CS and fed with diet supplemented with or without vitamin C. Thereafter, we looked at the sub-cellular distribution of c-Rel in lung sections of these animals. Consistent with the in vitro cell culture-based results, immunohistochemical analysis showed marked reduction in nuclear translocation of c-Rel in lung sections obtained from guinea pigs fed with vitamin C-supplemented diet compared to sections obtained from guinea

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**Fig. 4.** Effect of vitamin C on intracellular ROS level in CSE treated A549 cells. Intracellular ROS of control and CSE-treated cells that were either pretreated or not treated with vitamin C was measured by staining with ROS sensitive dye DCFH-DA. The fluorescence resulted from the oxidation of DCFH-DA was analyzed with a flow cytometer (BD Pharmingen, California) using approximately 10,000 cells. The mean fluorescence intensity (MFI) of DCF positive cells was plotted. Result represents the mean ± SD of three independent experiments. Mean value with different letters indicate significant difference among different treatments, whereas mean values with same letter have no significant difference. *P* < 0.01 was considered significant.

**Fig. 5.** Effect of vitamin C on nuclear translocation of c-Rel in guinea pig lung cells. Sections were made from lungs of CS-exposed guinea pigs that were either provided with vitamin C supplemented diet or not. Localization of c-Rel was carried out by immunohistochemical analysis using anti-c-Rel-antibody and FITC-tagged secondary antibody. Green fluorescence at the top panel indicates the sub-cellular distribution pattern of c-Rel. Panel in the middle shows the DAPI-stained nuclei and the bottom panel represents the merged image of the top and its respective middle panel.
pigs exposed to CS only (Fig. 5). This result clearly lends support to our in vitro study.

4. Discussion

A common feature in the pathogenesis of various smoking related diseases is inflammation. Given that NF-κB plays a vital role in inflammation, it can be an effective therapeutic target for inflammatory diseases (Karín et al., 2004; Rahman and Fazal, 2011; Tak and Firestein, 2001). Toward this we investigated the role of vitamin C in preventing CS-induced NF-κB activation in alveolar epithelial cells. Although inactivation of NF-κB can be achieved by different NF-κB inhibitors, the non-specific toxicity of these inhibitors is a major concern (Adams, 2001; Hideshima et al., 2002; Majumdar et al., 2002). On the other hand, vitamin C is nontoxic and pharmacologically safe in dietary consumption. Our results demonstrate that pretreatment of alveolar epithelial A549 cells with vitamin C abrogated CSE-induced IκBα degradation thereby affecting the nuclear translocation of c-Rel and subsequent NF-κB activation. Besides, the in vitro observations are also corroborated with in vivo animal data from CS-exposed guinea pig that were either treated or not treated with vitamin C. Like humans, guinea pigs cannot synthesize vitamin C and this inability makes it a more appropriate animal model compared to mouse for such studies. The capability of vitamin C to prevent CS-induced NF-κB activation even in the in vivo guinea pig model bolster the therapeutic potential of vitamin C against CS-induced NF-κB activation and increases the importance of this antioxidant in the physiological system.

Vitamin C has important role in metabolic processes, particularly it is important in blood and the fluids present in the extracellular space, where antioxidant enzymes are absent or present only in small quantities. Therefore, dietary supplementation of vitamin C is required for animals like human and guinea pigs that are incapable of synthesizing it. Our results in guinea pig model reveals that a dose of 1 mg/day of vitamin C is not sufficient to combat the CS-induced NF-κB activation, rather a higher dose of 5 mg/day is required for this purpose. Based on this result in guinea pig, it can be suggested that for humans a regular intake of around 15 mg of vitamin C/kg body weight/day may provide protection against CS-induced NF-κB activation. Our result shows that vitamin C pretreatment helps to reduce the level of ROS generated by CSE, thereby limiting the level of oxidative stress triggered by CSE. However it is not clear whether this prevention of NF-κB activation by vitamin C is exclusively due to its antioxidant property or it has the ability to modulate signaling pathways independent of its antioxidant property.

Quitting smoking is the best way of protecting oneself from the threat of CS-induced inflammatory diseases. However different approaches for cessation of smoking have limited success due to the highly addictive nature of the habit. In addition, therapeutic agents, such as nicotine patches, nicotine gum, bupropion and varenicline, have also been introduced to reduce the severity of withdrawal symptoms and cravings. However, these too are not free from side effects (Altintas et al., 2013; Einarson and Einarson, 1997; Gemenetzidis et al., 2009; Morales-Suárez-Varela et al., 2006). To this end, vitamin C may provide an alternative option to prevent the menace of CS-induced cellular damages and subsequent life threatening diseases.

5. Conclusion

The current study demonstrates that vitamin C, by impairing CS-induced degradation of IκBα and subsequent nuclear translocation of c-Rel, prevents CS(E)-induced NF-κB activation in alveolar epithelial cells. Since NF-κB plays vital role in inflammation and alveolar epithelial cells are major contributor toward developing pulmonary pathogenesis, vitamin C could potentially be an effective preventive agent against CS-induced lung inflammatory diseases for the better management of public health.

Competing interest

The authors declare no conflict of interest.

Acknowledgements

This work is funded by University Grant Commission, Government of India [Sanction number: 32-591/2006(SR)]. BD is supported with a fellowship funded by University of Calcutta.

References

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 I-κBε and not I-κBα. This degradation of I-κBε depends on IKKβ activity, which preferentially targets I-κBα. 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 I-κBα have a central role, the predominantly active signaling cascade in CS-induced NF-κB activation in the lung epithelial cells comprises of IKKβ-I-κBε-c-Rel/p50. Thus, this study uncovers a new axis of NF-κB activation wherein I-κBε and c-Rel have the central role.

**ORIGINAL ARTICLE**

**IKKβ–I-κBε–c-Rel/p50: a new axis of NF-κB activation in lung epithelial cells**

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The modified I-κB 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 I-κBε degradation. Consistent with this, Rajendrasozhan et al. showed the degradation of I-κBα 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 I-κBα 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 I-κBα degradation by IKKγ. Thus, the present study provides a new axis of NF-κB activation comprising IKKβ-I-κBε-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...