Toxicology Letters xxx (2013) xxx–xxx

Contents lists available at SciVerse ScienceDirect

Toxicology Letters

journal homepage: www.elsevier.com/locate/toxlet

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 I-κBε.
- 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

Article history:
Received 14 January 2013
Received in revised form 9 April 2013
Accepted 15 April 2013
Available online xxx

Keywords:
Cigarette smoke
NF-κB
Vitamin C
Oxidative stress

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 I-κBε 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 I-κBε 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, I-κBε 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.

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 I-κB (Hayden and Ghosh, 2004). When cells are activated by various extracellular signals, like tumor necrosis factor alpha (TNF-α), interleukin-1 α, lipopolysaccharide (LPS), I-κB 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 I-κBα 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

Abbreviations: CSE, cigarette smoke extract; CS, cigarette smoke; NF-κB, nuclear factor kappa-B; ROS, reactive oxygen species; EMSA, electrophoretic mobility shift assay; FACS, fluorescence activated cell sorter; DCFH-DA, 2′,7′-dichlorofluorescin diacetate; DCF, 2′,7′-dichlorofluorescin; DAPI, 4′,6-diamidino-2-phenylindole; AM, alveolar macrophase; PMN, polymorphonuclear cells.

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0378-4274/$ – see front matter © 2013 Published by Elsevier Ireland Ltd.
http://dx.doi.org/10.1016/j.toxlet.2013.04.009

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 N F-KB promoter (Promega Inc., E894A) using PolyFect reagent (Qiagen, Hilden, Germany) according to manufacturer's instruction.

2.2. Preparation of cigarette smoke extract

Cigarette smoke extract (CSE) were prepared from filter tipped 69 mm cigarette as described previously (Maity et al., 2009).

2.3. Electrophoretic mobility shift assay (EMSA)

EMSAs were performed using ^{32}P-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-diamidino-2-phenylindole (DAPI). 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 (DGF), by flow cytometer (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 Minitab1 6.

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 for 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.

Please cite this article in press as: Das, B., et al., Vitamin C forestalls cigarette smoke induced NF-κB activation in alveolar epithelial cells. Toxicol. Lett. (2013), http://dx.doi.org/10.1016/j.toxlet.2013.04.009