

# Inhibition of Cigarette Smoke Induced-inflammation and Oxidative Damage by Caffeic Acid Phenethyl Ester in A549 Cells

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## Abstract

**Introduction:** The main objective of this study was to evaluate the effect of antioxidative and antiapoptotic effects and underlying molecular mechanisms of caffeic acid phenethyl ester (CAPE) on human lung epithelial cells exposed to cigarette smoke (CS). **Materials and Methods:** Human alveolar epithelial A549 cells were exposed to CS and treated with various concentrations of CAPE for 24 h, and their effective concentrations were identified by cell viability assay (MTT). Antioxidant and anti-inflammatory effect of CAPE on nuclear erythroid related factor-2 (Nrf2) and nuclear factor- $\kappa$ B (NF- $\kappa$ B) protein levels were analyzed by Western blotting. Furthermore, caspase 8 gene expression level was analyzed by reverse transcription-quantitative polymerase chain reaction. **Results:** Low concentration CAPE pretreatment rescued 52% of CS-exposed A549 cells from death. CS upregulated gene expression level of caspase 8 by 4.28 fold. However, 2.5  $\mu$ M CAPE pretreatment increased caspase 8 level by 52%. CS exposure also elevated NF- $\kappa$ B (p65) protein level by 70%, however, CAPE pretreatment significantly reversed this activation. While CS exposure decreased Nrf2 protein levels by 48% as compared with the control group, CAPE pretreatment increased Nrf2 protein level two folds approximately according to CS group. **Discussion:** CAPE markedly decreased inflammatory transcription factor NF- $\kappa$ B and increased antioxidant response element Nrf2 protein expression levels in CS-exposed human alveolar cells. According to the data obtained from this study, CAPE could be used as a strategic alternative to support treatment of inflammatory and oxidative stress-induced lung diseases.

**Key words:** Apoptosis, caffeic acid phenethyl ester, cigarette smoke, oxidative stress

## INTRODUCTION

Cigarette smoke (CS) is quite harmful mixture which contains so many harmful chemicals together in its mixture including nicotine, tar, carbon monoxide, and heavy metals.<sup>[1]</sup> Therefore, CS is one of the main factors that led to the development of lung diseases including chronic obstructive pulmonary disease (COPD) and lung cancer.<sup>[2-4]</sup>

Recently, many studies have revealed that CS is the main reason of COPD, and this lung disease is the fifth leading cause of overall mortality in global.<sup>[5-7]</sup> The data obtained in recent studies have revealed that stable oxidant as peroxy nitrite radicals in CS cause oxidative stress, and apoptosis appears in affected cell.<sup>[8]</sup> Apoptosis called programmed cell death is a defense system that enables the removal of unwanted damaged and diseased cells.<sup>[9]</sup> P53 is a tumor suppressor

protein that plays a central role in cell growth, apoptosis, and cell cycle regulation.<sup>[10]</sup>

COPD-related airways and alveolar epithelial cells are the most important source of cytokines and proteases. During CS exposure, epithelial cells produce inflammatory mediators such as interleukin (IL)-8 and tumor necrosis factor (TNF)- $\alpha$ .<sup>[11]</sup> Therefore, airway epithelial cells play a significant role in the body defense system. Moreover, alveolar epithelial cells secrete cationic peptides such as defense in which acts as antimicrobial and tissue repair.<sup>[12]</sup>

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**Received:** 04-10-2016

**Revised:** 18-10-2016

**Accepted:** 27-10-2016

Nuclear erythroid related factor-2 (Nrf2) is a cytosolic protein that stimulates gene expression of antioxidant and detoxification in cells against oxidants such as CS.<sup>[13]</sup> It is found predominantly in the epithelium and alveolar macrophages (AMs) during pathogenesis of COPD. In a study, Itoh *et al.*<sup>[14]</sup> have reported that CS causes intense cell death on Nrf2<sup>-/-</sup> cells. Therefore, Nrf2 has a vital role in protecting the alveolus against oxidants.

Propolis is a honeybee product with a broad spectrum of biological properties such as anti-inflammation, antimicrobial, antioxidant, and antitumor activities in humans.<sup>[15]</sup> The characteristic constituents of propolis are flavonoids, esters of aliphatic and aromatic acids, alcohols, ketones, aldehydes, terpenes, chalcones, amino acids and sugars.<sup>[16-19]</sup> Caffeic acid phenethyl ester (CAPE) is the main active ingredients of propolis and it has a strong anti-inflammatory effect. This anti-inflammatory activity of CAPE occurs by then inhibition of inflammatory cytokines (such as TNF- $\alpha$  and IL-1) and by blocking NF- $\kappa$ B activation.<sup>[20]</sup>

In this study, antioxidative and anti-inflammatory properties and its underlying mechanisms of CAPE have been investigated in human lung alveolar epithelial cells against CS.

## MATERIALS AND METHODS

Purified CAPE ( $\geq 99\%$ ) was purchased from Sigma Chemical Co. (Germany) and stock solution was dissolved in ethyl alcohol. Quantitative MTT test was used to determine cell viability. All experiments were performed with cells at 70-80% confluence in tissue culture flasks (Sigma Chemical Co., USA).

### Cell culture and treatment

The human alveolar cells (A549) cells were supplied by Dr. J. Mazella (CNRS, France) cultured according to the instructions in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum and 100 IU/ml penicillin, and 100  $\mu$ g/mL streptomycin.<sup>[21]</sup> To grow A549 cells,  $1 \times 10^6$  ml<sup>-1</sup> cell were maintained at 37°C in a sterile incubator containing 5% CO<sub>2</sub>, 95% air and retired by passage 20 (Heraeus, Germany).

CS extract preparation and cell treatments were performed according to Barlas and Erdogan methods.<sup>[22]</sup> The study groups are follows: C, control (untreated); CS, tobacco cigarette smoke-exposed group; CAPE, caffeic acid phenethyl ester applied; +CAPE, CS exposed-CAPE treated cells.

### Cell viability assessment

For the dose-response, cells were treated with 0.5, 1, 2.5, 5, 10 and 20  $\mu$ M of CAPE for 24 h. In some experiments,

the medium also contained CS, in which case cells were pretreated for 1 h with CAPE. Then, the cells were exposed to CS-containing medium for 3 h. Following the exposure, the medium was changed with fresh complete medium containing CAPE for 24 h.

Cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide test (MTT) (Fluka, USA). Thereafter, supernatants were collected from wells and MTT reagent was added. After incubation (90 min at incubator), unreactive supernatants in wells were discarded, and 100  $\mu$ l of acidified isopropanol was added to the cultures. At the end of incubation, the absorbance value (A) of each well was determined using a spectrophotometer reader at 570 nm. Viability lost in A549 cells was determined by MTT test after stimulation with 10-100% CS. Consequently, 100% CS exposure for 3 h was used to stimulate the cells for this study.

### Real-time reverse transcription-polymerase chain reaction (RT-PCR) analyses

Total RNA was extracted using RNA isolation reactive (Trizol reagent-Sigma, USA). The RNA samples were reverse transcribed using a transcriptase kit (Thermo, Germany), which served as template for quantitative RT-PCR thermal cycler (Bio-Rad, South Korea). cDNA was used as a template for amplification using SYBR Green PCR amplification reagent (Sigma, USA) and gene-specific primers. Primer sequences for caspase 8 were: Reverse 5'-GCAAAAGCACGGGAGAAAG-3' and Forward 5'-GGATACAGCAGATGAAGCAG-3', for  $\beta$ -actin: Reverse 5'-CATCGTCACCAACTGGGACGAC-3' and Forward 5'-CTGTGGCCATCTCTTGCTCGAAG-3'.

### SDS-PAGE Western blot analysis

Cells were plated on 10 cm<sup>2</sup> dishes at a density of  $1 \times 10^6$  cells/ml in the presence or absence of agent and CS for 24 h. To prepare whole cell extracts, cells were washed with phosphate-buffered saline including zinc ion (1 mM) and suspended in lysis buffer (1.3M NaCl, 0.027 M KCl, 0.1 M Na<sub>2</sub> HPO<sub>4</sub> and 0.018 M KH<sub>2</sub>PO<sub>4</sub>; pH 7.4). Lysates were incubated for 30 min at 4°C and centrifuged at 12,000  $\times$ g for 20 min. The supernatants were collected as whole-cell extracts. The protein concentration was determined with bicinchoninic acid assay protein assay reagent (Thermo Sci., USA) using bovine serum albumin as a standard. Western blot analysis was performed with Pierce Fast Western Blot Kit included ECL substrate (Thermo Fischer, USA). Total cell lysates with equal protein content from control, CS, and CAPE-treated samples were resolved on 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels along with a prestained protein molecular weight standard (Bio-Rad, Germany). Proteins were then blotted onto polyvinylidene difluoride membranes and reacted with primary antibodies

(anti-nuclear factor- $\kappa\beta$ , anti-Nrf2, and anti- $\beta$ -actin (1/200). The secondary antibody was a peroxidase conjugated goat anti-rabbit IgG secondary antibody (1/4000). All antibodies were obtained from Thermo Sci. Coop., (USA). After antibody binding, the bands were visualized using a commercial kit in a ChemiDoc MP Imaging System (Bio RAD).

## Statistical analysis

The differences of the trials were obtained using one-way ANOVA followed by *post hoc* Duncan tests (SPSS 20.0 for Windows). Data obtained are shown here as the mean  $\pm$  standard error of mean. The band images obtained in Western blot analysis were determined using Image Lab 4.0.1 Software package program. The band intensities evaluated according the groups were presented by a graph.

## RESULTS

### Cell viability

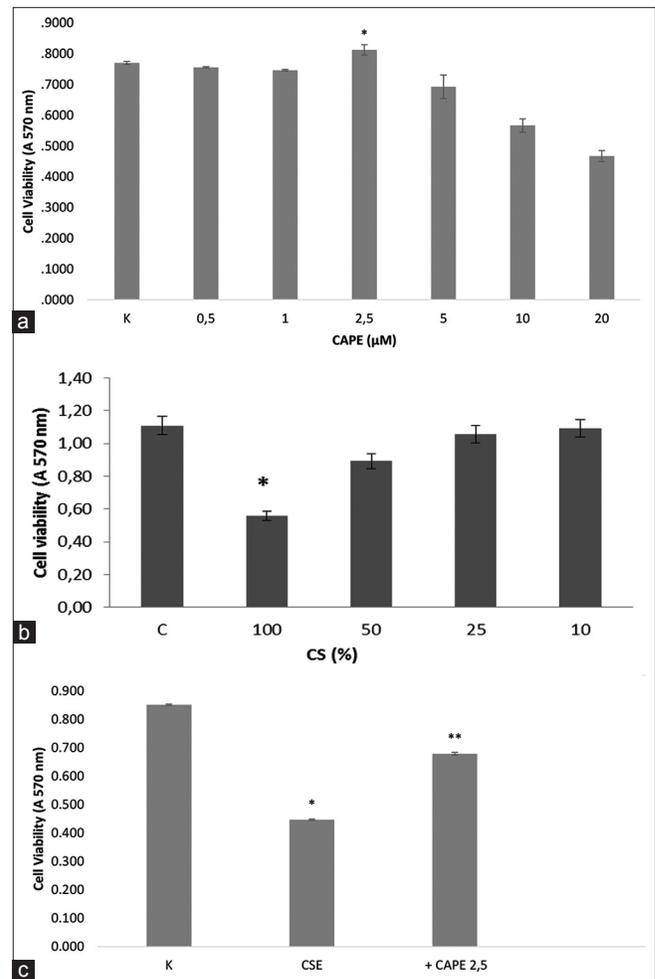
CAPE dose of more than 2.5  $\mu$ M had cytotoxic effects [Figure 1a]. Therefore, 2.5  $\mu$ M CAPE was used as a cell-protective concentration for further experiments. Concentration-response study demonstrated that at 100% CS exposure for 3 h reduced the cellular viability approximately 50% ( $0.56 \pm 0.01$ ) after 24 h incubation compared with control group ( $1.11 \pm 0.01$ ) ( $P \leq 0.05$ ) [Figure 1b]. On the other hand, CAPE pre-treatment recovered about 52% of cell loss ( $0.852 \pm 0.002$ ) ( $P \leq 0.05$ ) induced by CS exposure [Figure 1c].

### Expression levels of caspase 8

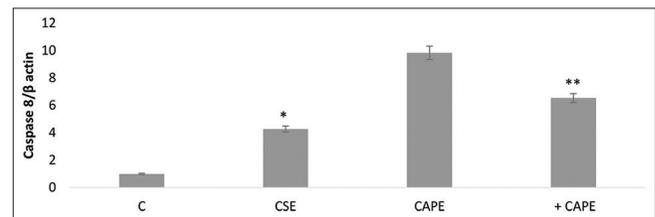
Another aim of the study was to investigate the efficacy of CAPE on apoptosis caused by CS exposure. According to data obtained, CS extract upregulated relative expression of caspase 8 by 4.28 fold, and 2.5  $\mu$ M CAPE pretreatment was found to increase caspase 8 induced apoptosis by 52% [Figure 2].

### Protein levels of Nrf2 and NF- $\kappa$ B

Protein analysis has demonstrated that CS exposure has altered both Nrf-2 and NF- $\kappa$ B protein levels in A549 cells [Figure 3a-c]. Since CS exposure elevated NF- $\kappa$ B (p65) protein level by 70%, CAPE pretreatment decreased this protein level by 57% compared to CS group. Conversely, CS exposure decreased the Nrf2 protein levels 48% as compared with the control group. However, CAPE pretreatment up-regulated Nrf2 protein level nearly by 200% compared to CS group.



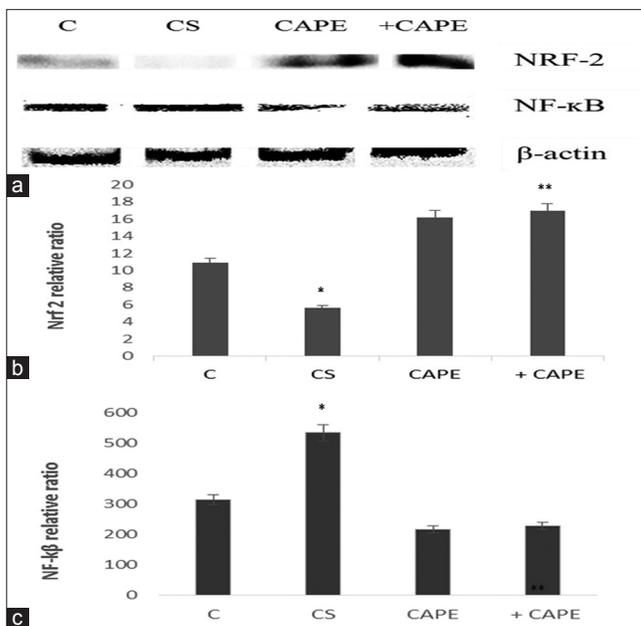
**Figure 1:** (a) Dose dependent effect of cigarette smoke (CS) on cells. (b) Dose dependent effect of caffeic acid phenethyl ester (CAPE) on cells. (c) Effects of CAPE on CS induced alveolar cells. The data are represented as mean  $\pm$  standard deviation of five experiments. \* $P \leq 0.05$  versus control group; \*\* $P \leq 0.05$  versus control group



**Figure 2:** Relative expression levels of caspase 8 gene.  $\beta$ -actin was used as housekeeping gene. Experiments shown are representative of three separate experiments with similar results. \* $P \leq 0.05$  versus control group; \*\* $P \leq 0.05$  versus cigarette smoke-treated group

## DISCUSSION

In this study, we analyzed molecular mechanisms underlying the anti-inflammatory effects of CAPE on human A549 lung epithelial cells exposed to CS. Its



**Figure 3:** Effects of caffeic acid phenethyl ester and cigarette smoke (CS) on protein levels of nuclear erythroid related factor-2 (Nrf-2) and nuclear factor- $\kappa$ B (NF- $\kappa$ B). (a) The band views of  $\beta$  actin, Nrf-2 and NF- $\kappa$ B protein expression. (b and c) Are relative ratios of Nrf-2 and NF- $\kappa$ B protein levels, respectively. \* $P \leq 0.05$  versus control group; \*\* $P \leq 0.05$  versus CS-treated group

already has been demonstrated that CAPE could protect the cells from various damages such as CS or oxidative stressors.<sup>[22-25]</sup> However, the underlying mechanisms have not been sufficiently demonstrated. Although low levels of CAPE protects lung alveolar cells from damages or inflammation, higher concentrations could be apoptotic or cytotoxic.<sup>[23,26]</sup>

Our data indicated that low-level CAPE pretreatment of CS-exposed A549 cells significantly rescued the cells from death by 52%. It was found in the present study that CS exposure markedly increased an inflammatory transcription factor NF- $\kappa$ B protein expression level. NF- $\kappa$ B activation plays a key role in the production of chemotactic cytokines, and CS is a potent activator of NF- $\kappa$ B. The previous studies demonstrated that persistent CS exposure induced airway inflammatory reactions, with elevated pro-inflammatory cytokine levels, which are generally regulated by pro-inflammatory gene transcription factors.<sup>[27,28]</sup> A study performed by Yang *et al.*<sup>[29]</sup> showed that CS-induced NF- $\kappa$ B levels in AMs. Moreover, Lixuan *et al.*<sup>[30]</sup> illustrated that CS exposure enhanced NF- $\kappa$ B subunit p65 transcription *in vivo* COPD inflammation model. Furthermore, Di Stefano *et al.*<sup>[31]</sup> reported NF- $\kappa$ B p65 subunits have significantly stimulated in patients with COPD. In our research, CAPE has significantly suppressed CS-induced inflammation dependent on NF- $\kappa$ B increase. It can be suggested that CAPE can protect the lung cells from CS stress by inhibiting NF- $\kappa$ B expression. CAPE has been stated to inhibit NF- $\kappa$ B activation and activities

of certain enzymes such as cyclooxygenase and xanthine oxidase.<sup>[32,33]</sup>

CS is known to cause oxidative stress in alveolar epithelial cells. Nrf2 is a transcription factor that plays a crucial role in the cellular protection against oxidative stress.<sup>[34]</sup> It was found in the present study that CS significantly decreased Nrf2 protein expression in the cell exposed to CS. However, CAPE pretreatment completely reserved CS-depleted Nrf2 expression. It is suggested CAPE treatment protects lung epithelial cells against CS-induced oxidative stress. It was reported by others that Nrf2 protein levels are increased in dose-dependent manner of CAPE.<sup>[35-37]</sup> These hypotheses are confirmed by our data showing increased protein on Nrf2 in response to CS-induced oxidative stress in A549 cells.

Another aim of the study was to investigate the efficacy of CAPE on apoptosis caused by CS exposure. Similarly to our data, in CS-induced apoptosis model of rats, it was demonstrated by Western blot analysis that CS significantly stimulated caspase 8 expressions in the lungs.<sup>[38]</sup> CS also increased expression of NF- $\kappa$ B p65 subunit and stimulated p50 subunit in the lung tissue. In another study, caspase 8 activity reported to increase after 20% CSE exposure in a time-dependent manner in CS-induced apoptosis in lung fibroblasts (MRC-5 cells).<sup>[39]</sup> Moreover, in an *in vivo* study, CAPE was shown to reduce Fas ligand (CD95) subunit protein levels and suppress caspase 8 expression in carbon tetrachloride-induced liver toxicity.<sup>[40]</sup> In addition, it was demonstrated that CAPE increased DNA strand breaks in dose and time dependent manner and stimulated Fas ligand induced-caspase 8 signaling pathway of apoptosis in human breast cancer cells.<sup>[41]</sup> It was found in our research that CAPE caused rises in expression level of caspase 8 but it did not lead to loss of cell viability. In addition, it can be concluded that increase in transcription of this gene may not be occurred in translational level.

## CONCLUSION

According to the data obtained in our study, CAPE was found to significantly suppress inflammation and oxidative stress induced by CS. CAPE increased expression level of caspase 8 but it did not cause cell loss. This stimulation could be tolerated via inhibition of NF- $\kappa$ B and stimulation of antioxidant Nrf2 by CAPE. Therefore, it can be concluded that CAPE can be used as a supportive agent for the treatment of tobacco smoke caused inflammation and oxidative stress related respiratory damage. However, there is a need for further research on the subject.

## ACKNOWLEDGMENT

The authors offer their thanks to Mustafa Kemal University (Hatay, Turkey) Scientific Research Projects Unit for the material support to carry out this study (Project ID: 388).

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**Source of Support:** Nil. **Conflict of Interest:** None declared.