

Caffeic acid phenethyl ester protects lung alveolar epithelial cells from cigarette smoke-induced damage

Fırat Barış BARLAS¹, Suat ERDOĞAN^{2*}

¹Department of Biochemistry, Graduate School of Natural and Applied Science, Ege University, İzmir, Turkey

²Department of Medical Biology, Faculty of Medicine, Trakya University, Balkan Campus, Edirne, Turkey

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Background/aim: To evaluate the influence of caffeic acid phenethyl ester (CAPE) on cigarette smoke (CS)-induced cell damage, oxidative stress, and inflammation in human alveolar epithelial cells.

Materials and methods: A549 alveolar epithelial cells were divided into control, CS exposure, CAPE, and CS+CAPE treatment groups. Undiluted CS-exposed medium (100%) and three dilutions (50%, 25%, and 10%) of CS-exposed media were applied to cultured A549 cells, which were analyzed after 3 h of incubation. Viability was measured by MTT assay, the gene expressions were evaluated by real-time PCR, and spectrophotometric techniques were used for biochemical assessments.

Results: While CS exposure markedly reduced cellular viability by 32% after 3 h of incubation, 2.5 µM CAPE treatments prevented CS-induced cell death by 40% in the cells. CS exposure triggered lipid peroxidation and depleted antioxidant capacity through inhibiting catalase activity and depleting glutathione levels. Moreover, CS increased nitric oxide production via upregulation of iNOS expression. CAPE treatment significantly restored antioxidant capacity and prevented lipid peroxidation. Cigarette smoke exposure induced inflammation by significantly upregulating TNF-α, IL-1β, and COX-2 mRNA expressions (3-, 2- and 25-fold, respectively). CAPE treatment of A549 cells significantly reversed the inflammation.

Conclusion: CAPE may potentially represent a new therapeutic option in the prevention of CS-induced lung damages.

Key words: Caffeic acid phenethyl ester, CAPE, inflammation, oxidative stress, cigarette smoke, A549

1. Introduction

Epithelial cells lining the respiratory tract are vulnerable to excessive proinflammatory response due to direct exposure to environmental factors such as cigarette smoke (CS). CS contains more than 4000 harmful chemical compounds per puff, of which 200 are highly toxic (1). Many of these chemicals induce an oxidative burden and lead to inflammatory reactions that could cause cellular damage in the lungs (2,3). Inhalation of CS resulted in lung damage by generation of oxidative stress, production of cytokines, chemokines, and other factors (4). Therefore, CS is significant as an etiologic factor in the progression of chronic degenerative lung disorders, such as chronic obstructive pulmonary disease (COPD) (2,5).

Caffeic acid phenethyl ester (CAPE) is derived from different plants and is a bioactive constituent found in bee propolis that may have pharmacological applications. Recently, a wide range of pharmacological properties were demonstrated for this substance, including

antiinflammatory, antioxidant, antiproliferative, and immunomodulatory activities (6–8). The prominent protective property of CAPE makes it a potential therapeutic compound against damage to the kidneys, brain, lungs, and other tissues or organs. CAPE is a specific and potent inhibitor of nuclear factor-κB (NF-κB) signaling (9) and a potent inhibitor of leukotriene biosynthesis; therefore, CAPE may have a potential therapeutic effect on inflammatory diseases (10). However, the effects of CAPE on CS-induced inflammation and oxidative events in the alveolar epithelium have not been extensively studied yet.

In this study, we explored whether CAPE can prevent CS-triggered proinflammatory cytokine release and oxidative stress in human-derived alveolar epithelial cell lines.

2. Materials and methods

In the present study, A549 cells were used to observe lung alveolar epithelial cell responses. CS was used to mimic

* Correspondence: suaterdogan@trakya.edu.tr

inhaled tobacco smoke and the effects of CAPE on CS-induced oxidative stress and inflammatory reactions on A549 cells were analyzed. Effects of CAPE and CS on cell proliferation and viability were assessed by MTT assay and trypan blue exclusion test. All reagents were of analytical grade and obtained from Sigma Chemical Co. (USA) unless otherwise stated.

2.1. Cell culture

The human adenocarcinoma epithelial cells (A549) were maintained in Dulbecco's modified Eagle medium (DMEM) complemented with 10% FBS, 100 IU/mL penicillin, and 100 µg/mL streptomycin. The cells were incubated at 37 °C in a humidified atmosphere containing 5% CO₂ (Heraeus, HeraCell 150, Germany). Exponentially growing A549 cells were used for all assays.

A549 cells were kindly provided by Dr J Mazella (CNRS, France) and cultured overnight at a density of 10×10^4 /well or 4×10^4 /well in sterile 12-well or 24-well plates, respectively (Costar, Corning Inc., USA). To quantify the concentration of exogenously applied CS in the culture medium over time, the medium was removed, and a fresh medium containing 10%–100% CS was added. The cells were incubated for 3 h.

CS was prepared according to the reported method with minor modifications (11). Commercial unfiltered cigarettes (Marlboro, Philips Morris, Inc., USA; 12 mg of tar and 0.9 mg of nicotine per cigarette) were smoked and drawn continuously by the apparatus. By application of a vacuum, mainstream smoke was drawn through a vessel containing 20 mL of DMEM that was prewarmed to 37 °C. Each cigarette was smoked for 5 min, and one cigarette was used per 20 mL of DMEM to generate a CS solution. Its pH was adjusted to 7.4 and it was filtered through a 0.22-µm pore filter and used within 30 min after preparation. The cells were exposed to CS-containing medium for 3 h. Final concentration of this solution is expressed as percent values, which was calculated with the following equation: (mL CS solution/total mL) \times 100.

2.2. MTT and trypan blue exclusion assays

To test the cytoprotective effects, the cells were pretreated with CAPE 1 h before CS exposure, and then cells were stressed with CS for 3 h. Cell viability was measured by both 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Fluka, USA) assay and trypan blue dye exclusion. In some cases, the remaining adherent cells were photographed using a CKX41 model Olympus phase contrast microscope (Japan). CAPE concentrations (2.5 µM CAPE and 100% CS) used for the treatments were chosen to obtain maximal effects. Concentrations of CAPE and CS were determined by dose-response studies.

2.3. Homogenate preparation

After CS exposure, cells were homogenized in ice-cold homogenization buffer (10 mM Tris, 1 mM EDTA, 25

mM MgCl₂, 0.1 mM dithiothreitol, 0.25 M sucrose, pH 7.4) containing a complete protease inhibitor cocktail. After centrifugation at 4 °C and 15,000 rpm for 10 min, the soluble part was reserved. Protein concentrations were analyzed using bovine serum albumin as a standard.

2.4. Determination of nitrite levels

The concentration of NO in culture media was determined as nitrite, using the Griess reaction as described previously (12).

2.5. Determination of glutathione levels

Nonenzymatic antioxidant glutathione (GSH) content of A549 cell homogenates was determined according to the method of Sedlak and Lindsay (13).

2.6. Assessment of lipid peroxidation

The lipid peroxidation level was evaluated by measuring the concentrations of cellular homogenate malondialdehyde (MDA) (14).

2.7. Measurement of catalase activity

Catalase activity in the cell homogenate was determined according to the method of Luck (15), and the activity was presented as unit k/mg protein, where k is the rate constant of a first-order reaction.

2.8. RNA isolation and real-time QRT-PCR analyses

A549 cells in culture are known to express proinflammatory cytokines such as TNF-α and IL-1β in response to stimuli that trigger inflammatory responses. To examine whether CAPE effectively inhibits oxidative stress-induced inflammation in our experimental conditions, A549 cells were exposed to 100% CS for 3 h. Total RNA from A549 cells was isolated using TRIzol reagent. RNA was reverse-transcribed using a transcriptase kit (Fermentas, Germany). Real-time PCR was carried out in a QPCR system (Stratagene Mx 3005P, Lenexa, USA). cDNA was used as a template for amplification using SYBR Green PCR amplification reagent and gene-specific primers. Primer sets used were from Thermo Electron Corporation (Germany): TNF-α forward: CAGAGGGAAGAGTTCCCCAG, reverse: CCTTGGTCTGGTAGGAGACG; IL-1β forward: 5'-GCAAGCGCTTCAGGCAGGCGGCG-3', reverse: 5'-GGTCATTCTCCTGGAAGGTCTGTGGGC-3'; iNOS forward: GGCCTCGCTCTGGAAAGAA, reverse: TCCATGCAGACAACCTT; COX-2 forward: 5'-TGAAACCCACTCCAAACACACAG-3', reverse: 5'-TCATCAGGCACAGGAGGAAG-3'. The amount of RNA was normalized to β-actin amplification in a separate reaction forward: CATCGTCACCAACTGGGACGAC, reverse: CGTGGCCATCTCTTGCTCGAAG.

2.9. Statistical analysis

The results are presented as the mean \pm standard error of at least three experiments. The level of significance was set at $P < 0.05$. Data obtained from various groups were statistically evaluated using one-way ANOVA followed by post hoc Duncan tests (SPSS 9.0 for Windows).

3. Results

3.1. Effect of CAPE treatment (incubation) on CS-exposed cellular viability

Concentration response course demonstrated that a CAPE dose of more than 2.5 μM had cytotoxic effects (Figure 1A). For this reason, 2.5 μM CAPE was used as a cell-protective concentration for further experiments. Viability of A549 cells decreased steadily in a concentration-dependent manner over the range of 50% to 100% following 3 h of CS exposure (Figures 1B and 1C). The data showed that 100% CS killed 32% of cells at the end of 3 h incubation, and CAPE prevented 40% of the cell death caused by CS (Figure 1C). Cells were evaluated with a microscope for morphologic changes. It was noted that many cells detached from the flask following CS exposure (Figure 2). Conversely, the majority of cells recovered after CAPE treatment (Figure 2, CS+CAPE).

There was a significant induction in nitrite levels in CS-exposed cells when compared to the control (Figure 3). CS similarly increased iNOS mRNA expression by 2.2-fold (Figure 4). A significant reduction in both NO production

and iNOS mRNA expression was observed in cells treated with 2.5 μM CAPE (Figures 3 and 4).

To determine the effect of CS exposure on oxidant and antioxidant homeostasis, we also measured reduced GSH levels in cells with or without CS. There was a significant (27%) reduction in GSH levels in CS-exposed cells when compared to the control. On the other hand, CAPE completely recovered the loss of GSH caused by exposure (Figure 3).

Lipid peroxidation levels (MDA) were measured in A549 cells as a marker of oxidative stress (Figure 3). MDA levels were compared in the CS+CAPE-treated group and the control group (nonexposed group). Significantly increased MDA levels were observed in the CS-exposed group ($P < 0.001$) in comparison to the control group. However, CAPE significantly ($P < 0.001$) reduced lipid peroxidation induced by CS.

Catalase is an important antioxidant enzyme actively involved in quenching harmful radicals contained in CS. The cells exposed to CS had significantly low levels of catalase activity (Figure 3), while 2.5 μM CAPE treatment for 3 h increased catalase activity by 50%.

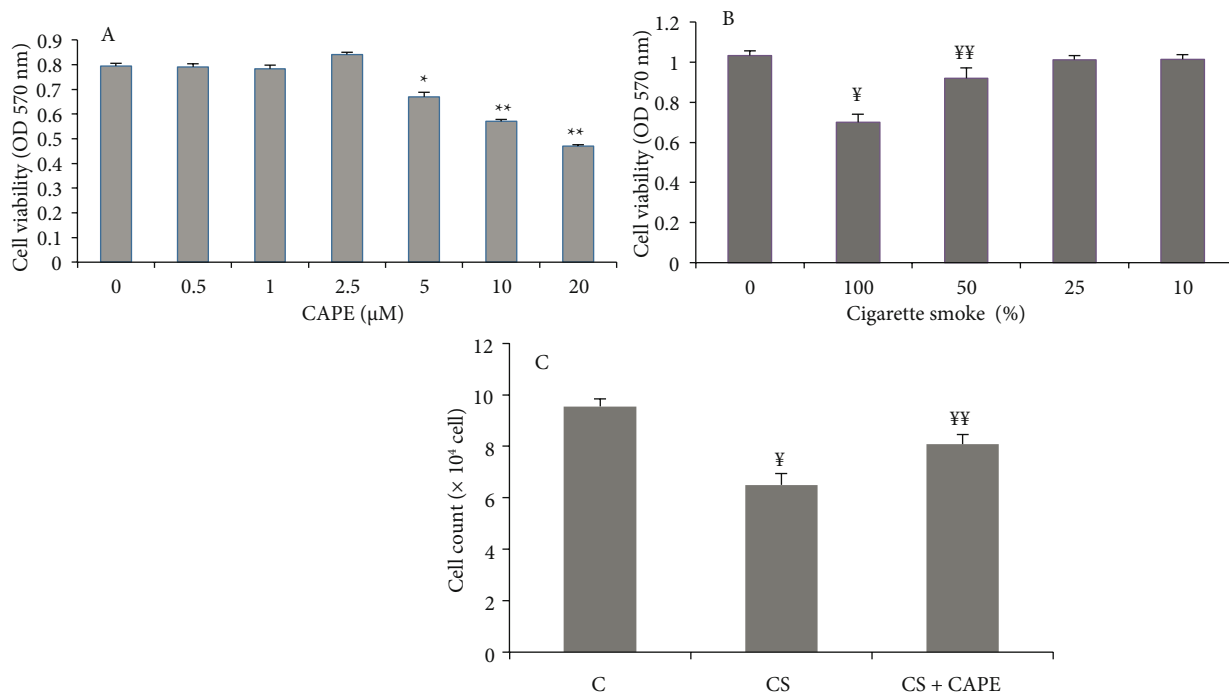


Figure 1. Effects of CAPE and cigarette smoke (CS) on A549 cell viability. Cells were seeded 24 h before the assay in 24-well plates at a density of 4×10^4 cells per well in complete medium. One hour before CS exposure, cells were pretreated with CAPE at indicated concentrations for 3 h, and cell viability was determined by MTT assay (A). The cells were exposed to 10%, 25%, 50%, or 100% CS-containing medium, respectively, for 3 h (B), and then the viability was determined using MTT test. The effect of CAPE (2.5 μM) on 100% CS exposure was estimated by trypan blue exclusion test after 3 h incubation (C). Data represent the means of three independent experiments and were compared using one-way ANOVA test. *: $P < 0.05$, **: $P < 0.01$ vs. control (0); ¥ : $P < 0.01$, ¥¥ : $P < 0.05$ vs. control (0). C: Control, CS: cigarette smoke, CS+CAPE: cigarette smoke + caffeic acid phenethyl ester.

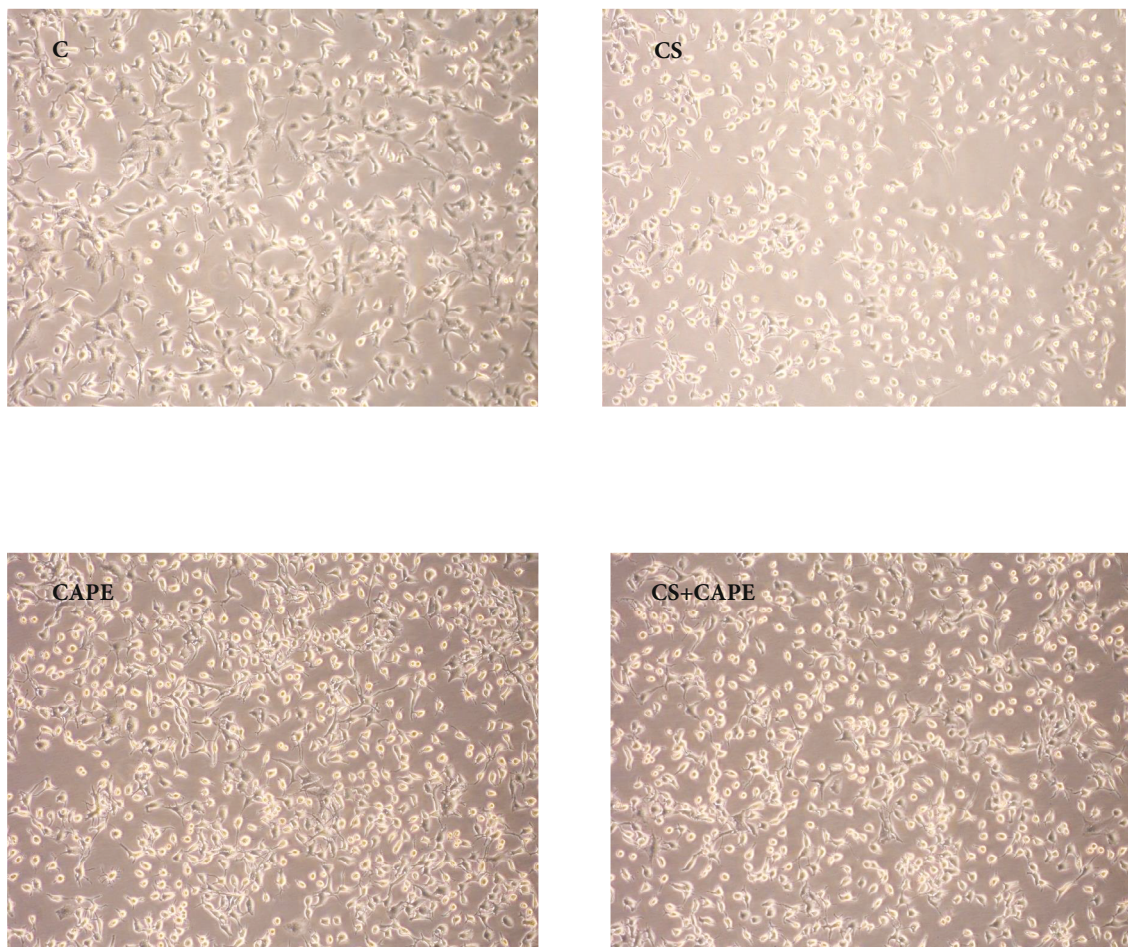


Figure 2. CS resulted in morphologic changes and viability loss of A549 cells. After treatment with 100% CS or 2.5 μ M CAPE for 3 h, cells were observed under inverted microscope. Closely adherent cells were seen in the control group (C). The cells showed lowered numbers after exposure to CS (SC). It was noted that some cells detached from the flask (CS). However, a majority of the cells were recovered after CAPE treatment (CS+CAPE). Phase contrast microscopy, original magnification, 40 \times . C: Control, CS: cigarette smoke, CS+CAPE: cigarette smoke + caffeic acid phenethyl ester.

3.2. Effect of CAPE on COX-2 mRNA expressions in A549 cells exposed to CS

As COX-2-dependent prostanoids play a central role in inflammation, the role of COX-2 mRNA expression was investigated. CS treatment upregulated COX-2 mRNA by 25-fold in A549 cells (Figure 4).

CS exposure significantly induced TNF- α (3 fold) and IL-1 β (2 fold) mRNA expression compared to the control cells. Additionally, CS-induced cytokine expression was reversed by 2.5 μ M CAPE treatments ($P < 0.001$) (Figure 4).

4. Discussion

The aim of this study was to evaluate the antiinflammatory and antioxidant properties of CAPE treatment on CS-induced airway epithelial injury. We demonstrated that CAPE could protect alveolar epithelial cells against

oxidative and inflammatory stress-induced damage of CS for the first time in the literature (16).

We hypothesized that CS caused cytotoxic effects because of the presence of very reactive compounds present in CS (2,17,18) or because of the production of cytoplasmic reactive oxygen/nitrogen species (17). Undeniably, studies have demonstrated that the cytotoxic capacity of CS is due to a number of chemical components (17–19). These results suggest that CAPE is an effective exogenous cell protective mediator against oxidative injury.

Disturbance of prooxidant and antioxidant balance has been reported to result in lung tissue injury in tobacco smokers (2,7,19). Excessively produced NO derived from inducible NOS (iNOS) has been demonstrated to play a key role in development of inflammation and oxidative stress in the lungs (20). In our study, induction of iNOS

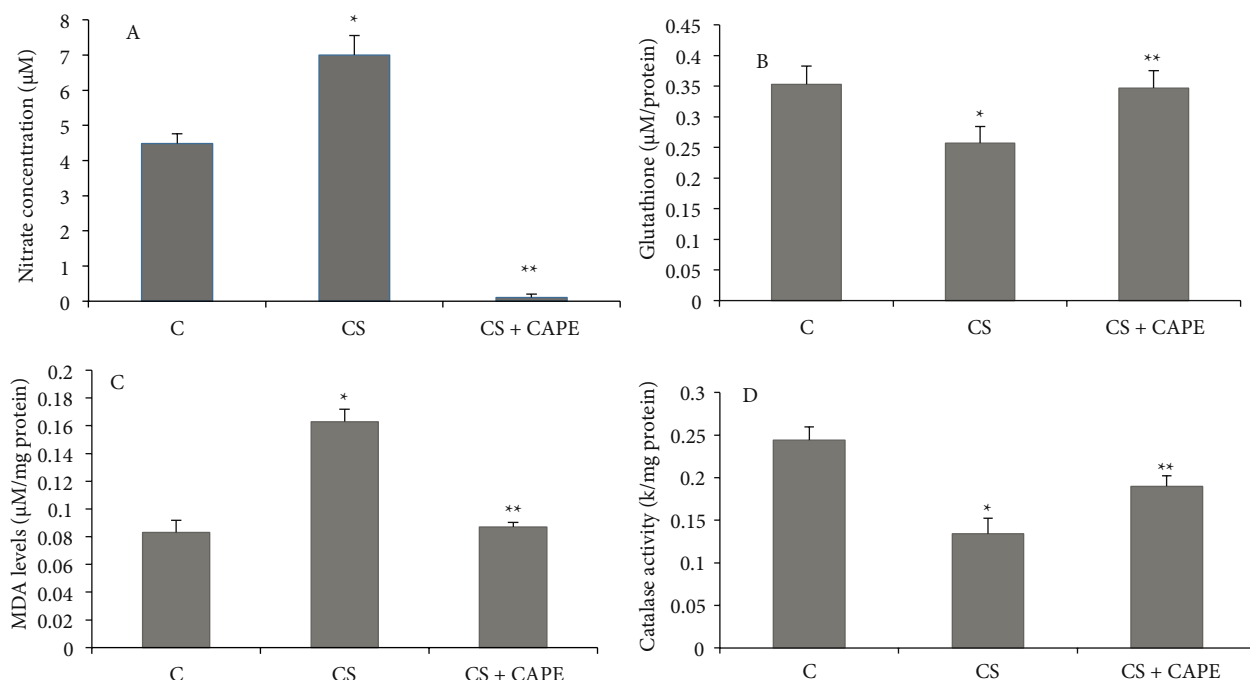


Figure 3. CAPE pretreatment enhanced antioxidant system and prevented lipid peroxidation induced by CS. Nitric oxide (NO) concentration was measured in culture medium as nitrate 3 h after incubation with or without 100% CS and 2.5 µM CAPE (A). Glutathione (B) and lipid peroxidation (MDA) (C) levels and catalase activity (D) were measured using cultured cell homogenates. CAPE was administrated 1 h before CS-exposed medium addition. Data represent one of three independent experiments with similar results. *: P < 0.01 vs. C (control); **P < 0.01 vs. CS (cigarette smoke).

expression by CS exposure and subsequent production of NO was significantly inhibited by CAPE pretreatment. The decrease in iNOS activity by CAPE administration may contribute to the high risk of lung disease in cigarette smokers.

In addition, GSH is a crucial intracellular antioxidant and provides an important defense in the lungs. Cigarette smoking has been proven to induce oxidative stress and reduce GSH homeostasis (2,7). We have shown that exposure of A549 cells to CS resulted in significant depletion of GSH concentration. As suggested previously by Rahman et al., CS-mediated reduction of GSH content might be due to the formation of GSH conjugates with the electrophilic β-carbonyl compounds in CS (21). Furthermore, our observation is consistent with earlier findings of CS's ability to induce oxidative stress by generating reactive oxygen species and decreasing cellular GSH levels (21). We have shown that CAPE dramatically blocked GSH depletion in cells exposed to CS. These effects could be dramatically inhibited by CAPE. This suggests that CAPE can provide a promising approach for the treatment or protection of CS-related pulmonary diseases (7,19).

It has been reported that CS increased the degree of lipid peroxidation via oxidative stress in human lung epithelial

cells and fibroblasts (22,23). We have demonstrated here that CS exposure caused lipid peroxidation in lung alveolar epithelial cells as measured by determining MDA concentrations. Administration of CAPE to CS-exposed cells significantly prevented lipid peroxidation, indicating that CAPE may protect the lungs from CS stress. This result is in agreement with a previous study highlighting the effect of CS exposure on the prevention of histopathological changes in rabbit airway epithelium (24).

In the present study, CS exposure significantly decreased activity of catalase, an antioxidant enzyme associated with H₂O₂ hydrolyses. Mak et al. (25) showed that erythrocyte catalase activity increased in patients with COPD, which is different from our findings. However, Tavilani et al. (26) and some others demonstrated that antioxidant enzyme might be downregulated during severe or chronic oxidant exposure (27). CAPE incubation significantly restored catalase activity in this study. The result suggests that CAPE would prevent CS-induced oxidative damage in lungs by supporting antioxidant defense system.

Since there is an active interplay between oxidative stress and inflammation, we also evaluated the antiinflammatory activity of CAPE in CS-exposed A549 cells. Previous studies demonstrated that persistent CS exposure induced airway

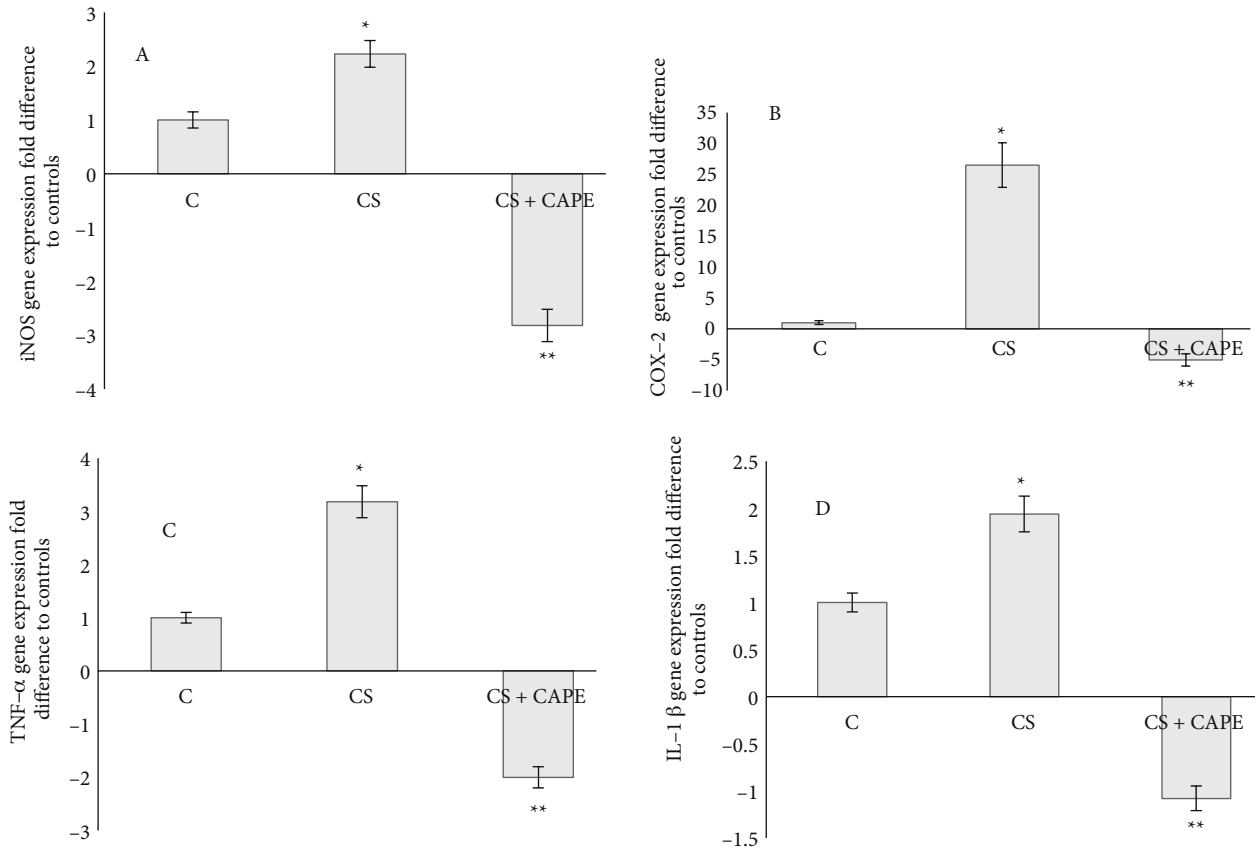


Figure 4. Real time RT-PCR analyses of iNOS, COX-2, TNF- α , and IL-1 β expressions in A549 cells. The mRNA levels of iNOS (A), COX-2 (B), TNF- α (C), and IL-1 β (D) were determined using QRT-PCR in order to evaluate possible protective effects of 2.5 μ M CAPE against 100% CS exposure. Results were expressed as a relative increase of mRNA expression compared with control cells. Experiments shown are representative of three separate analyses with similar results. *: P < 0.001 vs. control (C), **: P < 0.001 vs. CS. C: Control, CS: cigarette smoke, CS+CAPE: cigarette smoke + caffeic acid phenethyl ester.

inflammatory reactions, with elevated proinflammatory cytokine levels, which are generally regulated by proinflammatory gene transcription factors (7,28). TNF- α and IL-1 β secretion was shown to be induced by a wide variety of agents including stress, CS, cytokines, and free radicals (29). In the present study, CAPE application suppressed TNF- α and IL-1 β mRNA expressions that had been induced by CS. The proinflammatory cytokine TNF- α is the key proinflammatory mediator that initiates a rapid increase in mitochondrial reactive oxygen species and triggers necrosis and apoptosis (30). During inflammatory stimulation, translocation of NF- κ B from the cytosol into the nuclei of cells induces the expression of a large number of genes such as TNF- α , IL-1 β , acute phase proteins, and enzymes such as iNOS (7). CS promotes the induction of COX-2 and contributes to the proinflammatory effects of PGE₂ in the airways of COPD patients (31). CAPE may exert antiinflammatory properties through the suppression of COX-2 expression. These results propose that inflammation induced by CS could be suppressed

by CAPE through inhibition of the production of some inflammatory mediators.

Our data have demonstrated that CAPE has antioxidative and antiinflammatory properties associated with inhibition of inflammatory cytokine production, as well as iNOS and COX expressions in an alveolar cell culture model. Therefore, CAPE might play a protective role in degenerative lung diseases associated with cigarette smoking. It should be noted that this cell line may not accurately represent the in vivo situation, as the phenotype of immortalized cells often differs from that of the normal tissue. For instance, A549 cells are not as sensitive to cigarette smoke exposure as primary cultures (32). Thus, further in vivo studies should be performed to determine whether CAPE could provide a valuable therapeutic potential.

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