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Ghrelin Inhibits Interleukin-6 Production Induced by Cigarette Smoke Extract in the Bronchial Epithelial Cell Via NF-κB Pathway

Hao Wang,[1,2] Ting Yang,[1,2] Yongchun Shen,[1,2] Chun Wan,[1,2] Xiaoou Li,[1,2] Diandian Li,[1,2] Yang Liu,[3] Tao Wang,[1,2] Dan Xu,[1,2] Fuqiang Wen,[1,2] and Binwu Ying[4,5]

Abstract—Cigarette smoke (CS)-induced airway inflammation is the main pathogenesis of COPD. The present study was designed to evaluate whether ghrelin, a novel growth hormone-releasing peptide, can affect the pro-inflammatory cytokine interleukin-6 (IL-6) production induced by cigarette smoke extract (CSE) in the human bronchial epithelial cell line (16-HBE) and its possible mechanism. 16-HBE cells were pre-incubated with vehicle or ghrelin (0.1 to 1000 ng/mL) in a concentration-dependent manner, and then CSE (0 to 16 %) was added. The protein levels of IL-6 in the medium were determined by ELISA, and the mRNA expressions of IL-6 was detected by RT-PCR. We also detected the phosphorylation of IKKα/β/p65 protein and the degradation of inhibitory protein-κB (I-κB) by Western blot analysis. And the generation of reactive oxygen species (ROS) in 16-HBE was evaluated by labeling specific fluorescence probes DCFH-DA. 16-HBE Cells treated with CSE (8 %) exhibited significantly higher IL-6 production compared with cells treated with vehicle alone ($P<0.05$). Ghrelin suppressed CSE-induced IL-6 production at both mRNA and protein levels in a concentration-dependent manner ($P<0.05$). Moreover, ghrelin attenuated CSE-triggered NF-κB activation in 16-HBE, but the intracellular ROS level after application of CSE was not affected by ghrelin (0.1 to 1000 ng/mL). Together, these results suggest that ghrelin inhibits CSE-induced IL-6 production in 16-HBE cells by targeting on NF-κB pathway, but not by scavenging intracellular ROS.

KEY WORDS: ghrelin; cigarette smoke; interleukin-6; NF-κB pathway; bronchial epithelia.

INTRODUCTION

Cigarette smoke (CS) is the predominant pathogenetic factor in the development of chronic airway inflammatory diseases especially chronic obstructive pulmonary disease (COPD), and each puff of cigarette smoke contains about 5000 toxic compounds, which includes potent oxidants such as hydrogen peroxide (H_2O_2), hydroxyl, and organic radicals [1]. These toxic compounds contribute to the adverse effects of cigarette smoke on lung cells, for example, inflammation, oxidative stress, and proteinase production [2]. The inflammation process in lung is characterized by the production of histamine, bradykinin, leukotrienes, and a variety of cytokines by structural and migrating cells [3]. Among these pro-inflammatory cytokines, interleukin-6 (IL-6) is a multifunctional cytokine, considered to contribute to inflammation, vascular permeability, and cell proliferation and participate in the pathogenesis of lung inflammation-related diseases [4]. A recent investigation showed that IL-6 levels in the serum and sputum of AECOPD patients were higher than those of control patients [5].

Ghrelin is a hormone predominantly expressed by the X/A-like cells of the stomach, as an endogenous ligand for

Hao Wang, Ting Yang and Yongchun Shen contributed equally to this work.

[1] Division of Pulmonary Diseases, State Key Laboratory of Biotherapy of China, Sichuan University, Chengdu, Sichuan 610000, China
[2] Department of Respiratory Medicine, West China Hospital of Sichuan University, Chengdu, Sichuan 610041, China
[3] West China School of Medicine, Sichuan University, Chengdu, Sichuan 610041, China
[4] Department of Laboratory Medicine, West China Hospital of Sichuan University, Chengdu, Sichuan 610041, China
[5] To whom correspondence should be addressed at Department of Laboratory Medicine, West China Hospital of Sichuan University, Chengdu, Sichuan 610041, China. E-mail: Docbwy@126.com

growth hormone secretagogue receptor (GHS-R); ghrelin plays a critical role in stimulating the release of growth hormone (GH) from pituitary cells [6, 7]. Besides its effects on GH expression, ghrelin could reduce pancreatic and hepatic destruction accompanied with pulmonary and renal damages induced by pancreaticobiliary obstruction in rats by a neutrophil-dependent mechanism [8]. While in pulmonary system, elevated plasma ghrelin level has been founded in underweight patients with COPD both in Japanese and in Chinese, Moreover, plasma ghrelin correlated negatively with body mass index and plasma insulin levels, and correlated positively with tumor necrosis factor-α (TNF-α), forced expiratory volume in 1 s (FEV1), percent predicted residual volume and residual volume-to-total lung capacity ratio [9, 10]. Another study found that ghrelin administration can suppress airway inflammation by decreasing neutrophil accumulation in lungs and increase body weight in cachectic patients with chronic respiratory infection [11]. A previous study showed that smoking significantly increases plasma levels of ghrelin [12]. However, whether ghrelin has protective effect on airway exposed to cigarette smoke and its related mechanisms remain unknown. Therefore, we hypothesized that ghrelin may have anti-inflammatory and antioxidantive effects on the lung bronchial epithelial cells and the aim of this study was to demonstrate whether ghrelin could inhibit the production of IL-6 induced by cigarette smoke extract (CSE) and which mechanism might be associated in cultured human bronchial epithelial cell line 16-HBE.

MATERIALS AND METHODS

Reagents and Materials

Ghrelin was purchased from phoenix pharmaceuticals (Karlsruhe, Germany). Antibodies to phospho-p65, Phospho-IKKα/β (Ser176/180), IκB-α, and β-actin were from Cell Signaling Technology (Beverly, MA, USA). Enzyme-linked immunosorbent assays (ELISA) kit for human IL-6 was purchased from R&D Systems (Wiesbaden-Nordenstadt, Germany). One specific fluorescence probes, DCFH-DA (Beyotime Institute of Biotechnology Co., China), were used to identify the variations of cellular ROS.

Cell Culture

The human bronchial epithelial cell line (16-HBE) was bought from the American Type Culture Collection and cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10 % fetal bovine serum (FBS), 10 mM glutamine, 100 IU/mL penicillin, and 100 μg/mL streptomycin at 37 °C in a humidified atmosphere of 5 % CO_2. After reaching 90 % confluence, cells were subcultured and used for later experiments.

Cigarette Smoke Extract (CSE) Preparation

Cigarette smoke extract (CSE) in DMEM was prepared freshly for each experiment. Commercial, nonfilter cigarettes (Tianxiaxiu, Chengdu Cigarette Factor, Chengdu, China; 1.0 mg nicotine and 14 mg tar per cigarette) were used. CSE was prepared with a modification of the method of Oltmanns (Oltmanns et al., 2005). Briefly, cigarette smoke derived from one cigarette was drawn slowly into a 50-mL syringe and bubbled through 5 mL of DMEM. One cigarette yielded five draws of 50 mL of the syringe, and each individual draw took approximately 10 s to complete. The resulting solution was represented "100 %" strength, and then adjusted to pH 7.4 with concentrated NaOH and filtered before being diluted in DMEM to the required strength for application to 16-HBE cultures.

Evaluation of Cell Viability

16-HBE cells were exposed to 0 to 16 %CSE for 24 h. Cell viability was assessed by 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide (MTT) as described previously [13]. Briefly, 16-HBE cells were seeded on 96-well plates at a density of 5×10^3 cells/well. After treatments, cells were incubated with 1 mg/mL MTT for 4 h. The medium was removed, and the formation product solubilized with 100 μL dimethylsulfoxide (DMSO). The optical density (OD) was measured with 96-well plate reader (Bio-Rad, Hercules, CA, USA) at 570 nm.

Enzyme-Linked Immunosorbent Assays (ELISA)

16-HBE cells (1×10^4 cells) were cultured in DMEM without FBS in the absence or presence of ghrelin (0.1 to 1000 ng/mL) for 6 h and then exposed to CSE for 24 h. The concentration of IL-6 in the culture medium was detected by ELISA Kit according to the instructions of the manufacturer. The IL-6 concentration of unknown samples was counted using the standard curve. The results were expressed as picograms per milliliter of culture medium.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

16-HBE cells (1×10^6 cells) were seeded onto 100-mm cell culture dishes in DMEM with or without 0.1 to 1000 ng/mL ghrelin for 6 h (as our previous work [14]), and then exposed to 8 %CSE for 24 h. Total RNA was isolated with Trizol (Invitrogen, Paisley, UK) and reverse transcribed. The cDNA was amplified by PCR using the TaKaRa PCR kit. The following primers (Invitrogen, Carlsbad, CA, USA) were used: IL-6 sense: 5′-TGA ACT CCT TCT CCA CAA GC-3′; antisense: 5′-ATC CAG ATT GGA AGC ATC CA-3′, β-actin sense: 5′-TGG AGA AAA TCT GGC ACC AC-3′; antisense: 5′-GAG GCG TAC AGG GAT AGC AC-3′. The conditions for amplification were as follows: IL-6, 95 °C for 2 min for 1 cycle, 95 °C for 45 s, 58 °C for 45 s, and 72 °C for 1 min for 35 cycles, 72 °C for 2 min for 1 cycle; β-actin, 94 °C for 2 min for 1 cycle, 94 °C for 25 s, 57 °C for 25 s, and 72 °C for 25 s for 35 cycles, 72 °C for 2 min for 1 cycle. The PCR products were analyzed by 2 % agarose gel electrophoresis, and the expressions were measured by densitometry using Molecular Analysis Software (Bio-Rad). Relative quantitations of IL-6 gene expressions were normalized to β-actin with each sample.

Western Blot Analysis

Cells were pre-incubated with vehicle or 0.1 to 1000 ng/mL ghrelin for 6 h, then 8 %CSE was added for 1 h. Total protein was extracted by ice-cold lysis buffer containing 50 mM Tris–HCl, 150 mM NaCl, 1 % NP-40, 0.5 % sodium deoxycholate, 2 mM NaF, 2 mM EDTA, 0.1 % SDS, and a protease inhibitor cocktail tablet (Roche Applied Science, Indianapolis, IN, USA). Proteins were quantified using the Bradford method. Ten micrograms of total protein for each sample was separated by 10 % SDS polyacrylamide gels and transferred electronically to polyvinylidene difluoride (PVDF) membranes (Millipore Co., Bedford, MA, USA). After blocking in 5 % BSA at room temperature for 2 h, following antibodies were used: anti-human Phospho-IKKα/β (Ser176/180) (1:1000 dilution); anti-human phospho-p65 (1:1000 dilution); anti-human IκB-α (1:1000 dilution); anti-human β-actin (1:1000 dilution), and anti-rabbit or anti-mouse horseradish peroxidase-conjugated secondary antibodies (1:5000 dilution, Santa Cruz). Blots were subsequently washed 3×5 min in TBS-T20 and then developed by enhanced chemiluminescence (Millipore). The bands were visualized and quantified using quantity one imaging software (Bio-Rad). The Phospho-IKKα/β, phospho-p65, and IκB-α band intensity was adjusted by the β-actin band intensity.

Measurement of Reactive Oxygen Species (ROS)

Since ROS are natively nonfluorescent, we used one specific fluorescence probes DCFH-DA to label the intracellular ROS that its derivation can be detected by fluorescence microscope. The nonfluorescent DCFH-DA can be converted to red fluorescent ethidium by intracellular ROS. Briefly, 16-HBE cells were pre-incubated with vehicle or 0.1 to 1000 ng/mL ghrelin for 6 h, then 8 %CSE was added for 2 h. Then we collect the cells in tubes and removed the culture medium, then added the FBS-free DMEM containing 10 uM DCFH-DA, incubating at 37 °C for 20 min. FBS-free DMEM was then introduced into the tubes three times to wash away unreacted DCFH-DA. Fluorescence levels were acquired by using a fluorospectrophotometer at 525 nm absorbance.

Statistical Analysis

The data were expressed as mean±standard deviation (S.D.). This analysis was performed by SPSS software using one-way analysis of variance (ANOVA), followed by LSD significant difference test. A value of $P<0.05$ was considered statistically significant.

RESULTS

Cigarette Smoke Extract (CSE)-Induced IL-6 Expression in 16-HBE Cells at Both the mRNA and Protein Levels in a Time- and Dose-Dependent Manner

To investigate whether CSE can activate inflammatory reaction, we first indentified concentrations of CSE that would not induce cytotoxicity in 16-HBE cells. As shown by MTT assay, incubation with up to 8 %CSE for up to 24 h produced no measurable cytotoxicity as determined (vehicle=0.84±0.16 absorbance units versus 8 %CSE=0.82±0.12 absorbance units; $P>0.05$). Our results showed that CSE upregulated IL-6 expression in 16-HBE cells at both the mRNA and protein levels in a time- (from 0 to 12 h) and dose-dependent (from 0 to 8 %) manner (Figs. 1 and 2).

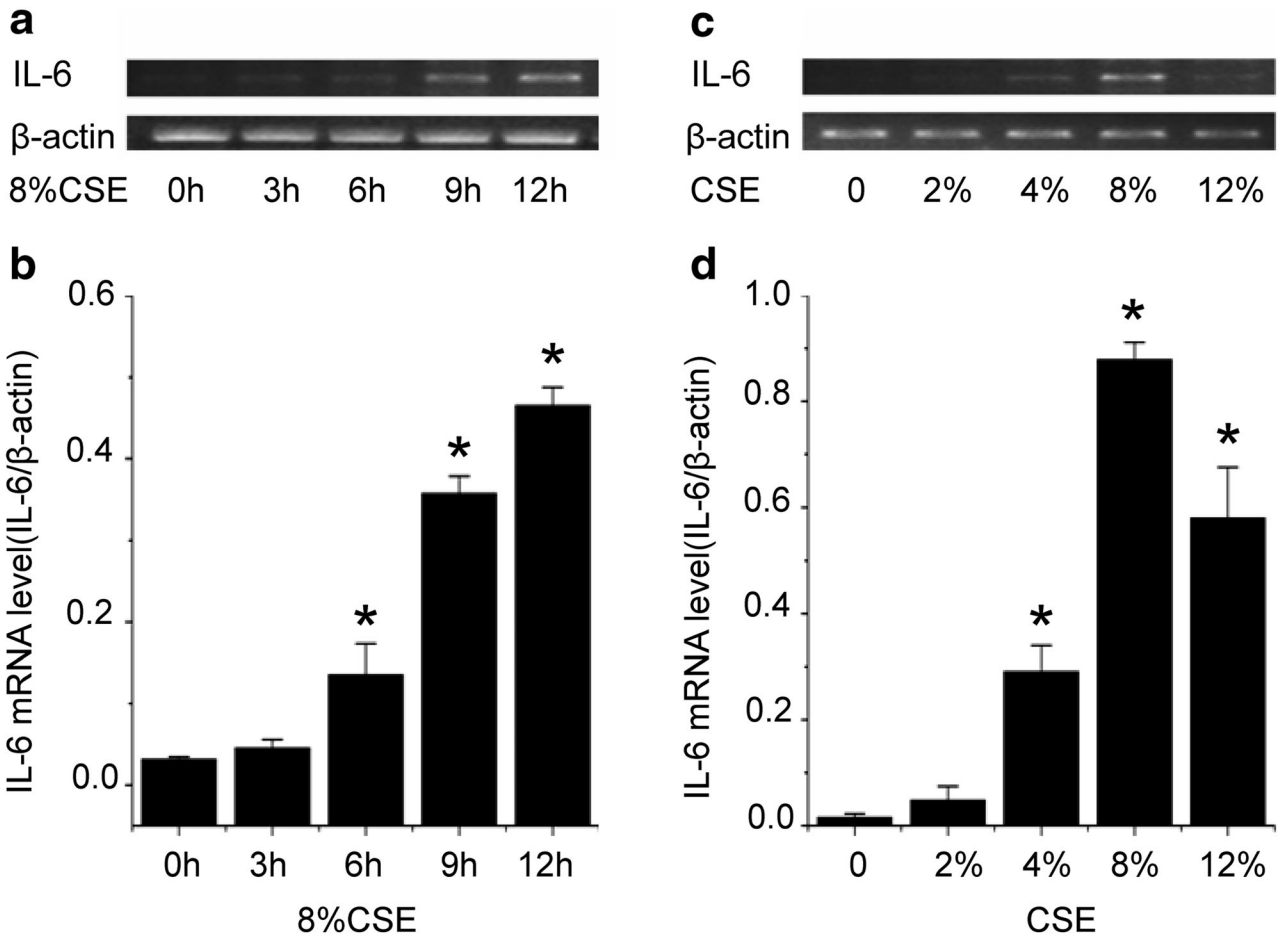

Fig. 1. CSE-induced IL-6 mRNA expression in 16-HBE cells. 16-HBE cells were treated with CSE for various times (**a**) and various doses (**c**) as indicated. IL-6 mRNA was detected by RT-PCR with β-actin as internal controls. Increased fold (means ± S.D.) for IL-6 mRNA normalized to β-actin in three repeated experiments with each triplicate is expressed as (**b**) and (**d**). *$P<0.05$ compared with sample without any treatment.

Ghrelin Inhibited CSE-Induced IL-6 Production in 16-HBE Cells

We examined whether ghrelin inhibited IL-6 mRNA expression and protein release evoked by CSE in 16-HBE cells. We firstly pretreated 16-HBE cells with vehicle or ghrelin for 6 h and then exposed to CSE for 24 h. The total RNA was extracted and IL-6 mRNA expressions were analyzed by RT-PCR. Increased IL-6 mRNA expression after CSE exposure was concentration-dependently attenuated by ghrelin (Fig. 3a, b). IL-6 levels in the media were assayed by ELISA. Ghrelin also inhibited CSE-induced IL-6 release in a concentration-dependent manner. These findings suggest that ghrelin can inhibit CSE-induced IL-6 production from 16-HBE in a concentration-dependent manner at both mRNA and protein levels. Although ghrelin alone slightly increased IL-6 mRNA expression and protein secretion levels in 16-HBE, there was no statistical significance ($P>0.05$) (Figs. 3 and 4).

CSE-Induced NF-κB Activation Including IKKα/β Phosphorylation, p65 Phosphorylation, and IκB-α Degradation in 16-HBE Cells was Inhibited by Ghrelin

To test whether ghrelin inhibits CSE-induced NF-κB activation in 16-HBE cells, we performed Western blotting to examine IKKα/β phosphorylation, p65 phosphorylation, and IκB-α degradation in 16-HBE cells. CSE-induced IKKα/β and p65 protein phosphorylation, which

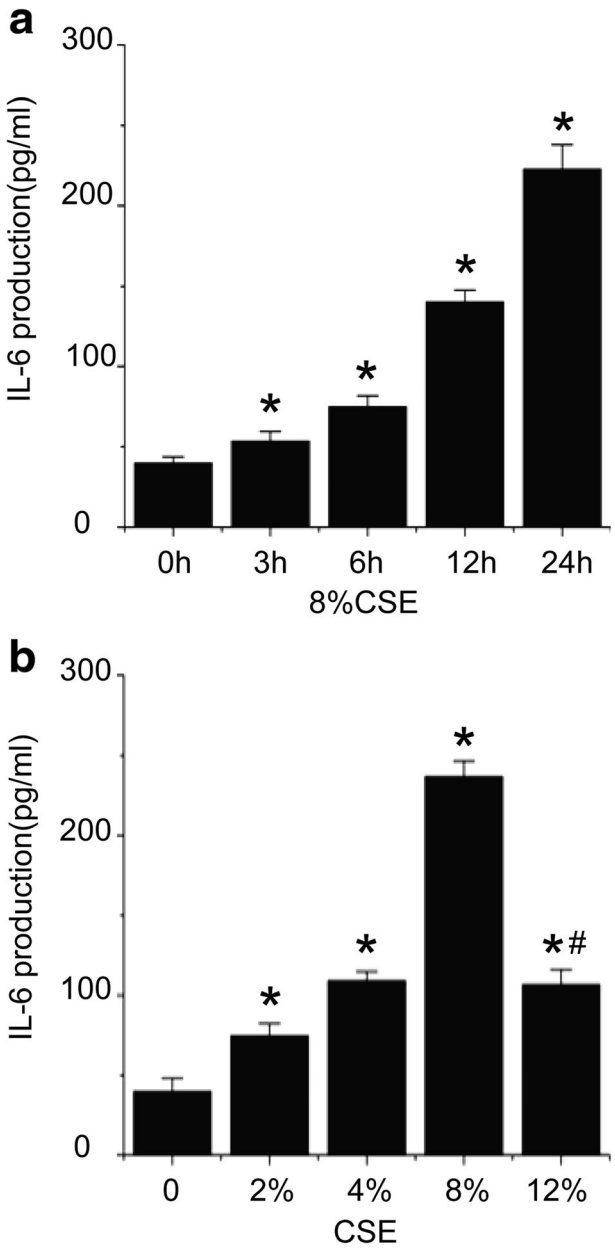

Fig. 2. CSE-induced IL-6 secretion in 16-HBE cells. 16-HBE cells were exposed to varying times (**a**) and concentrations (**b**) of CSE; IL-6 released in the medium was measured by ELISA. Data represent mean ± SD in three independent experiments. *$P<0.05$ compared with sample without any treatment. #$P<0.05$ compared with 16-HBE cell exposure to 8 %CSE

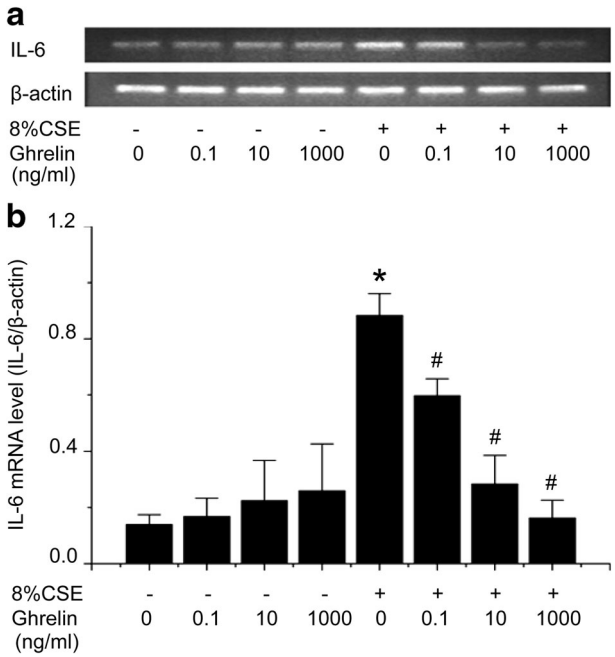

Fig. 3. Ghrelin inhibited IL-6 mRNA expression induced by CSE in 16-HBE cells. 16-HBE cells were pre-incubated with different concentrations of ghrelin for 6 h, and IL-6 mRNA expression was determined 12 h later in both unstimulated cells and cells treated with 8 %CSE by RT-PCR. Representative band was showed (**a**). Histograms represent mean ± SD of the relative intensity of the IL-6 bands normalized to β-actin (**b**). *$P<0.05$ compared with sample without any treatment. #$P<0.05$ compared with 16-HBE cell exposure to 8 %CSE. Data represent similar results from three independent experiments

was concentration-dependently attenuated by ghrelin (Fig. 5a–c). Meanwhile, after 1-h CSE exposure, the levels of IκB-α were markedly decreased in the cytoplasmic fraction of 16-HBE, compared to cells without exposure to CSE. In addition, CSE-induced IκB-α degradation was

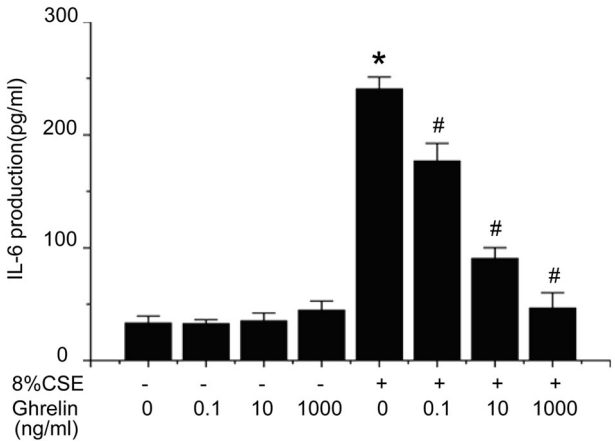

Fig. 4. Ghrelin inhibited IL-6 secretion induced by CSE in 16-HBE cells. 16-HBE cells were pretreated with or without ghrelin (G) at different concentration for 6 h and then exposed to 8 %CSE for 24 h, IL-6 released in the medium was measured by ELISA. Data represent mean ± SD in three independent experiments. *$P<0.05$ compared with sample without any treatment. #$P<0.05$ compared with 16-HBE cell exposure to 8 %CSE

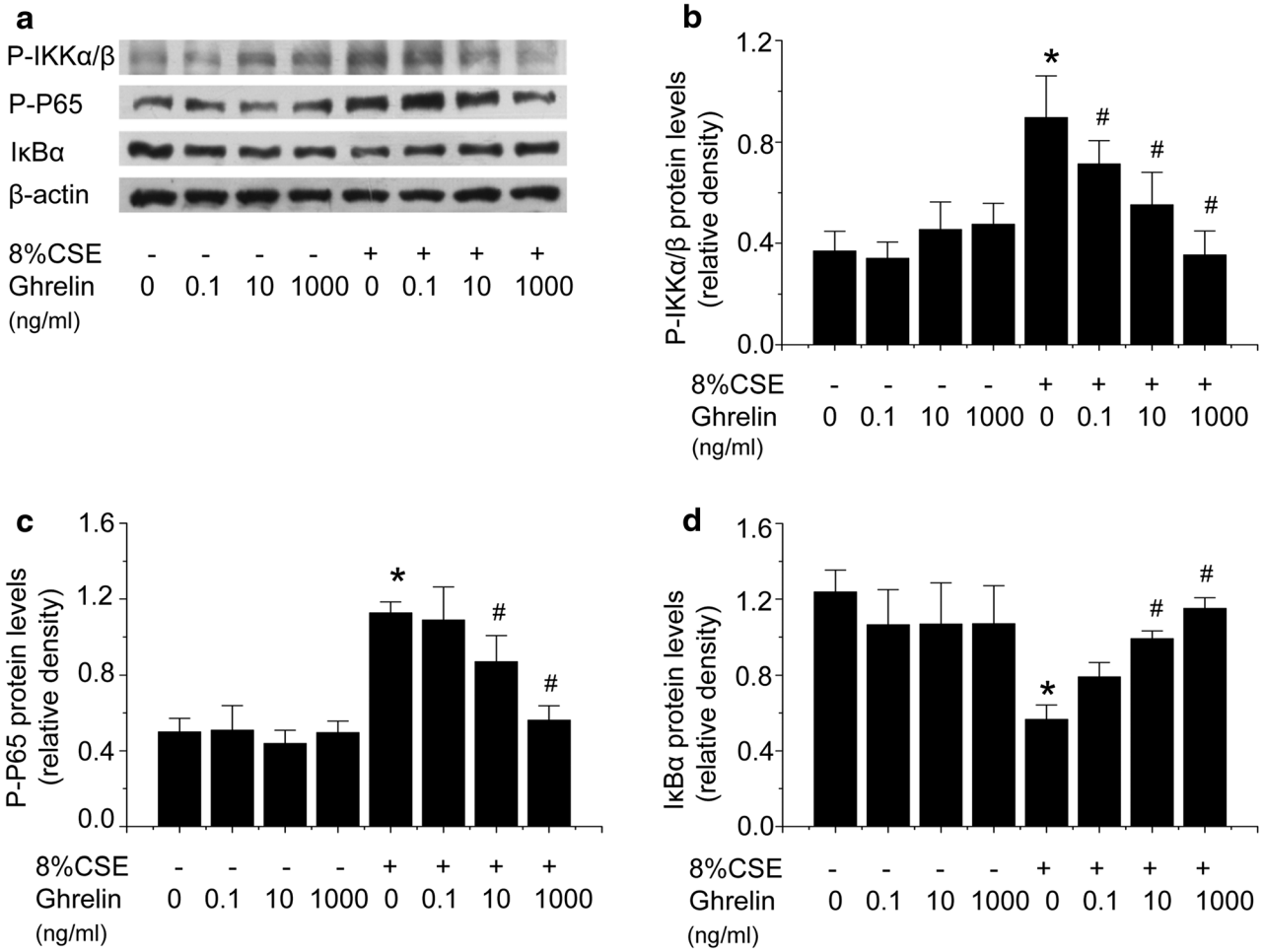

Fig. 5. Effect of ghrelin on CSE-induced NF-κB activation including p65 phosphorylation, IKKα/β phosphorylation, and IκB-α degradation in 16-HBE cells. 16-HBE cells were pretreated with or without different concentrations of ghrelin for 6 h and then exposed to 8 %CSE for 1 h. Total protein was extracted, and Western blot analysis was performed to detect phosphorylated p65 protein, phosphorylated IKKα/β, and the level of IκB-α protein (**a**). Histograms represent mean value and S.D. of the relative intensity of the P-IKKα/β (**b**), P-p65 (**c**), and IκB-α (**d**) protein bands normalized to β-actin.*$P<0.05$ compared with sample without any treatment. $^{\#}P<0.05$ compared with 16-HBE cell exposure to 8 %CSE. Data represent similar results from three independent experiments

also inhibited by ghrelin in a concentration-dependent manner (Fig. 5a, d).

Effect of Ghrelin on CSE-Induced Oxidative Stress in 16-HBE Cells

Previous studies demonstrated that CSE can activate oxidative stress such as production of reactive oxygen species (ROS), and ROS also can activate inflammatory signaling pathways [15]. To determine whether ghrelin might inhibit CSE-induced IL-6 release by eliminating intracellular ROS, we performed the Reactive Oxygen Species Assay Kit to detect ROS in 16-HBE cells. We found that CSE increased the levels of ROS in a concentration-dependent manner (Fig. 6a), but this was not affected by different concentration of ghrelin (from 0.1 ng/mL to 1000 ng/mL) (Fig. 6b).

DISCUSSION

The novel findings of the present study demonstrate that exogenous ghrelin is able to attenuate CSE-induced IL-6 expression in human bronchial epithelia cell line 16-HBE. We show for the first time that ghrelin inhibits CSE-induced pro-inflammatory cytokine IL-6 production in 16-HBE cells. Furthermore, ghrelin inhibits NF-κB activation

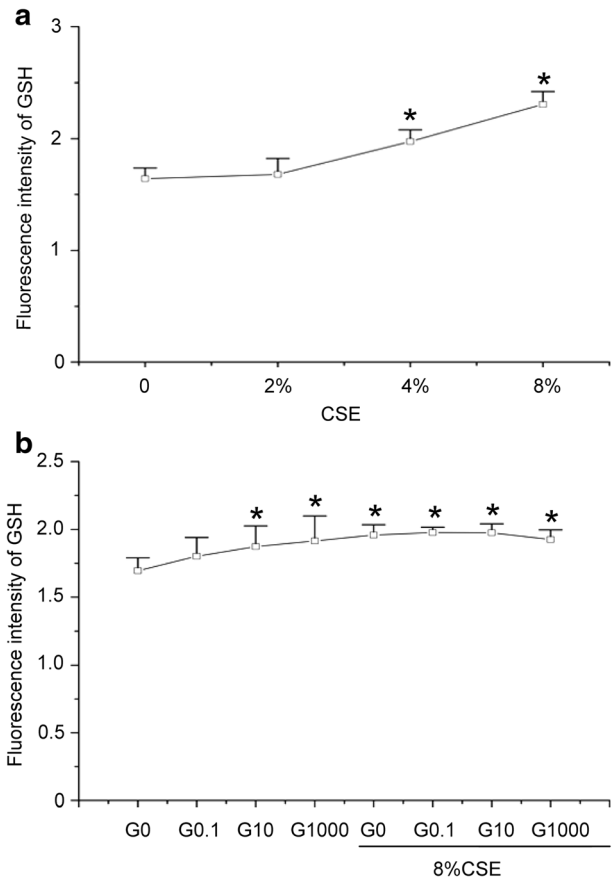

Fig. 6. Effect of ghrelin on CSE-induced intracellular ROS activation. 16-HBE cells were exposed to different concentration CSE, and ROS levels were measured 2 h latter (**a**). 16-HBE cells were pretreated with or without different concentrations of ghrelin for 6 h and then exposed to 8 %CSE for 2 h. The effect of ghrelin on CSE-induced increase in ROS level was measured (**b**). Data are means ± S.D. of three replicates from four independent experiments. *$P < 0.05$ compared with sample without any treatment

including p65 phosphorylation, IKKα/β phosphorylation, and IκB-α degradation in 16-HBE cells. These results show that ghrelin may act its anti-inflammatory role through NF-κB pathways.

Since firstly reported in 1999 [6], it is now widely accepted that ghrelin exerts multiple physiological functions such as the secretion of GH, the regulation of energy metabolism, the release of insulin, decreased mean arterial pressure, orexigenic regulation, and anti-inflammatory and anti-oxidative action [6, 16–18]. The functions of ghrelin have largely been linked to the release of GH dependent on activation of GHS-receptors within the anterior pituitary, and it has been demonstrated that GHS-receptors for ghrelin have been identified in the lung [19], suggesting that ghrelin may play a modulatory role in the lung. Recent researches demonstrate that ghrelin may be a new therapeutic strategy for the treatment of different pulmonary diseases. In severe sepsis-induced acute lung injury (ALI), ghrelin administration attenuates lung injury, increases pulmonary blood flow, decreases pro-inflammatory cytokines, and improves survival in sepsis [20]. Ghrelin treatment attenuates MCT-induced pulmonary hypertension (PH), pulmonary vascular remodeling, and RV hypertrophy in a rat model [21]. In persistent pulmonary hypertension of the newborn (PPHN) rat model, administration of ghrelin improves hypoxia-induced PH and attenuates pulmonary vascular remodeling via phosphorylation of glycogen synthase kinase 3β (p-GSK3β)/β-catenin signaling [22]. However, there is no report about potential actions of ghrelin on CS-induced airway inflammation. The present study is for the first time to explore the protective effects of ghrelin on CS-induced airway inflammation and oxidative stress, as well as the mechanisms.

Inflammatory events and oxidative stress induced by cigarette smoke in the airway epithelium are important pathogenesis of smoking-related pulmonary diseases. Circulatory IL-6 levels of COPD patients significantly increased after a 3-year follow-up compared to baseline condition and were related to mortality and severity of COPD [23]. Cigarette smoke extract dose-dependently induced IL-6 and IL-8 release and depleted glutathione concentration in primary human small airway epithelial cells (SAEC) through NF-κB pathway [24]. The airway epithelial cell line MM-39 and primary human bronchial epithelial cells were cultivated as air-liquid interface cultures and stimulated with volatile cigarette smoke, resulting in upregulation of mRNA and protein levels of IL-6 and IL-8 [25]. Yu et al. [3] used IL-6 knocked-out (KO) mice and WT mice receiving IL-6 antibody treatment to demonstrate that IL-6 deficient was associated with attenuated inflammation and injury in lung exposed to ozone or to aged and diluted cigarette smoke (ADSS)/ozone. A recent study also shows that IL-6 plays a critical role between CSE-induced airway inflammation and epithelial–mesenchymal transition (EMT) in HBE [26]. These indicate that IL-6 may play a critical role in CS-induced airway inflammatory diseases, especially COPD. In the present study, we found that CSE promoted IL-6 expression in 16-HBE cells at both the mRNA and the protein levels in a time- and dose-dependent manner. Cigarette smoke-mediated oxidative stress leads to increased transcription of pro-inflammatory cytokine genes, which are involved in pathogenesis of airway epithelium damage [15, 27]. We also found CSE-induced ROS production. And we suggested that downregulation of IL-6 and ROS levels may be effective ways to reduce airway inflammation.

Previous studies showed that ghrelin inhibited the expression of IL-6 in T lymphocytes and monocytes [28]. It was also reported that Ghrelin treatment attenuated levels of inducible nitric oxide (iNOS) protein and thiobarbituric acid reactive substance (TBARS) and increased glutathione (GSH) in rat suffering from gastric ischemic injury. Ghrelin also attenuated ROS generation in human polymorphoneuclear (PMN) cells in a dose-dependent manner [18]. Our study showed that CS-induced IL-6 release by 16HBE was effectively blocked by ghrelin. However, ghrelin did not reduce the level of intracellular ROS. Taken together, these findings suggest that ghrelin acts as an anti-inflammatory agent rather than an antioxidant agent (i.e., it does not scavenge ROS) in 16HBE. Our present study is consistent with our previous findings, ghrelin did not reduce the level of intracellular ROS in A549 cells treated with H_2O_2 [14].

NF-κB is a critical signaling molecule in cigarette smoke-induced inflammation. Volatile CS using humidified and warm air upregulated mRNA for IL-6 and IL-8 and protein release in airway epithelial cells, mediated by the mitogen-activated protein kinase (MAPK) p38 and the transcription factor NF-κB [25]. IL-6 expression is also directly regulated by NF-κB involving p38 MAPK activation [29]. Our group recently found that ghrelin inhibits H_2O_2-induced interleukin-8 production in A549 cells by targeting on NF-κB pathway [14]. Therefore, a potential mechanism of how ghrelin could modulate inflammatory response to CSE is blocking activation of the transcription factor NF-κB. As shown in our study, CSE exposure markedly increased IKKα/β phosphorylation, p65 phosphorylation, and IκB-α degradation. And ghrelin did not only attenuate CSE-induced phosphorylation of IKKα/β and p65 protein but also restore the level of I-κB. These results indicated ghrelin indeed inhibited NF-κB activation and displayed the anti-inflammatory function. This is consistent with the conclusion that ghrelin inhibits NF-κB activation in A549 cells [14].

There are potential limitations in our study. Previous studies found that ghrelin suppressed the expression of pro-inflammatory cytokines in human T lymphocytes and monocytes via acting on GHS-R [28]. Similarly, if ghrelin attenuates cigarette smoke-induced airway inflammation by interacting with specific receptors such as GHS-R should be investigated next. Meanwhile, further studies performed in vivo are needed to explore the specific effects of ghrelin on airway inflammation. Besides, it is a long way to translate our study findings into clinical practice. And we will continue to focus on these fields.

In summary, the present study demonstrates that ghrelin attenuates CSE-induced IL-6 production in a time and dose-dependent manner in human epithelial cell line 16HBE via regulating NF-κB pathway, but not via scavenging intracellular ROS. And recently, a phase II clinical trial showed that administration of ghrelin could successfully increase both food intake and appetite, and ameliorate weight loss in gastric cancer patients after total gastrectomy without significant side effects [30], which implied that ghrelin could be transferred into clinical practice effectively and safely. Combining with our findings, we suggested that ghrelin would also be introduced into clinical application as a therapeutic drug for cigarette smoke-related airway inflammatory diseases such as COPD in the future.

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Conflicts of Interest. The authors declare that they have no competing interests.

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