

Original Article

Carbocisteine attenuates TNF- α -induced inflammation in human alveolar epithelial cells *in vitro* through suppressing NF- κ B and ERK1/2 MAPK signaling pathways

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Aim: We previously proven that carbocisteine, a conventional mucolytic drug, remarkably reduced the rate of acute exacerbations and improved the quality of life in the patients with chronic obstructive pulmonary disease. In this study we investigated the mechanisms underlying the anti-inflammatory effects of carbocisteine in human alveolar epithelial cells *in vitro*.

Methods: Human lung adenocarcinoma cell line A549 was treated with TNF- α (10 ng/mL). Carbocisteine was administered either 24 h prior to or after TNF- α exposure. The cytokine release and expression were measured using ELISA and qRT-PCR. Activation of NF- κ B was analyzed with Western blotting, immunofluorescence assay and luciferase reporter gene assay. The expression of ERK1/2 MAPK signaling proteins was assessed with Western blotting.

Results: Carbocisteine (10, 100, 1000 μ mol/L), administered either before or after TNF- α exposure, dose-dependently suppressed TNF- α -induced inflammation in A549 cells, as evidenced by diminished release of IL-6 and IL-8, and diminished mRNA expression of IL-6, IL-8, TNF- α , MCP-1 and MIP-1 β . Furthermore, pretreatment with carbocisteine significantly decreased TNF- α -induced phosphorylation of NF- κ B p65 and ERK1/2 MAPK, and inhibited the nuclear translocation of p65 subunit in A549 cells. In an NF- κ B luciferase reporter system, pretreatment with carbocisteine dose-dependently inhibited TNF- α -induced transcriptional activity of NF- κ B.

Conclusion: Carbocisteine effectively suppresses TNF- α -induced inflammation in A549 cells *via* suppressing NF- κ B and ERK1/2 MAPK signaling pathways.

Keywords: carbocisteine; human alveolar epithelial cells; TNF- α ; NF- κ B; ERK1/2; cytokines; anti-inflammation

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Introduction

Carbocisteine (S-carboxymethylcysteine) has been conventionally prescribed as a mucolytic agent because of its capacity to reduce mucus viscosity by modifying mucus's biochemical properties. Consistent with literature reports^[1,2], we found that carbocisteine was effective in reducing the rate of acute exacerbations and improving quality of life in patients with

chronic obstructive pulmonary disease (COPD) in a large-scale multi-center clinical trial^[3].

Despite the lack of a free thiol group, carbocisteine harbors a thioether group that can be oxidized by reactive oxygen species (ROS)^[4]. Carbocisteine reportedly scavenged H₂O₂, HOCl, OH* and ONOO⁻ in cell-free medium, inhibited ROS production in rat neutrophils^[4], promoted GSH and HO-1 expression in cigarette smoke-exposed bronchial epithelial cells^[5], and ameliorated oxidant-induced 16HBE cell apoptosis^[6]. Furthermore, carbocisteine was capable of attenuating the synthesis of pro-inflammatory cytokines following rhinovirus or respiratory syncytial virus infection^[7,8]. It was speculated that the efficacy of carbocisteine in COPD was more closely associated

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with its anti-oxidant and anti-inflammatory effects^[9], which have been scarcely reported previously^[10], than with mucolytic activity.

Inflammation plays pivotal roles in the pathogenesis and progression of chronic respiratory diseases (particularly in COPD). Exacerbations of COPD have been associated with aggravated inflammatory responses, which increased the risks of morbidity and mortality^[11, 12]. Various transcription factors or kinases, including nuclear factor kappa B (NF- κ B) and mitogen-activated protein kinases (MAPKs), have been implicated in the pathogenesis of both stable and exacerbations of COPD^[13].

Tumor necrosis factor- α (TNF- α), a classical pro-inflammatory cytokine, plays an important role in promoting lung inflammation in COPD. The excessive release or spillover of TNF- α has been associated with the maintenance of neutrophilic airway inflammation and systemic manifestations, such as muscle wasting^[14, 15]. TNF- α has also been linked to COPD progression because circulating TNF- α levels were found to be inversely correlated with arterial partial pressure of oxygen (PaO₂) and increased with dyspnea severity^[16]. Mechanistically, TNF- α is a major inflammatory mediator of the lung and induces NF- κ B and MAPK activation in different respiratory cell lines^[17, 18]. Of these, alveolar epithelial cells serve as an important dynamic barrier against environmental stimuli, sepsis, and other illnesses affecting the lung. Alveolar epithelial cells may amplify inflammatory signals via inflammatory cell chemotaxis and inflammatory mediator release^[19]. Exploration of the effects on injured alveolar epithelial cells may provide greater insights into the mechanisms of action of medications (*ie*, mucolytics) in patients with COPD. We have previously revealed that carbocisteine could inhibit the inflammation induced by hydrogen peroxide in human lung alveolar type II epithelial cells (A549 cells)^[10]. However, limited information is available about the effects as well as molecular mechanisms of carbocisteine against TNF- α -induced inflammation.

Therefore, we sought to explore the impact of carbocisteine on the synthesis and release of inflammatory cytokines and the signaling pathways in cultured A549 cells and HEK293 cells. Our findings will offer new insight into clarifying the mechanisms responsible for the effects of carbocisteine and lend support to its clinical application.

Materials and methods

Materials

Fetal bovine serum and RPMI-1640 medium were purchased from Gibco BRI (Grand Island, NY, USA), and the Trizol and PrimeScript RT Master Mix (Perfect Real Time) kits were from Takara (Dalian, China). Primers and probes of tested genes were synthesized by Invitrogen Trading Co, Ltd (Shanghai, China). Human IL-6 and IL-8 ELISA kits were purchased from R&D Systems (Minneapolis, MN, USA), the BCA Protein Assay Kit and Super-Signal West Pico Trial Kit were from Thermo Scientific (Rockford, IL, USA), rabbit anti-pNF- κ B p65, anti-NF- κ B p65, anti-pI κ B- α , anti-I κ B- α , anti-pERK1/2, anti-ERK1/2 and anti-GAPDH monoclonal antibodies were

from Cell Signaling Technology Inc (Beverly, MA, USA), HRP-conjugated goat anti-rabbit antibodies were from EarthOx Life Science (Millbrae, CA, USA), and the Bright-GloTM Luciferase assay system was from Promega Corporation (Madison, WI, USA). All other materials were purchased from Sigma Chemical Inc (St Louis, MO, USA).

Cell culture

Cells of the human lung adenocarcinoma cell line A549 were incubated *in vitro* in RPMI-1640 medium containing penicillin (100 U/mL), streptomycin (100 μ g/mL) and 10% (*v/v*) fetal bovine serum (FBS). Human embryonic kidney (HEK-293) cells were co-transfected with pNF- κ B-TATA-F-LUCI (4.5 μ g) and pQCXIP-eGFP (0.5 μ g) plasmid (9:1) in a 60-mm dish and selected with 1 μ g/mL puromycin for one week. The cells expressing eGFP with NF- κ B reporter luciferase activity were cultured *in vitro* in DMEM medium supplemented with penicillin (100 U/mL), streptomycin (100 μ g/mL) and 10% (*v/v*) FBS. All cells were incubated at 37°C in a humidified incubator with 5% CO₂.

When the confluence reached 70%–80%, A549 cells and HEK293 cells were treated with carbocisteine with/without TNF- α for various time periods with various doses as follows. To examine the protective effect of carbocisteine on cytokine expression and release, cells were pre-treated with 10, 100, and 1000 μ mol/L carbocisteine for 24 h prior to or after exposure to TNF- α (10 ng/mL for 24 h). To determine intracellular signaling, A549 cells and HEK293 cells were pre-treated with 10, 100, and 1000 μ mol/L carbocisteine for 24 h before exposure to TNF- α (10 ng/mL for 30 min). The cells and cell culture medium were harvested for subsequent measurements.

RNA extraction and quantitative real-time polymerase chain reaction (qRT-PCR)

RNA was extracted from cultured A549 cells using Trizol reagent by following the manufacturer's protocol. cDNA synthesis and quantitative PCR were performed using standard procedures as described previously^[10]. Primers and probes of tested genes are listed in Table 1. Amplification was performed under the following conditions: 10 s at 95°C, followed by 40 cycles with 5 s at 95°C and 40 s at 60°C. Cycle threshold (Ct) values were determined by the fit point method from the exponential phase of each amplification. The difference in Ct values was calculated as follows: Δ Ct=Ct (test gene)–Ct (GAPDH), $\Delta\Delta$ Ct= Δ Ct (treated sample)– Δ Ct (untreated control). The relative expression of tested genes was calculated according to the formula: Relative expression= $2^{-\Delta\Delta$ Ct} [20].

Enzyme-linked immunosorbent assay (ELISA)

After treatment with carbocisteine, interleukin-6 (IL-6) and interleukin-8 (IL-8) in the cell culture supernatant were measured by ELISA according to the manufacturer's instructions.

Western blotting

Cellular proteins of A549 cells were extracted from treated A549 cells. An aliquot of proteins was electrophoresed on

Table 1. Specific primers of tested genes were used for qRT-PCR.

Gene	Primer/probe sequences
IL-6	Forward primer 5'-CGGGAACGAAAGAGAAGCTCTA-3' Reverse primer 5'-CGCTTGTGGAGAAGGAGTTCA-3' Probe 5'-FAM-TCCCCTCCAGGAGCCAGCT-3'-TAMRA
IL-8	Forward primer 5'-TTGGCAGCCTTCCTGATTC-3' Reverse primer 5'-TATGCACTGACATCTAAGTTCTTTAGCA-3' Probe 5'-FAM- CCTTGGCAAACCTGCACCTTACACA-3'-TAMRA
TNF- α	Forward primer 5'-AACATCCAACCTTCCCAAACG-3' Reverse primer 5'-GACCCTAAGCCCCAATTCTC-3' Probe 5'-FAM-CCCCCTCCTCAGACACCCTCAACC-3'-TAMRA
MCP-1	Forward primer 5'-CAAGCAGAAGTGGGTTCCAGGAT-3' Reverse primer 5'-AGTGAGTGTCAAGTCTTCGGAGTT-3' Probe 5'-FAM-CATGGACCCTTGACAAGCAAACC-3'-TAMRA
IP-10 (forward)	Forward primer 5'-GAAATTATCTGCAAGCCAATTT-3' Reverse primer 5'-TCACCCTTCTTTTTCATTGTAGCA-3' Probe 5'-FAM-TCCACGTGTTGAGATCA-3'-MGB
MIP-1 α	Forward primer 5'-CTGCATCACTTGCTGCTGACA-3' Reverse primer 5'-CACTGGCTGCTCGTCTCAAAG-3' Probe 5'-FAM-TTCAGCTACACCTCCCGGCAGATCC-3'-TAMRA
MIP-1 β	Forward primer 5'-AAACCTCTTGGCCACCAATACC-3' Reverse primer 5'-GAGAGCAGAAGGCAGCTACTAG-3' Probe 5'-FAM-TGAAGCTCTGCGTACTGCTCCTGTCT-3'-TAMRA
GAPDH	Forward primer 5'-CAAGCAGAAGTGGGTTCCAGGAT-3' Reverse primer 5'-AGTGAGTGTCAAGTCTTCGGAGTT-3' Probe 5'-FAM-CAAGCTTCCCGTCTCAGCC-3'-TAMRA

SDS-PAGE gel and transferred to polyvinylidene difluoride membranes. Membranes were blocked and subsequently incubated with antibodies against pNF- κ B p65 (Ser536), total NF- κ B p65, pI κ B- α (S32), total I κ B- α , pERK1/2 (T202/Y204), total ERK1/2 and GAPDH (1:1000). Following washing, the bound antibody was detected using horseradish peroxidase (HRP)-conjugated secondary antibodies (1:5000), and the bound complexes were detected using electrochemiluminescence (ECL). The relative intensities of bands were analyzed using an image analysis software program (Image J 1.44p, Wayne Rasband, National Institutes of Health, USA).

Immunofluorescence

Following treatment with carbocisteine alone, TNF- α alone, or carbocisteine plus TNF- α , A549 cells were washed with PBS, fixed with 4% paraformaldehyde solution for 30 min, permeabilized with 0.5% Triton X-100 for 15 min and blocked with 5% BSA for 20 min. This was followed by cell incubation with a rabbit monoclonal antibody against pNF- κ B p65 (1:100) overnight at 4°C. After conjugation with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (1:150) and 4',6-diamidino-2-phenylindole (DAPI) (10 μ mol/L), fluorescence was visualized with a Zeiss Axiovert 135 fluorescence microscope (Jena, Germany).

Luciferase assay of NF- κ B in HEK-293 cells

HEK293 cells, which were stably co-transfected with a NF- κ B-

responsive luciferase reporter and constitutively expressed GFP as an internal control, were grown in 96-well plates and treated with carbocisteine (24 h) followed by TNF- α (10 ng/mL, 30 min). Luciferase activity was determined using the Luciferase Assay System according to the manufacturer's instructions and normalized against the levels of GFP expression^[21].

Statistical analysis

Continuous data were expressed as the means \pm standard deviation from the indicated number of studies (*n*) and compared by using Student's *t*-test or one-way ANOVA analysis if appropriate. Comparisons of data with *P*<0.05 were considered statistically significant.

Results

Carbocisteine ameliorated TNF- α -induced IL-6, IL-8, TNF- α , MCP-1 and MIP-1 β expression in A549 cells

There is a wide array of cytokines and chemokines that have been implicated in various facets of the pathogenesis of COPD. IL-6 contributes to the pathogenesis of the autoimmune response observed in the lungs of the patients with more severe COPD; IL-8 plays a major role in neutrophil chemotaxis caused by alveolar macrophage-derived conditioned media of COPD; TNF- α is an important chemotactic protein for neutrophils^[22]; IP-10 is a chemokine strongly associated with viral infection of COPD^[23]; MCP-1 is involved in the migration of macrophages into the small airways, thus contributing to the inflammatory load associated with COPD^[24]; neutrophil-derived MCP-1, MIP-1 α , and MIP-1 β are involved in macrophage recruitment into inflamed tissue during COPD^[25]. MIP-1 β contributes substantially to the development of lung inflammation following exposure to tobacco smoke^[26]. The levels of IL-6, IL-8, TNF- α , IP-10, MCP-1, MIP-1 α and MIP-1 β were increased in the sputum and/or serum of patients with stable COPD or during exacerbation compared with controls^[23-25]. Therefore, these cytokines are cardinal markers of airway inflammation in COPD patients.

To investigate the protective effect of carbocisteine against TNF- α -induced inflammatory responses, we compared the relative abundance of all of these cytokines by using qRT-PCR. Treatment with TNF- α markedly up-regulated IL-6, IL-8, TNF- α , IP-10, MCP-1, MIP-1 α and MIP-1 β mRNA expression in A549 cells, whereas treatment with carbocisteine (before or after TNF- α stimulation) led to decreased IL-6, IL-8, TNF- α , MCP-1 and MIP-1 β mRNA expression in a dose-dependent manner (Figure 1). However, IP-10 and MIP-1 α expression levels remained relatively unchanged (data not shown).

Carbocisteine attenuated TNF- α -induced IL-6 and IL-8 production in A549 cells

Next, we quantified IL-6 and IL-8 release from A549 cells using ELISA assay. As a potent pleiotropic proinflammatory cytokine, TNF- α elicited a pronounced increase in the release of IL-6 and IL-8 in A549 cells, which could be markedly inhibited by carbocisteine (before or after TNF- α stimulation) in a dose-dependent manner (particularly for IL-8) (Figure 2).

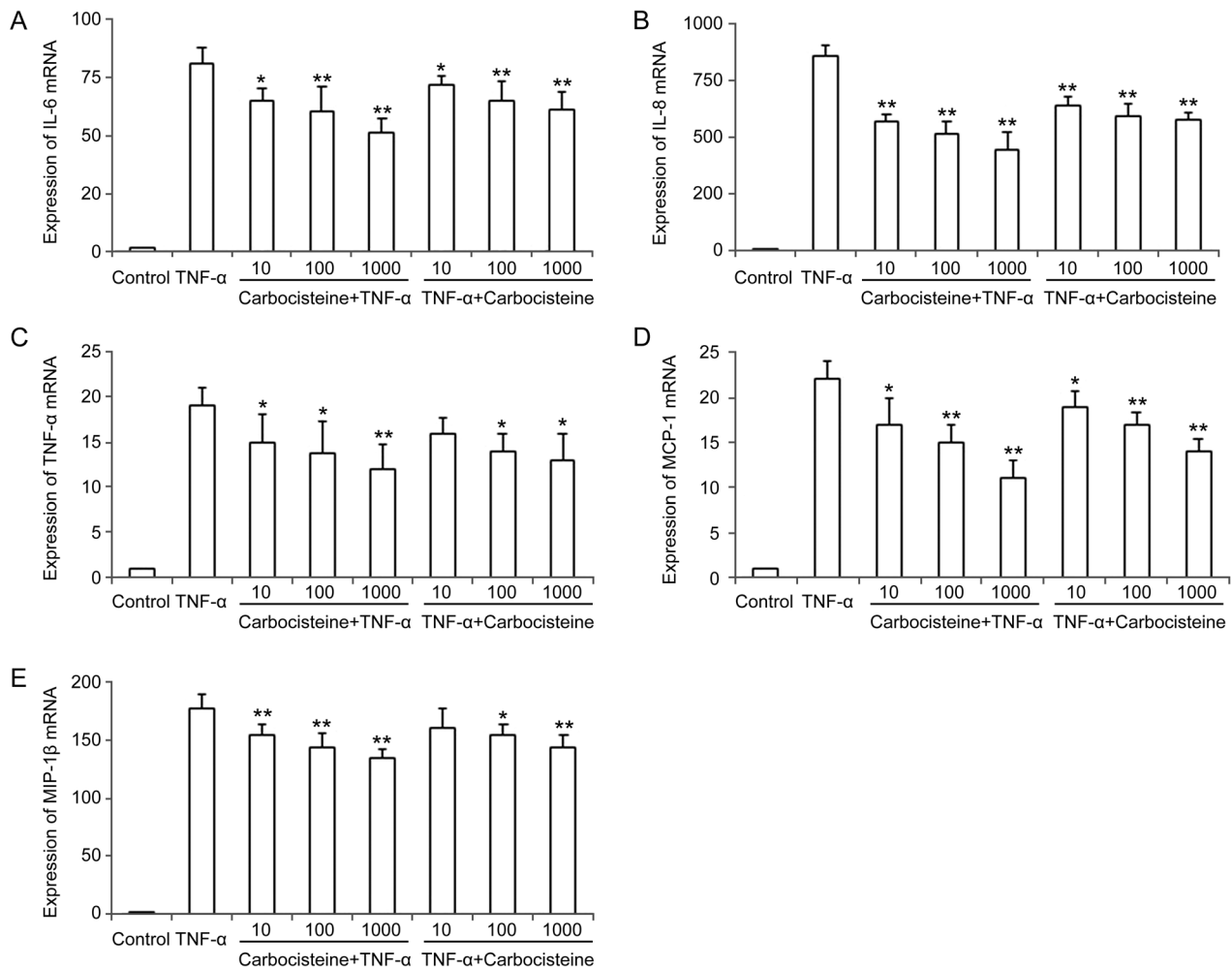


Figure 1. Effects of carbocisteine on the TNF- α -induced expression of the IL-6, IL-8, TNF- α , MCP-1 and MIP-1 β genes in A549 cells. Control group: same amount of vehicle (PBS); TNF- α group: 10 ng/mL; Carbocisteine+TNF- α group: pre-treated with 10, 100, and 1000 μ mol/L carbocisteine for 24 h, then exposure to 10 ng/mL TNF- α for 24 h; TNF- α +Carbocisteine group: pre-treated with 10 ng/mL TNF- α for 24 h, then exposure to 10, 100, and 1000 μ mol/L carbocisteine for 24 h. (A) IL-6, (B) IL-8, (C) TNF- α , (D) MCP-1, (E) MIP-1 β . Mean \pm SD. $n=3$. * $P<0.05$, ** $P<0.01$ compared with the TNF- α group.

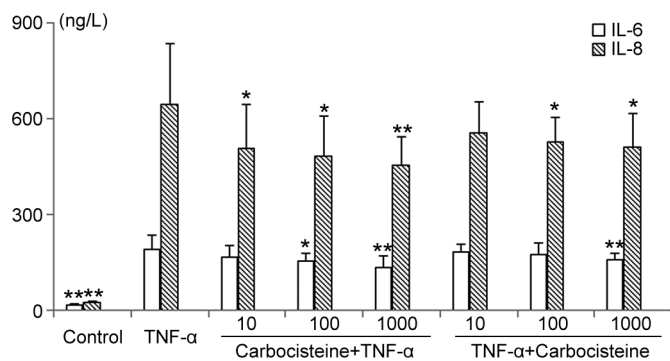


Figure 2. Effects of carbocisteine on TNF- α -induced IL-6 and IL-8 release in A549 cells. Control group: same amount of vehicle (PBS); TNF- α group: 10 ng/mL; Carbocisteine+TNF- α group: pre-treated with 10, 100, or 1000 μ mol/L carbocisteine for 24 h, then exposure to 10 ng/mL TNF- α for 24 h; TNF- α +Carbocisteine group: pre-treated with 10 ng/mL TNF- α for 24 h, then exposure to 10, 100, or 1000 μ mol/L carbocisteine for 24 h. Mean \pm SD. $n=6$. * $P<0.05$, ** $P<0.01$ compared with the TNF- α group.

Carbocisteine inhibited TNF- α -induced NF- κ B activation and I κ B- α degradation in A549 cells

Carbocisteine reportedly inhibited type A seasonal influenza virus-induced NF- κ B activation in human tracheal epithelial cells^[27]. To examine whether this inhibitory effect of carbocisteine also applied to A549 cells, we pre-treated A549 cells with three doses of carbocisteine (10, 100 and 1000 μ mol/L) for 24 h before exposure to TNF- α . Western blotting revealed that TNF- α significantly promoted NF- κ B p65 and I κ B- α phosphorylation (Figure 3A–3D) accompanied by I κ B- α degradation (Figure 3C, Figure 3E), which was in accordance with the translocation of the NF- κ B p65 subunit from the cytosol to the nucleus (Figure 3F). Pre-treatment with carbocisteine significantly reduced the levels of phosphorylated NF- κ B, nuclear NF- κ B protein and I κ B- α degradation, whilst the carbocisteine alone showed no effects on the phosphorylation of NF- κ B or I κ B- α degradation (Figure 3).

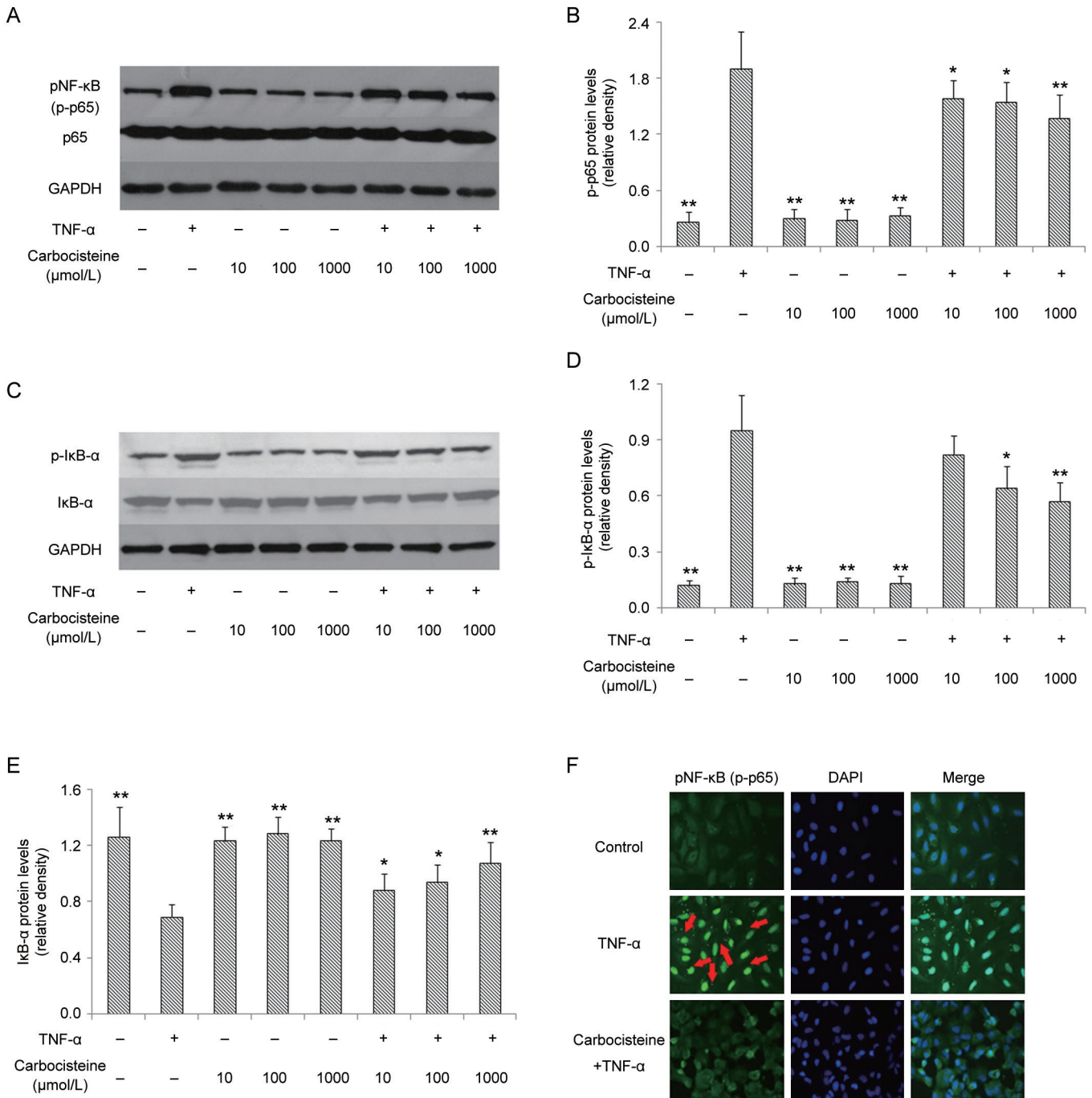


Figure 3. Carbocisteine inhibited TNF- α -induced NF- κ B activation, including p65, I κ B- α phosphorylation and I κ B- α degradation, in A549 cells. (A) Representative Western blots of p-p65, total p65 and GAPDH. (B) Histograms represented means and SD of the ratio of p-p65 protein bands normalized to the GAPDH protein. (C) Representative Western blots of p-I κ B- α , total I κ B- α and GAPDH. (D) Histograms represent means and SD of the ratio of p-I κ B- α protein bands normalized to the GAPDH protein. (E) Histograms represent means and SD of the ratio of I κ B- α protein bands normalized to the GAPDH protein. (F) Immunofluorescence analysis was performed for the identification of p65 translocation (red arrows show the translocation of the NF- κ B p65 subunit from the cytosol to the nucleus). Mean \pm SD. $n=3$. * $P<0.05$, ** $P<0.01$ compared with the TNF- α group.

Carbocisteine inhibited TNF- α -induced NF- κ B activation in HEK 293 cells

As a potent activator, TNF- α could directly activate NF- κ B signaling pathways in many cells, including HEK 293 cells^[28]. Consequently, we determined whether TNF- α -induced NF- κ B

activation could also be inhibited by carbocisteine in HEK 293 cells. As shown in a luciferase reporter assay, carbocisteine alone had no effects on the phosphorylation of NF- κ B, and the TNF- α -induced activation of NF- κ B was dose-dependently ameliorated by pre-treatment with carbocisteine (Figure 4).

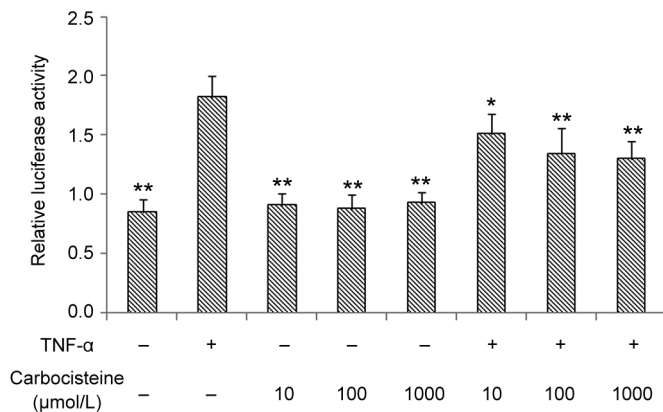


Figure 4. Carbocisteine attenuated TNF- α -induced NF- κ B activation in HEK293 cells. Mean \pm SD. $n=3$. * $P<0.05$, ** $P<0.01$ compared with the TNF- α group.

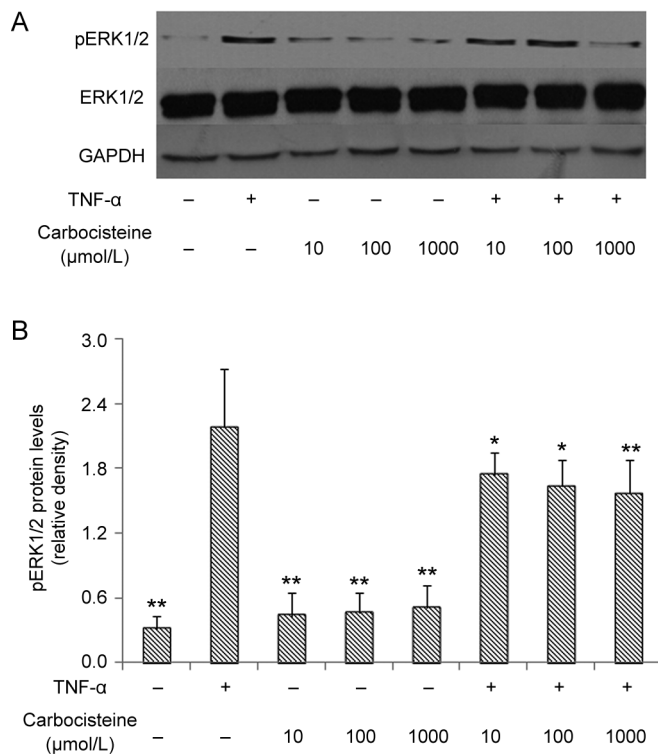


Figure 5. Carbocisteine inhibited TNF- α -induced ERK1/2 activation in A549 cells. (A) Representative Western blots of pERK1/2, total ERK1/2 and GAPDH. (B) Histograms represent the means and SD of the ratio of pERK1/2 protein bands normalized to the GAPDH protein. Mean \pm SD. $n=3$. * $P<0.05$, ** $P<0.01$ compared with the TNF- α group.

These findings were consistent with those of the A549 cell line.

Carbocisteine inhibited TNF- α -induced ERK1/2 MAPK activation in A549 cells

It has been reported that TNF- α could activate the ERK1/2 MAPK signaling pathway in A549 cells^[29]. As demonstrated

in Figure 5, ERK1/2 MAPK phosphorylation was observed at 30 min in response to TNF- α induction. Pre-treatment with carbocisteine significantly abolished ERK1/2 MAPK activation in a dose-dependent fashion in A549 cells compared with TNF- α alone.

Discussion

Pro-inflammatory cytokines (*ie*, TNF- α) play pivotal roles in triggering and amplifying the inflammatory response leading to lung tissue injury in COPD. In this study, we investigated the anti-inflammatory effects of carbocisteine using TNF- α -induced A549 cell and HEK 293 cell models. Carbocisteine significantly reduced IL-6 and IL-8 production, and attenuated IL-6, IL-8, TNF- α , IP-10 and MIP-1 β mRNA expression, which was accompanied by suppressed NF- κ B and ERK1/2 MAPK phosphorylation in TNF- α -stimulated A549 cells. In addition, by using an NF- κ B luciferase reporter system consisting of the stably transfected cell line HEK293/NF- κ B-luc, we also found that carbocisteine inhibited the TNF- α -induced transcriptional activity of NF- κ B.

TNF- α aggravates airway inflammation *via* the activation of inflammatory signaling pathways, such as NF- κ B and MAPKs. It has been demonstrated that various pathogens and cytokines activate NF- κ B signaling, and multiple inflammatory cytokines and chemokines are target genes of NF- κ B^[30]. MAPKs are evolutionarily conserved mediators in signaling pathways, which modulate embryogenesis, gene expression and cell functions. MAPKs also mediate pro-inflammatory gene transcription via phosphorylation^[31]. TNF- α plays pivotal role in triggering down-stream airway inflammation in many respiratory diseases, including COPD; therefore, the attenuation of TNF- α -mediated inflammatory responses might achieve considerable therapeutic outcomes.

Apart from the anti-inflammatory capacity reported previously^[10], carbocisteine could suppress IL-6 and IL-8 release and inhibit IL-6, IL-8, TNF- α , IP-10 and MIP-1 β mRNA expression after TNF- α stimulation. Our findings were consistent with a previous report which documented that carbocisteine reduced the levels of IL-1 β , IL-6, and IL-8 at 5 days following FluA virus infection and diminished baseline concentrations of these cytokines prior to infection in human airway epithelial cells^[27]. Similar findings have also been reported in human tracheal epithelial cells following rhinovirus or respiratory syncytial virus infection^[7, 8]. Furthermore, Nogawa *et al* showed that carbocisteine inhibited IL-1 β -induced IL-6 and IL-8 release in NCI-292 cells, as did N-acetylcysteine (NAC)^[4]. These findings further supported the cytoprotective effects of carbocisteine against airway inflammation in COPD. Because the exacerbation of COPD has been associated with a substantial release of pro-inflammatory cytokines and chemokines^[32, 33], the inhibitory effects of carbocisteine, as confirmed by previous studies and our team, may lend support to interpreting the reduced frequency of exacerbations in COPD.

We evaluated the roles of the NF- κ B and MAPK signaling pathways to explore the mechanisms by which carbocisteine modulates TNF- α -induced inflammation. Our results

showed that the inhibitory effects of carbocysteine were mainly attributed to the inactivation of NF- κ B (by inhibiting NF- κ B translocation from cytoplasm to nucleus and attenuating NF- κ B phosphorylation) and ERK1/2 MAPK (by attenuating ERK1/2 phosphorylation) following TNF- α stimulation. This was in line with the findings of Yamaya and colleagues, who also reported that carbocysteine inhibited FluA virus-induced inflammation partially by decreasing the activation of NF- κ B DNA-binding activity^[27]. In a pilot study that investigated the effects of carbocysteine on TNF- α -induced phosphorylation of p38 MAPK and JNK in A549 cells, we showed that carbocysteine conferred no effects on these signaling pathways. Furthermore, we also noted a dose-response inhibitory effect of carbocysteine on airway inflammation in this study. This suggested that greater doses of carbocysteine or other thiol-containing mucolytics would be needed to achieve better therapeutic outcomes, such as a reduction in the future risk of COPD exacerbation. This was consistent with the findings of the PANTHEON^[34] and HIACE^[35] studies, which utilized high-dose drugs, whilst administration of the relatively low-dose N-acetylcysteine failed to produce a favorable response^[36]. Further studies on different cell lines are needed to determine if longer durations of treatment with carbocysteine would lead to more favorable protective effects on airway inflammation and oxidative stress.

There are limitations that should be addressed. It has also been reported that carbocysteine harbors other anti-inflammatory targets, such as nuclear factor-erythroid 2-related factor (Nrf2), which is a redox-sensitive transcription factor in COPD^[37, 38] that can be activated by TNF- α in certain cell lines^[39]. Via the activation of Nrf2, carbocysteine attenuated inflammation *in vivo* after exposure to cigarette smoke following influenza virus infection^[40] and alleviated cigarette smoke extract (CSE)-induced oxidative stress *in vitro*^[5]. It remains to be further revealed whether Nrf2 is involved in the regulation of TNF- α -stimulated inflammation by carbocysteine. Our principal findings have not been replicated in animal (*in vivo*) studies. Furthermore, we could not verify the effects of prolonged use of carbocysteine on reducing COPD exacerbation because there has not been consensus on the duration of administration for *in vivo* studies. Further studies are warranted to confirm whether carbocysteine would antagonize inflammatory mediators other than TNF- α , the principal biomarker contributing to the amplification of airway inflammation.

In conclusion, we have demonstrated that carbocysteine was effective in suppressing inflammation in human alveolar epithelial cells upon stimulation with TNF- α *in vitro*. These effects are intimately associated with the inhibition of the NF- κ B and ERK1/2 MAPK signaling pathways, suggesting that carbocysteine harbors considerable therapeutic potential for the prevention and management of airway inflammation in COPD.

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Author contribution

Wei WANG and Jin-ping ZHENG designed research; Wei WANG, Wei-jie GUAN, Jin-ping ZHENG, Shao-xuan ZHU, Mao CHEN, and Nan-shan ZHONG contributed to study conception; Wei WANG, Rong-quan HUANG, and Yan-qing XIE performed research; Wei WANG performed statistical analysis; Wei WANG and Wei-jie GUAN drafted the manuscript.

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