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Ambroxol alleviates ventilator-induced lung injury by inhibiting c-Jun expression

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Abstract. – OBJECTIVE: Ventilator-induced lung injury (VILI) remains a challenge. This study was designed to investigate the effects of ambroxol on VILI and the underlying mechanisms in a rodent model.

MATERIALS AND METHODS: Male Wistar rats weighing 310-380 g were divided into four groups (n=8 per group): 1) saline only, 2) ventilation plus saline, 3) ventilation plus ambroxol (2 mg/kg), and 4) ventilation plus ambroxol (50 mg/kg). Rats in groups 1 and 2 were treated (i.p.) with 2.5 ml of saline once a day for six days and last injected 1 h prior to tracheotomy. Rats in groups 3 and 4 received ambroxol on the same schedule. Rats were ventilated for 90 minutes at a tidal volume (VT) of 30 ml/kg. The expression levels of c-Jun, a component of activator protein-1 (AP-1), and gamma-glutamylcysteine synthetase (γ-GCS), the rate-limiting enzyme in the synthesis of glutathione (gamma-glutamyl-cysteinyl-glycine, GSH), an endogenous antioxidant, were measured with immunohistochemical staining and *in situ* hybridization. Both AP-1 and GSH are involved in VILI.

RESULTS: Ambroxol at 50 mg/kg inhibited ventilation-induced lung inflammation, significantly elevated the ventilation-induced down-regulation of γ-GCS mRNA and protein, and significantly decreased the ventilation-induced up-regulation of c-Jun mRNA and protein. It has been reported that reactive oxygen species (ROS) can activate AP-1, leading to the production of pro-inflammatory cytokines and lung inflammation.

CONCLUSIONS: Ambroxol increases γ-GCS to promote GSH production, which in turn, inhibits ROS-dependent AP-1 activation and inflammation.

Key Words:
Ambroxol, Mechanical ventilation, Acute lung injury, Active protein-1, γ-Glutamylcysteine synthetase.

Introduction

Mechanical ventilation, commonly used to treat critically ill patients, often causes ventilator-induced lung injury (VILI) or aggravates a ventilator-associated lung injury (VALI), which can produce acute respiratory distress syndrome (ARDS)[1]. Many efforts have been made to reduce the risk of VILI and VALI, and ventilator strategies based on lower tidal volumes are the standard of care in Intensive Care Units[2]. However, the high morbidity and mortality rates associated with ARDS remain devastating problems[3,4]. The mechanisms of VILI have been investigated for the past 30 years but remain elusive. Imbalanced oxidative/antioxidative reactions are involved in the pathogenesis of VILI. Oxidative stress, caused by high levels of reactive oxygen species (ROS) and reactive nitrogen species, plays an important role in inflammatory responses by activating transcription factors such as activator protein-1 (AP-1)[5,6]. Mechanically ventilated lungs show increased AP-1 binding activity[7], and Fos, the main component of AP-1, is expressed in pulmonary epithelial cells undergoing mechanical stretch[8]. In epithelial cells, Jun-D, another component of AP-1, shows significantly increased recruitment to the 5'-flanking region of the F3 gene, which is implicated in the pathogenesis of lung injury[9]. These data indicate that AP-1 plays a key role in VILI. On the other hand, cytoprotective antioxidation is critical for cellular detoxification of ROS and reactive nitrogen species and for maintaining cellular redox homeostasis. Glutathione (gamma-glutamyl-cysteinyl-glycine, GSH) is an essential antioxidant tripeptide that protects mammalian cells against oxidants[10,11]. Pretreatment with the thiol antioxidants GSH and hyperoxia increases GSH in type II alveolar epithelial cells (A549), which in turn, inhibits the H_2O_2-induced cell injury and NF-kappa B binding that play key roles in lung inflammation.[12] The rate-limiting enzyme in GSH synthesis is gamma-glutamylcysteine synthetase (γ-GCS). Nitric oxide (NO) increases synthesis of the antioxidant GSH, and its protection against H_2O_2 in rat aortic endothelial cells depends on *de novo* biosynthesis of γ-GCS[13]. Consistent with *in vitro* studies, patients with idiopathic pulmonary fibrosis (IPF)[14] and lung allograft patients[15] have significantly less GSH

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in the epithelial lining fluid (ELF) of the lower respiratory tract than do normal subjects. Taken together, these data suggest that augmenting intracellular GSH might alleviate VILI. Ambroxol (2-amino-3,5-dibromo-N-[trans-4-hydroxycyclohexyl] benzylamine), a metabolite of bromhexine, shows antioxidant activities. Ambroxol significantly decreases heat- and H_2O_2-induced generation of conjugated dienes, the first lipid peroxidation product, in lung homogenates[16]. Moreover, ambroxol inhibits lipid peroxidation by 96% in rat liver mitochondria and by 74% in gastric mucosa and significantly decreases indomethacin-caused corpus gastric and antral lesions[17]. Further, a study showed that ambroxol treatment significantly decreased postoperative pneumonia and hypoxemia and increased oxygenation index within five days of operation in patients with acute cervical spinal cord injury[18]. In a clinical trial, ambroxol significantly decreased postnatally acquired pneumonia[19,20]. These data indicate that ambroxol may have protective effects on VILI. We hypothesized that ambroxol alleviates VILI by inhibiting AP-1 activity.

Materials and Methods

Animal Preparation

Male Wistar rats weighing 310-380 g (Laboratory Animal Center, Shanxi Medical University) were kept under controlled laboratory conditions (22°C, relative humidity 40-60%, 12 h alternate ligh-–dark cycles, food and water *ad libitum*) and were acclimatized to the environment for five days prior to experimentation. The animal protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of Shanxi Medical University [SCXK (Jin2009-0001)].

Experimental Design

Rats with tracheotomy were divided into four groups (n=8 per group): 1) saline only; 2) ventilation plus saline, 3) ventilation plus ambroxol (2 mg/kg), and 4) ventilation plus ambroxol (50 mg/kg). Rats in groups 1 and 2 were treated (i.p.) with 2.5 ml of saline once a day for 6 day prior to tracheotomy, and the last saline was given 1 h before tracheotomy. Rats in groups of 3 and 4 were treated with ambroxol on the same schedule.

Tracheotomy

Animals were anesthetized with urethane (1.2 mg/kg), and tracheotomies were performed under aseptic conditions. An endotracheal tube (2-mm inner diameter) was inserted and tightly tied to avoid air leaks. Rats were ventilated on an artificial animal ventilator (Model DH-150, Zhejiang University Medical Instrument Co., Ltd., Hangzhou, China) for 90 minutes at a tidal volume (V_T) of 30 ml/kg, an inspiration-to-expiration ratio (I:E) of 1:3, a pressure-controlled fractional-inspired oxygen concentration (FiO_2) of 21%, and a respiratory rate of 60 breaths a minute.

Lung Preparation

After mechanical ventilation, each rat was euthanized by abdominal aortic bloodletting. The right lung was removed and fixed in 10% neutral formalin for 24 hours and regularly embedded in paraffin. The tissues were sectioned at 6 μm for haematoxylin and eosin (HE) staining (Boster, Wuhan, China) with a standard protocol, immunohistochemical staining, and *in situ* hybridization. All sections were scrutinized by an investigator who was blinded to the experimental groups.

Immunohistochemical Staining for c-Jun and γ-GCS

A standard ABC method was used. Briefly, paraffin-embedded sections were deparaffinised with xylene and hydrated with an alcohol gradient. After the antigen was retrieved with boiled 0.01 M citrate buffer (Beyotime, Shanghai, China), endogenous peroxidase activity was blocked with 3% H_2O_2 and non-specific binding sites were blocked with 2% normal goat serum, the sections were incubated overnight at room temperature in rabbit polyclonal antisera against c-Jun (1:50; Boster Biological Technology, Ltd., Wuhan, China) or γ-GCS (1:50; Boster Biological Technology, Ltd., Wuhan, China). After incubation in secondary biotinylated goat-anti-rabbit antiserum (1:100) for 1 h at room temperature, sections were incubated in avidin and biotinylated horseradish peroxidase (HRP) complex (1:100) for another hour at room temperature. The reaction product was developed for 5 minutes in 0.05% 3,3'-diaminobenzidine (DAB) tetrahydrochloride (Sigma-Aldrich, St. Louis, MO, USA) containing 0.01% H_2O_2 in 0.1 M phosphate buffer. Sections were rinsed in three changes of phosphate-buffered saline (PBS) for 10 minutes each between incubations. Finally, the sections were counterstained with haematoxylin. Five sections randomly selected from each animal were analyzed under an Olympus CX21 microscope (Olympus

Optical Co., Ltd., Tokyo, Japan) to quantify the optical density of AP-1 and γ-GCS-like immunoreactive staining in the lung (BI-2000, Chendu Technology Co., Ltd., Chendu, China). Optical density was averaged for each rat and then for the group. Control sections were incubated as above, but the primary antibody was replaced with PBS, which produced no staining.

In Situ Hybridization of c-Jun and γ-GCS mRNA

In situ hybridization of AP-1 and γ-GCS mRNA were performed following the Boster protocol (Boster Biological Technology, Ltd., Wuhan, China). Briefly, the sections were deparaffinised and hydrated as above. Endogenous peroxidase activity was blocked by incubating sections in 3% H_2O_2 for 10 minutes. Next, the sections were digested for 30 minutes at 37°C with freshly prepared pepsin (1:10 in 3% citric acid) to expose mRNA. After being fixed in 1% paraformaldehyde for 10 minutes at room temperature, the sections were pre-hybridized for 4 hours at 38°C, then hybridized overnight at 38°C in a hybridization buffer containing three digoxin-labelled probes at 18-23 bps. The sections were next incubated with mouse anti-digoxin antibodies and developed with DAB. Sections were rinsed in PBS, distilled water, or saline-sodium citrate buffer between incubations. After being counterstained with haematoxylin, the sections were cover slipped, observed, and analysed as in the immunohistochemistry experiment. Positive staining presented as brown and yellow cytoplasm. Mismatched probes produced no staining (data not shown).

Statistical Analysis

Data are presented as the mean ± SD (Standard Deviation) and were analysed using one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls test for multiple comparisons between groups (Statistical Product and Service Solutions (SPSS) 13.0) (SPSS Inc., Chicago, IL, USA). $p<0.05$ was set as the level of statistical significance.

Results

Inflammatory Response

Unventilated control rats showed normal alveolar septa, complete bronchial epithelia, and no inflammatory cells. Ventilated, saline-injected rats displayed diffused pulmonary interstitial oedema, infiltration of inflammatory cells, broken alveoli, ruptured blood vessels, and bleeding in the alveolar cavity (Figure 1A). Ventilated rats given high-dosage (50 mg/kg) ambroxol exhibited pulmonary interstitial oedema and infiltration of inflammatory cells to a lesser extent than did ventilated rats given saline (Figure 1B). A lower dosage (2 mg/kg) of ambroxol did not alleviate the ventilation-induced inflammatory effect (data not shown).

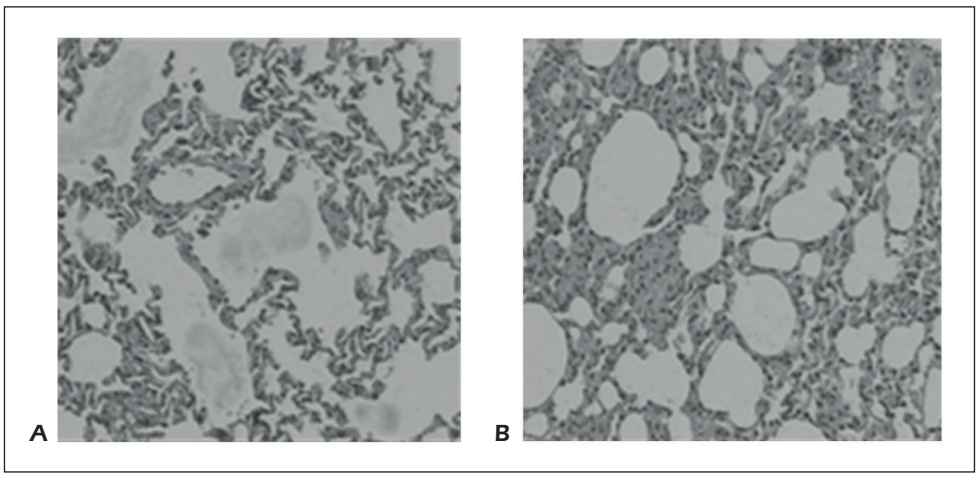

Figure 1. A, HE staining of lung sections in mechanically ventilated, saline-treated rats. Note that blood cells penetrated the alveolar space (magnification 100X). **B**, HE staining of lung sections from mechanically ventilated, ambroxol (50 mg/kg)-treated rats. Note the broken alveolar septa (magnification 100X).

Table I. Effect of ambroxol on ventilation-induced c-Jun and γ-GCS immunostaining measured by optical density (Mean ± SD).

Groups	C-Jun	γ-GCS
Saline only	0.23±0.06	0.61±0.08
Ventilation + saline	0.51±0.11*	0.30±0.04*
Ventilation + 2 mg/kg ambroxol	0.53±0.04*	0.34±0.00*
Ventilation + 50 mg/kg ambroxol	0.37±0.05*#	0.49±0.05*#

*p<0.01 compared to saline only; #p<0.01 compared to ventilation plus saline.

C-Jun and γ-GCS Protein Expression

As shown in Table I and Figure 2, c-Jun was significantly (p<0.01) up-regulated in rats on ventilation plus saline compared to saline alone (0.51 *vs.* 0.23). High-dosage (50 mg/kg) ambroxol treatment significantly decreased the optical density of c-Jun immunostaining from 0.51 to 0.37. Low-dosage (2 mg/kg) ambroxol had no effect on c-Jun expression. In contrast, γ-GCS was significantly (p<0.01) down-regulated in rats on ventilation plus saline compared to saline alone (0.30 *vs.* 0.61). High-dosage ambroxol significantly increased the optical density of γ-GCS immunostaining from 0.30 to 0.49. Low dosage showed no effect on γ-GCS expression. The data demonstrate that ambroxol significantly eases ventilation-induced c-Jun up-regulation and γ-GCS down-regulation.

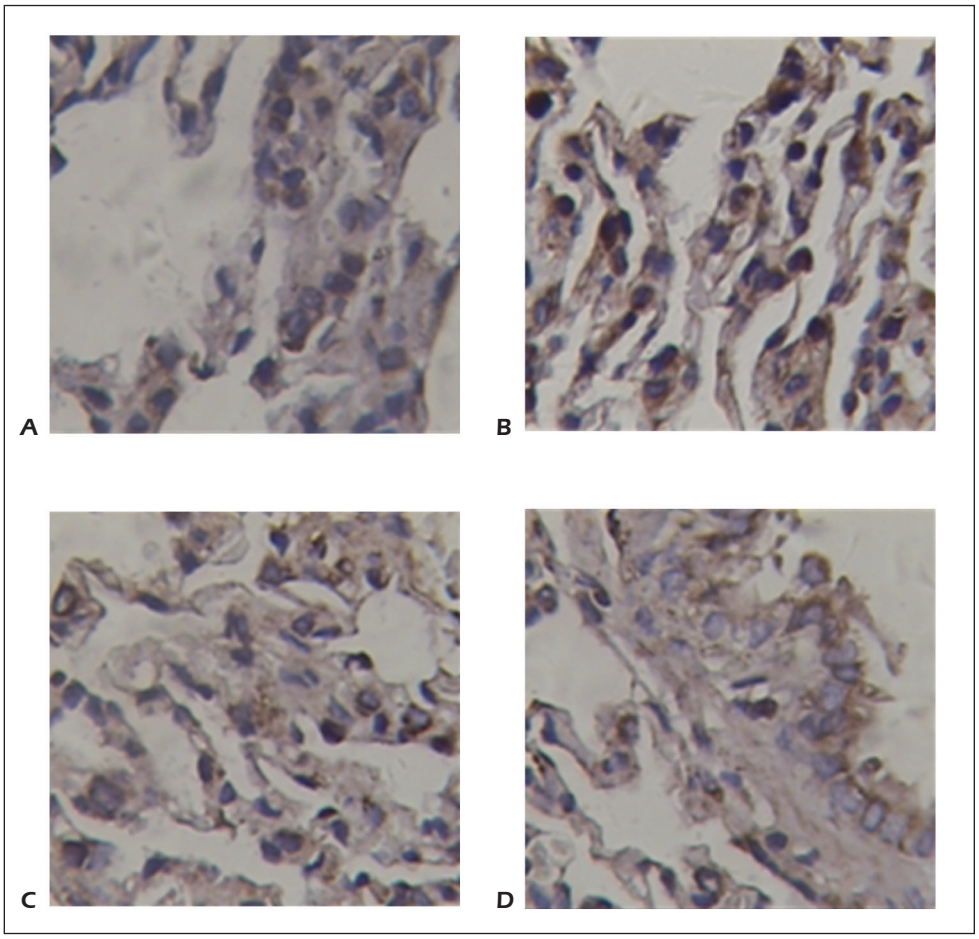

Figure 2. Immunohistochemical c-Jun (**A**, **B**) and γ-GCS staining (**C**, **D**) on lung sections from mechanically ventilated, saline-treated rats (B, D) or from ventilated, 50 mg/kg ambroxol-treated rats (**A**, **C**). **A**/**B**, Ambroxol treatment decreased the staining (brown colour) of c-Jun in **B** compared to saline control in A. **C**/**D**, Ambroxol treatment enhanced γ-GCS staining in **D** compared to saline control in **C** (magnification 400X).

Table II. Effect of ambroxol on ventilation-induced c-Jun and γ-GCS mRNA expression measured by optical density (Mean ± SD).

Groups	C-Jun mRNA	γ-GCS mRNA
Saline only	0.27± 0.04	0.72± 0.06
Ventilation + saline	0.63 ± 0.04*	0.31± 0.06*
Ventilation + 2 mg/kg ambroxol	0.58 ± 0.06*	0.34±0.07*
Ventilation + 50 mg/kg ambroxol	0.45 ± 0.02*#	0.49±0.02*#

*p<0.01 compared to saline only; #p<0.01 compared to ventilation plus saline.

C-Jun and γ-GCS mRNA Expression

As shown in Table II and Figure 3, c-Jun mRNA was significantly (p<0.01) up-regulated in rats on ventilation plus saline compared to saline alone (0.63 *vs.* 0.27). High-dosage (50 mg/kg) ambroxol significantly decreased the optical density of c-Jun mRNA in *in situ* hybridization staining from 0.63 to 0.45. The low dosage (2 mg/kg) had no effect on c-Jun mRNA expression. In contrast, γ-GCS mRNA was significantly (p<0.01) down-regulated in rats on ventilation plus saline compared to saline alone (0.31 *vs.* 0.72). High-dosage ambroxol significantly increased the optical density of γ-GCS mRNA from 0.31 to 0.49. Low dosage had no effect on γ-GCS mRNA expression. The data demonstrate that ambroxol significantly eases ventilation-induced c-Jun mRNA up-regulation and γ-GCS mRNA down-regulation.

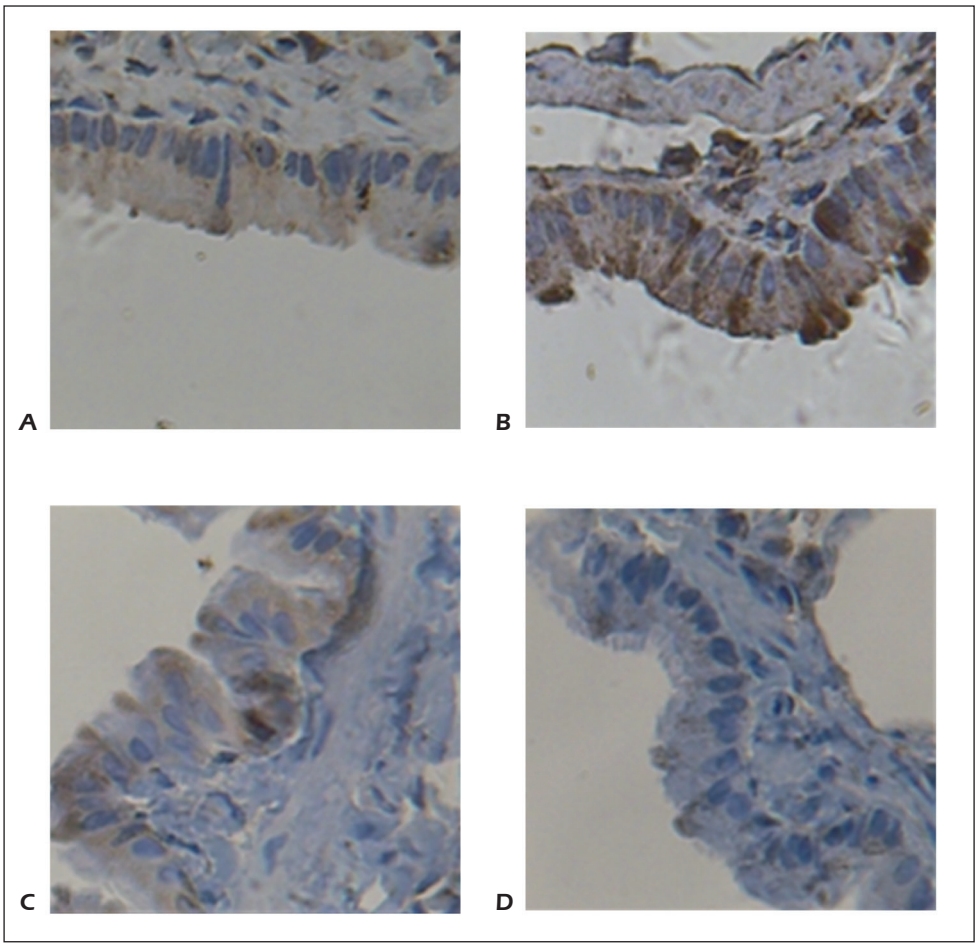

Figure 3. In situ hybridization of c-Jun mRNA (**A**, **B**) and γ-GCS mRNA (**C**, **D**) on lung sections from mechanically ventilated, saline-treated rats (**B**, **D**) or from ventilated, 50 mg/kg ambroxol-treated rats (**A**, **C**). **A**/**B**, Ambroxol treatment decreased c-Jun mRNA staining (brown colour) in **B** compared to saline control in **A**. **C**/**D**, Ambroxol treatment enhanced γ-GCS staining in D compared to saline control in **C** (magnification 400X),

Discussion

In the present study, mechanical ventilation induced significant acute lung injury, which was evidenced by diffused pulmonary interstitial oedema, inflammatory cell infiltration, alveolar and small blood vessel rupture, and alveolar haemorrhage. This result is consistent with previous reports[21]. Our study further demonstrates that ambroxol alleviates inflammatory responses and eases ventilation-induced c-Jun mRNA and protein up-regulation as well as γ-GCS mRNA and protein down-regulation. Ambroxol inhibition of inflammation has been reported[22]. In a previous study, ambroxol minimized lipopolysaccharide (LPS, i.p.)-induced leukocyte accumulation in rat lungs[23]. Further, in a mouse model of acute lung injury induced by intratracheally instilled LPS, ambroxol treatment decreased the synthesis of pro-inflammatory cytokines in bronchoalveolar lavage. Consistent with those studies, our data demonstrate that ambroxol treatment alleviates mechanical ventilation-caused lung inflammation. The mechanisms of ambroxol inhibition of inflammation have not been understood. The promoter region of the human γ-GCS heavy subunit gene contains a putative c-Jun homodimer (AP-1) binding site[24], suggesting that AP-1 may modulate γ-GCS expression. It was reported that Jun-containing AP-1 up-regulated γ-GCS mRNA and GSH synthesis[25]. However, it has also been reported that GSH pre-treatment decreased asbestos-induced up-regulation of AP-1 proto-oncogene mRNA, including c-Jun, JunB, c-Fos, and Fra-1, a close relative of the FOS gene product in terms of its structure and functional activity[26]. ROS may activate AP-1. For instance, cyclic strain induces monocyte chemotactic protein-1 (MCP-1) in endothelial cells by elevating intracellular ROS and subsequent AP-1 activation[27]. Hyperoxia significantly increases ROS generation, AP-1 activation, and interleukin-8 (IL-8) production in human alveolar epithelial cells and can be alleviated by antioxidant superoxide dismutases[28]. An anti-inflammatory and antioxidant chemical, piclamilast, significantly inhibits H_2O_2-induced gene expression, including IL-8, a C-X-C chemokine, through mechanisms involving AP-1 activation and c-Jun phosphorylation[29]. GSH is an essential antioxidant tripeptide that protects mammalian cells against oxidants[10,11]. Of note, in the present study, ambroxol significantly increased γ-GCS, the rate-limiting enzyme in GSH synthesis. The data indicate that ambroxol increases γ-GCS, which in turn, promotes GSH synthesis to inhibit ROS production and lead to suppression of AP-1 activity. In fact, the present study demonstrates that ventilation-caused up-regulation of AP-1 can be significantly alleviated by ambroxol treatment. AP-1 is composed mainly of Jun and FOS gene products, and it binds to DNA to modulate transcription of other genes. For example, AP-1 mediates nickel compound-induced production of tumour necrosis factor-alpha (TNF-α) in bronchial epithelial cells[30] and silica-induced TNF-α in macrophage cells[31]. AP-1 is also involved in TNF-α induction of IL-8 in lung epithelial cells[32]. IL-8 acts as a chemoattractant for neutrophils and other immune cells. Moreover, AP-1 participates in the TNF-α-induced expression of transforming growth factor beta-1 (TGF-β1) in lung fibroblasts[33]. TGF-β1 is a potent inhibitor of GSH synthesis in lung epithelial cells[34]. Together, these pro-inflammatory cytokines work to induce inflammation. Thus, ambroxol inhibition of c-Jun may contribute to ambroxol-produced alleviation of ventilation-caused lung inflammation, which is supported by evidence that ambroxol decreases cytokine production[22, 35].

Conclusions

Ambroxol increases γ-GCS to promote GSH production, which in turn inhibits AP-1 activation and inflammation.

Acknowledgements

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Conflict of Interests

The authors declared no conflict of interest.

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