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Cigarette smoke enhances β-defensin 2 expression in rat airways via NF-κB activation

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Running head: Cigarette smoke and β-defensin 2

Abstract

β-defensin 2 (BD-2), an antimicrobial peptide, participates in airway defense. Cigarette smoke (CS) is the major risk factor of chronic obstructive pulmonary disease. This study mainly aims to investigate the effect of CS on rat BD-2 (rBD-2) expression in rat airways.

Rats were exposed to CS treated with caffeic acid phenethyl ester (CAPE), a nuclear factor (NF)-κB inhibitor, or astragaloside IV (AS-IV), an active ingredient of Astragalus mongholicus. Besides the analysis of bronchoalveolar lavage fluid (BALF) and histological changes after CS exposure, rBD-2 expression was investigated with immunohistochemistry, RT-PCR and ELISA. Total glutathione and nitric oxide (NO) levels in rat lungs were also detected.

CS exposure markedly increased rBD-2 immunoreactivity, as well as rBD-2 mRNA and protein levels in rat airways, which were inhibited by CAPE treatment. Moreover, associated airway inflammation induced by CS was evidenced by histological changes, increased cell counts and proinflammatory cytokines in BALF, and NF-κB activation and high levels of total glutathione and NO, which were all reversed by AS-IV in a dose-dependent fashion.

CS exposure induces rBD-2 expression in rat airways via a NF-κB-dependent pathway, and AS-IV attenuates CS-induced airway inflammation due to its anti-inflammatory and antioxidant properties, at least partly through NF-κB inactivation.

Keywords: astragaloside IV; beta-defensins; nuclear factor kappa B; smoke

Introduction

β-defensins (BDs) is a family of cationic antimicrobial peptides, which plays an important role in mucosal defense [(1)]. Human BD-2 (hBD-2) has been documented to express at many muscosal surfaces, including airway epithelium [(2)]. In airways, hBD-2 mRNA is expressed at low levels in resting cells but is markedly induced by proinflammatory stimuli, including IL-1β, TNF-α, and Pseudomonas aeruginosa [(2, 3)]. Further, hBD-2 has been suggested to function as a chemotactic factors of T cells, dendritic cells and monocytes in airways [(4)]. These observations suggest that BD-2 may be an important part of airway inflammatory-immune response.

Cigarette smoke (CS) is a critical etiological factor of chronic obstructive pulmonary disease (COPD) [(5, 6)], and is also supposed to suppress airway defense system [(7, 8)]. However, little is known about the effect of CS on BD-2 expression. Very recently, Lee et al and Mahanonda et al reported that acrolein (a major component of cigarette smoke) and cigarette smoke extract, suppressed hBD-2 expression by sinonasal epithelial cells and gingival epithelial cells [(9, 10)] and another study by Herr and his colleagues indicated decreased levels of hBD-2 were found in pharyngeal washing fluid and sputum from current and former smokers with acute pneumonia compared with that from never smokers, and smoke exposure reduced in vitro hBD-2 expression in airway epithelium in response to bacterial stimulation [(11)]. However, Shibata et al recently reported mouse BD-2 (mBD-2) mRNA expression was enhanced in mouse lung tissues after 6-month CS exposure [(12)]. These findings neither suggested a determinate role of CS in BD-2 expression, nor directly described the effect of CS on BD-2 expression in airways. Therefore, for further determination of the effect of CS on BD-2 expression in airways and exploration of the underlying mechanisms, a CS-exposed rat model was used, with treatment of caffeic acid phenethyl ester (CAPE), an inhibitor of nuclear factor (NF)-κB.

In addition, astragaloside IV (AS-IV) is a new glycoside of cycloartane-type triterpene isolated from the root of Astragalus membranaceus Bunge, a Chinese medicinal herb, which has been reported to have protective effects on various animal and in vitro disease models [(13-17)]. As the effect of AS-IV on CS-induced airway inflammation is not clear, the

investigation of the effect of AS-IV on this process was also performed in this study.

Methods

Reagents

AS-IV, extracted from Astragalus membranaceus Bunge (purity≥95%, using the HPLC method), was purchased from Xi'an Honson Biotechnology Co., Ltd (Xi'an, China). AS-IV was suspended in a 1% carboxymethyl-cellulose (CMC) solution at different concentrations of 0.03% (low, AL), 0.06% (moderate, AM) and 0.12% (high, AH). CAPE (Sigma, St. Louis, MO) was dissolved in the dimethyl sulfoxide (DMSO) solution, and a dose of 30 mg/kg bodyweight [18] was used in the present study.

Animal Treatment

Male wistar rats (220±20g) were purchased from the Experimental Animal Center of Sichuan University. The animal study was approved by the Panel on Laboratory Animal Care of West China School of Medicine, Sichuan University. These animals were housed in a temperature- and humidity-controlled condition and kept on a 12-h light/dark cycle, with free access to food and water. Chambers and cages were washed every three days. Rats were randomly divided into 8 groups, with 5 rats per group, as follows: saline-treated group (SA group), AS-IV-treated groups (AH group), CS-exposed group (CS group), CS plus AS-IV groups (CS+AL, CS+AM, CS+AH groups); CAPE-treated group (CAPE group), and CS plus CAPE group (CS+CAPE group).

After a 1-week acclimatization period, rats were exposed to the smoke of 5 cigarettes (a total of 1.0 mg nicotine and 14 mg tar in each cigarette) for 30 min, twice daily, 6 days/week for 4 weeks, according to a modified procedure based on the methods described by Izzotti et al and Ou et al [19, 20]**.** Briefly, rats were exposed to the smoke generated by 5 commercial cigarettes for 15 mins in the sealed whole-body exposure chambers with the volume of approximate 1.2 cubic meter**,** and then the cigarette smoke was discharged through the vent-pipe for a more 15 mins, which was blocked before CS exposure. Control rats were exposed to the air following the same schedule. Rats were administered by gavage with AS-IV suspension (1ml, once daily) and intraperitoneally with CAPE (30 mg/kg, qod) 30 mins before CS exposure. After 4 weeks CS exposure, all rats were anestethised intraperitoneally with chloral hydrate (3 ml/kg) and sacrificed by exsanguinations on the

day after the last exposure.

Analysis of Bronchoalveolar Lavage Fluid (BALF)

The left lung was lavaged three times with 1.5 ml of saline and the recovery rate is approximately 80%. The lavage fluid was centrifuged, and the cell pellet was resuspended in PBS, then the total cell number was determined by counting on a hemocytometer. Differential cell counts (minimum of 300 cells per slide) were performed on cytospin-prepared slides stained with Diff-Quik. IL-1β and TNF-α concentrations in the BALF were measured according to the instruction of the ELISA kits (R&D system, Minneapolis, MN).

Histology and Immunohistochemistry (IHC)

The middle lobe of right lung, not lavaged, was immersed in 4% phosphate-buffered paraformaldehyde to complete fixation and then embedded in paraffin, sectioned (4 μm), and finally stained with hematoxylin and eosin (H&E) to evaluate the morphological changes of lungs, and alcian blue (AB)-periodic acid Schiff (PAS) to evaluate the area stained for intracellular mucous glycoconjugates, respectively. Moreover, IHC analysis was performed, using a SP-HRP kit (Santa Cruz, CA). Sections were stained with a polyclonal anti-rat BD-2 (rBD-2) antibody (Santa Cruz, CA) with a dilution of 1:250, developed with DAB, and counterstained with hematoxylin. The positive stained areas of AB-PAS and rBD-2 in rat airways were quantified by Image-Pro Plus 4.5 software (Media Cybernetics, Bethesda, MD) according to the method described before [(21)].

RT-PCR

For total RNA extraction, lung tissues were stored at -80 ℃ after snap freezing in liquid nitrogen, and extracted using Trizol reagent (Invitrogen, Carlsbad, CA), and amplified using a PCR single-step kit (Bioteke Corporation, Beijing, China), according to the manufacturer's instructions. For RT-PCR, primers for rBD-2 and β-actin were designed using the sequence data from GenBank and pro/primer design software Primer Express (version 1.0; Applied Biosysthems, Frost City, CA). Primer sequences (forward/reverse, 5' to 3') were as follows: rBD-2, ATT TCT CCT GGT GCT GCT GTC /AGT CCA CAA GTG CCA ATC TGT C; β-actin, CCT CAT GAA GAT CCT GAC CG/ ACC GCT CAT

TGC CGA TAG TG. The annealing temperature for each primer pair was 60°C to rBD-2 and 58°C to β-actin, respectively. The products were separated by agarose gel electrophoresis and visualized by Gelview (Bioteke). Semiquantitative densitometric analysis was performed with the Bio-Rad Universal Hood and Quantity One software (Bio-Rad, Hercules, CA).

ELISA and Western Blot

Lung tissues for ELISA and Western Blot were homogenized in lysis buffers detailly described in our former articles [22, 23]. Protein concentration was quantitated by the Bicinchoninic Acid (BCA) Method (Pierce, Rockford, IL). rBD-2 level in rat lungs was determined according to the instruction of the commercial ELISA kit (Adlitteram Diagnostic Laboratories). For Western Blot, 30 μg of isolated soluble protein were separated by polyacrylamide gel electrophoresis, transferred onto polyvinylidene difluoride (PVDF) membranes, then treated with primary antibodies to P65, IκBα and β-actin (Santa Cruz, CA). After washing, bound antibody was detected using anti-rabbit linked to horseradish peroxidase, and the bound complexes were detected by Chemiluminescent HRP Substrate Kit (Millipore Corp., Marlborough, MA). Relative protein expression levels were quantified by densitometric measurement of chemiluminescent reaction bands and normalized as a percentage of the control bands using the Bio-Rad Quantity One software.

Electrophoretic Mobility Shift Assay (EMSA)

The assay is based on that DNA-protein complexes migrate slower than unbound DNA or double-stranded oligonucleotides in a native polyacrylamide or agarose gel, resulting in a "shift" in migration of the labeled DNA band. The detection of bands was by "The LightShift™ Chemiluminescent EMSA kit" (Pierce Biotechnology, Inc, Rockford, IL, USA) that uses a non-isotopic method to detect DNA-protein interactions. Biotin end-labeled DNA duplex of sequence 5'-AGT TGA GGG GAC TTT CCC AGG C-3' and 3'-TCA ACT CCC CTG AAA GGG TCC G-5' containing a putative binding site for nuclear factor κB was incubated with the nuclear extracts. After the reaction the DNA-protein complexes were subjected to a 6% native polyacrylamide gel electrophoresis and transferred to a nylon membrane (Pierce Biotechnology, Inc, Rockford, IL, USA). As gel shift controls, lane 1: contained biotin-labeled control oligonucleotide duplex with a binding site of:

5’-TAGCATATGCTA-3’ and 3’-ATCGTATACGAT-5’. Lane 2: contained Epstein-Barr virus nuclear extracts (EBNA) and biotin-labeled control oligonucleotide. Lane 3: contained EBNA extract, biotin-labeled control oligonucleotide and unlabeled control oligonucleotide for competitive binding inhibition. After transfer the membrane was immediately cross-linked for 15 min on a UV transilluminator equiped with 312 nm bulbs. A chemiluminescent detection method utilizing a luminol/enhancer solution and a stable peroxide solution (Pierce, Rockford, IL) was used as described by the manufacturer, and membranes were exposed to x-ray films for 2-5 min before developing. The relative intensities of bands were analyzed using the Bio-Rad Quantity One software.

Oxidative Stress Assays

Nitric oxide (NO) and total glutathione (GSH) levels in rat lungs were determined using the commercial kits from Beyotime Institute of Biotechnology (Shanghai, China). The generation of NO was measured with the Griess reaction [(24)]. In brief, 100 ul of medium was mixed with 100 ul Griess reagent in a 96-well plate. Nitrite concentration was quantified by an automated microplate reader (Bio-Rad, Hercules, CA) at 540 nm from a $NaNO_2$-derived standard curve. Total GSH content of the lung tissue was performed with an optimized enzymatic recycling method using GSH reductase [(25)]. The formation of 2-nitro-5-thiobenzoic acid (TNB), directly proportional to the concentration of GSH, was analyzed densitometrically at 412 nm using the microplate reader (Bio-Rad).

Statistical Analysis

All date are expressed as mean ± SEM. One-way analysis of variance (ANOVA) followed by Student-Newman-Keuls test was used to compare the differences among multiple groups. $P<0.05$ for the null hypothesis was accepted as indicating a statistically significant difference. SPSS 13.0 software package (SPSS, Inc., Chicago, IL) was used for statistical analysis.

Results

CS exposure enhances rBD-2 expression in rat airways

In order to confirm the effect of CS exposure on rBD-2 expression in rat airways, sections of lung tissues were prepared and immunostained for rBD-2. After 4 weeks of CS exposure, significantly increased immunoactivity of rBD-2 was predominately detected in the surface epithelium of bronchi compared with the SA controls **(Fig 1A, B)**. Likewise, in the RT-PCR and ELISA tests, increased rBD-2 mRNA **(Fig 1D, E)** and protein **(Fig 1C)** levels were also detected in the lungs of CS-exposed rats relative to the SA controls.

CS exposure enhances transcriptional activity of NF-κB and degradation of cytoplasmic IκB

NF-κB is maintained as a latent form in the cytoplasm of cells where it is complexed to IκB inhibitor proteins, and NF-κB activation involves the phosphorylation and subsequent degradation of the inhibitory protein [(26)]. To prove that transcriptional regulation of gene expression by NF-κB pathway is enhanced by CS exposure, nuclear translocation of P65/RelA subunits of NF-κB, P65 DNA banding capacity and the degradation of IκB inhibitory protein were assessed. Nuclear localization of P65 and cytoplasmic IκB degradation were analyzed by Western Blot. Significantly increased nuclear P65, as well as decreased levels of cytoplasmic IκBα, was detected in the lungs of rats exposed to CS compared with the SA controls, indicating increased NF-κB activity after CS exposure **(Fig 2A, B, C)**. To further determine P65 DNA banding capacity, EMSA shift assays were performed. The EMSA analysis showed that significantly increased P65 DNA binding capacity in rats exposed to CS, while no banding was detected in the SA group **(Fig 2D, E).**

CAPE inhibits CS-induced rBD-2 expression in rat lungs

In order to address the role of NF-κB in CS-induced rBD-2 expression, CAPE, a NF-κB inhibitor, was used to pretreat the rats before CS exposure. In the RT-PCR and ELISA analysis, CAPE pretreatment almost totally reversed CS-induced rBD-2 expression at both mRNA and protein levels **(Fig 1).**

AS-IV attenuates CS-induced inflammatory reaction in rat airways

Four-week repeated CS exposure markedly induced airway inflammation in rats, evidenced

by airway histological change **(Fig 3)**, and increased inflammatory cell counts and proinflammatroy cytokine levels in BALF **(Fig 4)**.

AS-IV treatment significantly attenuated CS-induced airway inflammatory reaction, including less hyperplastic changes of bronchus epithelium, decreased alveolar wall thickening, reduced inflammatory cells in alveoli and bronchiole wall, and decreased goblet cell hyperplasia **(Fig 3).** Likewise, decreased inflammatory cell influx (total cell, neutrophil, lymphocyte, and macrophage) **(Fig 4A)** and proinflammatroy cytokines levels (IL-1β and TNF-α) **(Fig 4B)** in the BALF were also demonstrated by AS-IV treatment.

AS-IV abrogates CS-induced NF-κB activation and oxidative stress

Effect of AS-IV on NF-κB activation was assessed by P65 translocation and DNA binding capacity. Pretreatment of rats with AS-IV inhibited CS-induced P65 translocation and DNA binding capacity in a dose-dependent fashion **(Fig 2)**. NO and total GSH levels were used to assess oxidative stress induced by CS exposure. Four weeks CS exposure significantly increased NO level and decreased total GSH contents, however, AS-IV treatment could reverse this fashion dose-dependently **(Fig 5)**.

Discussion

The present study demonstrated 4-week repeated CS exposure enhanced rBD-2 expression and associated inflammatory response in rat airways. Moreover, CS-induced rBD-2 mRNA and protein expression could be significantly inhibited by CAPE treatment, indicating firstly CS-induced rBD-2 expression was dependent on NF-κB pathway. Further studies showed that AS-IV could dose-dependently abrogate CS-induced NF-κB activation and oxidative stress, and subsequently attenuate CS-induced airway inflammation.

CS, the major risk factor for COPD development, is a potent source of oxidants, with over 4700 chemical compounds and high concentration of oxidants in the gas phase [(27)]. Repeated CS exposure followed by disordered inflammation damage to the local immunity in lungs, renders individuals to some degree more vulnerable to significant infections. On the other hand, recurrent and chronic infections is a driving force in the development of COPD. Antimicrobial peptides are important effector molecules of the lung innate immune system, and β-defensins, especially BD-2, are the principal family of antimicrobial peptides in respiratory tract, which are mainly produced by airway epithelial cells [(11)]. Lee et al, Mahanonda et al 's studies recently demonstrated a suppressive effect of CS on hBD-2 expression by cultured human sinonasal and gingival epithelial cells. Furthermore, Herr et al reported decreased hBD-2 level in pharyngeal washing fluid and sputum from smokers with acute pneumonia [(9-11)], indicating CS exposure possibly inhibits airway epithelial hBD-2 production, which links cigarette smoking with increased susceptibility of respiratory tract to microbial infections. However, Shibata et al reported upregulated mBD-2 mRNA expression in lung tissues after 6-month CS exposure [(12)], and we further demonstrated in the present study that 4-week repeated CS exposure stimulated rBD-2 mRNA and protein expressions in rat airways.

As is well-known, besides bacteria, fungi and some enveloped viruses [(28)], cytokines, such as TNF-α and IL-1β, can also stimulate hBD-2 expression. Repeated CS exposure induces airway inflammatory reactions, with enhanced proinflammatory cytokines levels, which are generally regulated by proinflammatory gene transcription factors. NF-κB is a redox-sensitive and proinflammatory gene transcription factor, which plays a critical role in

CS-associated inflammatory-immune response [29-31]. However, going back to our data, the potential role of NF-κB pathway in the CS-induced rBD-2 expression remains not clear. As shown in the present study, NF-κB activation, evidenced by the increased p65 NF-κB nuclear translocation and DNA binding capacity, and decreased level of cytoplasmic IκB after CS exposure, was paralleled with CS-induced rBD-2 expression, as well as elevated TNF-α and IL-1β levels in the BALF after CS exposure. Cloning of rBD-2 gene, homologous to hBD-2 [32], has revealed that it is unique among defensin genes, having three binding sites of the transcription factor NF-κB in the promoter region [33], which indicates a structural involvement between BD-2 expression and NF-κB activation. Noticeablely, both TNF-α and IL-1β are NF-κB-dependent proinflammatory mediators and have been determined to be stimulators of BD-2 expression via modulation by NF-κB [34, 35], suggesting a regulatory role of NF-κB in BD-2 expression by stimuli. Overall, these evidences support a potential role of NF-κB pathway in CS-induced BD-2 expression. Moreover, in the present study, inhibition of NF-κB pathway by CAPE treatment reversed CS-induced rBD-2 expression at both mRNA and protein levels, further confirming NF-κB activation is required for CS-induced rBD-2 expression. It is thus concluded firstly that NF-κB-dependent mechanism is the key to explain the findings of CS-induced rBD-2 expression in rat airways. Taken together, although the antimicrobial role of BD-2 in innate immunity has been determined, our data may throw a new light on the role of BD-2 in CS-associated airway diseases, such as COPD.

Astragalus mongholicus is a crude drug widely used in Chinese traditional medicine. AS-IV, an active monomer of Astragalus membranaceus, has been suggested to be a potentially protective role in various disorders [13-17], with immunoregulatory and anti-inflammatory functions [36, 37]. Noticeablely, Du et al reported AS-IV suppressed the progression of airway inflammation, airway hyperresponsiveness, and airway remodeling (goblet cell hyperplasiain and subepithelial fibrosis) in a ovalbumin-induced murine model of chronic asthma [17], indicating the protective effects of AS-IV on airway inflammatory injury. CS is the major environmental risk factor for airway inflammatory injury, and oxidative stress (oxidant/antioxidant imbalance) is a major component of CS-associated airway inflammation, leading to bronchial and alveolar damage and lung cell death. In this

process, excessive production of endogenous NO can directly cause respiratory tract injury, and on the other side, the homeostasis of GSH, the principle antioxidant in the lung, is essential for normal lung function [38, 39]. Our study firstly reported that AS-IV administration attenuated inflammatory reaction induced by CS exposure in rat airways. Simultaneously, CS-induced NF-κB activation and oxidative stress, evidenced by increased NO and decreased GSH levels, were also abrogated by AS-IV treatment. Overall, AS-IV may prevent CS-induced airway epithelium lesion due to its anti-inflammatory and antioxidant properties, and this protective effect may be at least in part through inactivation of NF-κB signaling.

In summary, combining the previous studies with our present findings, the pathophysiological role of BD-2 in CS-associated disorders could possibly be different from its protective effects on microbial infections, which still needs more investigations to determine. Furthermore, AS-IV, as a new active monomer of Astragalus mongholicus, may play a protective role in mucosal defense through its anti-inflammatory and antioxidant actions, which results in more beneficial effects during lung injury induced by CS exposure.

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Figure Legends

Figure 1. CS exposure enhances rBD-2 expression in rat airways and CAPE inhibits CS-induced rBD-2 expression

(A) Representative photomicrographs of rBD-2 immuno-stained sections were shown. Bar= 50 μm. (B) Semiquatitative analysis of immuno-stained area of rBD-2 in rat airways. (C) rBD-2 protein concentrations in lung tissues. (D) Representative bands of RT-PCR analysis (E) The mean ratios of photodensity of rBD-2 band to that of β-actin control. * P<0.05 .

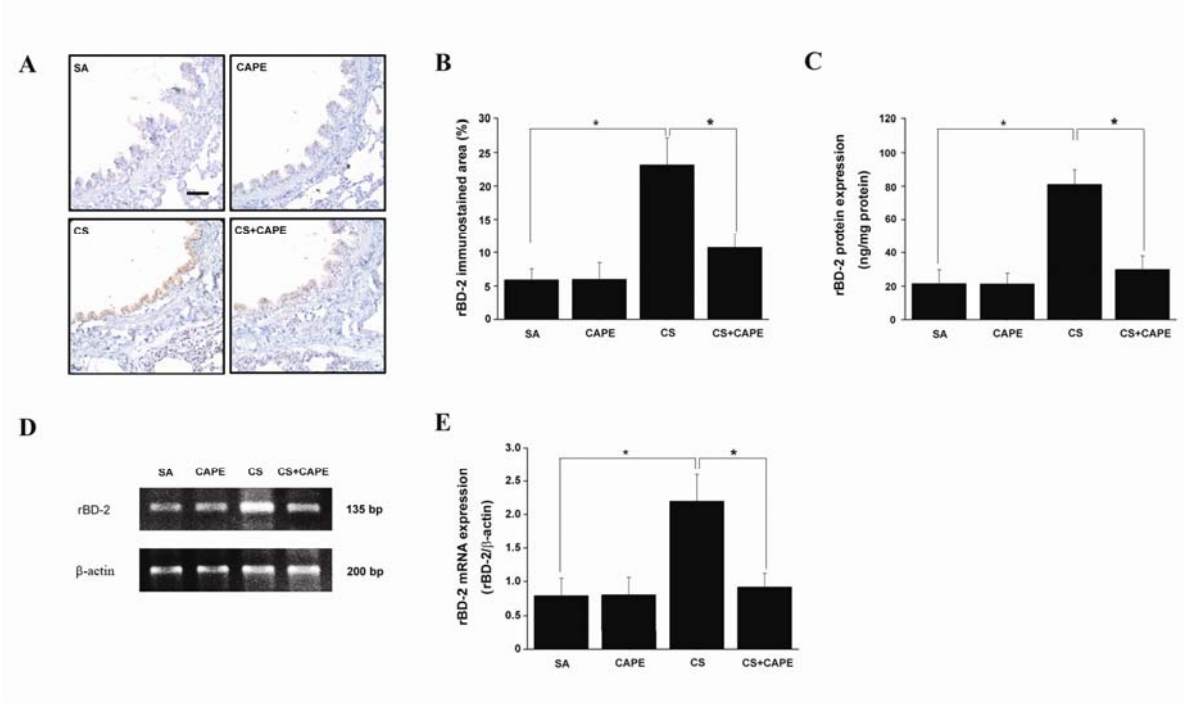

Figure 2. NF-κB activation after CS exposure and the effect of AS-IV on this process.

NF-κB activation was analyzed by Western Blot and EMSA. A) Representative bloting images of P65, IκBα and β-actin were shown, and densitometric analysis of P65 (B), IκBα (C) protein expression relative to the SA controls was presented. The results of EMSA were shown in (D). Lane 1: biotin-labeled control oligonucleotide; Lane 2: EBNA extract + biotin-labeled control oligonucleotide to act as positive control; Lane 3: EBNA extract + biotin-labeled control oligonucleotide + unlabeled control oligo nucleotide to act as negative control; Lane 4: nuclear protein extraction of cigarette smoke exposure group + biotin-labeled NF-κB oligonucleotide + unlabeled NF-κB oligonucleotide; Lane 5: biotin-labeled NF-κB oligonucleotide. and (E) DNA banding activity was quantitated as a

densitometric ratio of target band compared with EBNA control shifted band. * P<0.05 versus SA group, # P<0.05 versus CS group.

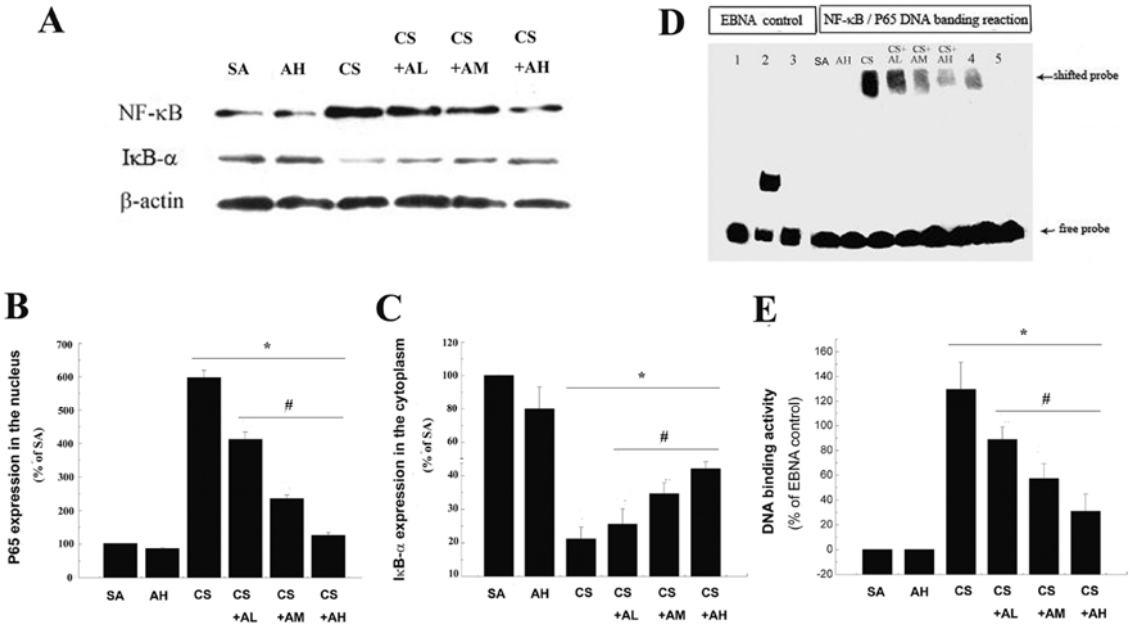

Figure 3. AS-IV attenuates CS-induced airway inflammation

Representative photomicrographs of HE and AB/PAS (insets) stained sections of lung tissues from each group were shown. Bar= 50 μm. (A) SA goup; (B)AH group; (C)CS group; (D) CS+AL group; (E) CS+AM group; (F) CS+AH group. (G) Semiquatitative analysis of AB/PAS-stained area in rat airways. * P<0.05 versus SA group, # P<0.05 versus CS group.

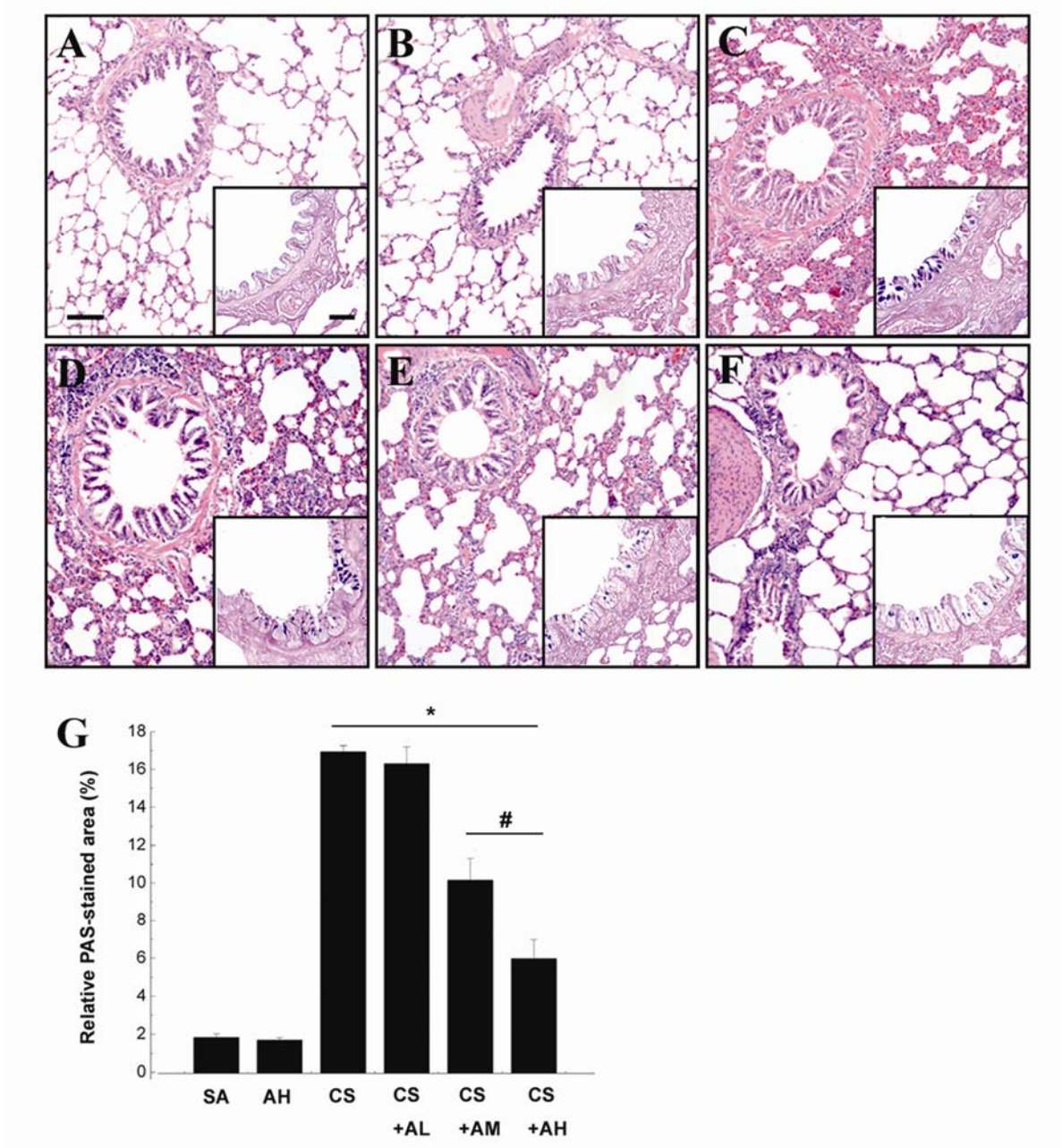

Figure 4. AS-IV decreases cell counts and proinflammatroy cytokines in BALF after CS exposure.

A) Total cells, neutrophils, lymphocytes, and macrophages in the BALF. B) IL-1β and TNF-α concentrations in the BALF. * $P<0.05$ versus SA group, # $P<0.05$ versus CS group.

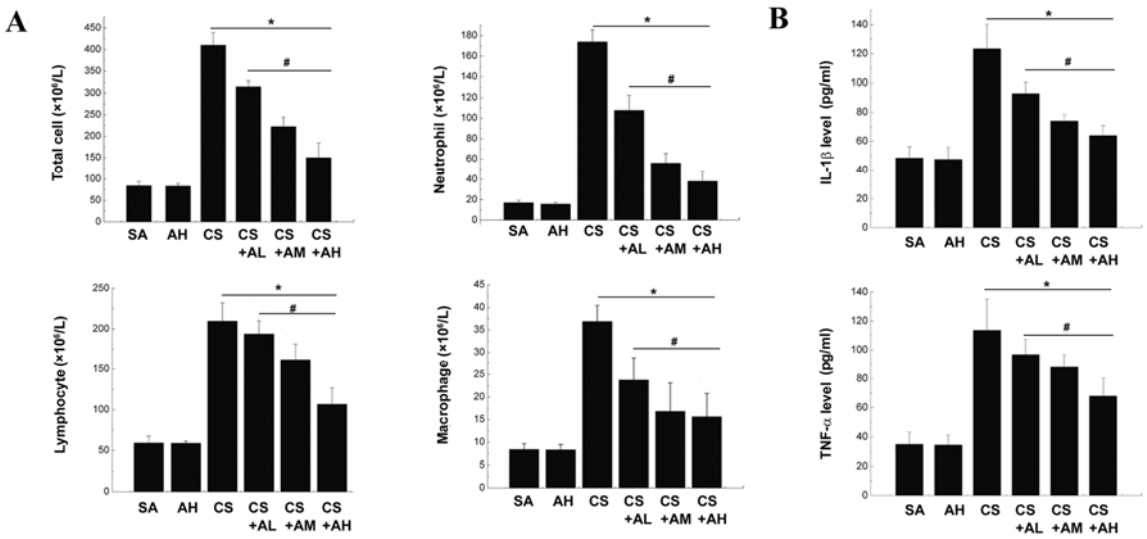

Figure 5. AS-IV reverses CS-induced total GSH and NO levels.

Alterations of NO level (A) and total GSH contents (B) in lung tissues were shown. * P<0.05 versus SA group, # P<0.05 versus CS group.