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Cigarette smoke enhances β-defensin 2 expression in rat airways *via* nuclear factor-κB activation

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ABSTRACT: β-defensin 2 (BD-2), an antimicrobial peptide, participates in airway defence. Cigarette smoke (CS) is a major risk factor for the development 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 and 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, reverse transcription 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 demonstrated by histological changes, increased cell counts and pro-inflammatory 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.

In conclusion, 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, β-defensin, nuclear factor-κB, smoke

The β-defensin (BDs) family of cationic antimicrobial peptides, plays an important role in mucosal defence [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 pro-inflammatory stimuli, including interleukin (IL)-1β, tumour necrosis factor (TNF)-α, and *Pseudomonas aeruginosa* [2, 3]. Furthermore, hBD-2 has been suggested to function as a chemotactic factor 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 aetiological factor of chronic obstructive pulmonary disease (COPD) [5, 6], and is also supposed to suppress airway defence system [7, 8]. However, little is known about the effect of CS on BD-2 expression. Very recently, LEE *et al.* [9] and MAHANONDA *et al.* [10] 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. Results from a study by HERR *et al.* [11] showed decreased levels of hBD-2 in pharyngeal washing fluid and sputum from current and former smokers with acute pneumonia compared with that from never smokers, and demonstrated that smoke exposure reduced *in vitro* hBD-2 expression in airway epithelium in response to bacterial stimulation. However, SHIBATA *et al.* [12] recently reported that mouse BD-2 (mBD-2) mRNA expression was enhanced in mouse lung tissues after 6-month CS exposure. These apparently contradictory findings neither suggested a defined 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, using treatment of caffeic acid phenethyl

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ester (CAPE), an inhibitor of nuclear factor (NF)-κB to delineate underlying pathways.

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%, assessed using high-performance liquid chromatography), was purchased from Xi'an Honson Biotechnology Co., Ltd (Xi'an, China). AS-IV was suspended in a 1% carboxymethylcellulose (CMC) solution at different concentrations: 0.03% (low, ''AL''), 0.06% (moderate; ''AM'') and 0.12% (high; ''AH''). CAPE (Sigma, St. Louis, MO, USA) was dissolved in the dimethyl sulfoxide (DMSO) solution, and a dose of 30 $mg \cdot kg^{-1}$ bodyweight was used [18].

Animal treatment

Male wistar rats (220±20 g) were purchased from the Experimental Animal Centre of Sichuan University (Chengdu, Sichuan, China). 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 3 days. Rats were randomly divided into eight groups, with five 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 and CS+AH groups); CAPE-treated group (CAPE group), and CS plus CAPE group (CS+CAPE group).

After a 1-week acclimatisation period, rats were exposed to the smoke of five cigarettes (a total of 1.0 mg nicotine and 14 mg tar in each cigarette) for 30 min, twice daily, 6 $days \cdot week^{-1}$ for 4 weeks, according to a modified procedure based on the methods described by IZZOTTI *et al.* [19] and OU *et al.* [20]. Briefly, rats were exposed to the smoke generated by five commercial cigarettes for 15 mins in the sealed whole-body exposure chambers with a volume of ~1.2 m^{-3}, and then the cigarette smoke was discharged through the vent-pipe for a further 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 (1 mL, once daily) and intraperitoneally with CAPE (30 $mg \cdot kg^{-1}$, *q.o.d.*) 30 mins before CS exposure. After 4 weeks of CS exposure, all rats were anaesthetised intraperitoneally with chloral hydrate (3 $mL \cdot kg^{-1}$) and sacrificed by exsanguinations on the day after the last exposure.

Analysis of bronchoalveolar lavage fluid

The left lung was lavaged three times with 1.5 mL of saline and the recovery rate was ~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 haemocytometer. Differential cell counts (minimum of 300 $cells \cdot slide^{-1}$) were performed on cytospin-prepared slides stained with Diff-Quik (Baxter Healthcare Corp., Deerfield, IL, USA). IL-1β and TNF-α concentrations in the bronchoalveolar lavage fluid (BALF) were measured according to the instruction of the ELISA kits (R&D system, Minneapolis, MN, USA).

Histology and immunohistochemistry

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 haematoxylin 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, immunohistochemistry (IHC) analysis was performed, using a SP-HRP kit (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Sections were stained with a polyclonal anti-rat BD-2 (rBD-2) antibody (Santa Cruz Biotechnology, Inc.) with a dilution of 1:250, developed with 3,3′-diaminobenzidine, and counterstained with haematoxylin. 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, USA) according to a previously described method [21].

Reverse transcription-PCR

For total RNA extraction, lung tissues were stored at -80°C after snap freezing in liquid nitrogen, and extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA), and amplified using a PCR single-step kit (Bioteke Corp., Beijing, China), according to the manufacturer's instructions. For reverse transcription (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, USA). Primer sequences (forward/reverse, 5′–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 for rBD-2 and 58°C for β-actin. The products were separated by agarose gel electrophoresis and visualised by Gelview (Bioteke Corp.). Semiquantitative densitometric analysis was performed with the Bio-Rad Universal Hood and Quantity One software (Bio-Rad, Hercules, CA, USA).

ELISA and western blot

Lung tissues for ELISA and western blot were homogenised in lysis buffers described in detail in our former articles [22, 23]. Protein concentration was quantitated using bicinchoninic acid (BCA; Pierce, Rockford, IL, USA). rBD-2 level in rat lungs was determined according to the instruction of a commercial ELISA kit (Adlitteram Diagnostic Laboratories, San Diego, CA, USA). For western blot, 30 μg of isolated soluble protein was separated by polyacrylamide gel electrophoresis, transferred onto polyvinylidene difluoride membranes, then treated with primary antibodies to P65, IκBα and β-actin (Santa Cruz Biotechnology, Inc.). 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, USA). Relative protein expression levels were quantified by densitometric measurement of chemiluminescent reaction bands and normalised as a percentage of the control bands using the Bio-Rad Quantity One software.

Electrophoretic mobility shift assay

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 labelled DNA band. The detection of bands was performed using a LightShift™ Chemiluminescent electrophoretic mobility shift assay (EMSA) kit (Pierce Biotechnology, Inc., Rockford, IL, USA) which uses a non-isotopic method to detect DNA–protein interactions. a biotin end-labelled 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 NF-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.). As gel shift controls, lane 1: contained biotin-labelled control oligonucleotide duplex with a binding site of: 5′-TAGCATATGCTA-3′ and 3′-ATCGTATACGAT-5′; lane 2: contained Epstein–Barr virus nuclear antigen (EBNA) extracts and biotin-labelled control oligonucleotide; lane 3: contained EBNA extract, biotin-labelled control oligonucleotide and unlabelled control oligonucleotide for competitive binding inhibition. After transfer, the membrane was immediately cross-linked for 15 min on a UV transilluminator equipped with 312 nm bulbs. A chemiluminescent detection method utilising a luminol/enhancer solution and a stable peroxide solution (Pierce Biotechnology, Inc.) 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 analysed using the Bio-Rad Quantity One software.

Oxidative stress assays

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

Statistical analysis

All data are expressed as mean±SEM. One-way 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, USA) 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

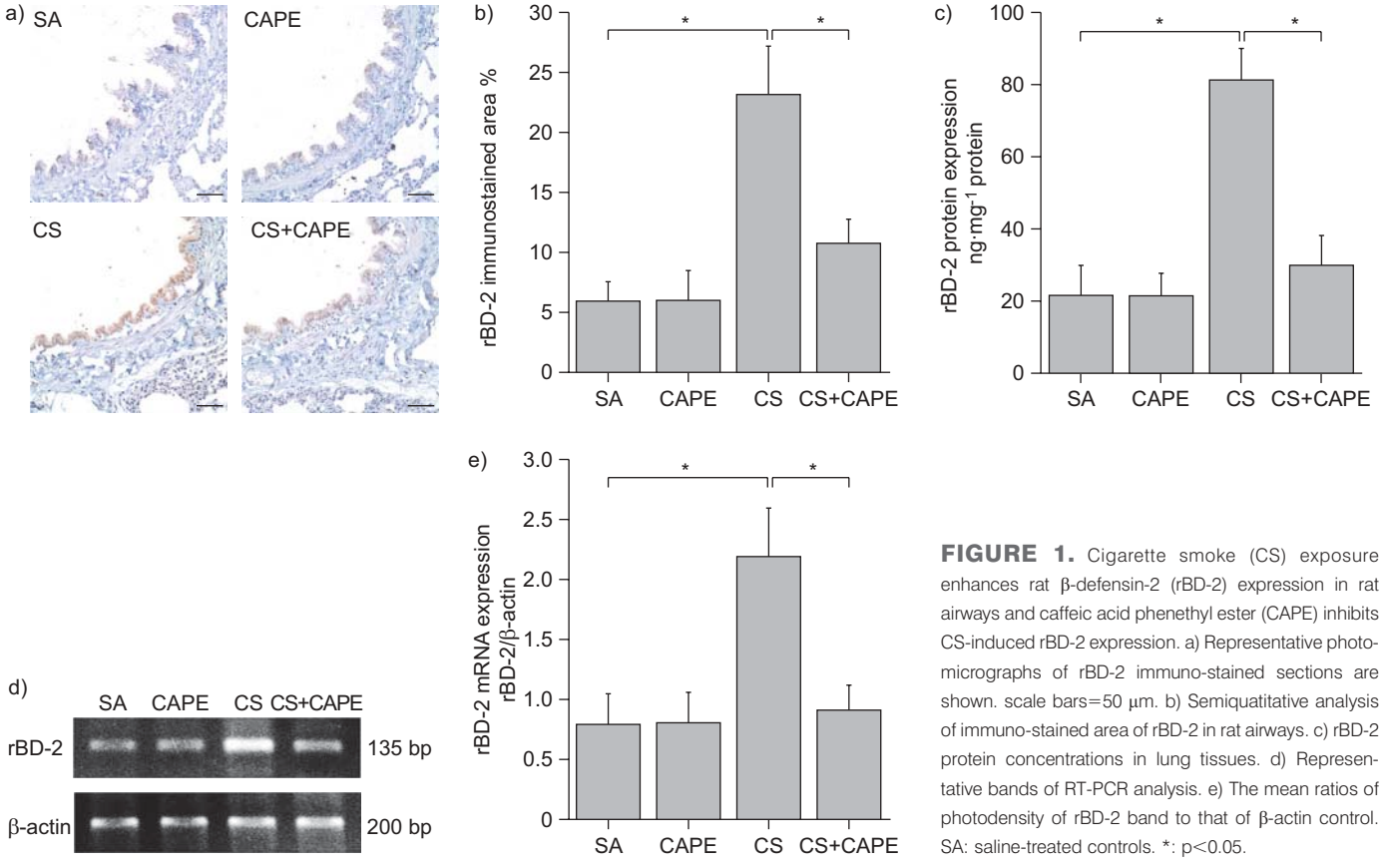


FIGURE 1. Cigarette smoke (CS) exposure enhances rat β-defensin-2 (rBD-2) expression in rat airways and caffeic acid phenethyl ester (CAPE) inhibits CS-induced rBD-2 expression. a) Representative photomicrographs of rBD-2 immuno-stained sections are shown. scale bars=50 μm. b) Semiquatitative analysis 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. SA: saline-treated controls. *: $p<0.05$.

predominately detected in the surface epithelium of bronchi compared with the SA controls (fig. 1a and b). Likewise, in the RT-PCR and ELISA tests, increased rBD-2 mRNA (fig. 1d and 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 localisation of P65 and cytoplasmic IκB degradation were analysed 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–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 and 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 pre-treat the rats before CS exposure. In the RT-PCR and ELISA analysis, CAPE pre-treatment 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

4-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 change of bronchus epithelium, decreased alveolar wall thickening, reduced inflammatory cells in alveoli and bronchiole wall, and decreased goblet cell hyperplasia (fig. 3). Likewise, decreased

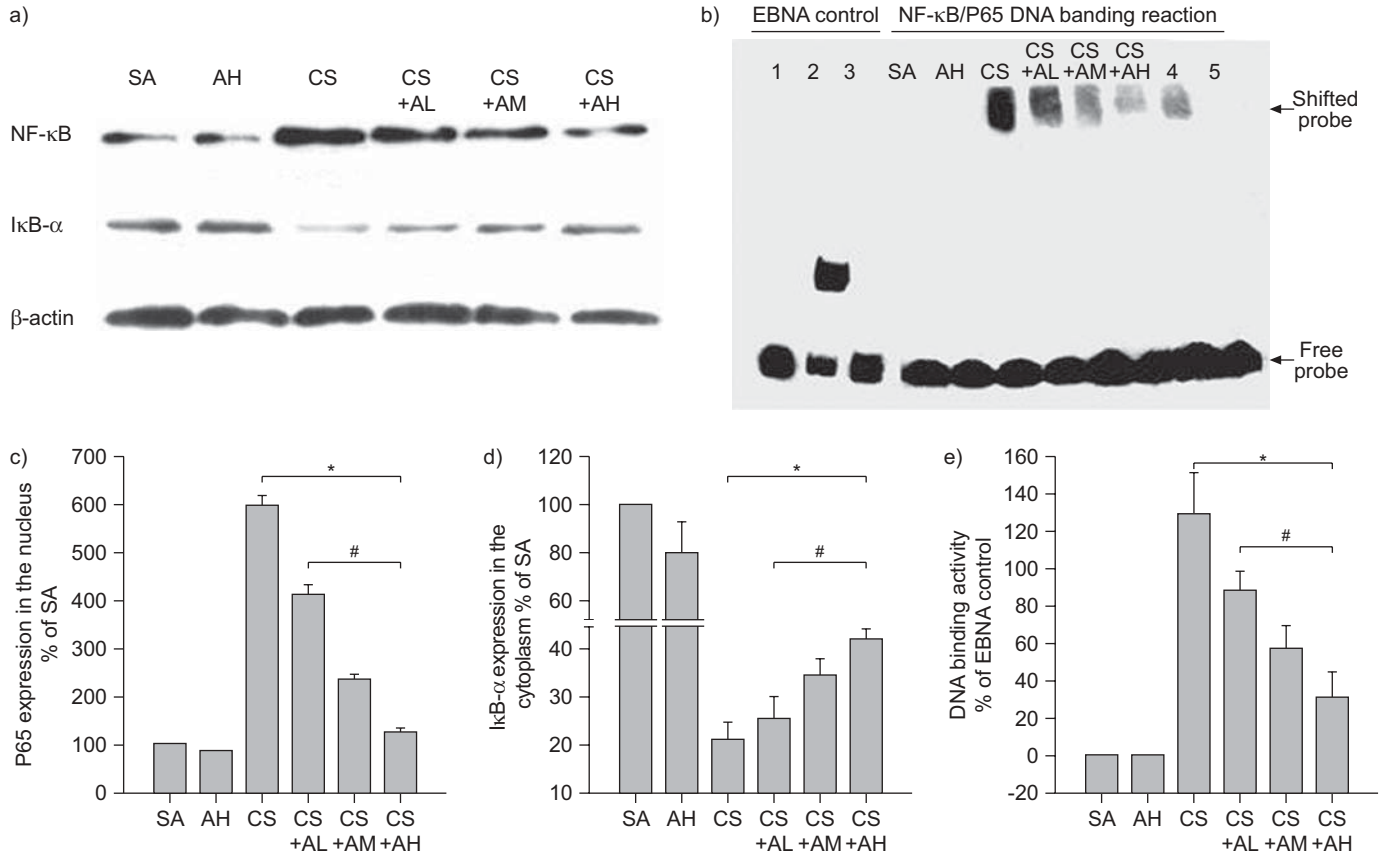
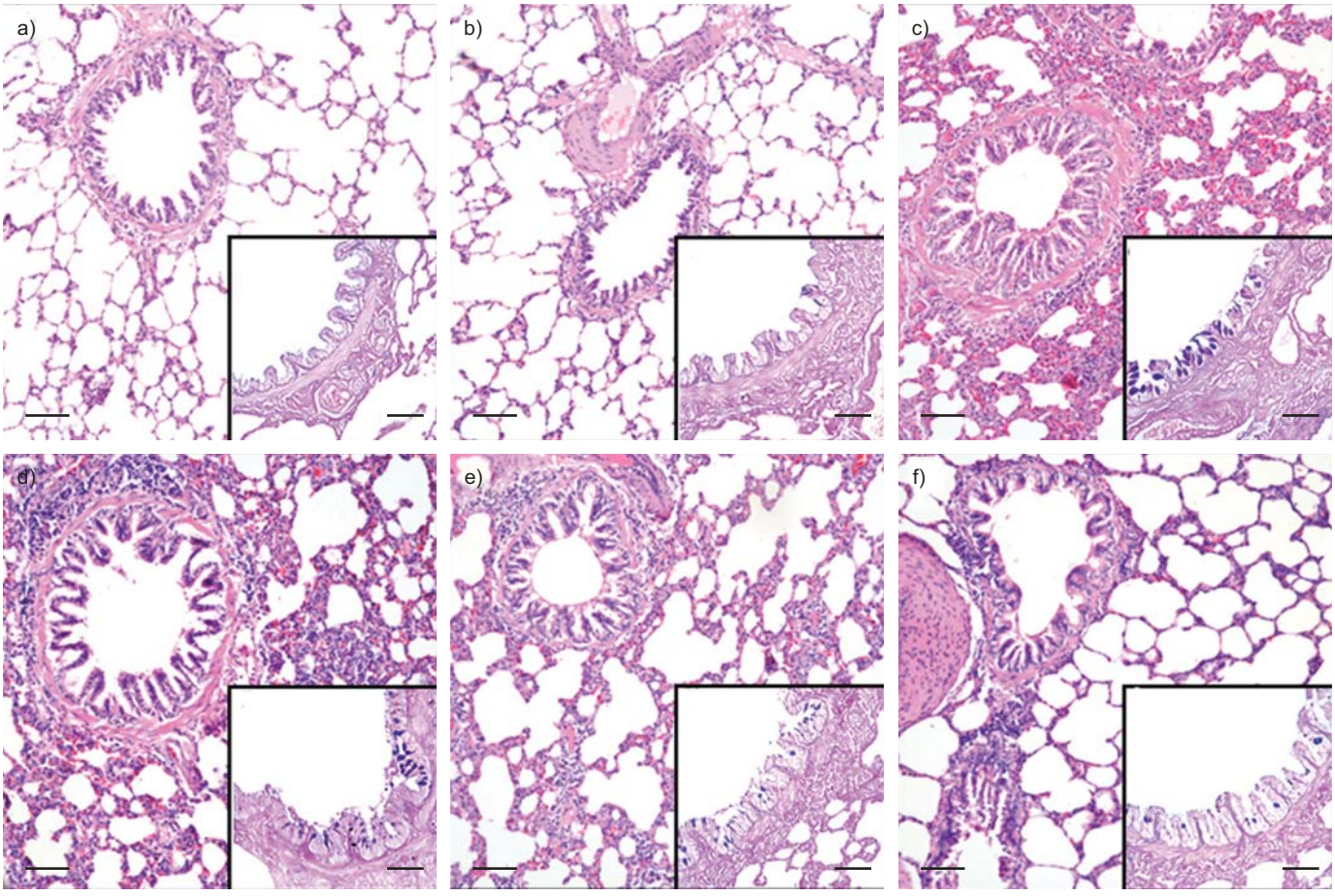


FIGURE 2. Nuclear factor (NF)-κB activation after cigarette smoke (CS) exposure and the effect of astragaloside IV (AS-IV) on this process. NF-κB activation was analysed by western blot and electrophoretic mobility shift assay (EMSA). a) Representative blotting images of p65, IκBα and β-actin are shown, and densitometric analysis of P65 (c) and IκBα (d) protein expression, relative to the saline-treated (SA) controls are presented. b) The results of EMSA are shown. Lane 1: biotin-labelled control oligonucleotide; lane 2: Epstein–Barr virus nuclear antigen (EBNA) extract + biotin-labelled control oligonucleotide to act as positive control; lane 3: EBNA extract + biotin-labelled control oligonucleotide + unlabelled control oligonucleotide to act as negative control; lane 4: nuclear protein extraction of CS exposure group + biotin-labelled NF-κB oligonucleotide + unlabelled NF-κB oligonucleotide; lane 5: biotin-labelled NF-κB oligonucleotide. e) DNA banding activity was quantitated as a densitometric ratio of target band compared with EBNA control shifted band. AL: 0.03% AS-IV; AM: 0.06% AS-IV; AH: 0.12% AS-IV. *: $p<0.05$ *versus* SA group; #: $p<0.05$ *versus* CS group.



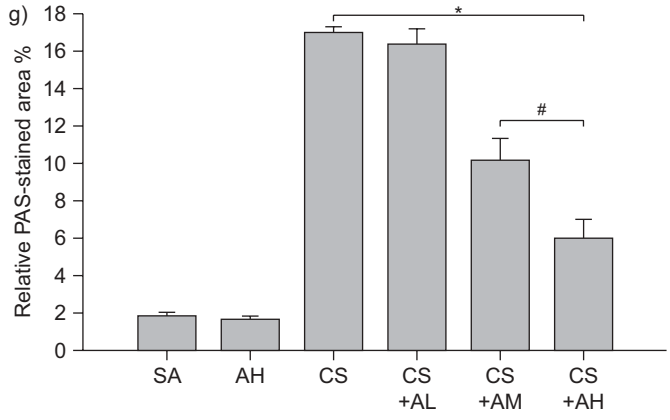


FIGURE 3. Astragaloside IV (AS-IV) attenuates cigarette smoke (CS)-induced airway inflammation. a–f) Representative photomicrographs of haematoxylin and eosin and alcian blue (AB)/periodic acid Schiff (PAS) (insets) stained sections of lung tissues from each group are shown. Scale bars=50 μm. a) Saline-treated (SA) goup; b) 0.12% AS-IV (AH) group; c) CS group; d) CS + 0.03% AS-IV (AL) group; e) CS + 0.06% AS-IV (AM) group; f) CS + 0.12% AS-IV (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.

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. Pre-treatment 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. 4 weeks of CS exposure significantly increased NO level and decreased total GSH content; however, AS-IV treatment could reverse this trend dose-dependently (fig. 5).

DISCUSSION

The present study demonstrated that 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 that CS-induced rBD-2 expression was dependent on NF-κB pathway. Further studies have showed that AS-IV could dose-dependently abrogate CS-induced NF-κB activation and oxidative stress and subsequently attenuate CS-induced airway inflammation.

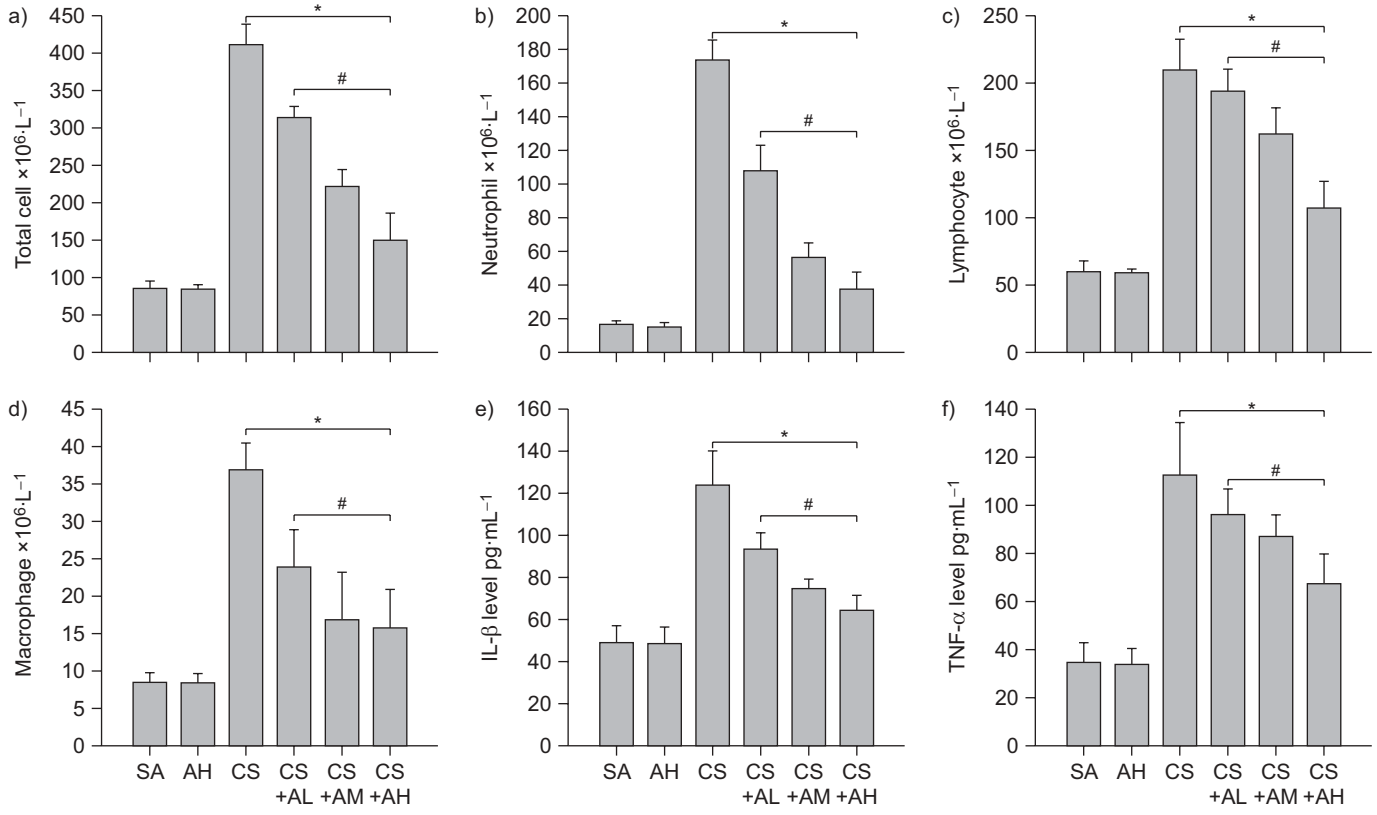


FIGURE 4. Astragaloside IV (AS-IV) decreases cell counts and proinflammatroy cytokines in bronchoalveolar lavage fluid (BALF) after cigarette smoke (CS) exposure. a) Total cells, b) neutrophils, c) lymphocytes and d) macrophages in the BALF. e) Interleukin (IL)-1β and f) tumour necrosis factor (TNF)-α concentrations in the BALF. SA: saline-treated; AL: 0.03% AS-IV; AM: 0.06% AS-IV; AH: 0.12% AS-IV. *: $p<0.05$ *versus* SA group; #: $p<0.05$ *versus* CS group.

CS, the major risk factor for COPD development, is a potent source of oxidants, with >4,700 chemical compounds and high concentrations 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. Alternatively, recurrent and chronic infections are a driving force in the development of COPD. Antimicrobial peptides are important effector molecules of the lung innate immune system, and BDs, especially BD-2, are the principal family of antimicrobial peptides in respiratory tract, which are mainly produced by airway epithelial cells [1]. Several studies have recently demonstrated a suppressive effect of CS on hBD-2 expression by cultured human sinonasal, gingival and airway epithelial cells *in vitro* and decreased hBD-2 levels in pharyngeal washing fluid and sputum from smokers with acute pneumonia *in vivo* [9–11], indicating that CS exposure possibly inhibits airway epithelial hBD-2 production, which links cigarette

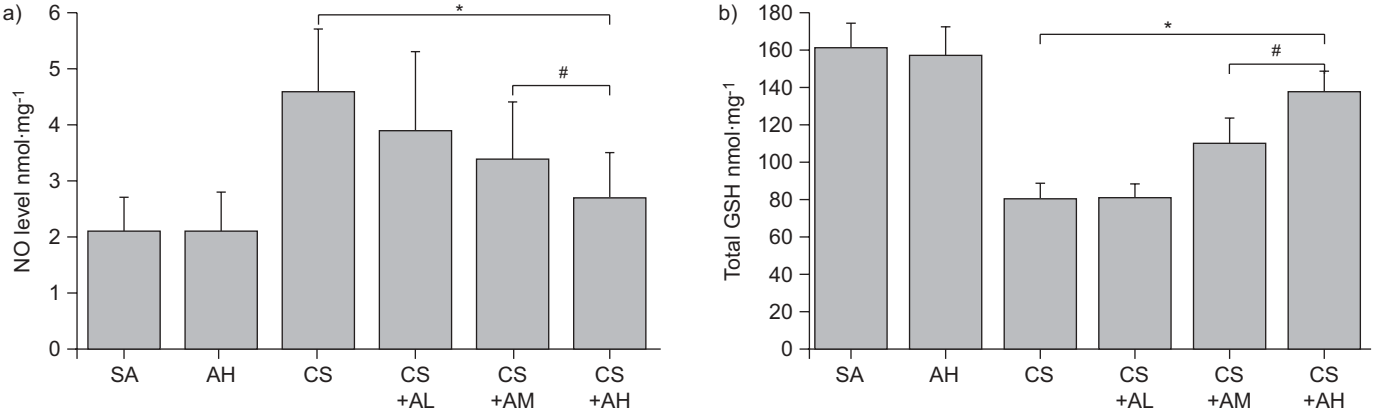


FIGURE 5. Astragaloside IV (AS-IV) reverses cigarette smoke (CS)-induced total glutathione (GSH) and NO levels. Change in NO level (a) and total GSH content (b) in lung tissues are shown. SA: saline-treated; AL: 0.03% AS-IV; AM: 0.06% AS-IV; AH: 0.12% AS-IV. *: $p<0.05$ *versus* SA group, #: $p<0.05$ *versus* CS group.

smoking with increased susceptibility of respiratory tract to microbial infections. However, SHIBATA *et al.* [12] reported upregulated mBD-2 mRNA expression in lung tissues after 6-month CS exposure, and we have further demonstrated, in the present study, that 4-week repeated CS exposure stimulated rBD-2 mRNA and protein expression in rat airways.

It is well-known that 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, with regards to the present data, the potential role of the NF-κB pathway in the CS-induced rBD-2 expression remains unclear. As shown in the present study, NF-κB activation, evidenced by the increased p56 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. Noticeably, 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 the NF-κB pathway by CAPE treatment, reversed CS-induced rBD-2 expression at both mRNA and protein levels, further confirming that NF-κB activation is required for CS-induced rBD-2 expression. It is thus concluded that the 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 have a potentially protective role in various disorders [13–17], with immunoregulatory and anti-inflammatory functions [36, 37]. Noticeablely, DU *et al.* [17] have reported that AS-IV suppressed the progression of airway inflammation, airway hyperresponsiveness and airway remodelling (goblet cell hyperplasiain and subepithelial fibrosis) in a ovalbumin-induced murine model of chronic asthma, 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; additionally, adequate levels of GSH, the principle antioxidant in the lung, are essential for normal lung function [38, 39]. Our study has reported that AS-IV administration attenuated the 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 epithelial injury due to its anti-inflammatory and antioxidant properties, and this protective effect may be at least in part through inactivation of NF-κB signalling.

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 requires more investigation. Furthermore, AS-IV, as a new active monomer of *Astragalus mongholicus*, may play a protective role in mucosal defence through its anti-inflammatory and antioxidant actions, which results in more beneficial effects during lung injury induced by CS exposure.

SUPPORT STATEMENT

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STATEMENT OF INTEREST

None declared.

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REFERENCES

1 Ganz T. Defensins: antimicrobial peptides of innate immunity. *Nat Rev Immunol* 2003; 3: 710–720.
2 Singh PK, Jia HP, Wiles K, *et al.* Production of β-defensins by human airway epithelia. *Proc Natl Acad Sci USA* 1998; 95: 14961–14966.
3 Harder J, Meyer-Hoffert U, Teran LM, *et al.* Mucoid *Pseudomonas aeruginosa*, TNF-α, and IL-1β, but not IL-6, induce human β-defensin-2 in respiratory epithelia. *Am J Respir Cell Mol Biol* 2000; 22: 714–721.
4 Yang D, Chertov O, Bykovskaia SN, *et al.* β-defensins: linking innate and adaptive immunity through dendritic and T cell CCR6. *Science* 1999; 286: 525–528.
5 Yoshida T, Tuder RM. Pathobiology of cigarette smoke-induced chronic obstructive pulmonary disease. *Physiol Rev* 2007; 87: 1047–1082.
6 Di Stefano A, Caramori G, Ricciardolo FL, *et al.* Cellular and molecular mechanisms in chronic obstructive pulmonary disease: an overview. *Clin Exp Allergy* 2004; 34: 1156–1167.
7 Glader P, Moller S, Lilja J, *et al.* Cigarette smoke extract modulates respiratory defence mechanisms through effects on T-cells and airway epithelial cells. *Respir Med* 2006; 100: 818–827.
8 Robbins CS, Bauer CM, Vujicic N, *et al.* Cigarette smoke impacts immune inflammatory responses to influenza in mice. *Am J Respir Crit Care Med* 2006; 174: 1342–1351.
9 Lee WK, Ramanathan M Jr, Spannhake EW, *et al.* The cigarette smoke component acrolein inhibits expression of the innate

immune components IL-8 and human β-defensin 2 by sinonasal epithelial cells. *Am J Rhinol* 2007; 21: 658–663.
10 Mahanonda R, Sa-Ard-Iam N, Eksomtramate M, *et al.* Cigarette smoke extract modulates human β-defensin-2 and interleukin-8 expression in human gingival epithelial cells. *J Periodontal Res* 2009; 44: 557–564.
11 Herr C, Beisswenger C, Hess C, *et al.* Suppression of pulmonary innate host defence in smokers. *Thorax* 2009; 64: 144–149.
12 Shibata Y, Abe S, Inoue S, *et al.* Altered expression of antimicrobial molecules in cigarette smoke-exposed emphysematous mice lungs. *Respirology* 2008; 13: 1061–1065.
13 Zhang WD, Chen H, Zhang C, *et al.* Astragaloside IV from Astragalus membranaceus shows cardioprotection during myocardial ischemia *in vivo* and *in vitro*. *Planta Med* 2006; 72: 4–8.
14 Liu H, Wei W, Sun WY, *et al.* Protective effects of astragaloside IV on porcine-serum-induced hepatic fibrosis in rats and *in vitro* effects on hepatic stellate cells. *J Ethnopharmacol* 2009; 122: 502–508.
15 Luo Y, Qin Z, Hong Z, *et al.* Astragaloside IV protects against ischemic brain injury in a murine model of transient focal ischemia. *Neurosci Lett* 2004; 363: 218–223.
16 Yu J, Zhang Y, Sun S, *et al.* Inhibitory effects of astragaloside IV on diabetic peripheral neuropathy in rats. *Can J Physiol Pharmacol* 2006; 84: 579–587.
17 Du Q, Chen Z, Zhou LF, *et al.* Inhibitory effects of astragaloside IV on ovalbumin-induced chronic experimental asthma. *Can J Physiol Pharmacol* 2008; 86: 449–457.
18 Maffia P, Ianaro A, Pisano B, *et al.* Beneficial effects of caffeic acid phenethyl ester in a rat model of vascular injury. *Br J Pharmacol* 2002; 136: 353–360.
19 Izzotti A, Balansky RM, Blagoeva PM, *et al.* DNA alterations in rat organs after chronic exposure to cigarette smoke and/or ethanol ingestion. *FASEB J* 1998; 12: 753.
20 Ou XM, Wen FQ, Uhal BD, *et al.* Simvastatin attenuates experimental small airway remodelling in rats. *Respirology* 2009; 14: 734–745.
21 Kim JH, Lee SY, Bak SM, *et al.* Effects of matrix metalloproteinase inhibitor on LPS-induced goblet cell metaplasia. *Am J Physiol Lung Cell Mol Physiol* 2004; 287: L127–L133.
22 Chen L, Wang T, Wang X, *et al.* Blockade of advanced glycation end product formation attenuates bleomycin-induced pulmonary fibrosis in rats. *Respir Res* 2009; 10: 55.
23 Liu DS, Liu WJ, Chen L, *et al.* Rosiglitazone, a peroxisome proliferator-activated receptor-γ agonist, attenuates acrolein-induced airway mucus hypersecretion in rats. *Toxicology* 2009; 260: 112–119.
24 Ing DJ, Zang J, Dzau VJ, *et al.* Modulation of cytokine-induced cardiac myocyte apoptosis by nitric oxide, Bak, and Bcl-x. *Circ Res* 1999; 84: 21–33.
25 Zhou XJ, Vaziri ND, Wang XQ, *et al.* Nitric oxide synthase expression in hypertension induced by inhibition of glutathione synthase. *J Pharmacol Exp Ther* 2002; 300: 762.
26 Hayden MS, Ghosh S. Signaling to NF-kappaB. *Genes Dev* 2004; 18: 2195–2224.
27 MacNee W, Rahman I. Is oxidative stress central to the pathogenesis of chronic obstructive pulmonary disease? *Trends Mol Med* 2001; 7: 55–62.
28 Donnenberg MS, Kaper JB. Enteropathogenic *Escherichia coli*. *Infect Immun* 1992; 60: 3953–3961.
29 Yang SR, Chida AS, Bauter MR, *et al.* Cigarette smoke induces proinflammatory cytokine release by activation of NF-κB and posttranslational modifications of histone deacetylase in macrophages. *Am J Physiol Lung Cell Mol Physiol* 2006; 291: L46–L57.
30 Shishodia S, Potdar P, Gairola CG, *et al.* Curcumin (diferuloylmethane) down-regulates cigarette smoke-induced NF-κB activation through inhibition of IkBα kinase in human lung epithelial cells: correlation with suppression of COX-2, MMP-9 and cyclin D1. *Carcinogenesis* 2003; 24: 1269–1279.
31 Liu X, Togo S, Al-Mugotir M, *et al.* NF-κB mediates the survival of human bronchial epithelial cells exposed to cigarette smoke extract. *Respir Res* 2008; 9: 66.
32 Jia HP, Mills JN, Barahmand-Pour F, *et al.* Molecular cloning and characterization of rat genes encoding homologues of human β-Defensins. *Infect Immun* 1999; 67: 4827–4833.
33 Liu L, Wang L, Jia HP, *et al.* Structure and mapping of the human β-defensin HBD-2 gene and its expression at sites of inflammation. *Gene* 1998; 222: 237–244.
34 Tsutsumi-Ishii Y, Nagaoka I. Modulation of human β-defensin-2 transcription in pulmonary epithelial cells by lipopolysaccharide-stimulated mononuclear phagocytes *via* proinflammatory cytokine production. *J Immunol* 2003; 170: 4226–4236.
35 Jang BC, Lim KJ, Paik JH, *et al.* Up-regulation of human β-defensin 2 by interleukin-1β 549 cells: involvement of PI3K, PKC, p38 MAPK, JNK, and NF-kappaB. *Biochem Biophys Res Commun* 2004; 320: 1026–1033.
36 Wang YP, Li XY, Song CQ, *et al.* Effect of astragaloside IV on T, B lymphocyte proliferation and peritoneal macrophage function in mice. *Acta Pharmacol Sin* 2002; 23: 263–266.
37 Zhang WJ, Hufnagl P, Binder BR, *et al.* Antiinflammatory activity of astragaloside IV is mediated by inhibition of NF-κB activation and adhesion molecule expression. *Thromb Haemost* 2003; 90: 904–914.
38 Vliet van der A, Eiserich JP, Shigenaga MK, *et al.* Reactive nitrogen species and tyrosine nitration in the respiratory tract. Epiphenomena or a pathobiologic mechanism of disease? *Am J Respir Crit Care Med* 1999; 160: 1–9.
39 Rahman I, MacNee W. Lung glutathione and oxidative stress: implications in cigarette smoke-induced airway disease. *Am J Physiol Lung Cell Mol Physiol* 1999; 277: L1067–L1088.