Hydroxysafflor Yellow A Attenuates Small Airway Remodeling in a Rat Model of Chronic Obstructive Pulmonary Disease

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Our previous studies found that hydroxysafflor yellow A (HSYA), an active ingredient in Carthamus tinctorius L., has anti-inflammatory and anti-fibrosis properties. In this study, we investigated the effect of HSYA on small airway remodeling (SAR) in a chronic obstructive pulmonary disease (COPD) rat model induced by cigarette smoke and lipopolysaccharide (LPS). SAR is a common lesion in COPD characterized by thickening of the airway wall, mainly by subepithelial fibrosis. In this study the thickness of the small airway was determined by total wall area/basement membrane perimeter (WAT/Pbm). Collagen deposition of the small airway was assessed by Masson's trichrome staining. HSYA significantly attenuated the thickening and collagen deposition of the small airway and inhibited transforming growth factor β1 (TGF-β1) mRNA and protein expression in COPD rat. In addition, HSYA inhibited the phosphorylation of p38 mitogen-activated protein kinases (MAPK) in the lung tissue of rat. HSYA can attenuate experimentally induced airway remodeling and this attenuation may be attributed to suppression of TGF-β1 expression.

Key words hydroxysafflor yellow A; airway remodeling; transforming growth factor-β1; p38 mitogen-activated protein kinase (MAPK); chronic obstructive pulmonary disease (COPD)

Chronic obstructive pulmonary disease (COPD), characterized by airflow limitation that is progressive and persistent, is a leading cause of death worldwide.5) Cigarette smoking and bacterial infection have been widely recognized as the main causes of COPD.2,3) The main pathological changes of the lung from COPD are emphysema and small airway remodeling (SAR).4) SAR, characterized by subepithelial fibrosis, goblet cell metaplasia and excessive mucus production, is now accepted as an important cause of airflow obstruction in COPD.5) No ideal medication has been able to effectively reverse the airway remodeling. Therefore the development of novel medications for the prevention of airway remodeling in COPD is urgently needed.

More recently, the role of transforming growth factor β1 (TGF-β1) in the pathogenesis of SAR in COPD has attracted more interests, particularly since genetic studies have demonstrated gene polymorphisms of the TGF-β superfamily is ubiquitously expressed and is secreted by many lung cell types including airway epithelial cells, vascular endothelial cells, smooth muscle cells, fibroblasts and inflammatory cells.6) TGF-β1 can increase deposition of extracellular matrix in the airways and can stimulate lung fibroblast differentiating into myofibroblast.7) It has been reported that intratracheal instillation of TGF-β1 in mice leads to airway remodeling with peribronchiolar collagen deposition and basal membrane thickening.8) Thus, it seems reasonable to hypothesize that targeting the TGF-β1 expression may provide a novel therapeutic method for attenuating SAR in COPD.

Safflower is the dried flower of Carthamus tinctorius L. and naturally distributed around the world.9) It has been used extensively in Chinese medicine for treating gynecological diseases and coronary heart disease. Safflor yellow (SY) is the active ingredient in the aqueous extract of the safflower and has been used in the treatment of cardiovascular diseases.10) Hydroxysafflor yellow A (HSYA) is the main active ingredient in SY. Our previous study has shown that HSYA can alleviate lung inflammatory response induced by lipopolysaccharide (LPS) or bleomycin in mice and can attenuate the development of lung fibrosis induced by bleomycin in rats.11–13) Additionally, it has also been reported that the aqueous extract of safflower could ameliorate unilateral ureteral obstruction-induced renal interstitial fibrosis by suppressing autocrine TGF-β114) and could protect against liver fibrosis by a decreased expression of TGF-β1.15) Fibrogenesis is an important hallmark of SAR in lung of COPD.16) These facts indicate that HSYA might possibly alleviate the formation of SAR in COPD. The present study was conducted to observe the effect of HSYA on SAR and TGF-β1 expression in COPD rats induced by cigarette smoke and LPS.

MATERIALS AND METHODS

Chemicals and Reagents Safflower which was identified by Professor Jiashi Li (Beijing University of Chinese Medicine) was provided by Huahuikaide Pharmaceutical Co., Ltd. (Shanxi, China), planted at Tacheng (Xinjiang Uyghur Autonomous Region, China). HSYA was isolated and purified by macroporous resin-gel column chromatography from the aqueous extract of Carthamus tinctorius L. as previously described.17) The molecular weight of HSYA is 612 (molecular structure Fig. 1). HSYA was dissolved in sterile 0.9% NaCl for subsequent use. Zhongnanhai brand cigarettes, containing 0.8mg nicotine and 10mg tar in each, were the product of the Beijing Tobacco Corporation in China. LPS (Escherichia coli O55:B5) was the product of Sigma Chemicals (St. Louis, MO, U.S.A.). LPS was dissolved in sterile 0.9% NaCl as a stock solution and the LPS working solution was freshly prepared by dilution with normal saline before use. Dexamethasone

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(DXM) was the product of the Tianjin Pharmaceutical Co., Ltd. TRIZOL reagent and M-MLV reverse transcriptase were the product of Invitrogen Co. (Carlsbad, CA, U.S.A.); the SYBR® Premix Ex Taq™ (Perfect Real Time) kit was the product of TaKaRa Bio Inc. (Shiga, Japan). Enzyme-linked immunosorbent assay (ELISA) kits were the product of Shanghai ExCell Biology, Inc. (Shanghai, China); the alpha-smooth muscle actin (α-SMA) primary antibody was the product of Santa Cruz Biotechnology (CA, U.S.A.). All other chemicals were analytical grade and were all made in China.

**HPLC Analysis of HSYA**

HPLC analyses were performed with an Apollo C18 column (250 mm × 4.6 mm, 5 μm; Grace Davison) on a LC-10AT HPLC chromatographic system with an SPD-6AV UV detector (Shimadzu, Kyoto, Japan). The mobile phase consisted of acetonitrile (A) and 0.1% trifluoroacetic acid (B) at a flow rate of 1.0 mL/min. The gradient elution program was as follows: an initial 0 min at 1% solvent A, 99% solvent B; then from 0 to 50 min, solvent A was linearly increased from 1% to 35%, and solvent B was linearly decreased from 99% to 65%; from 50 to 60 min, solvent A was linearly increased from 35% to 45%, and solvent B was linearly decreased from 65% to 55%. The optical absorbance was monitored at 405 nm and the column temperature was 30°C. The purity of HSYA was quantitatively determined by the area normalization method. The purity of HSYA for this research was 95.9% (Fig. 2).

**Animals and Experimental Procedure** Specific pathogen free mature male wistar rats weighing from 150 to 190 g were obtained from Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). (Certificate of Conformity: Beijing Experimental Animal Testing by certificate: 11400700013001). Animals were maintained in the animal department of An Zhen Hospital with controlled temperature (23±2°C) and humidity (60±10%), under a 12 h light/dark cycle. This study was approved by the Committee on the Ethics of Animal Experiments of Capital Medical University. All animal experiments were performed in accordance with the guidelines for the Brazilian College of Animal Experimentation (COBEA).

Passive cigarette smoke and intratracheal instillation of LPS was applied to establish the COPD rat model in this study. Before the experiment, the rats were adaptively bred for 1 week. The rats were stratified and randomly divided into seven groups according to body weight: sham group; HSYA blank group (76.8 mg/kg); COPD group; COPD+HSYA groups (30, 48, 76.8 mg/kg) and COPD+DXM (2 mg/kg) group. After being weighed, the rats were anesthesitized with sodium pentobarbital (20 mg/kg). The rats in the COPD, COPD+HSYA and COPD+DXM groups received intratracheal instillation of LPS 200 μL (1000 mg/L) twice, on the 1st and 14th day. The rats in the sham group and the HSYA blank group received the same volume of sterile saline instead of LPS. Everyday except the 1st and 14th day, the rats in the COPD, COPD+HSYA and COPD+DXM groups were exposed to cigarette smoke in a smoking box (80 cm × 60 cm × 50 cm) connected to a vacuum pump. The smoke from 6 cigarettes was pumped into the box and was kept for 60 min everyday for 4 weeks except the 1st and 14th day. The exposure to carbon monoxide concentrations from the cigarette smoke was measured by a CO detector (WT40, Weitai Technology Co., Ltd., Shanghai, China) and the CO concentration was maintained at 1000–1200 ppm. The rats in the COPD+HSYA and COPD+DXM groups were injected with HSYA or DXM (2 mg/kg) intraperitoneal (i.p.) daily except for the 1st and 14th day. The rats in the sham group were injected with sterile saline (1 ml).
group and the HSYA blank group were put into the same box as above and exposed to fresh air instead of smoke. Rats in the sham group received an equal volume of sterilised saline and rats in the HSYA blank group received HSYA i.p. (76.8 mg/kg). At the end of the experiment blood plasma was obtained from the abdominal aorta for TGF-\(\beta_1\) assay and the left lobe of the lung was taken and fixed in 4% paraformaldehyde for histological and immunohistochemical observation. The right lung was snap-frozen in liquid nitrogen for RNA and protein isolation.

**Histological Examination** After the animals were sacrificed the left lungs were fixed in 4% paraformaldehyde, and then dehydrated and embedded in paraffin. The samples were then sectioned at 5 \(\mu\)m and stained with HE or Masson’s trichrome according to conventional methods. The slides were evaluated under a light microscope (Nikon Eclipse 90i) and the histological analyses were performed blindly. In humans, small airways are usually defined as airways <2000 \(\mu\)m in internal diameter without cartilage. In this study, the airway of basement membrane perimeter (Pbm) <1000 \(\mu\)m was classified as small airways according to a report from Sapienza. Ten small airways from each group were selected in cross-sections defined by a ratio of minimal to maximal internal diameter of less than 0.5. The following morphological parameters were measured (previously described by Bai and colleagues). The area of airway wall (WAt) and basement membrane perimeter (Pbm) were determined by NIS-ELEMENTS quantitative automatic program (NIKON, Japan); Pbm was measured for the normalization of WAt. The ratios of WAt to Pbm (WAt/Pbm) were used to evaluate the thickness of the airway wall.

**Immunohistochemical Analysis** After deparaffinage with xylene and hydration with graded alcohol, the samples were incubated in citrate buffer (pH 6.0) at 96°C for 20 min to retrieve the antigen. After being washed with PBS three times, the samples were blocked with rabbit serum for 30 min. Then they were incubated with primary antibody against \(\alpha\)-SMA (1:200) at 4°C overnight. After being washed with PBS, the samples were incubated in biotinylated rabbit anti-goat antibody for 60 min at 37°C. To verify the binding specificity, some sections were also incubated exclusively with a primary antibody or exclusively with a secondary antibody. There was no positive staining in these sections. Digital images at 100X magnification were taken from 5 randomly selected fields for each section, and positive areas were integrated by NIS-ELEMENTS quantitative automatic program (NIKON, Japan).

**Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Analysis** Total RNA was extracted from frozen lung tissues using Trizol according to the manufacturer’s instructions. RNA purity and concentration were assayed with a NanoDrop 2000 devise (Thermo Scientific, Wilmington, DE, U.S.A.). The mRNAs were then reverse transcribed to cDNA using a RT-PCR kit with 2 \(\mu\)g RNA. The mRNA level of the target gene was quantified by real-time PCR using a SYBR® Premix Ex TaqTM kit on a Bio-Rad iCycler iQ5 Detection System. The primer pairs and expected lengths (in bp) were as follows (5’ to 3’): \(\beta\)-actin: AGG CCA ACC GTG AA AAG ATG and ACC AGA GGC ATA CAG GGA CAA (antisense), 101 bp; TGF-\(\beta_1\): GCC CTG GAA AGG GCT CAA CAC (sence) and TCC AAC CCA GGT CCT TCC TAA AGT C (antisense), 136 bp; \(\alpha\)-SMA: CGG GCT TTG CTG GTG CAC (sence) and GGT CAG GAT CCC TCT CTT GCT (antisense), 143 bp; collagen I (forward: AAA ACG GGA GGG CGA GTG (sence) and GGT CCC TCG ACT CCT ATG ACT TC (anti-
sense), 81 bp. PCR amplification conditions were as follows: initial denaturation at 95°C for 15 s followed by 40 cycles of denaturation at 95°C for 5 s and annealing at 61°C for 15 s. Relative mRNA level was calculated by the $2^{-\Delta\Delta Ct}$ method. All the results were normalized to the level of β-actin mRNA.

**Enzyme-Linked Immunosorbent Assay Analysis** The plasma active TGF-β1 concentration was measured with an ELISA Kit. For measurement of total TGF-β1 in the supernatant, latent TGF-β1 was converted to the activated form by acidification followed by a neutralization step according to the manufacturer’s instructions.

**Western Blot** Frozen lung tissue was homogenized in lysis buffer in an ice bath and homogenates were centrifuged at 12000 rpm for 15 min at 4°C to remove cellular debris. Protein concentration was determined using the bicinchoninic acid (BCA) method. Protein samples were separated by 10% SDS-PAGE and transferred onto a nitrocellulose membrane using the wet transferring method. In brief, the membrane was first blocked with 5% nonfat dry milk in Tris-buffered saline-Tween (TBST) at room temperature for 1 h, and then

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**Fig. 5.** Effect of HSYA on Collagen Deposition of Small Airway in Rats Stained with Masson's Trichrome (Original Magnification 200×)

(A) Sham group, (B) HSYA blank group, (C) COPD group, (D, E, F) COPD+HSYA (30, 48, 76.8 mg/kg), (G) COPD+DXM (2 mg/kg) (n=10).

**Fig. 6.** Effect of HSYA on α-SMA Expression in Lung Tissues by Immunohistochemistry Staining (Positive as Brown, Original Magnification 100×)

**Fig. 7.** Effect of HSYA on the Area of α-SMA-Positive Cells in Lung Tissues by Immunohistochemistry Staining

Data are presented as mean±S.D. n=10, *p<0.01 versus sham group, **p<0.01 versus COPD group, *p<0.05 versus COPD group.
hybridized with p38 MAPK and Phospho-p38 MAPK primary antibodies followed by IR Dye-conjugated secondary antibody (1:5000) for 1 h. The membranes were scanned and analyzed by an Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, NE, U.S.A.).

Statistical Analysis All data were expressed as mean±S.D. A one-way ANOVA with SNK multiple comparison tests was used to perform the statistical analysis with SPSS 19.0 software. Figures of results were generated with GraphPad Prism 5.0 software. A p value of less than 0.05 was considered statistically significant.

RESULTS

Effect of HSYA on SAR in Lung of Rat as Assessed by Hematoxylin and Eosin (HE) Staining The effect of HSYA on small airway pathological changes was assessed by HE staining (Fig. 3) and a quantitative evaluation of the thickness of the small airway was determined by WAt/Pbm (Fig. 4). The bronchial and alveolar epithelial structures were intact and no obvious inflammatory cell infiltration was found in the subepithelial tissue in the sham group or the HSYA blank group. Occlusion of the small airways, infiltration of inflammatory cells and thickening of the airway wall were found in the lungs of the COPD group. Mucus and/or inflammatory cell exudate inside some small airways (Fig. 3C black arrow) and emphysema were also observed in the lung of COPD rat. Infiltration of inflammatory cells in the small airway was attenuated in the COPD+HSYA group (Figs. 3D, E, F). WAt/Pbm was higher in the COPD group than in the sham group (p<0.01). WAt/Pbm was significantly lower in the COPD+HSYA group (Figs. 3D, E, F). WAt/Pbm was higher in the COPD group than in the sham group (p<0.01). WAt/Pbm was significantly lower in the COPD+HSYA group (Figs. 3D, E, F). WAt/Pbm was also higher in the COPD+DXM group than in the COPD group (p<0.01).

Effect of HSYA on Collagen Deposition of Small Airways in Rats as Assessed with Masson’s Trichrome Staining The effect of HSYA on collagen deposition of small airways was assessed by Masson’s trichrome staining (Fig. 5). The collagen is stained blue in Masson’s trichrome to distinguish it from other tissues. A slight collagen deposition was found in the margin of the airway wall in the sham group. Collagen deposition of the small airway increased significantly more in the COPD than in the sham group; however this deposition was attenuated in the COPD+HSYA groups. Collagen deposition of the small airway was also effectively attenuated in the positive control COPD+DXM group.

Effect of HSYA on Plasma TGF-β1 Concentration in Rats The plasma TGF-β1 levels increased significantly in the COPD group compared to that in the sham group. However, the TGF-β1 concentrations were attenuated in both the HSYA groups and the COPD+DXM group (Fig. 9).

Effect of HSYA on p-p38MAPK Level in Lung Tissue p38 MAPK is an intracellular signal transducer for inflammatory reactions and can be triggered by extracellular stimuli including cigarette smoke and LPS. Western blot analysis to
assess p38 MAPK activation in the lung tissue of the COPD group revealed that the level of phosphorylated p38 MAPK in the COPD group was markedly augmented and this augmentation was attenuated by both HSYA and DXM (Fig. 10).

**DISCUSSION**

COPD is a chronic inflammatory disease and is characterized by persistent airflow limitation. Of the two fundamental features: SAR and emphysema, SAR plays a more important role in airflow limitation in COPD. Our data in this study showed that an increased thickness of the airway wall as well as inflammatory exudates and mucus in COPD rats induced by cigarette smoke and LPS. A COPD rat model was established successfully.

In this study TGF-β1 in the mRNA and protein levels increased in COPD rats which is similar to others studies. It has reported TGF-β1 elevated in the plasma of COPD patients and increased the release of active TGF-β1 induced by cigarette smoke in rat tracheal explant. We found in this study that enhanced TGF-β1 expression correlated with the increase in airway wall thickness and collagen deposition and that the elevation of TGF-β1 in the mRNA and protein level was significantly inhibited in COPD rats after HSYA treatment. In addition, the decrease in TGF-β1 expression correlated with the reduction in small airway wall thickness and collagen deposition after HSYA treatment.

We speculate that HSYA reduces SAR by inhibiting the expression of TGF-β1, which is further supported by the suppression of collagen type I and α-SMA expression in the lung after HSYA treatment. We considered that HSYA inhibits α-SMA and collagen I elevation through inhibiting the expression of TGF-β1. Our previous study showed that SY (its main component is HSYA) inhibited morphological changes and the expression of α-SMA in lung fibroblast stimulated by TGF-β1. McMillan et al. reported that anti-TGF-β1 antibody treatment prevented the progression of airway remodeling in a mouse model for asthma. Moreover, it has also been reported that the aqueous extract of safflower could ameliorate renal interstitial fibrosis and liver fibrosis by suppressing TGF-β1 expression. These findings suggest that HSYA attenuates remodeling by inhibiting the expression of TGF-β1.

The attenuation of TGF-β1 expression is probably caused by the anti-inflammatory properties of HSYA. Although the mechanisms involved in the pathogenesis of SAR in COPD are largely unknown, chronic inflammation of the airways plays an important role. The inflammatory reaction and increased production of inflammatory cytokine caused by cigarette smoke and LPS are believed to be the pathogenesis of SAR. Cigarette smoke contains a complex mixture of thousands of chemicals and can directly damage airway epithelium and can induce inflammatory cells including macrophage and lymphocyte infiltration in the small airways. LPS can bind to Toll-like receptor 4 (TLR-4) on the surface of epithelial cells and can then activate the nuclear factor-kappa B (NF-κB) and p38 MAPK signal pathway, resulting in the transcription of pro-inflammatory cytokine genes. Inflammation induced by cigarette smoke and LPS can increase the pro-fibrotic cytokine TGF-β1 expression. TGF-β1 acts as a bridge between inflammatory injury and airway remodeling.

p38 MAPK is involved in the production of pro-inflammatory and profibrotic mediators which is activated by phosphorylation on Thr180 and Tyr182 in the Thr-Gly-Tyr motif. It is likely that the α isoform of p38 MAPK is the most important in the inflammatory response of COPD in the lungs. Underwood DC showed that obstructing p38 MAPK at the initial phase of the inflammatory response reduced renal fibrosis. We have found that HSYA effectively inhibited LPS-induced inflammatory signal transduction in A549 cells by suppressing the expression of TLR-4, TNFα, IL-1β and IL-6 and by inhibiting the phosphorylation of p38 MAPK. In this study, we found that p38 MAPK was activated in the lung of the COPD groups, which was also found by Marwick. Marwick examined rat lungs exposed to cigarette smoke and found enhanced p38 MAPK phosphorylation, associated with increased activation of the transcription factors NF-κB and AP-1 which regulate TGF-β1 gene expression. More importantly we observed that the phosphorylation of p38 MAPK in the lung was significantly inhibited and inflammatory cell infiltration of the small airway was alleviated after HSYA treatment. These results suggest that HSYA can attenuate airway remodeling and can inhibit the expression of TGF-β1, possibly...
through the inhibition of phosphorylation of p38 MAPK in the lung tissues.

In this study, dexamethasone was used as a positive control drug. The results of our preliminary experiment and formal experiment have shown that dexamethasone was sensitive to inhibit cigarette smoke/LPS-induced TGF-β1 high expression and p38 MAPK activation. We have noticed the fact that corticosteroid is refractory to airway inflammation in some COPD patients and animal models. We think this discrepancy could be mainly associated with the different process of establishment of COPD model.

In conclusion airway remodeling is a key pathological feature in COPD and early treatment to prevent airway remodeling is probably important for preventing the progression of COPD. Our preliminary data showed that HSYA can attenuate experimentally induced airway remodeling. This may be related to a decrease in the production of TGF-β in mRNA and protein level which may be attributed to the inhibition of phosphorylation of p38 MAPK in the lung tissues.

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