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Serotonin receptors 5-HTR2A and 5-HTR2B are involved in cigarette smoke-induced airway inflammation, mucus hypersecretion and airway remodeling in mice

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A B S T R A C T

Background: Cigarette smoke plays an important role in the pathogenesis of chronic obstructive pulmonary disease (COPD). Recently, elevated serotonin (5-HT) levels were found in the plasma of COPD patients. The role of 5-HT and its receptors in airway inflammation and remodeling induced by cigarette smoke is unclear.

Methods: BALB/c mice received the 5-HTR2A inhibitor ketanserin, the 5-HTR2B inhibitor RS-127445 or the natural 5-HTR2A/2B inhibitor quercetin intraperitoneally, then were exposed to cigarette smoke for 6 or 12 weeks. Control mice received placebo and were exposed to room air or cigarette smoke. Mice were sacrificed and bronchial alveolar lavage fluid (BALF) and lung tissue samples were collected.

Results: Immunohistochemistry and western blot confirmed an increase in both 5-HTR2A and 5-HTR2B expression in mouse lungs after exposure to cigarette smoke for 6 and 12 weeks. Cigarette smoke induced accumulation of macrophages and neutrophils and increased levels of inflammatory cytokines, including IL-1β and TNF-α, in BALF and lung tissue; these effects were inhibited by ketanserin, RS-127445 and quercetin. Pretreatment with 5-HT receptor antagonists suppressed the goblet cell hyperplasia induced by 6- or 12-week exposure to cigarette smoke, based on Alcian blue-periodic acid Schiff staining. After 12 weeks of cigarette smoke exposure, Masson's staining showed fibrosis surrounding the mouse airways, and inhibitor pretreatment significantly attenuated the thickening and collagen deposition around the small airways.

Conclusions: Our results suggest that cigarette smoke-induced airway inflammation and small airway remodeling are partially mediated by 5-HTR2A and 5-HTR2B, which could be a new therapeutic target for airway remodeling in COPD.

1. Introduction

Chronic obstructive pulmonary disease (COPD) is characterized by an abnormal persistent inflammatory response to cigarette smoke or other noxious particles [1]. These noxious insults lead to emphysema and airway remodeling, which is manifested by squamous and mucous metaplasia of the epithelium, smooth muscle hypertrophy, and airway wall fibrosis [2,3]. After 6 months of CS exposure, a substantial amount of collagen fiber was generated in the guinea pig airway leading to a significant increase in wall thickness [4]. Nicotine, an important component of cigarettes, was found to stimulate the production of inflammatory mediators and profibrogenic cytokines, induce fibroblast proliferation, increase the deposition of collagen, and promote fibrosis in a variety of cells and animal models [5]. No specific drugs have been found to reverse the pathogenic airway remodeling induced by cigarette smoke, reflecting its complexity.

Serotonin (5-hydroxytryptamine, 5-HT) is a neurotransmitter produced mainly by peripheral activated intestinal pigment cells, mast cells and platelets. Seven serotonin receptors (5-HTR1-7) have been identified thus far [6,7]. A number of studies have found that 5-HT receptor 2A (5-HTR2A) and 5-HT receptor 2B (5-HTR2B) exhibit important roles in the lung, and 5-HT regulates the activity of blood vessels and bronchi by activating 5-HTR2A/2B [8,9]. In addition, the level of free serotonin in serum is closely related to the clinical severity and the degree of lung function decline in asthmatic patients [10,11]. However, few studies have investigated the relationship between serotonin and COPD.

One study found that serotonin levels in the serum of patients with

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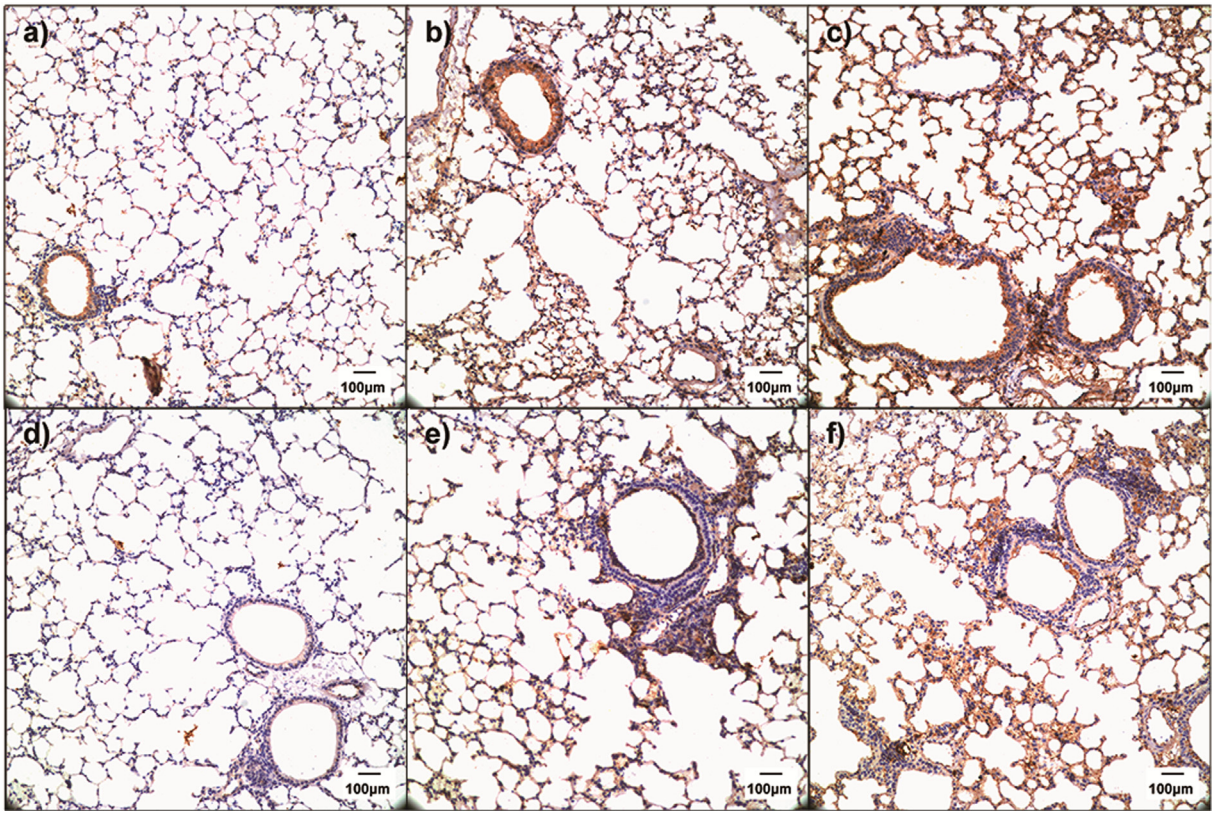

Fig 1. Cigarette smoke exposure increased the expression of 5-HT receptors (2A/2B) in mouse lungs. Immunohistochemistry showed that 6-week cigarette smoke exposure increased the expression of 5-HTR2A (b) and 5-HTR2B (e) in mouse lungs when comparing with control mice (a and b), and 12-week cigarette smoke exposure also boosted the expression of 5-HTR2A (c) and 5-HTR2B (f). Abbreviations: CS, cigarette smoke; 5-HTR2A, 5-hydroxytryptamine receptor 2A; 5-HTR2B, 5-hydroxytryptamine receptor 2B.

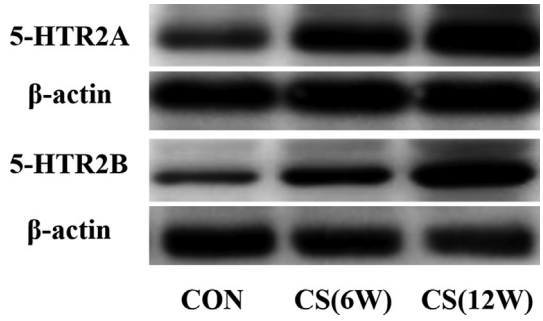

Fig 2. Cigarette smoke exposure increased the protein expression levels of 5-HT receptor (2A/2B) in mouse lungs. Western blot showed that both 6-week and 12-week cigarette smoke exposure enhanced the protein expression of 5-HTR2A and 5-HTR2B, and this trend was more significant at 12 weeks. Abbreviations: CON, control group; CS, cigarette smoke; 6 W, 6 weeks; 12 W, 12 weeks; 5-HTR2A, 5-hydroxytryptamine receptor 2A; 5-HTR2B, 5-hydroxytryptamine receptor 2B.

COPD were significantly higher than those of normal smokers and non-smokers [12]. Moreover, serum levels of serotonin in rats exposed to chronic cigarette smoke were significantly increased, but no significant changes were observed in alveolar lavage fluid and lung tissue [13]. 5-HT can also promote the proliferation of fibroblasts from the pulmonary arteries of rats exposed to hypoxic treatment—an environmental condition caused by cigarette smoke exposure [14]. Thus, we hypothesized that 5-HT may activate 5-HTR2A/2B during airway inflammation and airway fibrosis induced by cigarette smoke. In this study, we attempted to elucidate the biological role of 5-HT and 5-HTR2A/2B in airway inflammation and remodeling in mice exposed to cigarette smoke with or without pretreatment with receptor antagonists.

2. Materials and methods

2.1. Mice

Specific pathogen-free male BALB/c mice (6–8 weeks old, 20–25 g) were purchased from Dashuo Biological Technology (Chengdu, China). Animals were handled according to the ARRIVE guidelines [15]. The study protocol was approved by the Animal Ethics Committee of West China Hospital, Sichuan University. Mice were housed in a temperature- and humidity-controlled facility and kept on a 12-h light-to-dark cycle with free access to food and water. Mice were allowed to acclimate for one week prior to experiments. After one week of acclimation, mice were randomly divided into five groups (n = 12 per group) that received neither receptor antagonist nor cigarette smoke (control), cigarette smoke without antagonist, or one of the three receptor antagonists followed by cigarette smoke.

2.2. Pretreatment with serotonin receptor antagonists

Ketanserin (Selleckchem, Houston, TX, USA), RS-127445 (Selleckchem), and quercetin (Sigma-Aldrich, St. Louis, MO) were freshly prepared daily in an aqueous solution of 0.2% Tween [8], then immediately injected intraperitoneally into animals. One group of animals received 2 mg/kg qd ketanserin [16,17], another group received 1 mg/kg qd RS-127445 [18,19], and the third group received 50 mg/kg qd quercetin [20]. The remaining animals received only 0.2% Tween solution. At 30 min later, animals were exposed to cigarette smoke or room air as described in the next section.

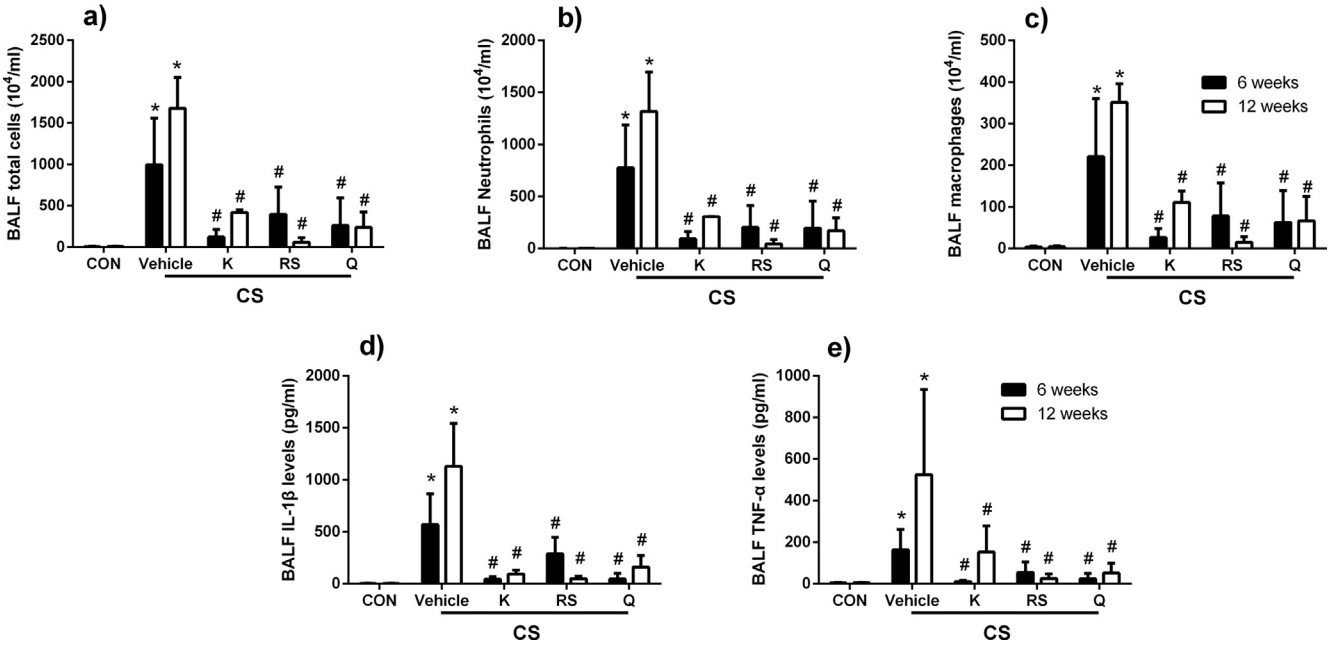

Fig 3. Serotonin receptor antagonist pretreatment suppressed cigarette smoke-driven inflammatory cell infiltration and pro-inflammatory cytokine secretion in BALF. BALF cell counting showed that both specific (ketanserin and RS-127445) and non-specific (quercetin) serotonin receptor antagonists alleviated 6-week or 12-week cigarette smoke-induced BALF cell infiltration (a), neutrophil infiltration (b) and macrophage infiltration (c). Besides, ELISA showed that these serotonin receptor antagonists suppressed cigarette smoke-induced IL-1β and TNF-α release in mouse BALF (d and e). Values are expressed as mean ± SD (4–6 animals per group). *P < 0.05 compared to the control group; #P < 0.05 compared to the cigarette smoke-exposed group. Abbreviations: CON, control group; CS, cigarette smoke; CS-C, cigarette smoke control group; K, ketanserin; RS, RS-127445; Q, quercetin.

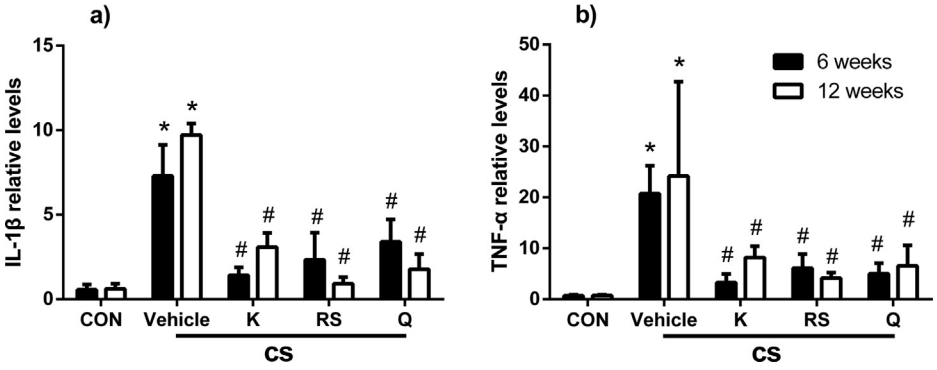

Fig 4. Pretreatment with serotonin receptor antagonists suppressed the cigarette smoke-induced increase in levels of mRNA encoding IL-1β and TNF-α in mouse lungs. RT-PCR showed that serotonin receptor antagonists attenuated cigarette smoke exposure-stimulated mRNA levels of IL-1β (a) and TNF-α (b). Values are expressed as mean ± SD (4–6 animals per group).*P < 0.05 compared to the control group; #P < 0.05 compared to the group exposed to cigarette smoke. Abbreviations: CON, control group; CS, cigarette smoke; CS-C, cigarette smoke control group; K, ketanserin; RS, RS-127445; Q, quercetin.

2.3. Cigarette smoke exposure

Experiments were performed according to a modified protocol from previously published work [21–23]. Briefly, mice assigned to be exposed to cigarette smoke were placed for 2 h in a ventilated plastic chamber filled containing 250 mg total particulate smoke matter per m^3 produced by a smoke generator (CH Technologies, Westwood, NJ, USA). This treatment was performed twice a day for six consecutive days. The smoke was composed of a 1:1 mix of mainstream cigarette smoke (Marlboro Red 85 mm, USA; 1.0 mg nicotine and 12 mg tar per cigarette) and fresh air. Airflow passing through the box was constant at 1.22 L/min. In parallel, the control group was exposed to room air only. After 6 or 12 weeks of exposure, mice (n = 6 mice/group/time point) were sacrificed via a lethal dose of sodium pentobarbital (100 mg/kg), which was given intraperitoneally. Then animals were exsanguinated *via* the abdominal aorta, and tissue samples were collected for further study.

2.4. Bronchoalveolar lavage fluid (BALF) and cell counts

Right lungs were lavaged three times with 0.5 ml of saline and 90% of the liquid was recovered. BALF samples were centrifuged at 1000 g for 5 min, and the supernatants were collected and stored at −80 °C for later use. The pelleted cells were resuspended in 0.2 ml of phosphate-buffered saline (PBS), and total cell number was determined using a hemocytometer. Differential cell counts were determined by cytospin preparations and stained with Wright–Giemsa (250 cells were counted for each mouse). An experienced investigator blinded to the experimental conditions performed all cell counts based on standard morphological criteria.

2.5. Pro-inflammatory cytokine detection in BALF

Levels of IL-1β and TNF-α were measured in BALF using commercially available ELISA kits (ExCell Bio, Shanghai, China) per the manufacturer's instructions. Absorbance was measured with a Bio-Rad 680 microplate reader (Hercules, CA, USA), and data were analyzed using Microplate Manager 5.2 (Bio-Rad).

2.6. Lung histopathology

Left lungs were fixed with 4% phosphate-buffered

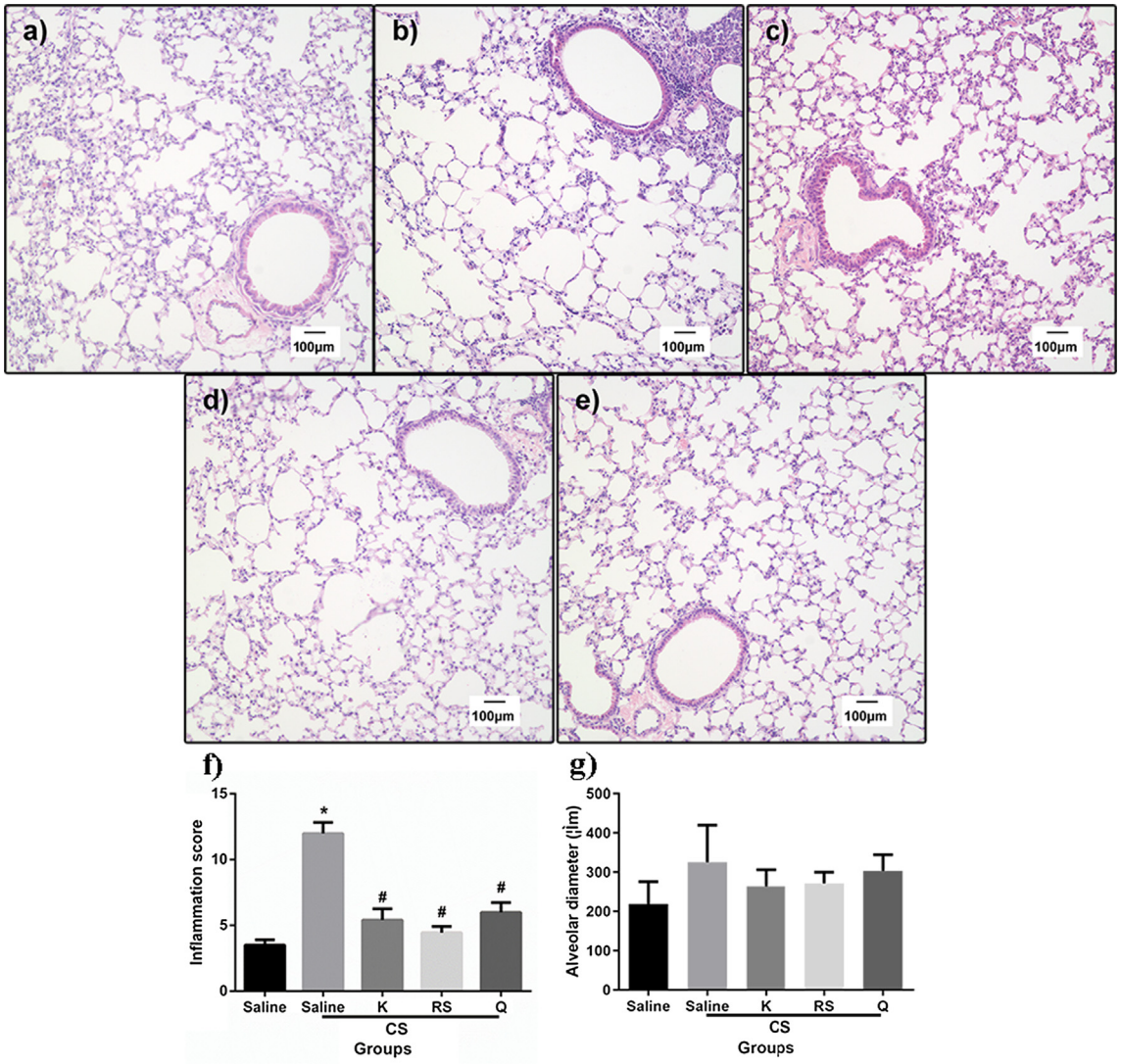

Fig 5. Pretreatment with serotonin receptor antagonists attenuated the pathohistological changes induced by cigarette smoke exposure after 6 weeks. H&E staining showed that both specific (ketanserin and RS-127445, c and d) and non-specific (quercetin, e) serotonin receptor antagonists suppressed 6-week cigarette smoke-induced epithelium thickening, lumen obstruction by mucus, cell debris, and peribronchial inflammatory cell infiltration (b) in mouse lungs, inflammatory scores (f) showed the similar trends, however, the emphysematous changes presented by alveolar diameters showed no statistic difference between different groups (g). Values are expressed as mean ± SD (4–6 animals per group). *P < 0.05 compared to the control group; #P < 0.05 compared to the CS group. Abbreviations: CON, control group; CS, cigarette smoke; CS-C, cigarette smoke control group; K, ketanserin; RS, RS-127445; Q, quercetin.

paraformaldehyde, embedded in paraffin, and sliced to a thickness of 4 mm. Paraffin sections were stained with hematoxylin and eosin solution (H&E), Alcian blue-periodic acid Schiff (AB-PAS), or Masson's stain to evaluate morphological changes, mucus secretion, or fibrosis in lungs. An experienced pathologist blinded to the experimental treatment of the samples determined a lung histopathology score for each sample based on the severity of lung lesions, including alveolar septal infiltrates, perivascular infiltrates, combined bronchus-associated lymphoid tissue hyperplasia, and peribronchiolar infiltrates as we previously described [24]. For each possible lesion, the score ranged from 0 to 4 (0: absent, 1: minimal, 2: mild, 3: moderate, and 4: marked), and the group histopathology scores were obtained by averaging the scores of individual mouse in each group. For evaluating the degree of emphysema in mice lung, vertical and horizontal lines were drawn in the H &E slice figures, and the number of alveolar across these lines was counted, then the average alveolar diameter was calculated with: (side length of the slice figures) / (number of alveolar) [25]. The percentages of positively stained areas by AB/PAS to the total airway epithelial areas were quantified by Image-Pro plus 4.5 software (Media Cybernetics, Bethesda, MD, USA). At least five complete airways per mouse were examined, and a density threshold was adopted. The optical density threshold was manually set to include all positive stained goblet cells [26]. Areas staining positively for Masson's stain were quantified using Image-Pro Plus 4.5 (Media Cybernetics, Bethesda, MD, USA).

2.7. Immunohistochemistry of 5-HTR2A/2B

Immunohistochemical staining for 5-HTR2A and 5-HTR2B protein was performed using an SPHRP kit (Santa Cruz Biotechnology, Santa Cruz, CA, USA). In brief, After deparaffinization and antigen unmasking, the slices were blocked with 5% goat serum in TBST for 1 h at room temperature, then they were incubated with anti-5HTR2A/2B (1:200 diluted with 5% gout serum in TBST) overnight at 4 °C, then washed with PBST for 3 times, 5 min per time. Then the slices were incubated with anti-biotin secondary antibodies (1:500 diluted with 5% TBST) for 30 min at room temperature. Then DAB kit was used to get an acceptable staining intensity. Areas staining positively for 5-HTR2A and

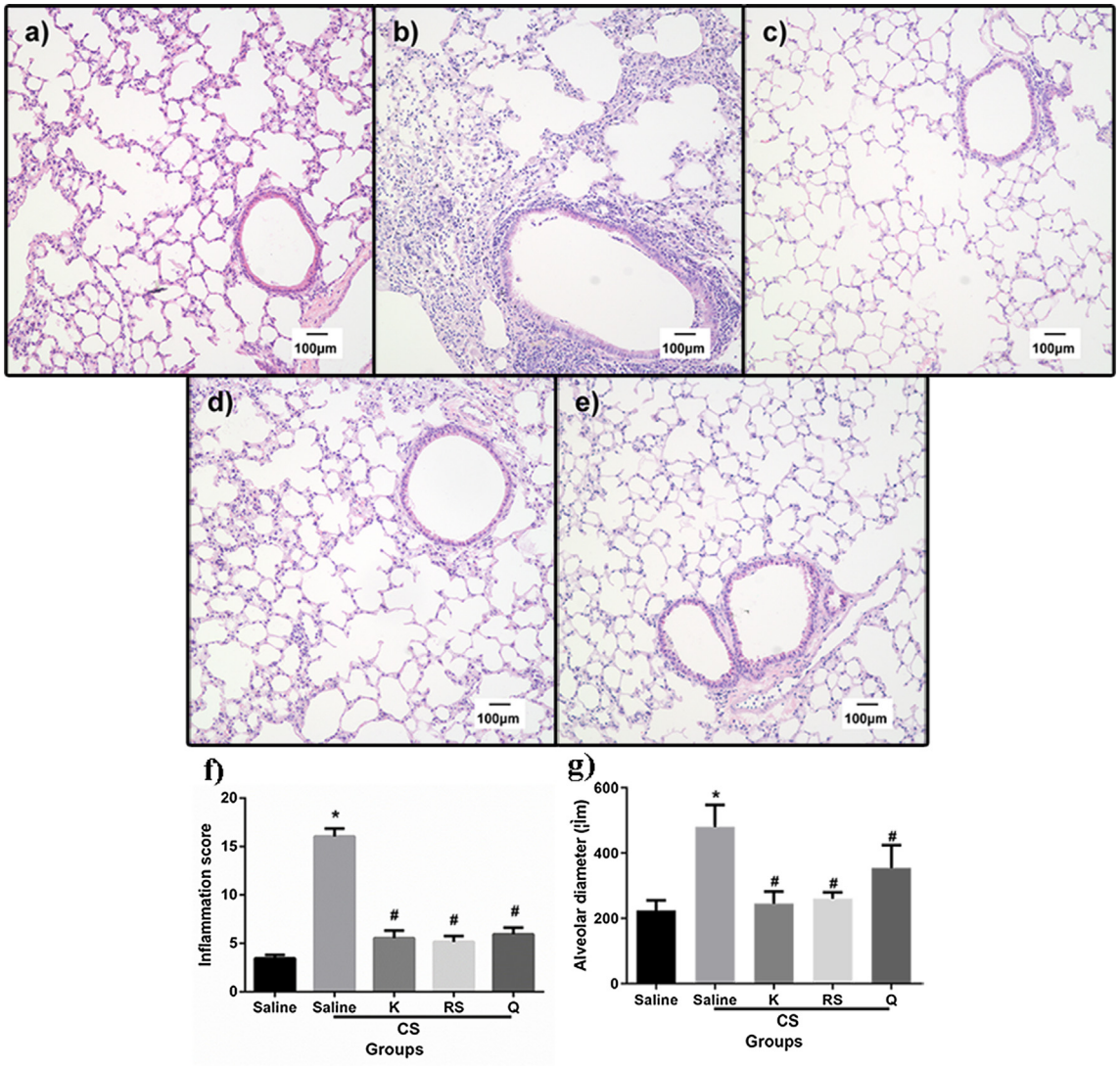

Fig 6. Pretreatment with serotonin receptor antagonists attenuated the pathohistological changes induced by cigarette smoke exposure for 12 weeks. H&E staining showed that both specific (ketanserin and RS-127445, c and 6d) and non-specific (quercetin, e) serotonin receptor antagonists suppressed 12-week cigarette smoke-induced epithelium thickening, lumen obstruction by mucus, cell debris, emphysematous change, and peribronchial inflammatory cell infiltration (b) in mouse lungs, inflammatory scores (f) and alveolar diameters (g). Values are expressed as mean ± SD (4–6 animals per group). *P < 0.05 compared to the control group; #P < 0.05 compared to the CS group. Abbreviations: CON, control group; CS, cigarette smoke; CS-C, cigarette smoke control group; K, ketanserin; RS, RS-127445; Q, quercetin.

5-HTR2B in mice lung were quantified by Image-Pro plus 4.5 software (Media Cybernetics, Bethesda, MD, USA).

2.8. Western blotting

Lower lobe of right lung tissue was lysed in RIPA buffer containing 50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 2 mM sodium fluoride, 2 mM EDTA, 0.1% SDS, and PMSF. Protein concentrations were determined by a BCA protein assay kit (Thermo Fisher Scientific, MA, USA). Total protein (20 μg) was resolved by 10% SDS polyacrylamide gel electrophoresis and transferred to a PVDF membrane. Membranes were blocked for 1 h at room temperature with 5% BSA in TBS-Tween, and incubated overnight at 4 °C with primary monoclonal antibodies (Cell Signaling Technology, Beverly, MA, USA) against ERK, p-ERK, JNK, p-JNK, p38, p-p38, and actin (1:1000 diluted with 5% BSA), then washed with TBST for 3 times, 5 min per time. Then the blots were incubated with HRP-linked anti-rabbit or anti-mouse IgG (Cell Signaling Technology, 1:10000 diluted with TBST) for 2 h at room temperature. After washed with TBST for 3 times, 5 min per time, blots were exposed to X-ray film with SuperSignal West Pico chemiluminescent substrate (Pierce, Rockford, IL, USA). The immune complexes were detected with chemiluminescent substrate Band intensities were quantified with QuantityOne software (Bio-Rad).

2.9. Reverse transcription-PCR (RT-PCR)

Total RNA was isolated from the upper lobe of the right lungs using EZNA® Total RNA kit I (Omega Bio-tek, Norcross, GA, USA). First-strand cDNA was synthesized from 10 μg of total RNA using the iScript cDNA Synthesis Kit (Bio-Rad) according to the manufacturer's instructions. cDNA was prepared with FastStart Essential DNA Green Master Mix (Roche), and real-time RT-PCR was conducted with the LightCycler® 96 PCR system (Roche Molecular Systems, Indianapolis, IN, USA) according to the manufacturer's specifications. The following primers (Invitrogen, Carlsbad, CA, USA) were used for PCR amplification: IL-1β, 5′-GTTGACGGACCCCAAAAG-3′ (forward) and 5′-GTGCTG CTGCGAGATTTG-3′ (reverse); GAPDH, 5′-AACTTTGGCATTGTGGA

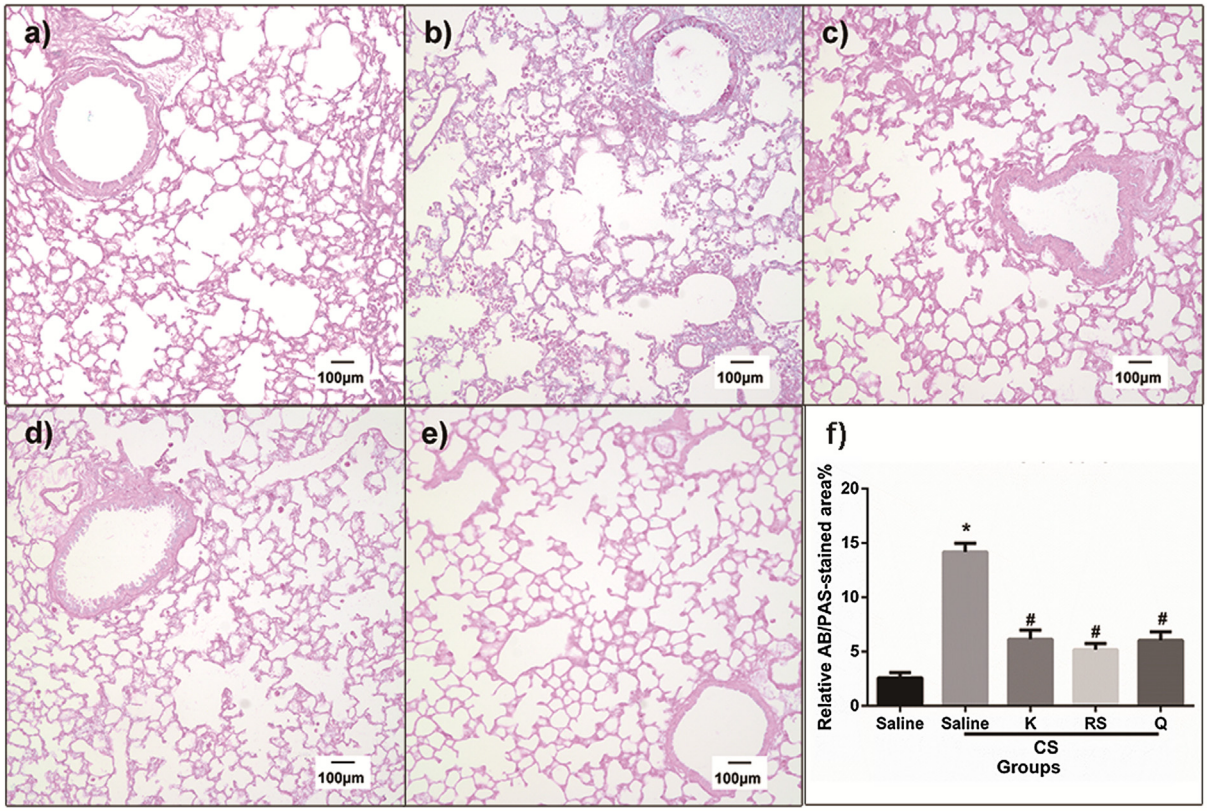

Fig 7. Serotonin receptor antagonist treatment attenuated the mucus hypersecretion induced by cigarette smoke exposure for 6 weeks. AB/PAS staining showed that both specific (ketanserin and RS-127445, c and d) and non-specific (quercetin, e) serotonin receptor antagonists suppressed 6-week cigarette smoke-induced mucus hypersecretion (b) in mouse lungs, AB/PAS-stained area showed the similar trends (f). Values are expressed as mean ± SD (4–6 animals per group). *P < 0.05 compared to the control group; #P < 0.05 compared to the CS group. Abbreviations: CON, control group; CS, cigarette smoke; CS-C, cigarette smoke control group; K, ketanserin; RS, RS-127445; Q, quercetin.

AGG-3′ (forward) and 5′-GTCTTCTGGGTGGCAGTGAT-3′ (reverse); MUC5AC, 5′-AACGGCAGTCCAAAATCC-3′ (forward) and 5′-GAAGGTT CCCAAACTCAAGG-3′ (reverse); and TNF-α, 5′-GTCGCTACATCACTGA ACCTCT-3′ (forward) and 5′-ATGACCCGTAGGGCGATTA-3′ (reverse). PCR reactions consisted of an initial incubation at 95 °C for 10 min, followed by a variable number of amplification cycles involving denaturation at 95 °C for 10 s, annealing for 15 s and extension at 72 °C for 15 s. A final extension was performed at 95 °C for 10 s, 65 °C for 60 s, and 97 °C for 1 s. The annealing temperature was 60 °C for all primer sets. For qualitative analysis, 35 PCR cycles were used.

2.10. Statistical analysis

All values are expressed as mean ± SD. Statistical analysis was performed using SPSS 13.0 (IBM, Chicago, USA). Mean differences were determined by one-way ANOVA followed by the least significant difference (LSD) test. A significant difference was accepted at $P \leq 0.05$ (two-tailed).

3. Results

3.1. Exposure to cigarette smoke for 6 or 12 weeks up-regulated serotonin receptor expression in mouse lung

The percentage of positively stained bronchial epithelial surface area was markedly increased after 6 or 12 weeks of cigarette smoke exposure, based on immunohistochemistry (Fig. 1). Western blot analysis confirmed these results by showing increased protein levels of both 5-HTR2A and 5-HTR2B (Fig. 2).

3.2. Serotonin receptor antagonist limited lung inflammation in mice exposed to cigarette smoke

After 6 or 12 weeks of CS exposure, total cell counts in BALF were determined as a measure of inflammatory response (Fig. 3a). Numbers were higher in smoke-exposed tissues than in control tissues, while pretreatment with ketanserin, RS-127445, or quercetin significantly decreased these cell numbers. Similar trends were observed in levels of the pro-inflammatory cytokines IL-1β and TNF-α (Fig. 3B and C). Pretreatment by serotonin receptor antagonists significantly decreased IL-1β and TNF-α levels at both time points. These data were further confirmed by RT-PCR (Fig. 4).

3.3. Serotonin receptor antagonist prevented histopathological changes in lungs of mice exposed to cigarette smoke

Histopathological changes in mouse airways were examined by H&E staining (Figs. 5 and 6). Cigarette smoke exposure for 6 or 12 weeks led to epithelium thickening, lumen obstruction by mucus and cell debris, and peribronchial inflammatory cell infiltration in the mouse airway. Inflammatory lesion scores were markedly higher in smoke-exposed mice than in control mice. These increases in score were attenuated by pretreatment with ketanserin, RS-127445, or quercetin. Emphysema also had been found in parts of the lung tissue of mice after cigarette smoke exposure for 12 weeks (Fig. 6g), not for 6 weeks (Fig. 5g). Three 5-HTR2A/2B antagonists all alleviated the emphysematous change in the 12 weeks cigarette smoke-induced model (Fig. 6g).

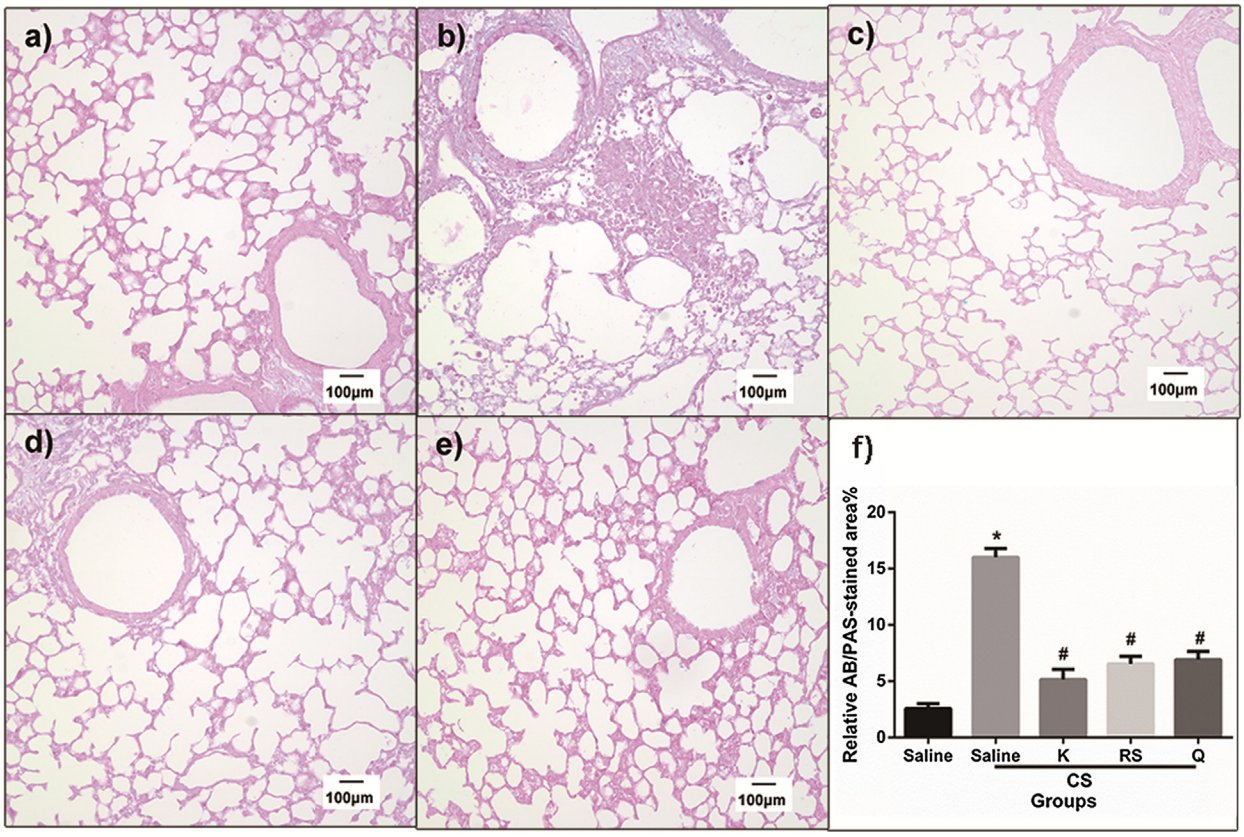

Fig 8. Serotonin receptor antagonist treatment attenuated the mucus hypersecretion induced by cigarette exposure for 12 weeks. AB/PAS staining showed that both specific (ketanserin and RS-127445, c and d) and non-specific (quercetin, e) serotonin receptor antagonists suppressed 12-week cigarette smoke-induced mucus hypersecretion (b) in mouse lungs, AB/PAS-stained area showed the similar trends (f). Values are expressed as mean ± SD (4–6 animals per group). *P < 0.05 compared to the control group; #P < 0.05 compared to the CS group. Abbreviations: CON, control group; CS, cigarette smoke; CS-C, cigarette smoke control group; K, ketanserin; RS, RS-127445; Q, quercetin.

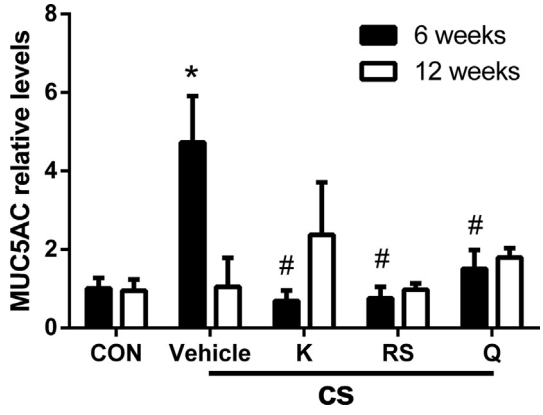

Fig 9. Pretreatment with ketanserin, RS-127445 and quercetin inhibited the increase in MUC5AC mRNA levels in mouse lungs after 6 weeks of cigarette smoke exposure. RT-PCR showed that serotonin receptor antagonist attenuated 6-week cigarette smoke-induced inceresed of MUC5AC mRNA, but this trend was absent in 12-week cigarette smoke exposure groups. Values are expressed as mean ± SD (4–6 animals per group).*P < 0.05 compared to the control group; #P < 0.05 compared to the group exposed to cigarette smoke. Abbreviations: CON, control group; CS, cigarette smoke; CS-C, cigarette smoke control group; K, ketanserin; RS, RS-127445; Q, quercetin.

3.4. Serotonin receptor antagonist decreased hypersecretion of mucus and fibrosis in the airway epithelium of mice exposed to cigarette smoke

Goblet cell hyperplasia was examined using AB/PAS staining (Figs. 7 and 8). Exposure to cigarette smoke for 6 or 12 weeks led to a prominent increase in the number of goblet cells along the airway surface epithelium. This increase in AB/PAS staining was significantly inhibited by each of the three receptor antagonists. Since MUC5AC is the predominant mucin gene expressed in goblet cells, the mRNA levels were examined by RT-PCR (Fig. 9). MUC5AC mRNA levels were markedly increased after 6 weeks, but not 12 weeks, of exposure to cigarette smoke. This increase was significantly attenuated by pretreatment with ketanserin, RS-127445, or quercetin. In contrast, Masson staining showed that the fibrosis surrounding bronchi increased after 12 weeks, but not 6 weeks, of exposure to cigarette smoke (Figs. 10 and 11). Inhibition of serotonin receptors blocked this effect.

3.5. Serotonin receptor antagonist inhibited cigarette smoke-induced activation of the MAPK pathway in lung

Cigarette smoke exposure led to activation of the MAPK pathway, as seen in increased levels of phosphorylated JNK and ERK in lungs from smoke-exposed mice (Fig. 12). Antagonist pretreatment prevented activation of these signaling molecules at 6 and 12 weeks, leading to near-baseline levels of p-JNK and p-ERK (Fig. 12). Cigarette smoke exposure did not affect p38 activation (data not shown).

4. Discussion

In this study, we found altered expression of two serotonin receptor isotypes, 5-HTR2A and 5-HTR2B, in the lungs of mice exposed to cigarette smoke for 6 or 12 weeks. Smoke exposure induced accumulation of macrophages and neutrophils coupled with increased levels of the

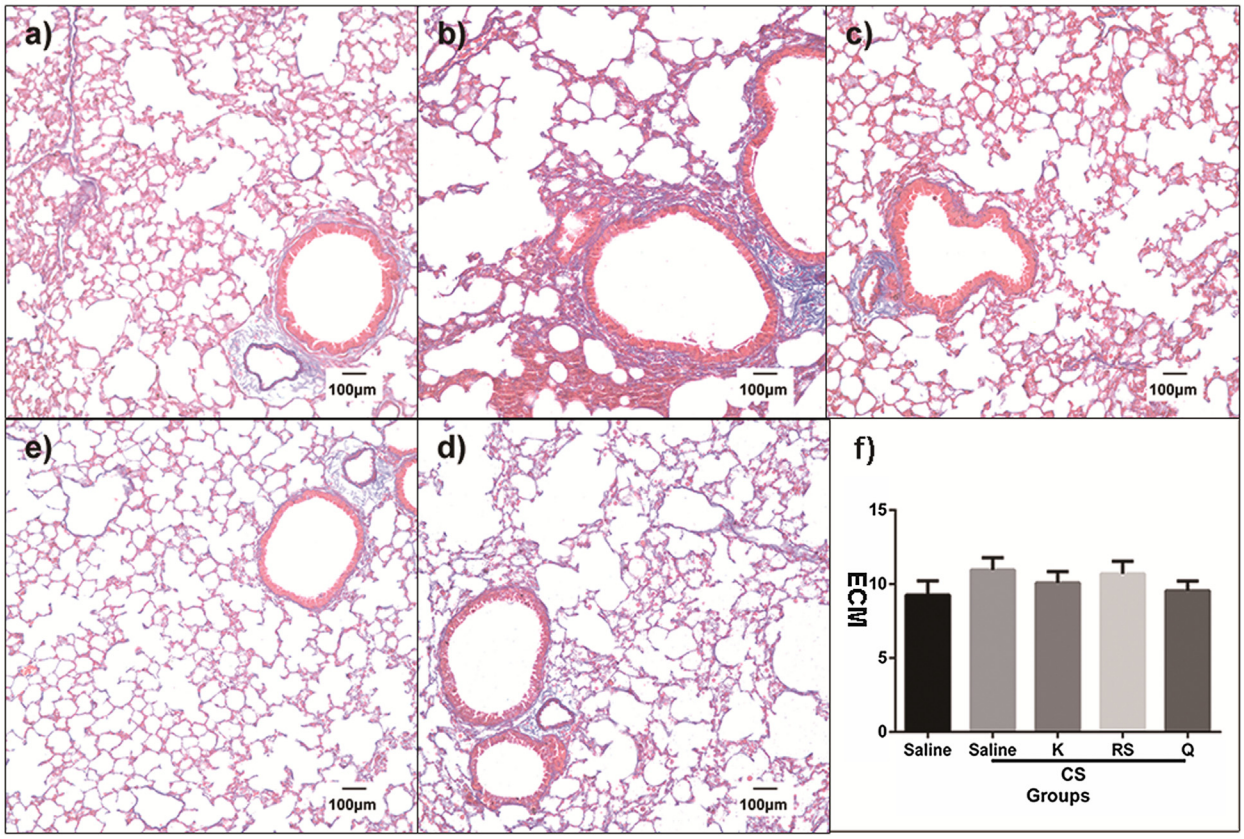

Fig 10. Cigarette smoke or serotonin receptor antagonist pretreatment did not affect the extracellular matrix deposition at 6 weeks. Masson staining showed 6-week cigarette smoke or serotonin receptor antagonist treatment (c, d and e) had no effect on the extracellular matrix deposition in mouse lungs, extracellular matrix scores showed the similar trends. Values are expressed as mean ± SD (4–6 animals per group). *P < 0.05 compared to the control group; #P < 0.05 compared to the CS group. Abbreviations: CON, control group; CS, cigarette smoke; CS-C, cigarette smoke control group; K, ketanserin; RS, RS-127445; Q, quercetin; ECM: extracellular matrix.

pro-inflammatory cytokines IL-1β and TNF-α in BALF and lung tissue. These increases were inhibited by pretreatment with the 5-HTR2A/2B antagonists ketanserin, RS-127445, or quercetin [27]. This pretreatment also suppressed smoke-induced goblet cell hyperplasia and fibrosis in airways. Finally, we showed that these pathogenic processes appear to involve MAPK pathways. Our results suggest that CS-induced airway inflammation, mucus hypersecretion, and small airway remodeling are partially mediated by the 5-HTR (2A/2B) pathway, and that 5-HT signaling may be altered in COPD.

Platelets are the main source of 5-HT in the periphery, which they release at sites of injury and inflammation. Tobacco smoke extracts prepared from both mainstream and side stream smoking have been associated with heightened platelet activation, aggregation, adhesion, and inflammation [28]. In our study, we found that expression of 5-HTR2A and 5-HTR2B increased after exposure to cigarette smoke for 6 or 12 weeks. Accumulating evidence suggests an important role for 5-HT signaling in the lung: asthma and pulmonary hypertension are associated with increased levels of 5-HT and downstream 5-HT receptor signaling [29,30]. Data implicating 5-HT and its receptors in fibrotic disorders such as lung fibrosis suggest that the expression and activation of 5-HTR2A and 5-HTR2B play a crucial role in the pathogenesis of COPD [31–33].

In our study, H&E staining of lung tissue from animals exposed to cigarette smoke for 6 or 12 weeks showed thickening of the airway epithelium, lumen obstruction by mucus and cell debris, and peribronchial inflammatory cell infiltration. Pretreatment with 5-HTR2A/2B antagonists inhibited the peribronchial inflammation. The ability of cigarette smoke to trigger inflammatory responses is supported by identification of a 5-HT analog in an aqueous extract of cigarette smoke that elevated levels of IL-8 mRNA and protein in human bronchial epithelial cells [34]. Our study also found that cigarette smoke increased neutrophil and macrophage infiltration, protein levels in BALF and levels of mRNAs encoding IL-1β and TNF-α in lung. These increases were inhibited by antagonist pretreatment. Actually, there was a limitation in this study. We investigated the effect of 5-HTR antagonists on cigarette smoke-induced airway inflammation based on the changes of BALF IL-1β and TNF-α. However, the other inflammatory cytokines including KC and macrophage inflammatory protein-2 (MIP-2) were not investigated.

Consistent with our findings, previous work showed that blockade of 5-HTR2A with ketanserin suppressed cigarette smoke-induced release of IL-8 by inhibiting p38, ERK1/2 MAPK, and Nrf2 signaling pathways in primary cultures of normal human bronchial epithelial cells, as well as in human bronchial epithelial cell lines [12]. Our study further determined that this blockade was due to suppression of cigarette smoke-induced phosphorylation of JNK, ERK1/2 MAPK, but not p38, in lungs. In previous work, we found that quercetin inhibited cigarette-induced inflammation and oxidative stress in lung tissue and airways by inhibiting NF-κβ and EGFR pathways [26]. Taken together, these data suggest that 5-HTR2A and 5-HTR2B trigger potentially several pathways to help mediate cigarette smoke-induced inflammatory processes.

Apart from inflammation, other characteristics of COPD pathological airway remodeling include hypersecretion of mucus, emphysema and a disproportionate increase in the extracellular matrix within the smooth muscle layer caused by fibrosis [2]. In our study, Exposure to CS for 6 weeks and 12 weeks led to a prominent increase in the numbers of goblet cells along the airway surface epithelium, which was

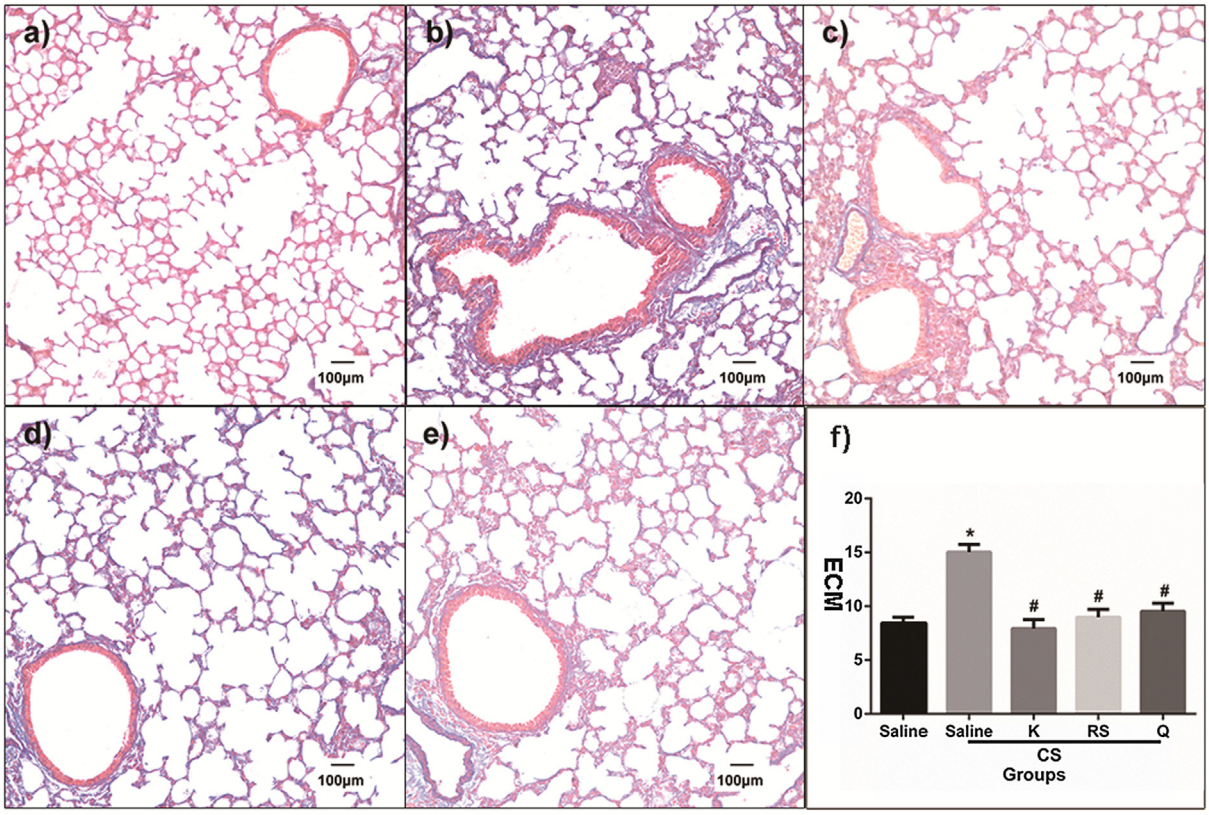

Fig 11. Serotonin receptor antagonist pretreatment attenuated the extracellular matrix deposition induced by cigarette smoke exposure for 12 weeks. Masson staining showed that both specific (ketanserin and RS-127445, c and d) and non-specific (quercetin, e) serotonin receptor antagonists suppressed 12-week cigarette smoke-induced extracellular matrix deposition (b) in mouse lungs, extracellular matrix scores showed the similar trends (f). Values are expressed as mean ± SD (4–6 animals per group). *P < 0.05 compared to the control group; #P < 0.05 compared to the CS group. Abbreviations: CON, control group; CS, cigarette smoke; CS-C, cigarette smoke control group; K, ketanserin; RS, RS-127445; Q, quercetin; ECM: extracellular matrix.

significantly inhibited by Ketanserin and RS-127445. And the mRNA level of Muc5ac was markedly increased after 6 weeks of CS exposure, while not 12 weeks, and this increase was significantly attenuated by Ketanserin and RS-127445. We also found fibrosis surrounding bronchi and emphysema after 12 weeks of cigarette smoke exposure, which was negated by pretreatment with 5-HTR2A/B antagonist. Consistent with our findings, patients and mice with lung fibrosis exhibit upregulated expression of 5-HT2A receptors [34,36], and 5-HT2A/B receptor antagonists exert antifibrotic effects in a wide array of tissue fibrosis animal models [32,35–38]. Few studies have explored the relationship between 5-HT and emphysema. Stoll et al. [39] Found there were no correlations between FEV1 and serum concentrations of 5-HT, neither in thegroup of all COPD patients nor in one of the GOLD stage subgroups. Further studies are needed to explore the role of 5-HT in emphysema and the related mechanisms.

In addition, consistent to our previous study which found Quercetin could inhibit cigarette induced mucus secretion of airway by inhibiting NF-κβ and EGFR pathway in rats [28], we also certified quercetin can inhibit cigarette smoke induced Goblet cell hyperplasia, mRNA expression of muc5ac and fibrosis surrounding bronchi in mice. While the quercetin is poorly specific as the 5-HTR antagonist, as it also activates or inhibits the activities of a number of proteins like estrogen receptors alpha (ERα) and beta (ERβ) [40,41].The reason we chose it in our study is that quercetin as a natural flavonoid may also work by antagonizing 5-HT2R [27]. Since flavonoids are present in food and medicinal plants, they are consumed by humans [42]. We explored the effect of quercetin on cigarette induced airway remodeling, providing an objective basis for the search for easy to access and safe drugs targeting 5-HT receptor. Even so, more specific 5-HTR antagonist should be investigated in further studies.

However, there was also a limitation in this study. As 5-HTR 2A/2B antagonists were delivered with the cigarette smoke exposure simultaneously, the post-treatment with these inhibitors was not evaluated. Actually the current study mimicked to treat the early stage COPD, implying that the interpretation at the early inflammatory initiation stage might attenuate the small airway remolding of COPD. Despite most of the clinical studies focused on the moderate or late-stage COPD patients, several large-scale clinical studies have also suggested that the drug treatment in the early stage of COPD is more effective to prolong the survival time and improve quality of life [43,44], suggesting that early treatment is meaningful for COPD. So our results suggested a great potential of 5-HTR2A /2B antagonist for anti-airway remodeling effect at the early stage. However, most COPD patients visiting clinics were at the exacerbation phase, which implying the moderate or late stage of COPD, thus in the future, the post-treatment of these antagonists in COPD-related airway remodeling should be examined.

5 Conclusion.

Following chronic cigarette smoke exposure for 6 and 12 weeks, mice exhibited enhanced peribronchial inflammation, goblet cell hyperplasia, and peribronchial fibrosis, and these effects involve the activity of 5-HT and its receptors (2A/2B). Regulation of 5-HT receptor activity appears to involve MAPK pathways. These data suggest that 5-HT and 5-HTR2A/2B are potentially novel therapeutic targets for airway inflammation, mucus hypersecretion and airway remodeling in COPD.

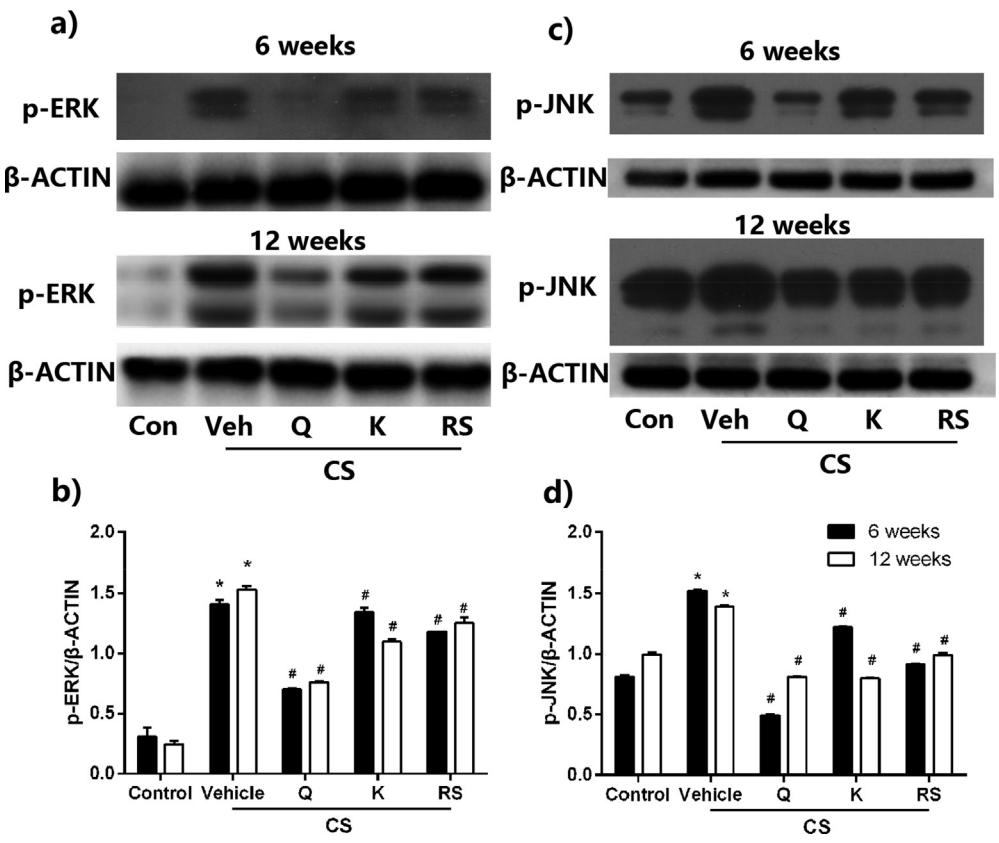

Fig 12. Serotonin receptor antagonists suppressed the cigarette smoke-induced phosphorylation of ERK and JNK in mouse lungs following smoke exposure for 6 or 12 weeks. Western blot showed that serotonin receptor antagonists suppressed 6-week or 12-week cigarette smoke-induced phosphorylation of ERK (a) and JNK (c). Blot densitometry showed the similar trends (b and d). Values are expressed as mean ± SD (4–6 animals per group). *P < 0.05 compared to the control group; #P < 0.05 compared to CS-C group. Abbreviations: CS, cigarette smoke; CON, control group; CS-C, cigarette smoke control group; 6 W, 6 weeks; 12 W, 12 weeks; K, ketanserin; R, RS-127445; Q, quercetin.

Author contributions

Ting Yang, Lei Chen and Fuqiang Wen designed the study. Ting Yang, Hao Wang, and Yuhao Li constructed the mouse model and performed measurements. Ting Yang, Hao Wang, Zijian Zeng, Yongchun Shen, Chun Wan, Yanqiu Wu, and Jiajia Dong collected and analyzed data. Ting Yang, Hao Wang, and Yuhao Li wrote the manuscript.

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Declaration of Competing Interest

The authors declare no conflicts of interest.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.intimp.2019.106036.

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