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Preliminary report

Tobacco smoking aggravates airway inflammation by upregulating endothelin-2 and activating the c-Jun amino terminal kinase pathway in asthma

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ABSTRACT

Background: Asthma is closely associated with tobacco smoking (TS) and is more difficult to effectively treat after exposure to TS.
Objective: To observe the effects of TS on the expression of endothelin-2 (ET-2) and airway inflammation in asthmatic rats and to explore the related mechanisms.
Methods: We established an animal model of asthma with ovalbumin (OVA)/$Al(OH)_3$ and subjected different animal groups to TS and/or dexamethasone/bosentan. The differences in the inflammatory cell infiltration, the pathological changes to the bronchial wall and the bronchial smooth muscle thickness, and the expression of ET-2, c-Jun amino terminal kinase (JNK1/2), malondialdehyde (MDA), and glutathione peroxidase (GSH) in the lung tissue and of interleukin (IL)-7 in bronchoalveolar lavage fluid (BALF) were assessed.
Results: Exposure to TS or OVA caused an obvious increase in the inflammatory cells in the BALF over what was observed in the control group. In asthma models, the expression of ET-1, JNK1/2, MDA, and GSH in the lung tissues, as well as that of IL-17 in the BALF, was increased. After treatment with dexamethasone/bosentan, the expression of IL-17, JNK1/2, MDA, and GSH decreased compared to the smoking group; airway inflammation and the staining intensity in the lung tissue were also reduced.
Conclusion: TS exposure can clearly exacerbate airway inflammation in asthmatic rats, while bosentan can alleviate airway inflammation through regulation of the ET-2/JNK1/2 signalling pathway.

1. Introduction

Asthma is a heterogeneous disease, affecting an estimated 300 million individuals worldwide. This disease is usually characterized by chronic airway inflammation. Asthma is defined by a history of respiratory symptoms such as wheezing, shortness of breath, chest tightness, and coughing that varies over time and in intensity; asthma is also associated with limitations in variable expiratory airflow [1]. Common risk factors for this disease include genetic predisposition, tobacco smoking (TS), infections, and allergens.

Some epidemiological studies suggest that active or passive TS is a major risk factor for developing asthma [2]. TS exposure can directly modulate the functions of airway epithelial, smooth muscle, or neural cells to evoke airway hyper-responsiveness (AHR). TS can also induce neutrophilic inflammation and airflow obstruction, enhance AHR, alter the airway inflammatory phenotype of asthma [3], exacerbate airway remodelling in asthma, and decrease response to glucocorticoid therapy [4,5]. However, the exact mechanisms underlying these TS-induced changes in asthma are unclear.

Endothelins (ETs) (including ET-1, ET-2, and ET-3) are a family of 21-amino acid-long peptides, which are uniquely powerful vasoconstrictors with a slow onset of action. It is likely that ET is synthesized in the different types of pulmonary cells and not just in the endothelial cells. Clinical studies have confirmed that both ET-1 and ET-3, as well as their precursors, are present in the airway epithelial cells and the submucosal glands [6]. Activation of endothelin receptors (ETRs) plays an important role in promoting the contraction of bronchial smooth muscles, plasma exudation increase, and mucus secretion [7–9]. Hey and Chanez P also reported that ET-1 could stimulate monocytes and macrophages to release tumour necrosis factor (TNF)-α, fibronectin, interleukin (IL)-1b and IL-6, all of which play important roles in the allergic inflammation of asthma [10,11], so ET-1 could be considered

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an important marker of allergic asthma. However, the interrelationship between ET-2 and asthma remains unclear, especially in asthma patients who smoke. Thus, in this study, we aimed to uncover the effect of TS exposure on ET-2 expression in asthma animal models and to explore the underlying mechanisms.

2. Materials and methods

2.1. Animals and reagents

Forty-two 6-week-old specific-pathogen-free Sprague-Dawley male rats (Animal Center of Xuzhou Medical University), weighing 150–180 g, were used. The following materials were used: chicken ovalbumin, a rabbit anti-ET-2 polyclonal antibody, a rabbit anti-JNK1/2 polyclonal antibody (Beijing Bi Olson Biotech Corp), an alkaline phosphatase-labelled goat anti-rabbit IgG (Beyotime Biotech Corp), a horseradish peroxidase (HRP)-labelled goat anti-rabbit IgG (GenScript Biotech. Co. Ltd.), bosentan (Patheon Company), Mount Lu cigarettes (0.8 mg nicotine, 10 mg tar), dexamethasone (Wuhan Binhu Shuanghe Pharmaceutical Company; batch 1607142111), a malondialdehyde (MDA) determination kit and a glutathione peroxidase (GSH) kit (Nanjing Institute of Biological Engineering), a diaminobenzidine (DAB) colour reagent kit (China Zhongshan Jinqiao Biotech Corp.), and a BCIP/NBT alkaline phosphatase staining kit (Beyotime Biotech Corp.). The rats were used for the study one week after their procurement. Our experiments were approved by the Xuzhou Medical University Ethics Committee for Animal Research and performed according to the National Institutes of Health Guidelines for the Use of Experimental Animals (No 2017-10-13).

2.2. Animal grouping and preparation of the animal models

Forty-two rats were randomly divided into seven groups, with six rats in each group: group a (normal control group), group b (TS group), group c (asthma group), group d (TS asthmatic group), group e (dexamethasone, TS asthmatic group), group f (bosentan, TS asthmatic group), and group g (dexamethasone, bosentan intervention TS asthmatic group). The rat asthma models were prepared by sensitizing and stimulating the rats with OVA, as previously described [12]. In groups c–g, 1 mg of freshly prepared OVA and 2 mL of fluid with $Al(OH)_3$ (100 mg) were intraperitoneally injected on days 1 and 8. The rats were then placed in an airtight container each day and stimulated with a 1% OVA normal saline solution using a spray nebulizer for 40 min per administration from day 15 to day 28 [9]. The group a rats were simultaneously treated with saline. In groups b, d, e, f, and g, the rats were exposed to 6 cigarettes 1 h per day in a homemade semi-closed smoke box for 28 days, as described previously by Al-Sawalha [13]. In groups e and g, dexamethasone (2 mg/(kg·d)) was intraperitoneally injected 30 min before every atomization from day 15 to day 28. In groups f and g, bosentan was administered by gavage (100 mg/(kg·d)) 30 min before each atomization from day 15 to day 28.

2.3. Bronchoalveolar lavage fluid (BALF) collection

The rats were killed 24 h after the last ovalbumin or saline challenge. The BALF was collected by perfusing both lungs after cannulating the trachea. The collected BALF was centrifuged at 3000 r/m for 10 min, and the liquid supernatant was collected and stored at −80 °C for subsequent testing for the interleukin-17 (IL-17), IL-6, and TNF-α concentrations in the BALF by enzyme-linked immunosorbent assays (ELISAs), following the manufacturer's instructions. The absorbance was read at 450 nm. In addition, the centrifugally collected cells were resuspended in 1 mL phosphate buffered saline for counting the cells by haemacytometer.

2.4. Histopathology and immunohistochemical staining

The right upper lung lobes of the rats were collected, after which they were fixed, rinsed, dehydrated, embedded and sectioned. Three paraffin sections were randomly selected from each rat for HE staining, with which any pathological changes were examined under a high-power microscope. The positive cells were identified by a brown-yellow colour in the nucleus, membrane or cytoplasm. The distribution of positive cells and the expression of ET-2 were observed under a microscope.

2.5. Western blot

Protein was extracted from 100 mg of the rat left lower lobe lung tissue of each rat. A BCA protein quantitative analysis kit was used to test the protein concentration. After electrophoresis, protein transfer, and blocking, the membranes were incubated overnight in a refrigerator at 4 °C with antibodies against ET-2 (1:500), JNK1/2 (1:200) and β-actin (1:1000). After washing the membranes, the secondary antibody, a goat anti-rabbit IgG labelled with horseradish peroxidase, was incubated with the membranes at room temperature for 1.5 h, and exposure of the membranes for detection of signal was performed with the BCIP/NBT kit. ImageJ software was used to analyse the grey value of the protein bands.

2.6. Measurement of oxidative stress markers

The lung tissue was processed into a homogenate and centrifuged at 3000 r/min for 15 min, and the upper serum solution was collected. The content of glutathione peroxidase (GSH) in the supernatant was measured by calorimetric enzymatic assays following the manufacturer's instructions. The content of MDA in the supernatant was detected by a thiobarbituric acid method by a strict adherence to the manufacturer's instructions.

2.7. Statistical analyses

The data were calculated and processed using SPSS23.0. All values are shown as the mean ± standard error of the mean. Comparisons of the inflammatory cells, oxidative stress markers and cytokine levels were analysed by one-way ANOVA. The results were considered statistically significant when $P < 0.05$.

3. Results

3.1. Inflammatory cells in the BALF

The leukocyte populations in the BALF of the TS group ($10.117 \pm 1.303 \times 10^6$/L), asthma group ($9.155 \pm 1.033 \times 10^6$/L), and TS asthmatic group ($11.55 \pm 1.524 \times 10^6$/L) were obviously higher than the values of the control groups ($8.282 \pm 0.736 \times 10^6$/L). The TS asthmatic group had the largest population of leukocytes, indicating that TS could exacerbate airway inflammation. After intervention with dexamethasone/bosentan, the leukocytes decreased in the dexamethasone TS asthmatic group ($10.583 \pm 1.788 \times 10^6$/L), the bosentan TS asthmatic group ($10.533 \pm 1.171 \times 10^6$/L), and the dexamethasone bosentan TS asthmatic group ($9.83 \pm 1.491 \times 10^6$/L), compared to the TS asthmatic group ($11.55 \pm 1.524 \times 10^6$/L). The changes in the populations of neutrophils and eosinophils were similar to those observed for the leukocytes. The differences were statistically significant ($P < 0.05$) (Table 1) (Fig. 1A).

3.2. IL-17, IL-6 and TNF-α levels in the BALF

The analysis of the ELISA findings showed that the expression of IL-17 significantly increased after exposure to TS and OVA in the TS group

Table 1
Leukocytes and differential cell counts in rats BALF ($\times 10^6$/L, n = 6, $\bar{x} \pm s$).

Groups	leukocytes	Neutrophiles	Eosinophiles
a	8.282 ± 0.736	3.35 ± 1.257	0.342 ± 0.318
b	10.117 ± 1.303*	4.178 ± 1.624*	2.278 ± 0.235*
c	9.155 ± 1.033*	4.433 ± 0.606*	1.818 ± 0.755*
d	11.55 ± 1.524*	5.783 ± 1.361*	2.928 ± 0.524*
e	10.583 ± 1.788#	5.355 ± 1.20#	2.287 ± 0.732#
f	10.533 ± 1.171#	4.877 ± 0.629#	2.640 ± 0.340#
g	9.83 ± 1.491#	4.458 ± 1.355#	1.695 ± 0.635#

Statistical analysis was performed with one-way ANOVA.*P < 0.05 *vs group a*; #P < 0.05 *vs group d*.
a = normal control group, b = tabacco smoking group, c = asthma group, d = tabacco smoking asthmatic group, e = dexamethasone tabacco smoking asthmatic group, f = bosentan tabacco smoking asthmatic group, g = dexamethasone bosentan intervention tabacco smoking asthmatic group.

Table 2
The levels of cytokines in BALF (n = 6, $\bar{x} \pm s$).

Group	IL-17 (ng/ml)	IL-6 (ng/ml)	TNF-α (ng/ml)
a	24.327 ± 1.516	16.667 ± 0.957	2.797 ± 0.601
b	34.977 ± 2.006*	33.683 ± 0.841*	6.005 ± 0.907*
c	38.667 ± 1.536*	41.607 ± 1.397*	8.512 ± 0.6574*
d	51.592 ± 2.895*	67.292 ± 1.787*	12.75 ± 2.147*
e	42.698 ± 2.265#	44.039 ± 1.212#	6.733 ± 0.976#
f	37.740 ± 1.876#	60.813 ± 1.076#	7.960 ± 0.696#
g	32.320 ± 2.005#	34.730 ± 1.243#	5.405 ± 0.393#

Statistical analysis was performed with one-way ANOVA.*P < 0.05 *vs* group a; #P < 0.05 *vs group d*.
a = normal control group, b = tabacco smoking group, c = asthma group, d = tabacco smoking asthmatic group, e = dexamethasone tabacco smoking asthmatic group, f = bosentan tabacco smoking asthmatic group, g = dexamethasone bosentan intervention tabacco smoking asthmatic group.

(34.977 ± 2.00 ng/ml), the asthma group (38.667 ± 1.536 ng/ml), and the TS asthmatic group (51.592 ± 2.895 ng/ml), compared with the control group (24.327 ± 1.516 ng/ml) ($P < 0.05$). The IL-17 level was the highest in the TS asthmatic group. After intervention with dexamethasone or/and bosentan, the levels of IL-17 decreased, especially in the dexamethasone bosentan TS asthmatic group. The changes in IL-6 and TNF-α were similar to those observed for IL-17 ($P < 0.05$; Table 2 and Fig. 1B).

3.3. Pathological changes

In the control group, we observed that the bronchial epithelial mucosa was intact and undamaged, with few inflammatory cells infiltrating under the bronchial mucosa, and the alveolar wall was smooth. In the TS group, the bronchial mucosa showed hypertrophy and thickening, with an infiltration of neutrophils. In the asthma group, the epithelial cells of the bronchial mucosa were deformed and exfoliated, the alveolar wall was congested and oedematous with an

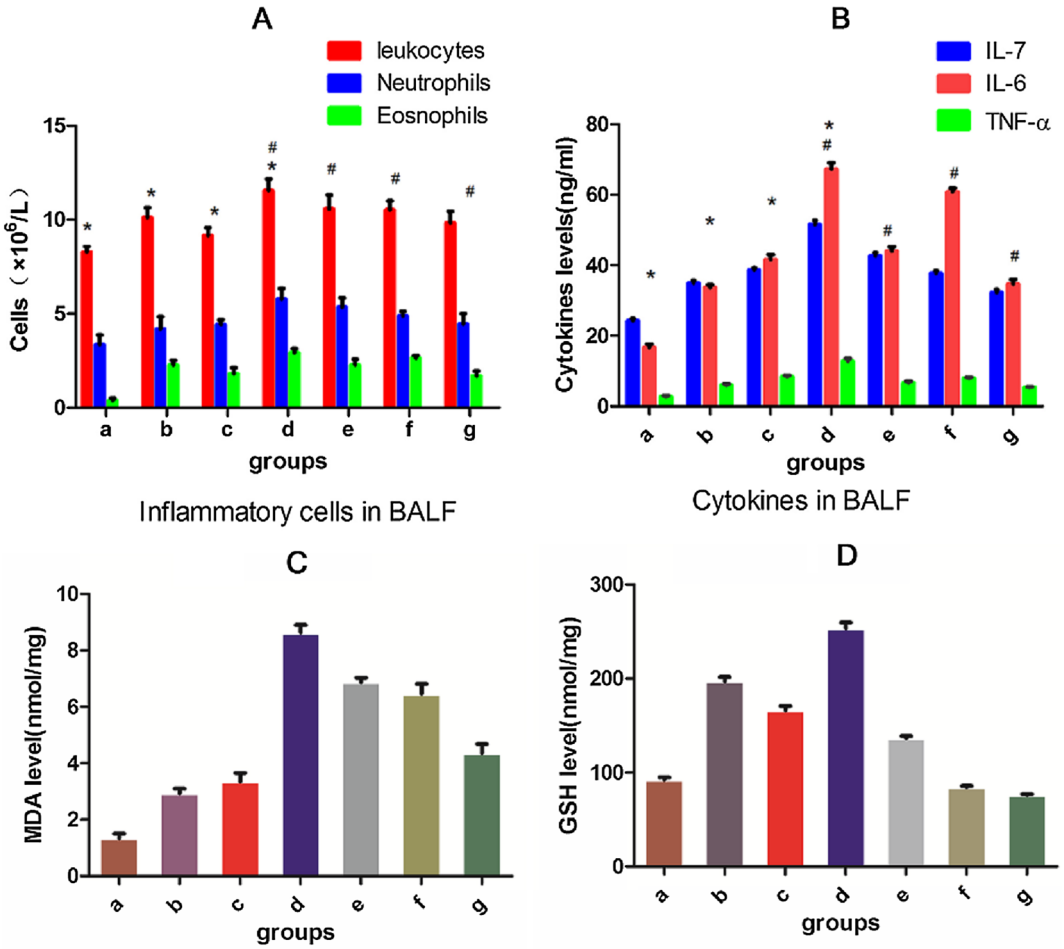

Fig. 1. A. Total number and classification of inflammatory cells in each group. B. IL-17 levels in the BALF samples from each group. C. MDA expression in the lung tissue from each group. D. GSH expression in the lung tissue from each group. a: normal control group; b: tobacco smoking group; c: asthma group; d: tobacco smoking asthmatic group; e: dexamethasone-tobacco smoking asthmatic group; f: bosentan-tobacco smoking asthmatic group; g: dexamethasone/bosentan-tobacco smoking asthmatic group.

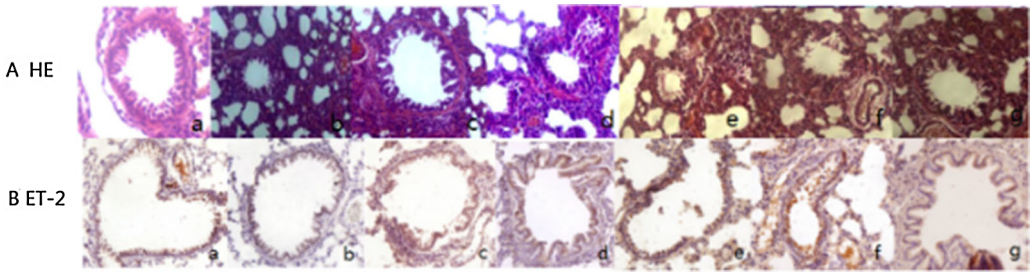

Fig. 2. A. Pathological changes in the lung tissue of the rats in each group (HE staining, ×40). B. Detection of ET-2 and JNK1/2 expression by immunohistochemistry.

infiltration of eosinophils, and the bronchial mucosa showed hypertrophy and thickening. In the TS asthmatic group, the continuity of the bronchial lumen was interrupted, the mucosal epithelium was necrotic and exfoliated, and the airway wall was thickened, with an extensive infiltration of lymphocytes, eosinophils, and neutrophils. In the dexamethasone TS asthmatic group and the bosentan TS asthmatic group, the alveolar wall edema and the inflammatory cell infiltration were reduced, while the bronchial mucosa epithelium was necrotic and exfoliated and the airway wall thickening was not obvious. In the dexamethasone bosentan TS asthmatic group, necrosis and exfoliation of the bronchial epithelium were not obvious, airway wall thickening was not obvious, and infiltration of inflammatory cells was significantly decreased (Fig. 2A).

3.4. Expression of ET-2 in the lung tissue

Many studies have demonstrated that the ET-2 protein can be expressed in the nucleus, the cytoplasm, and the cell membrane of alveolar epithelial cells and bronchial epithelial cells. Our results revealed that ET-2 expression was weaker in the lung tissues of the control group and stronger in the TS group, the asthma group and the TS asthmatic group, especially in the TS asthmatic group. After intervention with dexamethasone, ET-2 expression was found to have decreased slightly in the dexamethasone TS asthmatic group and the dexamethasone bosentan TS asthmatic group, while no decrease was noted after intervention with bosentan in the bosentan TS asthmatic group compared to the TS asthmatic group (Fig. 2B).

3.5. Changes in JNK1/2 expression at the protein level

Western blot analyses showed that the expression of JNK1/2 in the TS group, the asthma group and the TS asthmatic group was significantly increased, compared to that in the control group, which is similar to what was observed with ET-2. After intervention with dexamethasone in the dexamethasone TS asthmatic group, JNK1/2 expression decreased in the dexamethasone TS asthmatic group. Upon ETR inhibition with bosentan, the expression of JNK1/2 decreased in the bosentan TS asthmatic group and the dexamethasone bosentan TS asthmatic group, compared to the TS asthmatic group (Fig. 3).

3.6. MDA and GSH expression in the lung tissue

The expression of MDA and GSH was significantly higher after exposure to TS in the TS group and the TS asthmatic group, compared to the control group. After treatment with dexamethasone/bosentan, the expression of MDA and GSH also decreased in the dexamethasone TS asthmatic group, the bosentan TS asthmatic group and the dexamethasone bosentan TS asthmatic group, compared to the TS asthmatic group (Table 3).

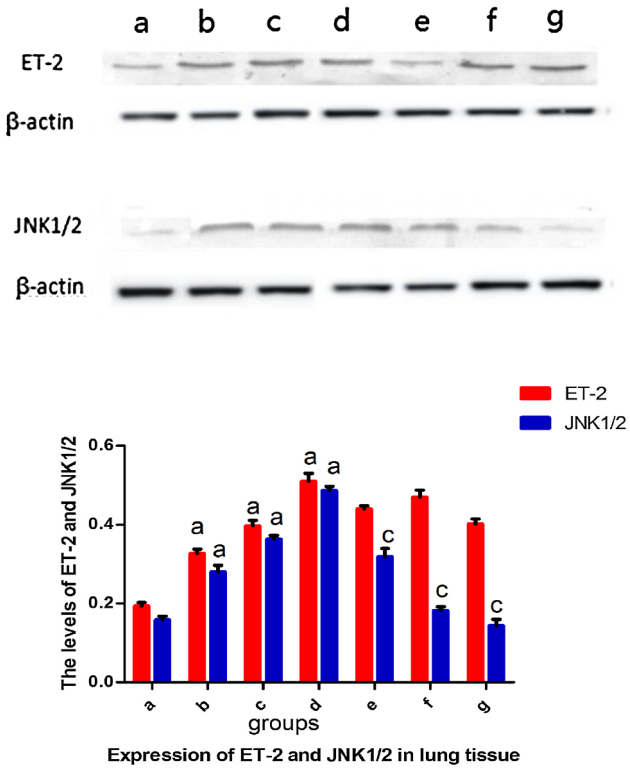

Fig. 3. Changes in the expression of the ET-2 and JNK1/2 proteins in the lung tissue of the rats in each group.

Table 3
The expression of MDA and GSH in lung tissue (n = 6, $\bar{x} \pm s$).

Groups	MDA (nmol/mg protein)	GSH (nmol/mg protein)
a	1.338 ± 0.164	92.383 ± 5.982
b	2.932 ± 0.160*	197.05 ± 11.335*
c	3.348 ± 0.305*	166.467 ± 11.027*
d	8.627 ± 0.274*	253.65 ± 14.881*
e	6.872 ± 0.16#	136.633 ± 5.341#
f	6.465 ± 0.349#	84.727 ± 2.614#
g	4.36 ± 0.318#	76.222 ± 2.461#

Statistical analysis was performed with one-way ANOVA.*P < 0.05 *vs* group a; #P < 0.01 *vs group d*.
a = normal control group, b = tabacco smoking group, c = asthma group, d = tabacco smoking asthmatic group, e = dexamethasone tabacco smoking asthmatic group, f = bosentan tabacco smoking asthmatic group, g = dexamethasone bosentan intervention tabacco smoking asthmatic group.

4. Discussion

TS is one of the most serious public health concerns, accounting for 6.1 million deaths worldwide; it was the second leading cause of deaths

in 2013 [14]. Many epidemiological investigations have showed that the severity of asthma is closely associated with TS. If a pregnant woman smokes during her term, or if children are exposed to passive or active smoking, the incidence of asthma in children will be increased [15,16]. The plasticity of embryonic development and epigenetic processes can be affected by maternal smoking during pregnancy, both of which are closely related to asthma [17]. Studies have shown that exposure to TS can exacerbate the symptoms of asthma, such as a higher risk of apnoea, deteriorated lung function, altered airway inflammation, and a decreased effect of glucocorticoids [18,19]. Simpson et al. clearly demonstrated that neutrophil-induced airway inflammation caused by TS could reduce the efficiency of glucocorticoids [20].

IL-17 is a key proinflammatory cytokine produced by Th17 cells; this cytokine plays an important role in neutrophil inflammation and participates in airway remodelling in asthma [21]. Thus, the level of IL-17 is closely related to the severity of asthma. TNF-α and IL-6, shown to be produced by mononuclear phagocytes from allergic asthmatics as early as the 1990s, play pivotal roles in chronic airway inflammation and structural remodelling [22]. In our study, we found that airway inflammation in the TS asthmatic group was more severe than that in the asthma model group. In addition, the levels of white cells, neutrophils, and inflammation mediators (IL-6, IL-17, and TNF-α) in the BALF showed a clear increase, indicating that TS can aggravate airway inflammation in asthmatic rats, especially neutrophilic inflammation. Treatment with bosentan or dexamethasone decreased the airway inflammation in the TS asthmatic rats. When both bosentan and dexamethasone were used, airway inflammation was reduced to an even greater extent. TS is a major exogenous source of oxidative stress, contributing to the subsistence and progression of the inflammatory response and disease chronicity in asthma. GSH and MDA are good indicators of the level of oxidative stress [23]. Our study showed that MDA and GSH expression increased after exposure to TS, which was reversed by exposure to bosentan/dexamethasone. Thus, bosentan and dexamethasone can alleviate asthma-associated inflammation.

The ETs (ET-1, ET-2, and ET-3) are a group of small (21 amino acids) vasoactive peptides with diverse paracrine/autocrine functions. The ETs bind to a minimum of two receptor subtypes: ET_A and ET_B receptors, which are seven transmembrane domain G protein-coupled receptors [9,24]. Activation of the ETRs plays an important role in airway inflammation, mucous secretion, release of inflammatory mediators and fibronectin, and studies about airway inflammation are the most. Shimoda et al have reported that TS can upregulate the expression of the ETRs in rat bronchial smooth muscle cells [5]. ET-1 has proinflammatory effects in the airways, being both a chemoattractant mediator, such as IL-6 and IL-8, and a granulocyte-macrophage colony-stimulating factor (GM-CSF) [22]. A previous study had reported that the level of ET-1 in BALF is significantly increased in asthmatic cats, compared to the control, and therefore, ET-1 can be used as one of the indicators for asthma diagnosis [25]. Finsnes reported that the concentrations of several proinflammatory mediators (TNF-α, IL-8, IL-4, IL-1b, IFN-γ, and ET-1) were decreased when animals with asthma were treated with ET receptor antagonist bosentan in 2001 [26]. Gamze et al. reported that after bosentan treatment of animals with emphysema, the concentrations of TNF-α, IL-1β, IL-4, and ET-1 in the BALF and the lung tissue decreased, as did the neutrophils and macrophages in the BALF [22]; thus, bosentan could be considered an inhibitor of airway inflammation. ET-2 is considered to be an effective inflammatory chemokine, such as the CC chemokine 2 (C/C motif chemokine ligand 2, CCL2), which exerts a low-level chemotactic effect on neutrophils via the mitogen-activated protein kinase (MAPK) pathway. Selective removal of the ET-2 gene causes morphological changes in the lung tissue, leading to hypoxemia and hypercapnia [27]. However, no previous studies have investigated ET-2 expression in asthma. Our study found that the expression of ET-2 in the airway epithelium increased after exposure to TS, especially in asthmatic rats.

JNK is a member of the MAPK family, which becomes phosphorylated through three different signalling pathways. The activation of JNK by translocation into the nucleus, through the activation of the AP-1 transcription factor, promotes gene expression, plays an important role in cell proliferation and differentiation and in tumour transformation, and participates in the stress and inflammatory processes [28,29]. TS can upregulate the expression of the 5-hydroxytryptamine receptor 2A (5-HTR2A) and the ETA and ETB receptors in the rat cerebrovascular system via the Raf/ERK/MAPK pathway [30]. Van der Velden demonstrated an important role for JNK1 in promoting house dust mite-induced fibrotic airway remodelling, independent of the recruitment of inflammatory cells, airway hyper-responsiveness, and mucus metaplasia [29]. Our study showed that the expression of JNK1/2 protein was higher in the TS and the asthma model groups, compared to the control group, which is consistent with previous experiments. Bosentan is a non-specific inhibitor of ETR, inhibiting the ETA and ETB receptors [31]. In our study, we found that the expression of JNK1/2 decreased in the TS asthmatic group after bosentan treatment, indicating that JNK1/2 activation by ET-2 alters the airway inflammatory phenotype of asthma, thereby exacerbating airway remodelling in asthma. Further studies are required to determine whether bosentan can be safely and efficiently used to treat refractory asthma.

5. Conclusion

The findings of our study showed that TS can increase the expression of ET-2 in the airway epithelium of asthmatic rats and aggravate neutrophil-induced airway inflammation in asthmatic rats. Therefore, smoking cessation is very important in asthmatic patients. Bosentan can reduce the effects of TS in asthmatic rats by inhibiting the ETRs. JNK1/2 is closely related to ET expression in the airway epithelia. Although the animal model does not represent a model of clinical disease, it provides a vehicle for studying the system in the absence of underlying inflammation. However, more preclinical and clinical trials are warranted to evaluate the use of bosentan for asthma care.

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Declaration of Competing Interest

We have no conflicts of interest to declare.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.intimp.2019.105916.

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