Soluble advanced glycosylation receptor is a potential target for the treatment of neutrophilic asthma

Xiaobo Zhang

First affiliated hospital of guangxi medical university <https://orcid.org/0000-0002-7972-863X>

Jun Xie

guangxi medical university

Hongmei Sun

guangxi medical university

Qing Wei

guangxi medical university

Ying Tao

guangxi medical university

Guangmin Nong (✉ ngmprof8525@126.com)

First Affiliated Hospital of Guangxi Medical University <https://orcid.org/0000-0002-3219-0895>

Research

Keywords: sRAGE, neutrophilic asthma, neutrophilic airway inflammation, airway remodeling, epithelial-mesenchymal transition

Posted Date: August 31st, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-64838/v1>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Abstract

Background

Neutrophilic asthma (NA) was a subtype of asthma. Soluble advanced glycosylation receptor (sRAGE) was considered to be associated with the neutrophilic airway. However, the role of sRAGE in NA still limited.

Methods

A NA mouse model was established and the levels of sRAGE in the bronchoalveolar lavage fluid (BALF) were measured by ELISA. Hematoxylin-eosin (HE) and Masson trichrome staining were used to identifying airway remodeling. Adeno-associated virus 9 (AAV9) overexpressed sRAGE and inhibitors for HMGB1, RAGE, and PI3K were used to intervene NA mouse model via tail-vein injection and intraperitoneally injection. Expressions of airway remodeling, EMT, and signaling markers were detected using qRT-PCR or western blotting. The levels of IL-17 and IL-6 in BALF were measured by ELISA. HMGB1 was applied to induce EMT of human bronchial epithelial cells (16HBE), then E-cadherin and vimentin expressions were examined after sRAGE, RAGE inhibitor, and PI3K inhibitor administration.

Results

sRAGE levels were significantly reduced in BALF and the airway remodeling was observed in the NA mouse model. AAV9-sRAGE significantly inhibited the neutrophilic airway inflammation, airway remodeling, and the expression of IL-17, IL-6, TGF- β 1, RAGE, PI3K, and EMT markers -E-cadherin and vimentin *in vivo*. HMGB1 inhibitor, RAGE inhibitor, and PI3K inhibitor upregulated E-cadherin level. Moreover, HMGB1 promoted the EMT process via RAGE/PI3K in 16HBE cells and sRAGE reversed HMGB1- induced EMT *in vitro*.

Conclusion

sRAGE levels decrease in the mouse model with NA. sRAGE treatment attenuates neutrophilic airway inflammation, airway remodeling, and EMT. This suggests sRAGE may yield benefits in the treatment of NA.

1. Background

Asthma is a common chronic airway inflammatory disease affecting more than 300 million people in the world [1]. Neutrophilic asthma (NA) is a subtype of asthma occurring in 15–25% of the asthma cases [2]. Some patients with NA exhibited a poor response to treatment and may have irreversible airflow obstruction [3]. A long-term cohort study has shown that children with asthma reported a 10-32-fold

increased risk of developing adult COPD which was also characterized by airway neutrophilic inflammation [4]. Airway remodeling is a common pathological process of asthma and COPD and it is also an important cause of airflow limitation. Studies confirmed that children with asthma have existed airway remodeling in early childhood. Therefore, better understanding the mechanism of airway remodeling in NA may pave the way for targeted treatment in the key asthma subtype and benefit to COPD management in the future.

Soluble advanced glycosylation receptor (sRAGE) as a predictive indicator of airway neutrophil inflammation and a biomarker for COPD, which was decreased in the blood and bronchoalveolar lavage fluid (BALF) of patients with COPD [5]. Moreover, it was negatively correlated with COPD grade and lung function [6, 7]. We hypothesized that sRAGE may be involved in the pathogenesis of NA and the common pathological process of NA and COPD.

To study the role of sRAGE in NA, we established a mouse model with NA to explore sRAGE expression in BALF and the function of sRAGE on airway remodeling by an adeno-associated virus 9 (AAV9) overexpressed sRAGE administration. We proposed that sRAGE overexpression may yield benefits in the treatment of neutrophil airway inflammation and airway remodeling for NA.

2. Materials And Methods

2.1 Animals

Female C57BL/6 mice aged 6–8 weeks with weight 18–20 g were purchased from Guangxi Medical University Animal Center. Mice were housed under specific pathogen-free conditions in separate cages with a relatively stable temperature (20–24 °C) and humidity (55 ± 10%) at 12-h light/dark cycles and given food and water freely. All animal experiments were approved by The Ethics Committee of The First Affiliated Hospital of Guangxi Medical University [2019 (KY-E-035)] and carried out following The National Institutes of Health Guide for the Care and Use of Laboratory Animals.

2.2 *Animal groups*

Mice were randomly divided into five groups (n = 6 in each group): normal control group (NC), NA group (NA), phosphate-buffered saline group (PBS), AAV9 overexpressed sRAGE group (NA + sRAGE) and AAV9-control group (NA + sRAGE control). The model of NA was established according to the protocol from Wilson *et al* [8] and our previous study (published in Chinese). Mice were sensitized by airway delivery of 100 µg ovalbumin (OVA; Grade II & V; Sigma-Aldrich; Merck KGaA) and 1 µg lipopolysaccharide (LPS; Sigma-Aldrich; Merck KGaA) in a total volume of 50 µl PBS on days 1, 7 and 14. The OVA + LPS mixture was instilled along the posterior oropharyngeal wall, and the mixed solution was inhaled into the airway, followed by a challenge with 1% OVA aerosol for 1 h from day 21 for 7 consecutive days. In the NA + sRAGE group and NA + sRAGE control group, mice were administered with AAV9-sRAGE (0.8E + 12 v.g./mL × 100 µL, Shanghai GeneChemCo., Ltd, China) and AAV9-control (0.8E + 12 v.g./mL × 100 µL, Shanghai GeneChemCo., Ltd, China) via tail-vein injection 1 week before sensitization and challenge,

respectively. HMGB1 inhibitor, RAGE inhibitor, and PI3K inhibitor were intraperitoneally injected on days 21, 23, 25, and 27 at 30 min before each OVA challenge. Mice in the PBS group received the equivalent amount of PBS treatment instead of OVA + LPS for sensitization and challenge. The NC group received no treatment.

2.3 Measurement of airway hyper-responsiveness (AHR)

Airway responses to aerosolized methacholine were measured using a lung function test instrument for the mouse (FinePointe Resistance and Compliance; Data Sciences International; Harvard Bioscience, Inc.). Mice were anesthetized with 1% pentobarbital sodium (50 mg/kg body weight) by intraperitoneal injection, and the trachea was cannulated with a needle, followed by mechanical ventilation. Airway resistance (R; cmH₂O.s/ml) was measured after aerosolization of 10 µl PBS and administration of increasing doses of aerosolized methacholine (3.125, 6.25, 12.5, 25 and 50 mg/ml in 10 µl; Sigma-Aldrich; Merck KGaA) sequentially. The results are presented as fold-increase of R (cmH₂O.s/ml) above the baseline and were calculated as follows: $[R(\text{response}) - R(\text{baseline})]/R(\text{baseline})$ [9].

2.4 Cell classification of BALF

Mice were sacrificed 24 h after the final aerosolization. The cervical dislocation was used for euthanasia and death was confirmed by the onset of rigor mortis, according to The National Institutes of Health Guide for the Care and Use of Laboratory Animals. The trachea was exposed, and a 22-gauge needle was used for endotracheal intubation. The lungs were subjected to bronchoalveolar lavage twice with 0.5 ml PBS (recovery rate $\geq 80\%$) and the total volume of BALF was 0.8 ml. Total and differential cell counts from BALF were determined by staining with Diff-Quick (Beijing Solarbio Science & Technology Co., Ltd.) for 1 min at room temperature. BALF was centrifuged at 160 x g for 10 min at 4°C and the supernatants were stored at -20°C for further experiments.

2.5 Enzyme-linked immunosorbent assay (ELISA)

ELISA kits were used to measure the levels of sRAGE (mlbio, China, cat.no.ml037657-1), IL-17 (BOSTER, China, cat.no.EK0431), and IL-6 (CUSABIO, China, cat.no. CSB-E04639m) in the BALF, according to the manufacturer's protocol.

2.6 Histopathological analysis

Lungs were fixed in 4% paraformaldehyde solution for 24 h at room temperature and subjected to gradient alcohol dehydration and paraffin-embedding, which were cut into 5-7-µm thick sections and subsequently stained with hematoxylin-eosin (HE) and Masson's trichrome. The sections were subsequently stained with hematoxylin at room temperature for 2–3 min and then with eosin at room temperature for 30–60 sec. The score of peribronchiolar inflammation was determined according to the infiltration of inflammatory cells as follows: 0, normal; 1, few inflammatory cells; 2, a layer of inflammatory cells; 3, two to four layers of inflammatory cells; and 4, more than four layers of inflammatory cells. An Olympus CX31 light microscope (Olympus Corporation) was used to evaluate the general inflammation and the airway morphology.

2.7 Cell culture

The human bronchial epithelial cell line (16HBE) was purchased from HuHeng Cell Center (Shanghai, China; cat.no.20170925-06) and maintained in KM Medium (ScienCell, USA; cat.no. 2101) in a humidified incubator at 37 °C with a 5% CO₂ atmosphere. The cell line used has been authenticated by the STR genotype test, and mycoplasma testing has been done. Cells generation 4 were used and the following culture groups were obtained: normal control (NC), sRAGE (1000 ng/ml, Biorbyt, United Kingdom; cat.no. PRO-601-10ug), HMGB1(300 ng/ml, 500 ng/ml, 1000 ng/ml; BD, USA; cat.no. 1690-HMB-050), HMGB1(1000 ng/ml) + sRAGE (1000 ng/ml), HMGB1 (1000 ng/ml) + RAGE inhibitor (FPS-ZM1, 250 nM; MCE, USA; cat.no. HY-19370), HMGB1 (1000 ng/ml) + PI3K inhibitor (LY294002, 10 μM; MCE, USA; cat.no. HY- 10108). 16HBE cells were treated with sRAGE, RAGE inhibitor and PI3K inhibitor for 10 min after the cells achieved a growth confluence of 80% and then treated with HMGB1 for 24 h. The mRNA expression of RAGE, PI3K, E-cadherin, and Vimentin were measured by RT-qPCR or western blotting, respectively.

2.8 Quantitative real-time reverse transcription PCR (RT-qPCR)

The lungs and cells were obtained for RNA extraction by Trizol (Invitrogen). cDNA was synthesized using the PrimeScript™ RT reagent Kit with gDNA Eraser (TaKaRa, Japan; cat.no.RR047A). qPCR was performed by ABI 7500 Real-Time quantitative instrument (Applied Biosystems, Thermo Fisher Scientific, Inc., MA, U.S.A.) with SYBR Premix EX Taq (Takara, Japan; cat.no. RR820A) using primers: glyceraldehyde-3-phosphate dehydrogenase (GAPDH): forward 5'CCTCTGCGCCCTTGAGCTAGGA3' and reverse 5'CACAAGAAGATGCGGCCGTCTC3'; TGF-β1: forward 5' TACGGCAGTGGCTGAACCAA3' and reverse 5' CGGTTTCATGTCATGGATGGTG3'; VEGF: forward 5' ACATTGGCTCACTTCCAGAAACAC3' and reverse 5' TGGTTGGAACCGGCATCTTTA3'; MMP-9: forward 5' GCCCTGGAACCTCACACGACA3' and reverse 5' TTGGAAACTCACACGCCAGAAG3'; α-SMA: forward 5' GACAATGGCTCTGGGCTCTGTA3' and reverse 5' TTTGGCCCATTTCCAACCATTA3'; E-cadherin: forward 5' CACCGATGGTGAGGGTACACAG 3' and reverse 5' GGCTTCAGGAATACATGGACAAAGA 3'; Vimentin: forward 5' AAAGCGTGGCTGCCAAGAA 3' and reverse 5' ACCTGTCTCCGGTACTCGTTTGA3'; RAGE: forward 5' AATGGTTCCCTCCTCCTTCC3' and reverse 5' TCCTTCCCTCGCCTGTTAGTT3'; PI3K: forward 5' CTACTGTAGCCAACAACAGCATGAA3' and reverse 5' AAGGTCCCATCAGCAGTGTCTC3'; GAPDH (human): forward 5'GCACCGTCAAGGCTGAGAAC3' and reverse 5'TGGTGAAGACGCCAGTGGA3'; E-cadherin (human): forward 5'AGGATGACACCCGGGACAAC3' and reverse 5'TGCAGCTGGCTCAAGTCAAAG3'; Vimentin (human): forward 5'AACCTGGCCGAGGACATCA3' and reverse 5'TCAAGGTCAAGACGTGCCAGA3'; RAGE (human): forward 5'CTGGAAGGAAGCAGGATGG3' and reverse 5'AAGGAAGAGGGAGCCGTTG3'; PI3K (human): forward 5'TGCAGCAGCCTTCAACAAAGA3' and reverse 5'AGCTACACAGTAGCCAGCACAGGA3'. PCR cycling was performed with denaturation at 95 °C for 30 s, annealing at 95 °C for 5 s, and extension at 60 °C for 34 s for 40 cycles. The mRNA levels were normalized to GAPDH. The fold-change for each gene was calculated by the $2^{-\Delta\Delta CT}$ method [10].

2.8 Western Blotting

The lung tissues and cells were lysed in a mixture of RIPA lysis buffer (Beyotime Inc., Nanjing, China) and protease inhibitors (Thermo Fisher Scientific, Waltham, MA, USA). The protein concentration of samples was measured using a BCA protein assay kit (Applygen, China). Equal amounts of the total proteins were loaded into 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electrotransferred onto PVDF membranes (Thermo Fisher Scientific, USA). Membranes were blocked with 5% skim milk for 1 h and incubated at 4 °C overnight with primary antibodies against the following proteins: TGF- β 1 (#41494, 1:1000, Signalway Antibody), VEGF (#32988, 1:1000, Signalway Antibody), MMP-9 (#49576, 1:1000, Signalway Antibody) α -SMA (#19245, 1:1000, Cell Signaling Technology), E-cadherin (#3195, 1:1000, Cell Signaling Technology), Vimentin (#5741, 1:1000, Cell Signaling Technology), GADPH (#21612, 1:5000, Signalway Antibody), followed by a secondary antibody (anti-rabbit IgG, 1:5000, Signalway Antibody) incubation at room temperature for 1 h. ECL kits (BL520A, Biosharp, China) were used to view protein bands and ImageJ software was applied for the analysis of the relative intensities of the target proteins.

2.9 Statistical analysis

Data were analyzed by GraphPad Prism (San Diego, CA, USA) and expressed as mean $\pm$ SD. The statistical significance was determined by unpaired Student's t-test or ANOVA test for multiple comparisons. For non-normally distributed data, the significance was performed using a non-parametric one-way ANOVA with a post hoc Kruskal-Wallis multiple comparisons test. A *p*-value of less than 0.05 was considered to be statistically significant.

3. Results

3.1 NA mouse model was established

A mouse model with NA was induced by OVA plus LPS sensitization followed by seven sequential daily OVA challenges (Fig. 1a). sRAGE and inhibitors were administered to NA mice as illustrated in Fig. 1b and 1c. As shown in HE staining, the control group manifested a normal bronchial lumen and alveolar structure, while NA mice exhibited a disordered structure with epithelial cell shedding, thickened bronchial wall, and basement membrane with multi-layer inflammatory cell infiltration around (Fig. 2a). Compared with the NC group and PBS group, airway resistance increased significantly in the NA group after methacholine challenge at doses of 12.5, 25, and 50 mg/mL ($P < 0.0001$) (Fig. 2b). The inflammatory cells in BALF significantly elevated (including total cells, neutrophils, and lymphocytes) in NA mice when compared with the levels in control mice and the PBS group (Fig. 2c).

Figure 1. Protocols for NA mouse model and inhibitor intervention. (a) Sensitization and challenge for mouse model with NA. (b) Protocols for sRAGE administration. (c) Protocols for HMGB1 inhibitor, RAGE inhibitor, and PI3K inhibitor intervention. i.v.: tail-vein injection, i.p.: intraperitoneally injection.

3.2 sRAGE level decreased in NA mouse

Compared with the control group, the sRAGE level was significantly decreased in NA mouse BALF determined by ELISA (Fig. 2d). Given it was negatively correlated with lung function in COPD [6, 7], we evaluated the relation of sRAGE and airway resistance in NA. It showed that there was an opposite trend between sRAGE levels in BALF and airway resistance, however, with no statistical differences (Fig. 2e). A study revealed that sRAGE may be a predictor of neutrophilic airway inflammation of asthma and COPD [5]. Our data verified that sRAGE was negatively related to BALF neutrophils, especially in the NA group with $r=-0.9217$ and $P=0.0078$ by Spearman correlation analysis (Fig. 2f).

Figure 2. sRAGE level decreased in NA mouse. (a) Representative images of HE stained lung tissue (magnification, $\times 100$). The increased number of inflammatory cells infiltrating around the bronchial wall in the NA group. (b) Airway resistance increased significantly in the NA mouse model analyzed by two-way ANOVA. (c) Inflammatory cells, especially total cells and neutrophils were significantly increased in the NA mouse BALF analyzed by one-way ANOVA. (d) sRAGE levels were assessed by ELISA. Kruskal-Wallis test was performed for analysis. (e) The opposite trend of BALF sRAGE expression and airway resistance in the NA group. (f) Negative correlations between BALF sRAGE and neutrophils using Spearman correlation analysis. $n=6$ in each group. Results were depicted as means $\pm$ SD. ns: no significant difference. $**P<0.01$, $***P<0.0001$, compared with the NC and NA group. NC: normal control group; NA: neutrophilic asthma group; BALF, bronchial alveolar lavage fluid. Mch: methacholine. Mac: macrophages. Neu: neutrophils. Lym: lymphocytes.

3.3 Overexpressed sRAGE decreased neutrophilic airway inflammation in NA mice

AAV-9 was used to overexpress sRAGE and then the transfection efficiency was assessed by the green fluorescent protein (GFP) expression. Compared to the NC and NA groups, the NA + sRAGE group exhibited a distinct expression of GFP (Fig. 3a). Further, the sRAGE administration inhibited the number of neutrophils in BALF cytology smears (Fig. 3b). More importantly, sRAGE sharply reduced the influx of total cells and neutrophils in NA mouse BALF (Fig. 3c). Compared with the airway showing increased inflammatory cell infiltration and thick basement membrane in NA mice, sRAGE overexpression significantly inhibited the airway inflammation and the thicknesses of the basement membrane (Fig. 3d). sRAGE intervention significantly inhibited the inflammation score in NA mice ($P<0.05$) (Fig. 3e). Interleukin (IL) -17 and IL-6 were reported as the inflammatory mediator of NA, in this study, sRAGE decreased IL-17 and IL-6 levels in BALF (Fig. 3f).

Figure 3. sRAGE reduced neutrophilic airway inflammation of NA mice. (a) Representative immunofluorescence images of lung tissue. (b) Representative images of cytology smears (magnification, $\times 100$). (c) Classified cell count of BALF in the mouse model. (d) Representative images of HE-stained lung tissue (magnification, $\times 100$). sRAGE inhibited the airway inflammation and the thicknesses of the basement membrane. (e) Inflammation score in HE-stained lung tissue. sRAGE decreased the score of the NA mouse model. (f) sRAGE administration inhibited IL-17 and IL-6 levels in

BALF. $n = 6$ in each group. Results were depicted as means $\pm$ SD. * $P < 0.05$ and **** $P < 0.0001$ using one-way ANOVA for analysis.

3.4 sRAGE reduced the airway remodeling of NA mice

Masson trichrome staining was widely used to identify collagen deposits which were proved to be the typical phenotype of airway remodeling. Compared to the NC group, NA mice exhibited more blue-stained collagen deposits around the bronchus and blood vessels (Fig. 4a). sRAGE administration highly decreased the collagen deposition, whereas the NA + sRAGE control group showed no reduction. Airway remodeling markers were detected by RT-qPCR (Fig. 4b) and Western Blotting (Fig. 4c). The result revealed that TGF- β 1 mRNA was increased in NA mice lung ($P < 0.05$), while VEGF, MMP-9, and α -SMA expression showed no elevation. Consistently, TGF- β 1 protein expression was increased in the NA group with VEGF, MMP-9, and α -SMA protein expression no change. Overexpression of sRAGE significantly decreased the mRNA and protein expression of TGF- β 1 ($P < 0.05$), suggesting sRAGE may inhibit airway remodeling in the NA mouse model.

Figure 4. Effects of sRAGE on airway remodeling of NA mice. (a) Representative images of Masson trichrome stained lung tissue (magnification, $\times 40$). sRAGE administration decreased the peribronchial collagen deposition. (b) mRNA expressions of TGF- β 1, VEGF, MMP-9, and α -SMA were examined using RT-qPCR. (c) TGF- β 1, VEGF, MMP-9, and α -SMA protein were detected by Western Blotting. $n = 6$ in each group. Results were depicted as means $\pm$ SD. ns: no significant difference. * $P < 0.05$, ** $P < 0.01$. One-way ANOVA was performed for analysis.

3.5 Effects of sRAGE on EMT and signaling pathway in NA mice

EMT is one of the mechanisms of airway remodeling. The expression of E-cadherin and vimentin were evaluated using RT-qPCR (Fig. 5a) and Western Blotting (Fig. 5b). Compared with the control mice, E-cadherin expression was decreased, while vimentin level was increased in the NA group. sRAGE administration markedly upregulated the level of E-cadherin and reduced the vimentin expression, indicating sRAGE regulated the EMT of NA mouse.

As reported that RAGE and PI3K participated in the EMT process of human airway epithelial cells. sRAGE as a decoy receptor competitively bound the ligands of RAGE (HMGB1, S100, etc) to inhibit the inflammatory effect. In this study, RAGE, PI3K, and HMGB1 expressions were elevated in the lung of NA mice measured by RT-qPCR, while the sRAGE administration inhibited the expressions (Fig. 5c, d). To determine whether sRAGE modulated EMT via RAGE/PI3K, we assessed E-cadherin and vimentin expressions after HMGB1 inhibitor, RAGE inhibitor, and PI3K inhibitor intervention (Fig. 5e, f). It showed that the mRNA and protein levels of E-cadherin were improved in the NA + HMGB1 inhibitor group, NA + RAGE inhibitor group, and NA + PI3K inhibitor group, whereas the administration of the inhibitor could not affect the vimentin expression. The data suggesting that RAGE/PI3K may partly participate in the EMT process of NA.

Figure 5. Effects of sRAGE on EMT and signaling pathway in NA mice. (a) The mRNA expression of E-cadherin and vimentin were examined using RT-qPCR. (b) E-cadherin and vimentin protein expressions were detected by Western Blotting. (c, d) The mRNA expression levels of RAGE, PI3K, and HMGB1. (e, f) mRNA and protein expressions of E-cadherin and vimentin were measured after inhibitor intervention. ns: no significant difference. $n = 6$ in each group. Results were depicted as means $\pm$ SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. One-way ANOVA was performed for analysis.

3.8 The effect of sRAGE on EMT in 16HBE cells

HMGB1 was reported to induce EMT of airway epithelial cells. We assessed the function of sRAGE on HMGB1-induced EMT in 16HBE cells. The results showed that the migration ability of 16HBE cells was enhanced in the HMGB1 group ($P < 0.0001$), while the sRAGE administration inhibited this effect ($P < 0.001$) (Fig. 6a, b). The mRNA expression of E-cadherin decreased and vimentin elevated in HMGB1 group (1000 ng/mL) ($P < 0.05$) (Fig. 6c). Compared to the HMGB1 group, E-cadherin mRNA was significantly elevated ($P < 0.0001$), while the vimentin level was decreased in the HMGB1 + sRAGE group ($P < 0.05$) (Fig. 6d).

Figure 6. The effect of sRAGE on EMT in 16HBE cells. (a, b) Representative images (magnification, $\times 40$) of wound-healing assay of 16HBE cells treated with sRAGE and HMGB1 after 24 hours post scratch. Results are expressed as the percentage of the recovered wound area. Migration index = migration distance of test group/migrating distance of the control group. (c) E-cadherin and vimentin mRNA was examined in HMGB1-induced 16HBE cells. (d) E-cadherin and Vimentin expressions were detected after sRAGE administration. $n = 6$ in each group. Data are presented as mean $\pm$ SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. One-way ANOVA was performed for analysis.

3.9 The signaling pathway involved in EMT of 16HBE cells

RAGE and PI3K mRNA expressions improved in 16HBE cells treated with HMGB1 ($P < 0.01$) (Fig. 7a), while the sRAGE administration inhibited the improvement. RAGE inhibitor and PI3K inhibitor were used to intervene in the HMGB1-induced 16HBE cells. It showed that E-cadherin mRNA was increased, while vimentin mRNA was declined in the HMGB1 + RAGE inhibitor group and HMGB1 + PI3K inhibitor group (Fig. 7b). Western Blotting demonstrated that HMGB1 down-regulated the protein expression of E-cadherin ($P < 0.001$) and up-regulated vimentin expression ($P < 0.05$) in 16HBE cells (Fig. 7c). However, the E-cadherin level was elevated ($P < 0.01$) with vimentin protein decreasing ($P < 0.01$) in HMGB1 + RAGE inhibitor group and HMGB1 + PI3K inhibitor group, suggesting that RAGE/PI3K may be involved in EMT of 16HBE cells.

Figure 7. The signaling pathway involved in EMT of 16HBE cells. (a) The mRNA expression of RAGE and PI3K was examined by RT-qPCR. (b) E-cadherin and Vimentin expressions were detected using RT-qPCR after RAGE inhibitor and PI3K inhibitor intervention. (c) The protein levels of E-cadherin and Vimentin were detected by Western Blotting. $n = 6$ in each group. Data are presented as mean $\pm$ SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. One-way ANOVA or Mann-Whitney test was performed for analysis.

4. Discussion

NA was first described in 1993 in a patient with sudden-onset fatal asthma [11]. With the deepening of research, it was gradually discovered that NA existed not only in the adult with severe asthma but also in children with mild and remission asthma. In recent years, the relationship between childhood asthma and COPD in adulthood has attracted much attention. Airway neutrophilic inflammation in asthma was considered to be related to airway remodeling and airflow limitation, suggesting NA and COPD may have a potential connection. sRAGE has been well understood in COPD and also be discussed in some studies with asthma. Here, we tried to explore the function of sRAGE in NA.

In the present study, a NA mouse model was established based on the work by Wilson *et al* [8] and our previous study (published in Chinese). NA mice exhibited increased AHR, an elevated number of neutrophils in BALF, thicken bronchial wall, and accumulated infiltration of inflammatory cells around the bronchial wall. Consistent with the sRAGE expression in patients with COPD and asthmatic patients with airway neutrophil inflammation [5], sRAGE was reduced in NA mouse BALF. Moreover, we found that the lower the sRAGE level in the BALF, the higher the airway resistance after the 50 mg/mL acetylcholine atomization excitation. Furthermore, the expression of sRAGE was significantly negatively correlated with the proportion of neutrophils in BALF. Studies have shown that serum sRAGE levels were inversely related to the severity of asthma in children [12], and asthmatic patients with higher airway neutrophils exhibited lower plasma sRAGE levels [13]. The research reported that sRAGE as a predictive indicator of airway neutrophils for it was related to airway neutrophilic inflammation both in patients with asthma and COPD [14]. Similar to the existing studies, the sRAGE level in NA mouse BALF was decreased and the reduced sRAGE was negatively correlated with the airway neutrophil inflammation, suggesting the loss of sRAGE may be involved in the development of NA.

Further, we transfected the NA mouse model with an AAV9-overexpressed sRAGE by tail vein injection. AAV is a common vector for gene manipulation because of its low immunogenicity, high transfection efficiency, and long-term stable gene expression. AAV9 is widely used in the research of respiratory for it is one of the most efficient serotypes to infect lung epithelium cells. Studies have reported that sRAGE reduced the neutrophil infiltration of lung tissue in LPS-induced acute lung injury mouse model [15] and the airway neutrophil inflammation in asthmatic mice [16]. Consistent with the existing research, overexpressed sRAGE not only significantly reduced the infiltration of inflammatory cells and inflammation score of lung tissue in NA mice but also sharply inhibited the inflammatory cells and neutrophils in BALF, which implying that sRAGE deficiency may participate in airway neutrophilic inflammation of NA mice. As reported, administration with mesenchymal stem cells overexpressing sRAGE reduced the expression of Th17 cells and IL-17 in the spleen of mice with rheumatoid arthritis [17]. Similarly, we found that IL-17 and IL-6 levels significantly decreased in BALF of AAV9-sRAGE overexpressed NA mice. Studies proposed that both IL-17 and IL-6 were involved in airway remodeling, especially the relationship between IL-17 and airway remodeling has been confirmed in asthma and COPD [18, 19], suggesting that sRAGE may be involved in airway remodeling of NA mice.

Airway remodeling is a common pathological process of asthma and COPD, which leads to progressive and irreversible damage to lung function [20, 21]. We explored the effect of overexpression of sRAGE on airway remodeling in the NA mouse model. The results showed that sRAGE administration significantly inhibited airway collagen deposition and TGF- β 1 expression in NA mice. TGF- β 1 can be secreted by neutrophils [22], and it was involved in the airway remodeling of asthma by mediating the differentiation, proliferation, survival, and apoptosis of airway structural cells [23]. It was also an important inducer of EMT which was one of the mechanisms of airway remodeling [24]. More importantly, TGF- β 1 was considered to be a key molecule connecting children asthma and COPD [25]. Limited animal models suggested that there was a potential relationship between sRAGE, TGF- β 1, and tissue remodeling [26]. We confirmed that sRAGE intervention declined the TGF- β 1 expression, suggesting that sRAGE may play a role in the airway remodeling through TGF- β 1 in NA.

EMT was one of the mechanisms of airway remodeling for it resulted in epithelial barrier destruction, subepithelial fibrosis, smooth muscle proliferation, and airway inflammation [27], it was also considered to be involved in airway remodeling of asthma and COPD [28–30]. In this study, epithelial marker E-cadherin expression was lost and interstitial marker vimentin was enriched in NA mice. However, AAV9-sRAGE restored the expressions of E-cadherin and Vimentin, implying that sRAGE overexpression may reduce airway EMT in NA mice. A study has revealed that sRAGE alleviated the Th17 cell response and airway neutrophilic inflammation in NA mice through competing with a RAGE ligand -HMGB1 [16]. RAGE was highly expressed in the lung tissues of asthmatic mice and was involved in the mucus hypersecretion and airway remodeling of asthma [31]. Moreover, RAGE inhibitor reduced airway inflammation and airway remodeling in asthmatic mice [32]. RAGE induced the activation of PI3K in human peripheral blood neutrophils while antagonizing RAGE/PI3K inhibited the adhesion activation of neutrophils [33]. In the present study, HMGB1, RAGE, and PI3K expressions were increased in NA mice, whereas the levels were inhibited by sRAGE administration. Besides, we observed the HMGB1-induced EMT in 16HBE cells *in vitro*, while this process was suppressed by sRAGE intervention. Furthermore, rescue assay showed that HMGB1 induced the EMT via RAGE and PI3K, suggesting sRAGE may regulate the EMT caused by HMGB1 through RAGE/PI3K in 16HBE cells.

Conclusions

In summary, we found that sRAGE expression was remarkably reduced in the NA mouse model. AAV9-sRAGE administration significantly inhibited the airway neutrophilic inflammation, airway remodeling, the expression of TGF- β 1, and EMT markers in the NA mouse model. Moreover, sRAGE reversed the EMT in 16HBE cells induced by HMGB1 *in vitro* via RAGE/PI3K. This suggests sRAGE overexpression may yield benefits in the treatment of neutrophilic airway inflammation and airway remodeling of NA.

Abbreviations

NA
neutrophilic asthma

COPD
chronic obstructive pulmonary disease
sRAGE
soluble advanced glycosylation receptor
RAGE
receptor for advanced glycation end products
AAV9
Adeno-associated virus 9
OVA
ovalbumin
LPS
lipopolysaccharide
PBS
phosphate-buffered saline
Mch
methacholine
H&E
hematoxylin-eosin
BALF
bronchial alveolar lavage fluid
RT-qPCR
real-time quantitative reverse transcription-polymerase chain reaction
EMT
epithelial-mesenchymal transition

Declarations

Ethics approval and consent to participate

The present study was approved by The Ethics Committee of The First Affiliated Hospital of Guangxi Medical University [approval no. 2019(KY-e-035)].

Consent for publication

Not applicable

Availability of data and material

Not applicable

Competing interests

The authors declare that they have no competing interests.

Funding

This study was supported by International Communication of Guangxi Medical University Graduate Education, Guangxi Natural Science Foundation (grant number: 2018GXNSFAA281256), Innovation Project of Guangxi Graduate Education (grant number: YCBZ2019045), Chen Xiaoping foundation for the development of science and technology of Hubei province (grant number: CXPJJH1 1900003-17) and Guangxi Health and Health Committee Project(grant number: Z20190762).

Author contributions

X.Z. performed and analyzed the experiments, conceived and wrote the manuscript; J.X. and H.S conducted the animal studies; Q.W. performed the molecular experiment and analyzed the data and Y.T. performed the molecular experiment and biostatistical analyses, G.N. designed and supervised the study, as well as critically revised the manuscript and had the primary responsibility for writing. All authors contributed to the writing of the manuscript.

Acknowledgements

No applicable.

References

1. Papi A, Brightling C, Pedersen SE, Reddel HK. Asthma. *Lancet*. 2018;391:783–800.
2. Seys SF, Lokwani R, Simpson JL, Bullens DMA. New insights in neutrophilic asthma. *Curr Opin Pulm Med*. 2019;25:113–20.
3. Gibson PG, Foster PS. Neutrophilic asthma: welcome back! *Eur Respir J*. 2019;54.
4. McGeachie MJ. Childhood asthma is a risk factor for the development of chronic obstructive pulmonary disease. *Curr Opin Allergy Clin Immunol*. 2017;17:104–9.
5. Sukkar MB, Wood LG, Tooze M, Simpson JL, McDonald VM, Gibson PG, et al. Soluble RAGE is deficient in neutrophilic asthma and COPD. *Eur Respir J*. 2012;39:721–9.
6. Pouwels SD, Klont F, Kwiatkowski M, Wiersma VR, Faiz A, van den Berge M, et al. Cigarette Smoking Acutely Decreases Serum Levels of the COPD Biomarker sRAGE. *Am J Respir Crit Care Med*. 2018.
7. Cazzola M, Puxeddu E, Ora J, Rogliani P. Evolving Concepts in Chronic Obstructive Pulmonary Disease Blood-Based Biomarkers. *Mol Diagn Ther*. 2019.
8. Wilson RH, Whitehead GS, Nakano H, Free ME, Kolls JK, Cook DN. Allergic sensitization through the airway primes Th17-dependent neutrophilia and airway hyperresponsiveness. *Am J Respir Crit Care*

Med. 2009;180:720–30.

9. Hou C, Kong J, Liang Y, Huang H, Wen H, Zheng X, et al. HMGB1 contributes to allergen-induced airway remodeling in a murine model of chronic asthma by modulating airway inflammation and activating lung fibroblasts. *Cell Mol Immunol*. 2015;12:409–23.
10. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2^{(-Delta Delta C(T))} Method. *Methods*. 2001;25:402–8.
11. Sur S, Crotty TB, Kephart GM, Hyma BA, Colby TV, Reed CE, et al. Sudden-onset fatal asthma. A distinct entity with few eosinophils and relatively more neutrophils in the airway submucosa? *Am Rev Respir Dis*. 1993;148:713–9.
12. Li Y, Wu R, Tian Y, Bao T, Tian Z. Fraction of exhaled nitric oxide and soluble receptors for advanced glycation end products are negatively correlated in children with recurrent wheezing. *Asian Pac J Allergy Immunol*. 2017;35:33–7.
13. El-Seify MY, Fouda EM, Nabih ES. Serum level of soluble receptor for advanced glycation end products in asthmatic children and its correlation to severity and pulmonary functions. *Clin Lab*. 2014;60:957–62.
14. Lyu Y, Zhao H, Ye Y, Liu L, Zhu S, Xia Y, et al. Decreased soluble RAGE in neutrophilic asthma is correlated with disease severity and RAGE G82S variants. *Mol Med Rep*. 2018;17:4131–7.
15. Zhang H, Tasaka S, Shiraishi Y, Fukunaga K, Yamada W, Seki H, et al. Role of soluble receptor for advanced glycation end products on endotoxin-induced lung injury. *Am J Respir Crit Care Med*. 2008;178:356–62.
16. Zhang F, Su X, Huang G, Xin XF, Cao EH, Shi Y, et al. sRAGE alleviates neutrophilic asthma by blocking HMGB1/RAGE signalling in airway dendritic cells. *Sci Rep*. 2017;7:14268.
17. Park MJ, Lee SH, Moon SJ, Lee JA, Lee EJ, Kim EK, et al. Overexpression of soluble RAGE in mesenchymal stem cells enhances their immunoregulatory potential for cellular therapy in autoimmune arthritis. *Sci Rep*. 2016;6:35933.
18. Robinson MB, Deshpande DA, Chou J, Cui W, Smith S, Langefeld C, et al. IL-6 trans-signaling increases expression of airways disease genes in airway smooth muscle. *Am J Physiol Lung Cell Mol Physiol*. 2015;309:L129-38.
19. Lai T, Tian B, Cao C, Hu Y, Zhou J, Wang Y, et al. HDAC2 Suppresses IL17A-Mediated Airway Remodeling in Human and Experimental Modeling of COPD. *Chest*. 2018;153:863–75.
20. Hirota N, Martin JG. Mechanisms of airway remodeling. *Chest*. 2013;144:1026–32.
21. Mostaco-Guidolin LB, Osei ET, Ullah J, Hajimohammadi S, Fouadi M, Li X, et al. Defective Fibrillar Collagen Organization by Fibroblasts Contributes to Airway Remodeling in Asthma. *Am J Respir Crit Care Med*. 2019.
22. Halwani R, Al-Muhsen S, Al-Jahdali H, Hamid Q. Role of transforming growth factor-beta in airway remodeling in asthma. *Am J Respir Cell Mol Biol*. 2011;44:127–33.

23. Ojiaku CA, Yoo EJ, Panettieri RA. Jr. Transforming Growth Factor beta1 Function in Airway Remodeling and Hyperresponsiveness. The Missing Link? *Am J Respir Cell Mol Biol*. 2017;56:432–42.
24. Su J, Morgani SM, David CJ, Wang Q, Er EE, Huang YH, et al. TGF-beta orchestrates fibrogenic and developmental EMTs via the RAS effector RREB1. *Nature*. 2020;577:566–71.
25. DeVries A, Vercelli D. Of pleiotropy and trajectories: Does the TGF-beta pathway link childhood asthma and chronic obstructive pulmonary disease? *J Allergy Clin Immunol*. 2018;141:1992–6.
26. Lu L, Zhang Q, Xu Y, Zhu ZB, Geng L, Wang LJ, et al. Intra-coronary administration of soluble receptor for advanced glycation end-products attenuates cardiac remodeling with decreased myocardial transforming growth factor-beta1 expression and fibrosis in minipigs with ischemia-reperfusion injury. *Chin Med J (Engl)*. 2010;123:594–8.
27. Hackett TL. Epithelial-mesenchymal transition in the pathophysiology of airway remodelling in asthma. *Curr Opin Allergy Clin Immunol*. 2012;12:53–9.
28. Bartis D, Mise N, Mahida RY, Eickelberg O, Thickett DR. Epithelial-mesenchymal transition in lung development and disease: does it exist and is it important? *Thorax*. 2014;69:760–5.
29. Sohal SS, Ward C, Walters EH. Importance of epithelial mesenchymal transition (EMT) in COPD and asthma. *Thorax*. 2014;69:768.
30. Ijaz T, Pazdrak K, Kalita M, Konig R, Choudhary S, Tian B, et al. Systems biology approaches to understanding Epithelial Mesenchymal Transition (EMT) in mucosal remodeling and signaling in asthma. *World Allergy Organ J*. 2014;7:13.
31. Xiong J, Zhao WQ, Huang GH, Yao LH, Dong HM, Yu CH, et al. [Receptor for advanced glycation end products upregulates MUC5AC expression and promotes mucus overproduction in mice with toluene diisocyanate-induced asthma]. *Nan Fang Yi Ke Da Xue Xue Bao*. 2017;37:1301–7.
32. Xiong J, Zhao W, Lin Y, Yao L, Huang G, Yu C, et al. Phosphorylation of low density lipoprotein receptor-related protein 6 is involved in receptor for advanced glycation end product-mediated beta-catenin stabilization in a toluene diisocyanate-induced asthma model. *Int Immunopharmacol*. 2018;59:187–96.
33. Toure F, Zahm JM, Garnotel R, Lambert E, Bonnet N, Schmidt AM, et al. Receptor for advanced glycation end-products (RAGE) modulates neutrophil adhesion and migration on glycosylated extracellular matrix. *Biochem J*. 2008;416:255–61.

Figures

Figure 1

Protocols for NA mouse model and inhibitor intervention. (a) Sensitization and challenge for mouse model with NA. (b) Protocols for sRAGE administration. (c) Protocols for HMGB1 inhibitor, RAGE inhibitor, and PI3K inhibitor intervention. i.v.: tail-vein injection, i.p.: intraperitoneally injection.

Figure 2

sRAGE level decreased in NA mouse. (a) Representative images of HE stained lung tissue (magnification, $\times 100$). The increased number of inflammatory cells infiltrating around the bronchial wall in the NA group. (b) Airway resistance increased significantly in the NA mouse model analyzed by two-way ANOVA. (c) Inflammatory cells, especially total cells and neutrophils were significantly increased in the NA mouse BALF analyzed by one-way ANOVA. (d) sRAGE levels were assessed by ELISA. Kruskal-Wallis test was performed for analysis. (e) The opposite trend of BALF sRAGE expression and airway resistance in the NA group. (f) Negative correlations between BALF sRAGE and neutrophils using Spearman correlation analysis. $n=6$ in each group. Results were depicted as means $\pm$ SD. ns: no significant difference. $**P < 0.01$, $****P < 0.0001$, compared with the NC and NA group. NC: normal control group; NA: neutrophilic asthma group; BALF, bronchial alveolar lavage fluid. Mch: methacholine. Mac: macrophages. Neu: neutrophils. Lym: lymphocytes.

Figure 3

sRAGE reduced neutrophilic airway inflammation of NA mice. (a) Representative immunofluorescence images of lung tissue. (b) Representative images of cytology smears (magnification, $\times 100$). (c) Classified cell count of BALF in the mouse model. (d) Representative images of HE-stained lung tissue (magnification, $\times 100$). sRAGE inhibited the airway inflammation and the thicknesses of the basement membrane. (e) Inflammation score in HE-stained lung tissue. sRAGE decreased the score of the NA

mouse model. (f) sRAGE administration inhibited IL-17 and IL-6 levels in BALF. $n=6$ in each group. Results were depicted as means $\pm$ SD. $*P < 0.05$ and $****P < 0.0001$ using one-way ANOVA for analysis.

Figure 4

Effects of sRAGE on airway remodeling of NA mice. (a) Representative images of Masson trichrome stained lung tissue (magnification, $\times 40$). sRAGE administration decreased the peribronchial collagen deposition. (b) mRNA expressions of TGF- β 1, VEGF, MMP-9, and α -SMA were examined using RT-qPCR. (c) TGF- β 1, VEGF, MMP-9, and α -SMA protein were detected by Western Blotting. $n=6$ in each group. Results were depicted as means $\pm$ SD. ns: no significant difference. $*P < 0.05$, $**P < 0.01$. One-way ANOVA was performed for analysis.

Figure 5

Effects of sRAGE on EMT and signaling pathway in NA mice. (a) The mRNA expression of E-cadherin and vimentin were examined using RT-qPCR. (b) E-cadherin and vimentin protein expressions were detected by Western Blotting. (c, d) The mRNA expression levels of RAGE, PI3K, and HMGB1. (e, f) mRNA and protein expressions of E-cadherin and vimentin were measured after inhibitor intervention. ns: no significant difference. $n=6$ in each group. Results were depicted as means $\pm$ SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. One-way ANOVA was performed for analysis.

Figure 6

The effect of sRAGE on EMT in 16HBE cells. (a, b) Representative images (magnification, $\times 40$) of wound-healing assay of 16HBE cells treated with sRAGE and HMGB1 after 24 hours post scratch. Results are expressed as the percentage of the recovered wound area. Migration index=migration distance of test group/migrating distance of the control group. (c) E-cadherin and vimentin mRNA was examined in HMGB1-induced 16HBE cells. (d) E-cadherin and Vimentin expressions were detected after sRAGE administration. $n=6$ in each group. Data are presented as mean $\pm$ SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. One-way ANOVA was performed for analysis.

Figure 7

The signaling pathway involved in EMT of 16HBE cells. (a) The mRNA expression of RAGE and PI3K was examined by RT-qPCR. (b) E-cadherin and Vimentin expressions were detected using RT-qPCR after RAGE inhibitor and PI3K inhibitor intervention. (c) The protein levels of E-cadherin and Vimentin were detected by Western Blotting. $n=6$ in each group. Data are presented as mean $\pm$ SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. One-way ANOVA or Mann-Whitney test was performed for analysis.