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ORIGINAL ARTICLE

Sevoflurane Prevents Airway Remodeling *via* Downregulation of VEGF and TGF-β1 in Mice with OVA-Induced Chronic Airway Inflammation

Qi-Ying Shen,[1,4] Ling Wu,[1] Chuan-Sheng Wei,[2] Yan-Nan Zhou,[1] and Hui-Mei Wu[3,4]

Abstract— Asthma is characterized by chronic airway inflammation, which is the underlying cause of airway remodeling featured by goblet cell hyperplasia, subepithelial fibrosis, and proliferation of smooth muscle. Sevoflurane has been used to treat life-threatening asthma and our previous study shows that sevoflurane inhibits acute lung inflammation in ovalbumin (OVA)-induced allergic mice. However, the effect of sevoflurane on airway remodeling in the context of chronic airway inflammation and the underlying mechanism are still unknown. Here, female C57BL/6 mice were used to establish chronic airway inflammation model. Hematoxylin and eosin (H&E), periodic acid-Schiff (PAS), and Sirius red (SR) staining were used to evaluate airway remodeling. Protein levels of α-SMA, VEGF, and TGF-β1 in lung tissues were detected by western blotting analyses and immunohistochemistry staining. Results showed that inhalation of sevoflurane inhibited chronic airway inflammation including inflammatory cell infiltration and pro-inflammatory cytokine production in BALF of the OVA-challenged mice. Meanwhile, sevoflurane suppressed airway thickening, goblet cell hyperplasia, smooth muscle hyperplasia, collagen deposition, and fiber hyperplasia in the lung tissues of the mice with airway remodeling. Most notably, sevoflurane inhibited the OVA-induced expressions of VEGF and TGF-β1. These results suggested that sevoflurane effectively inhibits airway remodeling in mouse model of chronic airway inflammation, which may be due to the downregulation of VEGF and TGF-β1in lung tissues. Therefore, our results indicate a potential role of sevoflurane in inhibiting airway remodeling besides its known suppression effect on airway inflammation, and support the use of sevoflurane in treating severe asthma in ICU.

KEY WORDS: asthma; airway inflammation; sevoflurane; airway remodeling.

INTRODUCTION

Allergic asthma is a kind of airway diseases characterized with chronic airway inflammation, airway hyperresponsiveness (AHR), and airway remodeling (AR) [1]. The chronic inflammatory response within the airways of asthmatics is associated with airway structural changes [2], which is the underlying cause of AR. Airway structural remodeling is an important feature for severe asthma, and is also correlated with AHR [3–5]. Five to ten percent of

[1] Department of Anesthesiology, The First Affiliated Hospital of Anhui Medical University, Jixi Road 218, Hefei, Anhui, China
[2] School of Basic Medical Sciences, Anhui Medical University, Meishan Road 81, Hefei, Anhui, China
[3] Anhui Geriatric Institute, The First Affiliated Hospital of Anhui Medical University, Jixi Road 218, Hefei, Anhui, China
[4] To whom correspondence should be addressed to Qi-Ying Shen at Department of Anesthesiology, The First Affiliated Hospital of Anhui Medical University, Jixi Road 218, Hefei, Anhui, China. E-mail: shenqiying-yy@163.com; and Hui-Mei Wu at Anhui Geriatric Institute, The First Affiliated Hospital of Anhui Medical University, Jixi Road 218, Hefei, Anhui, China. E-mail: wuhm@ahmu.edu.cn

patients with a severe form of the disease account for the higher morbidity and health costs resulted from asthma [6, 7].

The use of sedatives in asthmatic patients has a long history. Inhalation of volatile anesthetics, including sevoflurane, has been used as the last resort to the treatment of life-threatening asthma for several years [8]. In recent years, it has been reported, both in domestic and foreign countries, that long time and repeated inhalation of sevoflurane can be successfully used to treat asthmatic patients, which are refractory to routine therapeutic drugs, and especially useful to infants and young children with severe asthma and persistent state [9–11]. However, whether sevoflurane prevents AR during the management of asthma and the underlying mechanisms are still unclear.

AHR and continuous state of asthma are closely related to airway inflammation and AR [2]. Airway inflammation may cause airflow limitation by releasing mediators that act directly on airway smooth muscle (ASM) [12]. In order to restore lung homeostasis, a repair process will follow with injury, which will then results in AR. Therefore, the chronic inflammation may lead to structural changes, such as increased amount of ASM, deposition of extracellular matrix protein, and subsequent fibrosis [13]. We and others have shown that repeated inhalation of sevoflurane could inhibit the lung inflammation in ovalbumin (OVA)-induced allergic mice, including decrease of the inflammatory cells infiltrated in allergic lung tissue and modulated the imbalance of inflammatory cytokines [14–16]. As the AR is closely related with lung inflammation, and TGF-β1 and VEGF are closely related to collagen deposition, degradation of fibrous protein, and vascular proliferation [17–20], we hypothesize that inhalation of sevoflurane might inhibit AR, and this effect may be due to the downregulation of VEGF and TGF-β1.

In the present study, we employed an OVA-induced AR model of mice to investigate the effect of repeated administration of sevoflurane on AR, including proliferation of ASM cells, mucous cell metaplasia and hypersecretion, and airway fibrosis. And we detected the expressions of VEGF and TGF-β1in lung tissues.

MATERIALS AND METHODS

Animals

Female, specified pathogen-free C57BL/6 mice were purchased from Shanghai SLAC Laboratory Animal Co. Ltd. All experiments described in this study were approved by the Animal Care and Use Committee of Anhui Medical University.

Provocation Procedures and Experimental Protocol

The model setting procedures are illustrated in Fig. 1a as we previously reported [21, 22]. Mice were randomly divided into three groups: control mice treated with saline (Con group); asthmatic mice (OVA group); and asthmatic mice treated with 3% sevoflurane in oxygen-air mixture (OVA/SVF group). Allergic mice were sensitized and challenged with OVA. Mice from OVA/SVF group were placed in a chamber for inhalational anesthesia and exposed to 3% sevoflurane transmitted by oxygen-air mixture for 1 h a day, just after every provocation. One day after the last challenge, mice were injected with pentobarbital intraperitoneally and then sacrificed. The left lung was used for western blot analysis, and the right lung tissues were fixed in formalin for histopathological staining.

Bronchoalveolar Lavage Fluid and Cellular Analysis

The lungs of the animal were rinsed with ice-cold saline thrice. Total and differential cell counts were respectively performed by hemocytometer or Wright-Giemsa staining.

Morphometric Analysis of Airway Remodeling

Mucous cell metaplasia and hypersecretion were detected by periodic acid staining (PAS). Inflammatory cell infiltration and ASM cell proliferation were determined by hematoxylin and eosin staining (H&E). Collagen deposition was analyzed by Sirius red (SR) staining.

Plasma IgE and BALF Cytokine Analysis

The prodution of IL-10 and TNF-α in BALF, OVA-specific IgE in serum was measured by enzymelinked immunosorbent assay (ELISA) using specific kits (Cusabio,Wuhan, China). The limitations of detection for IL-10 and TNF-α were 0.8 pg/mL, 3.9 pg/mL, and 3.57 ng/mL, respectively. The cut-off for positive detection of OVA-specific IgE was $\geq 2.1 \times$ the optical density value of negative controls.

Immunohistochemistry

Lung tissue sections from every group were deparaffinized, rehydrated, and subjected to antigen retrieval. After being blocked, the sections were incubated with α-SMA (Sigma, St. Louis, MO, USA) and anti-mouse VEGF (Abcam, Cambridge, MA, USA), and after being washed, the

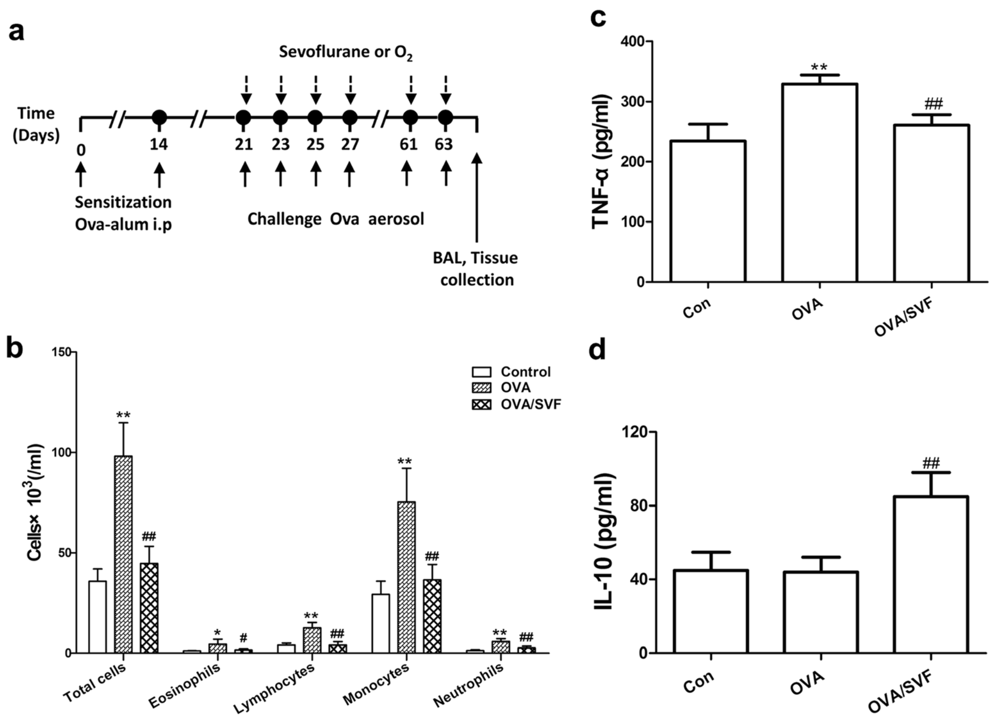

Fig. 1. Sevoflurane inhibits inflammatory cell infiltration and pro-inflammatory cytokine production in the BALF of allergic mice. **a** Experimental protocol, individual mice were sensitized with OVA or injected with saline on day 0 and day 14. On day 21, the mice were subjected to inhalation with 3% sevoflurane in oxygen-air or oxygen-air alone for 1 h and then challenged with aerosolized OVA or saline, every other day, for 6 consecutive weeks. The mice were sacrificed 24 h after the last challenge. **b** The number of total and differential cells in the BALF. **c** The level of TNF-α in the BALF. **d** The level of IL-10 in the BALF. Data are shown as the mean ± SEM of each group ($n = 7$ per group). $^*P < 0.05$, $^{**}P < 0.01$ *versus* the control; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *versus* the OVA group.

bound antibodies were detected with biotinylated secondary antibody, and then horseradish peroxidase (HRP)–conjugated streptavidin. The stained slices were visualized with DAB, and then counterstained with hematoxylin. All sections were analyzed under a bright-field microscopy.

Western Blotting Analyzes the Protein Level

The lung tissues of individual mice were homogenized in RIPA buffer (50 mM Tris–HCl, pH 7.4, 0.1% SDS, 1% Triton X-100, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA) supplemented with protease inhibitor cocktail (Roche, Indianapolis, IN, USA). After quantification of protein concentrations, the total proteins were separated by SDS-PAGE and transferred to PVDF membranes (Millipore, Billerica, MA, USA). After being blocked with 5% fat-free dry milk, the membranes were incubated with anti-α-SMA (Sigma), anti-VEGF (Abcam), anti-TGF-β1 (Abcam), and anti-GAPDH (KANGCHEN Biotech, Shanghai, China). The bound antibodies were detected with HRP-conjugated anti-rabbit IgG (Promega, Madison, WI, USA), and visualized using enhanced chemiluminescene (ECL, Thermo Scientific).

Statistical Analysis

Data are expressed as means ± standard error of the mean (SEM). Statistical significance was determined using analysis of variance (ANOVA) followed by a multiple comparison test with Bonferroni adjustment. P values $<$ 0.05 or $<$ 0.01 were considered significant.

RESULTS

Sevoflurane Inhibits Inflammatory Cell Infiltration and Pro-inflammatory Cytokine Production in BALF of Allergic Mice

As expected, there was a dramatic increase in the number of total cells recovered in the BALF of OVA-challenged mice when compared with mice that were challenged with saline (Fig. 1b). Further morphological assess revealed that the increase of total cells in BALF resulted from the infiltration of eosinophils, lymphocytes, monocytes, and neutrophils (Fig. 1b). However, this dramatic increase in inflammatory cell infiltration was notably inhibited by administration of sevoflurane (Fig. 1b).

Additionally, pro-inflammatory cytokine TNF-α was strongly induced after OVA challenge with comparison to that of control mice, while this induction was suppressed by sevoflurane treatment (Fig. 1c). Interestingly, anti-inflammatory cytokine IL-10 was found not to be induced in the OVA-challenged mice, but was severely elevated by sevoflurane (Fig. 1d).

Sevoflurane Prevents Airway Thickening and Goblet Cell Hyperplasia in the Lung Tissue of Allergic Mice

Next, lung tissue slices from each group were detected by H&E staining and PAS staining to evaluate airway thickening and goblet cell hyperplasia. Compared with that of the control group, OVA-challenged mice showed an obvious increase of the total area of the bronchial wall (Wat/Pbm) (Fig. 2a, b) and goblet cell hyperplasia (Fig. 2c). However, compared with the OVA-challenged group, the OVA/SVF groups showed significantly decreased Wat/Pbm and goblet cell hyperplasia (Fig. 2a–c).

Sevoflurane Suppresses Collagen Deposition in the Lung Tissue of Allergic Mice

Sirius red staining is routinely used for determination of collagen deposition. In the present study, we found that large amount of collagen deposition around the bronchioles after OVA challenge, while sevoflurane significantly suppressed this OVA-induced collagen deposition (Fig. 3).

Sevoflurane Treatment Prevents the Increase of Smooth Muscle Mass

Furthermore, we found that chronic OVA-challenged mice showed enhancement of the α-SMA stained area around the airway (Fig. 4a). Similarly, the results of western blot showed a significant increase of α-SMA protein level in OVA-induced mice, which was consistent with the results of immunohistochemistry (Fig. 4c). However, the protein level of α-SMA in the lungs of OVA-challenged mice that were treated with sevoflurane was significantly decreased (Fig. 4a, c). Qualification of α-SMA was accomplished by dividing the area of α-SMA staining cells by the perimeter of basement membrane (Wam/Pbm). OVA-challenged mice have greater α-SMA-positive cell mass than mice in the control group, and sevoflurane treatment leads to completely abrogation of the α-SMA cell mass increase (Fig. 4b).

Sevoflurane Decreased the Protein Level of VEGF and TGF-β1 in the Lung Tissue of Allergic Mice

AR has been indicated to be promoted by epithelial cell–derived VEGF [23]. Decreased VEGF expression leads to a significant reduction in goblet cell hyperplasia [24]. Our present study showed that the protein level of VEGF was significantly increased after OVA challenge by immunohistochemistry and western blot, while this increase was markedly inhibited by sevoflurane (Fig. 5a, b).

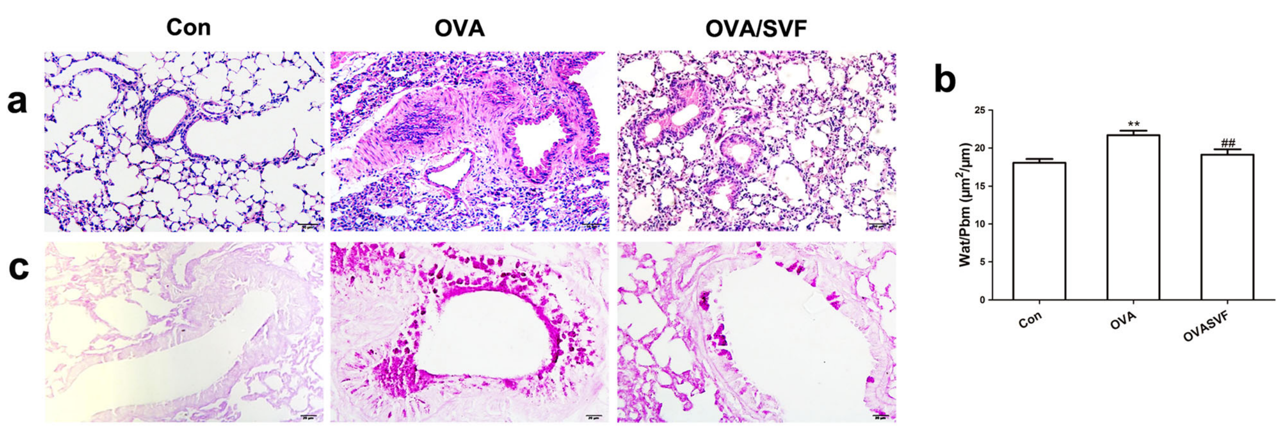

Fig. 2. Sevoflurane prevents airway thickening and goblet cell hyperplasia in the lung tissue of allergic mice. In the OVA/SVF group, the thickened airway wall is significantly inhibited compared with the OVA group (**a**, **b**); the airway goblet cell hyperplasia and hypersecretion were inhibited in the OVA/SVF group compared with the OVA group. **c** Pbm, perimeter of basement of membrane; Wat, total bronchial wall area. Data are shown as the mean ± SEM of each group ($n = 7$ per group). $^{**}P < 0.01$ *versus* the control; $^{\#\#}P < 0.01$ *versus* the OVA group.

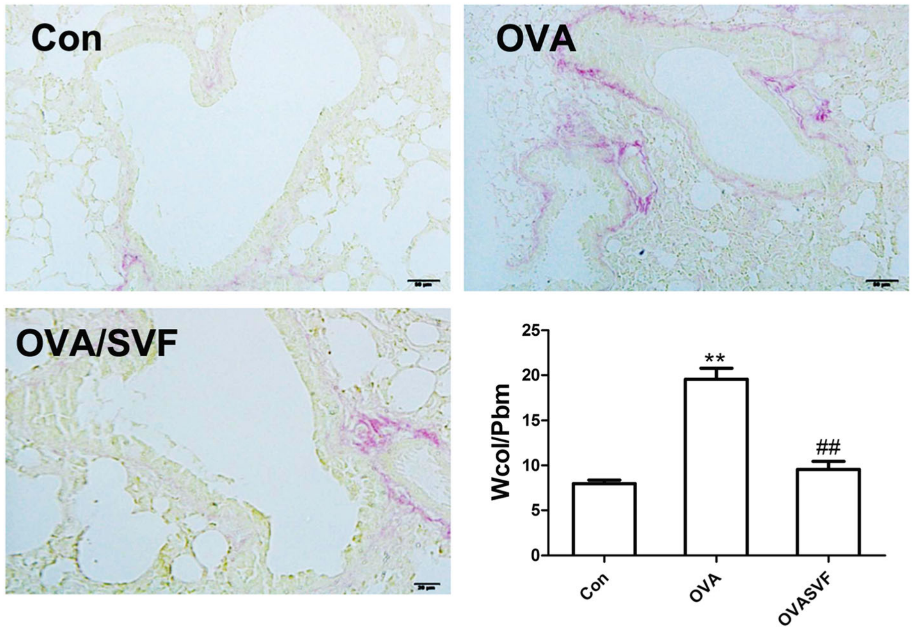

Fig. 3. Sevoflurane suppresses collagen deposition in the lung tissue of allergic mice. The area of collagen deposition around the airway wall in the OVA/SVF group was significantly decreased compared with the OVA group. Wcol, collagen area around the bronchial wall; data are shown as the mean ± SEM of each group (*n* = 7 per group). $^{**}P < 0.01$ *versus* the control; $^{\#\#}P < 0.01$ *versus* the OVA group.

On the other hand, TGF-β1, a pleiotropic cytokine that has been evidenced to be involved in the synthesis of matrix molecules in the ASM cells, has been implicated in the pathogenesis of AR [25]. Here, our western blotting analyses showed that the protein level of TGF-β1 in lung tissues was significantly higher in the OVA group, compared with those in the control group. However, the protein level of TGF-β1 was significantly downregulated in the OVA/SVF group compared with those in the OVA group (Fig. 5c).

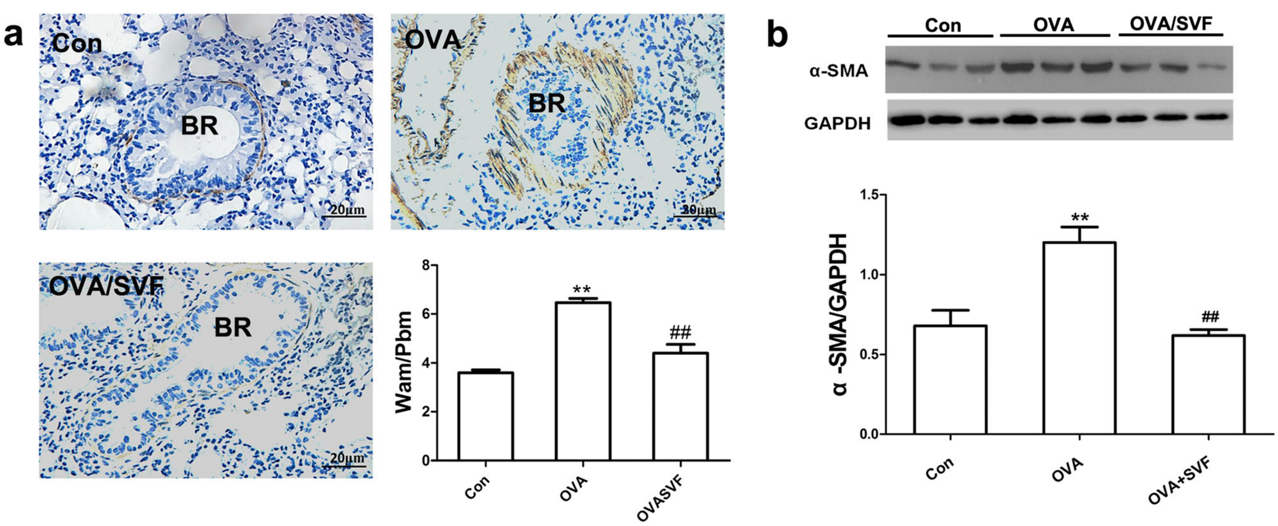

Fig. 4. Sevoflurane treatment prevents the proliferation of airway smooth muscle cells. Compared with the OVA group, smooth muscle hyperplasia and α-SMA expression were decreased in the OVA/SVF group (**a**, **b**); Wam, bronchial smooth muscle wall area. Data are shown as the mean ± SEM of each group (*n* = 7 per group). $^{**}P < 0.01$ *versus* the control; $^{\#\#}P < 0.01$ *versus* the OVA group.

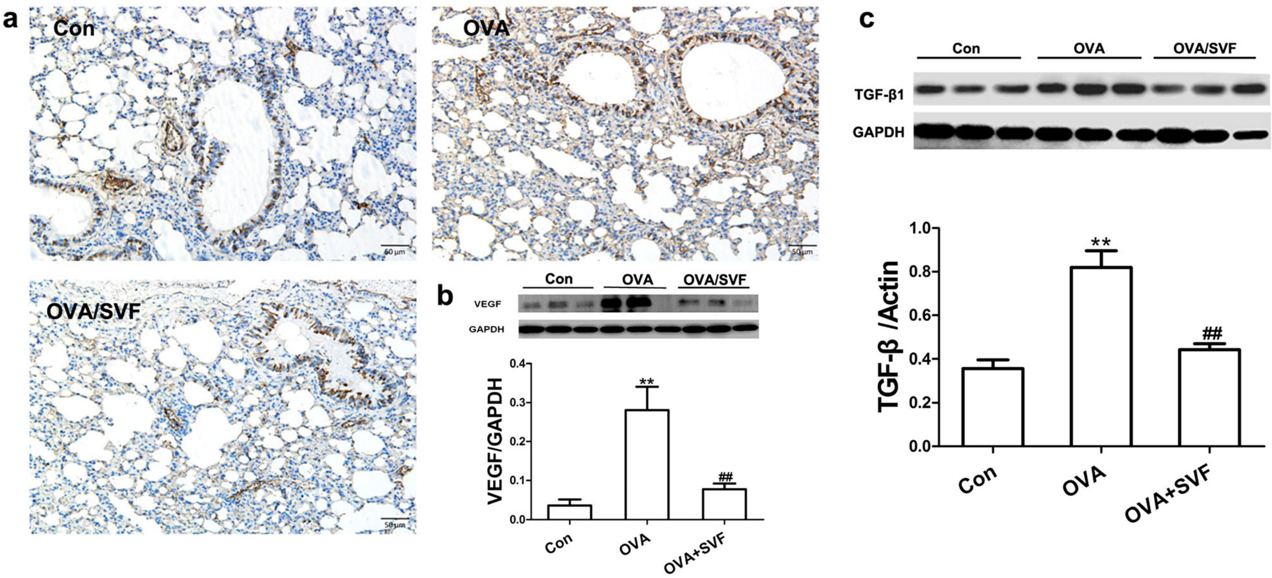

Fig. 5. Sevoflurane inhibits VEGF and TGF-β1expression in the lung tissue of allergic mice. Compared with the control group, VEGF expression in the lung tissues of the OVA group was significantly upregulated; compared with those in the OVA group, the levels of VEGF expression in the OVA/SVF group were reduced (**a**, **b**). Similarly, OVA-induced TGF-β1 expression in the lung tissues was inhibited by sevoflurane (**c**). Data are shown as the mean ± SEM of each group ($n = 7$ per group). $^{**}P < 0.01$ *versus* the control; $^{\#\#}P < 0.01$ *versus* the OVA group.

DISCUSSION

In the present study, we demonstrated that sevoflurane suppressed OVA-induced infiltrations of inflammatory cells and production of pro-inflammatory cytokine, while increased the level of anti-inflammatory cytokine. Concomitantly, sevoflurane inhibited the thickening of the airway wall, hyperplasia of airway goblet cells, proliferation of ASM, and over deposition of collagen around the airway wall in OVA-sensitized and challenged lung tissues of mice. Most notably, sevoflurane inhibited the expressions of VEGF and TGF-β1 induced by OVA, both are reported to promote AR. To our knowledge, this research was the first to investigate the effect of sevoflurane on AR in OVA-induced allergic mice.

Sevoflurane is commonly used inhaled anesthetic and has been shown to inhibit the AHR of both asthmatic animals and asthmatic patients [26, 27]. Especially, sevoflurane has been reported to be useful in controlling severe asthmatic status, which was resistant to normal treatment [10, 11]. In the present study, we found that sevoflurane significantly inhibited inflammatory cell infiltration and pro-inflammatory cytokine TNF-α production in the BALF of mice with chronic airway inflammation, but increased anti-inflammatory cytokine IL-10 production. Our previous study has showed that sevoflurane can inhibit acute airway inflammation [16], and here we further suggested that sevoflurane prevents OVA-induced chronic airway inflammation.

The chronic inflammatory response is the underlying cause of AR, and AR is closely related to AHR in asthmatic individuals. Therefore, we speculated that sevoflurane may inhibit AR. As expected, we found that sevoflurane could regulate indicators of AR, which is characterized by basement membrane thickening, goblet cell hyperplasia, myofibroblast collagen production, and smooth muscle cell proliferation. Furthermore, we demonstrated that sevoflurane downregulated OVA-induced SMC proliferation and the increased expression of α-SMA. α-SMA is the marker of SMCs, and is also the signal for activation and metaplasia of myofibroblast [28]. Activated myofibroblast is the key effector cell during fibrillation. Therefore, our present study suggested that sevoflurane may inhibit AR besides its suppression effect on airway inflammation.

Additionally, we also detected the effect of sevoflurane on the protein levels of TGF-β1 and VEGF, the two critical factors in the pathogenesis of AR [29, 30]. First, we found that sevoflurane effectively inhibited the upregulated expression of TGF-β1 induced by repeated OVA challenge. TGF-β1 is pivotal for most cells involved in AR, including SMCs [31–34]. It is an important mediator in mediating myofibroblast collagen production and regulating goblet cell hyperplasia and angiogenesis [35, 36], which are important characteristics for AR [37, 38]. On the other hand, VEGF is an efficient and multifunctional vascular regulator [39]. It can stimulate the proliferation and migration of vascular endothelial to form new vascular, and make great contributions to airway inflammation and AR in asthma [18, 40]. Our present study demonstrated that sevoflurane effectively decreased OVA-induced expression of VEGF. However, in the present research, the effect of sevoflurane on the VEGF and/or TGF-β1 signaling was not analyzed *in vitro*. Macrophages have been reported to play pivotal role in the pathogenesis of allergic airway inflammation, and they are the main source of VEGF and TGF-β1. Whether sevoflurane shows any effect on the secretion of VEGF and TGF-β1 from macrophages and exploring the underline mechanism in the airway inflammation will be the future study in our laboratory.

Together, our results displayed that repeated inhalation of sevoflurane could attenuate AR in OVA-induced mouse model of chronic airway inflammation. The underling mechanism might be that sevoflurane reverses the upregulated expression of TGF-β1 and VEGF, which are critical for AR. Therefore, our results further indicate a potential role of sevoflurane in inhibiting AR besides its known suppression effect on airway inflammation, and support the use of sevoflurane in treating severe asthma in ICU.

FUNDING

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COMPLIANCE WITH ETHICAL STANDARDS

Conflict of Interest. The authors declare that they have no conflict of interest.

Ethical Approval. All animal experiments described in this study were approved by the Animal Care and Use Committee of Anhui Medical University.

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