Article

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Methacholine Could Induce Asthma-Like Changes at the Molecular Level

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Background: The typical symptoms of asthma, including airway hyperresponsiveness (AHR) and airway remodeling, induce mechanical stress. Despite its crucial role in tissue development and pathophysiology, the role of mechanical stress in the development of asthma and its impact on the induction of AHR remain unclear. In this study, we aimed to investigate the potential contribution of mechanical stress to asthma by using a mouse model.
Methods: Healthy mice were challenged with methacholine (METH) and compared with an allergic airway inflammation model induced by ovalbumin (OVA). Different approaches, including immunofluorescence, immunohistochemical staining, Western blot analysis, and histopathology were employed to detect pathological changes in the lung tissue of the experimental mice. Transcriptome analysis was conducted to identify the genetic changes in AHR-related genes within the lung tissue of mice following METH treatment.
Results: METH treatment did not promote allergic phenotypes, but induced asthma-like changes in gene expression profiles. Affected genes were enriched in the immune response and inflammation-related genes, including genes involved in granulocyte migration and inflammatory reactions. Importantly, mechanical force perception ability was significantly enhanced in the OVA-induced allergic airway inflammation mouse lung, evidenced by increased expression of mechanoreceptor proteins.
Conclusions: Short-term bronchial constriction without inflammation may not lead to tissue remodeling. However, genes involved in asthma development remain active.

Keywords: methacholine; asthma; inflammation; airway hyperresponsiveness

Introduction

Asthma, one of the crucial human health concerns, affects approximately 260 million people annually, resulting in 455,000 deaths [1]. Unfortunately, preventive and therapeutic measures are limited because of the diverse nature of their pathogens.

Airway hyperresponsiveness (AHR), which induces mechanical stress during the contraction of airway smooth muscles [2,3], represents a typical symptom of asthma [4,5]. Computational analyses suggest that airway remodeling, another feature of asthma, also contributes to generating mechanical stress [6,7], indicating a connection between mechanical stress and asthma. Studies have revealed that healthy individuals display different levels of non-specific bronchial reactivity to stimuli [8], and AHR shows heritable features [9]. Moreover, asymptomatic asthma patients exhibit an increased level of non-specific bronchial reactivity [8]. Increased non-specific bronchial reactivity found in asymptomatic asthma patients may be a result of airway remodeling and inflammation, indicating the potential involvement of mechanical stress in the pathological mechanism of asthma. Further studies showed that bronchial contraction, even in the absence of additional inflammation, can induce thickening of the basement membrane and increase the number of goblet cells in asthma patients, thus indicating airway remodeling [10]. Mechanical compression of bronchial epithelial cells *in vitro* can induce asthma-related symptoms [11–13] and trigger the release of transforming growth factor-β in human umbilical vein endothelial cells [14]. This observation confirms the role of bronchoconstriction in the pathogenesis of asthma. However, in the absence of inflammation, the role of mechanical stress generated during simple bronchoconstriction [15] in inducing asthma symptoms and its involvement in the pathogenesis of asthma remain unclear, suggesting the need for further investigations.

Mechanosensitive ion channels play a significant role in the disease-related remodeling of tubular tissue [16,17], with Piezo1 emerging as a significant candidate. Piezo1, found in various tissues and organs, such as the blood vessels, skin, renal tubules, and lungs, has been associated with the pathogenesis of multiple diseases [18–21]. There is still no direct evidence correlating increased airflow to the activation or upregulation of Piezo1 gene expression in pneumocytes or airway cells. However, reports concerning other tubular tissues have implied at this possibility [22]. We speculate that Piezo1 protein expression is induced in the

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lung tissue of mice experiencing allergic inflammation as well as those exposed to simple mechanical stress. Previous research has reported similar levels of bronchoconstriction induced by allergens and methacholine (METH) [10]. Therefore, in this study, we used METH to induce bronchial constriction and the allergen ovalbumin (OVA) as a positive control to establish a mouse model, aiming to explore the molecular and pathological changes triggered by mechanical stress.

Materials and Methods

Experimental Animal

Male BALB/c mice (6–8 weeks old) were purchased from Jinan PengYue Experimental Animal Breeding Co., Ltd. and bred in a specific pathogen-free (SPF) facility with consistent temperature and humidity. They were maintained on a 12-hour light/dark cycle and provided food and water ad libitum.

Establishment of Mouse Model

The mice were fed adaptively for one week and then randomly divided into three groups, each containing twelve mice: the OVA group (positive control), the METH group, and the control group (negative control). The experimental mice underwent two challenges [23]: challenge A involved injecting 20 μg of OVA (A107820, aladdin, Shanghai, China) or METH (O135659, aladdin, Shanghai, China), dissolved in 200 μL of 10 μg alum (A110529, aladdin, Shanghai, China), intraperitoneally on days 0 and 14. However, the control group received 200 μL of phosphate buffer (PBS, G0002, Servicebio, Wuhan, China). On day 21, the mice were exposed to nebulization stimulation with OVA (10 mg/mL), METH (10 mg/mL), or PBS for 30 minutes daily for 3 days. After this, 6 mice in each group were euthanized utilizing a cervical dislocation method. Furthermore, in challenge B, started on day 25, the mice underwent a 30-minute OVA, METH, or PBS aerosol inhalation once every 2 days for one month. Following treatments, all mice were euthanized, and their lung tissues were surgically collected for histopathological examination. The study protocol was approved by the Medical Ethics Committee of Weifang Medical College (protocol code 2022YX024).

Immunofluorescence

The fresh lung tissue was fixed and subsequently embedded in paraffin. The tissues were cut into 10 μm paraffin sections and were then dewaxed, repaired, and blocked. The sections were incubated overnight with ratCD11b antibody (1:200, MA1-80091, Thermo Fisher Scientific, Waltham, MA, USA) at 4 °C. CD11b is a marker of lung monocytes and granulocytes that play a pivotal role in the development of asthma [24]. After washing, the sections were incubated with HRP-labeled goat anti-rat IgG (1:200, GB23302, Servicebio, Wuhan, China) at 25 °C for 50 minutes. Subsequently, the tissue sections were incubated with cyanine3-tyramide (1:500, G1223, Servicebio, Wuhan, China) at 25 °C for 10 minutes in the dark. In the next step, the tissue sections were cleaned and repaired followed by overnight incubation with anti-caspase-3 rabbit pAb (1:200, GB11767C, Servicebio, Wuhan, China) at 4 °C. Caspase-3 is an apoptosis marker [25]. After this, tissue sections were incubated with an Alexa Fluor 488 labeled goat anti-rabbit IgG (1:200, GB25303, Servicebio, Wuhan, China) at 25 °C for 50 minutes. Finally, these tissue sections were stained with DAPI. Observed using a Nikon Eclipse C1 fluorescence microscope, and imaged with NIKON digital sight DS-FI2 and NIS_F_Ver43000_64bit_E (Nikon, Tokyo, Japan).

Immunohistochemistry

The paraffin-embedded tissue sections were dewaxed, repaired, and blocked followed by overnight incubation with Piezo1 antibody (1:200, Cat no.15939-1-AP, Proteintech, Wuhan, China) at 4 °C. After washing, the sections underwent a 50-minute incubation with HRP-labeled goat anti-rabbit IgG (1:200, GB23303, Servicebio, Wuhan, China) at 25 °C. In the following step, they were exposed to the DAB chromogenic solution (G1211, Servicebio, Wuhan, China), and the staining time was monitored under a microscope. Finally, the sections were counterstained with hematoxylin and observed using Nikon Eclipse C1 light microscope , and imaged with NIKON digital sight DS-FI2 and NIS_F_Ver43000_64bit_E (Nikon, Tokyo, Japan).

Lung Histopathology

Paraffin-embedded tissue sections were stained with hematoxylin and eosin (G1076, Servicebio, Wuhan, China) reagent, and pathological changes in the lung tissue were observed utilizing a Nikon Eclipse C1 light microscope, and imaged with NIKON digital sight DS-FI2 and NIS_F_Ver43000_64bit_E (Nikon, Tokyo, Japan).

Western Blot Analysis

Lung tissue was homogenized in RIPA buffer (G2002, Servicebio, Wuhan, China) followed by centrifugation at 10,000 ×g for 10 minutes. The resultant supernatant was loaded onto an SDS polyacrylamide gel to resolve the proteins. Subsequently, proteins were transferred onto polyvinylidene fluoride membranes. After this, the membranes were blocked and incubated overnight with Piezo1 antibody (1:500, Cat no.15939-1-AP, Proteintech, Wuhan, China) and anti-beta Actin antibody (1:2000, GB15003, Servicebio, Wuhan, China) at 4 °C. The following day, membranes were washed with PBST and subsequently incubated with HRP-labeled goat anti-rabbit IgG (1:500, GB23303, Servicebio, Wuhan, China) at 25 °C for one hour. Finally, protein bands were visualized using an ECL

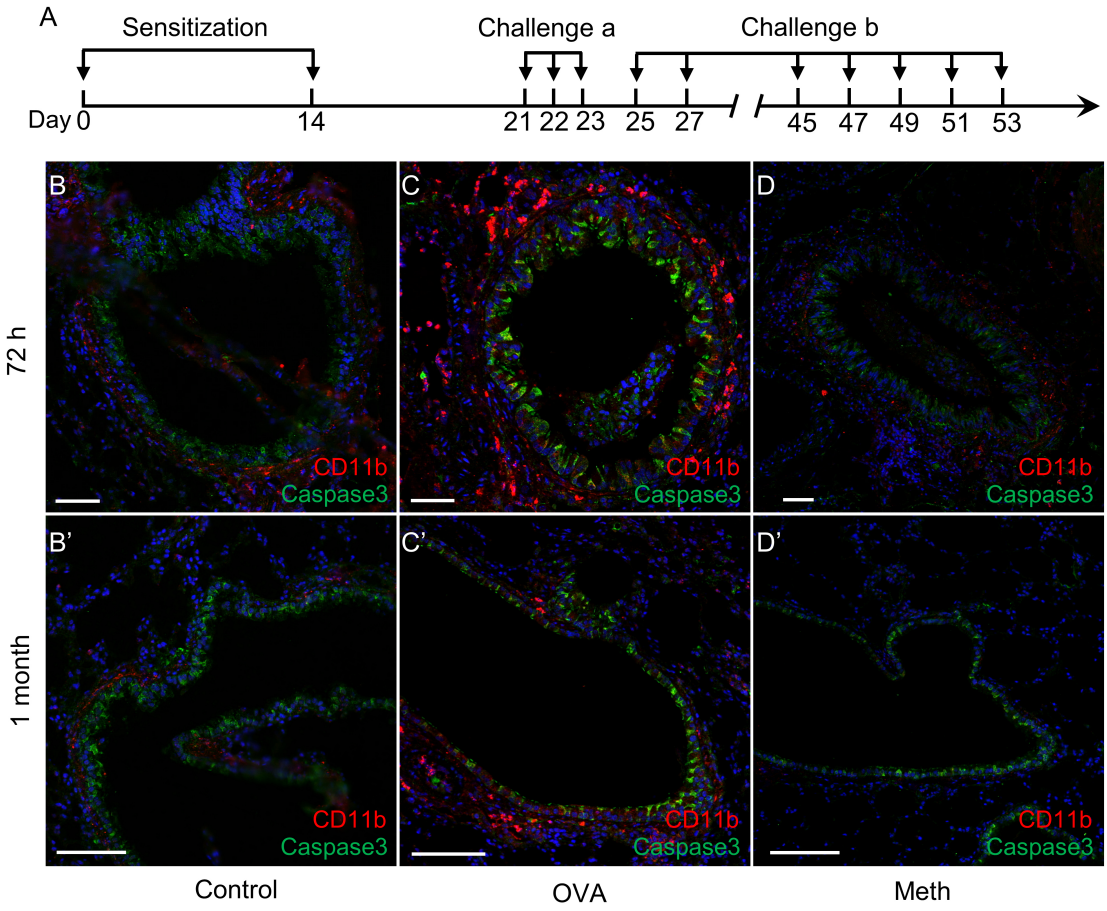

Fig. 1. Establishment of the mouse model. (A) Method for establishing mouse model. (B–D) and (B'–D') Double immunofluorescence staining of CD11b and caspase3 in mouse lung tissue. CD11b and caspase3 serve as markers for inflammatory cells and apoptotic signals, respectively. The representative images of the control (B), OVA (C), and METH (D) groups at 72 hours after mouse induction. The representative images of the control (B'), OVA (C'), and METH (D') groups one month after the nebulization challenge. Scale bar = 50 μm. OVA, ovalbumin; METH, methacholine; CD11b, cluster of differentiation 11b.

luminescent reagent (G2014, Servicebio, Wuhan, China), and their images were captured utilizing a chemiluminescence instrument (CLINX6100, Clinx, Shanghai, China).

Transcriptome Sequencing and Differential Expression Analysis

Total RNA was extracted from mouse lung tissue using the trizol (R0016, Beyotime, Shanghai, China) method and subsequently quantified using an ultramicro UV spectrophotometer (N50 Touch, Implen, Munich, Germany). The enriched mRNA was sequenced, and the raw data obtained were filtered and standardized. Differentially expressed genes were analyzed using a threshold of $|\log2 \text{ fold change}| > 1$ and $p\text{-value} < 0.05$. Furthermore, enrichment analysis of differential gene functions was performed, focusing on Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways.

Statistics Analysis

After normalization of related genes, statistical analyses were conducted using GraphPad Prism 8.4.0 software (GraphPad Software, Inc., San Diego, CA, USA). A two-way ANOVA was used to analyze the significant differences between the control and OVA of related genes. The WB results were analyzed using Image J 1.53e/Java 1.8.0_172 (National Institutes of Health, Bethesda, MD, USA) to assess the gray values of protein bands. The differences between the two groups were compared using a *t*-test. The p-value < 0.05 and <0.01 indicated significant and highly significant differences, respectively.

Results

Impact of Bronchoconstriction on Asthma Progression

Asthma is characterized by inflammation of the bronchial tubes. Therefore, to investigate whether bronchoconstriction can induce tissue inflammation, the lung

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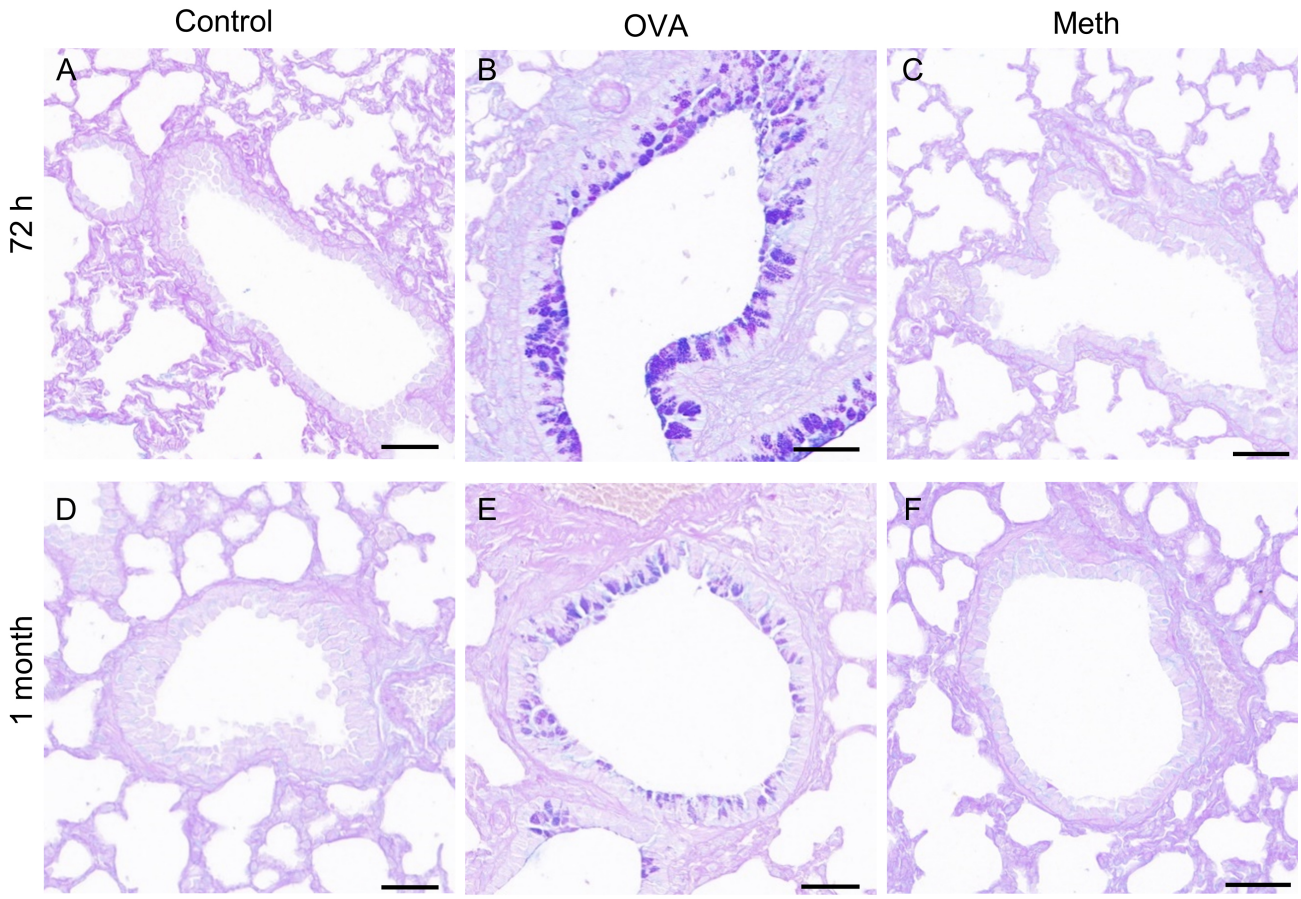

Fig. 2. Simple bronchoconstriction did not induce inflammation in the healthy respiratory tract. (A–C) respectively show the H&E staining of lung tissues after 72 hours of nebulization challenge with control, OVA, and METH groups. (D–F) respectively show the H&E staining of lung tissues after 1 month of nebulization challenge with control, OVA, and METH groups. Scale bar = 50 μm. H&E, hematoxylin and eosin.

tissues of mice were isolated and were maintained in three different conditions (Fig. 1A): specific pathogen-free condition (negative control), METH challenged, and OVA-challenged (positive control). The immunofluorescence results showed the accumulation of a large number of inflammatory cells within the lung tissue of the positive control group along with increased expression of apoptotic signaling proteins, after the short-term challenge. In contrast, METH-induced bronchoconstriction did not contribute to the accumulation of the corresponding inflammatory cells (Fig. 1B–D). However, similar effects were observed during the long-term challenge (Fig. 1B'–D'). A single staining image can be found in **Supplementary Fig. 1A–C**. Furthermore, photomicrographs revealed increased secretion in the airways within the OVA-exposed group, but no similar phenotype was observed in those exposed to normal conditions or METH (Fig. 2A–C). Mucus production and cell proliferation are two typical disease-related symptoms [26,27]. It was observed that bronchoconstriction in healthy animals did not trigger mucus production and cell proliferation even after mechanical stress induced for as long as one month (Fig. 2D–F).

Bronchoconstriction Induced Asthma-Related Genes

Previous research has demonstrated that mechanical stress alone does not cause significant changes in cell behavior. However, considering the observations in asthma patients [28], we inferred that bronchoconstriction is a risk factor for the development of asthma. Inducible changes may initially occur at the molecular level, they may vanish when the cells acquire corresponding capabilities. To explore the impact of bronchoconstriction at the molecular level, we conducted RNA sequencing analysis, and compared the transcription profiles of normal lungs and those under mechanical stress. There were significant effects of METH on mice at the molecular level. Both short- and long-term mechanical stresses contributed to alterations in the transcriptional profiles (Fig. 3A,B), and the animals could even be separated according to these changes. Furthermore, certain genes were upregulated under both short- and long-term mechanical stresses. In the short-term treatment group, 142 genes were upregulated and 48 were downregulated. However, in the long-term treatment group, 96 genes were upregulated and 57 were downregulated.

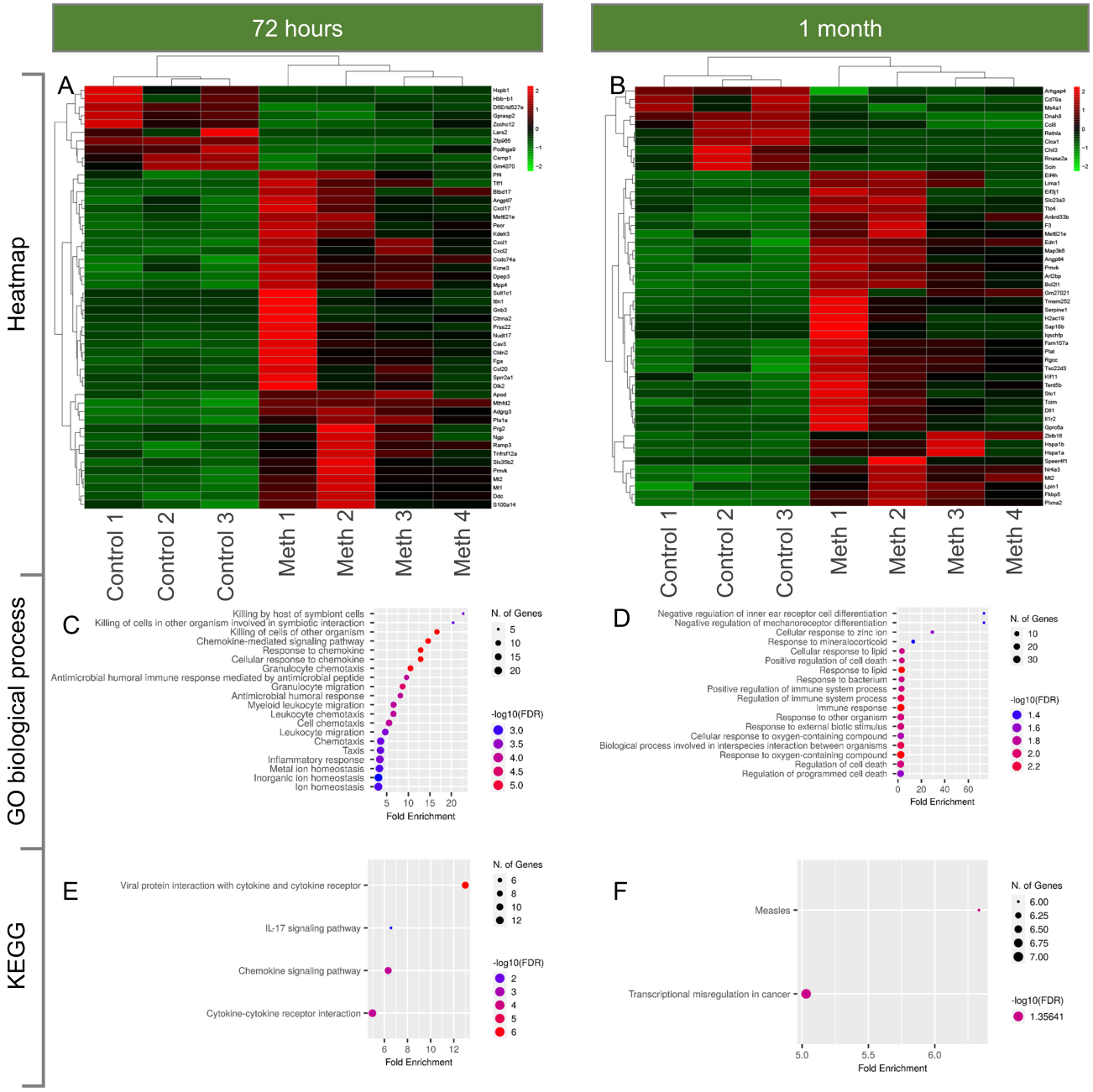

Fig. 3. Bronchoconstriction induced asthma-related genes. METH induced 190 and 153 genes to be differentially expressed (FC ≥ 2, $p < 0.05$) at 72 hours (A) and 1 month (B), respectively. (C) Short-term mechanical stress significantly upregulated genes involved in immune response and inflammation. (E) The IL-17 signaling pathway was enriched during mechanical stress. (D,F) Long-term mechanical stress did not exert a significant effect on inflammation. GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; IL-17, interleukin-17.

Furthermore, the function of the regulated genes was assessed using GO and KEGG pathways enrichment analyses. It was found that short-term mechanical stress significantly upregulated genes involved in the immune response and inflammation, such as granulocyte migration (Fig. 3C). Moreover, the KEGG analysis revealed enrichment of the interleukin-17 (IL-17) signaling pathway during mechanical stress (Fig. 3E).

Additionally, it was observed that long-term mechanical stress did not exert a significant effect on inflammation. However, genes involved in the cell cycle and immune response were significantly regulated. Moreover, GO analysis demonstrated the enrichment of "negative regulation of mechanoreceptor differentiation", (Fig. 3D). Similarly, KEGG analysis revealed the enrichment of genes involved in transcriptional misregulation during cancer (Fig. 3F).

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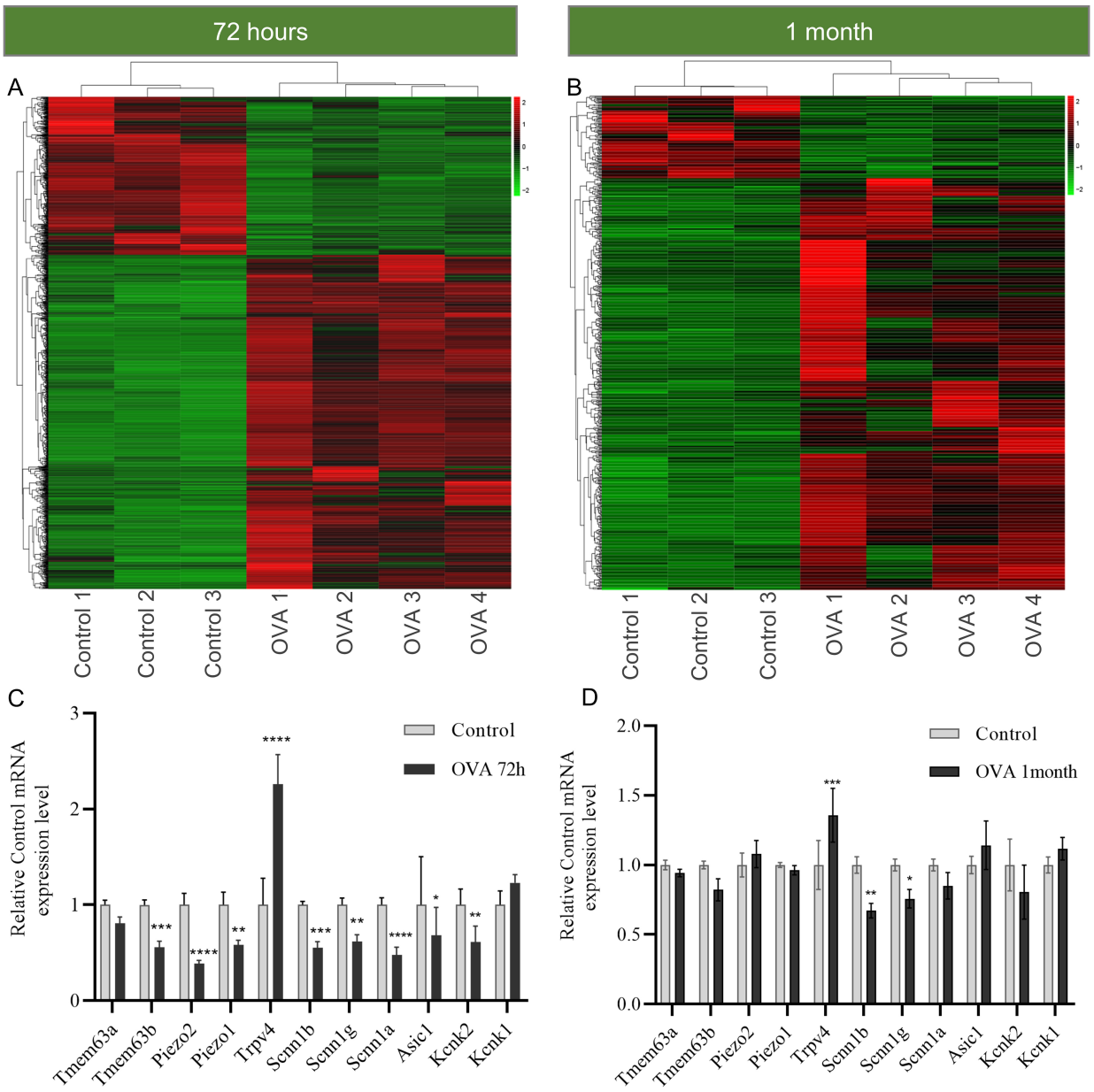

Fig. 4. Inflammation induced expression of mechanoreceptor. Differentially expressed genes in the short-term treatment group (A) and the long-term treatment group (B), were 1937 and 592 (FC $\geq$2, $p < 0.05$), respectively. (C,D) show statistical analysis of differentially expressed genes after 72 hours and 1 month of nebulization challenge, respectively (n = 4). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$ versus the control.

Inflammation Induced Expression of Mechanoreceptor

For transcriptional analyses, lung tissues were isolated from mice with allergic airway disease, serving as a positive control. This analysis revealed that most of the regulated genes were upregulated. In the short-term treatment group, 1314 genes were upregulated and 623 were downregulated (Fig. 4A). However, in the long-term treatment group, 493 genes were upregulated and 99 were downregulated (Fig. 4B). More genes were regulated by allergens than mechanical stress. Similarly, most of the genes were upregulated.

Importantly, transcriptional analyses revealed that genes involved in mechanosensitive ion channels were extensively downregulated after short-term allergen treatment (Fig. 4C). However, after long-term allergen induction, they were almost restored to their normal levels (Fig. 4D), particularly, the Piezo1 and Piezo2 genes recovered after long-term allergen treatment. Piezo1 is a well-known mechanoreceptor involved in the remodeling of tube structures. It has been recognized for its crucial role in the pathogenesis of several human diseases [16].

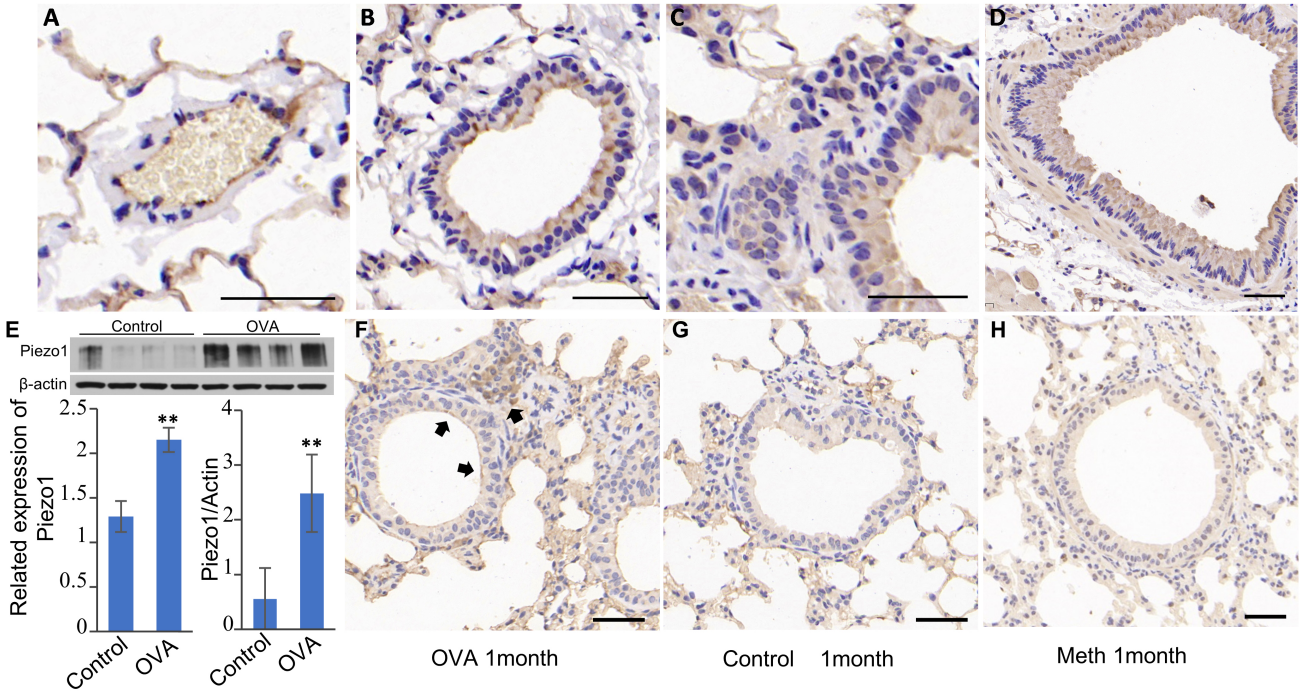

Fig. 5. Increased shear stress did not induce the expression of Piezo1 or the development of inflammation. (A–D) Immunohistochemical analysis of healthy mouse lungs stained for Piezo1. The distribution of Piezo1 in cells of pulmonary vessels (A), lymphoid cells (B), muscle (C), and pneumocytes (D). (E) Western blot and quantitative analysis of Piezo1 protein in lung tissue of inflammatory mice (n = 4) challenged with nebulization for 72 hours. (F–H) Immunohistochemical staining was performed to evaluate the expression of the Piezo1 protein in mouse lung tissue after 1 month of induction. The arrow represents the area with increased protein levels. Scale bar = 50 μm. $^{**}p < 0.01$ versus the control group.

Increased Shear Stress did not Induce the Expression of Piezo1 or the Development of Inflammation

To determine the location and pattern of Piezo1 expression in lung tissues, we examined its distribution in the lung tissue of healthy mice. Piezo1 was significantly expressed in the endothelial cells of pulmonary vessels (Fig. 5A), lymphoid cells (Fig. 5B), muscle cells, epithelial cells of airways (Fig. 5C), and pneumocytes (Fig. 5D). Moreover, the WB analysis revealed that under short-term induction of OVA, the positive control exhibited an increase in the levels of Piezo 1 protein in the lung tissue (Fig. 5E). However, there were no significant changes in Piezo1 in the METH or negative control groups (Fig. 5F–H).

Discussion

AHR is the excessive or premature contraction of airway smooth muscles caused by direct or indirect stimulation [29]. It is strongly associated with airway inflammation, smooth muscle functionality, and gene regulation [27,30,31]. Studies have shown that mechanical stress on airway cells *in vitro* results in the induction of various inflammatory protein genes [32] and the release of inflammatory signals [13,33], leading to the occurrence and exacerbation of airway inflammation. In this study, we used METH to induce frequent airway constriction in mice, leading to changes in gene expression profile. Using annotation and functional analysis of the differentially expressed genes, we found enrichment in asthma-related biological processes and signaling pathways, such as the inflammatory response and the interleukin-17 (IL-17) signaling pathway [34]. The effects of recurrent airway constriction on gene expression were further assessed through the inhibition of METH function [35], indicating the potential role of frequent airway constriction in asthma development.

In the development of AHR, chronic inflammation and persistent stimulation can lead to the proliferation and thickening of airway smooth muscle, resulting in structural changes in the airway wall. However, upon investigating cell proliferation, we found that cell proliferation was not induced by consecutive METH stress in lung tissue stained for phospho-histone H3 (PH3) (**Supplementary Fig. 2**) and pathological sections. This treatment alone failed to induce visible remodeling of lung tissue. Despite lacking observable tissue remodeling, it is possible that both cell proliferation and cell death were mildly enhanced, considering that an abnormal increase in cell number may be diminished by programmed cell death in healthy lungs.

Frequent airway constriction increases the occurrence of bronchial contractions and airflow within the respiratory tube. These signaling pathways are recognized by mechanosensitive ion channels. Abnormal activation of

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Piezo1, the mechanically sensitive cation channel, can cause pathophysiological changes in the respiratory system [36]. High-magnitude cyclic stretch and high tidal volume mechanical ventilation have been found to substantially upregulate the expression levels of Piezo1 and mediate signaling associated with lung injury [37]. In both animal models and patients with idiopathic pulmonary hypertension, Piezo1 is widely upregulated in pulmonary vascular endothelial cells. This upregulation leads to increased expression of Notch ligands and activation of Notch receptors, thereby resulting in the remodeling of the cardiovascular and pulmonary vascular systems [38]. In this study, we report a widespread distribution of Piezo1 in lung cells *in vivo*, indicating a possible role in sensing mechanical forces and regulating physiological processes in the lungs. Furthermore, we found significantly higher levels of Piezo1 protein expression in the lungs of allergic mice than those in the negative control group. However, since there is no effective approach to measure the activity of Piezo1 *in vivo*, we were unable to demonstrate increased Piezo1 activity under conditions of frequent airway constriction. Developing a method to monitor the cellular currents *in vivo* and establishing evidence of changes in Piezo1 activity under AHR are needed for further exploration.

Conclusions

The mechanical force generated by simple bronchoconstriction is not strong enough to cause tissue damage or induce the expression of mechanical force-related proteins in healthy lungs. However, the increased expression of disease-related genes, particularly those involved in cell secretion and proliferation, indicates that it remains a potential risk factor for asthma development in asymptomatic patients with AHR.

Availability of Data and Materials

All data included in this study are available upon request by contact with the corresponding author.

Author Contributions

XN and KTY contributed to the concept and designed the research study. XN and YKS performed the research. XN contributed to the analysis and interpretation of the data. ZQG and GHW provided help and advice on the experiments. XN, YKS, KTY, ZQG and GHW were involved in drafting the article or critically revising it for important intellectual content. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work in ensuring that questions related to its accuracy or integrity.

Ethics Approval and Consent to Participate

The study protocol was approved by the Medical Ethics Committee of Weifang Medical College (protocol code 2022YX024 and date of 25 November 2021).

Acknowledgment

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This research received no external funding.

Conflict of Interest

The authors declare no conflict of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.23812/j.biol.regul.homeost.agents.20243805.308.

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