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The relationship between Muc5ac high secretion and Munc18b upregulation in obese asthma

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Abstract. – OBJECTIVE: Mucus production and hypersecretion are important pathophysiological features of asthma. Airway mucus secretion is more serious in obese asthma. Therefore, it is of great significance to elucidate the mechanism of asthma airway mucus high secretion in improving the control of asthma and the prognosis of obese asthmatic patients.

MATERIALS AND METHODS: Obese asthmatic mice model was established to test the airway resistance and mucin secretion by hematoxylin-eosin (HE) staining. Munc18b and Muc5ac expression levels were determined by Western-blotting. Munc18b conditioned knockout mice were adopted to explore the mechanism of Muc5ac high secretion.

RESULTS: The mice weight increased in obese asthmatic model accompanied by elevated airway resistance. HE staining showed enhanced mucin secretion, which was correlated to weight and airway resistance. Munc18b and Muc5ac expressions significant upregulated in an obese asthmatic mouse model compared with normal control. Muc5ac expression failed to show elevation in Munc18b conditioned knockout mice.

CONCLUSIONS: Muc5ac high secretion was positively correlated with Munc18b upregulation in obese asthma. Munc18b participated in inducing Muc5ac high expression.

Key Words:
Obese asthma, Mucin, Muc5ac, Munc18b.

Introduction

In recent years, the number of obese people increased with the improvement of living standards. However, as an obesity-related disease, the relationship between asthma and obesity is not fully stated[1]. Mucus production and secretion hyperthyroidism are an important pathophysiological characteristic of asthma. Numerous mucins enter the airway in a short time, which exceeds the scavenging activity, leading to severe airway obstruction, increased airway resistance, and even death[2]. It was found that obese asthma induces a more severe mucus secretion[3], while mucus plug formation is the main cause of death in asthma mice[4]. Therefore, it is important to clarify the mechanism of airway mucus hypersecretion in obese asthma, which is of great significance in improving the control level of asthma and the prognosis of obese asthmatic patients.

Mucin is a class of glycoproteins consisting mainly of mucopolysaccharides which are common in knee synovial fluid[5]. Increased mucin production occurs in many adenocarcinomas, including pancreatic cancer, lung adenocarcinoma, breast cancer, ovarian cancer, and colon cancer. Mucin is also overexpressed in lung diseases, such as asthma, bronchitis, chronic obstructive pulmonary disease, and cystic fibrosis[6,7]. Mucin secreted by respiratory tract is mainly Muc5ac and Muc5b. Muc5b is the major type of mucin secreted by respiratory tract in the physiological condition that main plays a defensive function, while Muc5ac is a type of mucin that significantly increases under the external stimulus, thus participating in the physiological processes of various diseases[8]. Muc5ac mainly distributes in the cytoplasm or vacuole membrane. Airway mucin secretion and mast cell degranulation strictly control the balance of the lungs and the immune system. The previous study[9] showed that the Sec1 protein family (Munc18b and Munc18c) is associated with high secretion of Muc5ac. In addition, extracellular protein Munc18b is overexpressed in mouse airway epithelial cells, while its mechanism for high secretion in obese asthma is unclear.

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We investigated the expressions of Munc18b and Muc5ac in the obese asthmatic mouse model, the cell localization of Muc5ac expression, and the regulatory mechanism of Munc18b on Muc5ac. This study established mouse obese asthma model to measure airway resistance, observe mucin secretion by HE staining, and determine the expression levels of Munc18b and Muc5ac by Western-blotting. Finally, we applied Munc18b conditioned knockout mice to explore the mechanism of Muc5ac hypersecretion.

Materials and Methods

Main Materials and Reagents

Muc5ac and Munc18b primary antibodies were purchased from Abcam (Cambridge, MA, USA). PVA-aluminum hydroxide gel was bought from Sigma-Aldrich (St. Louis, MO, USA). HE staining kit and immunohistochemical kit were obtained from ZSbio (Beijing, China). Horseradish-peroxidase (HRP) labeled goat anti-rabbit secondary antibody was provided by Beyotime (Jiangsu, China). BCA protein quantification kit was obtained from Keygentec (Shanghai, China).

Main Instrument

Benchtop was provided by Boxun Industrial Co,. Ltd., (Shanghai, China). Gel imaging system was obtained from UVP Multispectral Imaging System (San Diego, CA, USA). PS-9 Semi-dry transfer electrophoresis meter was supplied by Jim-x Scientific (Dalian, China). Thermo-354 microplate reader was purchased from Thermo Fisher (Waltham, MA, USA).

Experimental Animals

Wild-type C57BL6/J mice in 6-8 weeks and SPF grade were purchased from our Model Animal Research Center. Munc18 mutant hybrid mice weighted 19-22 g were purchased from Cyagen Biosciences Inc (CA, USA) and Nanjing Biomedical Research Institute of Nanjing University (Nanjing, China). The mice were raised in a clean animal room with the temperature at 24°C, relative humidity at 60%, 12 h day/night cycle, and free eating and drinking.

Obese Asthmatic Model Establishment

The obese mouse model was established by high-fat diet for continuous 8 weeks. Asthma model was constructed by OVA sensitization method through PVA-aluminum hydroxide gel intraperitoneal injection on the 1st and 7th days, followed by 1% OVA atomization 15 min daily for 2 weeks from the 14th day, and atomization every other day for 4 weeks. The obese asthmatic model was established by two methods application together. The mice were weighted after 8 weeks' modeling. After pulmonary function measurement, the lung tissue was extracted for hematoxylin and eosin (HE) staining to confirm the formation of asthma inflammation.

Airway Resistance Test

The mice were anesthetized after tracheal intubation, and the airway resistance was measured by small animal lung function instrument.

Hematoxylin-eosin (HE) Staining

The lung tissue was fixed in 4% formalin and, then, subjected to conventional paraffin embedding and sliced. The slices were routinely dewaxed and stained with eosin. After differentiated by hydrochloric acid, the slice was stained with hematoxylin. After dehydrated by gradient ethanol and hyalinized by xylene, the slice was sealed by neutral balsam.

Vacuum Immunoblotting

The lung tissue was added with 1 ml guanidium buffer and homogenized after cutting into species. After incubated at 4°C overnight, the sample was centrifuged to obtain the supernatant. Next, the sample was added into the Slide-A-Lyzer Mini dialysis container and dialyzed in urea buffer at 4°C overnight to obtain the total protein. Next, the protein was quantified by BCA method and boiled at 95°C for 20 min together with 5×loading buffer. Then, the sample was diluted by alkylation buffer at 1:10 and incubated at room temperature avoid of light for 30 min. The TAE buffer was added to the agarose and electrophoresis at 90 V. Next, the gel was washed by 4×SSC buffer for 5 min and added with 10 mM DDT at room temperature for 20 min. The protein was transferred to NC membrane using vacuum device at room temperature and 15 cm Hg negative pressure for 4 h. After blocked by 5% BSA, the membrane was incubated with Muc5ac primary antibody at 4°C overnight. Then, the membrane was incubated in HRP labeled goat anti-rabbit secondary antibody at room temperature for 1 h. At last, the membrane was developed by chemiluminescence.

Western Blotting

Frozen lung tissues were homogenized and lysed with RIPA lysis buffer containing 10 mM-TrisCl (pH 7.2), 0.15 M NaCl, 0.1% SDS, 1.0% Triton X-100, 1% deoxycholate, 5 mMEDTA and 2 μl/ml protease inhibitor cocktail. Protein concentrations were determined using the BioRad protein assay kit (Hercules, CA, USA). 50 μg of protein were resolved by electrophoresison 10% SDS-PAGE gel. The proteins were then transferred onto a nitrocellulose membrane and non-specific binding blocked by incubating with 5% nonfat milk in TBST buffer (0.01 M Tris-Cl, pH 8.0, 0.15 M NaCl, 0.5% Tween-20) at room temperature for 1 h. The specific primary antibodies were diluted in TBST with 5% non-fat dry milk and incubated with the filter for 1.5 h at room temperature or overnight at 4°C. The detection was carried out using the SuperSignalWest Pico detection system. Monoclonal anti-β-actin antibody was used as the internal control.

Statistical Analysis

All data analyses were performed on SPSS 19.0 software (SPSS Inc., Chicago, IL, USA). The measurement data were presented as mean ± standard deviation and tested by normality test and homogeneity test of variance. The data were compared by *t*-test or one-way ANOVA. LSD was performed for post-hoc test. $p < 0.05$ was considered as statistical significance.

Results

The Changes of Mouse Weight

The mouse weight gradually increased in both of normal control and asthmatic model groups (Figure 1). Compared with normal control, the mouse weight slightly reduced in asthmatic model group. It significantly elevated in obese group and obese asthmatic group compared with normal control ($p < 0.05$).

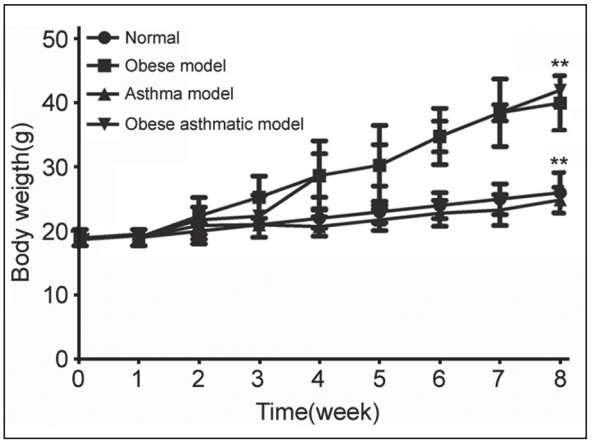

Figure 1. The changes of mouse weight. $^{**}p < 0.05$, compared with normal control.

Airway Resistance Measurement

As shown in Table I, the airway resistance in obese asthmatic group was higher than that in the other three groups. It markedly increased in the obese group and asthmatic group compared with normal control but lacked of statistical difference compared with each other.

The pathology Changes in Each Group

Airway HE staining revealed that inflammatory cell infiltration in airway was found in obese group, while mucus secretion exhibited no apparent changes compared with normal control (Figure 2). The mucus secretion markedly enhanced in asthmatic group compared with normal control ($p < 0.05$). The mice in the obese asthmatic group exhibited apparent morphologic changes accompanied by inflammatory cell infiltration and severe mucus hyper secretion.

Munc18b Expression Changes in Obese Asthma

We tested Munc18b expression in lung tissue. Compared with normal control, Munc18b level significantly upregulated in obese group and asthma group ($p < 0.05$, Figure

Table I. The changes of airway resistance (mean ± standard deviation).

Group	n	Airway resistance ($cmH_2O/mL/s$)
Normal control	10	0.296 ± 0.068
Obese model	10	$0.333 \pm 0.031^{**\#\#}$
Asthmatic model	10	$0.344 \pm 0.124^{**\#\#}$
Obese asthmatic model	10	$0.691 \pm 0.213^{**}$

$^{**}p < 0.05$, compared with normal control. $^{\#\#}p < 0.05$, compared with obese asthmatic group.

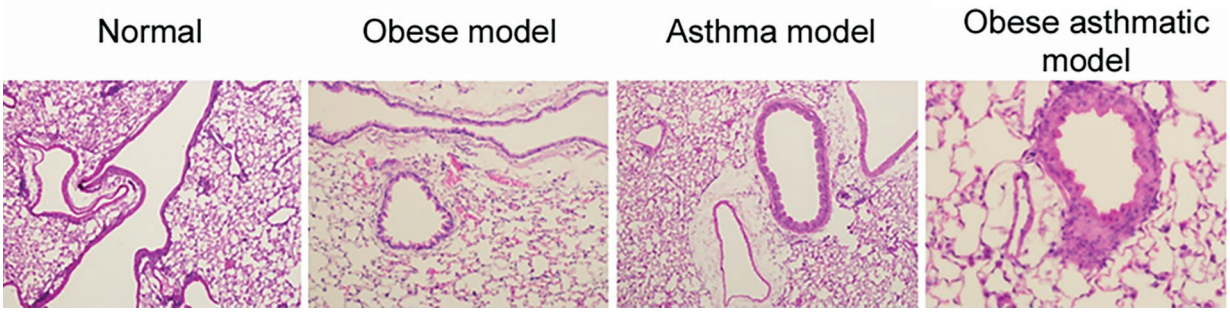

Figure 2. The pathology changes of airway detected by HE staining (×10).

3). Munc18b expression enhanced in obese asthmatic model group compared with simple obese or asthma group ($p < 0.05$).

Muc5ac Expression Changes in Obese Asthma

We also detected Muc5ac expression in lung tissue to investigate the relationship between Munc18b and Muc5ac. Compared with normal control, Muc5ac level significantly upregulated in obese group and asthma group ($p < 0.05$, Figure 4). Muc5ac expression enhanced in obese asthmatic model group compared with simple obese or asthma group ($p < 0.05$).

Muc5ac Expression in the Airway of Munc18b Conditioned Knockout Mouse

Since Muc5ac secretion increased accompanied with Munc18b in obese asthmatic mouse model, we proposed that Muc5ac elevation may be induced by Munc18b. Thus, we used Munc18b conditioned knockout mouse to establish obese asthmatic model. As shown in Figure 5, Muc5ac expression failed to show elevation in Munc18b conditioned knockout mice, suggesting that Muc5ac hypersecretion needed Munc18b participation.

Discussion

In recent years, the number of obese people increased year by year. Obesity and overweight can cause a series of chronic diseases, such as hypertension, diabetes, and respiratory sleep syndrome[10]. However, obese patients often exhibit poor response to the treatment of conventional asthma drugs, leading to the disease difficult to control. With the increasing incidence of obese asthma, elucidating the pathogenesis of obese asthma and establishing effective treatment according to the mechanism become an urgent requirement for asthma research. Mucus production and hypersecretion is an important pathophysiological characteristic of asthma. Numerous mucins enter the airway in a short time, which may cause severe airway obstruction, airway resistance increase, and even death as it exceeds the scavenging activity[11,12]. Mucus hypersecretion is more serious obese asthmatic airway, while mucus plug formation is the main cause of death in asthmatic mice. It is of great significance to improve the prognosis of obese asthmatic patients by elucidating the mechanism of mucus hypersecretion.

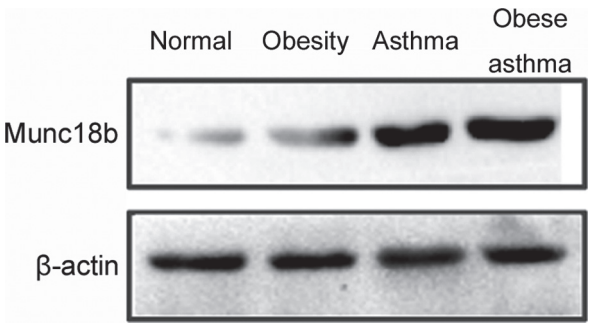

Figure 3. Munc18b expression changes in obese asthma.

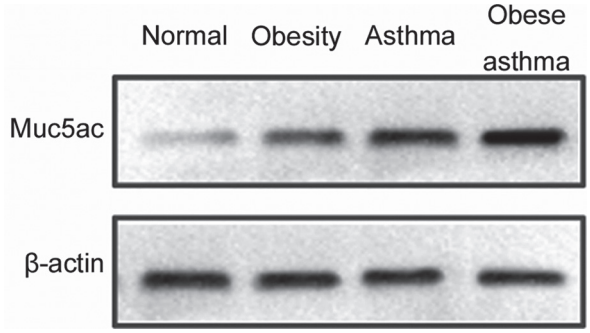

Figure 4. Muc5ac expression changes in obese asthma.

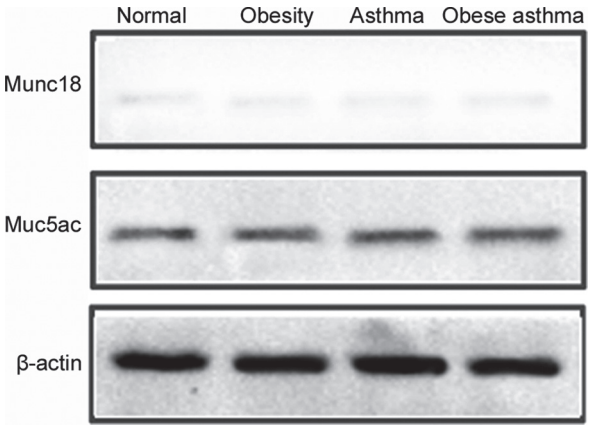

Figure 5. Muc5ac expression in the airway of Munc18b conditioned knockout mouse.

Airway mucus consists of 1% mucin and 99% water molecules[13]. Mucin is a highly glycosylated modified high molecular weight protein family produced by the epithelial tissue of multicellular animal. Respiratory secretory mucin mainly contains Muc5ac and Muc5b. Muc5b is the major mucin type that is secreted by the respiratory tract under the physiological condition, which mainly plays a protective role[14]. On the contrary, Muc5ac is a type of mucin that is significantly increased under exogenous stimulation and often involved in pathophysiological processes, such as cancer, asthma, and bronchitis, etc.[15].

The production and secretion of mucin in the airway secretory cells are in coordination with each other, thus cannot be found expression in the cells by immunochemistry[16]. Due to airway cell remodeling and mucus secretory cell hypertrophy in asthma patients, a large number of mucin is produced while the secretion does not increase, resulting in intracellular mucin accumulation. Once the allergen stimulates the initiation of the high secretory mechanism of mucin, the accumulated mucin is immediately secreted into the airway and causes spontaneous obstruction[9]. As we observed in the results, the obese asthmatic model mice exhibited mucus secretory cell hypertrophy and mucin synthesis. Vesicular transporters play an important role in cell vesicle transport, not only to help open Syntaxin, but also to provide space and support for the interaction between SNARE proteins. Therefore, it is an important regulatory protein for airway mucin secretion[17]]. Only the Sec1 protein family (Munc18a, Munc18b, and Munc18c) located in the cytoplasm and the Vps33 protein family located in vacuolar membranes, are theoretically functional in mucin secretion. However, previous studies have found that Vps33 is responsible for the transmission between endosomes and lysosomes. Munc18a is rarely expressed in the airway. Thus, only Munc18b and Munc18c may be involved in mucin secretion[18,19]. In 2012, it was demonstrated that airway mucin secretion in Munc18b mutant heterozygous mice was reduced by 50% compared with wild-type mice after ATP stimulation, whereas it was similar in Munc18b mutant heterozygote mice compared with wild-type mice without ATP stimulation, revealing that Munc18b may be involved in stimulating states and mucin secretion[20,21]. In this study, it was also observed that increased secretion of Muc5ac was associated with high expression of Munc18b. To demonstrate that Munc18b induced Muc5ac expression, we applied Munc18b conditioned knockout mice and confirmed that Munc18b conditioned knockout can block the overexpression of Muc5ac.

So far, the mechanism of mucin secretion in regulating SM protein type has not yet been elucidated. In recent years, the study of obese asthma mechanism focused on inflammation and susceptibility genes[22,23]. There is lack of report about the effects and mechanisms of airway mucus hypersecretion in obese asthma. In this study, Munc18b conditioned knockout mice were used to elucidate the mechanism of increased expression of Muc5ac. Cell experiment may elaborate the specific mechanism in the future. It would be more meaningful to fully explain the mechanism of increased mucin secretion if combined with cell experiments.

Conclusions

Muc5ac high secretion was positively correlated with Munc18b upregulation in obese asthma. Munc18b participated in inducing Muc5ac high expression.

Acknowledgements

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Conflict of Interest

The Authors declare that they have no conflict of interests.

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