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Expression Levels of Predominant Adipokines and Activations of STAT3, STAT6 in an Experimental Mice Model of Obese Asthma

Lei Chong[1], Liu Liu[2], Lili Zhu[3], Haiyan Li[3], Youyou Shao[3], Hailin Zhang[3], and Gang Yu[3]

[1] Institute of Pediatrics, National Key Clinical Specialty of Pediatric Respiratory Medicine, The Second Affiliated Hospital of Wenzhou Medical University, Wenzhou, Zhejiang, China
[2] Department of Pediatrics, The Fifth Affiliated Hospital of Zhengzhou University, Zhengzhou, Henan, China
[3] Discipline of Pediatric Respiratory Medicine, The Second Affiliated Hospital of Wenzhou Medical University, Wenzhou, Zhejiang, China

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ABSTRACT

Obese asthma is a new asthma phenotype. The underlying mechanisms are not clearly understood. Leptin and adiponectin are two predominant adipokines produced by adipose tissue. Studies have demonstrated a role of leptin on regulating the Janus kinase/signal transducer and ativator of transcription protein (JAK/STAT) signaling pathway and STAT3, STAT6 were known to have essential role on inflammatory cytokines production. However, whether STAT3 and STAT6 are activated and related to leptin merit further investigation. The aim of this study was to investigate the expression levels of leptin/adiponectin ratio and the activations of STAT3 and STAT6 in the lungs of obese asthma mice.

Experiments were carried out on male C57/B6J mice. The proteins in bronchoalveolar lavage fluid (BALF) were measured using ELISA. The expression levels of the transcriptional and translational factors in the lungs were examined using Quantitative Reverse Transcriptase Polymerase Chain reaction (qRT-PCR) and western blot.

The expression levels of leptin in the BALF of normal weight group, asthma group, obese group and obese asthma group were 2.032±0.133, 5.375±0.123, 5.418±0.165 and 7.486±0.168, respectively. The expression of leptin in obese asthma group was the highest (p<0.05)， while the expression of adiponectin the lowest (p<0.05). The expression level of P-STAT3 in the obese asthma group was 0.9244±0.014, and was significantly higher than three other groups (p<0.05). The expressions of P-STAT6 in three other groups were all significantly higher than normal weight group (p<0.05).

Our data suggest that the function of leptin on the pulmonary inflammation of obese asthma may be partly through activating the STAT3 signaling pathway.

Keywords: Adiponectin; Asthma; Leptin; Obesity; Signal transducer and activator of transcription protein

Corresponding Author: Gang Yu, MD;
Discipline of Pediatric Respiratory Medicine, The Second Affiliated Hospital of Wenzhou Medical University. No. 109, Wenzhou, 325027, Zhejiang, China. Tel: (+0577) 8800 2030, E-mail:ygangyu@sina.com,
* Lei Chong and Liu Liu devote equally to this article.

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INTRODUCTION

Asthma is a heterogenous disease. The pathogeneses and symptoms of asthma are affected by multiple factors. In the human studies it has become apparent that most people with asthma are overweight or obese[1]. Clinical reports have also pointed out that obesity caused increased prevalence of asthma and worsen the clinical outcomes.[2,3] However, obese asthma as a new asthma phenotype being proposed recently,[4, 5] the mechanisms are not clearly understood.

Obesity is a chronic low-grade systemic inflammation, characterized by the presence of circulating leukocytes, production of abnormal cytokines/chemokines and activation of inflammatory signaling pathways.[6-8] Obesity may also have an effect on lung inflammation. Therefore, it is important to understand the differences of airway inflammation between obese and lean subjects.

Adipokines, including leptin, adiponectin, resistin and adipsin, produced by adipose tissue exert their biological functions in an autocrine, paracrine and systemic manner.[9] Leptin affects food intake regulation and eventually glucose metabolism, lipometabolism, endocrine and immune functions, adipose tissue metabolism and energy expenditure. Most of the obese patients have elevated plasma levels of leptin.[10] On the contrary, adiponecin is decreased in obesity and inversely correlated with insulin resistance, glucose intolerance, dyslipidemia, and atherosclerosis[11]. Recently, Newson and colleagues[12] found that leptin and leptin/adiponectin ratio were positively associated with the severity of asthma according to a clinical follow-up survey. Besides, through evaluating the levels of leptin in exhaled breath condensate (EBC) from asthmatics, normal and overweight children, Bodini et al[13] found that EBC-leptin levels are significantly higher in the obese subjects and in asthmatic ones compared with healthy subjects. Therefore, leptin might translocate to the airway and play a role on the airway inflammation. However, the regulation of leptin on the airway inflammation is unknown.

Several studies found that signal transducer and activator of transcription 3, 6 (STAT3, 6) is essential for inflammatory cytokines production, and their activations are linked to the development of airway inflammation[14-16]. Furthermore, a few researches have demonstrated that leptin positively increased the STAT3 activation in inflammatory diseases including chronic inflammatory lung diseases.[17,18] Therefore, this study was designed to detect the expression levels of leptin, adiponectin, STAT3 and STAT6 mRNA and proteins in lungs and bronchoalveolar lavage fluid (BALF) as well as the activations of STAT3 and STAT6 protein in the lung tissues and evaluate the differences between the mRNA and protein expressions in obese and lean mice in experimental asthmatic model.

MATERIALS AND METHODS

Mice

40 3-4 weeks old, specific pathogen-free (SPF) C57/B6J male mice (Shanghai Slac Laboratory Animal Center, Shanghai, China) were used in the study. The mice were randomly divided into four groups (normal weight group, asthma group, obesity group and obese asthma group). All the animal procedures were approved by the Institutional Animal Care and Ethic Committee (N.: wydw2016-0159) and were consistent with the standards established by the Guide for the Care and Use of Laboratory Animals. Mice were maintained on a 12:12-h light cycle within individual static isolation cages that were autoclaved with bedding prior to use. Mice received autoclaved water from autoclaved water bottles.

Animal Model Establishment

Obesity group and obese asthma group were f ed with 45% high fat food (Medicience Ltd, Yangzhou, China), while normal weight group and asthma group were fed with ordinary mice food. After 12 weeks feeding, on days 1 and 13 from the 13th week, asthma group and obese asthma were given 0.1 mL 0.01% ovalbumin(OVA)/$Al(OH)_3$ suspension through i.p. injection for sensitization. Then they were challenged in an airtight box with a channel connected with nebulizer, which atomizing with 1% OVA aerosol for 30 min per day from days 25 to 32. Normal weight group and obesity group were sensitized and challenged by normal saline on the same schedule.[19]

BALF Collection and Detection

All mice were narcotized by 10% chloral hydrate and sacrificed within 24 h after the last challenge. After sacrifice, one side of the bronchus were ligated and

BALF was collected by flushing one side of the lungs with three separate normal saline through the trachea. The recycling rate was more than 80%. Total cell counts were determined for each BALF sample using a cytometer. The expressions of leptin and adiponectin were detected by ELISA.

Lung Tissues Isolation and Staining

Part of the non-lavage side of lungs was stored in the Ultra-low temperature freezer for mRNA and protein detection. And other part of non-lavage lung tissues was fixed in 4% formalin for less than a week and made into 4-μm-thick paraffin-embedded tissue sections, which then stained with hematoxylin and eosin (HE). The pathological and inflammatory changes in the lungs were observed under a light microscope (Olympus, Japan). The degrees of airway inflammation were scored by another person who was blind to the treatment of mice and were assessed according to the following histologic grading system (scored 0-4): absence of peribronchial inflammatory cells (0); a few scattered peribronchial inflammatory cells involving less than 25% of the circumference of the bronchus (1); focal peribronchial inflammatory cell infiltrate not completely surrounding a bronchus (2) (i.e., involving approximately 25–75% of the circumference of the bronchus); one definite layer of peribronchial inflammatory cells completely surrounding a bronchus (3); two or more layers of peribronchial inflammatory cells completely surrounding a bronchus (4).

Quantitative Reverse Transcriptase Polymerase Chain reaction (qRT-RCR)

The total RNA was extracted using a Trizol reagent (Thermo Fisher Scientific, USA) and reversely transcribed into cDNA using the RevertAid First Strand cDNA synthesis kit (Thermo Fisher Scientific, USA), according to the manufacturer's instructions. The mRNA expression levels were detected using a LightCycler480 SYBR Green I Master (Roche, Mannheim, Germany) in a 10 μL reaction volume containing 1 μL of cDNA and 0.5 μL primers. PCR was initiated with a 5min denaturation at 95°C, followed by 45 cycles of 95°C for 10s, 60°C for 10s and 72°C for 10s. Each reaction was run in triplicate. The relative quantity of mRNA was obtained using the $2^{-\Delta\Delta Ct}$ method. The primers were delineated in Table1.

Western Blot

Lung tissues were homogenized in RIPA lysis containing protease inhibitor. Aliquots of 70 μg of protein were separated using sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes, which were then blocked in 5% dry skim milk for 1 h. The primary antibodies were incubated overnight at 4°C with 1:1500 dilutions for STAT3 (Abcam, USA), 1:1000 for P-STAT3 (Abcam, USA), 1:1000 for STAT6 (Abcam, USA), 1:1000 for P-STAT6 (Abcam, USA) and 1:5000 for tubulin (Bioworld Technology, Shanghai, China). The secondary antibodies were goat anti-rabbit (Earthox Company, USA) used at 1:5000 dilution for 1 h at room temperature.

Protein concentrations were measured using the ChemiDoc XRS gel imaging system (Bio-Rad, USA). The target protein/ tubulin ratio was calculated for the comparison of each group.

Statistical Analysis

All results were presented as mean±SEM and analyzed by one-way ANOVA, with SPSS 17.0. For samples with unequal variances, Dunnett's T3 test was used instead. Statistical significance was determined as $p<0.05$.

RESULTS

The Body Weight of Mice

The high fat diet significantly increased the body weight of mice. After 16 weeks feeding, the mean body weights of normal weight group, asthma group, obesity group and obese asthma group were 30.92 g, 30.66 g, 39.70 g and 41.40 g, respectively. And the mean body weights of obesity group and obese asthma group were 20% higher than normal weight group and asthma group ($p<0.01$) (Figure 1).

Observation of Histopathological Changes and Inflammatory Cells Infiltration in Lungs

The normal weight group presented completed lung structures, regular bronchial lumen and intact mucous epithelium, with no or little inflammatory cells infiltration. The inflammatory cells infiltration of obesity group was significantly higher than normal weight group ($p<0.01$), but still lower than asthma group and obese asthma group ($p<0.01$) (Figure 2a).

Table 1. The design of primers for detecting mRNA expressions of predominant adipokines and signal transducer and activator of transcription 3, 6 (STAT3, 6) in the lungs of obese asthma mice

Gene	Primer
Beta-actin	F: 5'-GAGAGGGAAATCGTGCGTGACA-3' R: 5'-ACCCAAGAAGGAAGGCTGGAAA-3'
Leptin	F: 5'-CTTCACCCCATTCTGAGTTTGT-3' R: 5'-ATTCTCCAGGTCATTGGCTATC-3'
Adiponectin	F: 5'-GGGAACTTGTGCAGGTTGGATG-3' R: 5'-CTTCACCCCATTCTGAGTTTGT-3'
STAT3	F: 5'-GCAGCCAGCAAAGAGTCAC-3' R: 5'-GGTTCTCCACCACCTTCATT-3'
STAT6	F: 5'-GCAAGGGGCTAAGATGGACAA-3' R: 5'-CAAGGGTTCGCAGGACTTCATC-3'

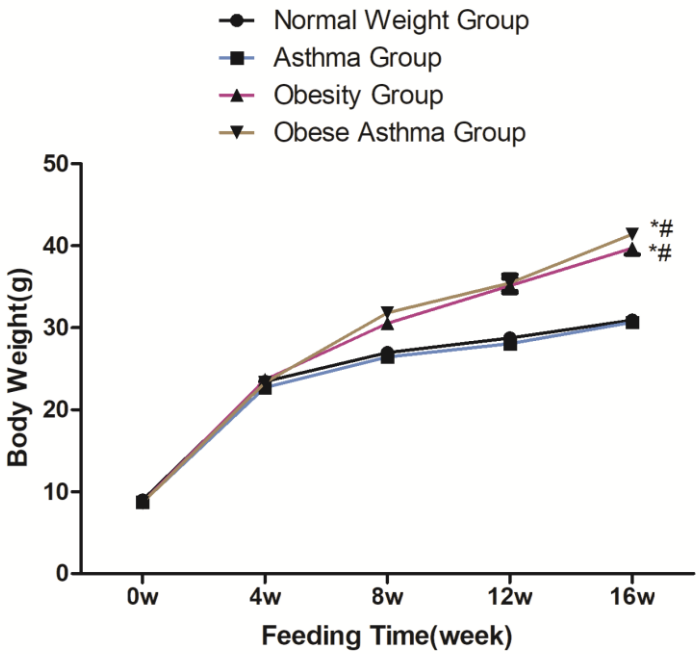

Figure 1. The body weight of mice in each group following the feeding week. The mean body weights of obesity group and obese asthma group were significantly increased from 8 weeks feeding, and were 20% higher than normal weight group and asthma group after 12 weeks feeding. Values are means±SEM; *n*=10 mice/group $^{*}p<0.01$ versus normal weight group, $^{\#}p<0.01$ versus asthma group.

The median inflammatory score in obesity group was 2. The asthma group and obese asthma group showed significantly higher inflammatory cells infiltration compared to normal weight group and obesity group ($p<0.01$) (Figure 2b).

The changes of the total cell counts in BALF between different groups were in consistent with the changes of airway inflammation. As shown in Figure 3, the total cell numbers in BALF of obesity group were significantly higher compared with normal weight group ($p<0.01$), but still lower than asthma group and obese asthma group ($p<0.01$).

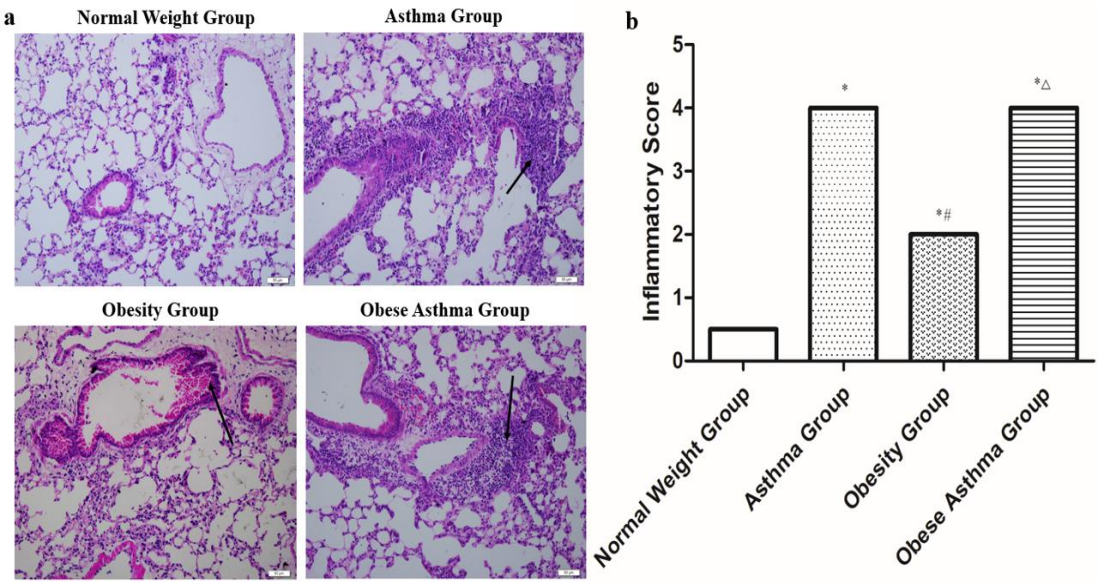

Figure 2. The histopathological changes in the lung tissues of mice from each group. (a) Representative photomicrographs of hematoxylin and eosin (HE) stains in sections of lungs from mice of the four groups (×200). There are a lot of inflammatory cells infiltration around bronchus and blood vessels in obese asthma group. (b) The inflammatory scores of airways from mice of the four groups. The asthma group and obese asthma group showed the highest inflammatory scores of airways. The airway inflammatory score of obesity group was significantly increased compared with normal weight group, but still lower than asthma group and obese asthma group. Calibration bars=50μm. Values are means±SEM; *n*=10 mice/group. $^{*}p$<0.01 versus normal weight group, $^{\#}p$<0.01 versus Asthma Group, $^{\triangle}p$<0.01 versus obesity group.

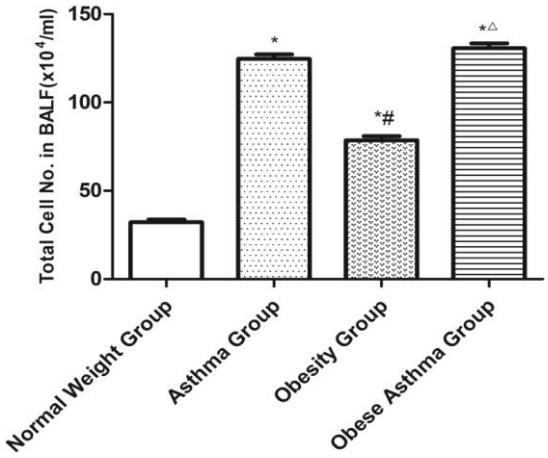

Figure 3. The total cell numbers in bronchoalveolar lavage fluid (BALF) of each group. The obese asthma group showed the highest total cell counts in BALF. No significant differences were found between asthma group and obese asthma group. The total cell numbers in BALF of obesity group were significantly higher compared with normal weight group, but still lower than asthma group and obese asthma group. Values are means±SEM; *n*=10 mice/group. $^{*}p$<0.01 versusnormal weight group, $^{\#}p$<0.01 versus asthma group, $^{\triangle}p$<0.01 versus obesity group.

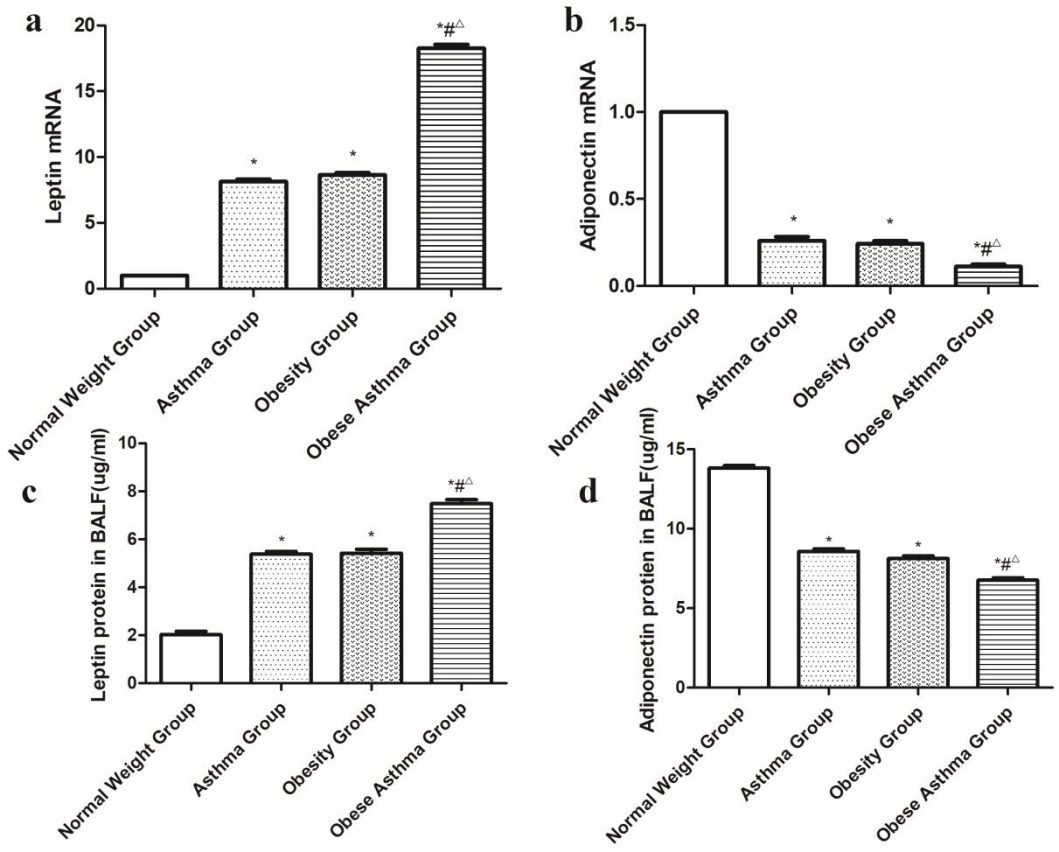

Figure 4. Expression levels of leptin mRNA (a) and adiponectin mRNA (b) in the lung tissues and leptin protein (c) and adiponectin protein (d) in BALF. Obese asthma group showed increased leptin mRNA and protein expressions and decreased adiponectin mRNA and protein expressions. The expressions of leptin mRNA and protein in asthma group and obesity group were significantly higher than normal weight group, but still lower than obese asthma group. The expressions of adiponectin mRNA and protein in asthma group and obesity group were significantly lower than normal weight group, but still higher than obese asthma group. Values are means±SEM; n=10 mice/group. *p<0.01 versus normal weight group, #p<0.01 versus asthma group, $^{\triangle}p$<0.01 versus obesity group.

Expression Levels of Leptin and Adiponectin mRNA and Protein in the Airways of Mice

Firstly, we detected the mRNA and protein expressions of adipokines, leptin and adiponectin in lung tissues and BALF. As shown in Figure 4, the obese asthma group exhibited the highest expression levels of leptin mRNA and protein (p<0.01), whereas the lowest expression levels of adipnectin mRNA and protein (p<0.01). The expressions of leptin mRNA and protein in asthma group and obesity group were significantly higher than normal weight group (p<0.01), but still lower than obese asthma group (p<0.01). While the expressions of adiponectin mRNA and protein in asthma group and obesity group were significantly lower than normal weight group (p<0.01), but still higher than obese asthma group (p<0.01).

Expression Levels of STAT3 and STAT6 mRNA in the Lungs of Mice

Next, the expressions of STAT3 and STAT6 mRNA in the lungs were examined. Similar with leptin expressions, both the STAT3 and STAT6 mRNA expressions in asthma group and obesity group were significantly higher compared with normal weight group (p<0.01) but still lower than obese asthma group (p<0.01) (Figure 5).

The Activation Status of STAT3 and STAT6 in the Lungs of Mice

Finally, to examine the effect of STAT3 and STAT6 in the airway inflammation, the expressions of STAT3, P-STAT3 and STAT6, P-STAT6 were detected. As shown in Figure 6, the STAT3, P-STAT3 protein expressions in all the three groups, asthma group, obesity group and obese asthma group, were significantly increased (p<0.01). Moreover, the expression levels of P-STAT3 in obese asthma group were even higher than asthma group and obesity group (p<0.01). However, although the STAT6, P-STAT6 protein expressions were also increased in asthma group, obesity group and obese asthma group, there were no significant differences of P-STAT6 among these three groups (p>0.05).

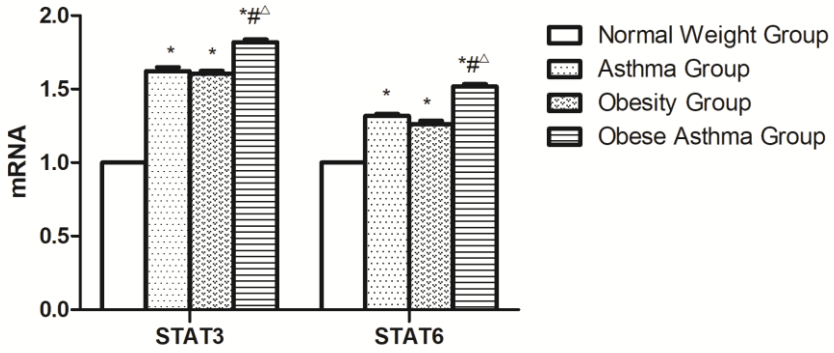

Figure 5. Expression levels of STAT3 mRNA and STAT6 mRNA in the lung tissues of mice from each group The expressions of STAT3 mRNA and STAT6 mRNA were both the highest in obese asthma group. Both the STAT3 and STAT6 mRNA expressions in asthma group and obesity group were significantly higher compared with normal weight group but still lower than obese asthma group. Values are means±SEM; n=10 mice/group. $^{*}p$<0.01 versus normal weight group, $^{\#}p$<0.01 versus asthma group, $^{\triangle}p$<0.01 versus obesity group.

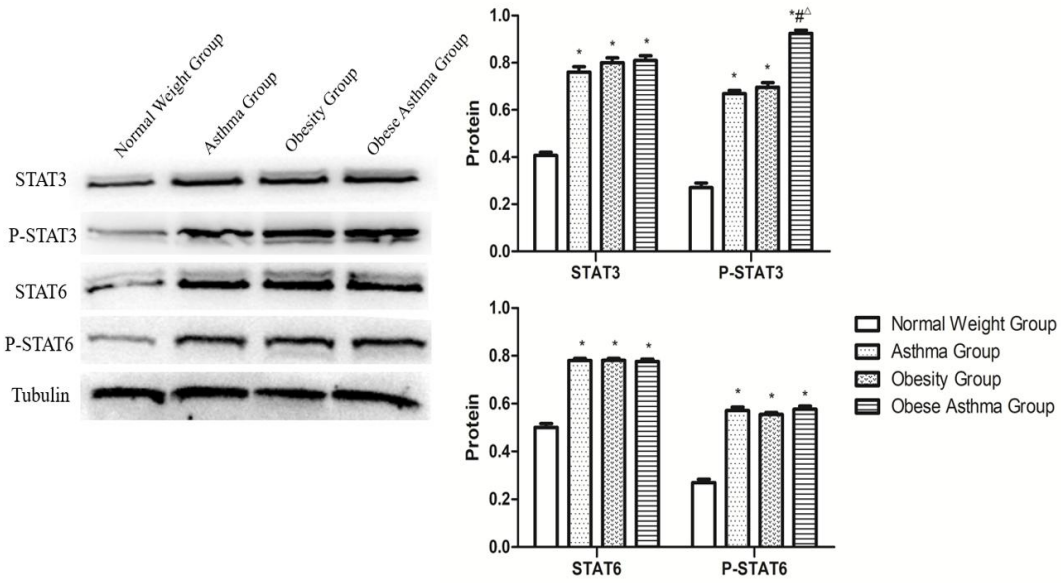

Figure 6. The activation status of STAT3 and STAT6 in the lungs of mice from each group. The activity of STAT3 was strongly activated in obese asthma group compared with three other groups. Although the STAT6, P-STAT6 protein expressions were also increased in asthma group, obesity group and obese asthma group, there were no significant differences of P-STAT6 among these three groups. Values are means±SEM; n=7 mice/group. $^{*}p$<0.01 versus normal weight group, $^{\#}p$<0.01 versus asthma group, $^{\triangle}p$<0.01 versus obesity group.

DISCUSSION

As the obesity epidemic has worsened, its impact on lung health and disease has become progressively evident. The interactions between obesity and the accompanying metabolic syndrome and diseases such as asthma have proven complex and often counterintuitive in human studies.[20,21] Hence, there is a growing need for relevant experimental approaches to understand the interactions between obesity and the lung. It is well known that obesity is a low-grade chronic pro-inflammatory condition and obesity-mediated changes in plasma adipokines have been associated with increased systemic and airway inflammation.[22,23] However, the underlying mechanisms were remains unclear.

In this study mice were fed with high fat diet or ordinary diet firstly, and then from the 13th week, ovalbumin (OVA) was used to sensitize and challenge mice. After the last challenge, mice were sacrificed with 10% chloral hydrate. Body weight were measured and then BALF and lung tissues were collected. With respect to the obese animal model, there was no unified standard.[24,25] We found that the mean body weight of the mice fed with high fat diet were 20% higher than the mice fed with ordinary mice diet, which was significantly different.

Airway and BALF inflammatory cells infiltration, airway hyperresponsiveness (AHR) and airway remodeling are the specific characteristics of asthma. In the present study, high fat diet induced obese mice showed inflammatory cells infiltration around pulmonary blood vessels and bronchus. OVA sensitization and challenge increased the degree of airway inflammation and total cells infiltration in BALF and induced epithelium rupture, airways smooth muscle hyperplasia and edema in both lean and obese groups, which was consistent with our previous study.[26]

Recently, multiple studies have demonstrated that obesity is a risk factor for asthma[27]. If obesity is the cause of asthma, it's reasonable to postulate that obesity related factors might have some role on the pathogenesis of asthma. Studies have found that, leptin, an important adipokine, not only played a central role in energy balance control but also has another important function: up-regulation of inflammatory responses.[28] Sood et al [29] verified that leptin concentration was negatively correlated with lung function in adults. Clinical researches also showed that the levels of leptin in EBC from asthmatic children were significantly higher than normal children.[30] On the other hand, adiponectin, another important adipokine produced by adipocytes, is significantly decreased in obese subjects. Conversely, weight loss promotes an increase on adiponectin levels.[31,32] Our data also showed that high fat diet induced obesity caused to the upregulation of leptin mRNA and the downregulation of adiponectin mRNA. OVA sensitization and challenge made the leptin/adiponectin ratio even higher. Additionally, the ratio of leptin/adiponectin proteins in BALF presented the same polarity, which confirmed the opposite role of leptin and adiponectin on the airway inflammation. However, no significant differences of leptin and adiponectin mRNA/protein have been found between asthma and obesity conditions, possibly due to the low concentrations in the lung tissues and BALF compared with blood.

The JAK-STAT pathway mediates important responses in immune cells. Activation of any of the four JAK family members leads to phosphorylation of one or more of seven STAT family members to exert their functional effect. Phosphorylation of STAT family members leads to their dimerization and translocation into the nucleus, in which they bind specific DNA sequences to activate functional gene transcription.[16,33,34] Regulation of JAKs and STATs therefore has a significant effect on signal transduction and subsequent cellular responses. Mathur and colleges[35] found that STAT3 is required for the expression of retinoic-acid-receptor-related orphan nuclear receptor gamma (ROR γ t) in T helper 17 cells (Th17) culture conditions and subsequently the development of Th17 cells, a factor recently shown to be critical for the development of asthma. Mathew et al[16] used a murine model of asthma in which in vitro–differentiated STAT6$^{-/-}$ antigen-specific Th2 cells were adoptively transferred into naive STAT6$^{-/-}$ and STAT6$^{+/+}$ mice followed by OVA challenge. They found that all of the features of asthma, including Th2 cell accumulation, Th2 and eosinophil-active chemokine production, and airway eosinophilia, mucus production, and hyperresponsiveness, seen in STAT6$^{+/+}$ mice, were dramatically absent in STAT6$^{-/-}$ mice that received STAT6$^{+/+}$ antigen-specific Th2 cells. All the above results suggested an important role of STAT3 and STAT6 on the airway inflammation of asthma. However, to our knowledge there is no study

investigating the role of STAT3 and STAT6 in the relationship between obesity and lung inflammation. The present study was therefore conducted to evaluate the expression levels and activations of STAT3 and STAT6 in the lungs of obese asthma mice.

Firstly, the transcriptional levels of STAT3 and STAT6 were detected, and as we expected, the expressions of STAT3 and STAT6 mRNA in the lungs of mice fed with high fat diet were significantly increased, which were comparable to the asthma group. Besides, obese asthma group expressed even higher STAT3 and STAT6 mRNA levels. Next, the translational levels of STAT3 and STAT6 were examined. Though no significant differences were found among the three groups, the expression levels of STAT3 and STAT6 proteins were all significantly upregulated in the lungs of mice challenged with OVA or fed with high fat diet or both. Finally, to evaluate the functional effects of STAT3 and STAT6, their phosphorylation forms were also detected. Consistent with its mRNA expression, P-STAT3 expression was the highest in the lungs of obese asthma mice. However, though the expressions of P-STAT6 in the lungs of asthma group, obesity group and obese asthma group were upregulated, no significant differences were found among the three groups. Therefore, these results indicated that the activation of STAT3 was increased and was associated with the airway inflammation of obese asthma. However, the mechanism underlying the increase of STAT3 activation in obese asthma warrants further investigation.

Furthermore, previous studies have demonstrated a role of leptin on regulating the JAK/STAT signaling pathway.[17,18] Therefore, together with our data, the function of leptin on the pulmonary inflammation may be partly through activating the STAT3 signaling pathway.

However, animal experiments have limitations. In this study we only observed the changes in the levels of tissues. Inhibition experiments are needed to evaluate the therapeutic effect targeting the leptin-STAT3 signaling pathway by using gene transfection model.

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2. This study has been approved by the Institutional Animal Care and Ethics Committee (Acceptance letter number: wydw2016-0159).

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