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Neutrophil elastase inhibitor suppresses oxidative stress in obese asthmatic rats by activating Keap1/Nrf2 signaling pathway

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Abstract. – OBJECTIVE: The aim of this study was to detect the oxidative stress response in the rat model of obesity, asthma and obese asthma. Meanwhile, we aimed to investigate the inhibitory effect of neutrophil elastase inhibitor (NEI) on cellular oxidative stress in the body and whether it exerted an effect on the oxidative stress response in obese asthma through the Kelch-like ECH-associated protein 1/nuclear factor E2-related factor 2 (Keap1/Nrf2) pathway.

MATERIALS AND METHODS: The obesity and asthma models were established using a total of 70 Sprague-Dawley (SD) rats. All rats were randomly divided into 7 groups. The rats with normal weight were divided into the control (CTR) group (n=10), asthma (ATM) group (n=10) and ATM+NEI group (n=10). Meanwhile, the obese rats were divided into the obesity (OBS) group (n=10), the OBS+NEI group (n=10), the OBS+ATM group (n=10) and the OBS+ATM+NEI group (n=10). After modeling, rats in NEI intervention groups were injected with Sivelestat (5 mg/kg) *via* the caudal vein twice a day for 1 week. The tests of cough sensitivity to capsaicin and bronchial responsiveness were performed 24 h after the last administration. Lung tissues of rats were collected for hematoxylin-eosin (HE) staining. Meanwhile, the levels of reactive oxygen species (ROS) in heart, lung and kidney tissues were detected *via* 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA). The activities of reduced glutathione (GSH), glutathione peroxidase (GSH-Px), H_2O_2 and total superoxide dismutase (T-SOD) in the heart, lung and kidney tissues were detected using the colorimetric method. The mRNA and protein expressions of Keap1 and Nrf2 messenger ribonucleic acid expressions in the heart, lung and kidney tissues were measured *via* Reverse Transcription-Polymerase Chain Reaction (RT-PCR) and Western blotting, respectively.

RESULTS: NEI significantly improved the symptoms and lung pathology in rats with asthma. The level of ROS in the heart, lung and kidney tissues of the OBS group, ATM group and OBS+ATM group was significantly increased. However, NEI markedly inhibited the level of ROS in rats with asthma. The activities of antioxidant stress-related enzymes (reduced GSH, GSH-Px, H_2O_2 and SOD) in the heart, lung and kidney tissues of the OBS group, ATM group and OBS+ATM group were significantly decreased. However, NEI markedly promoted the activities of the related antioxidant enzymes in oxidative stress response in asthma rats. Besides, the Keap1/Nrf2 signaling pathway in the heart, lung and kidney tissues of the OBS group, ATM group and OBS+ATM group was significantly inhibited, while NEI activated the Keap1/Nrf2 signaling pathway in rats with asthma.

CONCLUSIONS: NEI promotes the release of a variety of antioxidant factors, enhances the activity of antioxidant enzymes and improves the symptoms of rats with obese asthma. The possible underlying mechanism may be the activation of the Keap1/Nrf2 signaling pathway.

Key Words:
Neutrophil elastase inhibitor, Obese asthma, Signaling pathway, Oxidative stress.

Introduction

In recent years, the incidence rates of bronchial asthma and obesity in children have increased rapidly around the world[1-3]. Bronchial asthma is a kind of airway allergic inflammation. The acute asthmatic attack seriously affects the life quality of patients, and even leads to death in severe cases[4]. According to epidemiological studies[5,6], obesity is correlated with the incidence rate and severity of asthma. Studies[7,8] have found that the proportion of asthma in obese children is significantly higher than that of normal children.

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However, its underlying mechanism has not been clarified yet. Obese asthmatic patients usually respond poorly to conventional drugs for asthma, such as glucocorticoids and cholinergic drugs. Therefore, it is not easy to control the symptoms[9,10].

Oxidative stress refers to the excessive production of highly active molecules in the body, such as reactive nitrogen species (RNS) and reactive oxygen species (ROS). Due to harmful stimuli and the oxidative degree beyond scavenging ability, this may eventually lead to tissue damage. Active molecules produced by oxidative stress cause damage to the physiological functions of cellular proteins and nucleic acids through different pathways. It is the pathophysiological basis for the occurrence of a variety of diseases. In recent years, some studies have confirmed that there is an increase in the oxidative stress level of obese people[11,12]. At the same time, studies have indicated that the level of ROS in asthma patients is significantly higher than that of normal people[13-15]. Therefore, oxidative stress plays an important role in both obesity and asthma.

Kelch-like ECH-associated protein 1/nuclear factor E2-related factor 2 (Keap1/Nrf2) signaling pathway exerts an important place in the mechanism of anti-oxidative stress in the body. It is also an important member of the cellular defense system[16,17]. This pathway can be activated in various respiratory diseases, including asthma. Under non-stress conditions, Nrf2 interacts with its negative regulator Keap1 to form the Nrf2/Keap1 complex. Thus, Nrf2 is generalized and degraded *via* degradation of the Nrf2/Keap1 complex. Under stress conditions, the P-Nrf2/Keap1 complex transfers to the nucleus and binds to antioxidant response element (ARE), thereby activating a variety of antioxidant genes. In addition, the Keap1/Nrf2 pathway plays an important role in biological processes *in vivo*, such as anti-inflammation and anti-oxidative stress[18,19].

In the past, neutrophil elastase inhibitor (NEI) was often applied as adjuvant therapy for acute lung injury and systemic inflammatory response[20,21]. Animal experiments have confirmed that NEI possesses the effects of anti-inflammatory, anti-oxidative and anti-apoptotic[20,22]. In this work, NEI was used to intervene in experimental animals. Meanwhile, the inhibitory effect of NEI on oxidative stress in rats with high-fat-diet-induced obese asthma was determined. Moreover, whether NEI inhibited oxidative stress through the Keap1/Nrf2 pathway was further explored. Our study might provide experimental references for the pathogenesis of obese asthma and drug therapy for obese asthma.

Materials and Methods

Experimental Animals and Models

A total of 70 male Sprague-Dawley (SD) rats in clean grade weighing 120-130 g were provided by the Shandong University Animal Center. This study was approved by the Animal Ethics Committee of Jinan Maternity and Child Care Hospital Animal Center. Establishment of the obesity animal model: after feeding adaptively for 1 week, the rats were fed with high-fat diet for 6 weeks. The weight of the rat was measured once a week. The rats whose body weight was 20% more than that of those fed with normal diet were taken as experimental rats with obesity. Establishment of the asthma animal model: after feeding for 7 weeks, the rats were intraperitoneally injected with OVA suspension (1 mg OVA and 20 μg aluminum hydroxide dissolved in 0.5 mL 0.9% normal saline) (0.5 mL/rat), which was injected once again at 8 d. 10 mL OVA solution (10 g/L) was used for stimulation *via* atomization since the 15th day for 2 weeks (3 times a week, 30 min per time).

Experimental Grouping and Treatment

The rats were randomly divided into 7 groups. Rats with normal weight were divided into the control (CTR) group (n=10), asthma (ATM) group (n=10) and ATM+NEI group (n=10). Meanwhile, obese rats were divided into the obesity (OBS) group (n=10), the OBS+NEI group (n=10), the OBS+ATM group (n=10) and the OBS+ATM+NEI group (n=10). In the CTR group and the OBS group, OVA was replaced with normal saline for sensitization and stimulation. After modeling, rats in NEI intervention groups were injected with Sivelestat (5 mg/kg, Ono Pharmaceutical Co. Ltd, Osaka, Japan) *via* the caudal vein twice a day for 1 week. However, rats in other groups were treated with the corresponding volume of normal saline in the same way.

Capsaicin-Induced Cough Test

24 hours after the last administration, rats in each group were placed in an aerosol chamber and inhaled 10^{-4} mol/L capsaicin solution for 60 seconds. Coughing times of rats in each group were recorded within 2 minutes.

Pulmonary Function Test (Provocation Test)

Rats were anesthetized by intraperitoneal injection of 2% sodium pentobarbital (80 mg/kg). The trachea was intubated invasively. The tracheal intubation was placed on the back of the closed body tracing box of a small animal ventilator. Meanwhile, the tracheal intubation was connected to the body tracing box. After observing normal airway pressure waveform, the administration route of the common jugular vein was established to ensure a smooth passage. The body tracing box should be closed until the airway pressure was normal. After the force curve was stable, the following four doses of methacholine were given every 5 minutes: 0.025 mg/kg, 0.05 mg/kg, 0.1 mg/kg and 0.2 mg/kg. Total airway resistance (RL) and dynamic compliance after inhalation were detected.

Hematoxylin-Eosin (HE) Staining

After anesthesia with 7% chloral hydrate, the tissues were quickly removed from the rats. The collected tissues were washed with phosphate-buffered saline (PBS) and fixed in 4% paraformaldehyde, followed by dehydration with gradient alcohol and transparentization with xylene. After paraffin embedding, the tissues were sliced into 5 μm-thick sections, spread flat, placed on the glass slide and dried in an oven at 45°C. Subsequently, after deparaffinization with xylene, the sections were stained with HE, dehydrated with pure alcohol and transparentized again with xylene. The sections transparentized were added with Canada balsam and covered with cover glass, followed by observation and photography under a microscope.

Detection of ROS Level

The level of ROS in the heart, lung and kidney tissues of rats was detected *via* 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA). An appropriate number of tissues were prepared into single-cell suspension, and the total number of cells was not less than 10^6. DCFH-DA (10 μmol/L) was added into the single-cell suspension for incubation at 37°C for 45 min. 200 μL samples were taken for fluorescence detection. The protein concentration was measured using the bicinchoninic acid (BCA) method (Pierce, Rockford, IL, USA), and the level of ROS was expressed as fluorescence value/protein (mg).

Detection of Catalase (CAT), Reduced Glutathione (GSH), Glutathione Peroxidase (GSH-Px) and Total Superoxide Dismutase (T-SOD)

A proper number of tissues was accurately weighed, and the appropriate amount of normal saline was added. Mechanical homogenization was performed *via* an ice water bath. CAT, reduced GSH, GSH-Px and T-SOD in tissues were detected using the CAT assay kit (Leagene Biotech, Beijing, China), GSH assay kit, GSH-Px assay kit and T-SOD assay kit according to the instructions, respectively.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

An appropriate number of tissues was taken and homogenized. Total ribonucleic acid (RNA) was extracted from tissues using the TRIzol method (Invitrogen, Carlsbad, CA, USA). The extracted RNA was reversely transcribed into complementary deoxyribonucleic acid (cDNA) according to the instructions of the Reverse Transcription kit (TaKaRa, Otsu, Shiga, Japan). Primers used in this study were as follows: Keap1: F: AATGTTGACACGGAGGATTGG, R: ATCCGCCACTCATTCCTCTC. Nrf2: F: CTTCCATTTACGGAGACCCAC, R: GATTCACGCATAGGAGCACTG. β-actin: F: GAGACCTTCAACACCCCAGC, R: ATGTCACGCACGATTTCCC. RT-PCR was performed according to the instructions of SYBR green kit using the Step One Plus™ Real-Time PCR System. The relative expression level of the target gene was calculated using the $2^{-\Delta\Delta CT}$ method.

Western Blotting

Tissues were cut into pieces, and 1 mL radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime, Shanghai, China) was added to extract total protein. The protein concentration was measured using the BCA method. 10 μL protein samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). After that, the gel was removed and transferred onto polyvinylidene difluoride (PVDF) membranes (Roche, Basel, Switzerland) in an appropriate size soaked in methanol. The membrane was then sealed with 5% skim milk at room temperature for 1 h. Subsequently, the membrane was incubated with primary antibody, which was slowly shaken on a shaking table in a refrigerator at 4°C overnight. On the next day, the band was removed, and the membrane was incubated with horseradish peroxidase (HRP)-labeled secondary

antibody at room temperature for 2 h. Finally, the enhanced chemiluminescence (ECL) (GE Healthcare, Waukesha, WI, USA) solution was added for color development. Gray value of the band was detected and analyzed using the Image J software.

Statistical Analysis

All data were expressed as mean ± standard deviation. Statistical Product and Service Solutions (SPSS) 19.0 software (SPSS, Chicago, IL, USA) was used for all statistical analyses. *t*-test was used to compare the difference between the two groups. One-way analysis of variance was used for comparison among different groups, followed by Post-Hoc Test (Least Significant Difference). $p < 0.05$ was considered statistically significant.

Results

NEI Improved the Symptoms of Rats with Asthma

Rats in the CTR group had stable breath and smooth hair, with no abnormalities in the behavior and diet. After sensitization and stimulation *via* OVA, the rats in the ATM group and OBS+ATM group displayed the symptoms of acute asthma attack, such as dysphoria, walking around, hair sticking up and breath through the mouth and polypnea. There were stable weight and slow response at the same time. After NEI intervention, the above symptoms were significantly improved. Compared with the CTR group, the number of coughs in the ATM group and the OBS+ATM group increased significantly. After NEI intervention, the number of coughs was significantly decreased. (Figure 1A). There was no significant difference in lung resistance (RL) among the groups in the first excitation. In the second, third and fourth stimulation, RL in the ATM and OBS+ATM groups was significantly higher than that of the normal control group. However, RL in the ATM+NEI and OBS+ATM+NEI group was significantly decreased (Figure 1B). Compared with the CTR group, lung compliance ($C_{dyn)}$ in the ATM group and the OBS+ATM control group was remarkably decreased in the second, third and fourth stimulation. After NEI intervention, C_{dyn} increased significantly (Figure 1C).

NEI Lessened Pathological Damage in Rats with Asthma

In the CTR group, the rats had clear and complete bronchial and alveolar structures as well as regular lumen, without cilia shedding and inflammatory cell infiltration. In the ATM group and OBS+ATM group, the rats had edema in tracheal mucosa and incomplete epithelium. The lumen was filled with inflammatory secretion, and there was lumen stenosis and even occlusion. Meanwhile, the smooth muscle and basement membrane were significantly thickened. After NEI intervention, the above pathological changes were alleviated to different degrees (Figure 1D).

NEI Inhibited the ROS Level in Rats with Asthma

The production of ROS in the heart, lung and kidney tissues of rats was detected *via* DCFH-DA. The results revealed that the levels of ROS in the heart, lung and kidney tissues of the OBS group, ATM group and OBS+ATM group were significantly higher than those of the CTR group. The OBS+ATM group showed the highest level of ROS. After NEI intervention, the level of ROS was significantly declined in the OBS+NEI group, ATM+NEI group and OBE+ATM+NEI group. The decrease degrees of the OBS+NEI group and the ATM+NEI group were similar, which was the highest in the OBE+ATM+NEI group (Figure 2).

NEI Promoted the Activity of Related Antioxidant Enzymes in Oxidative Stress Response in Asthma

The activities of antioxidant stress-related enzymes (reduced GSH, GSH-Px, H_2O_2 and SOD) in the heart, lung and kidney tissues of each group were detected and compared. The results showed that the activities of reduced GSH, GSH-Px, H_2O_2 and SOD in the heart, lung and kidney tissues of the OBS group, ATM group and OBS+ATM group were markedly lower than the CTR group. After NEI intervention, the activities of antioxidant stress-related enzymes in the OBS+NEI group, ATM+NEI group and OBE+ATM+NEI group were increased. The increase degrees in the OBS+NEI group and ATM+NEI group were similar, which was significantly lower in the OBE+ATM+NEI group (Figure 3).

NEI Activated the Keap1/Nrf2 Signaling Pathway in Rats with Asthma

The changes in mRNA and protein expression levels of antioxidant genes (Keap1 and Nrf2) were detected. The results manifested that the

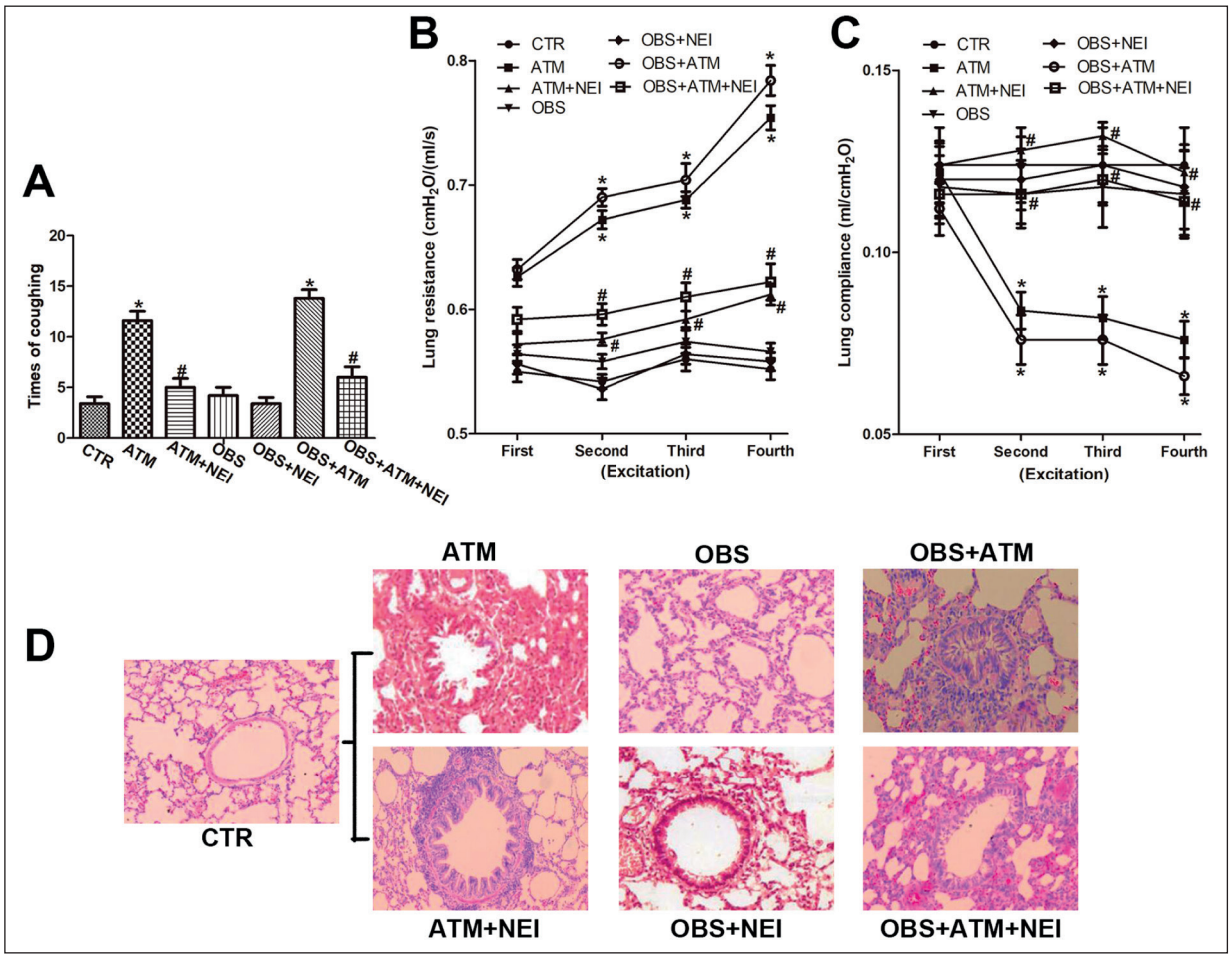

Figure 1. NEI improved the symptoms and alleviated the pathological damage of rats with asthma. ***A,*** Comparison of times of coughing in different groups. ***B,*** Analysis of lung resistance in different groups. ***C,*** Analysis of lung compliance in different groups. ***D,*** Representative images of hematoxylin and eosin staining of lung tissue (Magnification ×20). *$p < 0.05$ *vs.* CTR group, $^{\#}p < 0.05$ *vs.* ATM or OBS+ATM group.

mRNA and protein expression levels of Keap1 in the heart, lung and kidney tissues of the OBS group, ATM group and OBS+ATM group were remarkably higher than those of the CTR group. After NEI intervention, the mRNA and protein expression levels of Keap1 in the heart, lung and kidney tissues of the OBS+NEI group, ATM+NEI group and OBE+ATM+NEI group were

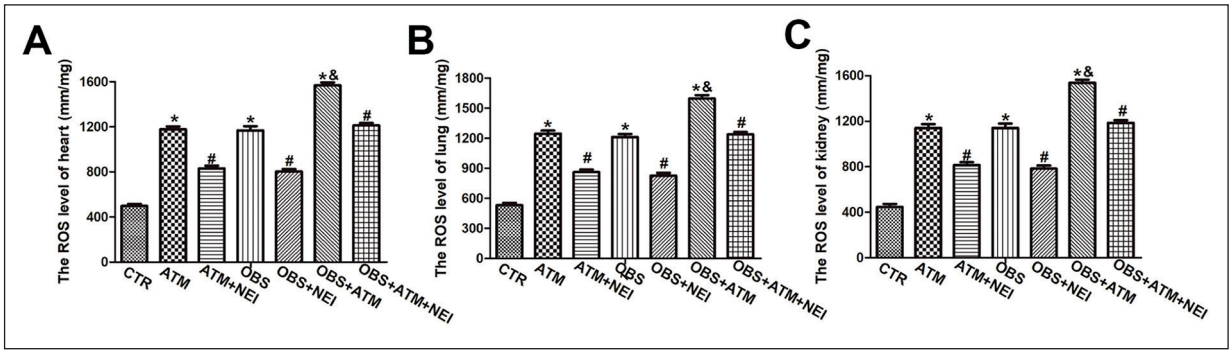

Figure 2. NEI inhibited the ROS level in rats with asthma. ***A,*** Analysis of ROS level of the heart in different groups. ***B,*** Analysis of ROS level of lung in different groups. ***C,*** Analysis of ROS level of kidney in different groups. *$p < 0.05$ *vs.* CTR group, $^{\#}p < 0.05$ *vs.* ATM or OBS or OBS+ATM group.

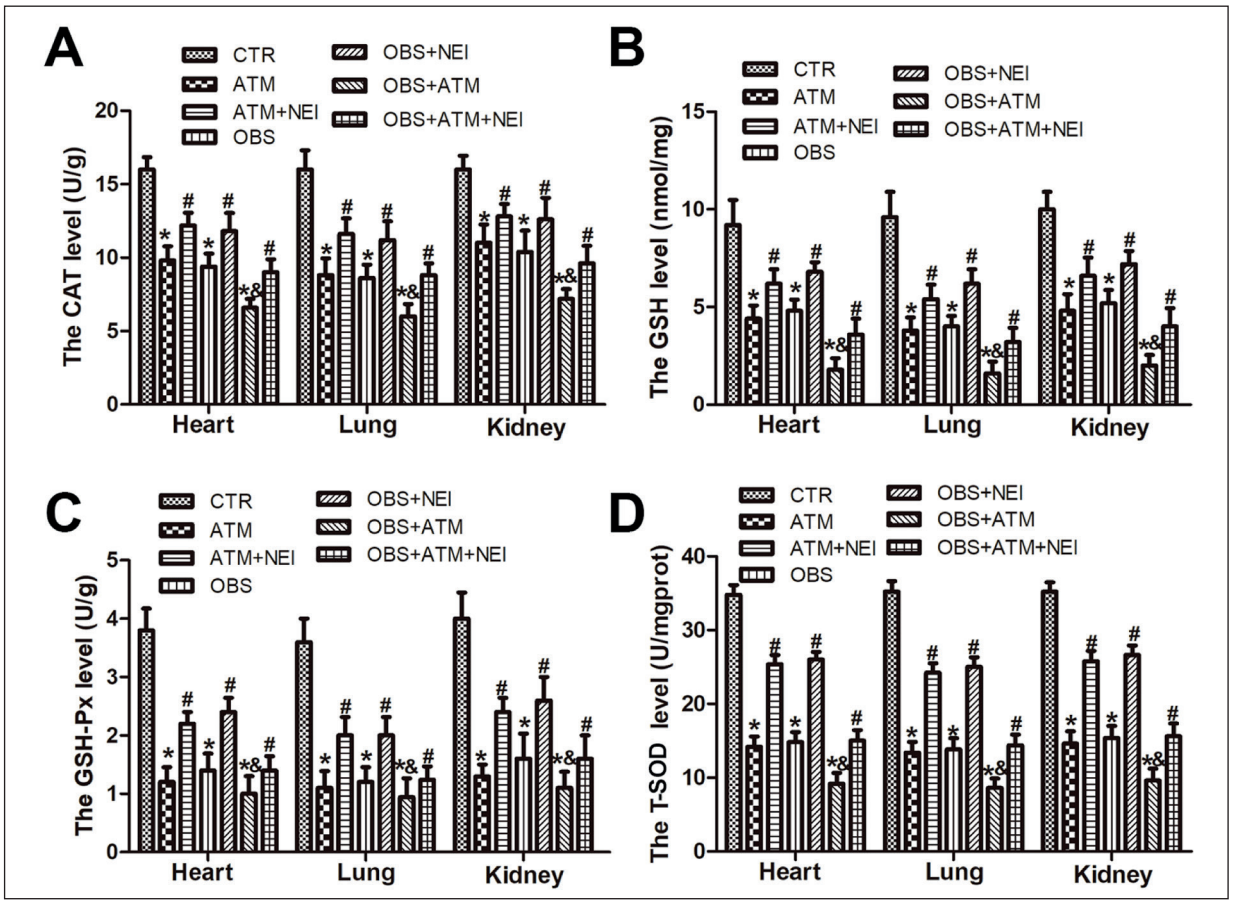

Figure 3. NEI promoted the activity of related antioxidant enzymes in oxidative stress response in asthma. ***A,*** Analysis of CAT level of the heart, lung and kidney in different groups. ***B,*** Analysis of reduced GSH level of the heart, lung and kidney in different groups. ***C,*** Analysis of GSH-Px level of the heart, lung and kidney in different groups. ***D,*** Analysis of T-SOD level of the heart, lung and kidney in different groups. $^{*}p < 0.05$ *vs.* CTR group, $^{\#}p < 0.05$ *vs.* ATM or OBS or OBS+ATM group. $^{\&}p < 0.05$ *vs.* ATM or OBS group.

remarkably declined. Moreover, the mRNA and protein expression levels of Nrf2 in the heart, lung and kidney tissues of the OBS group, ATM group and OBS+ATM group were significantly lower than those of the CTR group. After NEI intervention, the mRNA and protein expression levels of Nrf2 in the heart, lung and kidney tissues of the OBS+NEI group, ATM+NEI group and OBE+ATM+NEI group were significantly increased (Figure 4).

Discussion

In recent years, studies have found that smoking, infection and intracellular inflammatory factors result in the imbalance between oxidation and anti-oxidation in the body. This may eventually lead to the occurrence and development of bronchial asthma[23,24]. Obesity is a global epidemic disease currently. The level of oxidative stress in obese people is significantly increased and higher than that of non-obese people[11,12]. It is known that obesity is a risk factor for various human diseases, including asthma, heart disease and cancers[25-27]. Moreover, obesity aggravates the asthma status and reduces the therapeutic effect of traditional glucocorticoids on asthma patients[28]. In recent years, the correlation between obesity and asthma, as well as its mechanism has been widely studied[28]. High respiratory rate and low vital capacity in obese people increase the risk of asthma and severe asthma. At the same time, weight gain-induced lipid metabolic disorders and endocrine changes in adipose tissues also aggravate asthma. In this study, it was found that the levels of ROS in the heart, lung and kidney tissues of rats in the OBS group, ATM

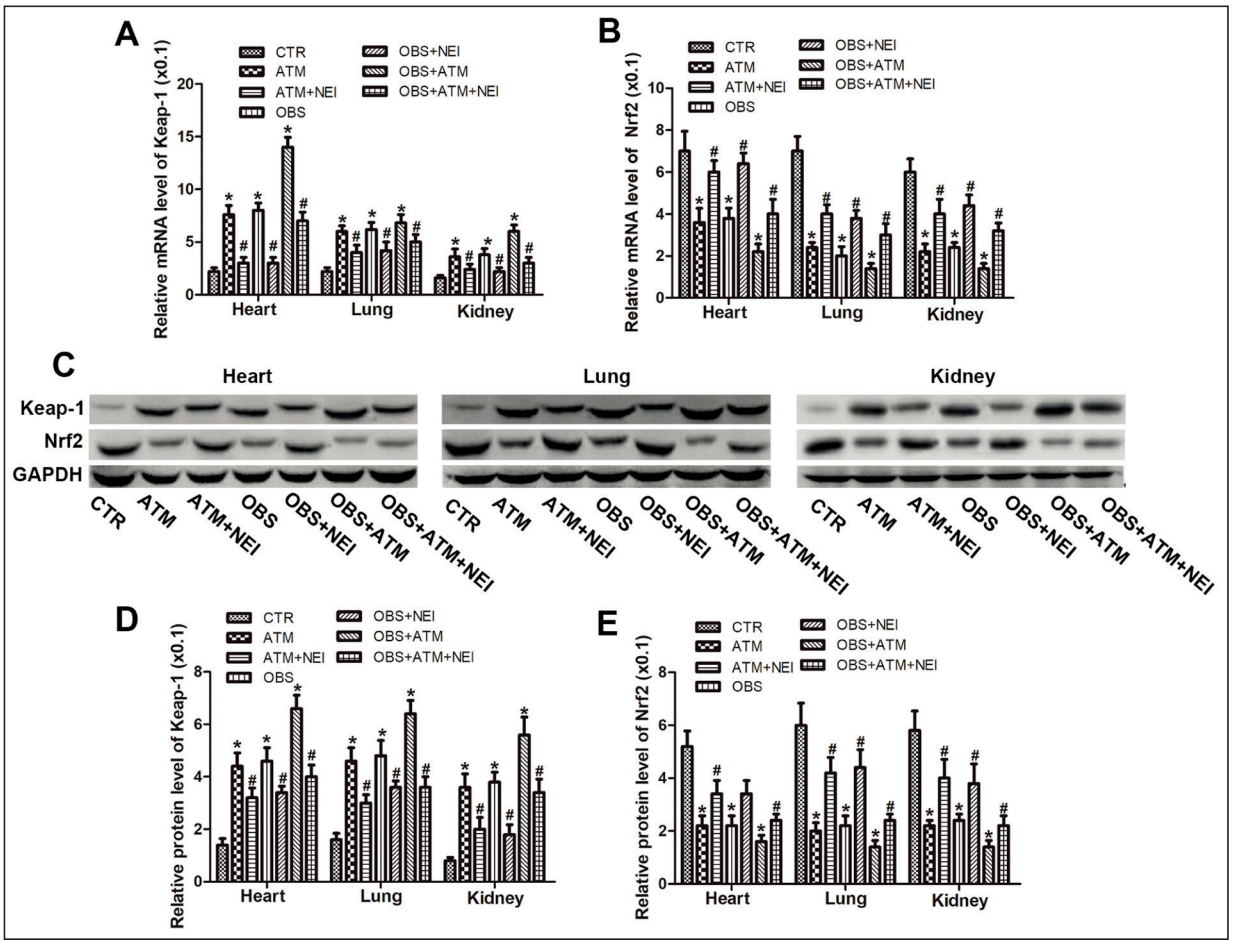

Figure 4. NEI activated the Keap1/Nrf2 signaling pathway in rats with asthma. ***A,*** Analysis of Keap-1 mRNA level of the heart, lung and kidney by RT-PCR in different groups. ***B,*** Analysis of Nrf2 mRNA level of the heart, lung and kidney by RT-PCR in different groups. ***C,*** Western blotting showed the Keap-1 and Nrf2 protein level in different groups. ***D,*** Analysis of Keap-1 mRNA level of the heart, lung and kidney by RT-PCR in different groups. ***E,*** Analysis of Nrf2 protein level of the heart, lung and kidney by Western blotting in different groups. $^{*}p < 0.05$ *vs.* CTR group, $^{\#}p < 0.05$ *vs.* ATM or OBS or OBS+ATM group.

group and OBS+ATM group were significantly higher than the CTR group. Meanwhile, it was also significantly higher in the OBS+ATM group than the OBS group and ATM group, displaying significant differences. Besides, the activities of antioxidant stress-related enzymes in the heart, lung and kidney tissues of rats in the OBS group, ATM group and OBS+ATM group were remarkably lower than those of the CTR group, which was the lowest in the OBS+ATM group. This indicated that obesity increased the level of ROS in rats with asthma by reducing the activity of antioxidant stress-related enzymes.

Keap1/Nrf2 signaling pathway plays an important role in protecting cells from oxidation and external damage. Keap1 is a multi-domain repressor of the Keap family[16,17]. In this study, the mRNA and protein expression levels of Keap1 in the heart, lung and kidney tissues of rats in each group were detected. It was found that the mRNA and protein expression levels of Keap1 in the heart, lung and kidney tissues of the OBS group, ATM group and OBS+ATM group were remarkably higher than those of the CTR group, which were the highest in the OBS+ATM group. This suggested that the expression of Keap1 gene was up-regulated under pathological conditions of obesity and asthma. Meanwhile, obesity and asthma exerted a synergistic effect. Nrf2 is an important nuclear transcription factor, which is the most important cellular defense mechanism for regulating excessive ROS. It also regulates the expression of a variety of antioxidant genes and proteins[29,30]. In this work, the

mRNA and protein expression levels of Nrf2 in the heart, lung and kidney tissues of rats in each group were also detected. Results demonstrated that the mRNA and protein expression levels of Nrf2 in the heart, lung and kidney tissues of the OBS group, ATM group and OBS+ATM group were remarkably lower than those of the CTR group, which were the lowest in the OBS+ATM group. This suggested that the expression of the Nrf2 gene was down-regulated under pathological conditions of obesity and asthma, in which obesity and asthma also exerted a synergistic effect.

NEI possesses anti-oxidative, anti-inflammatory and anti-apoptotic effects[21,22]. In this research, NEI was used to intervene rats in the OBS group, ATM group and OBS+ATM group. It was found that the levels of ROS in the heart, lung and kidney tissues of the OBS+NEI group, the ATM+NEI group and the OBE+ATM+NEI group were significantly decreased. At the same time, the content of antioxidant defense system components and activity of related enzymes (reduced GSH, GSH-Px, H_2O_2 and SOD) were significantly increased. These results proved that NEI could reduce the level of ROS in the inflammatory response caused by obesity and asthma in rats. The underlying mechanism might be the release of antioxidant molecules (reduced GSH and GSH-Px) and increased activity of antioxidant enzymes (H_2O_2 and SOD). At the same time, after NEI intervention, the mRNA and protein expression levels of Keap1 in the heart, lung and kidney tissues of rats were remarkably declined in the OBS+NEI group, ATM+NEI group and OBE+ATM+NEI group. Similarly, after NEI intervention, the mRNA and protein expression levels of Nrf2 in the heart, lung and kidney tissues of rats were markedly increased in the OBS+NEI group, ATM+NEI group and OBE+ATM+NEI group. The above results suggested that the effects of NEI in promoting the release of antioxidant factors and enhancing the activity of antioxidant enzymes might be achieved by activating the Keap1/Nrf2 signaling pathway.

The effect of NEI in reducing the level of ROS in the heart, lung and kidney tissues of the OBS+ATM group was stronger than the OBS group and the ATM group. However, its effect on increasing the activity of antioxidant factors in the heart, lung and kidney tissues of the OBS+ATM group was weaker than the OBS group and the ATM group. These findings indicated that NEI, in addition to activating the Keap1/Nrf2 signaling pathway, might also reduce the level of ROS in rats of the OBS+ATM group through other regulatory pathways.

Conclusions

We found that NEI promotes the release of a variety of antioxidant factors, enhances the activity of antioxidant enzymes and improves the symptoms of rats with obese asthma. The possible underlying mechanism is very likely to be realized by activating the Keap1/Nrf2 signaling pathway.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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