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Adiponectin alleviates exacerbation of airway inflammation and oxidative stress in obesity-related asthma mice partly through AMPK signaling pathway

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ABSTRACT

Adiponectin plays a role in asthma and obesity, but its effects and mechanism in obesity-related asthma remain elusive. This study aimed to evaluate the effects of adiponectin on airway inflammation and oxidative stress and to determine its mechanism in obesity-related asthma. Male C57BL6/J mice fed with a high-fat diet to induce obesity were sensitized and challenged with ovalbumin to induce asthma, and treated with adiponectin (1 mg/kg) and AMP-activated protein kinase (AMPK) inhibitor compound C (20 mg/kg) twice before the first ovalbumin challenge. We found exogenous adiponectin significantly reduced airway resistance, inflammatory infiltration in lung tissue, and cell counts in bronchoalveolar lavage fluid. Adiponectin inhibited great levels of eotaxin, myeloperoxidase, tumor necrosis factor-α, 8-hydroxy-2′-deoxyguanosine, and nitric oxide in obesity-related asthma mice. Moreover, we found increased nuclear factor kappa B p65, inducible nitric oxide synthase and B-cell lymphoma 2 protein expression were down-regulated with adiponectin administration. Additionally, adiponectin elevated the lower levels of pAMPK and AMPK activity in lung tissue. These protective effects of adiponectin were reversed after treatment with the AMPK inhibitor compound C. Thus, we conclude that adiponectin alleviates exacerbation of airway inflammation and oxidative stress in a murine model of obesity-related asthma partly through AMPK signaling pathway.

1. Introduction

Epidemiological data show that the incidence of asthma has increased 2.0- and 2.3-fold in obese children and adults, respectively [1,2]. Obesity is also associated with the development, severity, and control of asthma, which may be mediated by increased airway inflammation and airway hyper-responsiveness [3,4]. Current data suggest that obesity may induce oxidative stress [5], including increased inducible nitric oxide synthase (iNOS) expression and subsequently elevation of nitric oxide (NO) levels [6,7]. Asthma is also associated with increased oxidative stress [3].

Adiponectin, reduced in obesity, is predominantly an anti-inflammatory adipokine and has anti-oxidative effects [8]. Shore et al. found that adiponectin was reduced in a murine model of sensitized and challenged mice with ovalbumin (OVA) [9]. It is possible that adiponectin plays a role in allergic responses, as studies indicated that adiponectin attenuated allergic airway inflammation and airway hyperresponsiveness in OVA -challenged mice [9]. One Study has also reported that adiponectin can reduce inflammation and suppress oxidative stress in vivo [10]. Despite reports on the protective effects of adiponectin in pulmonary diseases, the mechanisms of adiponectin on airway hyper-responsiveness, airway inflammation, and oxidative stress are not fully understood, especially in asthmatic individuals with obesity.

Adiponectin exists in different higher-order complexes, including a high molecular weight form, a low molecular weight form, and a trimeric form, all of which have different roles in metabolic homeostasis [11]. Adiponectin plays a physiological role by activating receptors such as adiponectin receptor 1 (AdipoR1), adiponectin receptor 2 (AdipoR2), T-cadherin, and calreticulin, some of which are expressed on airway epithelial cells in the lung [8,12–14]. AdipoR1 and AdipoR2 directly interact with adiponectin to mediate its effects by activating

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AMP-activated protein kinase (AMPK) and peroxisome proliferator-activated receptor alpha, respectively [10]. AMPK is a crucial energy sensor in regulation of cellular metabolism, and energy metabolism is important in obesity [15]. AMPK is also reported to be a regulator of the inflammatory function of macrophages, accelerating macrophage polarization towards an anti-inflammatory phenotype [16]. Studies showed that adiponectin inhibited tumor necrosis factor-α (TNF-α) production and antagonized the inflammatory effects of TNF-α, which might be an important target for treating obesity-related asthma [17]. Additionally, the nuclear factor kappa B (NF-κB) pathway is an important signaling pathway during the inflammatory process [18].

In the present study, a mouse model of allergic asthma with obesity was well-established to investigate the effects of adiponectin and its mechanism. We measured total adiponectin in this study, allowing for comparisons with other studies. We hypothesized that adiponectin might attenuate the exacerbation of asthma with obesity by activating AMPK after binding to the adiponectin receptors, with subsequently inhibiting TNF-α/NF-κB pathway. Furthermore, we used a selective AMPK inhibitor compound C [19] (also known as dorsomorphin) to confirm the mechanism of adiponectin. Finally, we found that adiponectin protected against airway inflammation, airway hyper-responsiveness and oxidative stress possibly via activation of AMPK in this mouse model of obesity-related asthma.

2. Materials and methods

2.1. Animals and diet

All animal procedures and experimental protocols were performed in accordance with the ethical principles in animal research adopted for animal experimentation by the Wenzhou Medical University and were approved by the institutional committee for ethics in animal research of the Wenzhou Medical University (protocol wydw2013-0146). Male C57BL6/J mice were provided by Shanghai Laboratory Animal Center (Shanghai, China). All mice entered experimental protocols at 3–4 weeks of age and weighing 10–12 g. Mice were housed five per cage on a 12 h light-dark cycle at a constant temperature (22–25 °C) and fed for 12 weeks with either a standard chow diet (70% carbohydrate, 20% protein, 10% fat) or a high-fat diet (35% carbohydrate, 20% protein, 45% fat) (Medicience Ltd. Jiangsu, China). The high-fat diet was used to induce obesity in mice [20].

2.2. Murine model of OVA-induced asthma and treatments

Mice were randomly divided into eight groups and ten mice in each group: lean mice group (control mice, L); OVA-sensitized lean mice group (asthma mice, SL); OVA-sensitized lean mice treated with adiponectin group (SL + ADPN); OVA-sensitized lean mice treated with adiponectin and compound C group (SL + ADPN + COM); obesity mice group (obesity mice, O); OVA-sensitized obesity mice group (obesity-related asthma mice, SO); OVA-sensitized obesity mice treated with adiponectin group (SO + ADPN); and OVA-sensitized obesity mice treated with adiponectin and compound C group (SO + ADPN + COM).

We previously developed a mouse model of asthma [21,22]. Briefly, mice were actively sensitized with an intraperitoneal injection that contained 10 μg of ovalbumin (Sigma-Aldrich, St. Louis, MO) mixed with 20 mg Al $(OH)_3$ gel in 0.1 mL normal saline on days 1 and 7 of the 9th week. From days 25 to 32, sensitized mice were challenged in a sealed container filled with ovalbumin aerosol (1 mg/mL) for 30 min once per day (Fig. 1). Aerosols were generated with an Inqua Neb PLUS (Pari, Starnberg, Germany) and delivered to the container using bias flow of medical air. Non-sensitized mice (L and O groups) were identically sensitized and challenged with normal saline for use as controls.

The adiponectin treatment groups received adiponectin 1 mg/kg (Kang Peptide Biological Technology (Beijing) Co., Ltd., China) by caudal vein injection at 48 h and 24 h before the first ovalbumin challenge. The dose for adiponectin was determined from preliminary experiments and the study of Yao et al. [23]. The compound C treatment groups were additionally given compound C 20 mg/kg (BML-275, Enzo, Switzerland) intraperitoneally at 1 h before adiponectin [24]. Non-treatment groups (L and O groups) received normal saline at the same dose and in the same way (Fig. 1).

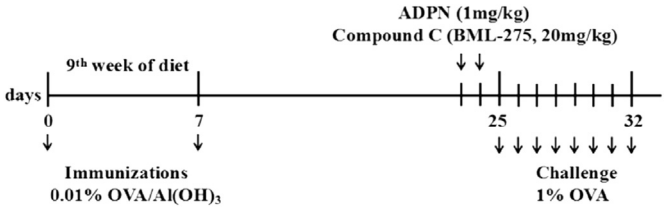

Fig. 1. Schematic representation of ovalbumin (OVA) immunizations and challenges as well as the time-course of adiponectin (ADPN) and compound C treatments. Mice were actively sensitized with an intraperitoneal injection (0.1 mL) of 0.01% ovalbumin/Al$(OH)_3$ on days 1 and 7 of the 9th week. From days 25 to 32, sensitized mice were challenged in a sealed container filled with 1% OVA aerosol for 30 min once a day. As controls, non-sensitized mice (Group L and Group O) were identically sensitized and challenged with normal saline.

Within 24 h after the last challenge, mice were anesthetized with chloral hydrate (0.01 mL/g injected intraperitoneally). Body weight, liver weight, epididymal fat and naso-anal length (the distance from the anus to the tip of the nose) were measured after sacrificing the mice. Lee's obesity index calculated by the formula [Body weight (g)^(1/3) / Naso-anal length(cm)] was measured in all mice [25]. Peripheral blood was collected from the eyeball and centrifuged twice at 3000 rpm for 10 min at 4 °C. The supernatant was stored at −80 °C. Collection of bronchoalveolar lavage fluid (BALF) was obtained as described below. Lung tissues were obtained and stored at −80 °C.

2.3. Measurement of lung function

Total airway resistance was measured within 24 h after the last challenge by the forced oscillation technique [26]. All mice were anesthetized as above described and ventilated at a frequency of 150 breaths/min with a tidal volume (VT) of 0.2 mL and a positive end-expiratory pressure (PEEP) of 2 cmH_2O by a specialized ventilator (FlexiVent; SCIREQ, Montreal, Canada). Baseline airway resistance was measured after aerosolized saline. Then the mice were challenged by an increasing dose of aerosolized-methacholine (Into Industrial Club in Tokyo, Japan) (10, 20, 40, 60, 80 mg/mL) every 3 min. Aerosols were generated with an ultrasound nebulizer and delivered to the inspiratory line of the FlexiVent.

2.4. Cell counts in BALF

Right lungs were washed by twice flushing 1 mL phosphate-buffered saline (PBS) after ligation of the left bronchus. PBS was instilled and then slowly withdrawn through the tracheal cannula. The recovery rate > 80% was eligible. The fluid recovered after each instillation was centrifuged at 3000 rpm for 15 min at 4 °C. BALF supernatant was stored at −80 °C. The cell pellet from BALF was resuspended in 100 μL PBS, 30 μL of which was used for total cell counts using a hemocytometer (BioRad, Shanghai, China), and differential (Diff-Quick stain) cell counts were calculated after counting 200 leukocytes under light microscopy in 50 μL of cell suspension.

2.5. Histological analysis

The left lung was removed and post-fixed by immersion for at least 24 h in 4% paraformaldehyde, after which it was macroscopically examined and then cut transversally into slices of approximately 4 mm before being embedding in paraffin. Paraffin sections were stained with

hematoxylin-eosin and evaluated for peribronchial and perivascular infiltration using an Olympus microscope (magnification, ×200) connected to a digital camera (CX21 FS1; Olympus, Tokyo, Japan).

For each mouse, the extent of peribronchial and perivascular infiltrate was assessed semi-quantitatively using the following histological grading system [27]: absence of peribronchial inflammatory cells (score 0); a few scattered peribronchial inflammatory cells involving < 25% of the circumference of the bronchus (score 1); focal peribronchial inflammatory cell infiltrate not completely surrounding a bronchus (involving approximately 25–75% of the circumference of the bronchus) (score 2); one definite layer of peribronchial inflammatory cells completely surrounding a bronchus (score 3); and two or more layers of peribronchial inflammatory cells completely surrounding a bronchus (score 4). For each lung section, mean peribronchial inflammatory score was calculated by adding the scores of all individual bronchioles in the lung section and averaging the score by the number of bronchioles present in the lung section.

2.6. Adiponectin, AdipoR1, AdipoR2, AMPKα1, and AMPKα2 mRNA expression

Lung tissue (~30 mg per mouse) was analyzed using quantitative polymerase chain reaction (PCR) for adiponectin, adiponectin receptors (Adipor1 and Adipor2), AMPKα1, and AMPKα2 mRNA expression. Total RNA was extracted from lung homogenates using TRIzol reagent (Invitrogen, Carlsbad, CA). RNA concentration was determined using an automatic microplate reader (ELX808IU; Bio-Tek, Winooski, VT). Total RNA (~2 μg) was reverse-transcribed with Moloney murine leukemia virus reverse transcriptase and oligo (dT) 18 primer (Invitrogen) to generate cDNA. Synthesized cDNA was amplified using real-time PCR (Light-Cycler 480; Roche Diagnostics, Indianapolis, IN) with SYBR Green master mix (Thermo Fisher Scientific, Waltham, MA) and specific primers (Invitrogen).

The following primers were used: Adiponectin, forward: 5′-CCTGG AGAGAAGGGAGAGAAAG-3′, reverse: 5′-CTGAGCGATACACATAAG CGG-3′; AdipoR1, forward: 5′-AGAGCATCTTCCGCATCCACACA-3′, reverse: 5′-GAGAAACTGAGGCAGAGCA-CCGC-3′; AdipoR2, forward: 5′-TAGAATACACACAGAGACGGGCAAC-3′, reverse: 5′-ATGAAAAGGA AAGGCAGAGAATGGC-3′; AMPKα1, forward: 5′-GATGAGATTA-CAGA AGCCAAA-3′, reverse: 5′-GAGGGAGGTGACAGATGAG-3′; AMPKα2, forward: 5′-TCCTCCATCAGGTTCTTT-3′, reverse: 5′-GACTTGGGCTTC GTTGTG-3′; and β-actin, forward: 5′-TGAGAGGGAAATCGTGCGT GAC-3′, reverse: 5′-GCTCGTTGCCA-ATAGTGATGACC-3′.

Samples were pre-incubated at 95 °C for 5 min followed by 45 cycles of denaturation at 95 °C for 10 s, annealing at 60 °C for 10 s, and extension at 70 °C for 10 s. Quantification was performed using the comparative 2-(ΔΔCt) method as follows: ΔΔCt = (target gene Ct of experimental group − reference gene Ct of experimental group) − (target gene Ct of control group − reference gene Ct of control group). Expression levels for target genes were normalized to β-actin for each sample.

2.7. AMPKα, phosphorylated AMPKα (pAMPKα), NF-κB p65, iNOS, and B-cell lymphoma 2 (Bcl-2) protein expression

Lung tissue (~50 mg per mouse) was homogenized in SDS lysis buffer containing protease inhibitors, centrifuged, and then the cell nucleus was broken using ultrasound to obtain protein extracts. Protein concentrations in the supernatant were determined using a BCA protein assay kit (Beyotime Institute of Biotechnology, China), with the nucleoprotein being extracted following the manufacturer's instruction. Proteins were resolved using 10% or 12% SDS-PAGE and the electrotransfer of proteins was performed in a semi-dry device (Bio-Rad Laboratories, Hercules, CA). Proteins were then transferred to polyvinylidene difluoride membranes (Millipore, USA). Membranes were blocked with 5% non-fat dried milk for 2 h before being incubated for 14–18 h at 4 °C with 1:1 000 dilutions of the specific antibodies (AMPKα, pAMPKα, NF-κB p65, iNOS, Bcl-2, and β-actin) (Cell Signaling Technology, USA) separately. Blots were incubated with the secondary antibody (HRP, Goat Anti-Rabbit IgG(H + L), Earthox, USA) (1:5000 dilution) for 2 h at room temperature and assayed using a ChemiDoc XRS gel imaging system (Bio-Rad Laboratories, USA) (Target protein gray value/β-actin gray value).

2.8. Enzyme-linked immunoabsorbent assay

Total adiponectin, TNF-α, immunoglobulin E (IgE), and 8-hydroxy-2′-deoxyguanosine (8-OHdG) levels in serum and total adiponectin, TNF-α, eotaxin, and myeloperoxidase (MPO) levels in BALF were measured using enzyme-linked immunoabsorbent assay (ELISA) kits (Beyotime Institute of Biotechnology). 8-OHdG, NO, total antioxidant capacity (TAOC), eotaxin, and MPO levels from lung homogenates were measured using commercially available ELISA kits, following the manufacturer's instructions (Beyotime Institute of Biotechnology).

2.9. Statistical analysis

Values were expressed as mean ± standard deviation (SD). One-way analysis of variance (ANOVA) followed by Dunn's post-test for post-hoc t testing was used as appropriate. Pearson's correlation coefficient was used in correlation analyses. Analyses were performed in GraphPad (version 6.01 for Windows, GraphPad Software, San Diego, CA, USA) and SPSS 18.0. A P-value < 0.05 was considered as statistically significant.

3. Results

3.1. Physical characteristics

At 8th week, mice fed the high-fat diet exhibited a significant increase (≥20%) in body weight compared with mice receiving standard chow (Fig. 2A). It was found that a mouse model of obesity was well-established at 8th week. Mice in obesity group and obesity-related asthma group showed increased naso-anal length, liver weight, epididymal fat (Table 1) and Lee's obesity index compared with lean mice (Fig. 2B).

3.2. Effect of adiponectin on airway hyper-responsiveness in obesity-related asthma

Dietary obesity and OVA-induced asthma affected baseline lung function in this mouse model. Baseline airway resistance of obesity-related asthma mice was significantly increased compared with either asthma mice or obesity mice (Fig. 2C). Airway resistance was increased gradually in each group with an increasing dose of aerosolized-methacholine (Fig. 2D). At the same dose of aerosolized-methacholine, airway resistance was increased markedly in obesity-related asthma mice compared with asthma mice. The total airway resistance showed improvement with administration of exogenous adiponectin in obesity-related asthma mice, which was inhibited by compound C treatment (Fig. 2C–D).

3.3. Effect of adiponectin on inflammatory cell recruitment in BALF

Total cells in BALF were increased significantly in asthma mice, obesity mice, and obesity-related asthma mice compared with control mice (Fig. 3A). Obesity-related asthma mice showed increased numbers of neutrophils (Fig. 3B) and decreased eosinophils (Fig. 3C) in BALF compared with asthma mice. With exogenous adiponectin treatment, total cell counts, neutrophil and eosinophil numbers were decreased in asthma mice and obesity-related asthma mice (Fig. 3A–C). These effects of adiponectin were reversed with compound C treatment (Fig. 3A–C).

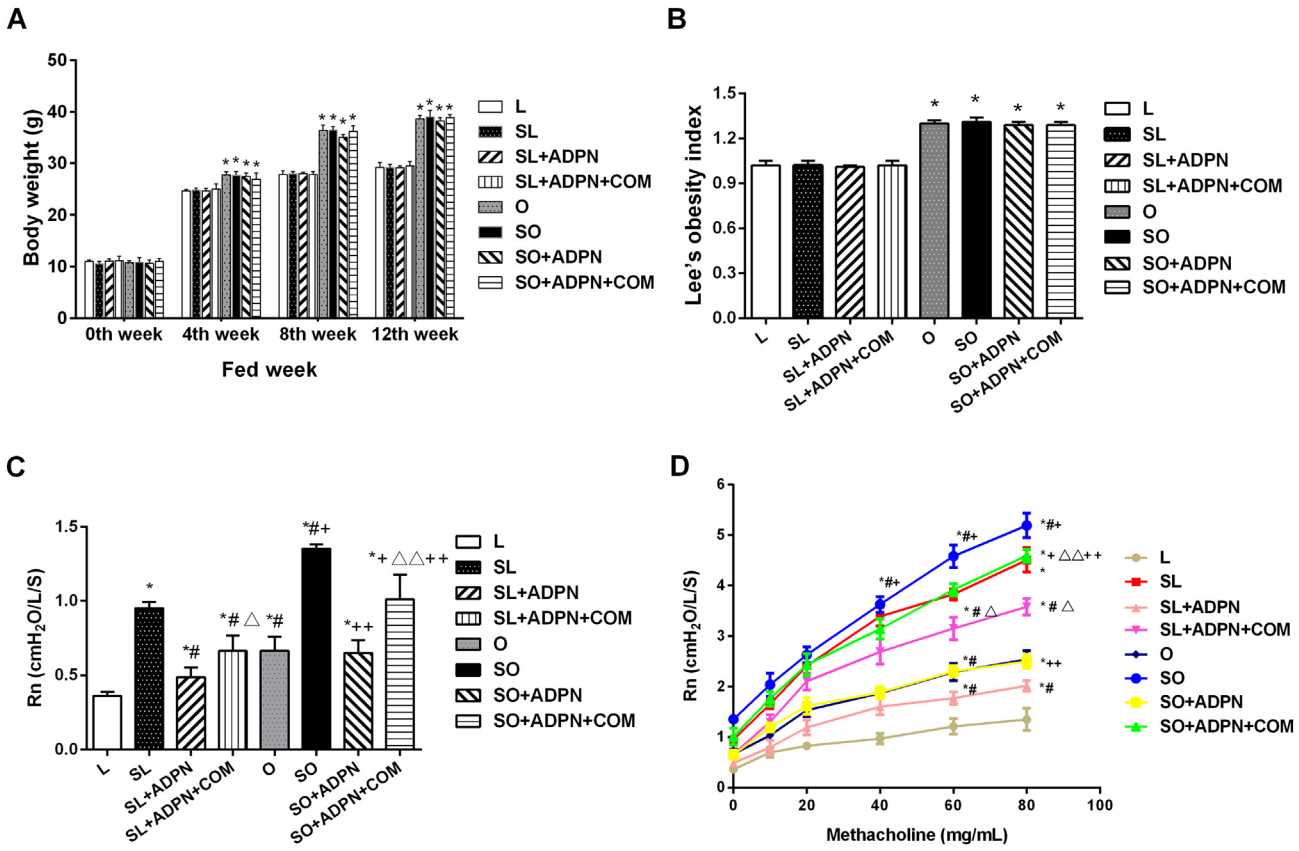

Fig. 2. Body weight and Lee's obesity index of mice fed with different diet; Effect of adiponectin on the total airway resistance (Rn). (A) Body weight of mice fed with different diet. Mice were weighed every four weeks, at 8th week mice fed the high-fat diet exhibited a significant increase (≥20%) in body weight compared with mice receiving standard chow. $^{*}P < 0.05$ as compared to mice fed with standard chow diet. (B) Lee's obesity index of mice fed with different diet. Lee's obesity index was calculated by Body weight(g)^(1/3) / Nasoanal length(cm). $^{*}P < 0.05$ as compared to mice fed with standard chow diet. (C) Baseline total airway resistance. (D) Methacholine-challenged total airway resistance. Airway resistance was measured by using the forced oscillation technique after the mice were anesthetized then ventilated and challenged by an increasing dose of aerosolized-methacholine (10, 20, 40, 60, 80 mg/mL). Values are means ± SD. $^{*}P < 0.05$ as compared to L group; $^{\#}P < 0.05$ as compared to SL group; $^{\triangle}P < 0.05$ as compared to SL + ADPN group; $^{+}P < 0.05$ as compared to O group; $^{++}P < 0.05$ as compared to SO group; $^{\triangle\triangle}P < 0.05$ as compared to SO + ADPN group.

Table 1
Body length, liver weight and epididymal fat of mice.

Group (n = 10)	Liver weight (g)	Epididymal fat weight (g)	Naso-anal length (cm)
L	1.45 ± 0.05	0.60 ± 0.06	9.52 ± 0.08
SL	1.46 ± 0.05	0.60 ± 0.10	9.50 ± 0.06
SL + ADPN	1.46 ± 0.05	0.58 ± 0.11	9.52 ± 0.08
SL + ADPN + COM	1.48 ± 0.08	0.56 ± 0.09	9.54 ± 0.05
O	1.64 ± 0.05*	2.88 ± 0.29*	9.96 ± 0.05*
SO	1.63 ± 0.14*	3.07 ± 0.41*	9.96 ± 0.09*
SO + ADPN	1.60 ± 0.07*	2.94 ± 0.23*	9.94 ± 0.05*
SO + ADPN + COM	1.60 ± 0.10*	2.88 ± 0.19*	10.02 ± 0.12*

Liver weight, epididymal fat weight and naso-anal length of mice was measured after mice were sacrificed.

* $P < 0.05$, compared with L group.

3.4. Effect of adiponectin on inflammatory cell recruitment in lung tissue

The semiquantitative histopathological score (Fig. 3D) was accordance with the histological analysis of lung tissue in the following (Fig. 3E). The control mice (L group) challenged with normal saline showed normal tissue, with no or few inflammatory cells throughout the peribronchial and perivascular areas (Fig. 3E). Obesity-related asthma mice (SO group) showed a significant influx of extensive inflammatory cells into the connective tissue surrounding the bronchial and bronchiolar segments, which was more obvious than either asthma mice (SL group) or obesity mice (O group) (Fig. 3E). This inflammatory response was alleviated with exogenous adiponectin treatment, which was reversed by compound C treatment (Fig. 3E).

3.5. Effect of adiponectin on eotaxin and MPO levels in BALF and lung tissue, and serum IgE level

Eotaxin and MPO levels in BALF and lung tissues were significantly increased in asthma mice and obesity-related asthma mice compared with control mice (Fig. 4A–D). The eotaxin level in obesity mice was markedly lower than in asthma mice, but there was no significant difference in eotaxin levels between asthma mice and obesity-related asthma mice (Fig. 4A, C). The MPO level was increased significantly in obesity-related asthma mice compared with asthma mice (Fig. 4B, D). With adiponectin treatment, eotaxin and MPO levels were decreased in both obesity-related asthma mice and asthma mice. This change was reversed with compound C treatment.

Serum level of IgE was significantly increased in asthma mice and obesity-related asthma mice compared with either control mice or obesity mice, respectively (4.86 ± 0.41 vs. 3.00 ± 0.26, 4.43 ± 0.34 vs. 3.46 ± 0.38 IU/uL; $P < 0.01$). This biomarker supported that the allergic mouse model was well-established. Neither exogenous adiponectin nor compound C treatment had any effect on IgE level among groups ($P > 0.05$).

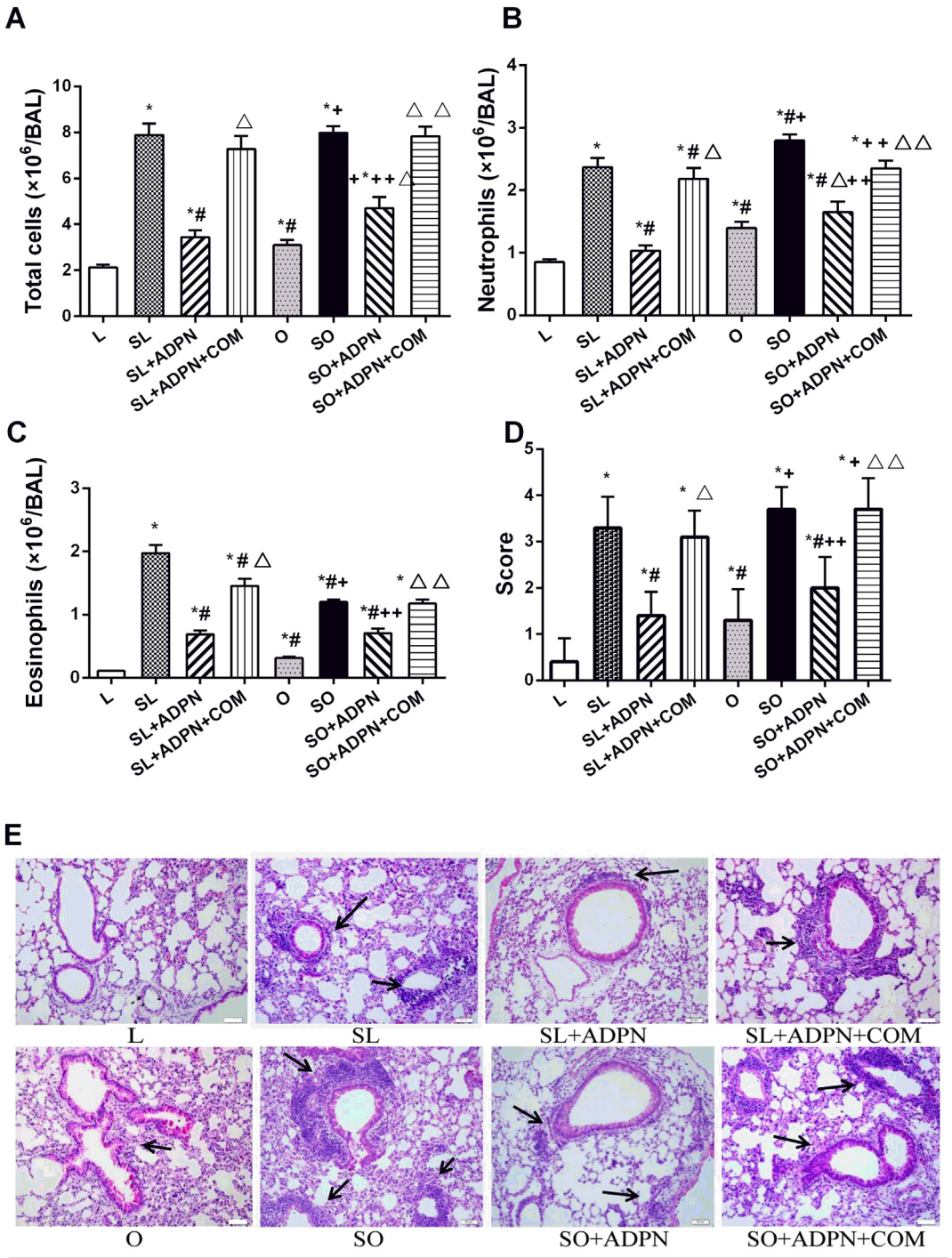

Fig. 3. Effect of adiponectin on pulmonary cell recruitment in BALF and lung tissue. (A) Numbers of total cells in BALF. (B) Numbers of neutrophils in BALF. (C) Numbers of eosinophils in BALF. Data are means ± SD. $^{*}P < 0.05$ as compared to L group; $^{\#}P < 0.05$ as compared to SL group; $^{\triangle}P < 0.05$ as compared to SL + ADPN group; $^{+}P < 0.05$ as compared to O group; $^{++}P < 0.05$ as compared to SO group; $^{\triangle\triangle}P < 0.05$ as compared to SO + ADPN group. (D) Semiquantitative pathology score of lung tissue (described in Materials and methods section). Data are means ± SD of ten mice per group. (E) Representative HE-stained (light microscopy, ×200) lung tissue slides of eight groups.

3.6. Effect of adiponectin on 8-OHdG, NO, and TAOC levels in lung tissue and serum

8-OHdG levels in serum (Fig. 5A) and lung tissue (Fig. 5B) were significantly increased in obesity-related asthma mice compared with either asthma mice or obesity mice, which was down-regulated with exogenous adiponectin treatment and reversed with compound C treatment. The NO level in lung tissue (Fig. 5C) changed in a similar manner to 8-OHdG. Adiponectin significantly increased the TAOC level (Fig. 5D) in lung tissue of obesity-related asthma mice, which was reduced compared with asthma mice, and this effect of adiponectin was inhibited by compound C treatment.

The pAMPKα/AMPKα ratio was considered to show AMPK activity. The 8-OHdG level in blood serum was positively correlated with the 8-OHdG level in lung tissue ($r = 0.7371$; $P < 0.000\ 1$). The 8-OHdG level in lung tissue was inversely correlated with AMPK activity (Fig. 5E). The TAOC level in lung tissue was positively correlated with AMPK activity (Fig. 5F). These findings suggested that obesity-related

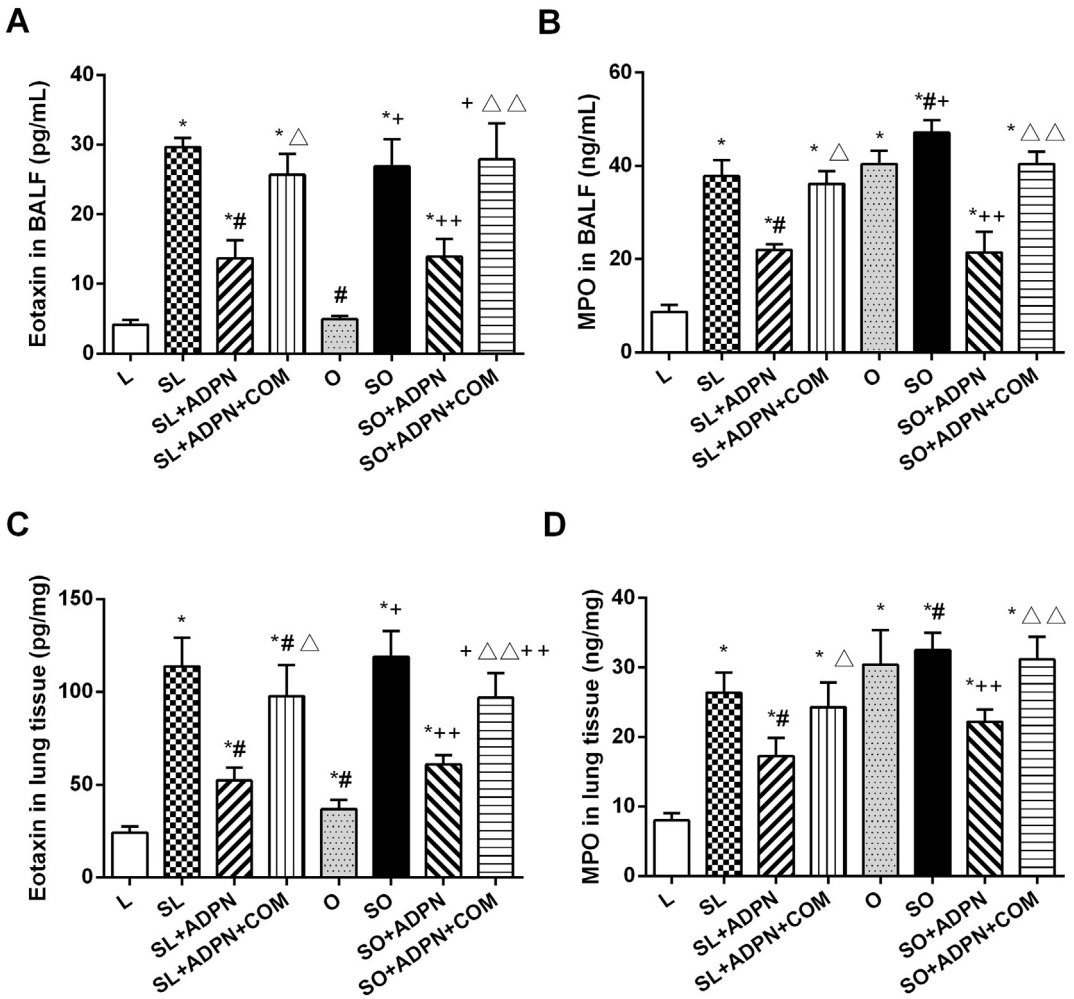

Fig. 4. Effect of adiponectin on eotaxin and myeloperoxidase (MPO) levels in BALF and lung tissue. (A) Levels of eotaxin in BALF. (B) Levels of MPO in BALF. (C) Levels of eotaxin in lung tissue. (D) Levels of MPO in lung tissue. All above inflammatory cytokines were measured by ELISA analysis. Results were obtained from three independent experiments. Values are means ± SD. $^{*}P < 0.05$ as compared to L group; $^{\#}P < 0.05$ as compared to SL group; $^{\triangle}P < 0.05$ as compared to SL + ADPN group; $^{+}P < 0.05$ as compared to O group; $^{++}P < 0.05$ as compared to SO group; $^{\triangle\triangle}P < 0.05$ as compared to SO + ADPN group.

asthma mice had airway and systematic oxidative stress, which was inversely correlated with AMPK activity.

3.7. Adiponectin and TNF-α levels in blood serum and BALF

Serum level of adiponectin was significantly decreased in obesity-related asthma mice compared with either asthma mice or obesity mice (Fig. 6A), which was up-regulated with exogenous adiponectin treatment. There was no difference in adiponectin level after treating with compound C. Serum TNF-α level (Fig. 6B) was markedly increased in obesity-related asthma mice compared with either asthma mice or obesity mice, which was down-regulated with exogenous adiponectin treatment, and reversed with administration of compound C.

Adiponectin and TNF-α levels in BALF were higher than in blood serum (Fig. 6A–B). Changes in adiponectin and TNF-α levels of BALF were observed to occur at almost the same time as changes in blood serum. The TNF-α level in BALF was inversely correlated with the adiponectin level in blood serum (Fig. 6C) as well as in BALF (Fig. 6D).

3.8. Adiponectin, adiponectin receptors (AdipoR1 and AdipoR2), AMPKα1, and AMPKα2 mRNA expression in lung tissue

Adiponectin and adiponectin receptors mRNA expression (Fig. 7A–C) were significantly decreased in obesity-related asthma mice compared with control mice, obesity mice and asthma mice. AdipoR1 mRNA expression (Fig. 7B) was higher than AdipoR2 mRNA expression (Fig. 7C) in lung tissue. Both adiponectin and adiponectin receptors mRNA levels were up-regulated with exogenous adiponectin treatment. With compound C treatment, adiponectin mRNA level was decreased but were still higher than before adiponectin treatment. There were no significant differences in adiponectin receptors mRNA levels after the mice treated with compound C (Fig. 7B–C). No significant differences were found in AMPKα1 and AMPKα2 mRNA levels after the mice treated with adiponectin or compound C ($P > 0.05$).

3.9. Effect of adiponectin on AMPKα, pAMPKα, NF-κB p65, iNOS, and Bcl-2 protein expression in lung tissue

There were no differences in AMPKα protein expression among groups (Fig. 8A). pAMPKα was significantly decreased in obesity-related asthma mice compared with control mice, obesity mice and asthma mice (Fig. 8A). pAMPKα was increased with the administration of exogenous adiponectin, and was reversed by compound C treatment. The AMPK activity (pAMPKα/AMPKα ratio) was found to change in the same manner as pAMPKα expression (Fig. 8B).

With exogenous adiponectin treatment, NF-κB p65, iNOS, and Bcl-2 protein expression (Fig. 9A–C) were all decreased, which were increased significantly in obesity-related asthma mice, but this effect was

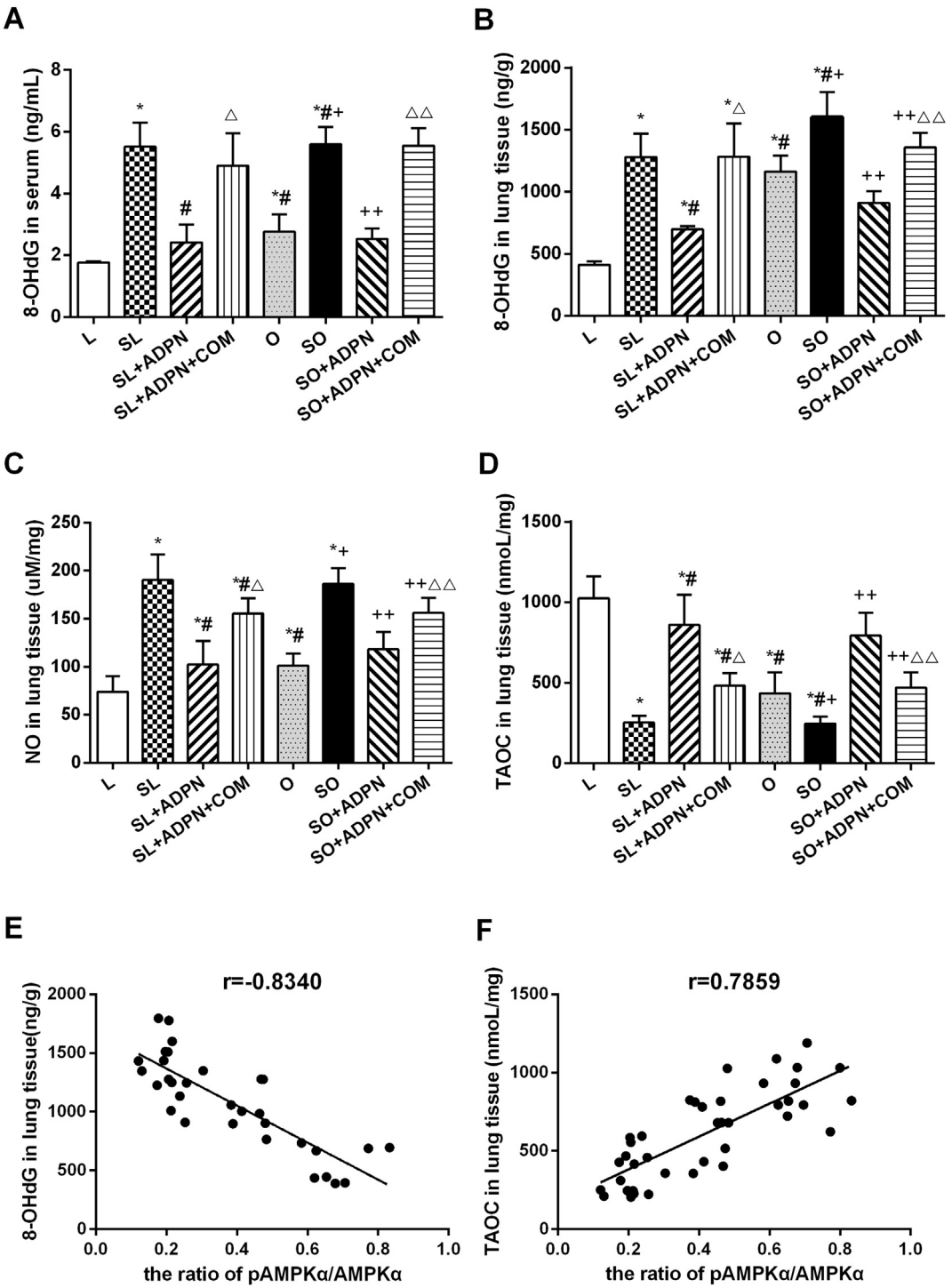

Fig. 5. Effect of adiponectin on 8-hydroxy-2′-deoxyguanosine (8-OHdG), nitric oxide (NO), and total antioxidant capacity (TAOC); the correlation analysis between the 8-OHdG, TAOC levels in lung tissue and the AMPK activity. (A) 8-OHdG level in blood serum. (B) 8-OHdG level in lung tissue. (C) NO level in lung tissue. (D) TAOC level in lung tissue. 8-OHdG level in blood serum and lung tissue, NO level and TAOC level in lung tissue were measured by ELISA kits. Values are means ± SD. $^{*}P < 0.05$ as compared to L group; $^{\#}P < 0.05$ as compared to SL group; $^{\triangle}P < 0.05$ as compared to SL + ADPN group; $^{+}P < 0.05$ as compared to O group; $^{++}P < 0.05$ as compared to SO group; $^{\triangle\triangle}P < 0.05$ as compared to SO + ADPN group. (E) The correlation between 8-OHdG level in lung tissue and AMPK activity. (F) The correlation between TAOC level in lung tissue and AMPK activity. The pAMPKα/AMPKα ratio was considered to show AMPK activity.

inhibited with compound C treatment. iNOS and Bcl-2 protein expression were lower than NF-κB p65 in lung tissue. There was a negative correlation between NF-κB p65 and AMPK activity (Fig. 9D), iNOS, and Bcl-2 protein expression in lung tissue ($r = -7028$, $r = -0.7271$; $P < 0.000\ 1$). NF-κB p65 protein expression was positively correlated with Bcl-2 protein expression in lung tissue ($r = 0.6903$; $P < 0.000\ 1$).

4. Discussion

The current study provided the first direct evidence to demonstrate that the role of adiponectin and its mechanism in obesity-related asthma. We found that IgE level significantly increased in asthma mice and obesity-related asthma mice which indicated a mouse model of obesity-related allergic asthma was well-established. Airway resistance,

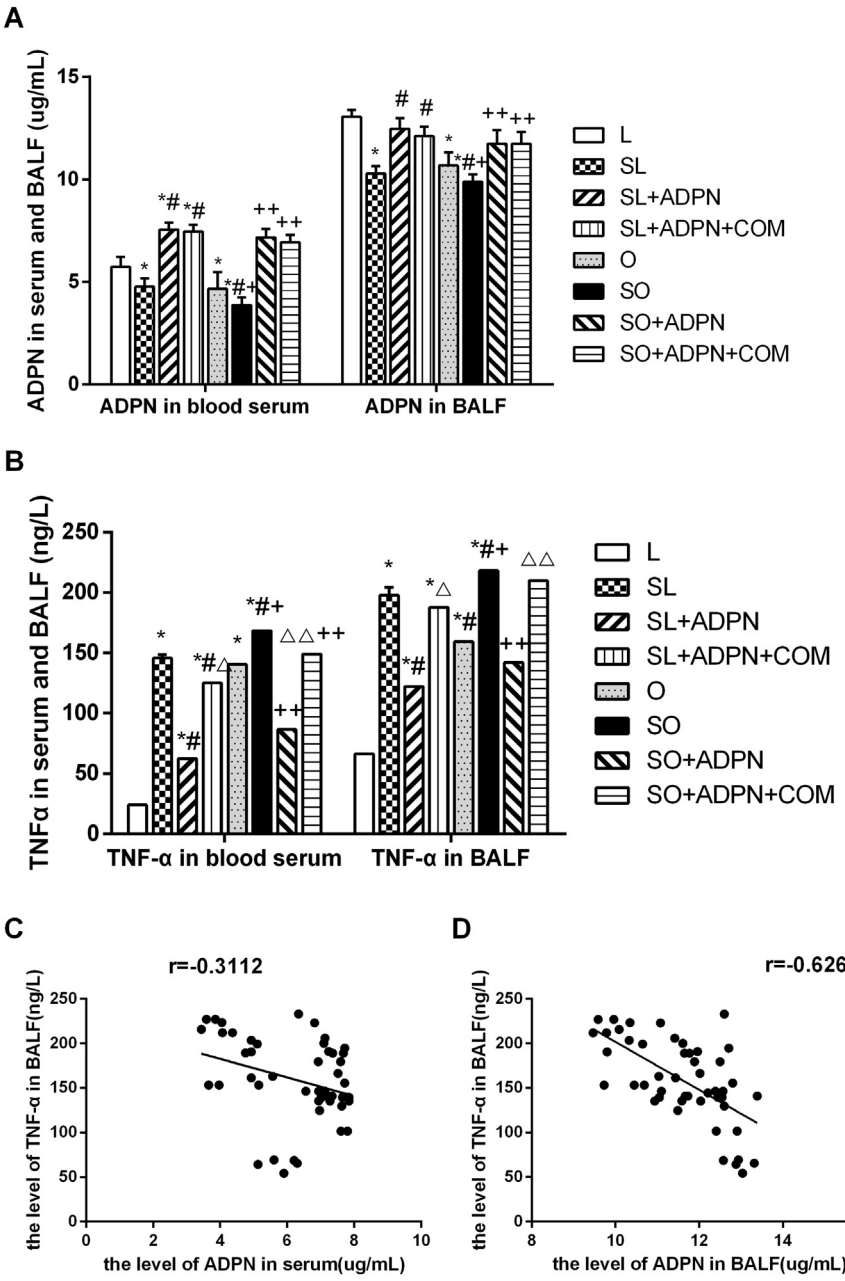

Fig. 6. The total adiponectin (ADPN) and TNF-α levels in blood serum and BALF; the correlation analysis between ADPN and TNF-α levels. (A) Total ADPN level in blood serum and BALF. (B) TNF-α level in blood serum and BALF. Total ADPN and TNF-α levels were measured by ELISA kits. Values are means ± SD. $^{*}P < 0.05$ as compared to L group; $^{\#}P < 0.05$ as compared to SL group; $^{\triangle}P < 0.05$ as compared to SL + ADPN group; $^{+}P < 0.05$ as compared to O group; $^{++}P < 0.05$ as compared to SO group; $^{\triangle\triangle}P < 0.05$ as compared to SO + ADPN group. (C) The correlation between TNF-α level in BALF and the level of ADPN in serum. (D) The correlation between TNF-α level in BALF and the level of ADPN in BALF.

airway inflammation, and oxidative stress were exacerbated in obesity-related asthma mice compared with asthma mice. These findings lend support for the suggestion that obesity is an independent risk factor for asthma. We found that adiponectin treatment could reduce exacerbation of obesity-related asthma, relieve inflammation, and improve AMPK activity while decrease iNOS, Bcl-2, NF-κB p65 levels in lung of obesity-related asthma mice. In addition, the AMPK inhibitor, Compound C, inhibiting AMPK kinase activity, was found to reverse the effects of adiponectin. These results suggested that the beneficial effects of adiponectin in obesity-related asthma were likely to be AMPK-dependent.

The adiponectin level in serum and BALF as well as adiponectin receptors (AdipoR1 and AdipoR2) mRNA expression in lung tissue were significantly decreased in obesity-related asthma mice. These findings align with the study by Shore et al. [9]. With exogenous adiponectin treatment, both the adiponectin level and adiponectin receptors mRNA expression were increased. Adiponectin improved the total airway resistance in obesity-related asthma mice, which was significantly higher than asthma mice, indicating that diet-related obesity might increase sensitivity to allergens [28]. Moreover, the exogenous ADPN treatment inhibited airway inflammation and oxidative stress in the mice with obesity-related asthma.

Inflammatory cells, especially eosinophils, play a crucial role in the pathogenesis of asthma. Eosinophil accumulation was observed in the lungs of the mice with obesity-related asthma, which further induced eosinophilic lung inflammation [29,30]. We observed a significant increase in extensive inflammatory cells surrounding the bronchial and bronchiolar segments in obesity-related asthma mice. It has been reported that adiponectin deficiency increases allergic airway inflammation which is associated with accumulation of eosinophils in the airways [31]. Cumulative studies have demonstrated that the protein hormone adiponectin can result in beneficial anti-inflammatory effects [32,33]. In the present study, we found that the population of eosinophil cells was significantly affected by adiponectin in obesity-related asthma mice. In addition, neutrophilic inflammation was observed at a greater extent in obesity-related asthma mice compared with asthma

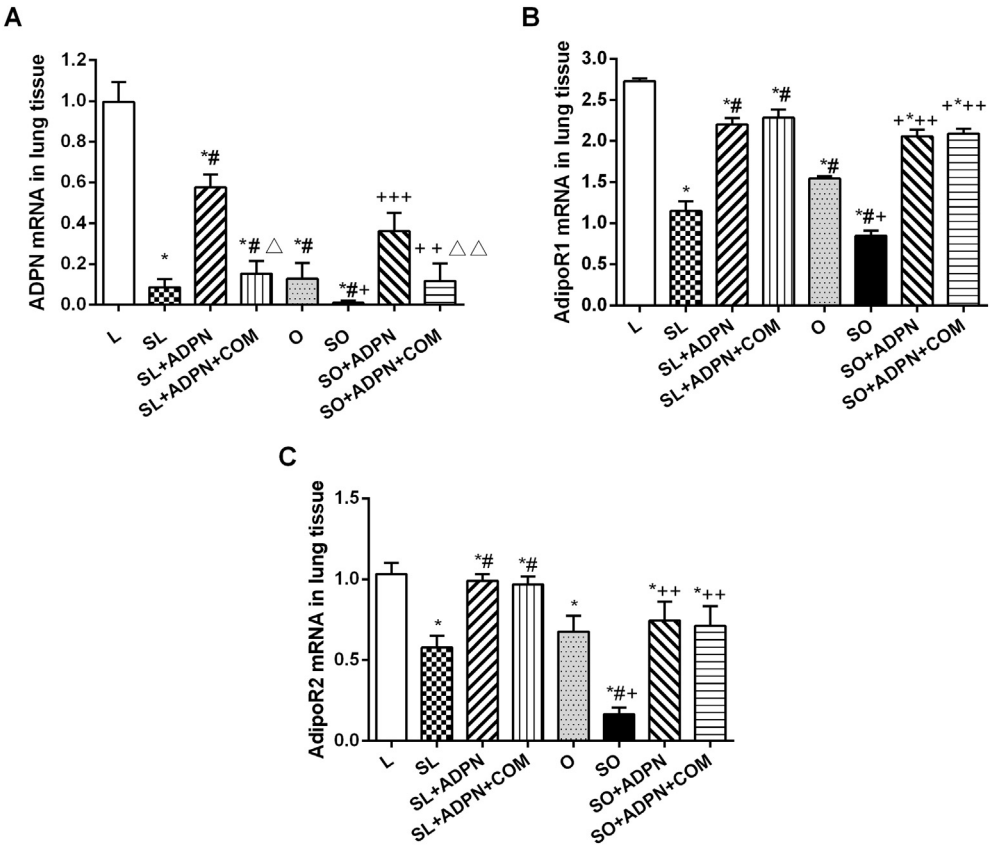

Fig. 7. Expression of adiponectin (ADPN) and adiponectin receptors (AdipoR1 and AdipoR2) genes in lung tissues. (A) Relative expression of mRNA for ADPN in lung tissue. (B) Relative expression of mRNA for AdipoR1 in lung tissue. (C) Relative expression of mRNA for AdipoR2 in lung tissue. Relative expression of mRNA for ADPN, AdipoR1 and AdipoR2 were quantified by quantitative RT-PCR analysis. Values are means ± SD. $^{*}P < 0.05$ as compared to L group; $^{\#}P < 0.05$ as compared to SL group; $^{\triangle}P < 0.05$ as compared to SL + ADPN group; $^{+}P < 0.05$ as compared to O group; $^{++}P < 0.05$ as compared to SO group; $^{\triangle\triangle}P < 0.05$ as compared to SO + ADPN group.

mice that mainly showed eosinophilic inflammation. Leiria et al. suggested there might be a neutrophilic inflammatory process involved in obesity-related asthma [34]. Some studies indicated that obesity might delay the migration of eosinophils into the airway lumen, allowing these cells to remain longer in lung parenchyma, accounting for the relatively lower eosinophils in BALF of obesity-related asthma mice compared to asthma mice [35]. Similar to the phenomenon observed in eosinophil cells, adiponectin treatment also reduced the elevation of neutrophil cells in lungs of obesity-related asthma mice. Our findings suggested that eosinophils and neutrophils, both of which contribute to allergic airway responses, were the possible cellular target of adiponectin. Further studies exploring the exact cellular types that adiponectin targets in obesity-related asthma model are needed.

Eotaxin and MPO were secretory products of eosinophils and neutrophils, respectively. Both of the two biomarkers were significantly increased in asthma mice and obesity-related asthma mice. And the

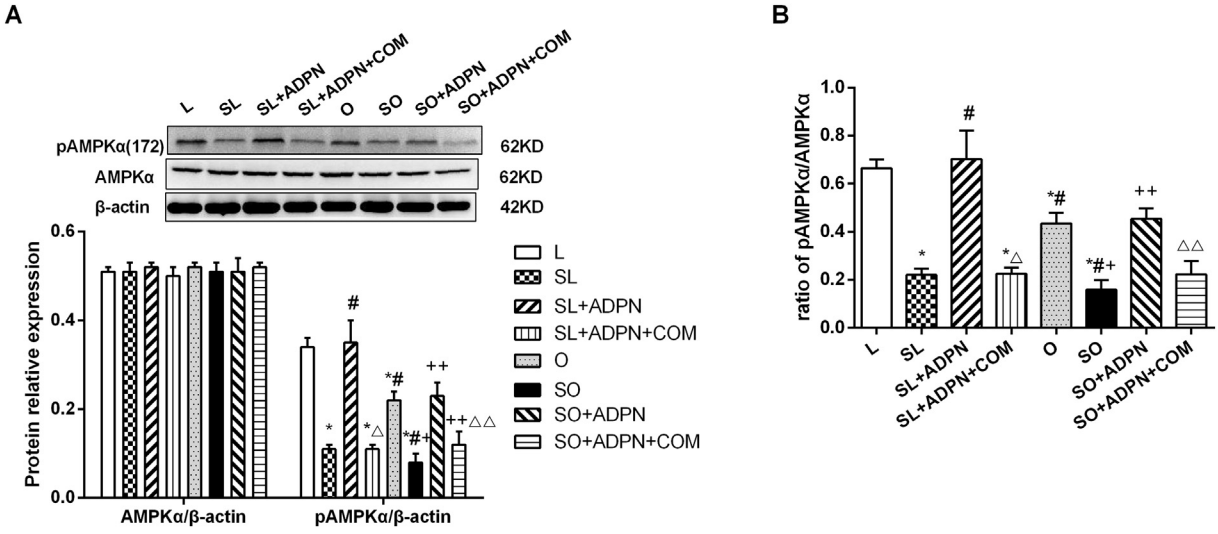

Fig. 8. Effect of adiponectin on AMPKα, pAMPKα protein expression and AMPK activity (the ratio of pAMPKα/AMPKα ratio) in lung tissue. (A) AMPKα and pAMPKα protein expression in lung tissue. Image is representative of three different blots. (B) AMPK activity (pAMPKα/AMPKα ratio) in lung tissue. AMPKα and pAMPKα protein expression were analyzed by western blotting using AMPKα and pAMPKα antibodies. The same blot was reprobed with anti-β-actin for loading control. Values are means ± SD. $^{*}P < 0.05$ as compared to L group; $^{\#}P < 0.05$ as compared to SL group; $^{\triangle}P < 0.05$ as compared to SL + ADPN group; $^{+}P < 0.05$ as compared to O group; $^{++}P < 0.05$ as compared to SO group; $^{\triangle\triangle}P < 0.05$ as compared to SO + ADPN group.

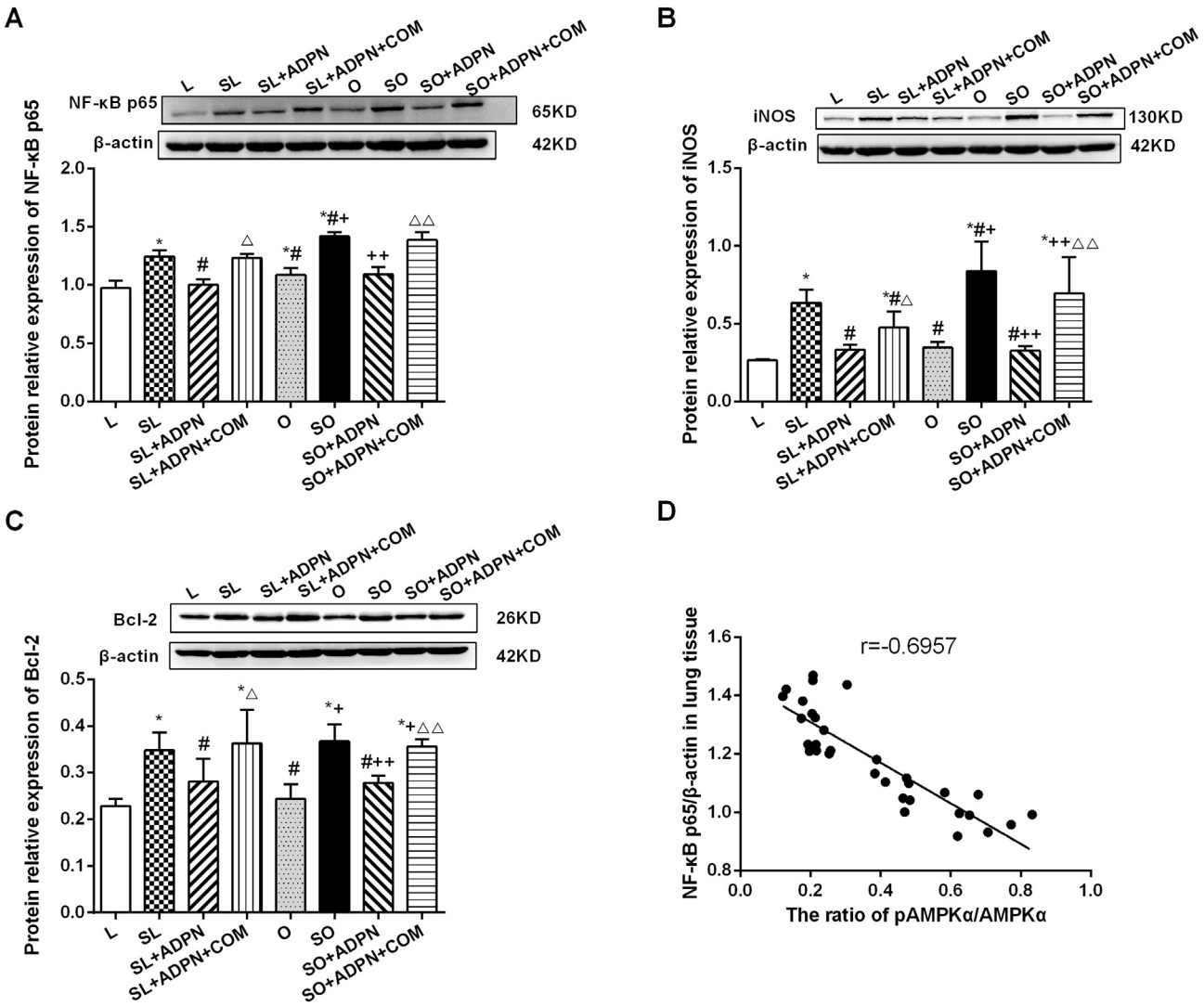

Fig. 9. Effect of adiponectin on NF-κB p65, iNOS, and Bcl-2 protein expression in lung tissue; the correlation analysis between NF-κB p65 and AMPK activity (the ratio of pAMPKα/AMPKα). (A) NF-κB p65 protein expression in lung tissue. (B) iNOS protein expression in lung tissue. (C) Bcl-2 protein expression in lung tissue. NF-κB p65, iNOS, and Bcl-2 protein expression were analyzed by western blotting using the specific antibodies (NF-κB p65, iNOS and Bcl-2). Image is representative of two different blots. The same blot was reprobed with anti-β-actin for loading control. Values are means ± SD. $^{*}P < 0.05$ as compared to L group; $^{\#}P < 0.05$ as compared to SL group; $^{\triangle}P < 0.05$ as compared to SL + ADPN group; $^{+}P < 0.05$ as compared to O group; $^{++}P < 0.05$ as compared to SO group; $^{\triangle\triangle}P < 0.05$ as compared to SO + ADPN group. (D) The correlation between NF-κB p65 protein expression in lung tissue and AMPK activity. The pAMPKα/AMPKα ratio was considered to show AMPK activity.

MPO level in obesity-related asthma mice was significantly higher than asthma mice. All above data suggested that obesity-related asthma mice possibly had neutrophilic inflammation while OVA-induced asthma mice mainly showed eosinophilic inflammation. Yamamoto et al. demonstrated that adiponectin attenuated eotaxin-induced eosinophil adhesion activity [36]. We found that adiponectin treatment alleviated pathological changes in lung tissue, reduced eosinophils and total cell numbers in BALF, down-regulated eotaxin and MPO levels, suggesting that adiponectin facilitated cell migration into the airway lumen, accelerating the clearance of pulmonary inflammation in obesity-related asthma mice. This partly explained why adiponectin appeared to provide a protective effect against lung inflammation in obesity-related asthma mice.

We further confirmed that the mechanism of adiponectin on the anti-inflammatory process was that adiponectin promoted apoptosis of inflammatory cells. Bcl-2 is an inhibitory apoptosis gene in the promoter region which contains NF-κB binding sites, including DNA-binding subunit p50 and transactivation subunit p65 [37–39]. NF-κB regulates Bcl-2 expression by raising Bcl-2 expression directly through transcription and promoting Bcl-2 expression through TNF-α [38,40]. In this study, NF-κB p65 and Bcl-2 protein expression in lung tissue as well as TNF-α levels in BALF and serum were significantly increased, all of which resulted in inflammatory response in obesity-related asthma mice. Studies have found that adiponectin down-regulates pro-inflammatory pathways including NF-κB signaling and TNF-α [41,42]. Our findings confirmed that adiponectin inhibited NF-κB p65 and reduced the expression of Bcl-2 as well as TNF-α level in obesity-related asthma mice, finally resulting in inflammatory cell apoptosis. These findings align with previous studies that adiponectin inhibits TNF-α expression, including suppression of TNF-α-induced iκB-α phosphorylation and subsequent NF-κB activation kinase, and relieves inflammation [43–45].

Evidence suggests that oxidative stress plays a key role in the pathogenesis and amplification of inflammation in asthma, as Dut R et al. reported that asthma was associated with oxidative stress both in the systemic circulation and in the airways [46]. Moreover, obesity is related with increased oxidative stress and systemic inflammation. We found diet-induced obesity had increased airway and systematic

oxidative stress to asthma mice, evaluated using 8-OHdG, NO, iNOS and TAOC levels in blood serum or lung tissue [47,48]. Motoshima et al. suggested that adiponectin could suppress oxidative stress [49]. We found that adiponectin inhibited the expression of iNOS and NO overproduction as well as NF-κB p65 expression in obesity-related asthma mice. The promoter region of the iNOS gene has many binding sites for transcription factors, including NF-κB. The molecular mechanisms for iNOS gene transcription occur mainly via activation of the transcription factor NF-κB [50]. Findings of the current study suggested that adiponectin inhibited NF-κB p65 binding to the iNOS promoter region, which possibly explained the reduced oxidative stress in obesity-related asthma mice with administration of adiponectin.

It has been well established that AMPK is a downstream effector of adiponectin. Adiponectin directly interacts with AdipoR1 and mediates its effects by activating AMPK [1]. Activation of AMPK can inhibit inflammatory processes [51]. We observed, in this work, that phosphorylated AMPK was significantly inhibited in lung tissue derived from obese asthmatic mice compared with the control group, and adiponectin administration induced significant increased activation of AMPK, which was reversed using an AMPK inhibitor (compound C). Similarly, almost all the protective effects of adiponectin in obesity-related asthma mice were inversely regulated with administration of this AMPK inhibitor. These findings suggested that the beneficial role of adiponectin in anti-inflammatory and antioxidant processes was, at least in part, dependent on AMPK activation, and that the AMPK signaling pathway could be an important regulator in this mouse model of obesity-related asthma.

In conclusion, adiponectin alleviates airway hyper-responsiveness, airway inflammation and oxidative stress in a murine model of obesity-related asthma. Adiponectin may protect against obesity-related asthma via activating the AMPK pathway, a suggestion supported by findings using the AMPK inhibitor. Thus, the findings of the present study suggest that adiponectin can be used as a new pharmacological agent for treating asthma in obese individuals.

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Declarations of interest

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