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The protective effect of oleanolic acid on NMDA-induced MLE-12 cells apoptosis and lung injury in mice by activating SIRT1 and reducing NF-κB acetylation

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ABSTRACT

Overactivation of the *N*-methyl-D-aspartate (NMDA) receptor promotes oxidative stress, aggravates the inflammatory response and induces excitotoxic lung injury. NMDA is a synthetic agonist that selectively activates the NMDA receptor. Oleanolic acid (OA) is a natural anti-inflammatory and antioxidant compound. This study investigated the effect and possible mechanism of OA on NMDA-induced acute lung injury (ALI) in mice. OA pretreatment alleviated NMDA-induced histological lung changes and ameliorated pulmonary oedema and pulmonary permeability. At the same time, OA inhibited inflammatory cell infiltration and decreased the levels of tumour necrosis factor (TNF)-α, interleukin (IL)-6 and IL-1β in the lung and bronchoalveolar lavage fluid (BALF). OA markedly decreased malondialdehyde (MDA) production and increased the superoxide dismutase (SOD) and glutathione (GSH) contents of the lung in vivo. Meanwhile, we first found that NMDA increased LDH activity and decreased cell viability, and induced oxidative stress and apoptosis in mouse lung epithelial (MLE)-12 cells. By employing SRT1720 and sirtinol, the activator and inhibitor of sirtuin 1 (SIRT1), we found that SRT1720 partially eliminated the increase in ROS , and sirtinol further promoted the increase in ROS caused by NMDA. OA increased MLE-12 cells viability and attenuated oxidative stress after NMDA challenge in vitro. OA suppressed NMDA-induced MLE-12 cells apoptosis, while sirtinol inhibited the effect of OA. In addition, OA significantly upregulated the levels of SIRT1, nuclear-related factor 2(Nrf2) and Bcl-2 protein and downregulated the levels of acetylated nuclear factor-kappa B (NF-κB), NLRP3 and Bax protein. In conclusion, OA attenuated NMDA-induced excitotoxic lung injury, potentially through its anti-inflammatory, antioxidative stress and anti-apoptotic effects. The mechanism may be related to activating SIRT1 and reducing NF-κB acetylation.

1. Introduction

Acute lung injury (ALI) is a clinically critical disease with a complicated pathogenesis. ALI is characterized by lung epithelium injuries, overactivation of neutrophils, upregulation of pro-inflammatory cytokines, and increased reactive oxygen species (ROS) production [1,2]. Many direct and indirect factors can cause ALI, and there are currently no effective treatment measures or drugs for ALI.

In recent years, the role of the *N*-methyl-D-aspartate (NMDA) receptor in lung tissue has attracted increasing attention. The NMDA receptor (NMDAR) is the key receptor mediating glutamate (Glu) excitotoxicity and has three subunits: NR1, NR2 and NR3. NR1, the functional subunit, is widely expressed in lung tissue, alveolar macrophages and neutrophils [3–5]. Abnormal activation of the NMDAR is involved in the occurrence and development of many diseases. The NMDAR mediates oxidant lung injury [6] and regulates inflammatory processes in nervous and non-nervous tissues, such as neuritis [7] and renal injury [8]. In a pathological state, NMDAR activation, high Ca^{2+} influx and ROS production can cause cell necrosis or apoptosis [9]. The NMDAR is highly expressed in the lung tissues of septic rats and lipopolysaccharide (LPS)-induced mice. NMDAR blockade using MK801 reduces pro-inflammatory cytokine levels and the oxidative stress response and alleviates lung injury [10,11]. Our previous research found that NMDAR activation is involved in bleomycin-induced pulmonary fibrosis [12]. Memantine, another NMDA receptor antagonist, has been shown to ameliorate pulmonary inflammation in a mouse model of chronic obstructive pulmonary disease (COPD) [13]. NMDA is a synthetic agonist that selectively activates the NMDAR, and its

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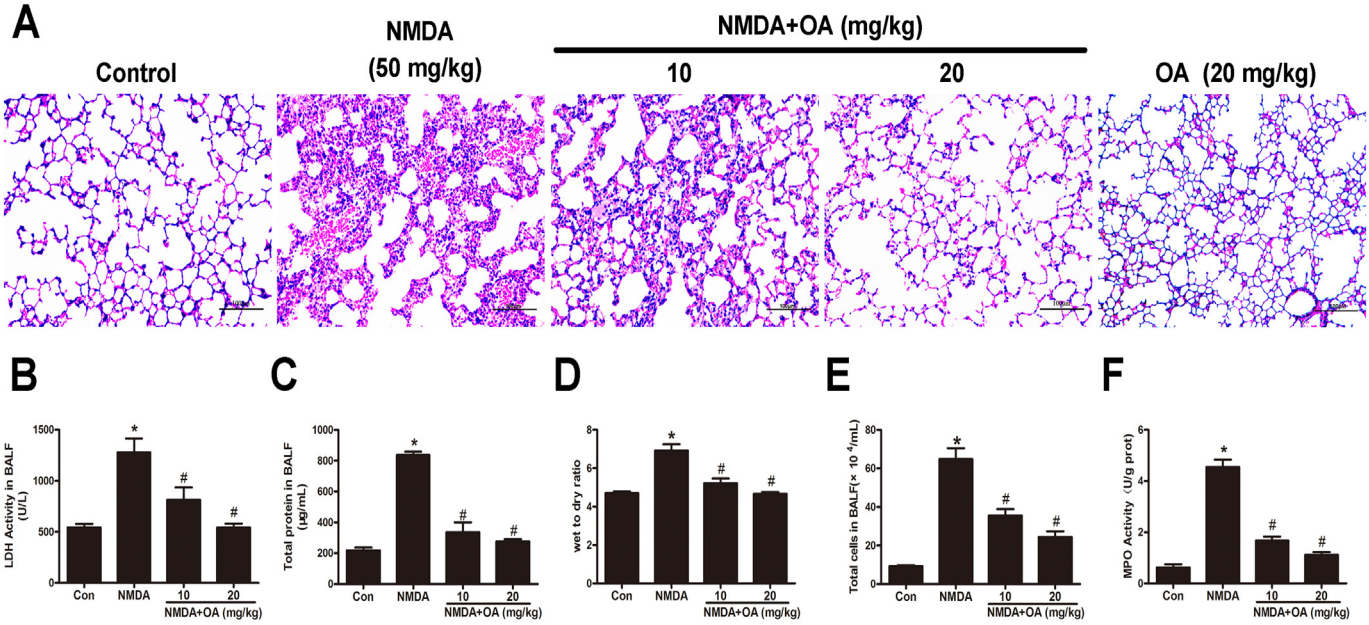

Fig. 1. OA alleviated NMDA-induced lung injury in mice. (A) Histopathological examination was performed using H&E staining (×200). Bar: 100 μm (n = 5). (B) The activity of LDH in BALF (n = 4). (C) Total protein in BALF (n = 5). (D) W/D ratio (n = 5). (E) Total cells (n = 5). (F) MPO activity in lung tissues was determined (n = 6). Values represent the mean ± SEM, *P < 0.05 vs. the control group, #P < 0.05 vs. the NMDA group.

intraperitoneal injection can induce ALI in mice [14]. These studies suggest that Glu-NMDAR signalling may play an important role in many acute or chronic lung diseases.

Oleanolic acid (OA) is a pentacyclic triterpenoid compound that is a natural active component of many plants and has strong anti-inflammatory and antioxidative activities [15–17]. OA can reduce the production of pro-inflammatory factors and ROS and improve lung morphofunction in mice with paraquat-induced ALI [18]. OA has a protective effect on silica-induced lung injury and fibrosis by inhibiting the transcriptional activity of nuclear factor-kappa B (NF-κB) [19]. Inhibition of the activation and transcriptional activity of NF-κB is an effective way by which OA exerts its anti-inflammatory effect [20]. In recent years, there has been interest in the study of natural plant therapy for Glu-induced neurotoxicity. Some natural compounds exhibit protective effects on NMDA-induced excitatory neurotoxicity in cortical neurons [21,22]. However, the protective effect of OA on NMDA-induced excitatory lung injury remains unclear. Therefore, inhibition of excitatory lung injury with plant-derived compounds is a possible therapeutic strategy.

Silent information regulator 1 (SIRT1; also known as sirtuin 1), a type III deacetylase, regulates multiple biological processes, including apoptosis, stress and inflammation through the deacetylation of histones and transcription factors [23]. SIRT1 negatively regulates the transcriptional activity of NF-κB by deacetylating the RelA/p65 subunit [24,25]. Lung SIRT1 expression is decreased in the lung tissue of patients with asthma and LPS-induced ALI rats [26,27], and SIRT1 may be a therapeutic target in COPD and pulmonary fibrosis [28,29].

This study investigated the potential effect of OA on excitatory lung injury induced by NMDA in mice and its possible mechanisms. This study may be useful for the development of plant treatments for NMDA-induced excitotoxic lung injury.

2. Materials and methods

2.1. Animals and experimental protocol

Sixty BALB/c mice (male, 18–20 g, SPF) were purchased from Hunan Jingda Laboratory Company (Hunan, Changsha). The animals were fed under standard experimental conditions of a 12-h light/dark cycle at a temperature of (25 ± 2 °C) and relative humidity of (55 ± 10%). After one week of adapting to laboratory conditions, the mice were randomly divided into four groups as follows: (1) control group (n = 15); (2) NMDA group (n = 15); (3) NMDA + OA (10 mg/kg/d) group (n = 15); (4) NMDA + OA (20 mg/kg/d) group (n = 15). Animals in the latter two groups were treated with OA by gavage (Santa Cruz, Dallas, USA) for 5 days. Twenty-four hours after the last dose on the 5th day, the mice were treated with an intraperitoneal injection of NMDA (50 mg/kg; Sigma; St. Louis, MO, USA). The mice in the other two groups were given the same volume of vehicle (1% sodium carboxymethyl cellulose) by gavage before intraperitoneal administration of 0.9% saline or NMDA. The mice were then sacrificed for further measurements. All procedures were performed according to the Guide for the National Institutes of Health (NIH) Guide for the care and use of animals in laboratory experiments, and the experimental protocol was approved by the Medical Ethics Committee of Jishou University (NO. 2016008).

2.2. Histopathological examination

Tissue from the lower lobe of the right lung was fixed with 4% buffered formalin solution overnight, dehydrated, embedded in paraffin, sliced continuously at a thickness of 4 μm, and stained with H&E. Slides were observed for morphological changes using a Leica photographic microscope (Leica Microscope Ltd., Wetzlar, Germany) (200 × magnification).

2.3. BALF analysis

The mice were anaesthetized with intraperitoneal sodium pentobarbital (30 mg/kg). Then, the right main bronchus was ligated, and the left lung was lavaged with 0.8 ml of 0.9% saline solution via the tracheal cannula three times. BALF was collected and separated by centrifugation (1200 rpm, 10 min, and 4 °C), and the supernatant was stored at −80 °C for further analysis. LDH activity and total protein in the supernatant were determined by an LDH cytotoxicity assay kit and BCA protein assay kit, respectively, according to the manufacturer's instructions (Nanjing Jiancheng Bioengineering Institute, China). The cell precipitate was resuspended in 100 μl phosphate-buffered saline

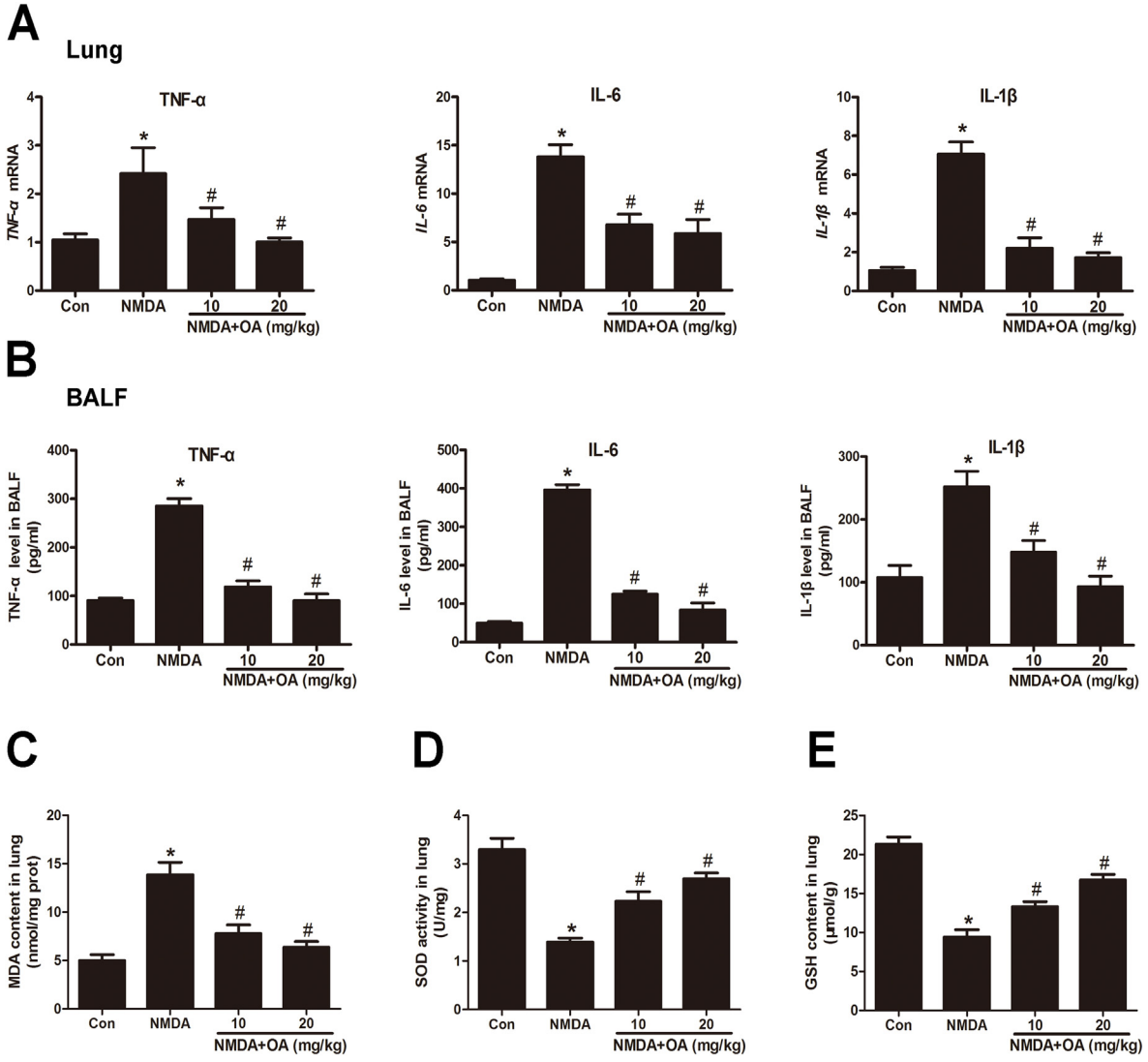

Fig. 2. OA reduced NMDA-induced inflammatory responses and oxidative stress in mice. (A) The levels of *TNF-α*, *IL-6* and *IL-1β* mRNA in lung homogenate were determined with real-time PCR (n = 6). (B) The levels of TNF-α, IL-6 and IL-1β protein in BALF were determined by enzyme-linked immunosorbent assay (ELISA). (n = 5). (C) MDA content, (D) SOD activity and (E) GSH content in lung tissues were determined (n = 6). Values represent the mean ± SEM, *P < 0.05 vs. the control group, #P < 0.05 vs. the NMDA group.

(PBS) and counted via a haemocytometer to obtain the total number of inflammatory cells.

2.4. W/D ratio assay

After weighing the lung, it was dried in an oven at 60 °C for 7 days to a constant weight. The W/D ratio was calculated to evaluate tissue oedema.

2.5. Determination of the levels of MPO, MDA, SOD and GSH

Lung tissues or MLE-12 cells were homogenized with 50 mM PBS (pH 7.4). The supernatants were collected after centrifugation at 3500 rpm for 15 min at 4 °C to determine the content of MPO, MDA, SOD and GSH using commercially available kits (Nanjing Jiancheng Bioengineering Institute, China) according to the manufacturer's instructions.

2.6. Real-time PCR

Total RNA from lung tissues was extracted using Trizol reagent (Invitrogen, USA). cDNA synthesis was performed with a reverse transcription kit (Fermentas, USA). SYBR Green quantitative PCR was performed on a Bio-Rad real-time PCR system (CFX96 Touch ™, Bio-Rad, USA). The conditions for the reaction were 40 cycles of 95 °C for 60 s, 95 °C for 30 s, 60 °C for 15 s, and 72 °C for 30 s, followed by 72 °C for 10 min. The primers for the targeted genes were as follows: TNF-α: 5′-GGA ACT GGC AGA AGA GGC ACT C-3′, 5′-GTA GAC AGA AGA GCG TGG TGG C-3′; IL-6: 5′-CTT GGG ACT GAT GCT GGT GAC A-3′, 5′-GCC TCC GAC TTG TGA AGT GGT A-3′; IL-1β: 5′-GCA CTA CAG GCT CCG AGA TGA A-3′, 5′-GTC GTT GCT TGG TTC TCC TTG T-3′; β-actin: 5′-TTC CAG CCT TCC TTC TTG-3′, 5′-GGA GCC AGA GCA GTA ATC-3′. Each PCR was conducted in triplicate, and β-actin was used as an endogenous control following the $2^{-\Delta\Delta CT}$ method.

2.7. Determination of inflammation levels

The concentrations of cytokines TNF-α, IL-1β and IL-6 in the supernatants of BALF were detected by ELISA kits according to the manufacturer's instructions (CUSABIO, China).

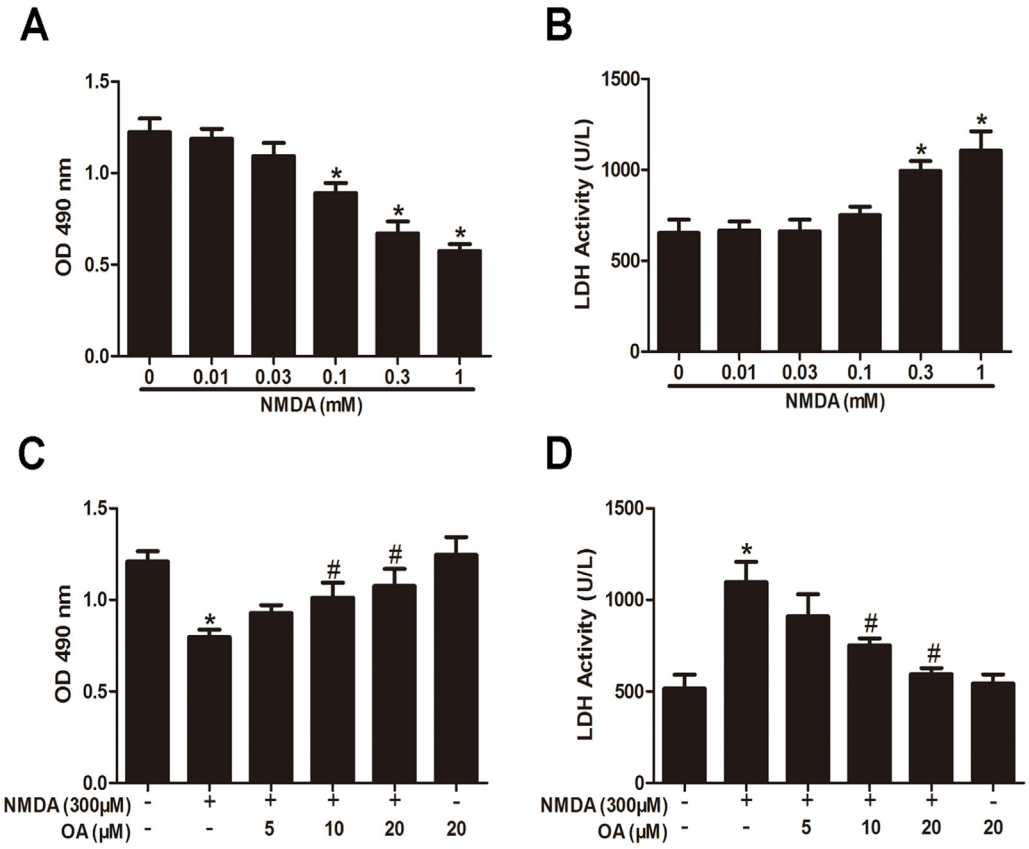

Fig. 3. OA promotes cell viability upon NMDA injury. (A) Effect of different concentrations of NMDA for 24 h on the cell viability of MLE-12 cells (n = 3, *P < 0.05 vs. the 0 mM NMDA group). (B) Effect of different concentrations of NMDA for 24 h on LDH activity in the supernatant of cultured MLE-12 cells (n = 3, *P < 0.05 vs. the 0 mM NMDA group). (C) Cell viability effects of different concentrations of OA on MLE-12 cells with or without NMDA treatment for 24 h (n = 5, *P < 0.05 vs. the vehicle group, #P < 0.05 vs. the NMDA group). (D) Effects of different concentrations of OA on MLE-12 cells with or without NMDA treatment for 24 h on LDH activity in the supernatant (n = 3, *P < 0.05 vs. the vehicle group, #P < 0.05 vs. the NMDA group). Values represent the mean ± SEM.

2.8. Western blot analysis

Lung tissues or MLE-12 cells were lysed by RIPA plus appropriate protease inhibitors (Auragene, Changsha, China). Protein content was measured using a BCA kit. Proteins (30 μg/lane) were separated by 8–10% SDS-PAGE electrophoresis (120 V for 90 min) and transferred onto a PVDF membrane (Millipore, USA). Membranes were incubated in 5% BSA blocking buffer at room temperature for 2 h and incubated at 4 °C overnight with primary antibodies targeting Sirt1, Bcl-2, Bax, tubulin (Proteintech), ac-NF-kB p65 (CST, Danvers, USA), Nrf2, NLRP3(Bioss, Beijing, China). After the membranes were washed with 0.5% TBS-Tween three times for 30 min, they were incubated with a secondary antibody (Boster, Wuhan, China) at room temperature for 1 h. The protein blots were detected using enhanced chemiluminescence (ECL) reagent. The band intensity of the target protein was analysed using ImageJ software. Tubulin was used as a loading control.

2.9. Cell culture of MLE-12 cells

MLE-12 cells (ATCC, USA) were cultured in DMEM (GIBCO, USA) basal medium supplemented with 10% foetal bovine serum (FBS, GIBCO, USA) and placed in a cell culture chamber with 5% CO_2 at 37 °C. Cell cultures were split when confluency reached 80–90%, and the medium was replaced every other day. MLE-12 cells were treated with NMDA (300 μM, Sigma, USA) alone or with the indicated concentration of OA (10 μM, 20 μM) (Santa Cruz, Dallas, USA) for 24 h. DMSO (0.1%) was used as the vehicle in the control group and NMDA group. Cells and culture medium were collected for further examination.

2.10. Cell viability assay

MTT (Sigma, USA) assay was performed to measure cell viability. Cells were seeded into 96-well culture plates (5×10^3 per well), and the growth media were supplemented with the indicated concentrations of NMDA (0, 0.01, 0.03, 0.1, 0.3 or 1 mM) for 24 h. Then, the growth media were replaced with serum-free medium containing MTT (5 mg/ml) for 4 h. The medium was then removed, and DMSO (150 μl) was added to each well. The absorbances of the resulting formazan were measured at 490 nm using a microplate reader (Thermo Fisher Scientific, USA) according to the manufacturer's instructions.

2.11. LDH activity assay

Cells were seeded into 96-well culture plates (5×10^3 per well), and the growth media were supplemented with the indicated concentrations of NMDA (0, 0.01, 0.03, 0.1, 0.3 or 1 mM) for 24 h. The supernatant was collected to measure LDH activity according to the manufacturer's instructions (Nanjing Jiancheng Bioengineering Institute, China). The optical density was measured at 450 nm using a microplate reader (Thermo Fisher Scientific).

2.12. Determination of ROS levels

ROS were determined by fluorescence analysis (DCFH-DA assay, Sigma, USA). Cells were stained with 10 μM dichloro-dihydro-fluorescein diacetate (DCFH-DA) at 37 °C for 20 min. DCF fluorescence intensities were determined by Varioskan Flash (Thermo Fisher Science, USA) excitation and emission wavelengths of 488 and 525 nm. Relative ROS content was normalized to the total protein content.

2.13. Cell apoptosis analysis

Cell apoptosis was detected by flow cytometry analysis using a PI staining kit following the manufacturer's instructions (FACSalibur, BD, UA). Briefly, cells were digested with 0.25% trypsin (without EDTA) and collected after centrifugation (1200 rpm, 10 min, and 4 °C). Cell suspensions were fixed in 70% cold ethanol overnight (−20 °C). After

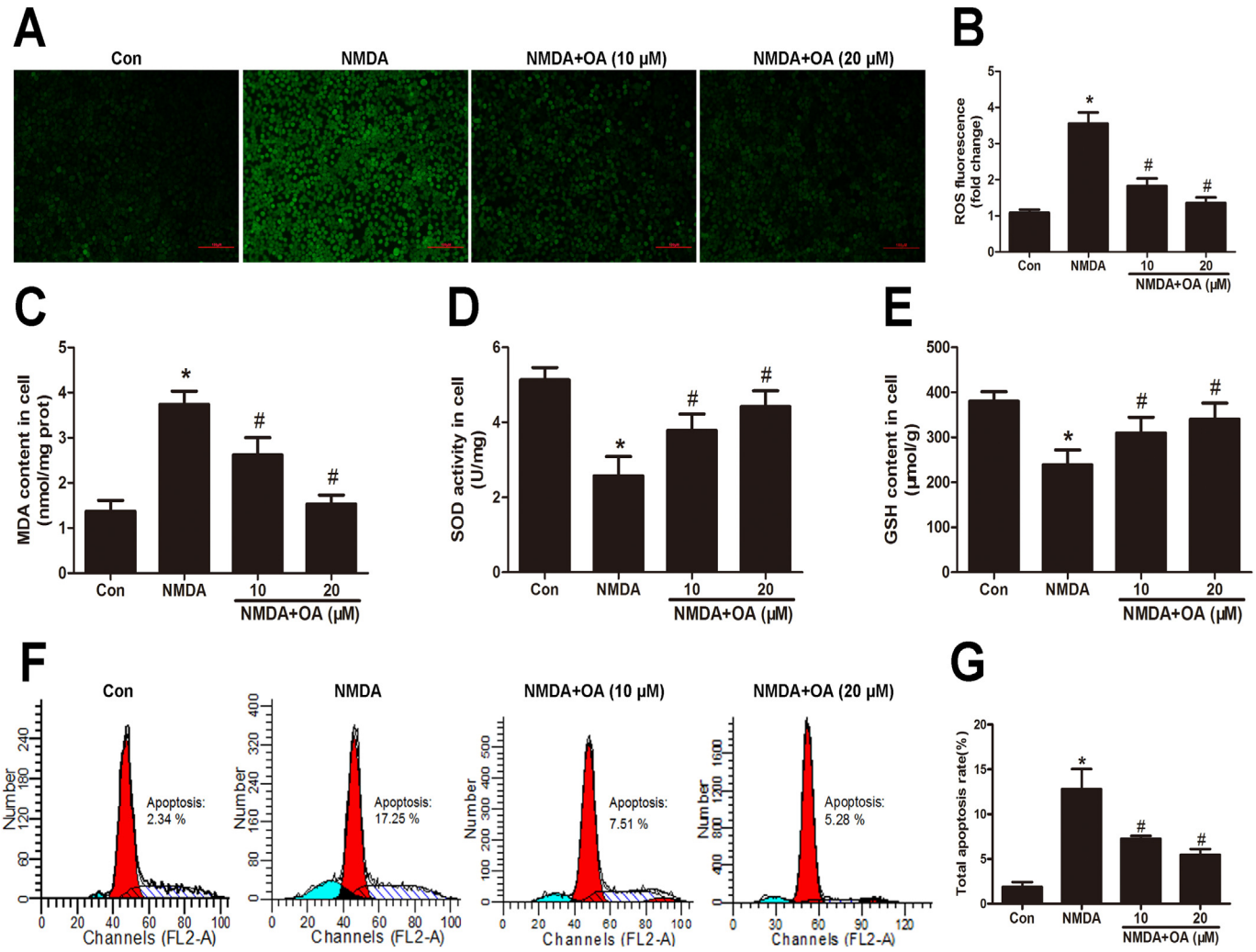

Fig. 4. OA blocks NMDA-induced oxidative stress and apoptosis in MLE-12 cells. (A, B) ROS production in MLE-12 cells was determined (n = 3). Bar: 100 μm (C) MDA content in MLE-12 cells was determined (n = 5). (C) SOD activity in MLE-12 cells was determined (n = 5). (E) GSH content in MLE-12 cells was determined (n = 5). (F) Apoptotic cells were identified by propidium iodide (PI) staining (n = 3). (G) Quantitative histogram of total apoptotic cells. Values represent the mean ± SEM. *P < 0.05 vs. the control group, #P < 0.05 vs. the NMDA group.

centrifugation, cells were washed once with 1 ml PBS, treated with 500 μl PBS containing 50 μg/ml PI, and incubated at 4 °C for 30 min before testing. Data were analysed using Modifit LT software (VSH, USA).

2.14. Statistical analysis

Data were expressed as the means ± SEM, and statistical analysis was analysed using SPSS 18.0 software (Chicago, IL, USA). The significance of the difference was determined by one-way analysis (ANOVA) followed by Tukey's method. Statistical significance was assigned at P < 0.05.

3. Results

3.1. Effect of OA on NMDA-induced lung injury in mice

Haematoxylin and eosin (H&E)-stained lung sections revealed that the NMDA group exhibited more inflammatory cell infiltration and alveolar wall destruction than the control group (Fig. 1A). OA pretreatment significantly attenuated the pathological changes induced by NMDA. Importantly, 20 mg/kg OA alone had no effect on normal mice and did not cause lung injury. OA pretreatment reduced lactate dehydrogenase (LDH) activity in the bronchoalveolar lavage fluid (BALF) of mice exposed to NMDA (Fig. 1B). NMDA induced pulmonary permeability changes and increased the BALF protein concentration, but OA pretreatment significantly reduced the protein concentration (Fig. 1C). The lung wet-to-dry (W/D) ratio, which reflects the effect of NMDA on pulmonary oedema, was higher in the group stimulated with NMDA than in the control group, and pretreatment with OA decreased the W/D ratio (Fig. 1D). NMDA increased the total number of inflammatory cells in BALF, which was reduced by OA pretreatment (Fig. 1E). Myeloperoxidase (MPO) activity, an important indicator of neutrophil infiltration, in lung tissue of NMDA-induced ALI mice was significantly reduced by OA pretreatment (Fig. 1F). These findings suggest that OA alleviates NMDA-induced lung injury.

3.2. Effect of OA on NMDA-induced inflammatory responses and oxidative stress in mice

Next, we analysed the effect of OA on the NMDA-induced inflammatory response and oxidative stress. The levels of tumour necrosis factor (TNF)-α, interleukin (IL)-6 and IL-1β in the lung tissues and BALF in the NMDA group were higher than those in the control group, but the increase in these pro-inflammatory cytokines was inhibited by OA pretreatment (Fig. 2A, B). Oxidative stress is an important factor in the occurrence and development of lung injury. Malondialdehyde (MDA) is

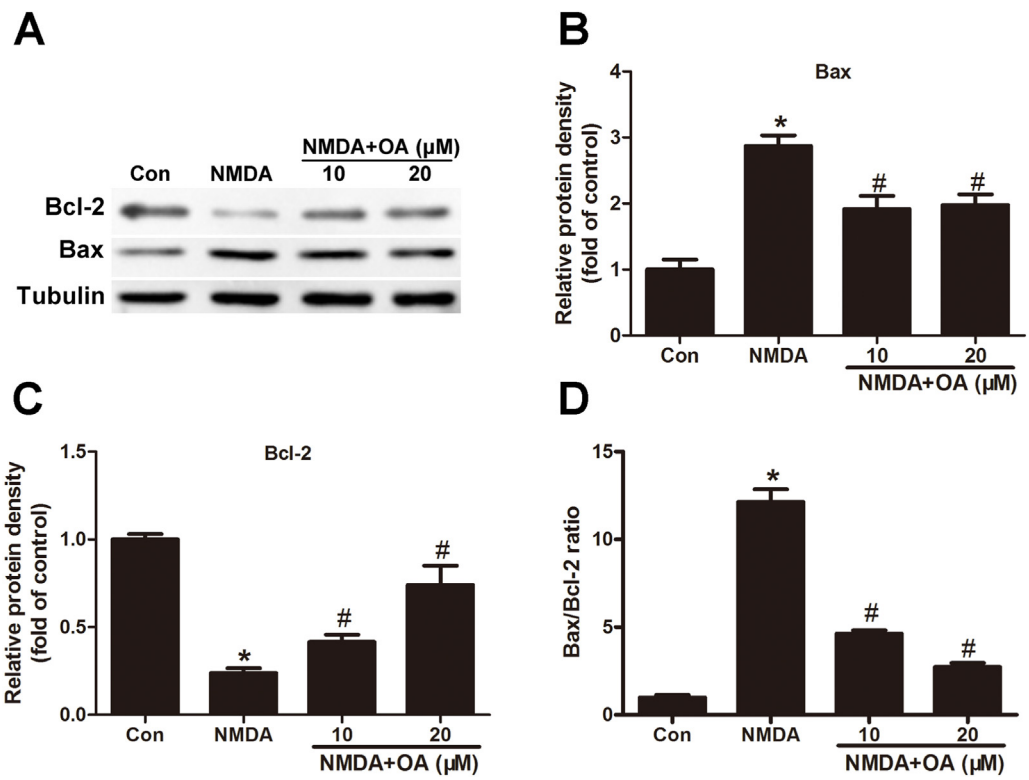

Fig. 5. Effects of OA on Bax and Bcl-2 protein expression. (A) The protein expression of Bax and Bcl-2 in MLE-12 cells was detected by Western blotting (n = 3). (B) Quantitative histograms of Bax protein expression. (C) Quantitative histograms of Bcl-2 protein expression. (D) The ratio of Bax/Bcl-2 is expressed as the fold change compared with the control. Values represent the mean ± SEM. *P < 0.05 vs. the control group, #P < 0.05 vs. the NMDA group.

a marker of lipid peroxidation, and the detection of MDA content can reflect oxidative stress in lung tissue. Here, the NMDA group had greater MDA production in the lung tissue than the control group, and this increase was significantly decreased by OA pretreatment (Fig. 2C). Antioxidant enzymes such as superoxide dismutase (SOD) and glutathione (GSH) are important substances that protect against oxidative stress. OA pretreatment evidently attenuated the SOD and GSH depletion caused by NMDA in mice (Fig. 2D, E). These results indicate that OA treatment reduces inflammatory responses and oxidative stress in NMDA-induced lung injury mice.

3.3. Effect of OA on the survival rate of NMDA-induced MLE-12 cells

We initially evaluated the effect of NMDA stimulation on the mouse lung epithelial cell line, MLE-12. We treated MLE-12 cells with increasing concentrations of NMDA for 24 h. MTT assays revealed that NMDA at a concentration of 0.1 mM significantly decreased cell viability (Fig. 3A). Furthermore, LDH activity in the supernatants of cultured MLE-12 cells was significantly increased after exposure to 0.3 and 1 mM NMDA (Fig. 3B). Next, we found that OA significantly increased cell viability of NMDA-treated MLE-12 cells in a concentration-dependent manner (Fig. 3C) and reduced LDH activity (Fig. 3D). In addition, 10 and 20 μM OA effectively protected against NMDA injury; therefore, these concentrations were used in later in vitro studies.

3.4. Effects of OA on NMDA-induced oxidative stress and apoptosis in MLE-12 cells

ROS produce excessive aggravation of the inflammatory response. We found that OA attenuated the production of ROS in MLE-12 cells induced by 0.3 mM NMDA for 24 h (Fig. 4A, B). As shown in Fig. 4C, the MDA content was markedly increased in MLE-12 cells after NMDA treatment, and OA markedly reduced MDA content. The levels of SOD and GSH were significantly lower in the NMDA treatment group than in the control group, and OA significantly increased SOD activity and GSH content (Fig. 4D, E). Then, we detected MLE-12 cells apoptosis by flow cytometry. NMDA significantly promoted MLE-12 cells apoptosis, but OA inhibited NMDA-induced apoptosis of MLE-12 cells (Fig. 4F, G). These findings indicate that OA alleviates NMDA-induced oxidative stress and apoptosis in MLE-12 cells.

3.5. Effects of OA on Bax and Bcl-2 expression

Bcl-2 family members include both anti-apoptotic and pro-apoptotic proteins. Compared to the control group, the group stimulated with NMDA exhibited anti-apoptotic factor Bcl-2 downregulation and pro-apoptotic factor Bax upregulation, thus increasing the Bax/Bcl-2 ratio. OA significantly suppressed the expression of Bax, upregulated the expression of Bcl-2 and reversed the ratio of Bax/Bcl-2 (Fig. 5). This effect of OA may be a way in which OA prevents MLE-12 cells from undergoing NMDA-induced apoptosis.

3.6. Effects of SIRT1 on NMDA-treated MLE-12 cells

MK801 is a noncompetitive channel antagonist of the NMDAR. The expression of SIRT1 was downregulated by 3 mM NMDA in MLE-12 cells (Fig. 6A, B), and MK801 (50 μM) partially reversed this decline. To evaluate the role of SIRT1 in NMDA-induced oxidative stress, the SIRT1-specific activator SRT1720 (10 μM) or the SIRT1 inhibitor sirtinol (10 μM) were applied to cells treated with NMDA. SRT1720 partially eliminated the increase in ROS, and sirtinol further promoted the increase in ROS caused by NMDA (Fig. 6C, D). Neither SRT 1720 (10 μM) nor sirtinol (10 μM) alone affected ROS production (data not shown). We further observed the effect of OA on NMDA-induced apoptosis in MLE-12 cells. OA suppressed NMDA-induced MLE-12 cells apoptosis, while sirtinol inhibited the effect of OA (Fig. 6E, F). In combination with the animal experiments, the results suggest that the protective effect of OA on NMDA-induced lung injury is at least partly attributed to the upregulation of SIRT1.

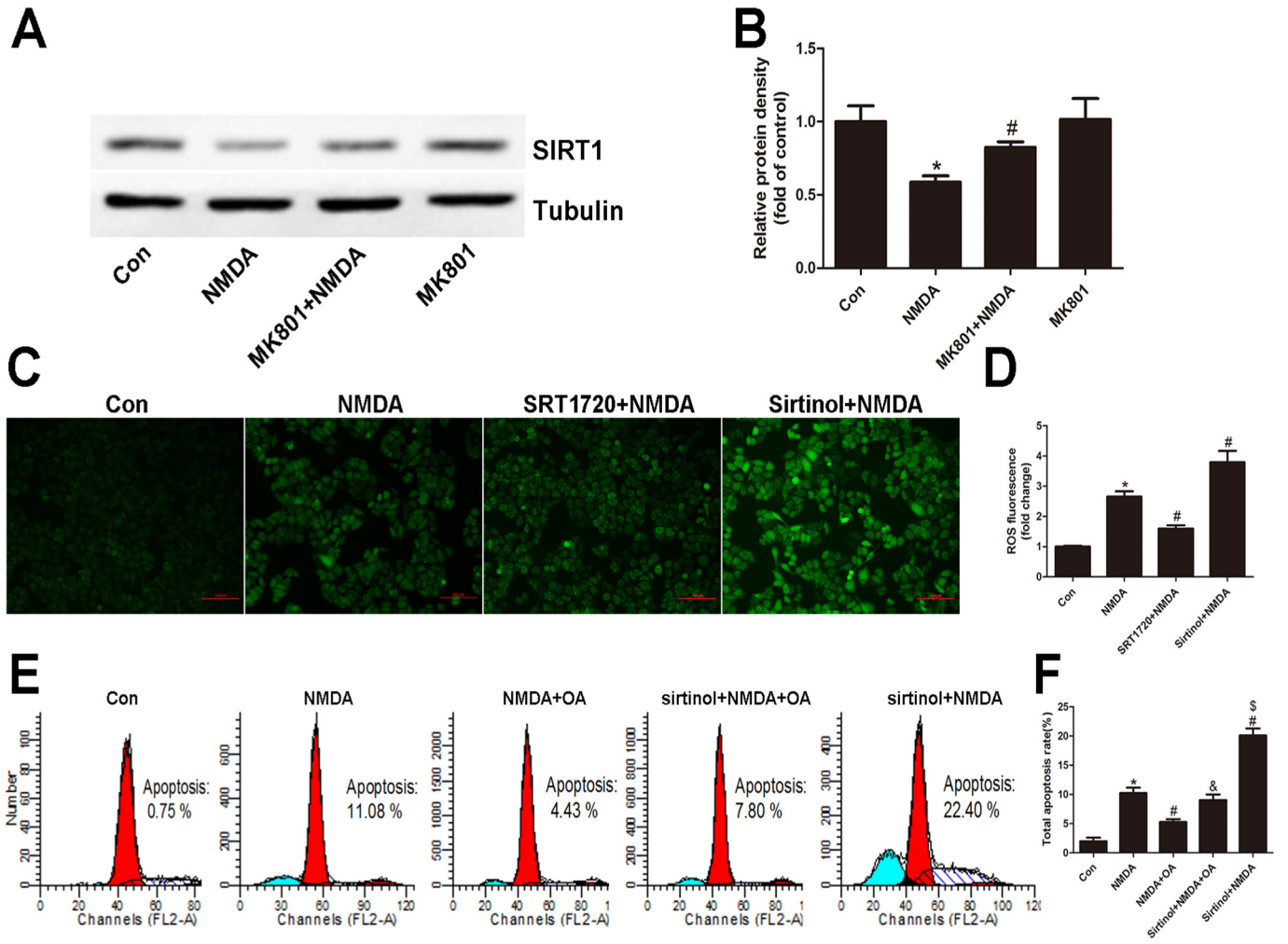

Fig. 6. Effect of SIRT1 on NMDA-treated MLE-12 cells.
(A) MLE-12 cells were treated with 300 μM NMDA for 24 h or pre-incubated with 50 μM MK801 for 30 min before 300 μM NMDA treatment. SIRT1 protein expression in MLE-12 cells was detected by Western blotting. (B) Quantitative histograms of SIRT1 protein expression (n = 3). (C, D) MLE-12 cells were treated with 10 μM SRT1720 or sirtinol, the activator and inhibitor of SIRT1, respectively, for 30 min before 300 μM NMDA treatment for 24 h. ROS production was determined. (Bar: 100 μm, n = 3). (E) MLE-12 cells were treated with NMDA (300 μM) alone or with OA (20 μM) for 24 h or pre-incubated with 10 μM sirtinol for 30 min before 300 μM NMDA treatment. Apoptotic cells were identified by PI staining. (F) Quantitative histogram of total apoptotic cells (n = 3). Values represent the mean ± SEM. *P < 0.05 vs. the control group, $^{\#}$P < 0.05 vs. the NMDA group, $^{\&}$P < 0.05 vs. the NMDA + OA group, $^{\$}$P < 0.05 vs. the sirtinol + NMDA + OA group.

3.7. Effects of OA on the expression of SIRT1, Nrf2, ac-p65 and NLRP3 in the lungs and MLE-12 cells

To further evaluate whether OA affects the expression of SIRT1-related regulatory protein in NMDA-induced lung injury, we detected the levels of SIRT1, nuclear -related factor 2 (Nrf2), acetylated NF-κB/p65 (ac-p65) and NLRP3 in lung tissues (Fig. 7A, B) and MLE-12 cells (Fig. 7C, D) by Western blot. SIRT1 and Nrf2 protein expression in the NMDA-treated group was lower than in the control group, and OA pretreatment attenuated this reduction. Ac-p65 and NLRP3 protein expression was higher in the NMDA group than in the control group, while OA pretreatment effectively reduced the expression of ac-p65 and NLRP3 caused by NMDA treatment. These results indicate that the effect of OA on NMDA-induced lung injury might be related to SIRT1 upregulation and reduced NF-κB p65 acetylation.

4. Discussion

Glu is an important excitatory neurotransmitter in the central nervous system and plays an important role in many physiological processes in learning, memory and synaptic plasticity. However, excessive extracellular Glu concentrations can over activate its receptors, leading to uncontrolled and sustained depolarization of neurons, known as Glu excitatory neurotoxicity [30], which ultimately leads to cell injury or death. Excitatory toxicity is involved in many acute diseases, such as stroke and seizures, as well as chronic neurodegenerative diseases, such as Parkinson's disease, Alzheimer's disease, and amyotrophic lateral sclerosis. In recent years, excessive release of endogenous Glu has become an important factor in peripheral organ injury [31].

Studies have shown that the NMDAR is expressed in the lung and that NMDAR-mediated Glu excitotoxicity is involved in the process of different lung injuries, but the mechanism is not yet clear. NMDA exhibited pro-inflammatory properties by upregulating pro-inflammatory cytokines such as IL-1β and TNF-α and enhancing leukocyte recruitment in retinal ganglion cells [32]. The increase in Glu may lead to overactivation of NMDARs and accelerate apoptosis of beta cells induced by hyperglycaemia [33]. Interventions including NMDAR1 siRNA and the NMDAR antagonist MK801 have been shown to reduce the production of pro-inflammatory cytokines in 6-hydroxydopamine-

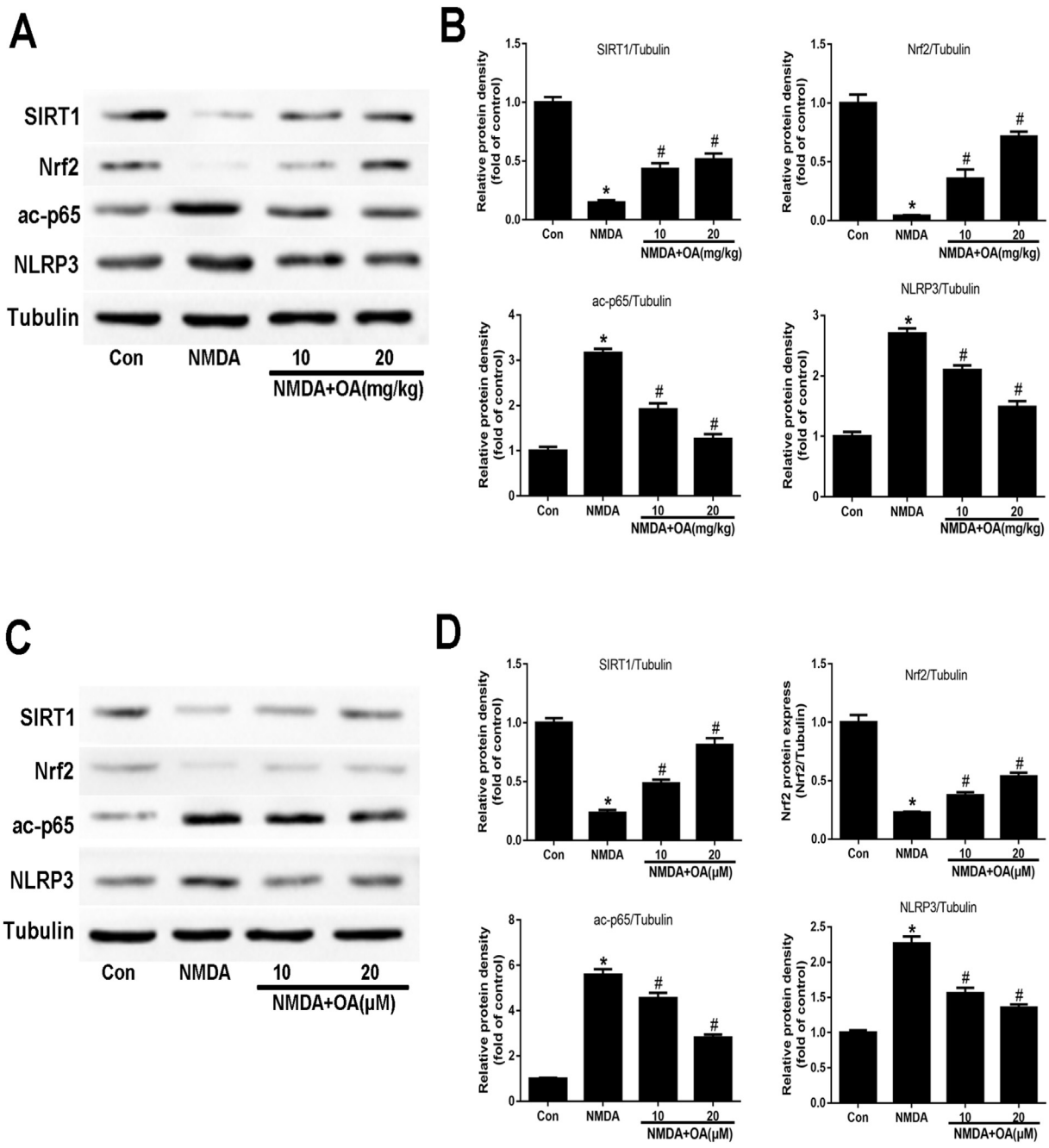

Fig. 7. Effects of OA on the protein expression of SIRT1, Nrf2, ac-p65 and NLRP3. (A) The protein expression of SIRT1, Nrf2, ac-p65 and NLRP3 in the lungs was detected by Western blotting (n = 3). (B) Quantitative histogram of protein expression. (C) The protein expression of SIRT1, Nrf2, ac-p65 and NLRP3 in MLE-12 cells was detected by Western blotting (n = 3). (D) Quantitative histogram of protein expression. Values represent the mean ± SEM, *P < 0.05 vs. the control group, #P < 0.05 vs. the NMDA group.

stimulated PC12 cells [34]. MK801 attenuated the oxidant lung injury induced by paraquat or xanthine oxidase [6] and ameliorated hyperoxia-induced lung injury in neonatal rats [35]. In this study, we found that OA could alleviate NMDA-induced lung permeability, pulmonary oedema and inflammatory cell infiltration and decrease the W/D ratio and protein level in the lung, suggesting that OA has a protective effect on NMDA-induced excitotoxic lung injury.

Inflammation plays a dominant role during the progression of ALI. Activation of neutrophils and monocytes releases inflammatory mediators to aggravate inflammation reactions. We found that NMDA caused a significant increase in inflammatory cytokine levels of TNF-α, IL-6 and IL-1β in mice. OA has been reported to inhibit the activation of protein kinase C (PKC)-Z and NF-κB p65 in the process of myocardial hypertrophy and play an anti-inflammatory role [20]. Furthermore, OA ameliorated colitis in mice by inhibiting the differentiation of Th17 cells and activating NF-κB [36]. Our study showed that OA effectively inhibited the accumulation of neutrophils in the lung and reduced the levels of inflammatory factors TNF-α, IL-6 and IL-1β, suggesting that OA may play an important role in NMDA-induced ALI mice by inhibiting the inflammatory response.

In the process of oxidative stress, a large number of ROS are produced, which causes a dynamic imbalance of oxidation and antioxidant systems, leads to lipid peroxidation and promotes cell death. Antioxidant enzymes SOD and GSH can reduce ROS accumulation and MDA formation and protect cells against oxidative damage [37]. In neurological diseases, excessive Glu activates NMDARs, which leads to

oxidative stress, mitochondrial dysfunction and neuronal death [38]. The NMDAR antagonist memantine significantly alleviated MDA content and oxidative stress in lung tissue [39,40]. In our study, administration of OA decreased MDA content and ROS production and increased SOD and GSH activity. These results revealed that OA possesses protective effects against NMDA-induced toxicity by inhibiting oxidative stress.

Alveolar epithelial cell damage is considered to be an important link leading to lung injury and fibrosis [41]. Decreased pulmonary surfactant synthesis in pulmonary epithelial cells during NMDA-induced lung injury is one of the reported mechanisms of Glu-induced lung injury [14]. Apoptosis of lung epithelial cells leads to destruction of the pulmonary epithelial barrier, increased alveolar exudation and aggravated pathological damage [42]. Glu induces different intracellular cytotoxic signals, and Bcl-2 family proteins are the main arbiter of mitochondrially mediated apoptosis [43]. The ratio of the anti-apoptotic protein Bcl-2 and the pro-apoptotic protein Bax plays an important role in regulating cell death [44]. Neuronal apoptosis induced by NMDA is associated with the Bcl-2 family [45]. Here, NMDA induced lung epithelial cell injury and increased apoptosis. However, OA upregulated Bcl-2 protein expression and inhibited Bax protein levels, thereby decreasing the Bax/Bcl ratio, and retarded lung epithelial cell apoptosis, which may be another mechanism by which OA inhibits NMDA-induced excitotoxic injury.

The NF-κB signalling pathway is involved in pulmonary diseases such as ALI, COPD and pulmonary fibrosis by promoting the expression of various cytokines [46–48]. The NF-κB signalling pathway is the target of recent therapeutic strategies for ALI [49]. Inflammasome are the core of inflammatory response , NF-κB mediates the activation of NLRP3 inflammasome. NMDA induces nitric oxide synthase (NOS) activation and nuclear p65 translocation in A549 cells [50]. The selective SIRT1 activator SRT1720 significantly attenuated LPS-induced lung injury [51]. The functional recovery of the olfactory bulb after excitatory injury induced by NMDA was shown to be related to an increase in SIRT1 expression [52]. Nrf2 is one of the important antioxidant transcription factors regulated by SIRT1. Nrf2 mediates the expression of downstream cytoprotective factor HO-1. Activation of Nrf2/HO-1 pathway has been shown to alleviate LPS-induced pulmonary inflammation [53]. We found that OA significantly increased the expression of SIRT1 and Nrf2 protein, and degraded the expression of ac-p65 and NLRP3 inflammasome, suggesting that OA inhibits excitotoxicity induced by NMDA, which may be related to upregulation of SIRT1/Nrf2 signal and the inhibition of NF-κB inflammatory signalling pathway activation.

In conclusion, OA effectively alleviated lung injury, reduced lung epithelial cell apoptosis and played a protective role in NMDA-induced lung injury in animal models and NMDA-challenged cell models. The mechanism of OA may be related to anti-inflammatory, antioxidative stress and anti-apoptosis processes involving upregulation of SIRT1 and reduction of NF-κB p65 acetylation. However, we cannot rule out the possibility that OA may provide protection to the lung through other mechanisms. Other supportive experiments are needed for further investigation.

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Conflicts of interest

The authors confirm that they have no conflicts of interest.

References

[1] W.L. Lee, G.P. Downey, Neutrophil activation and acute lung injury, Curr. Opin. Crit. Care 7 (1) (2001) 1–7.
[2] K. Jiang, S. Guo, C. Yang, et al., Barbaloin protects against lipopolysaccharide (LPS)-induced acute lung injury by inhibiting the ROS-mediated PI3K/AKT/NF-κB pathway, Int. Immunopharmacol. 64 (2018) 140–150.
[3] K.G. Dickman, J.G. Youssef, S.M. Mathew, S.I. Said, Ionotropic glutamate receptors in lungs and airways: molecular basis for glutamate toxicity, Am. J. Respir. Cell Mol. Biol. 30 (2) (2004) 139–144.
[4] L.H. Shang, Z.Q. Luo, X.D. Deng, et al., Expression of N-methyl-D-aspartate receptor and its effect on nitric oxide production of rat alveolar macrophages, Nitric Oxide. 23 (4) (2010) 327–331.
[5] C.D. Collard, K.A. Park, M.C. Montalto, et al., Neutrophil-derived glutamate regulates vascular endothelial barrier function, J. Biol. Chem. 277 (17) (2002) 14801–14811.
[6] S.I. Said, H. Pakbaz, H.I. Berisha, S. Raza, NMDA receptor activation: critical role in oxidant tissue injury, Free Radic. Biol. Med. 28 (8) (2000) 1300–1302.
[7] V.V. Costa, S.J.L. Del, R.F. Rocha, et al., N-methyl-d-aspartate (NMDA) receptor blockade prevents neuronal death induced by Zika virus infection, MBio 8 (2) (2017).
[8] C.S. Lin, S.F. Hung, H.S. Huang, M.C. Ma, Blockade of the N-methyl-D-aspartate glutamate receptor ameliorates lipopolysaccharide-induced renal insufficiency, PLoS One 10 (7) (2015) e0132204.
[9] A.A. Kritis, E.G. Stamoula, K.A. Paniskaki, T.D. Vavilis, Researching glutamate - induced cytotoxicity in different cell lines: a comparative/collective analysis/study, Front. Cell. Neurosci. 9 (2015) 91.
[10] Z. Zhe, B. Hongyuan, Q. Wenjuan, W. Peng, L. Xiaowei, G. Yan, Blockade of glutamate receptor ameliorates lipopolysaccharide-induced sepsis through regulation of neuropeptides, Biosci. Rep. 38 (3) (2018).
[11] Pauli V. da CAA, V.C. Saciura, et al., N-methyl-D-aspartate glutamate receptor blockade attenuates lung injury associated with experimental sepsis, Chest 137 (2) (2010) 297–302.
[12] X. Li, C. Li, Y. Tang, Y. Huang, Q. Cheng, X. Huang, F. Zhao, C. Hao, D. Feng, J. Xu, J. Han, S. Tang, W. Liu, S. Yue, Z. Luo, NMDA receptor activation inhibits the antifibrotic effect of BM-MSCs on bleomycin-induced pulmonary fibrosis, Am. J. Physiol. Lung Cell. Mol. Physiol. 315 (2018) L404–404L421.
[13] Q. Cheng, L. Fang, D. Feng, S. Tang, S. Yue, Y. Huang, J. Han, J. Lan, W. Liu, L. Gao, Z. Luo, Memantine ameliorates pulmonary inflammation in a mice model of COPD induced by cigarette smoke combined with LPS, Biomed. Pharmacother. 109 (2019) 2005–2013.
[14] L. Shen, L. Li, H. She, S. Yue, C. Li, Z. Luo, Inhibition of pulmonary surfactants synthesis during N-methyl-D-aspartate-induced lung injury, Basic Clin. Pharmacol. Toxicol. 107 (3) (2010) 751–757.
[15] Y. Yin, Y. Zhang, H. Li, et al., Triterpenoids from fruits of Sorbus pohuashanensis inhibit acetaminophen-induced acute liver injury in mice, Biomed. Pharmacother. 109 (2018) 493–502.
[16] W. Zhang, J. Feng, B. Cheng, Q. Lu, X. Chen, Oleanolic acid protects against oxidative stress-induced human umbilical vein endothelial cell injury by activating AKT/eNOS signaling, Mol. Med. Rep. 18 (4) (2018) 3641–3648.
[17] S. Su, G. Wu, X. Cheng, et al., Oleanolic acid attenuates PCBs-induced adiposity and insulin resistance via HNF1b-mediated regulation of redox and PPARγ signaling, Free Radic. Biol. Med. 124 (2018) 122–134.
[18] R.S. Santos, P.L. Silva, G.P. Oliveira, et al., Effects of oleanolic acid on pulmonary morphofunctional and biochemical variables in experimental acute lung injury, Respir. Physiol. Neurobiol. 179 (2–3) (2011) 129–136.
[19] H.B. Peng, R.X. Wang, H.J. Deng, Y.H. Wang, J.D. Tang, F.Y. Cao, J.H. Wang, Protective effects of oleanolic acid on oxidative stress and the expression of cytokines and collagen by the AKT/NF-κB pathway in silicotic rats, Mol. Med. Rep. 15 (2017) 3121–3128.
[20] H. Gao, H. Liu, T. Tang, et al., Oleanonic acid ameliorates pressure overload-induced cardiac hypertrophy in rats: the role of PKCζ-NF-κB pathway, Mol. Cell. Endocrinol. 470 (2018) 259–268.
[21] Z. Tian, S.B. Liu, Y.C. Wang, X.Q. Li, L.H. Zheng, M.G. Zhao, Neuroprotective effects of formononetin against NMDA-induced apoptosis in cortical neurons, Phytother. Res. 27 (12) (2013) 1770–1775.
[22] X. Yang, P. Si, H. Qin, L. Yin, L.J. Yan, C. Zhang, The neuroprotective effects of SIRT1 on NMDA-induced excitotoxicity, Oxidative Med. Cell. Longev. 2017 (2017) 2823454.
[23] S. Chung, H. Yao, S. Caito, J.W. Hwang, G. Arunachalam, I. Rahman, Regulation of SIRT1 in cellular functions: role of polyphenols, Arch. Biochem. Biophys. 501 (1) (2010) 79–90.
[24] F. Yeung, J.E. Hoberg, C.S. Ramsey, et al., Modulation of NF-kappaB-dependent transcription and cell survival by the SIRT1 deacetylase, EMBO J. 23 (12) (2004) 2369–2380.
[25] A.A. Sauve, C. Wolberger, V.L. Schramm, J.D. Boeke, The biochemistry of sirtuins, Annu. Rev. Biochem. 75 (2006) 435–465.
[26] Y. Wang, D. Li, G. Ma, et al., Increases in peripheral SIRT1: a new biological characteristic of asthma, Respirology 20 (7) (2015) 1066–1072.
[27] J. Ye, M. Guan, Y. Lu, D. Zhang, C. Li, C. Zhou, Arbutin attenuates LPS-induced lung injury via Sirt1/Nrf2/NF-κBp65 pathway, Pulm. Pharmacol. Ther. 54 (2019) 53–59.
[28] H. Yao, J.W. Hwang, I.K. Sundar, et al., SIRT1 redresses the imbalance of tissue inhibitor of matrix metalloproteinase-1 and matrix metalloproteinase-9 in the development of mouse emphysema and human COPD, Am. J. Physiol. Lung Cell. Mol. Physiol. 305 (9) (2013) L615–L624.
[29] H. Chu, S. Jiang, Q. Liu, Y. Ma, X. Zhu, M. Liang, X. Shi, W. Ding, X. Zhou, H. Zou, F. Qian, P.W. Shaul, L. Jin, J. Wang, Sirtuin1 protects against systemic sclerosis-related pulmonary fibrosis by decreasing proinflammatory and profibrotic processes, Am. J. Respir. Cell Mol. Biol. 58 (2018) 28–39.

[30] S.E. Proskurina, K.A. Petrov, E.E. Nikolsky, Influence of the activation of NMDA receptors on the resting membrane potential of the postsynaptic cell at the neuromuscular junction, Acta Nat. 10 (3) (2018) 100–102.
[31] M. Wang, Z. Luo, S. Liu, L. Li, X. Deng, F. Huang, L. Shang, C. Jian, S. Yue, Glutamate mediates hyperoxia-induced newborn rat lung injury through N-methyl-D-aspartate receptors, Am. J. Respir. Cell Mol. Biol. 40 (2009) 260–267.
[32] T. Nakazawa, Mechanism of N-methyl-D-aspartate-induced retinal ganglion cell death, Nippon Ganka Gakkai Zasshi 113 (11) (2009) 1060–1070.
[33] X.T. Huang, C. Li, X.P. Peng, et al., An excessive increase in glutamate contributes to glucose-toxicity in β-cells via activation of pancreatic NMDA receptors in rodent diabetes, Sci. Rep. 7 (2017) 44120.
[34] J. Yan, J. Sun, L. Huang, Q. Fu, G. Du, Simvastatin prevents neuroinflammation by inhibiting N-methyl-D-aspartic acid receptor 1 in 6-hydroxydopamine-treated PC12 cells, J. Neurosci. Res. 92 (5) (2014) 634–640.
[35] F. Tang, S. Yue, Z. Luo, D. Feng, M. Wang, C. Qian, X. Zhen, Y. Duan, Role of N-methyl-D-aspartate receptor in hyperoxia-induced lung injury, Pediatr. Pulmonol. 40 (2005) 437–444.
[36] G.D. Kang, S. Lim, D.H. Kim, Oleanolic acid ameliorates dextran sodium sulfate-induced colitis in mice by restoring the balance of Th17/Treg cells and inhibiting NF-kappaB signaling pathway, Int. Immunopharmacol. 29 (2) (2015) 393–400.
[37] A.M. Rousta, S.M. Mirahmadi, A. Shahmohammadi, et al., Protective effect of sesamin in lipopolysaccharide-induced mouse model of acute kidney injury via attenuation of oxidative stress, inflammation, and apoptosis, Immunopharmacol. Immunotoxicol. (2018) 1–7.
[38] N. Khatri, M. Thakur, V. Pareek, S. Kumar, S. Sharma, A.K. Datusalia, Oxidative stress: major threat in traumatic brain injury, CNS Neurol. Disord. Drug Targets 17 (9) (2018) 689–695.
[39] Y. Li, Y. Liu, X. Peng, et al., NMDA receptor antagonist attenuates bleomycin-induced acute lung injury, PLoS One 10 (5) (2015) e0125873.
[40] L. Ma, W. Liu, D. Feng, et al., Protective effect of NMDA receptor antagonist memantine on acute lung injury in mice, Zhong Nan Da Xue Xue Bao Yi Xue Ban 39 (1) (2014) 12–16.
[41] Q. Wang, H. Zhu, W.G. Zhou, X.C. Guo, M.J. Wu, Z.Y. Xu, J.F. Jiang, C. Shen, H.Q. Liu, N-acetylcysteine-pretreated human embryonic mesenchymal stem cell administration protects against bleomycin-induced lung injury, Am J Med Sci 346 (2013) 113–122.
[42] W. Xie, Q. Lu, K. Wang, J. Lu, X. Gu, D. Zhu, F. Liu, Z. Guo, miR-34b-5p inhibition attenuates lung inflammation and apoptosis in an LPS-induced acute lung injury mouse model by targeting progranulin, J. Cell. Physiol. 233 (2018) 6615–6631.
[43] Z. Zhang, J. Liu, C. Fan, L. Mao, R. Xie, S. Wang, M. Yang, H. Yuan, X. Yang, J. Sun, J. Wang, J. Kong, S. Huang, B. Sun, The GluN1/GluN2B NMDA receptor and metabotropic glutamate receptor 1 negative allosteric modulator has enhanced neuroprotection in a rat subarachnoid hemorrhage model, Exp. Neurol. 301 (2018) 13–25.
[44] J. Fan, S. Yu, Y. Cui, G. Xu, L. Wang, Y. Pan, H. He, Bcl-2/Bax protein and mRNA expression in yak (Bos grunniens) placentomes, Theriogenology 104 (2017) 23–29.
[45] L. Yang, X.B. Li, Q. Yang, et al., The neuroprotective effect of praeruptorin C against NMDA-induced apoptosis through down-regulating of GluN2B-containing NMDA receptors, Toxicol. in Vitro. 27 (2) (2013) 908–914.
[46] J. Dong, W. Liao, H.Y. Peh, T. WSD, S. Zhou, W. WSF, Ribosomal protein S3 gene silencing protects against cigarette smoke-induced acute lung injury, Mol. Ther. Nucleic Acids 12 (2018) 370–380.
[47] L. Yang, D. Li, Y. Zhuo, S. Zhang, X. Wang, H. Gao, Protective role of liriodendrin in sepsis-induced acute lung injury, Inflammation 39 (5) (2016) 1805–1813.
[48] A. Guzel, S. Kayhan, S. Tutuncu, et al., Attenuation of bleomycin induced lung fibrosis by erdosteine and inhibition of the inducible nitric oxide synthase, Bratisl. Lek. Listy 116 (3) (2015) 196–202.
[49] H. Zhang, S. Chen, M. Zeng, et al., Apelin-13 administration protects against LPS-induced acute lung injury by inhibiting NF-κB pathway and NLRP3 Inflammasome activation, Cell. Physiol. Biochem. 49 (5) (2018) 1918–1932.
[50] L. Li, L. Shen, H. She, S. Yue, D. Feng, Z. Luo, Nitric oxide-induced activation of NF-κB-mediated NMDA-induced CTP:phosphocholine cytidylyltransferase alpha expression inhibition in A549 cells, Cell Biol. Toxicol. 27 (1) (2011) 41–47.
[51] C. Fu, S. Hao, X. Xu, et al., Activation of SIRT1 ameliorates LPS-induced lung injury in mice via decreasing endothelial tight junction permeability, Acta Pharmacol. Sin. (2018).
[52] C. Marin, C. Langdon, I. Alobid, M. Fuentes, M. Bonastre, J. Mullol, Recovery of olfactory function after excitotoxic lesion of the olfactory bulbs is associated with increases in bulbar SIRT1 and SIRT4 expressions, Mol. Neurobiol. (2019), https://doi.org/10.1007/s12035-019-1472-y.
[53] Y. Huang, L. Huang, G. Zhu, Z. Pei, W. Zhang, Downregulated microRNA-27b attenuates lipopolysaccharide-induced acute lung injury via activation of NF-E2-related factor 2 and inhibition of nuclear factor κB signaling pathway, J. Cell. Physiol. 234 (5) (2019) 6023–6032.