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DL-3-n-butylphthalide attenuates lipopolysaccharide-induced acute lung injury via SIRT1-dependent and -independent regulation of Nrf2

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ABSTRACT

The acute respiratory distress syndrome (ARDS), a devastating clinical syndrome, is one of the most severe complications of acute lung injury (ALI). Despite of decades of clinical trials and supportive ventilation strategies, the incidence and mortality of ALI/ARDS remain high. DL-3-n-butylphthalide (NBP) is a synthesized raceme of L-3-n-butylphthalide which has been approved to possess various activities. In the current study, we aimed to investigate the effect of NBP on ALI in lipopolysaccharide (LPS)-treated mice. We found that 10 mg/kg and 50 mg/kg NBP significantly prevented LPS-induced increase of W/D ratio of lung, histological injury of lung, infiltration of inflammatory cells, release of pro-inflammatory cytokines and chemokines, and oxidative damage. Sirtuin 1 (SIRT1) and nuclear factor (erythroid-derived 2)-like 2 (Nrf2) expression was increased by NBP in lung of LPS-treated mice. Knockout of SIRT1 significantly aggravated LPS-induced ALI. Moreover, the absence of SIRT1 notably inhibited NBP-induced protective effects against LPS-induced increase of W/D ratio of lung, histological injury of lung, infiltration of inflammatory cells, release of pro-inflammatory cytokines and chemokines, and oxidative damage. However, knockout of SIRT1 did not completely inhibit NBP-induced upregulation of Nrf2 and attenuation of ALI. The results demonstrated that NBP could activate Nrf2 antioxidant signaling in a SIRT1-dependent and SIRT1-independent manner, resulting in the amelioration of oxidative stress, inflammation and pulmonary edema. The data highlights the importance of SIRT1/Nrf2 signaling in the protective effects of NBP.

1. Introduction

The acute respiratory distress syndrome (ARDS), a devastating clinical syndrome, is one of the most severe complications of acute lung injury (ALI) [1,2], with substantial morbidity and limited long-term quality of life [3,4]. Based on the principles of Berlin definition, ARDS is characterized by acute onset of mild to severe hypoxemia, radiographic diagnosis of bilateral opacities, and pulmonary edema which could not be explained entirely by overload of fluid or cardiac diseases [4]. Animal model of ALI, which was first described in 1967, is a widely used experimental setting for the research of ARDS [5,6]. Severe inflammatory response within the lung plays a critical role in the acute phase of ALI [2,7]. This inflammatory process results in disruption of epithelial and endothelial barrier, an influx of excessive neutrophils and other inflammatory cells, overproduction of inflammatory and cytotoxic mediators, such as tumor necrosis factor (TNF) α, interleukin (IL)-1β, and monocyte chemoattractantprotein-1 (MCP-1), increase of vascular permeability, pulmonary edema, and destruction of gas exchange [8,9]. Despite of decades of clinical trials and supportive ventilation strategies, the incidence and mortality of ALI/ARDS remain high [3]. It is urgently needed to explore novel strategies for treatment of ALI/ARDS.

Among a number of risk factors, sepsis is a main cause of ALI/ARDS [10,11]. Lipopolysaccharide (LPS) released by Gram-negative bacteria is an important source of sepsis and plays a crucial role in promoting innate immune response and inflammation [12]. It is considered that LPS is ubiquitously present in numerous pollutant particles, including air pollution, smoke of cigarette, and dusts of organic chemicals [13,14]. LPS is usually used to establish animal model of ALI/ARDS as LPS exposure can initiate ALI/ARDS-like pathological processes, including release of lymphocytes, interstitial and alveolar pulmonary edema, and damage of vessels [15].

DL-3-n-butylphthalide (NBP) is a synthesized raceme of L-3-n-butylphthalide which is extracted from seeds of *Apium graveolens* Linn [16]. NBP had been approved by the State Food and Drug Administration of China for the prevention and treatment of ischemic stroke in 2002 [17]. NBP has been shown to exhibit neuroprotective effects through multiple biological activities, including antioxidation and anti-

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inflammation [18]. NBP could reduce ischemic cerebral injury and attenuate impairment of cognition in dementia patients and animal models of Alzheimer's disease [19]. In rats, NBP can decrease apoptosis of hippocampal neurons, reduce the reactivity of astrocyte and attenuate deficits of spatial memory [20,21]. Inhibition of oxidative injury is crucial for NBP-induced protection against ischemic neuropathologic alterations [22].

Up to our knowledge, there is no evidence whether NBP could protect against ALI which is closely associated with oxidative damage. Hence, the present study aimed to investigate effect of NBP on ALI in LPS-treated mice and to explore the molecular mechanisms underlying NBP-induced effects. It is of significance that NBP exhibits evident protective effects against LPS-induced ALI and provides an experimental basis for the potential medicinal use of NBP in the intervention of ALI.

2. Materials and methods

2.1. Reagents and chemicals

NBP, with a purity of 99.6%, was extracted from celery seed and obtained from Shijiazhuang Pharmaceutical Group NBP Pharmaceutical Co., Ltd. (Shijiazhuang, China). NBP was diluted in corn oil. LPS (*Escherichia coli* 055:B5) was obtained from Sigma (St. Louis, MO, USA). Mouse ELISA kits for the measurement of TNF-α, IL-6, KC and MIP-2 were purchased from R&D Systems Inc. (Minneapolis, MN, USA). Assay kits for MDA, SOD and GPX were obtained from Beyotime Biotechnology (Shanghai, China). Antibodies against SIRT1, Nrf2, and β-actin were purchased from Cell signaling technology (Danvers, MA, USA).

2.2. Animals

Male SIRT1 knockout (KO) mice (7–8 weeks old) were obtained from the Jackson Laboratory (Bar Harbor, ME, USA). Mice were housed in a temperature (23 ± 2 °C) and humidity (65 ± 2%)-controlled environment under a 12/12 h light/dark cycle with free access to food and water. All animal experiments were carried out according to the Guide for Care and Use of Laboratory Animals and approved by the Animal Care and Use Committee of Shanxi Provincial People's Hospital. Background information of the mice was described previously [23].

2.3. Animal treatment

Protocol 1: 32 male C57BL/6 mice were randomly divided into four groups including control group, LPS group, LPS + 10 mg/kg NBP group, and LPS + 50 mg/kg NBP group. To induce ALI model, mice were intranasally treated with 15 mg/kg of LPS in 50 μl PBS. 10 mg/kg NBP, 50 mg/kg NBP or equal volume of corn oil were given to the animals 1 h before LPS administration. The dose of NBP was chose based on previous studies [24] and our preliminary results. 6 h after the LPS treatment, mice were narcotized using isoflurane and blood samples, lung tissues and bronchoalveolar lavage fluid (BALF) were collected.

Protocol 2: 16 wild type mice and 16 SIRT1 KO mice were randomly divided into LPS group and LPS + 50 mg/kg NBP group. To induce ALI model, mice were intranasally treated with 15 mg/kg of LPS in 50 μl PBS. 50 mg/kg NBP or equal volume of corn oil were given to the animals 1 h before LPS administration. 6 h after the LPS treatment, mice were narcotized using isoflurane and blood samples, lung tissues and BALF were collected.

2.4. Histopathological examination

In each group, six sections of lung tissues were collected and fixed in 4% paraformaldehyde in PBS at room temperature overnight. The sections were then processed for routine embedding in paraffin. The paraffin-embedded sections of lung tissues were used for standard staining hematoxylin and eosin (HE). Histopathological examination was performed using a light electric microscope (Olympus, USA).

2.5. Homogenate of tissues and biochemical determination

10% (*w/v*) homogenates of lung tissues in saline were obtained and centrifuged at 3000 rpm for 20 min at 4 °C. The supernatant was used for subsequent determination of MDA level, SOD and GPX activities. The parameters of oxidant products and antioxidant enzymes were expressed as per mg protein of lung tissue. Levels of TNF-α, IL-6, KC and MIP-2 in BALF were measured using ELISA assay kits according to the manufacturer's instructions.

2.6. W/D ratio of lung

"Wet" weights of right lung tissues were obtained immediately after the sacrifice of the mice. Then, the tissues were dried at 70 °C for 72 h and the "dry" weights of lung tissues were obtained. The lung wet weight was divided by dry weigh to obtain lung W/D ratio.

2.7. Cells count in BALF

Total cells in BALF were counted using hemocytometer. Cells were centrifuged to differentiate neutrophils and macrophages which were stained with Diff-Quik Stain reagent. The numbers of neutrophils and macrophages in BALF were also counted.

2.8. RNA extraction and quantitative real-time polymerase chain reaction

Isolation of total RNA from lung tissues was performed using Trizol reagent (Invitrogen, USA). Extracted RNA was quantified using NanoDrop ND-2000. cDNA was synthesized using the Revert Aid First Strand cDNA Synthesis Kit (Thermo Scientific, USA) according to the manufacturer's instructions. Quantitative RT-PCR (RT-qPCR) was carried out using the SYBR green Master Mix (Takara, Dalian, China) on a Bio-rad CFX96 Detection System (Bio-rad, USA). $2^{-\Delta\Delta Ct}$ method was used to evaluate the level of mRNA and relative mRNA expression was normalized to GAPDH which was used as the internal standard.

2.9. Western blotting analysis

50 mg lung tissues were lysed in 500 μl RIPA lysis buffer containing protease inhibitor. Concentration of extracted protein was determined using BCA method (Thermo Fisher Scientific, Rockford, IL, USA). Equal volume of protein supernatant and SDS loading buffer were mixed. Protein mixture was separated using SDS-PAGE gel electrophoresis. After transfer of protein onto a PVDF membrane (Millipore, Billerica, MA, USA), blocking was performed using 5% non-fat milk for 1 h at °C. Then, the blots were probed with specific antibodies, including SIRT1 (1:1000), Nrf2 (1:1000), and β-actin (1:1000). Subsequently, the blots were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies. Finally, the protein bands were detected by enhanced chemiluminescence (ECL) kit (Thermo Fisher Scientific, Rockford, IL, USA) and analyzed using Image J software (National Institutes of Health, Bethesda, MD, USA).

2.10. Statistical analysis

All data were expressed as the mean ± SD. Graph Pad Prism 6.0 software was used for statistical analysis. Statistical evaluation of the data was performed using a one-way analysis-of-variance (ANOVA) test, followed by Tukey's post hoc test. The p value < 0.05 was defined as statistically significant.

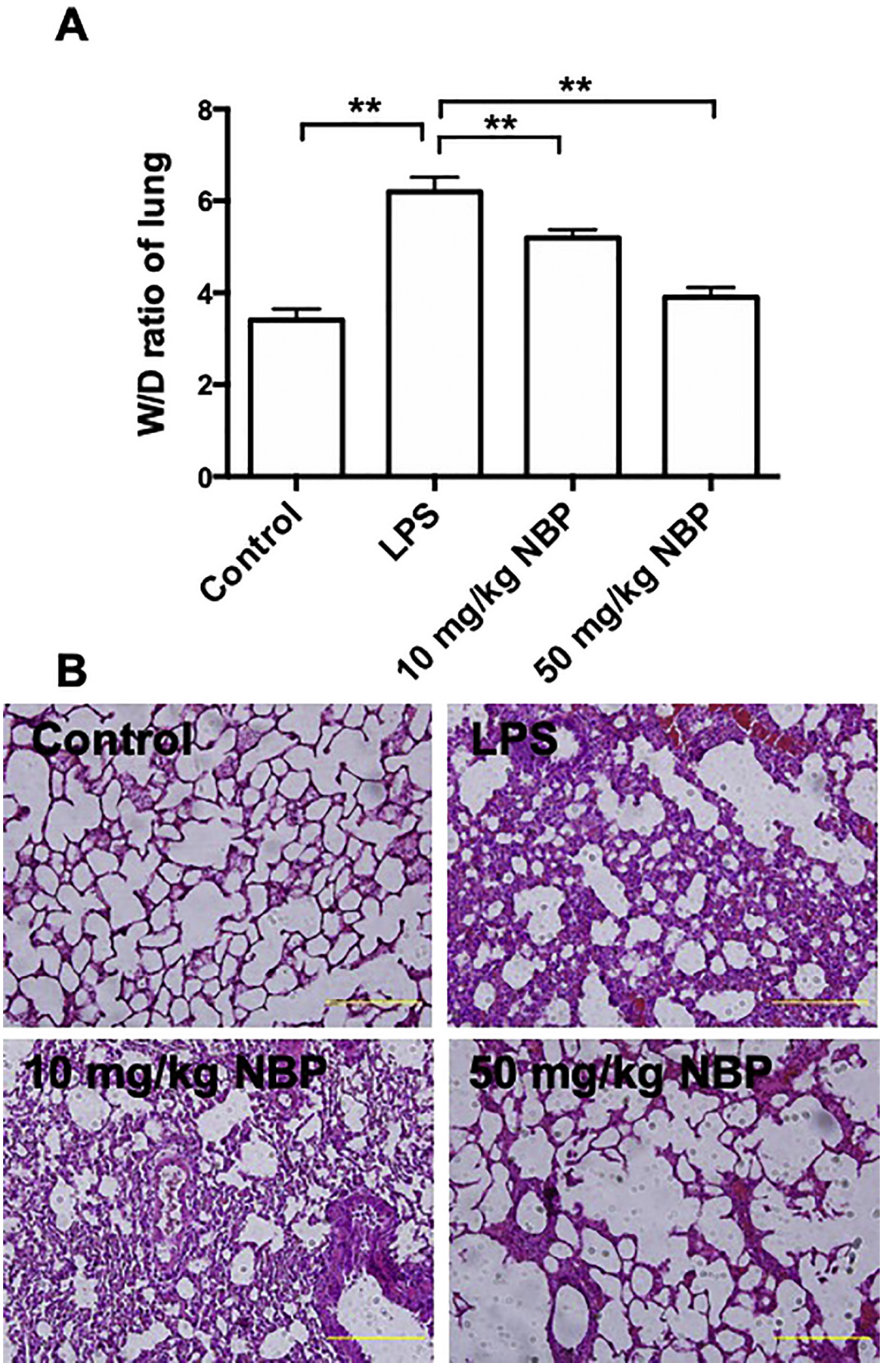

Fig. 1. NBP ameliorated LPS-induced pulmonary edema in mice.
(A) W/D ratio of lung tissues. (B) Histopathological examination of lung tissues. $^{**}p < 0.01$.

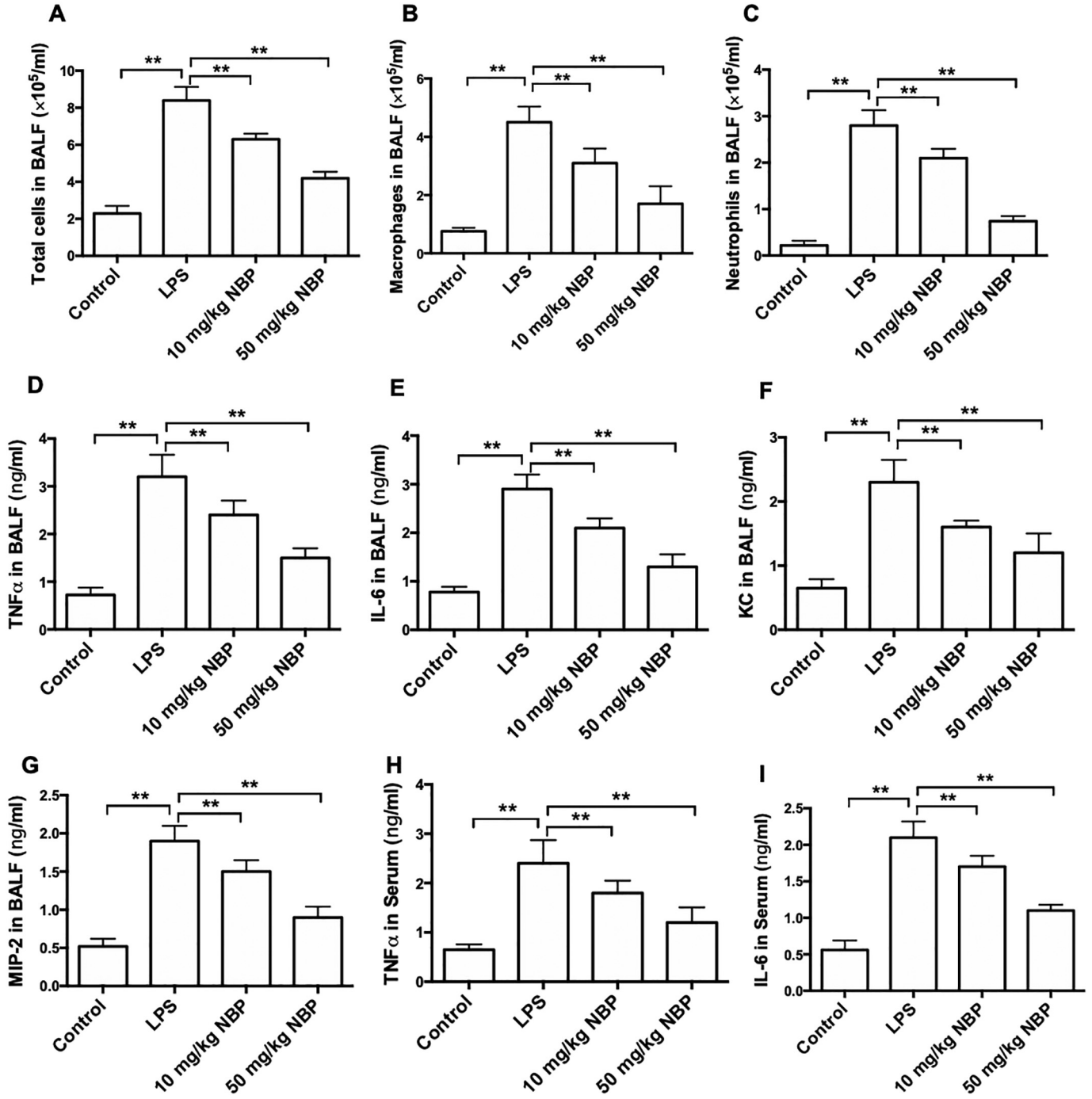

Fig. 2. NBP ameliorated LPS-induced accumulation of inflammatory cells, release of cytokines in lung, and increase of cytokines in serum.
(A) Total cells in BALF. (B) Number of macrophages in BALF. (C) Number of neutrophils in BALF. (D) TNFα content in BALF. (E) IL-6 content in BALF. (F) KC content in BALF. (G) MIP-2 content in BALF. (H) TNFα content in serum. (I) IL-6 content in serum. $^{**}p < 0.01$.

3. Results

3.1. NBP ameliorates LPS-induced pulmonary edema in mice

W/D ratio of lung tissue was a classical index for pulmonary edema. In the study, we showed that LPS induced a significant increase of W/D ratio of lung tissue in mice (Fig. 1A). 10 mg/kg and 50 mg/kg NBP significantly reduced W/D ratio of lung tissue in LPS-treated mice (Fig. 1A). Histopathological examination was also performed to evaluate LPS-induced acute injury of lung. As shown in Fig. 1B, LPS resulted in a notable destruction of pulmonary alveoli and drastic release of inflammatory cells in mesenchyme and pulmonary alveoli. The treatment of 10 mg/kg and 50 mg/kg NBP significantly reduced LPS-induced histopathological injury of lung tissue (Fig. 1B). NBP nearly completely reversed LPS-resulted changes of histology (Fig. 1B). These results demonstrated that NBP could effectively ameliorate LPS-induced ALI.

3.2. NBP ameliorates LPS-induced accumulation of inflammatory cells and release of cytokines and chemoattractants in lung and increase of cytokines in circulation

LPS-induced ALI was usually characterized by accumulation of

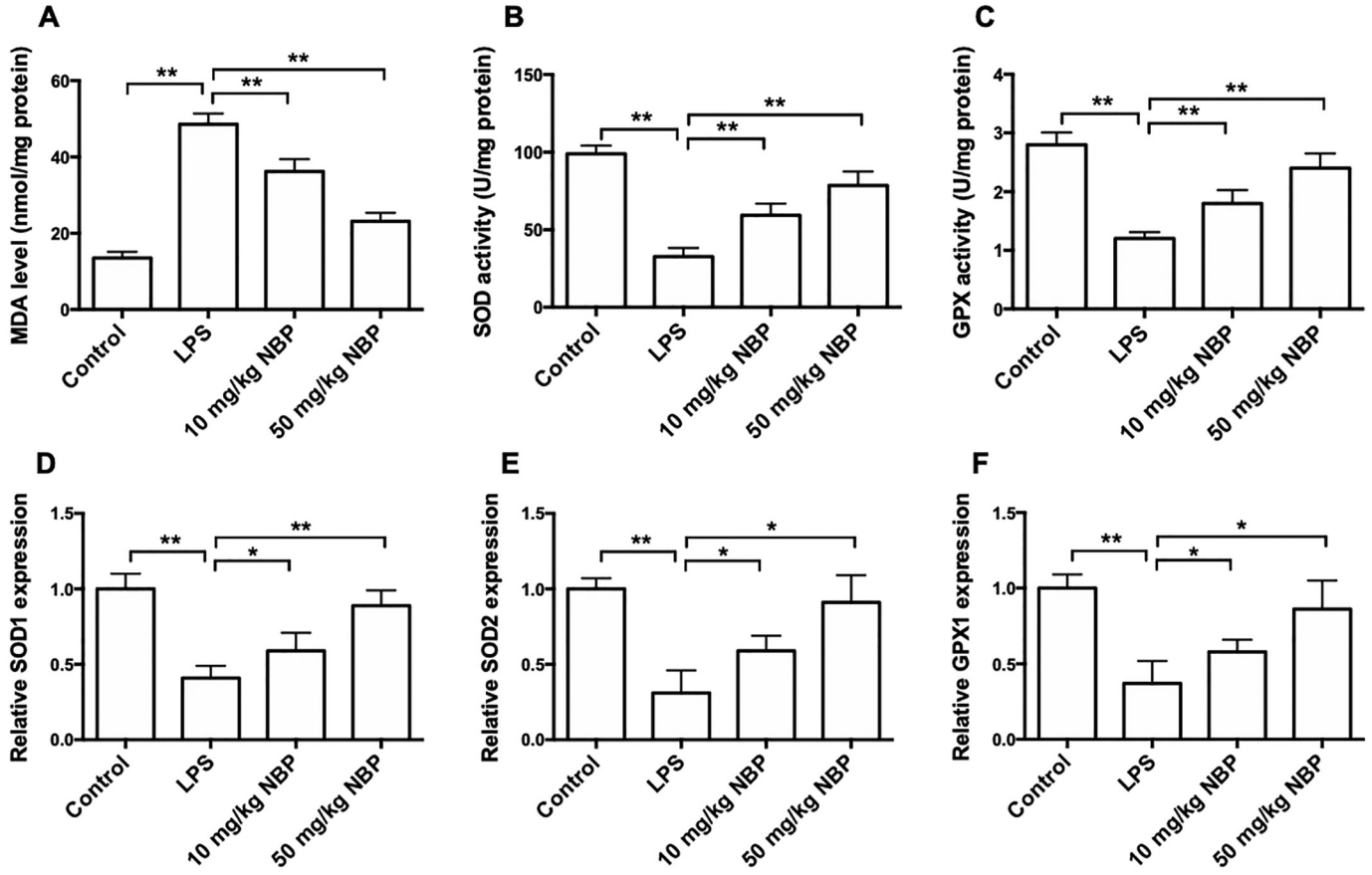

Fig. 3. NBP ameliorated LPS-induced oxidative injury in lung of mice.
(A) MDA level in homogenates of lung. (B) SOD activity in homogenates of lung. (C) GPX activity in homogenates of lung. (D) Relative mRNA expression of SOD1 in lung tissue. (E) Relative mRNA expression of SOD2 in lung tissue. (F) Relative mRNA expression of GPX1 in lung tissue. $^{*}p < 0.05$. $^{**}p < 0.01$.

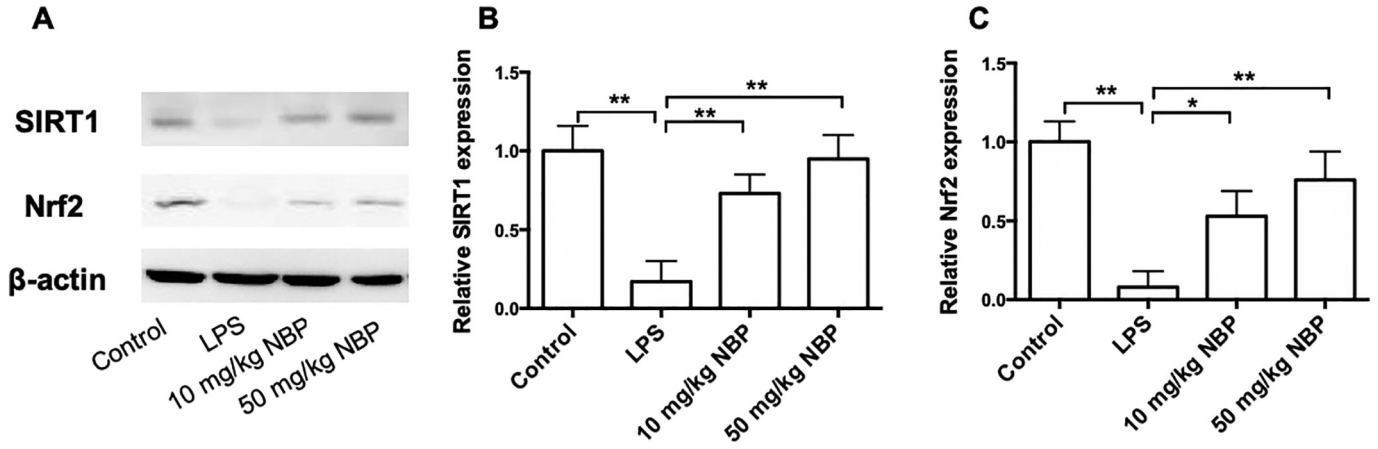

Fig. 4. NBP ameliorated LPS-induced inhibition of SIRT1/Nrf2 signaling.
(A) Representative image of protein expression of SIRT1 and Nrf2 in lung tissue. (B) Statistical analysis of SIRT1 protein expression. (C) Statistical analysis of Nrf2 protein expression. $^{*}p < 0.05$. $^{**}p < 0.01$.

inflammatory cells and release of cytokines in lung and systemic inflammation. In this study, we showed that LPS-induced increase of the number of total cells (Fig. 2A), macrophages (Fig. 2B), and neutrophils (Fig. 2C) in BALF significantly was inhibited by 10 mg/kg and 50 mg/kg NBP. In addition, LPS-induced increase of pro-inflammatory cytokines, including TNFα (Fig. 2D) and IL-6 (Fig. 2E), and chemoattractants which mediates polymorphonuclear neutrophils migration and infiltration, including KC (Fig. 2F) and MIP-2 (Fig. 2G), was inhibited by NBP. We also determined levels of cytokines in circulation of LPS-treated mice. LPS resulted in an evident increase of serum levels of TNFα (Fig. 2H) and IL-6 (Fig. 2I). The increase of serum levels of TNFα (Fig. 2H) and IL-6 (Fig. 2I) was notably inhibited by NBP. The results indicated that NBP ameliorated LPS-induced inflammation, as evidenced by reduced accumulation of inflammatory cells, decrease of cytokines and chemoattractants in lung and reduction of serum cytokines.

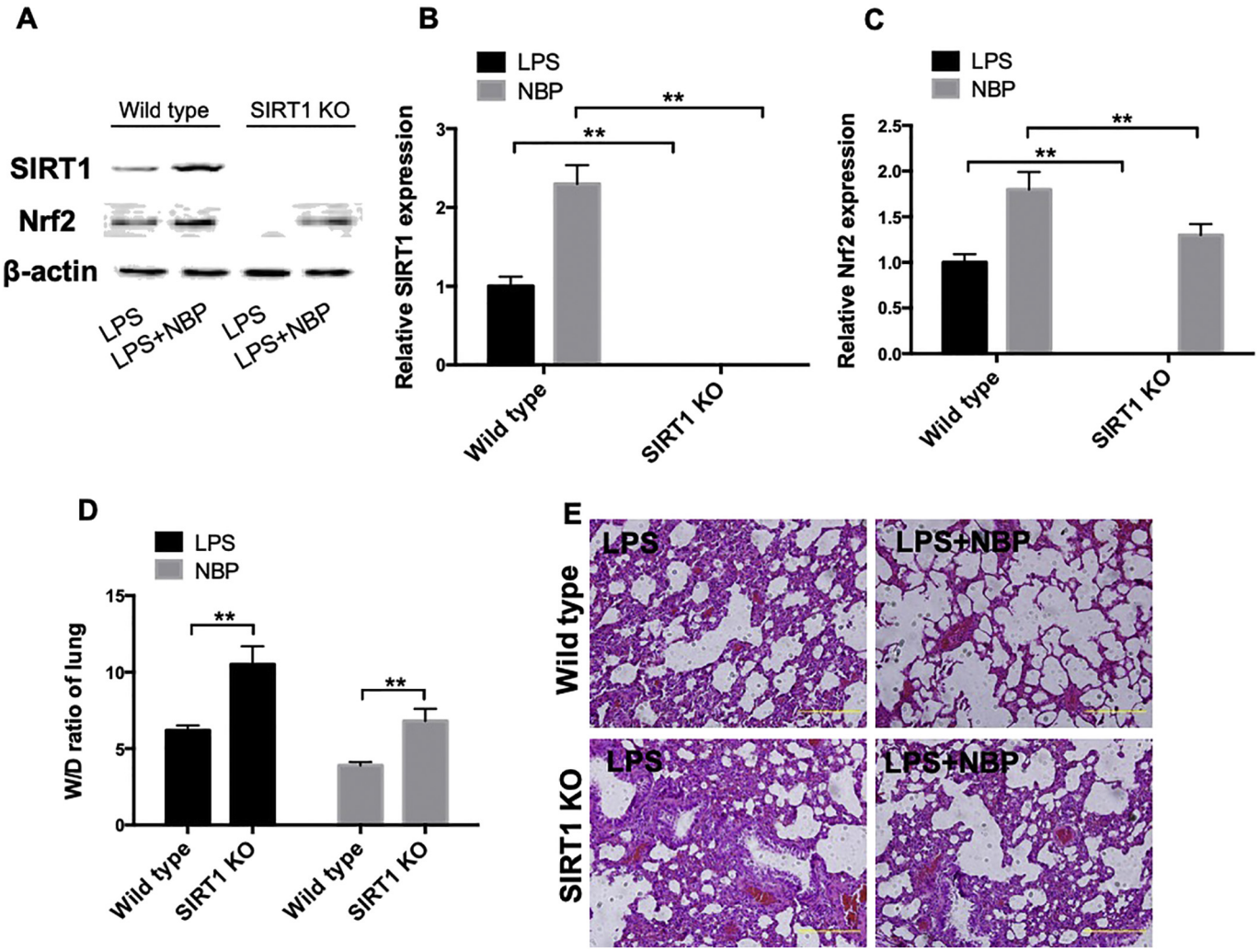

Fig. 5. Knockout of SIRT1 inhibited NBP-induced protective effects against LPS-induced pulmonary edema.
(A) Representative image of protein expression of SIRT1 and Nrf2 in lung tissue. (B) Statistical analysis of SIRT1 protein expression. (C) Statistical analysis of Nrf2 protein expression. (D) W/D ratio of lung tissues. (E) Histopathological examination of lung tissues. $^{**}p < 0.01$.

3.3. NBP ameliorates LPS-induced oxidative injury in lung of mice

Oxidative damage is closely associated with LPS-induced ALI. In this study, we also testify the effect of NBP on oxidative state in LPS-induced ALI mouse model. We showed that LPS resulted in a notable increase of MDA level (Fig. 3A), and decrease of SOD (Fig. 3B) and GPX (Fig. 3C) activities in lung tissues of mice. 10 mg/kg and 50 mg/kg NBP remarkably reduced the level of MDA and increased the activities of SOD and GPX in lung of LPS-treated mice. Additionally, mRNA expression of SOD1 (Fig. 3D), SOD2 (Fig. 3E), and GPX1 (Fig. 3F) in lung was significantly reduced by LPS. NBP remarkably prevented the decrease of mRNA expression of SOD1 (Fig. 3D), SOD2 (Fig. 3E), and GPX1 (Fig. 3F) in lung of LPS-treated mice. The results revealed that LPS-resulted oxidative damage was attenuated by NBP, which effect may be pivotal for the protective effects against ALI.

3.4. NBP ameliorates LPS-induced inhibition of SIRT1/Nrf2 signaling

We then investigated the possible mechanism underlying NBP-induced upregulation of antioxidation and inhibition of inflammation in the context of LPS toxicity. SIRT1 and Nrf2 are key regulators of redox balance. We explored the effect of NBP on SIRT1 and Nrf2 expression in LPS-treated mice. As shown in Fig. 4, the protein expression of SIRT1 and Nrf2 in lung was notably reduced by LPS. 10 mg/kg and 50 mg/kg NBP prevented the reduction of SIRT1 and Nrf2 expression in lung (Fig. 4).

3.5. Knockout of SIRT1 inhibited NBP-induced protective effects against LPS-induced pulmonary edema

To verify whether upregulation of SIRT1 and Nrf2 was involved in the protective effects of NBP against LPS-induced ALI, we used SIRT1-KO mice. As shown in Fig. 5A–C, knock out of SIRT1 significantly reduced the expression of Nrf2 in lung of LPS-treated mice. In LPS-treated SIRT1-KO mice, NBP did not affect the expression of SIRT1, but increased the expression of Nrf2 (Fig. 5A–C). The results demonstrated that NBP could regulate Nrf2 expression both in a SIRT1-dependent and SIRT1-independent manner. SIRT1-KO aggravated LPS-induced increase of W/D ratio of the lung (Fig. 5D). NBP-induced reduction of W/D ratio of the lung was inhibited by knockout of SIRT1 (Fig. 5D). Moreover, LPS-induced histological changes, including destruction of pulmonary alveoli and drastic release of inflammatory cells in mesenchyme and pulmonary alveoli, were promoted by SIRT1-KO (Fig. 5E). SIRT1-KO suppressed NBP-induced attenuation of histological injury of lung in mice (Fig. 5E). The results indicated that SIRT1/Nrf2 signaling was involved in NBP-induced protective effects against LPS-induced ALI.

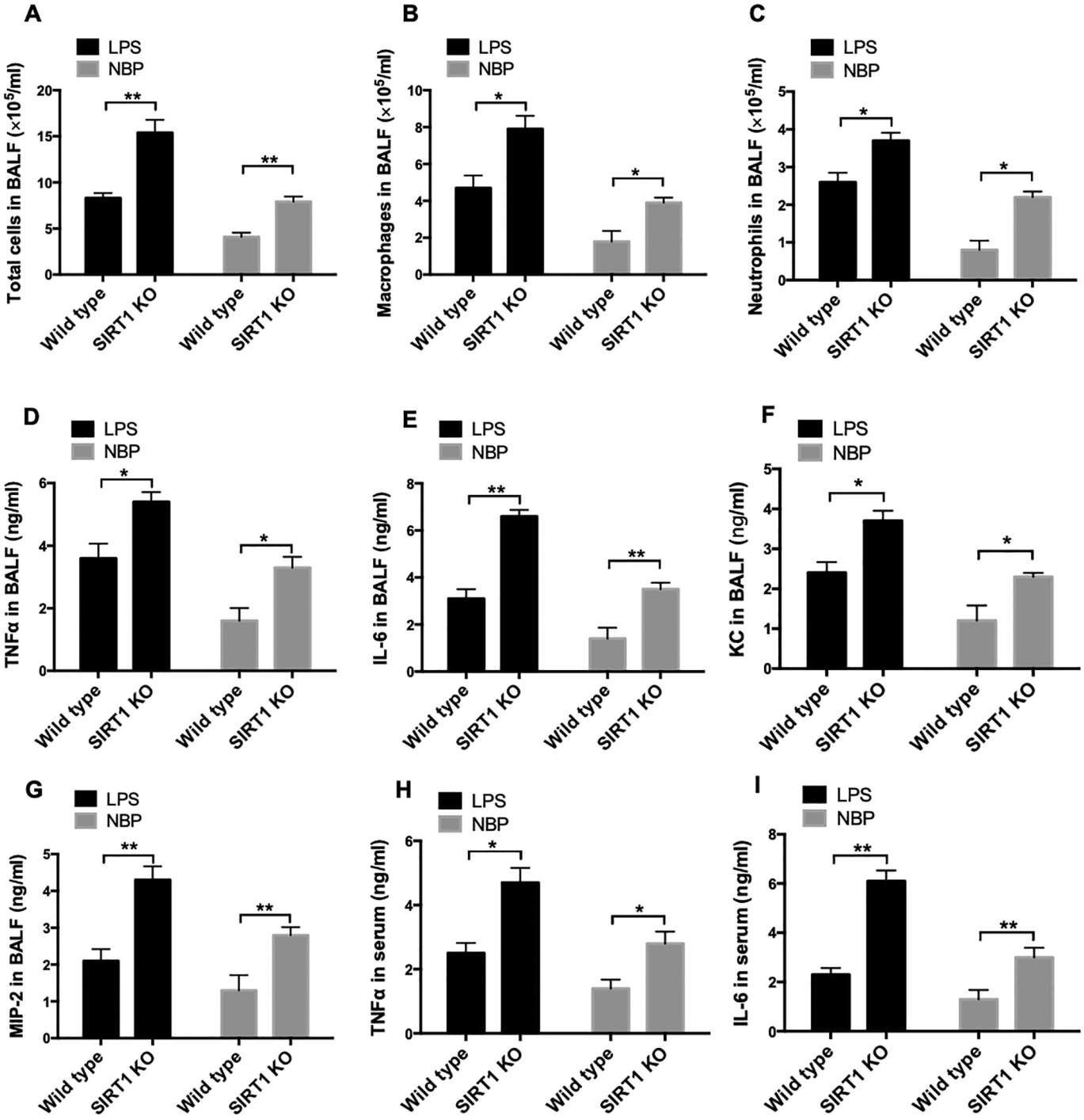

Fig. 6. Knockout of SIRT1 inhibited NBP-induced protective effects against accumulation of inflammatory cells and release of cytokines.
(A) Total cells in BALF. (B) Number of macrophages in BALF. (C) Number of neutrophils in BALF. (D) TNFα content in BALF. (E) IL-6 content in BALF. (F) KC content in BALF. (G) MIP-2 content in BALF. (H) TNFα content in serum. (I) IL-6 content in serum. *$p < 0.05$. **$p < 0.01$.

3.6. Knockout of SIRT1 inhibited NBP-induced protective effects against accumulation of inflammatory cells and release of cytokines

We also evaluated the effect of knockout of SIRT1 on accumulation of inflammatory cells and release of cytokines in lung of LPS-treated mice. As shown in Fig. 6A–G, knockout of SIRT1 aggravated LPS-induced increase of the numbers of total cells, macrophages, and neutrophils, and levels of TNFα, IL-6, KC, and MIP-2 in BALF. NBP-resulted decrease of accumulation of inflammatory cells and release of cytokines were significantly inhibited by knockout of SIRT1 (Fig. 6A–G). Moreover, knockout of SIRT1 aggravated LPS-induced increase of TNFα and IL-6 levels in serum (Fig. 6H–I). NBP-resulted decrease of TNFα and IL-6 levels in serum was significantly inhibited by knockout of SIRT1 (Fig. 6H–I). The results indicated that upregulation of SIRT1 was involved in NBP-induced protective effects against accumulation of inflammatory cells and release of cytokines.

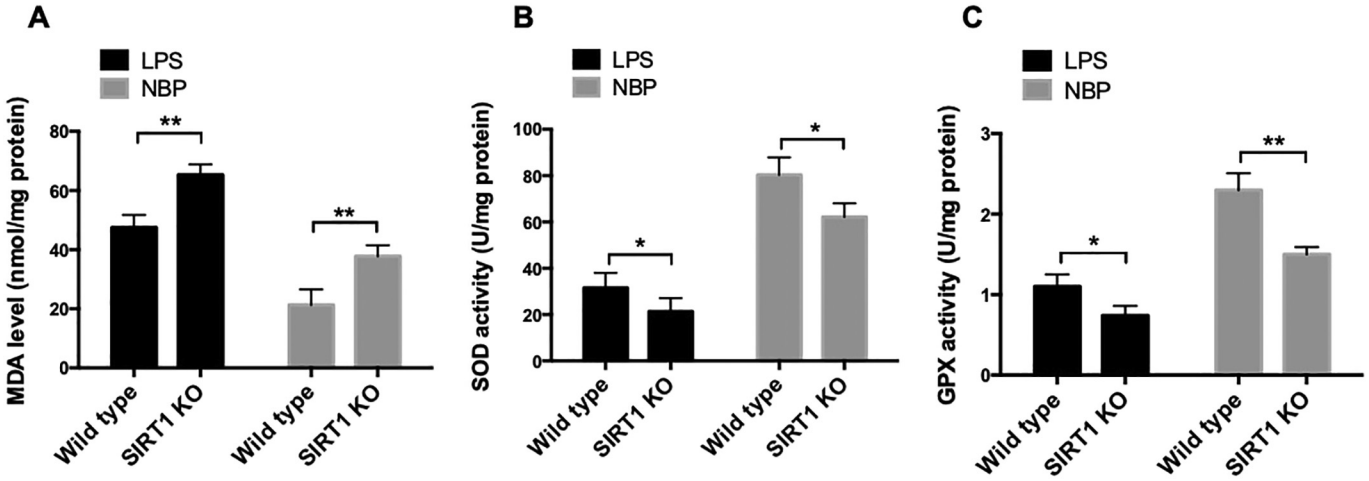

Fig. 7. Knockout of SIRT1 inhibited NBP-induced protective effects against LPS-induced oxidative injury.
(A) MDA level in homogenates of lung. (B) SOD activity in homogenates of lung. (C) GPX activity in homogenates of lung. $^{*}p < 0.05$. $^{**}p < 0.01$.

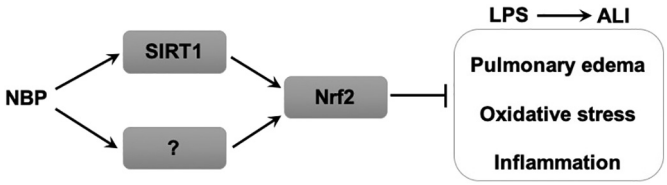

Fig. 8. Proposed molecular mechanism of NBP-induced protective effects against LPS-induced ALI.

3.7. Knockout of SIRT1 inhibited NBP-induced protective effects against LPS-induced oxidative injury

The effect of knockout of SIRT1 on oxidative damage in lung of LPS-treated mice was also assessed. As shown in Fig. 7, knockout of SIRT1 aggravated LPS-induced increase of MDA level, and decrease of SOD and GPX activities in lung tissue. Additionally, NBP-induced decrease of MDA level and increase of SOD and GPX activities were significantly, but not completely, inhibited by knockout of SIRT1 (Fig. 7). The results indicated that upregulation of SIRT1 was involved, at least partly, in NBP-induced protective effects against LPS-induced oxidative injury.

4. Discussion

In the present study, we evaluated the effect of NBP on LPS-induced ALI in mice. To the best of our knowledge, our study, for the first time, found that NBP protected against LPS-induced increase of W/D ratio of lung, histological injury of lung, increased infiltration of inflammatory cells, increased release of inflammatory cytokines and chemoattractants, and increase of oxidative stress in lung. It is demonstrated that NBP is a beneficial agent for the intervention of LPS-induced ALI.

Severe edema and dramatic inflammation are hallmarks of ALI. As a major component in the outer membrane of Gram-negative bacteria, LPS could initiate drastic inflammatory response and severe edema in lung of animals, which was believed to be a well-known animal model of ALI [25]. LPS-induced infiltration of inflammatory cells and release of pro-inflammatory cytokines and chemokines, including TNFα, IL-6, KC and MIP-2, could cause histological injury of lung tissue [26,27]. Pro-inflammatory cytokines and chemokines can activate polymorphonuclear neutrophils, which plays a critical role in LPS-caused ALI [27,28]. Several studies have reported the anti-inflammatory activity of NBP. For example, Wang et al. found that NBP could inhibit nucleotide binding oligomerization domain-like receptor family, pyrin domain containing 3 inflammasome, which effect was associated with the mitigation of Alzheimer's-like pathology [29]. Liao et al. reported that NBP protected against doxorubicin-induced neuroinflammation and behavioral changes [30]. In addition, NBP was also shown to improve LPS-induced depressive-like behavior in rats [31]. These literature focuses on the inhibitory effect of NBP on neuroinflammation. In this study, we found that NBP significantly inhibited the infiltration of polymorphonuclear neutrophils and reduced the release of pro-inflammatory cytokines and chemokines. These results indicated that the anti-inflammatory activity was critical for NBP-induced biological effects, including the attenuation of ALI.

SIRT1 is a nicotinamide adenine dinucleotide-dependent histone deacetylase, dysregulation of which has been reported to be involved in multiple pathophysiological processes, including inflammation, DNA damage, apoptosis, and oxidative stress [32–34]. Activation of SIRT1 has been verified to protect against lung injury and knockdown of SIRT1 could promote the inflammatory cytokine release [35,36]. Moreover, SIRT1 could regulate oxidative state through modulation of Nrf2 antioxidant signaling, which effect has been shown to attenuate lung injury in animal model [37,38]. Oxidative stress plays a predominant role in LPS-induced lung injury [39]. In the present study, we also investigated the role of SIRT1/Nrf2 signaling in the protective effects of NBP in vivo. We found that NBP upregulated SIRT1 and Nrf2 expression in lung of LPS-treated mice. Knockout of SIRT1 decreased Nrf2 expression and aggravated LPS-induced pulmonary edema, infiltration of inflammatory cells and release of pro-inflammatory cytokines. Knockout of SIRT1 significantly, but not completely inhibited NBP-induced protective effects against LPS-induced ALI. In the context of SIRT1-KO, NBP could upregulate Nrf2 expression, indicating that NBP regulated Nrf2 expression in a SIRT1-dependent and SIRT1-independent manner. This effect may explain why knockout of SIRT1 did not completely inhibit the protective effects of NBP. Anyway, we, for the first time, found that SIRT1 was involved in NBP-induced protection against lung injury. Previous reports also suggest that Nrf2 may be a crucial regulator that mediates NBP-induced pharmacological effects. The above mentioned literature showed that upregulation of Nrf2 was associated with the inhibitory effect of NBP on neuroinflammation [29,31]. Nrf2 activation is important for NBP-induced neuroprotection [40,41]. Based on the data in our study, we suggest that SIRT1-dependent and SIRT1-independent regulation of Nrf2 is a central mechanism for the pharmacological effects of NBP.

Overall, we revealed that NBP could effectively protect against LPS-induced ALI through anti-inflammatory and antioxidant activities. NBP could activate Nrf2-controlled antioxidant signaling in a SIRT1-dependent and SIRT1-independent manner, resulting in the amelioration of oxidative stress, inflammation and pulmonary edema (Fig. 8). The data

highlights the importance of SIRT1/Nrf2 signaling in the protective effects of NBP.

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Declaration of competing interest

The authors declare that there are no conflicts of interest.

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