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Original Research Article

Salvianolic acid A regulates Nrf2 and NF-κB pathways to alleviate lipopolysaccharide-induced pneumonia cell damage

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Abstract

Purpose: *To examine the regulatory roles of salvianolic acid A (SA-A) in lipopolysaccharide-induced pneumonia cell damage.*
Methods: *Cell proliferation in lung epithelial cells BEAS-2B was determined using CCK-8 assay while cell apoptosis was assessed by flow cytometry. The levels of TNF-α, IL-1β and IL-6 were determined using enzyme-linked immunosorbent assay (ELISA). Protein expressions were evaluated using western blot, while the levels of superoxide dismutase (SOD), myeloperoxidase (MPO), and malondialdehyde (MDA) were assessed using their corresponding ELISA kits.*
Results: *Cell apoptosis was enhanced after LPS induction ($p < 0.001$), but this change was offset after SA-A treatment ($p < 0.05$). Inflammation was increased after LPS stimulation ($p < 0.001$), but this change was offset following SA-A treatment ($p < 0.05$). Oxidative stress was heightened after LPS induction ($p < 0.05$), but reversed following SA-A treatment ($p < 0.05$). Finally, LPS stimulation enhanced endoplasmic reticulum stress and mitochondrial dysfunction ($p < 0.001$), which was significantly reversed by SA-A treatment ($p < 0.05$).*
Conclusion: *SA-A regulates Nrf2 and NF-κB pathways, inhibits oxidative stress, inflammation-stimulated ER stress, and mitochondrial dysfunction. It also alleviates LPS-induced pneumonia cell damage, thus indicating its potentials for development as a therapeutic agent for the treatment of pneumonia.*

Keywords: *Salvianolic acid A, Inflammation, Oxidative stress, Lipopolysaccharide, Pneumonia*

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INTRODUCTION

Acute lung injury (ALI) is a disease that generates acute respiratory distress syndrome, has a high mortality rate, and threatens human health [1]. This condition has gained worldwide attention in clinics [2]. The major manifestations of ALI are increased pulmonary vascular permeability, damage to the alveolar-capillary system, and excessive activation of macrophages and neutrophils, and these changes eventually result in respiratory function impairment [3]. There has been significant progress in the drug treatment of ALI, but many

drug resistance problems still exist. Therefore, the development of novel drugs for ALI treatment is of pivotal importance.

Salvianolic Acid A (SA-A) is a bioactive polyphenol extracted from the root of *Salvia miltiorrhiza*, a traditional Chinese herb with multiple functions [4]. Studies have revealed that SA-A is a multi-target drug with diversified pharmacological effects, including anti-inflammatory, antioxidant, anti-cancer and anti-fibrosis activities. For example, SA-A activates the Akt/GSK-3β/Nrf2 pathway and suppresses the NF-κB pathway to reduce oxidative stress in 5 out of 6 nephrectomized rats [5]. Furthermore, SA-A regulates Bax/Bcl-2 ratio and calcium release to reduce endoplasmic reticulum stress and mitochondrial-mediated apoptosis in hepatocyte LO2 cells [6]. Besides, SA-A inactivates the TAGLN2-mediated PI3K/Akt pathway to improve the malignant progression of glioma and strengthen temozolomide sensitivity [7]. Interestingly, in human non-small cell lung cancer, SA-A affects the PI3K/Akt/mTOR pathway to improve vasculogenic mimicry formation [8]. However, the special regulatory functions of SA-A in pneumonia progression requires further investigation.

This work investigates the regulatory roles of SA-A on lipopolysaccharide-induced pneumonia cell damage.

EXPERIMENTAL

Cell line and cell culture

Human normal lung epithelial cells BEAS-2B were acquired from Procell Life Science & Technology Co. Ltd (Wuhan, China). The RPMI-1640 (GIBCO, Grand Island, NY, USA) containing 10 % fetal bovine serum (FBS, Gibico, USA), and 1 % penicillin/streptomycin was utilized to culture BEAS-2B cells in a moist incubator at 37 °C, 5 % CO_2. Lipopolysaccharide (LPS) (5 μg/mL) or SA-A (20 or 40 μM; Solarbio, Beijing, China) was utilized to treat BEAS-2B cells for 24 h.

CCK-8 assay

Cell counting kit-8 (CCK-8; Dojindo Laboratories, Kumamoto, Japan) was employed in this assay. The BEAS-2B cells (1×10^4 cells/well) were placed into the 96-well plate for 48 h. Thereafter, CCK-8 solution (10 μL) was added into each well and the absorbance measured using a spectrophotometer (Thermo Fisher Scientific, MA, USA) at 450 nm.

Flow cytometry

The cell apoptosis was evaluated using the Annexin-V-PI Apoptosis Detection kit (BD Biosciences, Franklin Lakes, NJ, USA). The BEAS-2B cells were re-suspended after washing in phosphate-buffered saline and stained with annexin V-fluorescein isothiocyanate (FITC, 5 μL, 50 μg/mL). The cells were counterstain with propidium iodide (5 μL, 50 μg/mL) in the dark. Cell apoptosis was tested under flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA).

Enzyme-linked immunosorbent assay (ELISA)

Levels of TNF-α (ab181421; Abcam, Shanghai, China), IL-1β (ab214025), and IL-6 (ab178013) in cell supernatant were assessed using the corresponding ELISA kits. The levels of superoxide dismutase (SOD), myeloperoxidase (MPO), and malondialdehyde (MDA) were measured with the corresponding ELISA kits (Thermo Fisher Scientific, Waltham, Ma, USA).

Western blot

Total proteins from BEAS-2B cells were isolated by radioimmunoprecipitation assay (RIPA) lysis buffer (Thermo Fisher Scientific, Inc.). Protein electrophoresis was performed using 10 % sodium doceyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Separated proteins were transferred to polyvinylidene diflouride (PVDF) membranes (Beyotime, Shanghai, China) and post blocked with 5 % skimmed milk. Primary antibodies were added into the membranes at 4 °C for 12 h followed by secondary antibodies (1:2,000; ab7090). Blots were evaluated using chemiluminescence detection kit (Thermo Fisher Scientific, Inc.).

Primary antibodies employed were: p-P65 (1:2000; ab86299; Abcam, Shanghai, China), P65 (0.5 μg/mL; ab16502), p-IκBα (1:10,000; ab133462), IκBα (1:1,000; ab32518), Nrf2 (1:1,000; ab137550), GRP78 (1 μg/ml; ab21685), CHOP (5 μg/ml; ab11419), Cytochrome C (1:5,000; ab133504) and β-actin (1:1,000; ab8227; the internal reference).

Statistical analysis

Statistical analysis was performed using SPSS 22.0 statistical software (IBM Corp., Armonk, NY, USA). All determinations were done in triplicate and the data displayed as mean ± standard deviation (SD). Group comparison was carried out using independent Students' *t*-test and one-way analysis of variance (ANOVA). $P < 0.05$ was considered statistically significant.

RESULTS

SA-A reduced LPS-induced pneumonia cell apoptosis

The molecular structural formula of SA-A is displayed in Figure 1 A. Cell viability was significantly reduced after LPS treatment, but this effect was ameliorated after SA-A treatment (Figure 1 B). Cell apoptosis increased after LPS induction, but this change was offset after SA-A treatment (Figure 1 C). These results indicated that SA-A treatment decreased cell apoptosis in LPS-induced pneumonia.

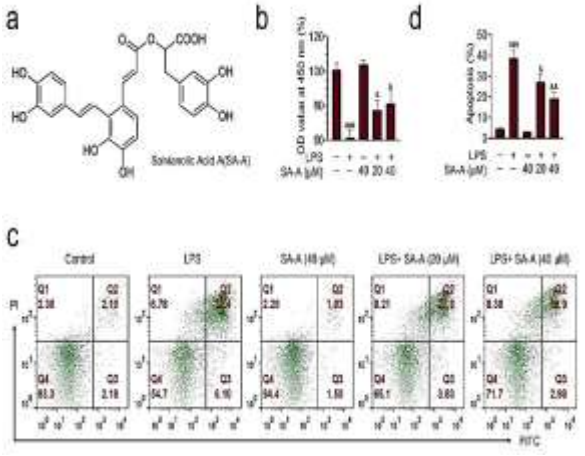

Figure 1: SA-A reduced LPS-induced pneumonia cell apoptosis. (a) Molecular structural formula of SA-A, (b) Cell viability measured using CCK-8 assay. (c-d): Cell apoptosis measured using flow cytometry. $^{###}P$ < 0.001 compared with control group, $^{\&}p$ < 0.05, $^{\&\&}p$ < 0.01 compared with LPS group

SA-A relieved LPS-stimulated inflammation in pneumonia cells

The TNF-α, IL-1β and IL-6 levels were elevated after LPS stimulation, but these effects were offset after SA-A treatment (Figure 2 A). Levels of NF-κB pathway related proteins (p-p65/p65 and p-IκBα/IκBα) were up-regulated after LPS induction, but these effects were reversed after SA-A treatment (Figure 2 B). This shows that SA-A relieved LPS-stimulated inflammation in pneumonia cells.

SA-A decreased LPS-triggered oxidative stress in pneumonia cells

The MDA, MPO and SOD levels increased after LPS treatment, but these effects were reversed after SA-A treatment (Figure 3 A). Additionally, the Nrf2 protein expression was decreased after LPS stimulation, but this change was reversed after SA-A treatment (Figure 3 B). This shows that SA-A decreased LPS-triggered oxidative stress in pneumonia cells.

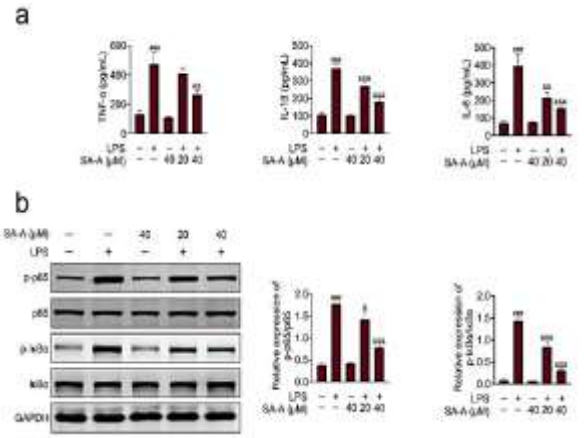

Figure 2: SA-A relieved LPS-stimulated inflammation in pneumonia cells. (a): The levels of TNF-α, IL-1β and IL-6 measured through using ELISA; (b): The protein expressions (p-p65, p65, p-IκBα and IκBα) determined using western blot. $^{###}P$ < 0.001 compared with control group, $^{\&}p$ < 0.05, $^{\&\&}p$ < 0.01, $^{\&\&\&}p$ < 0.001 compared with LPS group

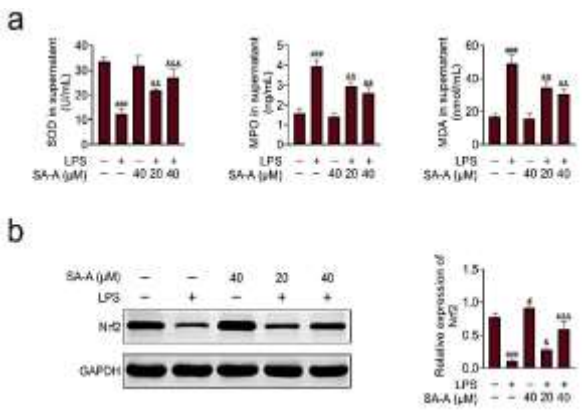

Figure 3: SA-A decreased LPS-triggered oxidative stress in pneumonia cells. (a) MDA, MPO and SOD levels evaluated through corresponding commercial kits; (b) Protein expression of Nrf2 measured through using western blot. $^{\#}P$ < 0.05, $^{###}p$ < 0.001 compared with control group; $^{\&}p$ < 0.05, $^{\&\&}p$ < 0.01, $^{\&\&\&}p$ < 0.001 compared with LPS group

SA-A modulated LPS-mediated ER stress and mitochondrial dysfunction

Protein expressions of GRP78 and CHOP increased after LPS induction, but these changes were offset after SA-A treatment (Figure 4 A). Similarly, cytochrome C protein expression was enhanced after LPS induction, but this effect was relieved after SA-A treatment (Figure 4 B). This shows that SA-A modulated LPS-mediated ER stress and mitochondrial dysfunction.

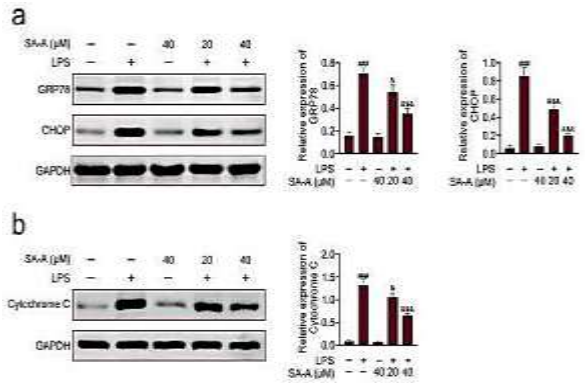

Figure 4: SA-A modulated LPS-mediated ER stress and mitochondrial dysfunction. (a-b) Protein expressions of GRP78, CHOP and Cytochrome C measured using western blot. $^{###}P < 0.001$ compared with control group; $^{\&}p < 0.05$, $^{\&\&\&}p < 0.001$ compared with LPS group

DISCUSSION

Many constituents from herbs have been found to modulate various mechanisms in the progression of ALI. For example, *Elsholtzia bodinieri* Vaniot modulates inflammation, pyroptosis, oxidative stress and macrophage polarization to improve ALI in mice [9]. In addition, isorhapontigenin regulates the Nrf2 signaling pathway to relieve LPS-stimulated ALI [10]. Similarly, salviplenoid A modulates the NF-κB and Nrf2 signaling pathways to improve inflammation in ALI progression [11]. SA-A has been shown to exhibit various pharmacological activities which are useful in many diseases. One of such is its effects on PPAR-γ to alleviate LPS-stimulated ALI in mice [12]. In this study, BEAS-2B cells treated with LPS were utilized to mimic a pneumonia cell model, and it was discovered that cell apoptosis was enhanced after LPS induction. However, this change was attenuated after SA-A treatment.

Previous evidence has revealed that inflammation and oxidative stress are intimately associated with ALI progression. In the progression of pneumonia, there is release of pro-inflammatory cytokines including tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-6 [13]. In addition, the activated neutrophils propagate inflammation and cause damage by generating reactive oxygen species (ROS) and proteolytic enzymes [14]. In this study, the inflammation and NF-κB pathway were strengthened after LPS stimulation, but this effect was offset after SA-A treatment. Likewise, the oxidative stress and Nrf2 pathway were heightened after LPS addition, but this effect was also reversed after SA-A treatment.

The endoplasmic reticulum (ER) plays a variety of roles in cells. In conditions of cellular stress and inflammation, unfolded protein response (UPR) is stimulated by ER stress so as to alleviate stress and regain ER homeostasis [15]. In pathological conditions such as ALI, sepsis, and infection, ER stress brings about large accumulation of incorrect protein structures, and there are three affected ER transmembrane proteins which include ATF6, IRE1, and PERK. Glucose regulated protein (GRP78) combines with the three ER transmembrane proteins in ER stress-free, and dissociates from these UPR sensors in ER stress [16]. Furthermore, CHOP has been discovered to be a pivotal molecule in ER-stimulated cell apoptosis [17]. The enhanced permeability of mitochondrial outer membrane results in release of substances in the membrane gap, which migrate into the cytoplasm; the most important of which is cytochrome C which initiates the Caspase reaction and then causes cell apoptosis [18]. In this study, SA-A modulated LPS-mediated ER stress and mitochondrial dysfunction. However, regulatory impacts of SA-A on pneumonia progression requires further studies since only few mechanisms were investigated.

CONCLUSION

Salvianolic acid A regulates Nrf2 and NF-κB pathways, inhibits oxidative stress, inflammation-mediated ER stress, and mitochondrial dysfunction. Furthermore, it alleviates LPS-induced pneumonia cell damage. However, more work is required to investigate the other regulatory functions of SA-A in pneumonia, and determine fully its potentials for the management of pneumonia.

DECLARATIONS

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Ethical approval

None provided.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Hai Lin and Jinrong Yi designed the study and carried them out, supervised the data collection, analyzed the data, interpreted the data, prepared the manuscript for publication and reviewed the draft of the manuscript. All authors read and approved the manuscript.

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