18β-glycyrrhetinic Acid Protects against *Staphylococcus aureus* Infection by Regulating the NF-κB Pathway

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**ABSTRACT**

**Background:** 18β-glycyrrhetinic acid (18β-GA) is reported to possess various pharmacological properties of which anti-inflammatory activities has been widely explored. However, the role of 18β-GA in *Staphylococcus aureus* (SA) infection has not been investigated. The aim of the present study was to explore the effects of 18β-GA on the SA infection especially the SA-induced Acute lung injury (ALI) and its related mechanisms. **Material and methods:** We infected the mice or cells with SA and then detected the survival rates of mice, bacterial burden and production of proinflammatory cytokines both *in vitro* and *in vivo*. We then detected the High-mobility group box 1 (HMGB1) expression by RT-qPCR and Western blotting. The effects on NF-κB activation was also determined by Western blotting and luciferase assay. **Results:** 18β-GA could significantly improve the survival rate of SA-infected mice, reduce bacterial burden, suppress infiltration of inflammatory cells and reduce secretion of IL-1β, IL-6 and TNF-α both in lung tissues and cells. 18β-GA treatment decreased high-mobility group box 1 (HMGB1) expression induced by SA infection and neutralizing of HMGB1 could improve the survival rate of mice induced by SA, implying that 18β-GA protected SA infection through down-regulating the HMGB1/NF-κB activation. Finally, we demonstrated that 18β-GA inhibited the NF-κB activation. **Conclusion:** Taken together, our preliminary study suggested that 18β-GA provided protective effects against SA infection via its anti-inflammatory properties possibly through down-regulating the HMGB1/NF-κB activation. **Key words:** 18β-glycyrrhetinic acid, *Staphylococcus aureus*, Acute lung injury, HMGB1, NF-κB.

**INTRODUCTION**

*Staphylococcus aureus* (SA) is a gram-positive bacterium that can cause a wide spectrum infection from mild infection of superficial skin to severe infection such as sepsis or septic shock.¹ Lung is a common site of SA infection. Acute lung injury (ALI) is a complex syndrome characterized by hypoxemia, extensive pulmonary edema, tiny atelectasis, intrapulmonary hemorrhage and severely impaired gas exchange.² The mechanisms involved in ALI including uncontrolled and excessive production of inflammatory mediators including cytokines, chemokines, adhesion molecules and bioactive lipid products.³ The primary cause of ALI is bacterial infection, in which SA is one of the most common pathogens. Despite the progress and improvements made in treatment, the mortality of pneumonia with ALI is still high. Strategies such as reducing bacterial load and attenuating exaggerated inflammation are under extensive evaluation.⁴
18β-glycyrrhetinic acid (18β-GA), a major component of *Glycyrrhiza glabra* has been demonstrated to have several pharmacological effects including anti-inflammatory, anti-ulcer, anti-viral, antioxidant and hepatoprotective properties.\(^5\)\(^8\) 18β-GA has been found to inhibit the LPS-induced inflammatory response in macrophages and increase the survival rate in LPS-induced ALI.\(^9\)\(^10\) Even in Meticillin-resistant *Staphylococcus aureus* (MRSA) infection, 18β-GA could also attenuate the skin lesion and decrease the virulence gene expressions.\(^11\) 18β-GA also exhibits the protective activities in inflammatory liver injury.\(^12\)\(^-\)\(^14\) 18β-GA has been shown to attenuate the inflammation in SA-induced ALI.\(^15\) However, the anti-inflammatory effects and molecular mechanisms were unclear.

High-mobility group box 1 (HMGB1) is one of the members of Damage-associated molecular pattern (DAMP) and is associated with delayed and sustained release during infection.\(^16\) HMGB1 is highly released after SA infection and anti-HMGB1 could ameliorate the pulmonary damage,\(^17\) suggesting that HMGB1 might be a target in patients with pneumonia caused by SA.

The main objective of our study is to explore the effects of 18β-GA on the SA infection, especially the induced ALI and the underlying mechanisms. Our study demonstrated that 18β-GA provided protective effects against SA infection via its anti-inflammatory properties possibly through down-regulating the HMGB1/NF-κB activation.

**MATERIALS AND METHODS**

**Chemicals and Mice**

18β-glycyrrhetinic acid (18β-GA) was obtained from Sigma-Aldrich (St. Louis, MO, USA) and dissolved in dimethyl sulfoxide (DMSO). Stock solutions were prepared at a concentration of 20 mg/ml and were diluted in medium to the appropriate concentrations indicated in each experiment. C57BL/6 mice (Shanghai Laboratory Animal Company, Shanghai, China) weighing between 16–18 g, age of 6-8 weeks were used. Mice were housed in a specific pathogen-free facility at Zhejiang University School of Medicine. Animals were treated in accordance with the Guide for the Care and Use of Laboratory Animals (6th edition, National Academies Press). This study was approved by the Animal Experimental Ethical Inspection of the First Affiliated Hospital, College of Medicine, Zhejiang University (Permit number is 2017-475).

**Bacterial Strains and Growth Conditions**

The SA strain was a kind gift from professor FengXu, the Second Affiliated Hospital, Zhejiang University School of Medicine.\(^1\) The bacteria were allowed to grow at 37°C in Tryptone Soya Broth (TSB) medium. SA stocks were kept at -80°C in TSB medium supplemented with 50% (vol/vol) of glycerol. Overnight cultures of SA were re-inoculated into fresh TSB and grown to a log phase and then quantified according to a OD600-based bacterial growth curve and Colony forming units (CFU) assay.\(^18\)

**Cell Culture and Treatment**

Human macrophage RAW264.7 cells and human liver hepatoma cells (HepG2) were obtained from American Type Culture Collection (ATCC, University Boulevard, Manassas, VA) and were cultured in DMEM supplemented with 10% FBS, 100 U/mL penicillin and 100 mg/mL streptomycin in a 5% CO₂ humidified incubator at 37°C. All the cells were pretreated with 18β-GA or DMSO at a final concentration of 20 μM 2 h before infected with SA at a Multiplicity of infection (MOI) of 10 and then continued culture for the indicated times.

**Mice Infection Model**

A SA-induced ALI mice model was established as previously reported.\(^19\)-\(^21\) In brief, mice were randomly divided into three groups: group PBS: mice were intraperitoneally (i.p.) injected with DMSO 2 h prior to mock infection with PBS; group PBS+SA: mice were i.p. injected with DMSO 2 h prior to infection with SA (1×10⁸ CFU); group 18β-GA + SA: mice were i.p. injected with 50 mg/kg 18β-GA 2 h prior to SA infection. Mice were anesthesiaded by 1.5% pentobarbital (5 μL/g per mouse) and then 40 μL of SA or phosphate buffered saline (PBS) was inoculated directly intratracheal (i.t.) into the mice.

All the mice were used to perform survival rate, histological and molecular biological examination. The survival rate experiment was performed by examining them every 6 h up to 3 days.

To determine the role of HMGB1 in SA infection, mice were intraperitoneally injected with either 600 μg anti-HMGB1 neutralizing antibody (Santa Cruz Biotechnology, Santa Cruz, CA) or non-immune rabbit IgG in 500 μL PBS 1 h before SA infection.

**Histopathological Changes Examination**

Lungs and livers of the mice were fixed in 4% paraformaldehyde for 24 h at 4°C, then embedded in paraffin. 4 μM sections were performed using a rotary microtome
ALT and AST Leakage

The production of ALT and AST were determined with commercial kits purchased from the Institute of Biological Engineering of Nanjing Jiansheng (Nanjing, China) according to the manufacturer’s instructions.

Western Blotting

Cells were rinsed with cold PBS and total cellular lysates were prepared using the RIPA buffer (CST, Danvers, MA) supplemented with phenyl methyl sulfonyl fluoride (PMSF, USA) and protease inhibitor cocktail. The protein concentrations were measured with the BCA Protein Assay Kit (CWBio). The protein samples (20 μg) were separated by 10% SDS-PAGE and transferred to PVDF membranes (Millipore, Billerica, MA, USA). The membranes were blocked with 5% skimmed milk for 1 h at room temperature and probed with primary antibodies against phosphorylated or total proteins for p65, p38, JNK, ERK42/44 and HMGB1 (CST, Danvers, MA). After washing with TBST for three times, membranes were incubated with the HRP-conjugated secondary antibodies (Lianke, Hangzhou, China). ECL reagent were used to detect the immunoreactive bands on a digital image system (FluorChem E; Proteinsimple, Santa Clara, CA, USA).

Luciferase Assay

RAW264.7 cells were seeded in 96-well plate at a density of 1x10^4 per well 1 day prior to transfection. Cells were transient transfected with NF-κB luciferase reporter plasmid with Lipofectamine 2000 (Invitrogen, Carlsbad, CA) following the manufacturer’s instructions. After 24 h post transfection, cells were pretreated with 18β-GA or DMSO at a final concentration of 20 μM 2 h before infected with SA. After 24 h, cells were collected, and the luciferase activities were determined by using the Bright-Glo luciferase assay system (Promega Corp., Madison, WI).

Statistical Analysis

All data were analyzed using GraphPad Prism 5 (GraphPad InStatSoftware, San Diego, CA, USA). Results were presented as mean ± SEM with at least three indepen-

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dent experiments. The Student t-test was used to estimate the significant differences between groups. Overall survival rates were calculated using the Kaplan-Meier method. The log-rank test was utilized to compare the survival rates between groups. *P<0.05.

RESULTS

18β-GA Protects Mice against SA-induced ALI and the Secondary Liver Injury

To evaluate the effect of 18β-GA on SA infection, C57BL/6 mice were pre-treated with 18β-GA 2 h before infected with SA. First, we checked the survival rate of the mice after SA infection. As shown in Figure 1A, 18β-GA treatment significantly improved the survival rate compared with PBS-treated mice (P=0.0237). We speculated that this might be resulted from the decreased bacterial load and attenuated inflammation. As shown in Figure 1B, lung tissues from mice treated with 18β-GA showed lower bacterial counts at 12 h post SA infection (P=0.043). Furthermore, 18β-GA attenuated the SA-induced increase of TNF-α, IL-1β and IL-6 mRNA expression in lung and protein secretion in BALF (Figure 1C, P<0.05). With the challenge of SA, lung tissues were significantly damaged with various histopathologic changes including interstitial edema and hemorrhage, alveolar wall thickening and notable infiltration of neutrophils in the lung parenchyma and alveolar spaces. While 18β-GA treatment relieved the lung damage (Figure 1D) accompanied with the lower neutrophils infiltration (Figure 1E, P=0.0074). We also investigated the liver histopathology. As shown in Figure 1F, SA infected mice showed the infiltration with cells at the peripheral regions of the blood vessels and 18β-GA reduced the cells infiltration.

18β-GA Exhibits Antimicrobial activity and Inhibits Proinflammatory Cytokine Production in SA Infected RAW264.7 Cells

To evaluate the effects of 18β-GA on SA infection in vitro, RAW264.7 macrophages were implied for SA infection. First, cells were pre-treated with different concentrations of 18β-GA before infected with SA for 6 h and then bacterial numbers were determined. 18β-GA significantly inhibited bacterial growth compared with control medium in a dose-dependent manner (Figure 2A). Consistent with the in vivo results, pretreatment of 18β-GA significantly decreased the IL-1β, IL-6 and TNF-α expression and production (Figure 2B, P<0.05).

18β-GA Stabilizes HepG2 Liver Cells During SA Challenge

We also detected the leakage of ALT and AST in culture medium from HepG2 cells exposed to SA infection with or without 18β-GA treatment. As presented in Figure 2C, 18β-GA significantly inhibited the leakage of ALT and AST (P=0.028 and 0.018, respectively) compared with the PBS-treated cells after SA challenge.

HMGB1 Expression was Down-Regulated after 18β-GA Treatment

HMGB1 has been reported to increase sepsis and is positively correlated with the severity of sepsis in animals. Thus, we detected the HMGB1 expression both in lung tissues and in RAW264.7 cells. As shown in Figure 3A-3D, the mRNA and protein levels of HMGB1 in tissues and cells were remarkably increased after SA
Therefore, to clarify the potential mechanism about its anti-inflammatory activity, the effects of 18β-GA on the activation of NF-κB and MAPK signaling pathways were investigated. As shown in Figure 4A, SA infection significantly increased the phosphorylation of p65, p38, JN and ERK42/44 at 15, 30, 60 or 120 min, as compared with control group. However, 18β-GA treatment only markedly decreased the phosphorylation of p65 at 60 or 120 min. We also assessed the NF-κB activation using the luciferase reporter assay. After exposure to 18β-GA, NF-κB activity was significantly decreased (Figure 4B, P<0.0001). These results suggested that alleviated effects of 18β-GA on SA-induced inflammatory response were at least partially through regulation of NF-κB activation.

18β-GA Inhibited the activation of NF-kB in RAW264.7 Cells

It is generally acknowledged that NF-κB is responsible for the transcription of pro-inflammatory genes and plays critical roles in the pathogenesis of ALI. Many studies have shown that reducing or blocking the activation of NF-κB and MAPK signaling pathway could attenuate the lung injury and inflammation in ALI.
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Of these, pneumonia is one of the most prevalent diseases induced by SA infection with high mortality rates up to 8%. SA infection induced ALI and more severely, the Acute respiratory distress syndrome (ARDS), is a leading cause of morbidity and mortality in critically ill patients. Current treatments for ALI or ARDS are lacking, so it is important to explore the other possibilities.

18β-GA has been reported to have several pharmacological effects including anti-tumor, anti-inflammation, anti-radiation and so on. Cao et al. reported 18β-GA inhibits the initiation and progression of gastric tumors by ameliorating the inflammatory microenvironment through downregulation of COX-2 expression and inhibiting Wnt-1 expression. 18β-GA significantly attenuates UV-induced skin photoaging mainly by virtue of its anti-oxidative and anti-inflammatory properties.

The anti-inflammatory effects of 18β-GA have been revealed in recent years. Studies have found 18β-GA exhibits anti-inflammatory activities through inhibition of MIP-1α production in acute propionibacterium acnes-induced inflammatory liver injury and inhibition of LPS/D–galactosamine-induced liver injury and prevention of free fatty acid-induced hepatic lipotoxicity. 18β-GA also has been demonstrated to inhibit the LPS-induced inflammatory response in macrophages and increase the survival rate in LPS-induced ALI. In accordance with these studies, we revealed that 18β-GA attenuated the inflammation in the SA induced ALI model.

Clinical and experimental studies have shown that ALI is an early and fatal complication of septic shock and the TLR2/4-mediated NF-κB activation could be an early molecular event leading to ALI during septic shock. NF-κB is a critical transcription factor in TLR-mediated signaling pathways and plays a critical role in regulation of expressions of a number of genes, including inflammatory cytokines such as HMGB1, TNF-α and IL-1β. 18β-GA has been found to attenuate NF-κB activation to alleviate the inflammation.
we found the same phenomenon that 18β-GA inhibited the activation of NF-κB.

CONCLUSION
Taken together, we demonstrated that 18β-GA protects mice against lung and liver injury after SA infection. The possible mechanisms are related to the decreased pro-inflammatory factors and HMGB1 expressions which might through the inhibition of NF-κB-regulated inflammation signaling pathways. Thus, 18β-GA may have the potential to serve as a useful therapeutic agent for treating ALI caused by bacterial infection.

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CONFLICT OF INTEREST
The authors declare no conflict of interest.

ABBREVIATIONS
18β-GA: 18β-glycyrrhetinic acid; SA: Staphylococcus aureus; ALI: Acute lung injury; HMGB1: High-mobility group box 1.

REFERENCES
18β-glycyrrhetinic acid (18β-GA) is reported to possess various pharmacological properties. In this study, we explored the effects of 18β-GA on the SA infection especially the SA-induced acute lung injury and its related mechanisms. Mice or cells were infected with SA both in vitro and in vivo and the survival rates of mice, bacterial burden and production of proinflammatory cytokines were detected. The expression of high-mobility group box 1 (HMGB1) and NF-κB activation was further determined. We found that 18β-GA provided protective effects against SA infection via its anti-inflammatory properties possibly through down-regulating the HMGB1/NF-κB activation, indicating that 18β-GA may have the potential to serve as a useful therapeutic agent for treating ALI caused by bacterial infection.