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Original Article

Schizandrin B protects LPS-induced sepsis via TLR4/NF-κB/MyD88 signaling pathway

Jianjun Xu[1], Caijiao Lu[1], Zhengjun Liu[1], Peng Zhang[1], Hailei Guo[1], Tingting Wang[2]

[1]Burn Wound Center, [2]Department of Infectious Internal Medicine, The First Affiliated Hospital of Wenzhou Medical University, Wenzhou 325000, P. R. China

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Abstract: Schizandrin B (*Sch B*) is the main component isolated from Schizandra fruit (*Schisandra chinensis*). While *Sch B* is established as having antioxidant, anti-proliferation and anti-inflammatory properties, but its activity in sepsis remains unclear. In the present study, we investigated the anti-inflammatory effects of *Sch B* in sepsis. Our experimental results demonstrated that *Sch B* inhibited production of IL-1β, TNF-α, IL-6 and HMGB1 by LPS-activated RAW264.7 cells. Moreover, *Sch B* suppressed expression of iNOS, reduced production of PGE2, blocked expression of MyD88 and TLR4, suppressed the activity of NF-κB and decreased phosphorylation of MAPKs in LPS-activated RAW264.7 cells. Administration of *Sch B* also reduced production of IL-1β and TNF-α, attenuated infiltration of inflammatory cells and tissue damage in lung, liver and kidney, and enhanced survival rate of LPS-challenged mice. Taken together, our data suggest that *Sch B* has anti-inflammatory properties against LPS-induced inflammation and sepsis. *Sch B* could protect against LPS-induced sepsis via the TLR4/NF-κB/MyD88 signaling pathway, and potentially be a novel anti-inflammatory and immunosuppressive drug candidate for treating sepsis.

Keywords: Schizandrin B, sepsis, anti-inflammatory, TLR4/NF-κB/MyD88 signaling pathway

Introduction

Sepsis is a systemic inflammatory syndrome that is induced by infection and involves damage to multiple organs and tissues [1]. It results from an excessive inflammatory response to an invading organism rather than being a direct result of the pathogen itself [1, 2]. Worldwide, approximately 18 million people get sepsis each year and the incidence is growing annually [3]. Despite many therapeutic strategies being trialed, mortality remains high at more than 30% [4]. Therefore, there is an urgent need for therapies that prevent initiation and progression of sepsis.

The most frequently used model for sepsis is the immune response induced by the endotoxin, lipopolysaccharide (LPS). LPS is a component of Gram-negative bacterial cell walls that can trigger a biological inflammatory response and disrupt the immune function of various organs [5]. As a pathogen-associated molecular pattern (PAMP), LPS can be recognized by Toll-like and related receptors on innate immune cells such as neutrophils and macrophages [1, 6, 7]. The transient excessive activation of NF-κB, and mitogen-activated protein kinase (MAPK) and IFN regulatory factor (IRF) mediated signaling pathways result in acute inflammation [6, 8]. Simultaneously, a family of pro-inflammatory cytokines including tumor necrosis factor-α (TNF-α), interleukin (IL)-1β, interleukin-6 (IL-6), interleukin-12 (IL-12), nitric oxide (NO) and prostaglandin E2 (PGE2) are released with resultant tissue damage and organ failure [9]. These host responses to overwhelming inflammation are considered to be the primary reason for the high mortality of sepsis.

Several anti-inflammatory treatments such as anti-endotoxin antibodies, interleukin-1 receptor (IL-1R) antagonists and tumor necrosis factor antagonists have been investigated in clinical or experimental trials of sepsis [10, 11]. However, the effectiveness of these agents remain less than ideal [3]. Therefore, there is an

urgent need for safe and effective treatments for sepsis.

Sch B is a dibenzocyclooctadiene derivative isolated from Schizandra fruit (*Schisandra chinensis*). It is the most important of these derivatives, and is regarded as a potential candidate drug for many diseases [12, 13]. Recent studies have shown that *Sch B* has a variety of pharmacological effects against inflammation, oxidation and tumors [14-16]. However, while there has been considerable investigation into these activities, the specific effects of *Sch B* on sepsis and the mechanisms underpinning these remain inadequately understood. In the present study, we investigated the protective effects of *Sch B* and the underlying mechanism on LPS-induced sepsis model. We found that *Sch B* exerted significant protective effects on LPS-induced sepsis by inhibiting the production of NO and PGE2, and pro-inflammatory cytokines. This process was regulated by the MyD88/TLR4/NF-κB signaling pathway. *Sch B* was found to protect against LPS-induced tissue damage and to enhance the survival rate of LPS-challenged mice. Our results suggest that *Sch B* might be a potential agent for the treatment of sepsis.

Materials and methods

Cells and cell culture

The murine macrophage cell line (RAW264.7) was purchased from the Cell Bank of Shanghai Academy of Science. The cells were cultured in RPMI-1640 medium supplemented with antibiotics (100 U/mL penicillin and 100 U/mL streptomycin) and 10% (v/v) FBS in a humidified atmosphere of 5% CO_2 at 37°C and passaged every 2-3 days to maintain growth.

Measurement of NO

The concentration of NO in the culture supernatant was determined as nitrite by the Griess reagent. RAW264.7 cells were seeded in a 96-well plate at a concentration of 1×10^5 cells/mL, and were allowed to acclimatize overnight. The cells were pre-treated with concentrations of *Sch B* ranging from 0 to 16 μM for 1 h, and were then treated with LPS 1 μg/mL for 24 h. Cell culture supernatant was collected and the concentration of NO assayed using the Griess reagent according to the manufacturer's protocol (Sigma Aldrich, St. Louis, MO, USA). The concentration of nitrite was converted into sodium nitrite concentration as a standard.

MTT assay for cell viability

Cells were seeded in a 96-well plate at a concentration of 1×10^5 cells/mL, and were allowed to acclimatize overnight. The cells were pre-treated with various concentrations of the *Sch B* ranging from 0 to 16 μM for 1 h, and then treated with LPS 1 μg/mL for 24 h. Cell viability was measured by the ability of viable cells to reduce MTT to formazan based on the ability of living cells to utilize Thiazole Blue and convert it into purple formazan, which absorbs light at 570 nm and could be determined by spectrophotometrically. The results are normalized to the untreated control.

Cytokine measurement

The production of IL-1β, TNF-α, IL-6, PGE2 and HMGB1 were measured by ELISA. RAW264.7 cells were seeded in 96-well plate at a concentration of 1×10^5 cells/mL, and were allowed to acclimatize overnight. The cells were pre-treated with various concentrations (0 μM to 16 μM) of *Sch B* for 1 h, and then treated with LPS 1 μg/mL for 24 h. Cell culture supernatant was used for determination of cytokine concentration by ELISA according to the manufacturer's protocol.

Real-time PCR

Trizol reagent (Invitrogen) was used for total RNA extraction following the manufacturer's instructions. Quantitative PCR was performed using SYBR Green qPCR Master Mixes (Takara, China). The expression of mRNA was normalized using β-actin as endogenous control. All the samples were amplified in triplicate and each experiment was repeated three times. The fold change in expression level was calculated using the $2^{-\Delta\Delta Ct}$ method. Primers of MyD88 were as follows: 5'-TCA TGT TCT CCA TAC CCT TGG T-3' (forward); 5'-AAA CTG CGA GTG GGG TCA G-3' (reverse). Primers of TLR4 were as follows: 5'-ATG GCA TGG CTT ACA CCA CC-3' (forward); 5'-GAG GCC AAT TTT GTC TCC ACA-3' (reverse). Primers of β-actin were as follows: 5'-GTG ACG TTG ACA TCC GTA AAG A-3' (forward); 5'-GCC GGA CTC ATC GTA CTC C-3' (reverse).

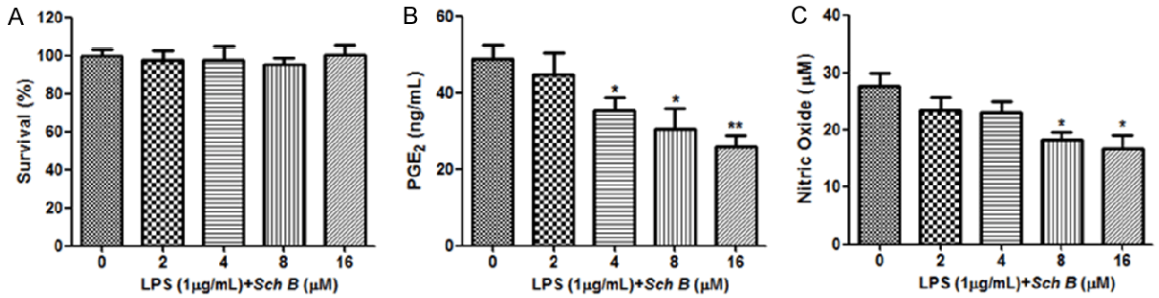

Figure 1. Effect of *Sch B* on cell viability, and PGE2 and NO production by LPS-activated RAW264.7 cells. Cells were incubated with different concentrations of *Sch B* for 1 h, and then treated with LPS (1 μg/mL) for 24 h. Cell viability was determined by MTT assay (A). PGE2 concentrations in cell culture supernatant were measured by PGE2 ELISA (B). NO concentrations in cell culture supernatant were measured by the Griess reaction (C). The data are presented as the mean ± SD of three independent experiments. Statistical significance was assessed by one-way ANOVA followed by Tukey post-hoc test and represented as follows: $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. LPS alone.

Western blotting

Cells were lysed by RIPA buffer with protease and phosphatase inhibitors as per the manufacturer's protocol (Sigma Aldrich, St. Louis, MO, USA). Nucleus and cytosol proteins were isolated using a nuclear protein isolation-translocation assay kit and BCA protein assay reagent (Thermo Scientific, Waltham, MA, USA), respectively, according to manufacturers' instructions. Cellular protein extracts were separated by electrophoresis using 10% SDS polyacrylamide gel and were electro-blotted onto PVDF membranes. The membranes were incubated with blocking solution at room temperature for 1 h, followed by incubation overnight with primary antibodies (1:1000) at 4°C. Blots were washed three times with Tris-buffered saline/Tween 20 (TBST) and incubated with a 1:5000 dilution of horseradish peroxidase conjugated secondary antibody at room temperature for 1 h. Blots were again washed three times with TBST and developed using an ECL chemiluminescence substrate (Thermo Scientific, Waltham, MA, USA). Band intensities were quantified using Alphaview SA software (Alpha Innotech Corporation, San Leandro, CA, USA).

Animals and experimental sepsis

Female C57BL/6 mice were purchased from the Vital River Laboratories (Beijing, China). The animals received LPS 50 mg/kg body weight in sterile PBS by intraperitoneal injection. In the treatment groups, mice were given *Sch B* 20 mg/kg body weight or 40 mg/kg body weight by intraperitoneal injection 1 h before the LPS injection. Mice in the control group were given an equivalent volume of DMSO 1 h before LPS injection. Blood samples were collected for 4 h after LPS injection for NO determination. The survival states of different groups were recorded at different intervals.

Histopathology and biochemistry analysis

In a separate experiment, female C57BL/6 mice were treated with *Sch B* (20 or 40 mg/kg body weight) or an equivalent volume of vehicle (DMSO) and then LPS (50 mg/kg body weight) as outlined in the previous section. After 20 h, all mice were sacrificed. Tissues (lung, liver and kidney) were collected and fixed with 4% formaldehyde, embedded in paraffin, and then sliced and stained with hematoxylin and eosin (H&E). Blood samples were collected and the concentrations of IL-1β and TNF-α measured.

Statistical analysis

All results are expressed as mean ± standard deviation (SD) of three independent experiments. Statistical analysis was performed using GraphPad Prism 5 software (San Diego, CA, USA). The *in vitro* and *in vivo* data were both assessed using one-way ANOVA followed by Tukey post-hoc analysis. Survival rates between groups were analyzed using the log-rank test. A significant difference was set at $^{*}P < 0.05$ and $^{**}P < 0.01$.

Results

Sch B reduces PGE2 and NO production in LPS-activated RAW264.7 cells

Initially, we investigated the toxic effects of *Sch B* on RAW264.7 cell survival. Our MTT assay

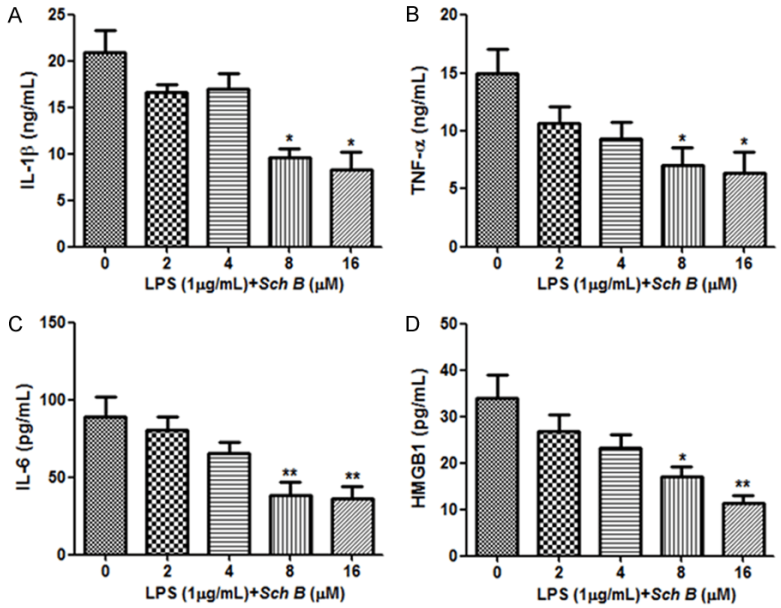

Figure 2. Effect of *Sch B* on production of IL-1β, TNF-α, IL-6 and HMGB1 by LPS-activated RAW264.7 cells. Cells were incubated with different concentrations of *Sch B* for 1 h, and then treated with LPS (1 μg/mL) for 24 h. The concentration of IL-1β (A), TNF-α (B), IL-6 (C) and HMGB1 (D) in cell culture supernatant was measured by ELISA. The data are presented as the mean ± SD of three independent experiments. Statistical significance was assessed by one-way ANOVA followed by Tukey post-hoc test and represented as follows: $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. LPS alone.

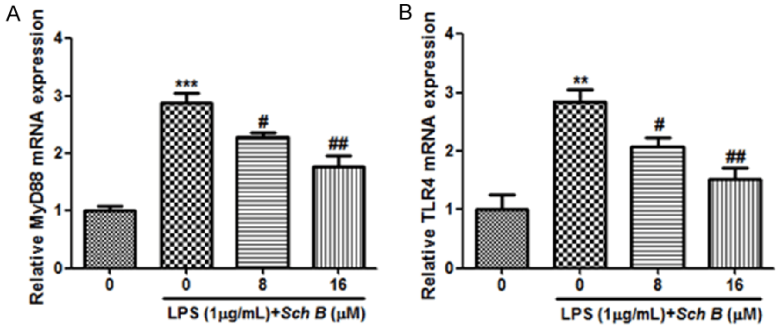

Figure 3. Effect of *Sch B* on the mRNA expression of TLR4/NF-κB/MyD88 pathway by LPS-activated RAW264.7 cells. Cells were incubated with different concentrations of *Sch B* for 1 h, and then were treated with LPS (1 μg/mL) for 30 min. Statistical significance was assessed by one-way ANOVA followed by Tukey post-hoc test and represented as follows: $^{**}P < 0.01$ vs. control; $^{\#}P < 0.05$ vs. LPS alone.

indicated that cell survival did not differ significantly when the cells were pretreated with *Sch B* 0 to 16 μM before exposure to LPS (**Figure 1A**). However, our subsequent experiments showed that pre-treatment with *Sch B* significantly inhibited PGE2 and NO production by LPS-stimulated RAW264.7 cells in a concentration-dependent manner (**Figure 1B** and **1C**, respectively).

Sch B inhibits LPS-induced production of pro-inflammatory cytokines in RAW264.7 cells

IL-1β, TNF-α and IL-6 are pro-inflammatory cytokines that play core roles in the pathological process of sepsis. The DNA-binding nuclear protein, HMGB1, is also a crucial pro-inflammatory cytokine and plays an important role in several inflammatory diseases. In our experiment, pre-treatment with *Sch B* decreased production of IL-1β, TNF-α, IL-6 and HMGB1 by LPS-activated RAW264.7 cells in a concentration-dependent manner (**Figure 2A-D**).

Sch B inhibits the MyD88/TLR4/NF-κB signaling pathway in LPS-induced RAW264.7 cells

To elucidate the molecular mechanism underlying the anti-inflammatory action of *Sch B*, we evaluated the MyD88/TLR4/NF-κB signaling pathway related mRNA and protein. Together with those results (**Figures 3**, **4**), the phosphorylation of NF-κB, IκB, p38 and ERK 1/2 in RAW264.7 cells were markedly increased after LPS stimulation. However, pre-treatment with *Sch B* (8 and 16 μM) significantly suppressed LPS-induced phosphorylation of NF-κB, IκB, p38 and ERK 1/2 in RAW264.7 cells. Furthermore, *Sch B* significantly inhibited the activation of MyD88 and TLR4 by LPS-activated RAW264.7 cells.

Sch B enhances the survival rate of LPS-challenged mice and inhibits the production of IL-1β and TNF-α in mouse serum

Overwhelming production of pro-inflammatory cytokines and mediators leads to tissue dam-

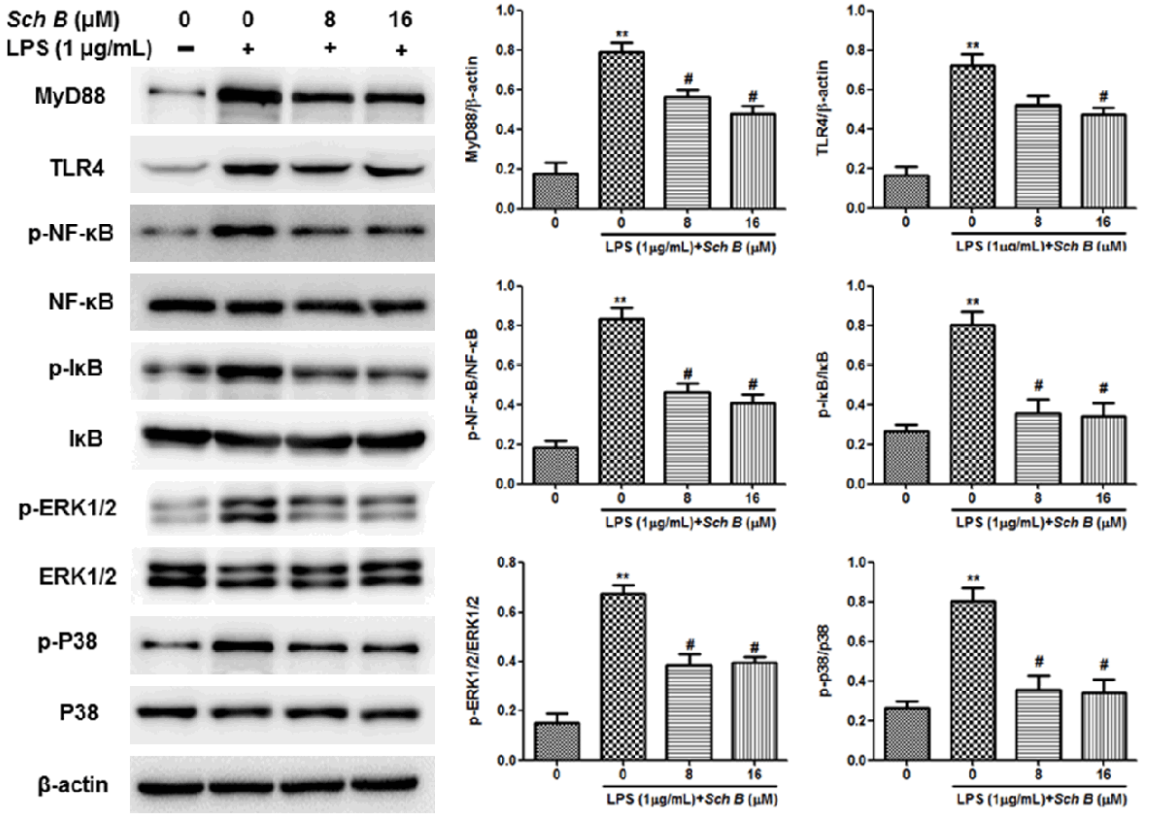

Figure 4. Effect of *Sch B* on the protein expression of TLR4/NF-κB/MyD88 pathway by LPS-activated RAW264.7 cells. Cells were incubated with different concentrations of *Sch B* for 1 h, and then were treated with LPS (1 μg/mL) for 30 min. Statistical significance was assessed by one-way ANOVA followed by Tukey post-hoc test and represented as follows: $^{**}P < 0.01$ vs. control; $^{\#}P < 0.05$ vs. LPS alone.

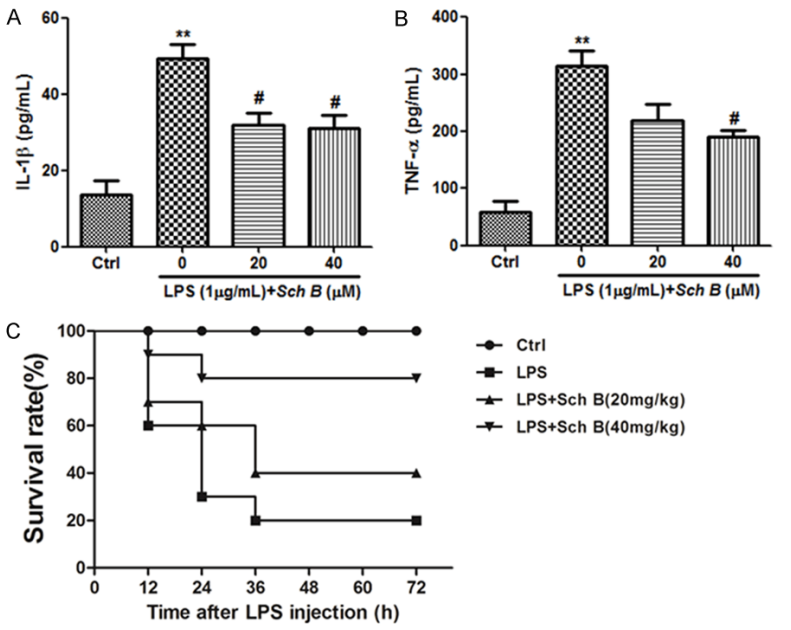

Figure 5. Effect of *Sch B* on mortality, and on serum IL-1β and TNF-α concentrations in LPS-challenged mice. Female C57BL/6 mice were injected with *Sch B* (20 or 40 mg/kg intraperitoneally) or vehicle (DMSO) 1 h before LPS injection (50 mg/kg intraperitoneally). Blood was sampled 4 h after LPS injection. Concentrations of IL-1β (A) and TNF-α (B) in mouse serum were measured by ELISA. Survival was recorded at different intervals (C). Each group contained 10 mice. Statistical significance was assessed by Log-Rank test and represented as follows: $^{**}P < 0.01$ vs. DMSO; $^{\#}P < 0.05$ vs. LPS alone.

age or death. In this experiment, mice were pre-treated with *Sch B* (20 or 40 mg/kg, i.p.) for 1 h, and then injected with LPS (50 mg/kg, i.p.). The results showed that administration of *Sch B* significantly decreased the production of IL-1β and TNF-α in serum in the 4 hours following LPS challenge (**Figure 5A**

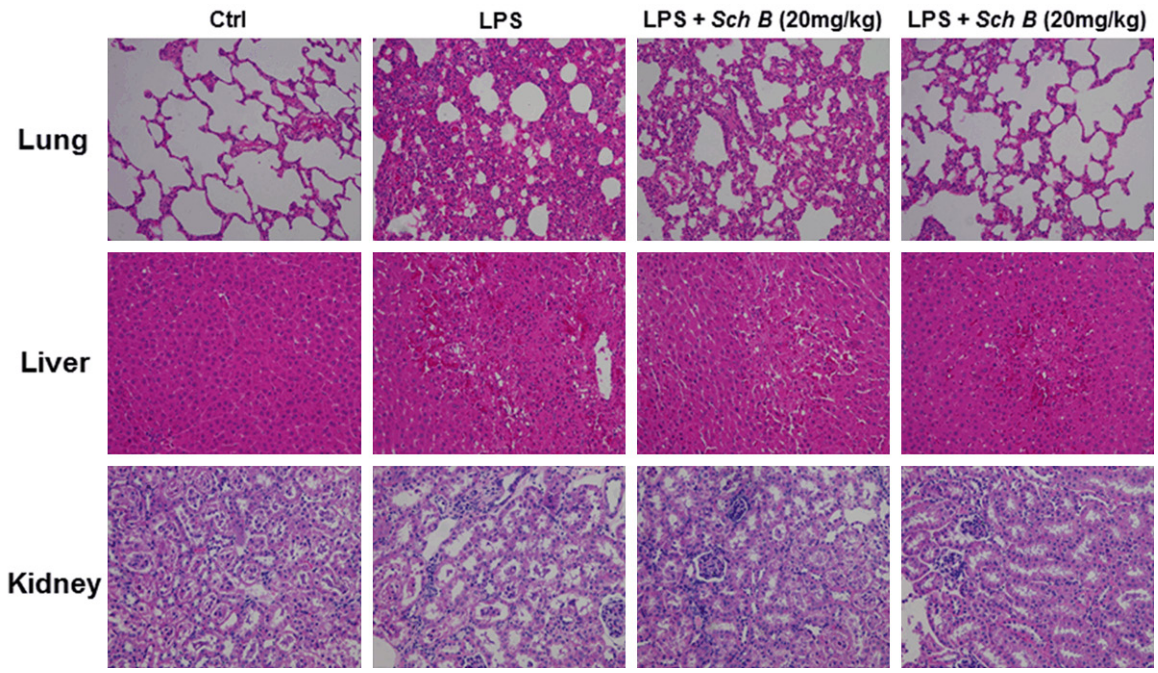

Figure 6. Effect of *Sch B* on lung, liver and kidney damage in LPS-challenged mice. Female C57BL/6 mice were injected with *Sch B* (20 or 40 mg/kg intraperitoneally) or vehicle (DMSO) 1 h before LPS injection (50 mg/kg intraperitoneally). Samples of lung, liver and kidney tissue were harvested 20 h after LPS injection. The results show H&E-staining of lung, liver or kidney tissue sections from the indicated group (×200). The figure is a representative of three independent experiments.

and **5B**). Moreover, *Sch B* also increased survival rate compared to LPS alone in the 72 h following LPS (**Figure 5C**).

Sch B exhibits protective effects against LPS-induced tissue damage in LPS-challenged mice

To further explore the protective effect of *Sch B* on the sepsis, we also investigated the tissue injury in LPS-challenged mice. Mice were pretreated with *Sch B* (20 or 40 mg/kg, i.p.) for 1 h, and then injected with LPS (50 mg/kg, i.p.) to induce experimental sepsis. The lung, liver and kidney samples were collected and examined by using Hematoxylin Eosin (HE) staining. LPS administration significantly increased inflammatory cell infiltration in lung, liver and kidney tissue samples (**Figure 6**). In the lung, alveolar wall thickness was increased and the number of pulmonary alveoli reduced in LPS-challenged mice compared with controls. Administration of *Sch B* repressed alveolar wall swelling and attenuated the decline in the number of pulmonary alveoli in LPS-challenged mice. In the liver, *Sch B* suppressed LPS-induced infiltration of inflammatory cells into the cavities of the hepatic tissue. In the kidney, renal tubular epithelial cells were sloughed, and brush borders and renal epithelial cells were decreased after LPS injection. The administration of *Sch B* inhibited the sloughing of tubular epithelial cells and diminished brush borders and epithelial cells in the kidney.

Discussion

Sepsis kills around one in every three patients affected, and effective treatment options are lacking. Most of the organ and tissue damage of sepsis results from an excessive and dysfunctional inflammatory response involving cytokines [17]. Unfortunately, the effects of recently therapeutic strategies which targeted on the overwhelming pro-inflammatory cytokines produced in sepsis were not ideal [18]. Therefore, new therapies or medicine for sepsis are urgently needed [4]. *Sch B* is an effective derivative isolated from Schizandra fruit (*Schisandra chinensis*), a wildly used traditional Chinese herb [19]. Several studies have reported the definite anti-inflammatory effect of *Sch B* [14]. Therefore, we further tested the protective effects of *Sch B* on sepsis.

As reported previously, overexpression of NO [20] and PGE2 contribute to the pathogenesis of many inflammatory diseases [21]. Excessive expression of the pro-inflammatory cytokines, TNF-α, IL-6, and IL-1β, characterize the initial phase of sepsis [22]. They rapidly initiate a systemic inflammatory response leading to stimulation of an adaptive immune response and a cytokine storm [23-25]. In this study, we investigated the effect of *Sch B* on LPS-induced NO and PGE2 production using macrophages in non-cytotoxic doses. We found that *Sch B* reduced production of both NO and PGE2 in LPS-stimulated RAW264.7 cells in a concentration-dependent manner. Similar findings were observed for the pro-inflammatory cytokines, IL-1β, TNF-α, IL-6 and HMGB1. Therefore, we proved that *Sch B* inhibited the inflammatory process in LPS-induced RAW264.7 cells by reducing the expression of NO, PGE2, and pro-inflammatory cytokines.

The Toll-like receptor 4 (TLR4)-mediated MyD88/IKκ/NF-κB pathway is a signaling pathway necessary for inflammatory responses [26, 27]. Briefly, LPS stimulates and binds to TLR4, a transmembrane receptor generally expressed in normal and macrophage cells [28, 29]. This induces the activation of MyD88-dependent intracellular signaling [7, 27]. IL-1 receptor-associated kinases (IRAKs) and tumor-necrosis factor receptor-associated factor 6 (TRAF6) are then recruited upon ligand stimulation. TRAF6 further activates the transforming growth factor-β-activated kinase 1 (TAK1) complex, which then activates the IKK complex and mediates NF-κB activation. TAK1 could also activate the MAP kinase family, including c-Jun NH2-termina kinase (JNK), p38 MAPK and extracellular signal-regulated kinase (ERK) [18, 30, 31]. NF-κB is an important transcription factor that controls the expression of many pre-inflammatory cytokines, including TNF-α, IL-6 and IL-1β [18, 32]. In our research, phosphorylation of NF-κB, IκB, p38 and ERK 1/2 in RAW264.7 cells was markedly increased after LPS stimulation, but was significantly suppressed by *Sch B* (8 and 16 μM). *Sch B* also significantly inhibited activation of MyD88 and TLR4 in the LPS-activated RAW264.7 cells. Taken together, these results indicated that the potential mechanism for the protective effects of *Sch B* on LPS-stimulated RAW264.7 cells was related to inhibition of the MyD88/TLR4/NF-κB signaling pathway.

In the animal model, overwhelming production of pro-inflammatory cytokines and mediators result in tissue damage or death. As our results show *Sch B* has protective effects against LPS-induced inflammation in macrophages. Therefore, we further examined whether *Sch B* affected the death rate in LPS-induced sepsis, and if it affected production of IL-1β and TNF-α in LPS-challenged mice. We showed that administration of *Sch B* (20 or 40 mg/kg intraperitoneally) significantly suppressed IL-1β and TNF-α serum concentrations after LPS challenge. Moreover, treatment with *Sch B* markedly increased the survival of LPS-challenged mice. As lung, liver and kidney tissues might be injured from the severe inflammatory response induced by LPS, we investigated the protective effects of *Sch B* on these organs in mice. *Sch B* (20 or 40 mg/kg intraperitoneally) decreased alveolar wall swelling and lessened the decline in the number of pulmonary alveoli in lung tissue, suppressed inflammatory cells in the cavities of liver tissue, and inhibited the sloughing of tubular epithelial cells while diminishing brush borders and epithelial cells in the kidney.

In conclusion, we found that *Sch B* exerted significant protective effects against LPS-induced sepsis by inhibiting NO and PGE2 production, and the expression of pro-inflammatory cytokines through the MyD88/TLR4/NF-κB signaling pathway. *Sch B* may also protect against LPS-induced tissue damage and enhance the survival rate of LPS-challenged mice. Our results provide evidence that *Sch B* might be a potential agent for the treatment of sepsis.

Acknowledgements

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Disclosure of conflict of interest

None.

Address correspondence to: Tingting Wang, Department of Infectious Internal Medicine, The First Affiliated Hospital of Wenzhou Medical University,

Wenzhou 325000, P. R. China. E-mail: dragonfly_wang@126.com

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