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Original Article

Effects of Tanshinone IIA sodium sulfonate on LPS-induced acute lung injury in mice

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Abstract: Objective: The study investigated the protective effect of Tanshinone IIA sodium sulfonate (TSS) on lipopolysaccharide (LPS)-induced acute lung injury (ALI), and the underlying mechanisms. Methods: Male C57/black mice were subjected to LPS (10 mg/kg, i.p.) administration to induce acute lung injury. Half an hour before LPS injection, mice were pretreated with TSS (30 mg/kg, i.p.). One hour, six and twelve hours later, mice were sacrificed. Lung edema and inflammatory cell infiltration were observed. Expression of pro-inflammatory factors (TNFα, IL-1β, IL-6) were measured by RT-PCR and ELISA in lung tissues. Markers of autophagy including LC3 and Beclin-1 expression were determined by RT-PCR and Western blot. Number of autophagosomes was observed by electron microscope. Results: Our results showed that TSS did not ameliorate the LPS-induced lung edema, but significantly reduced pro-inflammatory cell infiltration. LPS increased mRNA and protein expression of inflammatory factors (TNFα, IL-1β, IL-6) at all observed time. TSS markedly reduced the protein expression of IL-6 but not TNFα and IL-1β. In addition, LPS increased tissue autophagic levels (expression of Beclin-1, LC3 and number of autophagosomes) and TSS treatment further increased the autophagic levels. Conclusion: TSS exerts certain anti-inflammation and enhancing autophagy effects in LPS-induced ALI.

Keywords: Tanshinone IIA sodium sulfonate, lipopolysaccharide, acute lung injury, autophagy

Introduction

Acute lung injury (ALI)/acute respiratory distress syndrome (ARDS), is a severe clinical complication caused by stress situation such as trauma, burns and sepsis, is characterized with respiratory distress, refractory hypoxemia, and no cardiogenic pulmonary edema, along with bilateral pulmonary infiltrates and reduction in respiratory system compliance [1-3]. Even with the continuous efforts and success in treatment of ALI, clinical outcome still proofed high rates of morbidity and mortality in ALI patients [4].

One of the pathophysiological mechanism in ALI is loss of the lung's gas exchange surface area, leading to refractory hypoxemia [3]. Strategy to improve the gas exchange by mechanical ventilation is now a standard treatment in ALI in clinic [5]. However, due to that the ventilation itself could induce ALI, it is still arguable with this therapy [6, 7]. Second mechanism is the recruitment of neutrophils and other mediators of acute inflammation into the airspace [3]. Anti-inflammatory therapies are reasonably explored [8-11]. However, this therapy is also notoriously difficult to solve the problem, partly because ALI is not an inflammatory disease but a label describing acute respiratory failure occurring de novo as a result of a wide variety of conditions. Third mechanism is inflammatory exudate inactivates surfactant leading to collapse of distal lung parenchyma. New approach is undergoing to be explored through stem cell therapy, which could promote repair of ALI-induced cell damage [12, 13]. However, serious adverse events of cell therapy for respiratory diseases are also the inevitable question [14].

Tanshinone IIA sodium sulfonate (TSS) is one of the major active constituents of Salvia miltiorrhiza Bunge, which has been widely used in

clinical treatment for hundreds of years in China for the treatment of various diseases [15-17]. It is considered to have the function of activating blood circulation and removing blood stasis, entering the “heart”, “pericardium”, and “liver” channels according to the theory of traditional Chinese medicine (TCM) and has been used to successfully treat various circulatory disturbance-related diseases including vasodilatation, anticoagulation, anti-inflammation, and free radical scavenging [18, 19]. It has been reported that TSS attenuates seawater aspiration-induced acute pulmonary edema [20] and reduces lethality and acute lung injury in LPS-treated mice [21]. Anti-inflammatory effects of TSS also have been widely investigated in animal model or in cell line [22, 23]. However, the anti-inflammatory effects of TSS on ALI still require further investigation.

Recently, autophagy has been reported to play a role in the development of ALI [24]. It has been reported that autophagy protects against ischemia/reperfusion-induced lung injury through alleviating blood-air barrier damage [25], decreasing chlorine-induced mitochondrial injury and lung inflammation [26] and maintaining the integrity of endothelial barrier in LPS-induced lung injury [27]. On the other hand, inhibition of autophagy could ameliorate acute lung injury [28]. Excessive autophagy also has been reported to contribute to the ALI [29]. It is still controversial what is the real role of autophagy in ALI and there is still no observation of the effects of TSS on the autophagy level in ALI.

The present study is designed to observe the effects of TSS on LPS-induced ALI, focusing on the tissue edema, cell infiltration, inflammatory factors expression and tissue autophagic levels, which may provide basic evidence for clinical application of TSS on ALI patients.

Materials and methods

Experimental animals

Male C57 mice, weighing 25 to 30 g were purchased from Shanghai Laboratory Animal Center. All experiments were performed in accordance with the National Institutes of Health Guidelines for the Use of Laboratory Animals (NIH, publication number 85-23, revised 1996), which were approved by and performed according to guidelines for the care and use of animals established by Soochow University. Mice were randomly divided into seven groups according to receiving or not receiving TSS after LPS treatment for different time: (1) Saline control group (n=10), in which mice received saline 0.5 hours before the second saline administration; (2) LPS 1-hour group (n=10) in which mice received LPS (10 mg/kg) administration for 1 hour; (3) TSS + LPS 1-hour group (n=10), in which TSS (30 mg/kg) was administered 0.5 hours before LPS (10 mg/kg) injection for 1 hour. (4) LPS 6-hour group (n=10) in which mice received LPS (10 mg/kg) injection for 6 hours; (5) TSS + LPS 6-hour group (n=10), in which TSS (30 mg/kg) was administered 0.5 hours before LPS (10 mg/kg) injection for 6 hours. (6) LPS 12- hour group (n=10) in which mice received LPS (10 mg/kg) injection for 12 hours; (7) TSS + LPS 12-hour group (n=10), in which TSS (30 mg/kg) was administered 0.5 hours before LPS (10 mg/kg) injection for 12 hours [10, 21]. In all groups, administration was via intraperitoneal injection, saline was applied as negative control. At each time point, mice were sacrificed by neck broken, lung tissues were sampled for direct measurement of wet weight or were frozen for further investigation.

Wet-to-dry weight ratio (W/D)

At the end of the experiment, mice left lungs (n=10, respectively) were weighed, then dried to constant weight at 50°C for 72 h and weighed again. The ratio of W/D was finally calculated by dividing the wet weight by the dry weight.

Histological analysis

Part of mice right lungs were samples and fixed with 4% paraformadehyde and cut into 5-μm slices, which were stained with hematoxylin-eosin for morphological analysis. Numbers of cell nucleus were counted from 50 visual fields of ten independent slides.

Ultrathin sections of the right lung tissues were prepared and stained with uranyl acetate and lead citrate. The autophagosomes in the tissues were examined with a transmission electron microscope (CM120, Philips, Holland). Six samples were examined to calculate the percentage of autophagosomes in the cells.

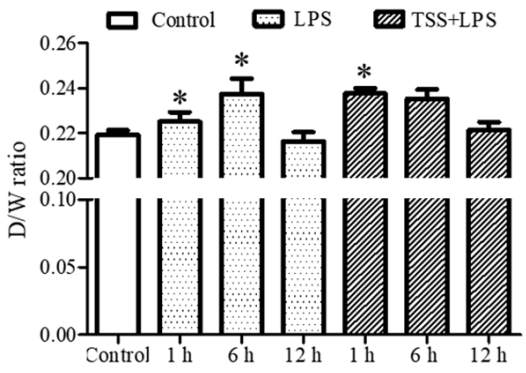

Figure 1. Ratio of wet to dry weight. LPS: Lipopolysaccharide; TSS: Tanshinone IIA sodium sulfonate. Blank column indicates control group, dot column indicates LPS treatment at all observed time points, diagonal column indicates TSS pretreatment at all observed time points, n=10 in each group. *P<0.05 vs. control group.

Real-time PCR

Total RNA was isolated from part of frozen right lung (about 20 mg) tissues by guanidinium isothiocyanate-acid phenol extraction and quantified by measuring the absorbance at 260 nm. One microgram of total RNA was used for reverse transcription under 95°C for 15 minutes. Mice TNFα, IL-1β, IL-6, Beclin-1 and LC3 mRNAs were quantified by real time PCR (prism7000; Applied Biosystems, Foster, California). The primer pairs for TNFα cDNA were 5'-GCCAGGAGGGAGAACAGAAACTC-3' (forward) and 5'-GGCCAGTGAGTGAAAGGGACA-3' (reverse). The primer pairs for IL-1β cDNA were 5'-TCCAGGATGAGGACATGAGCAC-3' (forward) and 5'-GAACGTCACACACCAGCAGGTTA-3' (reverse). The primer pairs for IL-6 cDNA were 5'-CCACTTCACAAGTCGGAGGCTTA-3' (forward) and 5'-CCAGTTTGGTAGCATCCATCATTTC-3' (reverse). The primer pairs for Beclin-1 cDNA were 5'-ATGGAGGGGTCTAAGGCGTC (forward) and 5'-TGGGCTGTGGTAAGTAATGGA (reverse). The primer pairs for LC3 cDNA were 5'-CGCTTGCAGCTCAATGCTAAC-3' (forward) and 5'-TCTCTCACTCTCGTACACTTCG-3' (reverse). The primer pairs for GAPDH cDNA were 5'-AAATGGTGAAGGTCGGTGTGAAC-3' (forward) and 5'-CAACAATCTCCACTTTGCCACTG-3' (reverse). Condition for RT-PCR is denature 30s at 95°C, amplification 40 cycles (95°C 5 s, 60°C 34 s), extension 7 min at 72°C. After calculation, relative expressions of each target mRNA were obtained. Expressions of TNFα, IL-1β, IL-6, Beclin-1 and LC3 mRNAs were normalized to GAPDH mRNA, and were compared with control group, respectively.

ELISA

Frozen right lung tissues (around 50 mg) were homogenized to measure TNFα, IL-1β, IL-6 content (Biolegend, Inc. Shanghai, China) by using a commercially available enzyme-linked immunosorbent assay (ELISA) kits. All steps were performed according to the manufacturer's instructions. Data were normalized according to the concentration of protein measured by BCA method.

Western blot analysis

Frozen right lung tissues (around 50 mg) were homogenized with RIPA buffer (50 mm Tris, pH 7.0, 150 mM NaCl, 1% Triton-X-100) containing phenylmenthanesulfonyl fluoride (R&D Systems Inc., Minneapolis, US). Homogenates were centrifuged at 12,000* g for 10 minutes at 4°C. Same amount of cell protein were separated by SDS-PAGE and transferred to PVDF membranes (Hybond TM-ECL; Amersham Pharmacia Biotech, Inc.). The membranes were blocked in 5% nonfat milk in PBS and 0.1% Tween-20 at room temperature. The blots were then incubated with primary antibody: anti-Beclin-1 antibody (1:1000, abcam, Inc.), anti-LC3 (1:1000, abcam, Inc.), or anti-GAPDH (1:1000, Santa Cruz Biotech, Inc.). Then the membranes were incubated for 1 hour with a secondary antibody (HRP-conjugated anti-rabbit Ig-G, 1:2000). Excess antibody was washed off with TBS-T three times (15 minutes each) before incubation of enhanced chemiluminescent reagent (ECL, R&D Systems Inc, Minneapolis, USA) for 1min. Subsequently, X-ray film was used to detect membrane signal. Immunoreactive bands were detected by the analysis of X-ray films using the software of Image J. The quantity of target proteins is normalized by GAPDH expression.

Statistical analysis

The SPSS 18.0 software was used for statistical analysis. Data were presented as the mean ± S.E.M. Grouped data were analyzed using a one-way analysis of variance followed by the Student-Newman-Keuls test. A *P* value <0.05 was considered to be statistically significant.

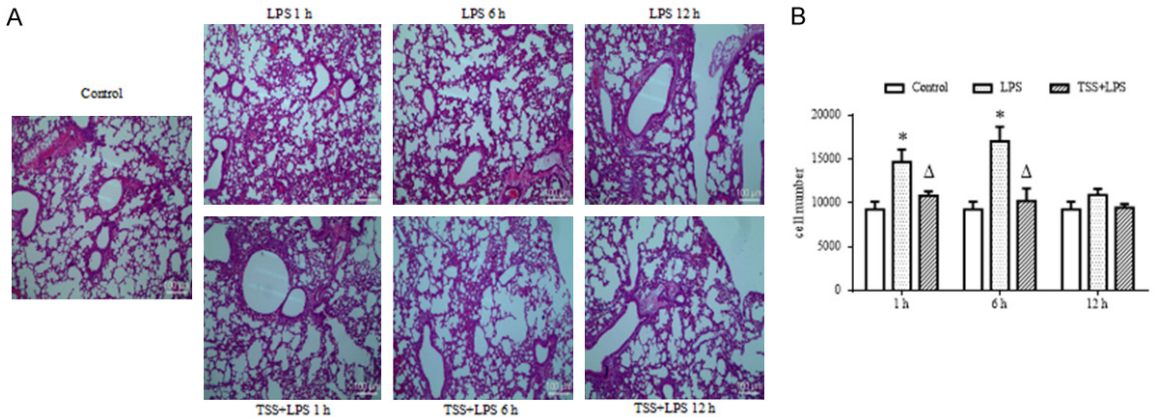

Figure 2. Histological analysis of each group. A. Representative HE staining of each group, B. Number of cell in each group. LPS: Lipopolysaccharide; TSS: Tanshinone IIA sodium sulfonate. Blank column indicates control group, dot column indicates LPS treatment at all observed time points, diagonal column indicates TSS pretreatment at all observed time points, n=10 in each group. Each bar presents the mean ± SEM. *P<0.05 vs. control group. ΔP<0.05 vs. LPS group at each corresponding time point.

Results

Effect of TSS on LPS-induced lung edema

To observe the effect of TSS on acute LPS-induce lung edema, wet to dry weight across different time points were investigated. Our results showed that only at 6-hour time point, LPS significantly increased ratio of wet to dry weight compared with control group (P<0.05, **Figure 1**), indicating LPS-induced lung edema occur around 6 hours. TSS treatment at 1-hour also markedly increased the ratio compared with control group (P<0.05, **Figure 1**), indicating TSS may induce early phase of lung edema, while LPS may lead to later phase lung edema (**Figure 1**).

Effect of TSS on LPS-induced cell infiltration

Histological analysis showed LPS induced increase of cell infiltration at 1-hour and 6-hour compared with control group (P<0.05, **Figure 2**), but not at 12-hour, indicating LPS induced cell infiltration is time dependent, which is only manifest at the onset of ALI. TSS treatment markedly reduced LPS-induced increase of cell infiltration both at 1-hour and 6-hour compared with LPS group (P<0.05, **Figure 2**), suggesting TSS has anti-cell-infiltration effect in response to LPS insult.

Effect of TSS on LPS-induced expression of inflammatory factors

To investigate the effects of TSS on inflammatory factors, the expressions of TNFα, IL-1β, IL-6 in lung tissue were determined by RT-PCR and ELISA. Data demonstrated that LPS increased both mRNA and protein expressions of TNFα, IL-1β and IL-6 compared with the control group at all observed time points (P<0.05, **Figure 3**). TSS treatment had no effects on LPS-induced increase of TNFα mRNA and protein expression. TSS inhibited LPS-induced increase of IL-1β protein expression only at 1-hour time point. However, Pretreatment with TSS significantly suppressed LPS-induced increase of IL-6 mRNA and protein expression at all observed time points compared with the LPS group (P<0.05, **Figure 3C**, **3F**). These results suggest that TTS may exert certain anti-inflammatory effect.

Effect of TSS on LPS-induced tissue autophagic level

LPS induced increase of mRNA and protein expressions of autophagic markers Beclin-1 and LC3 at all observed time points compared with control group (P<0.05, **Figure 4**). Pretreatment with TSS further increased the mRNA and protein expressions of Beclin-1 and LC3 at all observed time points compared with LPS group (P<0.05, **Figure 4**).

In addition, by transmission electron microscope observation, we confirmed that LPS increased the number of autophagosomes at all observed time points compared with control group (P<0.05, **Figure 5**). TSS pretreatment further increased the number of autophagosomes at all observed time points compared with LPS

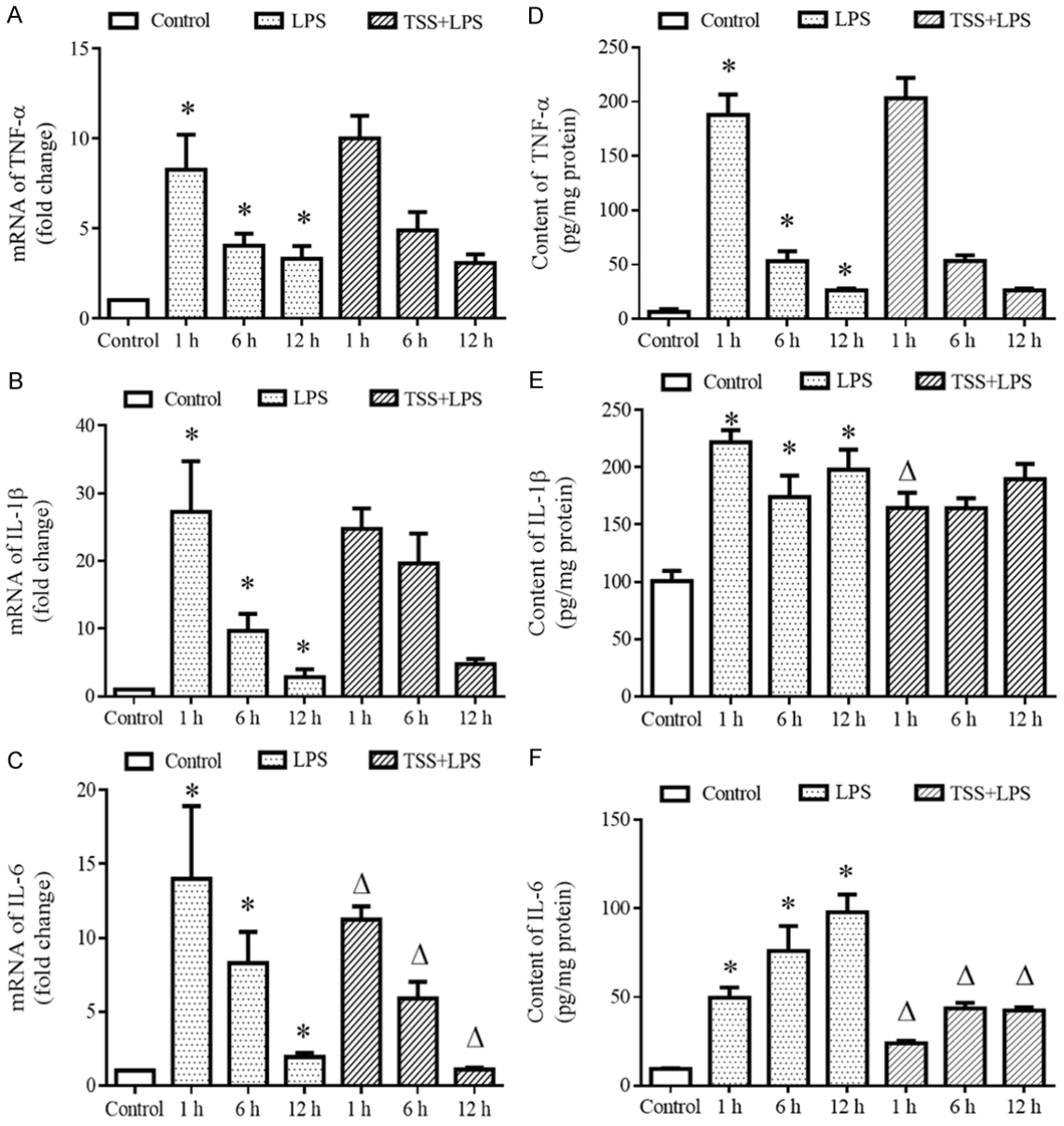

Figure 3. Expression TNFα, IL-1β and IL-6 in lung tissue. A. mRNA of TNFα expression. B. mRNA of IL-1β expression. C. mRNA of IL-6 expression. D. Protein content of TNFα. E. protein content of IL-1β. F. Protein content of IL-6. LPS: Lipopolysaccharide; TSS: Tanshinone IIA sodium sulfonate. Blank column indicates control group, dot column indicates LPS treatment at all observed time points, diagonal column indicates TSS pretreatment at all observed time points, n=10 in each group. Each bar presents the mean ± SEM. $^{*}P<0.05$ vs. control group. $^{\Delta}P<0.05$ vs. LPS group at each corresponding time point.

group which ($P<0.05$, **Figure 5**). These results indicate that TSS could further activate the tissue autophagic level.

Discussion

The major finding of our present study is that TSS has anti-cell infiltration, anti-inflammation effects, as well as activation of autophagy in response to LPS insult, but has no effect on edema.

ALI, one complication of sepsis, is still a major and increasing cause of mortality and morbidity throughout the world [30, 31]. Researchers are trying to explore the reliable therapy to overcome this worldwide problem. New drugs to prevent lung tissue damage in ALI is one neces-

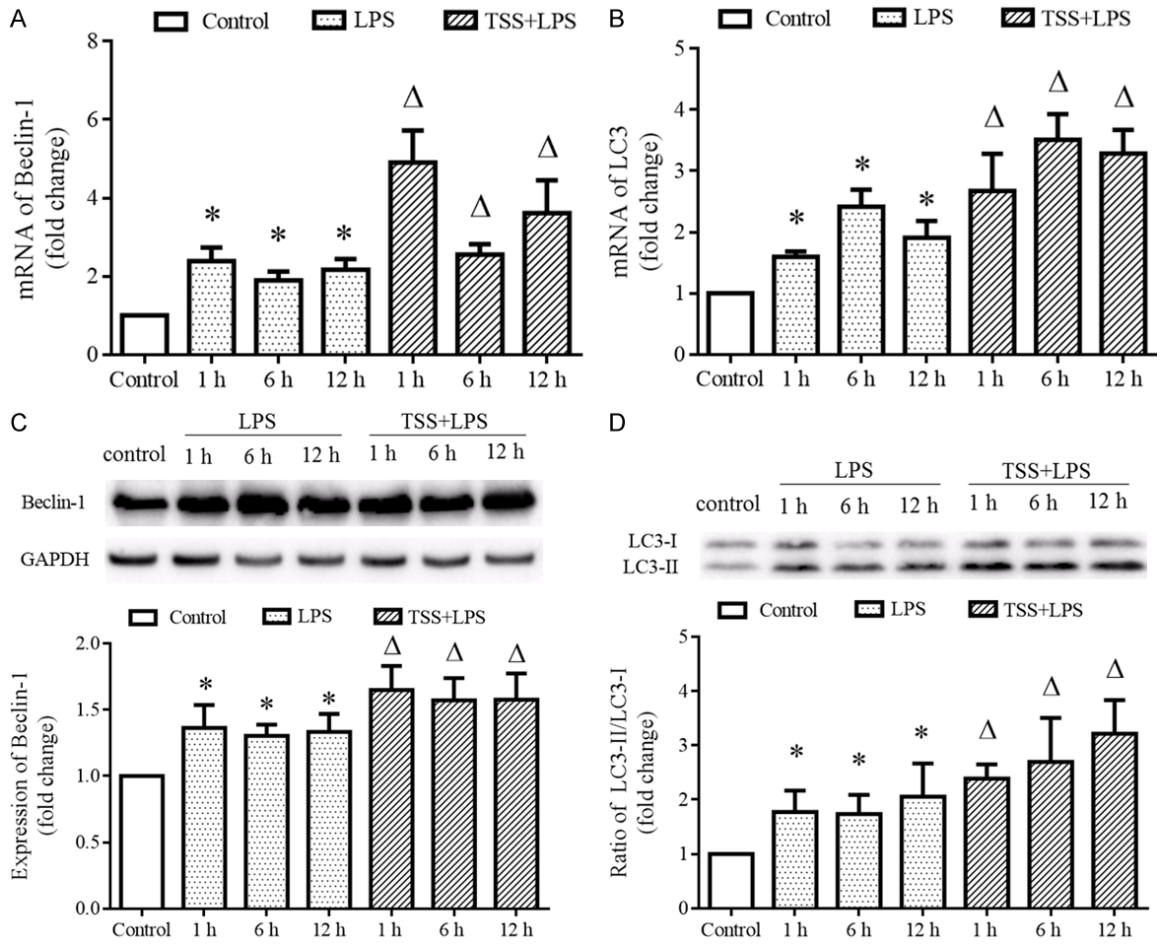

Figure 4. Expression of autophagic markers Beclin-1 and LC3. A. mRNA expression of Beclin-1 in each group. B. mRNA expression of LC3 in each group. C. Protein expression of Beclin-1 in each group, upper is the representative blots of Beclin-1 and GAPDH; lower is the densitometric analysis of Beclin-1 expression normalized to GAPDH. D. Protein expression of LC3 in each group, upper is the representative blots of LC3; lower is the densitometric analysis of LC3 expression normalized to GAPDH. LPS: Lipopolysaccharide, TSS: Tanshinone IIA sodium sulfonate. Blank column indicates control group, dot column indicates LPS treatment at all observed time points, diagonal column indicates TSS pretreatment at all observed time points, n=10 in each group. Each bar presents the mean ± SEM. *P<0.05 vs. control group. ΔP<0.05 vs. LPS group at each corresponding time point.

sary treatment. Among all of the drugs, TSS has been explored to protect LPS-induced lung injury in rats [32]. Later, it has been reported to played an important role in the prevention against LPS-induced ALI in mice [21]. As evidenced by reduction of lethality and the improvements of lactate dehydrogenase (LDH) content (one index for lung injury), as well as significantly suppressed LPS-induced lung edema [21]. However, our present study demonstrated that LPS-induced lung edema is time dependent under present ALI model, which occurs only at the early time (less than 12 hours). Under such circumstance, our results showed that TSS did not exert any protective effect on LPS-induced lung edema, on the contrary, it could accelerate the occurrence of lung edema in response to LPS stimulation (**Figure 1**). This discrepancy between our observation with previous may be due to the different models (female Kun-Ming mice in previous study and male C57 mice in our observation) and different degree of severity (50 mg/kg in previous study and 10 mg/kg in our observation) of ALI induced by LPS. Therefore, effects of TSS on ALI, especially on the lung edema is still questionable, further investigation is still needed.

It is well-known that the pathologic process of ALI involves the infiltration of inflammatory cells into lungs, which released large amounts of inflammatory cytokines, leading to increased

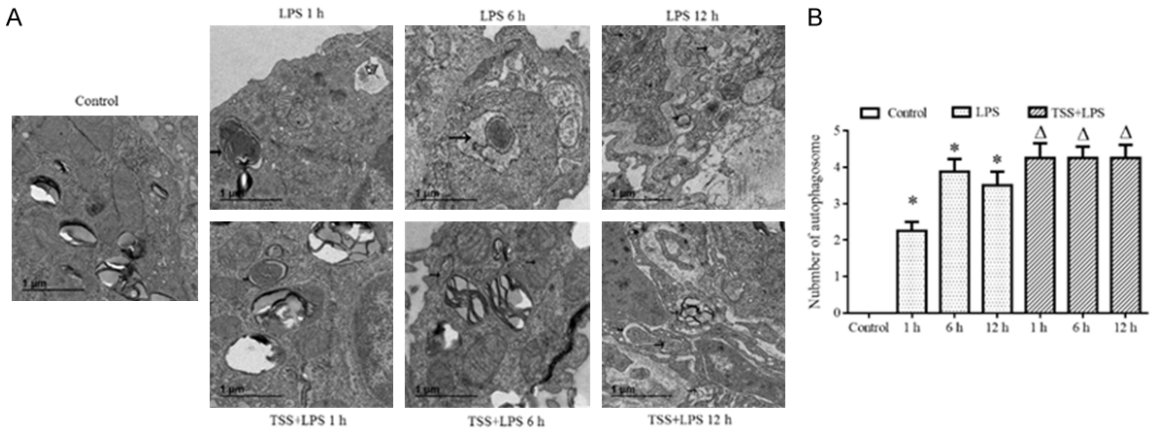

Figure 5. Autophagosome formation in each group. A. Representative transmission electron microscope images of each group, black arrows indicate autophagosome. B. Number of autophagosomes in each group. LPS: Lipopolysaccharide, TSS: Tanshinone IIA sodium sulfonate. Blank column indicates control group, dot column indicates LPS treatment at all observed time points, diagonal column indicates TSS pretreatment at all observed time points, n=10 in each group. Each bar presents the mean ± SEM. $^{*}P<0.05$ vs. control group. $^{\Delta}P<0.05$ vs. LPS group at each corresponding time point.

capillary permeability and interstitial edema [2, 3]. Solid evidence has demonstrated that inhibition of release of inflammatory cytokines could ameliorate the development of ALI. Since TSS has been reported to have anti-inflammatory property [33, 34], based on the pathogenesis of ALI, it is reasonable to apply TSS as an anti-inflammatory drug. Previous study all demonstrated that TSS could inhibit LPS-induced cell infiltration, expression of inflammatory effects both in rat [32] and mice model [21], and TSS may promote hypoxia-inducible factor-1α protein degradation via the proteasomal pathway in LPS-stimulated macrophages, thereafter suppress expression of inflammatory factors, such as TNF-α, IL-6 and IL-1β [22]. Our present study also demonstrated that TSS could markedly suppress LPS induced cell infiltration in lung tissue at all observed time points, which is consistent with previous studies. However, although our results also showed LPS increased both mRNA and protein expressions of inflammatory factors, such as TNF-α, IL-6 and IL-1β at all observed time points, but marked inhibitory effects of TSS only was observed on the expression of IL-6, but not expression of inflammatory factors, such as TNF-α and IL-1β. This discrepancy may be due to different tissues or models. In the present study we used male C57 mice and other studies used rat or female Kun-Ming mice, and we checked these inflammatory factors in the lung tissues and other study checked these factors in macrophages or serum. Our present study at least supported that TSS has certain anti-inflammatory in LPS-induced ALI.

Autophagy is one of the innate immune defense mechanisms against microbial challenges. Previous in vitro and in vivo models of sepsis demonstrated that autophagy was activated initially in sepsis, followed by a subsequent phase of impairment [35]. Role of autophagy in sepsis is well reviewed recently [36], and authors proposed that autophagy plays a protective role in sepsis according to the existing evidence [37, 38], although contradictory findings also have been reported [39, 40], and they concluded that autophagy increases transiently upon encounter of septic insult, this initial increase is followed by a prolonged decline of autophagic flux, contributing to organ dysfunction.

Experimental induction of autophagy by rapamycin and activated protein C protects against sepsis-induced acute lung injury by mitigating apoptosis and pro-inflammatory cytokine [41]. In addition, it has been reported that the protective effect of GAPDH (glyceraldehyde-3-phosphate dehydrogenase) against sepsis-related lung injury is mediated by ATG12-dependent autophagy enhancement [42, 43]. Our present study observed that autophagic level is continuously elevated in response to LPS stimulation, at least lasting for 12 hours, results did not show any suppression of au-

tophagy with prolonged time. The reason may be due to dosage of LPS applied in the present is mild, or due to rich blood supply of lung tissue (while we observed suppression of autophagy in intestine with prolonged time, data not shown). Importantly, our present study clearly demonstrated that TSS could further activate autophagic level in response to LPS stimulation, which is consistent with our previous study [44] observed that TSS ameliorates ischemia-reperfusion induced myocardial injury may be via enhancing autophagy.

It should be note that in the present study we do not obtain the data of the improvement of TSS on LPS-induced lung edema, which may be due the limitation of observing time. Long term treatment with TSS on LPS induced lung injury should be carried out in the future observation. Especially, our present study does not observe the effects of TSS on LPS-induced lung cell apoptosis. In addition, mechanism of TSS to activate autophagy needs to be investigated and the precise role of elevated autophagy levels in the present model also needs to be clarified.

In conclusion, our present study demonstrates that protective effects of TSS in LPS-induced ALI may via certain anti-inflammation and enhancing autophagy.

Disclosure of conflict of interest

None.

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