Article

Salidroside Regulates Inflammatory Response in Raw 264.7 Macrophages via TLR4/TAK1 and Ameliorates Inflammation in Alcohol Binge Drinking Induced Liver Injury

Running Title: The anti-inflammatory effects of salidroside

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Abstract: The current study was designed to investigate the anti-inflammatory effect of salidroside (SDS) and the underlying mechanism by using lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophages *in vitro* and a mouse model of binge drinking induced liver injury *in vivo*. SDS downregulated protein expression of toll-like receptor 4 (TLR4) and CD14. SDS inhibited LPS-triggered phosphorylation of LPS-activated kinase 1 (TAK1), p38, c-Jun terminal kinase (JNK), and extracellular signal-regulated kinase (ERK). Degradation of IκB-α and nuclear translocation of nuclear factor (NF)-κB were effectively blocked by SDS. SDS concentration-dependently suppressed LPS mediated inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) protein levels, as well as their downstream products, NO. SDS significantly inhibited protein secretion of interleukin (IL)-1β. Additionally C57BL/6 mice were orally administrated SDS for continuous 5 days, followed by three gavages of ethanol every 30 min. Alcohol binge drinking caused the increasing of hepatic lipid accumulation and serum transaminases levels. SDS pretreatment significantly alleviated liver inflammatory changes and serum transaminases levels. Further investigation indicated that SDS markedly decreased protein level of IL-1β in serum. Taken together, these data implied that SDS inhibits liver inflammation both *in vitro* and *in vivo*, and may be a promising candidate for the treatment of inflammatory liver injury.

Keywords: salidroside; inflammation; alcoholic liver injury; TLR4; TAK1

Introduction

Inflammation is an early response to tissue injury and foreign pathogens, and then recovered the normal tissue structure and function. A normal inflammatory response regulates expression of pro-inflammatory and anti-inflammatory proteins. During these inflammation responses macrophages are essential cells that bridged innate and adaptive immunity.

The transcription nuclear factor (NF)-κB is the key molecule in host defense and inflammatory responses against microbial and viral infections [1]. In an inactive status, NF-κB normally residues in the cytoplasm as a heterotrimer consisting of p50, p65 and IκB proteins. When NF-κB is activated by inflammatory stimuli, such as LPS, upstream IκB kinase (IKK) is phosphorylated and activated via upstream of TGF-β activated kinase 1 (TAK1) [2]. And then IκB is undergoing phosphorylation and degradation, while NF-κB is translocated from cytoplasm into the nucleus. Once there, NF-κB

binds to DNA and induces further pro-inflammatory gene expression and inflammatory response [3, 4]. Therefore, the inhibition of the NF-κB pathway may have a beneficial effect in anti-inflammation.

The mitogen-activated protein kinases (MAPKs) are a family of serine/threonine protein kinases, including c-Jun NH_2-terminal kinase (JNK), extra-cellular signal-regulated protein kinase (ERK) and p38, playing an essential role in in the regulation of cellular responses to cytokines and stresses [5]. Activation of MAPKs signaling pathways leads to the synthesis and release of pro-inflammatory mediators by activated macrophages during the inflammatory response [6].

Using the *in vitro* model of RAW 264.7 macrophages, recent studies have suggested that LPS could stimulate inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) through the ERK and JNK signaling pathway [7], and other reports proved the activation of MAPK and NF-κB by LPS [8-10]. The crosstalk between NF-κB and MAPK pathways in response to inflammatory signal regulates many genes in inflammatory response [11, 12].

Salidroside (SDS, p-hydroxyphenethyl-β-D-glucoside, structure shown in Fig. 1A) is one of the active components of *a highly valued medicinal herb* called Rhodiola sachalinensis, *which grows at high altitude and in cold regions, such as Changbai mountain [13].* SDS displays a broad spectrum of pharmacological effects and has been used a long history of folk use [14]. It has been reported that SDS possesses various pharmacological properties including resisting anoxia, anti-aging, anti-cancer, anti-oxidative, hepatoprotective and cardioprotective effects [15, 16]. *In vivo*, SDS could protect against acetaminophen-induced toxicity in C57BL/6 mice [17]. Recently Chang et al reported SDS could alleviate ethanol-induced acute gastric ulcer and H_2O_2-induced gastric epithelial cell damage through the MAPK/NF-κB pathway [18]. Several literatures reported that SDS regulated cytokine responses via NF-κB and ERK activation in LPS-stimulated RAW 264.7 macrophages *in vitro* and mice challenged with LPS *in vivo* [19, 20]. However, there was no available research on the effect of SDS on inflammatory response on TLR4/TAK1 pathway in LPS-stimulated RAW 264.7 cells and binge drinking induced liver inflammation. Thus the aim of the present study was to explore the anti-inflammatory mechanism of SDS *in vitro* and *in vivo*.

2 Results

2.1 Effect of SDS on Cell Viability.

To examine the toxicity of SDS under experimental conditions, Raw 264.7 cells were treated with various concentrations of SDS. The cell viability was analyzed by MTT assay. LPS (1 μg/ml) itself only numerically affect the viability of RAW 264.7 cells (p>0.5, Fig. 1B). SDS (200-3.12 μM) did not display any cellular toxicity on RAW264.7 cells in presence or absence of LPS within 24 h (Fig. 1B and 1C).

Figure 1. Effect of SDS on the cell viability. (A) Chemical structure of salidroside. (B) Cells were cultured with various concentrations of SDS for 24 h. (C) Cells were incubated with SDS in the presence of LPS (1 μg/ml) except normal group for 24 h. Cell viability was determined by MTT assay. Data are expressed as mean±SD of three independent experiments.
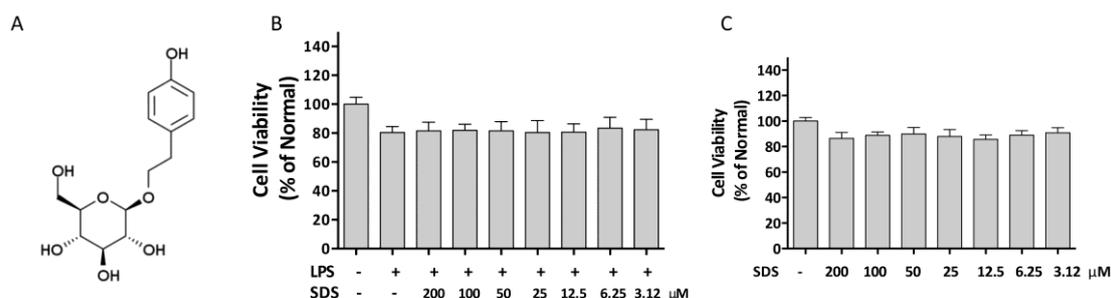

Figure 1

2.2 Effect of SDS on LPS-Induced iNOS and COX-2 protein expression.

In the inflammation process, iNOS and COX-2 are pivotal enzymes in the inflammation process. We measured the protein levels of iNOS and COX-2 as well as production of NO in RAW 264.7 macrophages stimulated with LPS for 24 h. Protein expression of iNOS and COX-2 as well as NO release were minimal in normal cells, respectively (Fig. 2A and 2B), but their levels were remarkably upregulated with LPS for 24 h. The induction of iNOS and COX-2 were dramatically suppressed by SDS pre-treatment in a concentration-dependent manner.

Figure 2. Effect of SDS on LPS-induced expression of iNOS，COX-2，iNOS，COX-2 protein and NO production. RAW 264.7 cells were pretreated with various concentrations of SDS for 1 h, followed by stimulating with LPS (1 µg/mL) for 24 h. (A) Representative pictures of Western blots and (C, D) quantitative analysis of blots. Each immunoreactive band was digitized and expressed as a ratio of α-tubulin levels. (B) NO level was measured by Griess reaction. L-NIL (N6-(1-Iminoethyl)-lysine, hydrochloride, 50 µM) was used as positive control. Data are expressed as mean±SD of three independent experiments. ** $p < 0.01$, *** $p < 0.001$, versus LPS-stimulated cells.

Figure 2
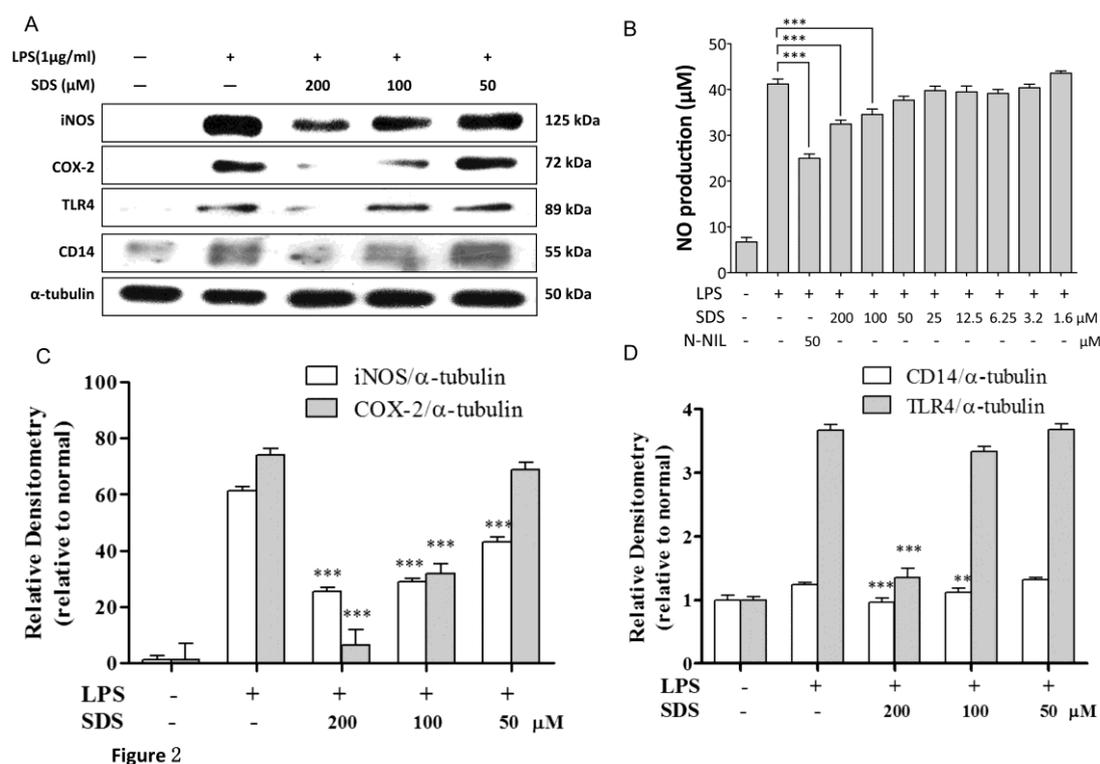
2.3 Effect of SDS on LPS-Induced TLR4 and CD14 expression.

The inhibition of iNOS and COX-2 expression by SDS in LPS-stimulated RAW 264.7 cells indicates that SDS influences the events in LPS signaling, such as the suppression of LPS binding to the cell surface receptors CD14/TLR4. Therefore, we tested whether SDS affects protein expression of TLR4 and CD14. As shown in Fig. 2A, we found that protein expression of TLR4 and CD14 significantly increased in LPS-stimulated RAW 264.7 cells compared with normal cells. Pretreatment with 200 µM SDS significantly decreased the expressions of TLR4 and CD14, compared to those in LPS-stimulated cells.

2.4 Effect of SDS on LPS-induced phosphorylation of TAK1 and MAPK.

TAK1 is the upstream signaling molecule of NF-κB, which regulates inflammatory genes such as COX-2, INOS and proinflammatory cytokines. The effect of SDS on the LPS-induced phosphorylation of TAK1 was investigated using Western blot. We found that an increase in p-TAK1

expression was detected with LPS for 30 min. However, pre-treatment with SDS inhibited this increase in TAK1 phosphorylation (Fig. 3A).

MAPKs are involved in the regulation of pro-inflammatory mediator expression in LPS-treated RAW 264.7 cells [21]. We were analyzed the phosphorylation of three MAPK signaling molecules including ERK, JNK, and p38 MAPK. Our results confirmed that phosphorylation of ERK, JNK, and p38 MAPK were significantly increased by treatment with LPS. Pretreatment with SDS inhibited the phosphorylation of ERK only at a higher concentration of 200 μM, and markedly prevented the LPS-induced increasing of JNK and p38 phosphorylation in a concentration-dependent manner (Fig. 3A).

Figure 3. Effect of SDS on LPS-induced phosphorylation of TAK1 and MAPK. RAW 264.7 cells were pretreated with various concentrations of SDS for 1 h, followed by stimulating with LPS (1 μg/mL) for 30 min. (A) Representative pictures of Western blots and (B, C) quantitative analysis of blots. Data are expressed as mean±SD of three independent experiments. *** $p < 0.001$, versus LPS alone.
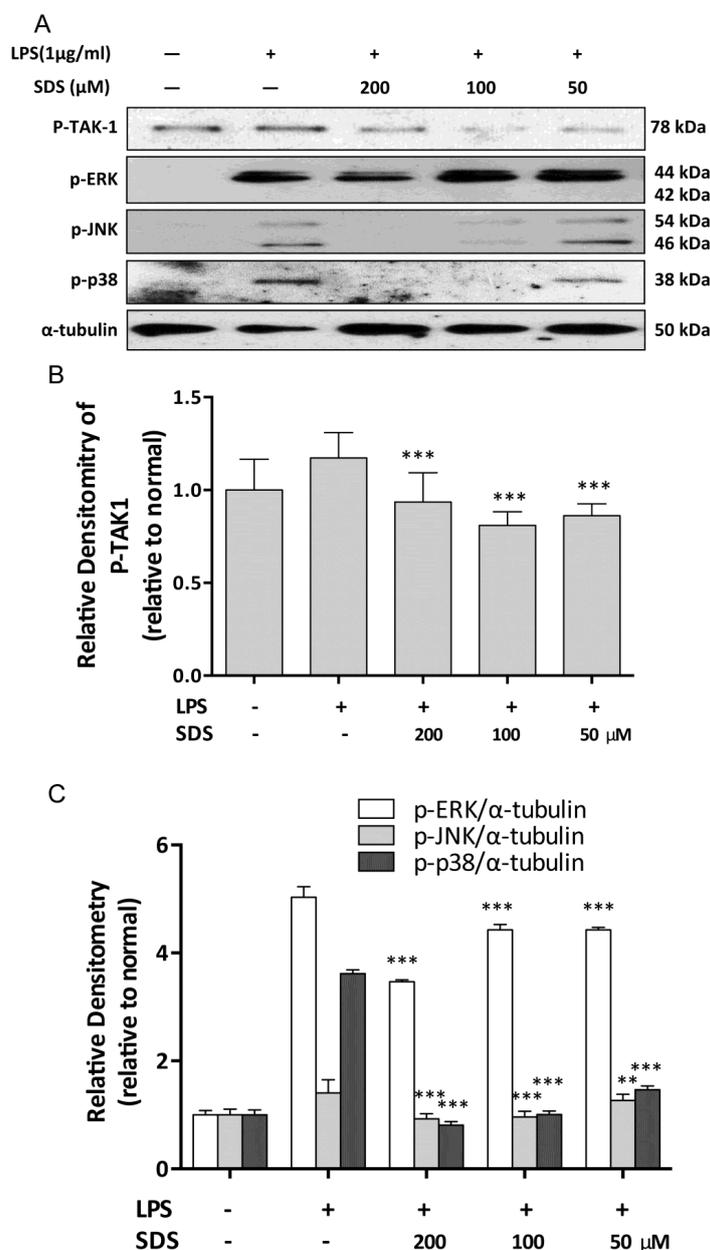

Figure 3

2.5 Effect of SDS on LPS-induced IκB degradation and NF-κB nuclear translocation.

NF-κB is a key upstream regulator in the production of proinflammatory mediators in LPS-stimulated RAW 264.7 cells, such as iNOS and COX-2 [22]. Therefore, we next investigated whether SDS has an inhibitory ability on the NF-κB signaling pathway. As shown in Fig. 4, LPS stimulation triggered the cytosolic IκB-α degradation and nuclear translocation of NF-κB p65 subunit. However, SDS pretreatment significantly attenuated the cytosolic level of IκB-α and the nuclear level of NF-κB p65 subunit, compared with LPS-primed RAW 264.7 cells. SDS exerted similarly anti-inflammatory effects when determining IL-1β mRNA being challenged with LPS. Specifically, incubation of cells with LPS resulted in a significant time-dependent induction of IL-1β mRNA and reach peak at 6 h (Fig. 5). Pretreatment with SDS blocked LPS-induced expression of IL-1β mRNA in RAW 264.7 cells at 1 h, and then recovered to the same level in LPS-stimulated cells. It indicated that SDS might transiently inhibit the secretion of proinflammatory cytokines induced by LPS.

Figure 4. Effect of SDS on LPS-induced IκB-α degradation and NF-κB activation in RAW 264.7 cells. RAW 264.7 cells were pretreated with various concentrations of SDS for 1 h, followed by stimulated with LPS (1μg/mL) for 30 min. The cells were harvested, and then protein was prepared for the detection of NF-κB p65 from in cytosol (Cy) or nuclear (Nu). α-tubulin (Cy) and Topo I (Nu) are loading controls of corresponding cellular fractions. Data are expressed as means±SD of three independent experiments.   *p < 0.05, ** p < 0.01, *** p < 0.001, versus LPS alone.
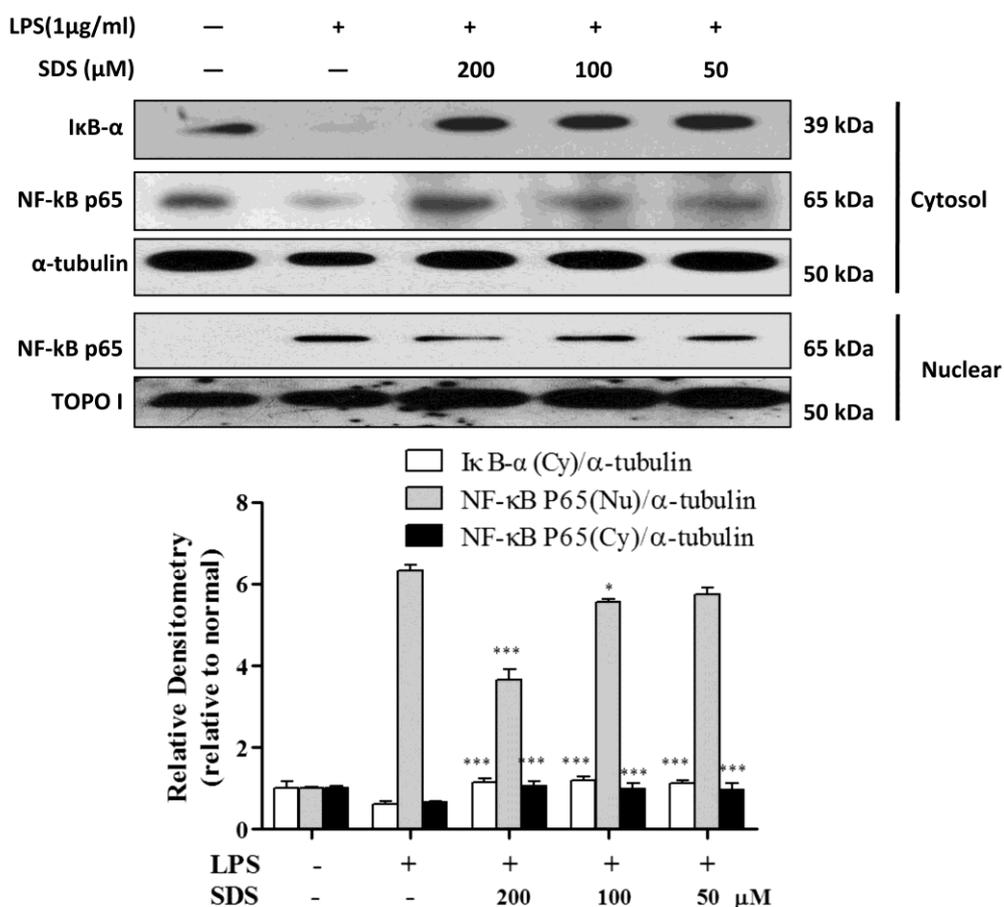

Figure 4

Figure 5. Effect of SDS on IL-1β mRNA. RAW 264.7 cells were pretreated with various concentrations of SDS for 1 h, followed by stimulated with LPS (1 μg/mL) for 1, 3 or 6 h. Figures were selected as representative data from three independent experiments.
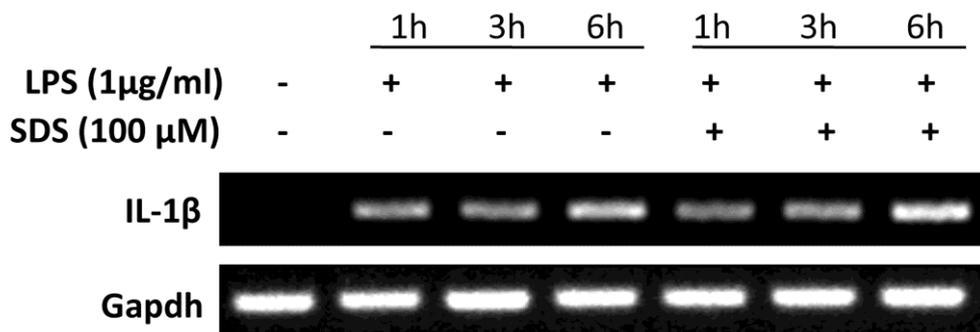
		1h	3h	6h	1h	3h	6h
LPS (1µg/ml)	-	+	+	+	+	+	+
SDS (100 µM)	-	-	-	-	+	+	+

IL-1β

Gapdh

Figure 5

2.6 Effect of SDS on binge drinking induced liver injury.

Binge drinking can create an inflammatory response in the liver. So we investigated the contribution of SDS to alcohol-induced liver injury in vivo in a model of acute alcohol intoxication caused by binge drinking. Alcoholic hepatitis is characterized by the hepatocytes injury and inflammation. Mice received ethanol showed marked ballooning of the hepatocytes in the centrolobular area. The increase histologic findings of hepatocyte injury in alcohol-fed animals were associated with enhanced blood levels of AST and ALT (Fig. 6A and 6B). The elevation in serum transaminase levels suggested the early stage of liver damage. SDS successively suppressed AST and ALT levels, as well as hepatocyte damage. As a result alcohol caused a marked increase in Oil red O staining of lipid droplets. Pretreatment with SDS prior to alcohol ingestion significantly blunted alcohol-induced liver steatosis and enhanced transaminase levels.

Figure 6. Effect of SDS on binge drinking induced liver injury. C57BL/6 mice were orally administrated SDS (100 mg/kg) for 5 days, followed by three gavages of dextrin maltose or ethanol every 30 min. (A) Representative H&E staining and Oil Red O staining of liver tissues, (B) serum ALT and AST, (C) serum IL-1β protein. Black arrows represent damaged hepatocytes. Values represent means ± SD. *p < 0.05, ** p < 0.01, *** p < 0.001, versus ethanol-fed group.
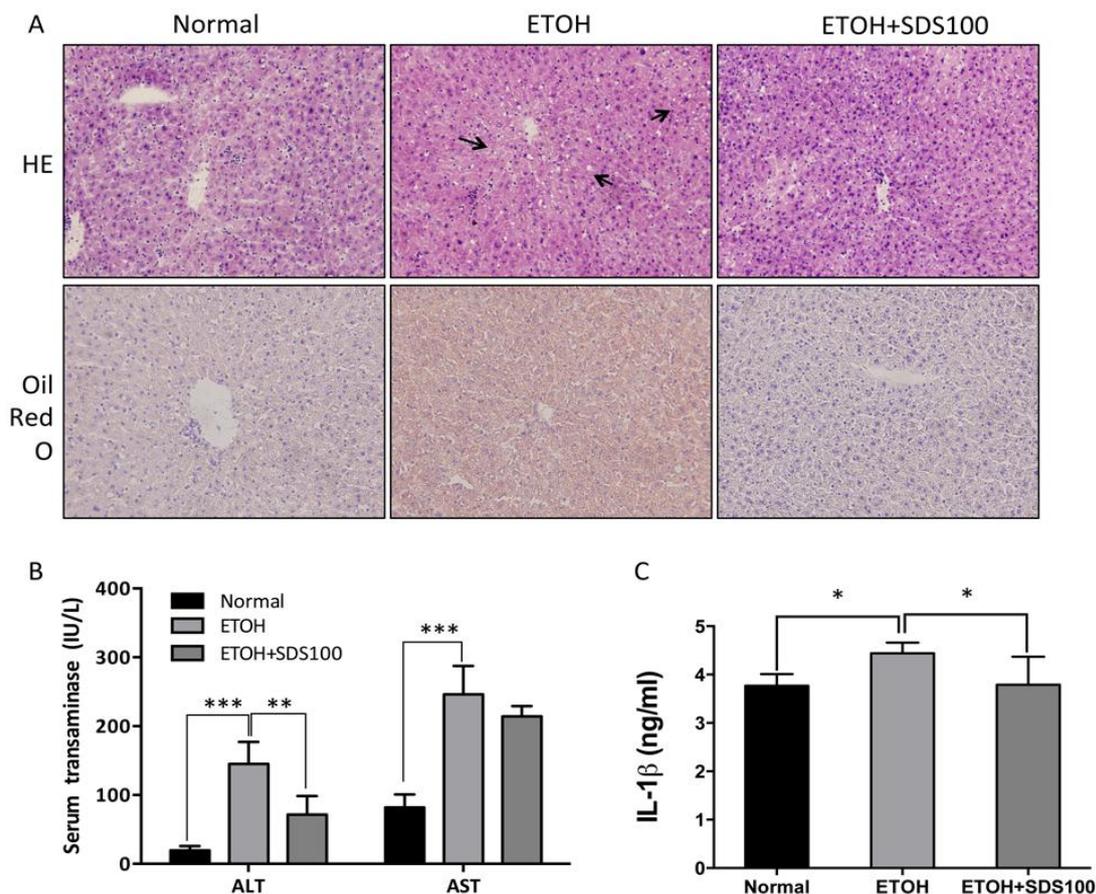
Figure 6

IL-1β is a potent proinflammatory cytokine that is elevated in alcoholic liver injury [23]. IL-1β plays a pivotal role on the expression of proinflammatory genes by activation of intracellular signaling pathways, such as NF-κβ [24]. IL-1β was induced in mice fed ethanol, but it was greatly reduced in SDS pretreated mice, indicating the decrease of IL-1β may also impact hepatocyte survival.

3 Discussion

Inflammation is a host response to various stimuli that leads to the release of a large amount of inflammatory mediators [25]. To exploit the concept that SDS exhibited anti-inflammatory through blocking the endotoxin-induced expression of the TAK1 and thereby blocking the NF-κB signaling cascade in macrophages, firstly we determined the expression of TLR4, CD14, IL-1β, and COX-2 as well as iNOS protein levels and the release of NO in LPS-primed RAW 264.7 macrophages.

iNOS is induced by various inflammatory stimuli including bacterial endotoxic LPS and inflammatory cytokines in macrophages. COX-2 is an inflammation-induced enzyme. At the sites of inflammation, it is highly expressed [26]. This suggested that iNOS and COX-2 plays a key role in inflammation. Our data showed that pretreatment with non-toxic concentrations of SDS (50, 100 and 200 μM) caused a dose-dependent inhibition of COX-2 and iNOS at protein expression, which was consistent with previously reported literatures [20].

In LPS signaling, LPS binds its major receptor CD14 and then initiate the intracellular signaling cascades. The interaction of LPS with LPS-binding protein (LBP) and its transfer to cellular CD14 activate macrophages [27]. Binding of LPS to Myeloid differentiation 2 (MD-2) triggers the recruitment of the adaptors myeloid differentiation factor 88 (MyD88) and Toll-interleukin-1R domain-containing adapter inducing interferon-β (Trif) [28]. CD14 catalyzes TLR4-MD-2-LPS

complex to initiate LPS-TLR4 signaling. Finally, TLR4-MD2 dimerization stimulates the intracellular pathway that eventually results in activation of NF-kB and the production of pro- inflammatory cytokines [29]. As shown in Fig.2A, our data showed that protein expression of TLR4 and CD14 increased by LPS stimulation, while SDS successfully blocked LPS-TLR4 signaling pathway. These results suggest that inhibition of iNOS and COX-2 by SDS might be related with suppression of LPS-TLR4 signaling pathway.

Upon LPS recognition, MyD88 in turn recruits IRAK4 and IRAK1, and then IRAK4 induces IRAK1 phosphorylation. Then phosphorylated IRAK1 leads to the phosphorylation of TAK1 [30]. TAK1 is a mitogen-activated protein kinase kinase kinase (MAP3K) and acts as upstream of NF-κB -inducing kinase (NIK) [31]. And NF-κB also regulates COX-2 and iNOS expression. The activation of NF-κB requires phosphorylation of upstream IκB kinase (IKK) via upstream of TAK1. Our results showed that SDS pretreatment inhibited the increase in TAK1 phosphorylation firstly (Fig. 3), and then eventually prevented nuclear translocation of NF-κB (Fig.4). And blockade of NF-κB activity w SDS leads to the inhibition of NF-κB target genes production, including proinflammatory cytokine, IL-1β (Fig. 5).

MAPKs are responsible for the regulation of many genes involved in inflammatory mediators production. Upon LPS stimulation, JNK, ERK and p38 MAPK are activated via phosphorylation of both tyrosine and threonine residues and play different roles in different signaling capacities [32]. Our data revealed that SDS effectively blocked the signal transduction by MAPK molecules in LPS-stimulated macrophages (Fig. 6), which was in line with previous report on THP-1 cells [33].

Acute alcohol consumption enhanced circulating levels of LPS in alcoholics with alcoholic liver diseases [34]. Therefore, during development of ALD, hepatic response to gut derived endotoxin (LPS) is a critical step [35]. And preexposure of alcohol can prime Kupffer cells to LPS stimulation, resulting in the increased release of proinflmmatory cytokines [36]. In regard to this, we applied the mice model of binge drinking induced liver injury to mimic liver inflammation with hepatocyte injury to further investigate anti-inflammatory mechanism of SDS. Similar to the results obtained in vitro, SDS administration effectively alleviated alcohol-induced hepatocyte damage and proinflammatory cytokine secretion.

In summary, this study demonstrated that SDS inhibited LPS-induced inflammatory mediators by attenuating the LPS-dependent induction of TLR4-CD14 and the subsequent phosphorylation of TAK1, and lead to inhibit NF-κB and MAPKs signaling pathway, eventually suppressing its proinflammatory target gene (Fig. 7). Future studies will need to identify underlying mechanism responsible for beneficial effects of SDS in alcohol binge drinking induced liver inflammation, our results suggest that SDS may be therapeutically useful for the treatment of hepatic inflammation associated with an activation of TAK1.

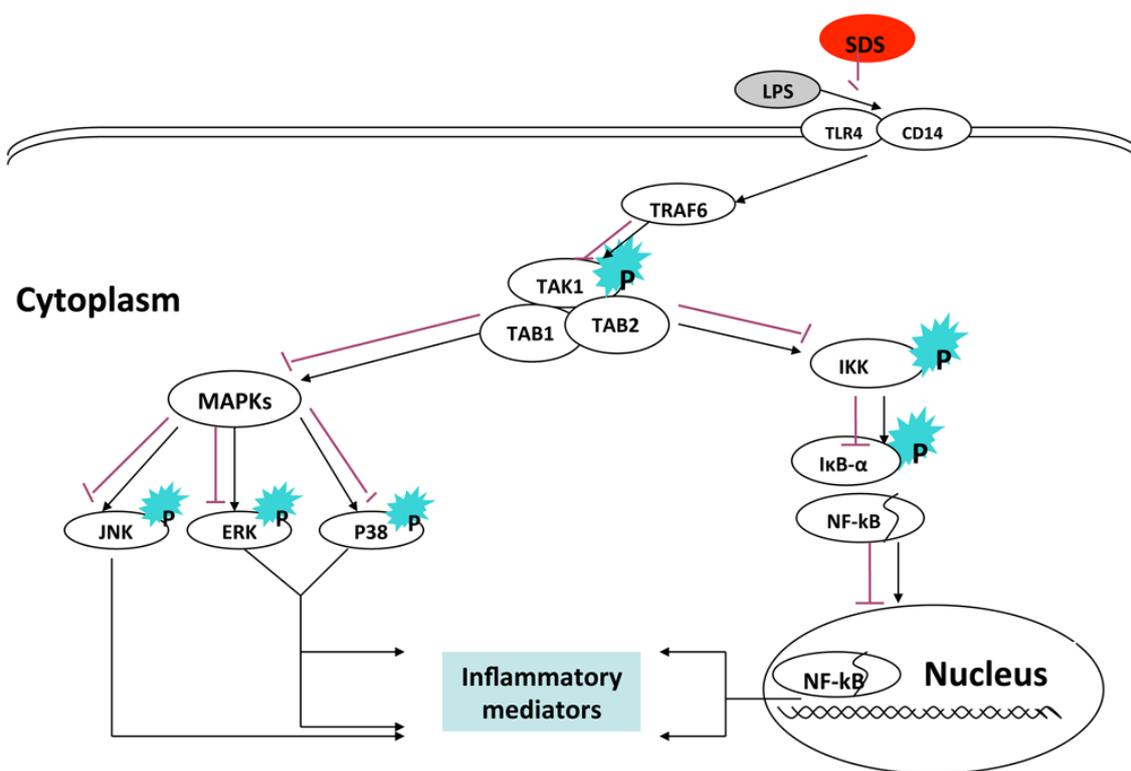**Figure 7.** A proposed mechanism of anti-inflammatory effects of SDS.

Figure 7

4. Materials and methods

4.1 Materials.

SDS was obtained from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). The purity of SDS reached 99%. All cell culture reagents were from Gibco, Stockholm, Sweden. Lipopolysaccharide (LPS) and dimethyl sulfoxide (DMSO) were purchased from Sigma Chemicals Co. (St Loius, MO, USA). All reagents were purchased from Beyotime Institute of Biotechnology (Jiangsu, China). Anti-iNOS, anti-p-TAK1, anti-P-JNK, anti-P-ERK and anti-P-p38 antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). Anti COX-2, anti-CD14, anti-TLR4, anti-NF-κB P65, anti-IκBα and anti-topoisomerase I were purchased from Santa Cruz Biotechnology (CA, USA) and anti-α-tubulin was purchased from Sigma (St.Louis, MO, USA).

4.2 Cell Culture.

RAW 264.7 cells, a mouse macrophage cell line, were cultured in Dulbecco's Modified Eagle Medium (DMEM) medium containing 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin and incubated at 37°C in 5% CO_2.

4.3 Measurement of cell viability.

After pretreated with different concentrations of SDS (0-200 μM) for 1 h, RAW 264.7 cells were followed by stimulations absence or presence of LPS (1 μg/ml) for 24 h. Subsequently cells were incubated with MTT solution (5 mg/ml) for another 3 h. Then the content of formazan was measured in DMSO by using a microplate reader at 570 nm.

4.4 Quantification of NO production.

The nitrite accumulated in medium supernatant was measured as NO production based on the Griess reaction as described previously [27].

4.5 Binge drinking protocol.

Twenty week-old male C57BL/6 mice were obtained from Animal Division of Jilin University (Jilin, China). Procedures were carried out according to the criteria of the "Guide for the Care and Use of Laboratory Animals" published by the USA National Institutes of Health (1996) and approved by Animal Research Committee of the University. Animals were maintained at ambient temperature and humidity under an artificial 12 h light/12 h dark cycle.

Mice were randomly divided into 3 groups: normal group, ethanol group and ethanol plus SDS group. Mice were fasted for 4 h and then received 3 gavages of equivalent calories of dextrin maltose or ethanol (3.5 g/kg) every 30 min, and then mice were sacrificed 6 h later [37]. Before gavaging ethanol, the mice in ethanol plus SDS group were orally administrated SDS (100 mg/ml) for five continuous days. The doses of SDS were determined according to our previous experiments [17, 38]. Livers were harvested, snap frozen in liquid nitrogen, and stored at −80°C until they were used.

4.6 Blood biochemistry and IL-1β ELISA assay.

Serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were detected using an Autodry Chemistry Analyzer (SPOTCHEM™ SP4410, Arkray, Kyoto, Japan). Serum IL-1β levels were determined by Murine IL-1β Standard ABTS ELISA Development Kit (PeproTech Inc., Rocky Hill, NJ, USA) according to the manufacturer's protocol.

4.7 RT-PCR analysis.

Total RNA was prepared from mice livers by use of the Beyozol reagent，and the cDNA was reverse transcribed from 1 μg of total RNA as described previously [39]. RT-PCR was performed using primers specific for the mouse IL-1β and housekeeping gene glyceraldehydes-3-phosphate dehydrogenase (GAPDH). The primers were 5′-GTACATCAGCACCTCACAAG (sense) and 5′-CACAGGCTCTCTTTGA-ACAG (anti-sense) for IL-1β; 5′-ATGGTGAAGGTCGGTGTGAA (sense) and 5′-CGCTCCTGGAAGATGGTGAT (anti-sense) for GAPDH.

4.8 Histochemistry.

Livers removed from mice were rapidly frozen in optimal cutting temperature (OCT) medium and were stored at -80°C until use. Serial sections of 5 μm were cut and were fixed in an iced mixture of acetone:methanol (v:v,1:1). The hematoxylin and eosin-stained sections were evaluated by lightmicroscopy. Lipid accumulation was detected using Oil Red O staining as described by Kanuri et al [40].

4.9 Western blotting analysis.

After pretreated with various concentrations of SDS for 1 h, RAW 264.7 cells were stimulated with 1 μg/ml LPS for 24 hours for iNOS, COX-2, CD14 and TLR4 detection. For the detection of NF-κB p65, IκB-α, p-TAK1, p-ERK p-JNK and p-p38, the cells were only treated with LPS for 30 min. Whole cell lysates were prepared using ice-cold cell lysis buffer (Beyotime, Jiangsu, China). Nuclear and cytosol fractions were prepared from cells using the reagent-based method (Beyotime). Protein concentration was determined using BCA Protein Assay Kit (Beyotime).

Cell lysates were separated by 10% SDS-polyacrylamide gel electrophoresis and electrotransferred to polyvinylidene difluoride (PVDF) membranes. The membrane was incubated in blocking solution (5% nonfat milk in PBS with 0.1% Tween 20) at room temperature for 1 h, and then incubated overnight at 4°C with specific primary antibodies, followed by incubation with horseradish peroxidase (HRP)-conjugated secondary antibodies (Santa Cruz Biotechnology) for 1 h at room temperature. Immunoreactive protein was visualized by the BeyoECL plus kit (Beyotime).

To correct for potential unequal loading, blots were stripped and reproved with antibodies to topoisomerase I (Topo I) for nuclear fractions and α-tubulin for cytoplasmic fractions. The relative intensity of each band was quantified by densitometric analysis using Quantity One software (Bio-Rad, USA).

4.10 Statistical Analysis.

All data were expressed as mean±SD. Data analysis was performed with one-way ANOVA and Tukey's multiple comparison tests by using the GraphPad Prism program (GraphPad Software, USA). Statistical significance was defined as p values less than 0.05.

5 Conclusions

The suppression of LPS-induced inflammatory mediators by SDS is mediated by TAKI phosphorylation, leading to inhibition of NF-κB and MAPK phosphorylation, eventually suppressing its proinflammatory target gene.

ABBREVIATIONS: ALT, alanine aminotransferase; AST, aspartate aminotrans-ferase; ERK, extracellular signal regulated kinase; iNOS, inducible nitric oxide synthase; COX-2, cyclooxygenase-2; IκB-α, inhibitory kappa B-α; IKK, IκB kinase; IRAK, interleukin-1 receptor-associated kinase; JNK, c-Jun terminal kinase, LPS, lipopolysaccharide; LBP, lipopolysaccharide-binding protein; MAPK, mitogen-activated protein kinases, MD-2, myeloid differentiation-2, MyD88, myeloid differentiation primary response gene 88; NF-κB, nuclear factor-κB; TAK1, TGF-beta activated kinase 1; TLR4, toll-like receptor 4; TRAF6, TNF receptor associated factor 6; TRIF, toll/IL-1 receptor domain containing adaptor inducing interferon-β.

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