Int J Clin Exp Pathol 2018;11(10):4917-4925
www.ijcep.com /ISSN:1936-2625/IJCEP0082837

Original Article

Schisandrin B attenuates lipopolysaccharide-induced activation of hepatic stellate cells through Nrf-2-activating anti-oxidative activity

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Received July 20, 2018; Accepted August 25, 2018; Epub October 1, 2018; Published October 15, 2018

Abstract: Activated hepatic stellate cells (HSCs) are known to have a potential role in increasing the deposition of ECM and elevating proliferation in liver fibrosis, which can be driven by lipopolysaccharide (LPS). Schisandrin B (SB) is a dibenzocyclooctadiene derivative of Schisandra chinensis with anti-oxidative stress activity, but its effective target is unknown. Here, we have evaluated whether SB is protective against the LPS-induced activation of HSCs and have explored the underlying anti-oxidative stress mechanisms of SB. HSCs were treated with SB 1 h prior to LPS, and then incubated for indicated time. Nrf-2 in HSCs was inhibited genetically. The simultaneous effects on Nrf-2 activity, oxidative stress, cell proliferation, and ECM deposition were examined. SB decreased LPS-induced cell proliferation, fibrosis, and oxidative stress in HSCs. We further demonstrated that the protective effects of SB in LPS-induced HSCs activation involve the modulation of Nrf-2. SB, specifically targeting Nrf-2, attenuates the oxidative stress in HSCs. SB also reduces LPS-induced fibrosis and cell viability in HSCs. In addition, Nrf-2 may serve as a therapeutic target for infections or periods of chronic oxidative stress and may help with future drug discovery.

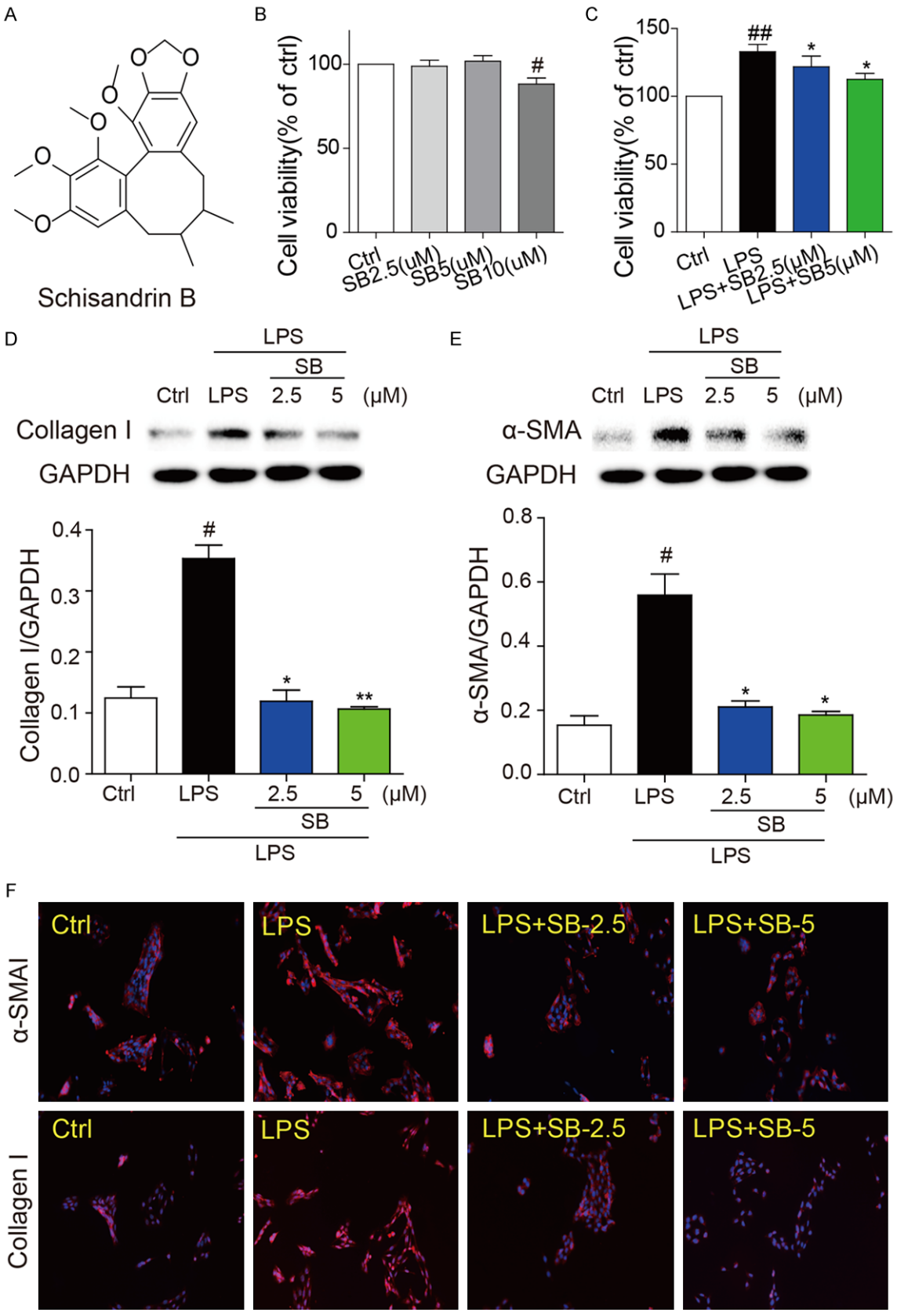
Keywords: Schisandrin B, hepatic stellate cell, lipopolysaccharide, oxidative stress, nuclear factor erythroid 2-related factor 2

Introduction

Hepatic fibrosis is regarded as a frequent and repeated process existing in liver cells which results from various direct and indirect stressors, and which may lead to cirrhosis and even liver cancer [1, 2]. Liver fibrosis results from an imbalance between the accumulation and deposition of excessive extracellular matrix (ECM) [3]. Activated hepatic stellate cells (HSCs) are well known for their potential role in increasing the deposition of ECM and for elevating proliferation in liver fibrosis [4]. Lipopolysaccharide (LPS), the major cell wall component of gram-negative bacteria, has been identified as a main factor that leads to liver fibrosis and the activation of HSCs [5].

Previous studies have shown that LPS-induced liver injury is caused by the inflammatory response, while oxidative stress also involved in these physio-pathological conditions [6]. Oxidative stress is manifested by the aberrant production of reactive oxygen species (ROS) and the failure of the antioxidant defense system [7]. Recently, it has been documented that nuclear factor erythroid 2-related factor 2 (Nrf-2) and its downstream proteins are recognized as remarkable signaling mediators in the antioxidant defense mechanism to prevent liver cell fibrosis [8].

The multifactorial nature of liver fibrosis and HSCs activation requires a multi-pronged approach to neutralize the toxicity. Recently, Schisandrin B (SB, **Figure 1A**), a dibenzocyclooctadiene derivative of Schisandra chinensis [9, 10], was shown to improve carbon tetrachloride (CCl4)- and acetaminophen-induced liver injury [11, 12]. Although the mechanisms of these protective activities of SB are not known, these may, at least in part, be mediated through the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B
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SB2.5(uM)
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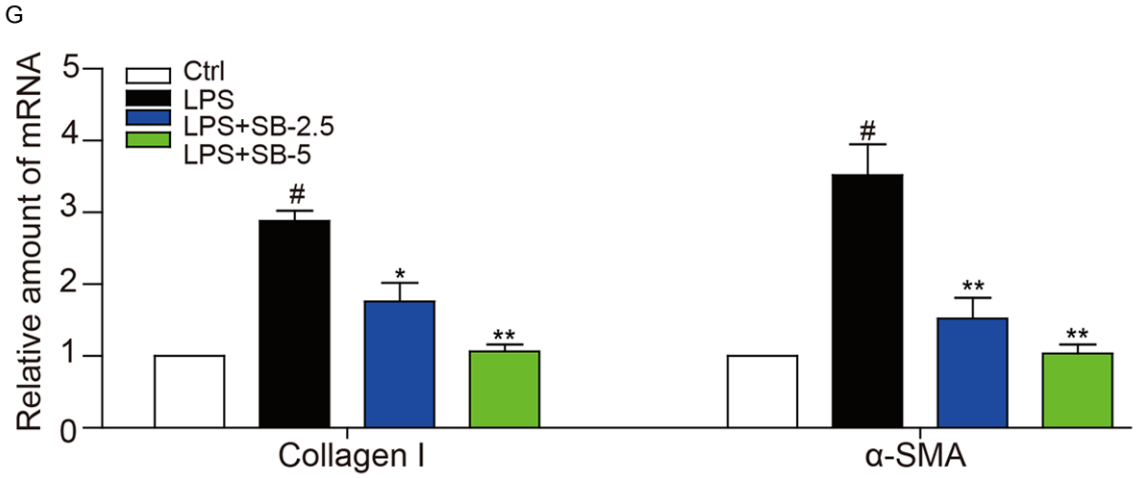

Figure 1. SB attenuated LPS-induced HSCs activation. (A) The structures of Schisandrin B. (B) Effects of SB (incubated for 72 h) on cell viability by MTT assay. (C) Effects of SB on the proliferation of HSCs treated with LPS for 24 h. HSCs were pretreated with SB (2.5 or 5 μM) for 1 h and then were incubated with LPS (100 ng/mL) for the indicated time. (D, E) Exposed to LPS for 24 h. Western blot analysis detected the Col-1 (D) and α-SMA (E) proteins. (F) Exposed to LPS for 24 h. Immunofluorescence staining of HSCs for Col-1 (red), and α-SMA (red) in LPS-treated cells pretreated with SB prior to LPS exposure. [blue = DAPI]. Representative micrographs are shown. (G) Exposed to LPS for 6 h. The mRNA expression of Col-1 and α-SMA were carried out. (n>3 for each experiment; *P<0.05, **P<0.01, vs. the LPS group, #P<0.05, ##P<0.01 vs. the Ctrl group).

induction of Nrf-2 [11, 12]. These findings suggest that SB may be an ideal therapeutic drug for liver fibrosis. Nevertheless, it is unclear if SB can function as a potential regulator in LPS-related oxidative stress and the activation of HSCs. Thus, the purpose of the present study was to investigate the protective effect and related molecular mechanism of SB against LPS-induced oxidative stress and the activation of HSCs.

Material and methods

Reagents

LPS was purchased from Sigma-Aldrich (St. Louis, MO, USA). The SB compound was provided by Aladdin and dissolved in DMSO for the cell experiments. The final concentration of SB on cells was 2.5 μg/ml and 5 μg/ml, 0.1% DMSO. Antibodies against Collagen 1, α-SMA, Lamin B, and GAPDH were purchased from Santa Cruz Technology (Santa Cruz, CA). Antibodies against Nrf-2, HO-1, NQO-1, and GCLC were from Cell Signaling Technology (Danvers, MA).

Cell culture and treatment

HSCs were isolated from male Sprague-Dawley rats (450-500 g) as described previously [13]. Briefly, after in situ perfusion of the liver with 2-step pronase-collagenase digestion, the HSCs were separated from other nonparenchymal cells by density-gradient centrifugation using OptiPrep (Axis-Shield, 1114542). All treatments were initiated 24 h after isolation unless otherwise indicated. Before the cells were treated with LPS (100 ng/ml) and SB, the cells were serum starved (DMEM with 0.25% FBS) overnight. All treatments were given for 24 h in 0.25% FBS-containing DMEM medium. Cells treated with 0.1% DMSO were used as a control. Cells treated with LPS also had 0.1% DMSO. All the experiments were repeated at least 3 times.

Measurement of cell viability by MTT assay

Cell viability was assessed using the MTT assay. HSCs were plated in 96-well plates at 5,000 cells per well and then treated with or without LPS for 24 h. After being incubated with MTT for 3 h, the reduction of MTT to purple fomazan was detected by a microplate reader at 540 nm. Cell viability was calculated using the formula: cell viability = Atreated/Acontrol × 100%.

Immunofluorescence cell staining

HSCs were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, and stained. Col-1 and α-SMA staining was performed

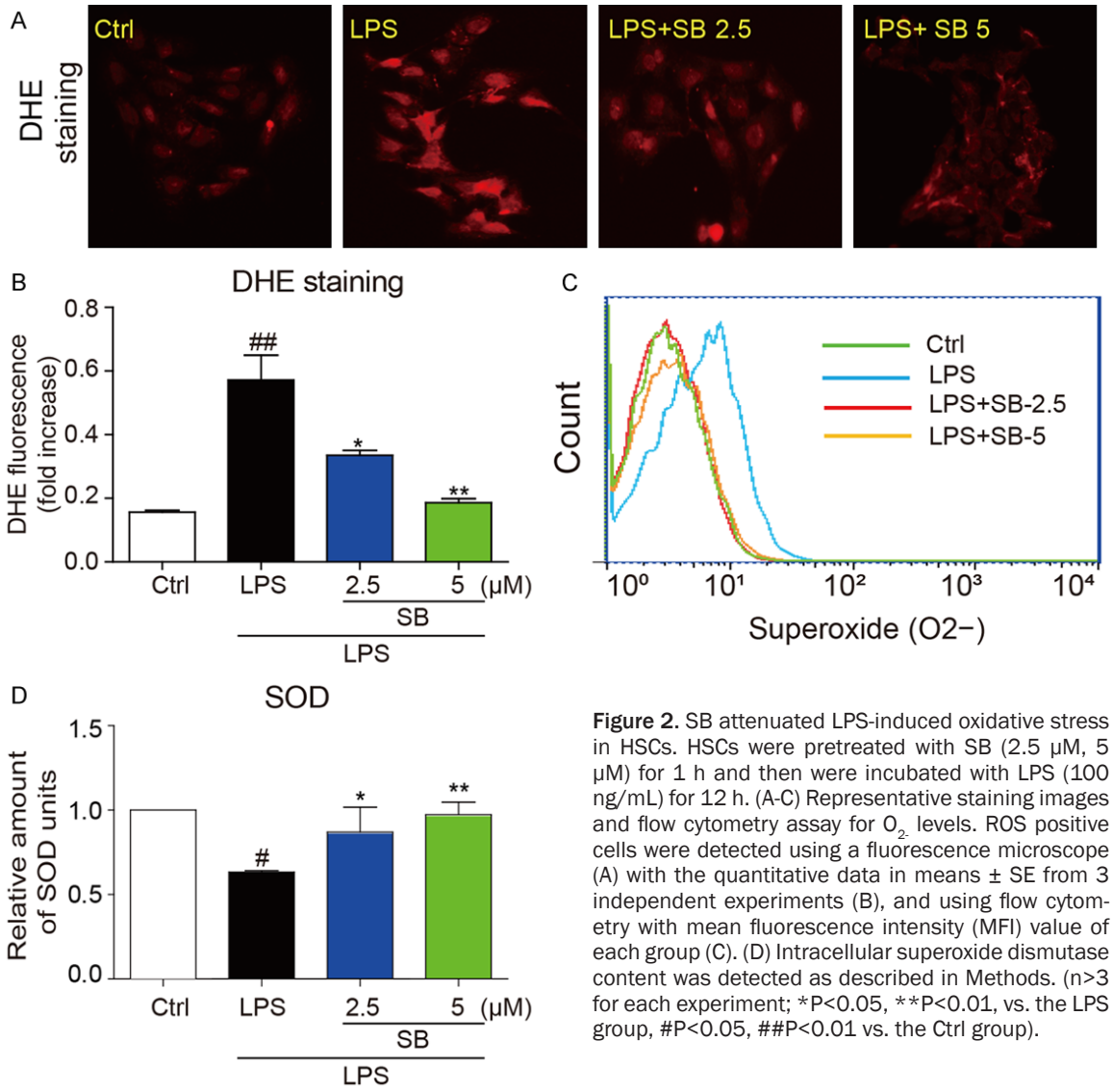

Figure 2. SB attenuated LPS-induced oxidative stress in HSCs. HSCs were pretreated with SB (2.5 μM, 5 μM) for 1 h and then were incubated with LPS (100 ng/mL) for 12 h. (A-C) Representative staining images and flow cytometry assay for O_{2}^{-} levels. ROS positive cells were detected using a fluorescence microscope (A) with the quantitative data in means ± SE from 3 independent experiments (B), and using flow cytometry with mean fluorescence intensity (MFI) value of each group (C). (D) Intracellular superoxide dismutase content was detected as described in Methods. (n>3 for each experiment; *P<0.05, **P<0.01, vs. the LPS group, #P<0.05, ##P<0.01 vs. the Ctrl group).

by incubating slides with anti-Col-1 or an anti-α-SMA antibody (1:200) overnight at 4°C and then detected by a PE-conjugated secondary antibody at 1:200 dilution. Cells were counterstained with a DAPI nuclear stain. Images were captured (original magnification 400; Nikon, Tokyo, Japan).

To analyze ROS generation and oxidative stress in HSCs, we utilized three assays. First, we stained HSCs for dihydroethidium (DHE) (**Figure 2A**, **2B**). DHE forms a red fluorescent product (ethidium) upon reaction with superoxide anions and intercalates with DNA. Next, we detected the ROS production with a confirmation using flow cytometry (**Figure 2C**). And lastly, we measured SOD levels in HSCs (**Figure 2D**).

Determination of oxidative stress

To analyze the oxidative stress level, we utilized three assays. First, we stained the HSCs for dihydroethidium (DHE) to evaluate superoxide (O_{2}^{-}) production as described previously [14]. Next, we detected the ROS production using 2 μM DHE, and the fluorescence intensity was acquired using flow cytometry (BD, CA) as described previously [14]. And lastly, using commercially available kits (Beyotime Biotech, Nantong, China), we measured the SOD levels in HSCs.

Determination of Nrf-2 activation by western blot analysis

Using the Nuclear and Cytoplasmic Protein Extraction Kit (KeyGEN Biotech, Nanjing, China),

Table 1. Sequences of primers for real-time qPCR assay used in the study

Gene	Species	FW	RW
HO-1	Rat	TCTATCGTGCTCGCATGAAC	CAGCTCCTCAAACAGCTCAA
NQO-1	Rat	ACCTTGCTTTCCATCACCAC	CAAAGGCGAAAACTGAAAGC
GCLC	Rat	ATGCAGTATTCTGAACTACC	ACAAACTCAGATTCACCTAC
Collagen 1	Rat	CGAGTATGGAAGCGAAGGTT	ACGCTGTTCTTGCAGTGATA
α-SMA	Rat	TGACCCAGATTATGTTTGAG	AGATAGGCACGTTGTGAGTC
β-actin	Rat	AAGTCCCTCACCCTCCCAAAAG	AAGCAATGCTGTCACCTTCCC

the nuclear extracts in HSCs were prepared as described previously [15]. And then the protein concentrations were determined.

siRNA-induced gene silencing

Nrf-2 gene silencing in cells was achieved by transfecting cells with siRNA (5'-GGGUAAG-UCGAGAAGUGUUTT-3') using LipofectAMINE™ 2000 (Invitrogen, Carlsbad, California). Knockdown was verified by western blotting.

Western blotting

HSCs were lysed, and protein amounts were separated by 10% SDS-PAGE and were electro transferred to PVDF membranes. Each membrane was blocked for 1.5 h with a Tris-buffered saline solution containing 0.05% Tween 20 and 5% non-fat milk. PVDF membranes were then incubated with the specific primary antibodies. Immunoreactive bands were detected by incubating membranes with horseradish peroxidase-conjugated secondary antibodies and visualisation using enhanced chemiluminescence (Bio-Rad). The amounts of the proteins were analysed using ImageJ analysis software version 1.38e and were normalised to their respective controls.

Real-time quantitative PCR

Total RNA was isolated from HSCs using TRIzol (cat. no. 15596026). RT and qPCR were carried out using a two-step Platinum SYBR Green qPCR SuperMix-UDG kit (cat. no. 11733046; Thermo Fisher Scientific, Inc., Waltham, MA, USA). An Eppendorf Mastercycler (Eppendorf, Hamburg, Germany) was used for the qPCR analysis. Primers for genes including Collagen 1, α-SMA, HO-1, NQO-1, GCLC, and β-actin were obtained from Thermo Fisher Scientific, Inc. The primer sequences are listed in **Table 1**. The mRNA expression levels of the target genes were normalized to β actin.

Statistical analysis

All data represented 3 independent experiments and were expressed as the means ± SEM. Statistical analyses were performed using GraphPad Pro. Prism 5.0 (GraphPad, San Diego, CA). Student t-tests or one-way ANOVAs followed by multiple comparisons tests with Bonferroni corrections were employed to analyse the differences between the sets of data. A P value <0.05 was considered significant.

Results

SB reversed LPS-induced HSCs activation

As shown in **Figure 1B**, SB significantly inhibited cell proliferation at the concentrations of 10 μM for HSCs. Thus, we selected 2.5 and 5 μM for our study. To evaluate the effect of SB on LPS induced-HSCs activation, we determined the cell viability of HSCs. In accordance with previous studies [16], LPS statistically stimulated HSCs proliferation (**Figure 1C**), indicating that LPS could increase the activation of HSCs. The treatment of SB reduces LPS related-cell viability (**Figure 1C**).

Increased production of ECM is critical for sustaining HSC activation and fibrosis [17]. Upon sustained LPS treatment for 24 h, the protein expression of ECM including Col-1 (**Figure 1D**), and α-smooth muscle actin (α-SMA) (**Figure 1E**) was stimulated. The pretreatment of SB dose-dependent decreased Col-1 (**Figure 1D**), and α-SMA (**Figure 1E**) expression. These results were also verified by staining cells for Col-1, and α-SMA (**Figure 1F**). As shown in **Figure 1G**, LPS stimulated the mRNA expressions of Col-1 and α-SMA, which were reduced by SB pretreatment in a dose dependent manner. These findings strongly suggest SB attenuated LPS-related fibrosis in HSCs, and the inhibition of the liver fibrosis protein expression by SB may be associated with the decreased viability of HSCs.

SB prevented LPS-induced oxidative stress in HSCs

Building on our findings, we explored whether the modulation of oxidative stress is involved

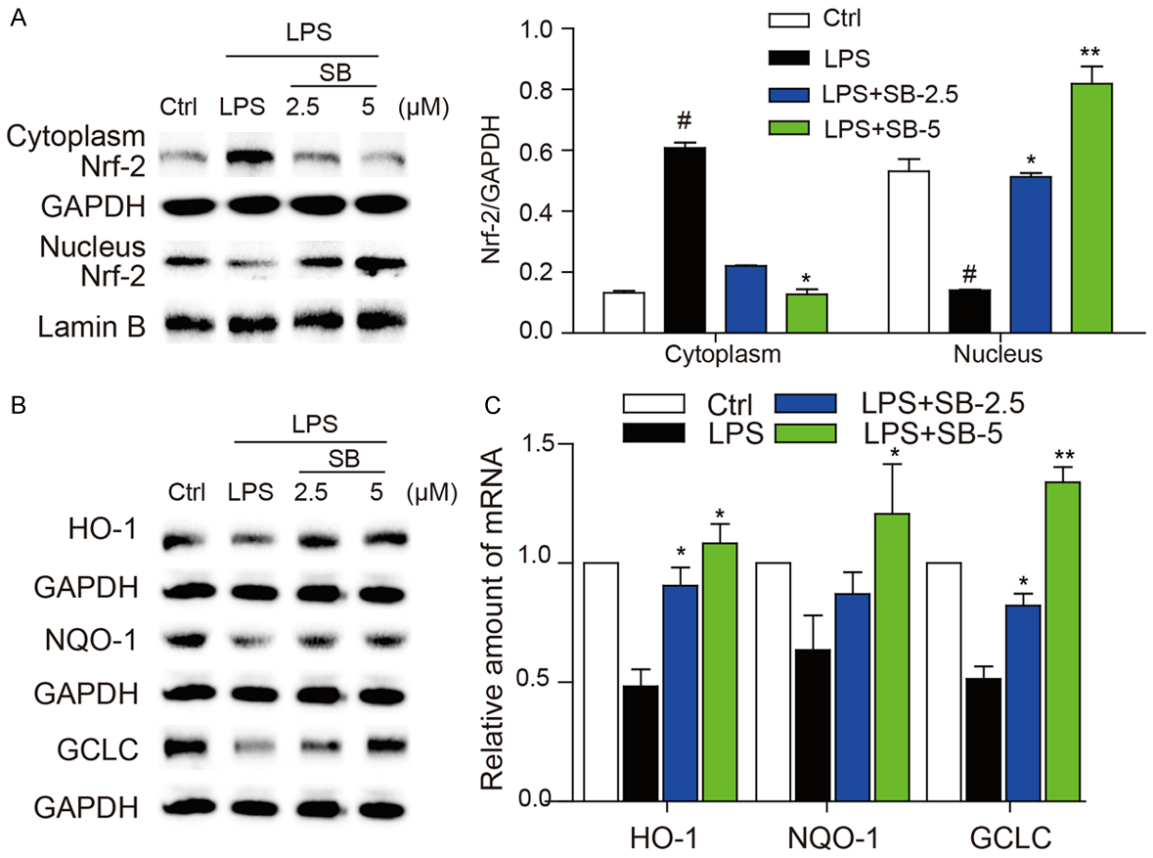

Figure 3. SB induced the expression of anti-oxidant response proteins in LPS challenged HSCs. HSCs were pretreated with SB (2.5 or 5 μM) for 1 h and then were incubated with LPS (100 ng/mL) for the indicated time. A. Exposed to LPS for 8 h. Western blot analysis detected the protein of nuclear and cytoplasm proteins of Nrf-2. B. Exposed to LPS for 8 h. The western blot analysis detected the apoptosis markers of HO-1, NQO-1, and GCLC. C. Exposed to LPS for 6 h. The mRNA expression of HO-1, NQO-1, and GCLC were carried out. (n>3 for each experiment; *P<0.05, **P<0.01 vs. the LPS group, #P<0.05, ##P<0.01 vs. the Ctrl group).

in the effects of SB. Oxidative stress has been linked to the onset of liver fibrosis. Recent studies also show that oxidative damage induced by reactive oxygen species (ROS) from LPS plays a critical role in HSCs activation [18]. To analyze ROS generation and oxidative stress in HSCs, we utilized three assays. First, we stained HSCs for dihydroethidium (DHE) (**Figure 2A**, **2B**). DHE forms a red fluorescent product (ethidium) upon reaction with superoxide anions and intercalates with DNA. Next, we detected the ROS production with a confirmation using flow cytometry (**Figure 2C**). And lastly, we measured SOD levels in HSCs (**Figure 2D**). All three measures showed increased oxidative stress levels in LPS-induced HSCs (**Figure 2A-D**). However, SB treatment markedly attenuated LPS-induced HSCs oxidative stress. These findings are in alignment with recent reports of SB inhibiting ROS in activated LPS-challenged macrophages [19].

SB induced the expression of Nrf-2 and its downstream anti-oxidant genes in LPS challenged HSCs

Studies further showed that SB may mediate this anti-oxidant activity through the induction of nuclear factor (erythroid-derived 2)-like 2 (Nrf-2) and Nrf-2-driven antioxidant response genes [19]. Under normal conditions, Nrf-2 exists in the cytoplasm. Once exposed to stressors or inducers, Nrf-2 translocates into the nucleus to promote the expression of its downstream anti-oxidant genes, such as glutamate-cysteine ligase catalytic (GCLC) subunit, heme oxygenase-1 (HO-1) and NADPH quinine oxidoreductase (NQO-1) [8]. To determine whether the same mechanism is reducing LPS-induced oxidative injury in HSCs, we assessed Nrf-2 and Nrf-2-driven NQO-1, HO-1 and GCLC [20]. Using western blot, we found that LPS significantly down-regulated the nucleus Nrf-2 protein ex-

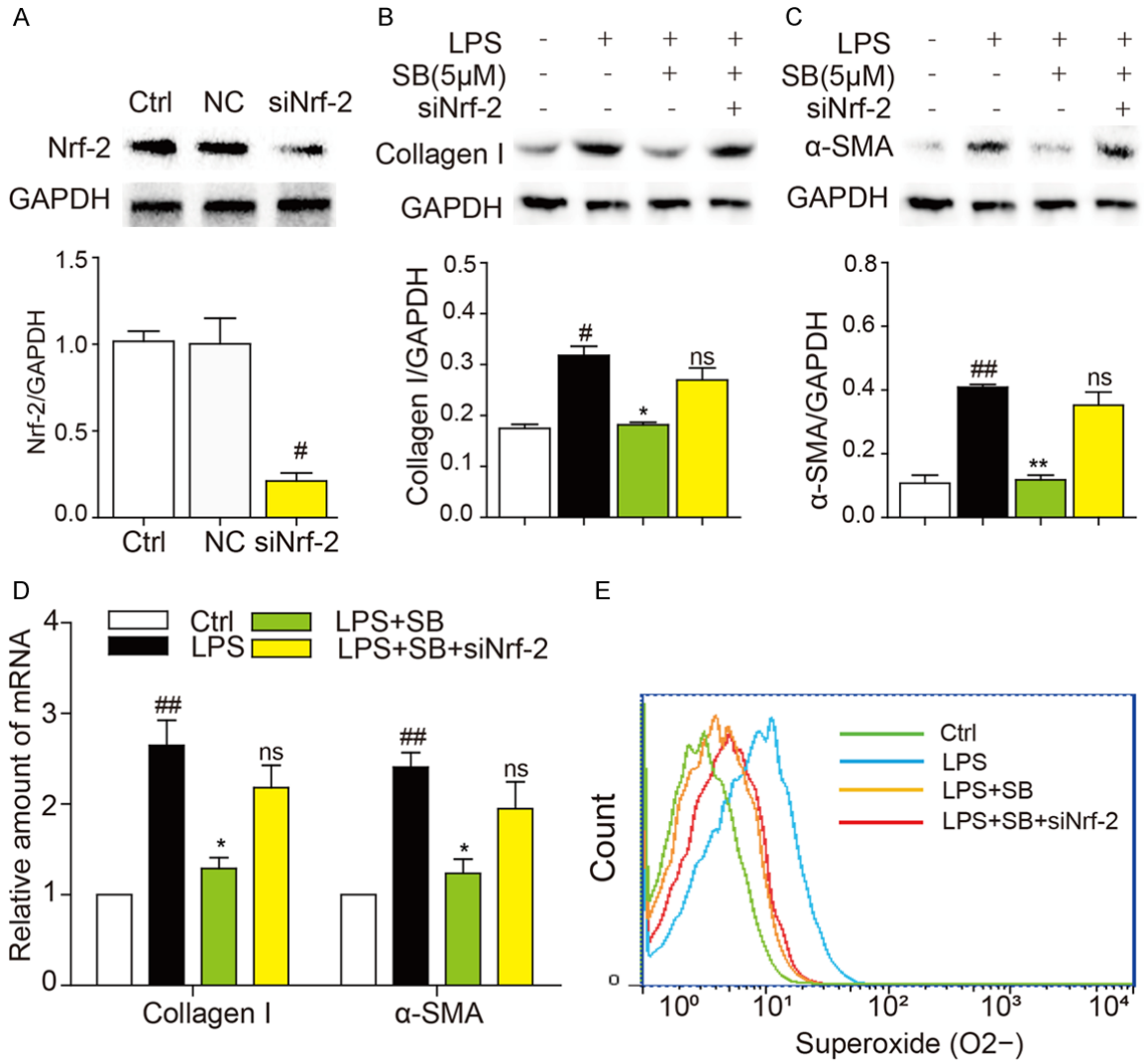

Figure 4. Protective effects of SB involve modulation of Nrf-2. (A) Western blot analysis of Nrf-2 following siRNA transfection in HSCs. HSCs were pretreated with SB (5 μM) for 1 h and then were incubated with LPS (100 ng/mL) for indicated time. (B, C) Exposed to LPS for 24 h. Western blot analysis detected the effect of Nrf-2 knockdown on LPS-induced Col-1 (B) and α-SMA (C) expression. (D) Exposed to LPS for 6 h. detected the effect of Nrf-2 knockdown on LPS-induced Col-1 and α-SMA mRNA level. (E) Exposed to LPS for 12 h. Using flow cytometry with a mean fluorescence intensity (MFI) value of each group. (*P<0.05, **P<0.01, vs. LPS group; ns = not significant vs. LPS group; #P<0.05, ##P<0.01, vs. Ctrl group).

pression and increased cytoplasm Nrf-2 DNA-binding activity in HSCs. Pretreatment of HSCs with SB significantly increased nucleus Nrf-2 protein expression and reduced Nrf-2 cytoplasm Nrf-2 protein expression (**Figure 3A**). Consistent with Nrf-2 activation, a western blot (**Figure 3B**) and RT-qPCR (**Figure 3C**) analysis showed that HO-1, NQO-1, and GCLC were all induced when HSCs were treated with SB (**Figure 3B** and **3C**). This antioxidant response by SB was associated with reduced perturbation of ROS generation in HSCs.

The protective effects of SB in LPS-induced HSCs activation involve modulation of Nrf-2

To investigate whether Nrf-2 pathway modulates the protective effects of SB in LPS-induced HSCs activation, we knocked down the expression of Nrf-2 (**Figure 4A**) prior to SB treatment. Compared with a scrambled vector, the transfection of cells with a specific siRNA reduced protein abundance by more than 70% (**Figure 4A**). SB treatment reduced Col-1 (**Figure 4B**) and α-SMA (**Figure 4C**) protein expression in

normalizing LPS-induced HSCs, while, SB was not able to inhibit these protein expressions in the Nrf-2-knockdown of HSCs (**Figure 4B**, **4C**), indicating that the anti-fibrosis action of SB is Nrf-2-dependent. These results were also verified by RT-qPCR for Col-1 and α-SMA (**Figure 4D**). Using flow cytometry, we also detected the ROS production in this system. Consistent with ECM expression, SB pretreatment could not reduce superoxide generation in Nrf-2-knockdown HSCs (**Figure 4E**). These findings strongly suggest the protective effects of SB in LPS-induced HSCs activation involve the modulation of Nrf-2.

Discussion

Activated hepatic stellate cells (HSCs) are well known for their potential role in increasing the deposition of ECM and elevating proliferation in liver fibrosis [4, 21]. During liver injuries, quiescent HSCs that store vitamin A in lipid droplets (LDs) and reside in the spaces of Disse convert to an activated phenotype and are depleted of vitamin A [4]. The activation process of quiescent HSCs can be driven by various stimuli, including lipopolysaccharide (LPS) [22]. LPS, the classic ligand for Toll-like receptor 4 (TLR4) [23], has been found to be associated with hepatic fibrogenesis through direct interactions with HSCs [22]. However, the molecular mechanisms underlying the effects of LPS on HSC activation are poorly understood.

Recently, it has been reported that SB plays an important role in liver diseases in response to extracellular stimuli, such as CCl4 and acetaminophen [11, 12]. Here in our study, we observed a similar effect in HSCs of LPS treatment. SB decreased the expression of the profibrosis genes and proteins Col-1 and α-SMA in the HSCs subjected to LPS (**Figure 1**). Oxidative stress is a well-known mechanism of LPS induced hepatic injury, and the redox imbalance produced may result in depletion of endogenous antioxidant proteins [24]. We found that SB treatment markedly attenuated LPS-induced HSCs oxidative stress (**Figure 2**).

Nrf-2 contributes to LPS-induced liver injury via the antioxidant response and other nuclear proteins, such as HO-1, NQO1 and GCLC to mount strong antioxidant and cytoprotective responses [25]. It was reported that SB reduces neurocytoma oxidative stress via the activation of the Nrf-2 pathway [26]. As observed in our study, the treatment of SB in HSCs significantly increased Nrf-2 activation, HO-1, NQO1 and GCLC expression (**Figure 3**), indicating that activation of Nrf-2 may be a therapeutic strategy for reducing oxidative stress. To validate the importance of Nrf-2, we used siRNA-silencing Nrf-2 knockdown in HSCs. We found that the expression of Col-1 and α-SMA and ROS production could not be reduced by SB in Nrf-2 knockdown HSCs (**Figure 4**), suggesting that augmenting the antioxidant defense system becomes necessary, especially during infections or periods of chronic oxidative insult.

Although these are promising findings, the mechanism of Nrf-2 induction by SB is enigmatic. The currently accepted mechanism of Nrf-2 regulation appears to be carried out primarily through post-translational mechanisms and involve the modulation of a binding protein called Keap 1 (Kelch-like Erythroid-cell-derived protein with CNC homology (ECH)-Associated Protein 1) [8, 24]. Whether SB alters keap1-Nrf-2 interaction opens up exciting possibilities for the treatment of liver fibrosis.

In summary, a natural compound, SB, specifically targets Nrf-2 and attenuates the oxidative stress in HSCs. SB also reduces LPS-induced fibrosis and cell viability in HSCs. These findings provide support for future research and the potential use of SB for the treatment of liver fibrosis. In addition, our data indicate Nrf-2 as a therapeutic target for infections or periods of chronic oxidative stress treatment and future drug discovery.

Acknowledgements

The project was supported by research grants from the Zhejiang Provincial Program of Chinese Medical and Health Science Funding (20-172A141), the Zhejiang Provincial Program of Medical and Health Science Funding (2017KY-679), the Zhuji City Natural Science Funding, and the Zhejiang Pharmaceutical Association Science Funding (2016ZYY30).

Disclosure of conflict of interest

None.

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