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

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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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A

B

C

D

E

F

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

<table>
<thead>
<tr>
<th>Gene</th>
<th>Species</th>
<th>FW</th>
<th>RW</th>
</tr>
</thead>
<tbody>
<tr>
<td>HO-1</td>
<td>Rat</td>
<td>TCTATCGTGTGCATCAGTAAC</td>
<td>CAGCTCTCAAAACAGCTCAA</td>
</tr>
<tr>
<td>NQO-1</td>
<td>Rat</td>
<td>ACCTGCTTCTCATACACCAC</td>
<td>CAAAGGCAGAAGCTAACAGG</td>
</tr>
<tr>
<td>GCLC</td>
<td>Rat</td>
<td>ATGCAGATTCTGACCTTACC</td>
<td>ACAACTCAGATTCCACTAC</td>
</tr>
<tr>
<td>Collagen 1</td>
<td>Rat</td>
<td>CGAGTATGGAAGCGGAGGTT</td>
<td>AGCGTGTTCTTGCAGTGATA</td>
</tr>
<tr>
<td>α-SMA</td>
<td>Rat</td>
<td>TGACCGAGATTGAGTTGAGG</td>
<td>AGATAGGGCACGTTGAGTGC</td>
</tr>
<tr>
<td>β-actin</td>
<td>Rat</td>
<td>AAGTCCTCTACACCTCCAAAAG</td>
<td>AAGCAATGCTGTACCCCTCCC</td>
</tr>
</tbody>
</table>

**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...
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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, hem 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 expression. However, SB treatment markedly attenuated LPS-induced oxidative stress. These findings are in alignment with recent reports of SB inhibiting ROS in activated LPS-challenged macrophages [19].
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expression 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 HSCs.

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).
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

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Disclosure of conflict of interest

None.

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