Glucocorticoids alleviates lipopolysaccharide-induced acute liver injury associated with promoting the expression of NTCP and BSEP

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Abstract: Background: Methylprednisolone (MP) is a glucocorticoid steroid administered usually in severe sepsis and septic shock. However, the role and molecular mechanisms underlying hepatoprotective effect of MP in sepsis-induced acute liver injury (ALI) is largely unknown. The aim of the present study was to investigate the effect of MP in a mouse model of lipopolysaccharides (LPS)-induced ALI. Methods: A total of 96 male C57BL/6J mice were randomly divided into four groups (n=24 per group) according to the treatment including vehicle (control group) or LPS only (LPS group) or LPS with 2 mg/kg MP (LPS+2 mg/kg MP group) or LPS with 20 mg/kg MP (LPS+20 mg/kg MP group) three times at 1 h, 24 h, 48 h after LPS treatment. The livers and serum were collected for further analysis. Serum levels of total bilirubin (TBIL), total bile acid (TBA), alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured at 24 h, 48 h or 72 h after LPS administration. Histological examinations were performed on liver, and liver sections were stained with hematoxylin & eosin (HE). Furthermore, the expression of Interleukin-6 (IL-6), Tumour Necrosis Factor-α (TNF-α), sodium-taurocholate co-transporting polypeptide (NTCP) and bile salt export pump (BSEP) in liver was analyzed by reverse transcription quantitative polymerase chain reaction (RT-qPCR) and western blot. Results: MP therapy protects mice against LPS-induced ALI at the dose of 2 mg/kg and 20 mg/kg. The serum TBIL, TBA, ALT, AST levels were significantly decreased in LPS+2 mg/kg MP group and LPS+20 mg/kg MP group compared with LPS group. However, there is no significant difference between the LPS+2 mg/kg MP group and the LPS+20 mg/kg MP group. Furthermore, LPS administration suppresses the expression of NTCP and BSEP, which is significantly reversed by MP therapy. Moreover, LPS-induced higher expression of IL-6 and TNF-α was significantly suppressed by MP administration. Conclusion: MP protects mice against LPS-induced ALI and improves bile acid secretion and transportation, partially due to the upregulated expression of NTCP and BSEP.

Keywords: Methylprednisolone, sepsis, liver injury, NTCP, BSEP, mouse

Introduction

Sepsis is defined as life-threatening organ dysfunction caused by a dysregulated host response to infection [1]. Although the clinical management of sepsis has made important progress, sepsis mortality rate remains high. Liver is a vulnerable organ in sepsis. Liver dysfunction is associated with poor prognosis in patients with sepsis [2, 3]. Sepsis-induced acute liver injury (ALI) is characterized by hyperbilirubinemia [4]. Conjugated hyperbilirubinemia reflects intrahepatic cholestasis [5]. Thus, improving cholestasis contributes to improvement in the prognosis of patients with sepsis-induced ALI.

Bile acid metabolism is closely related to cholestasis-associated liver injury. Sodium-taurocholate co-transporting polypeptide (NTCP) and bile salt export pump (BSEP) are two key transporters for hepatic bile acid uptake and excretion. The expression of Ntcp and Bsep are markedly downregulated in livers of lipopolysaccharide (LPS)-treated animal and human liver slices [6-8] as well as in inflammation-induced icteric cholestasis [9]. LPS induces higher levels of tumor necrosis factor (TNF)-α and interleukin (IL)-1β [10], as well as hepatic damage in rats [11]. Furthermore, TNF-α and IL-1β suppress the expression of Ntcp and Bsep in mice [12, 13]. All these studies imply that regulation of bile acid metabolism and inflam-
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Severe sepsis may result in decreased production of cortisol and glucocorticoid tissue resistance. So, exogenous glucocorticoids (GCs) are commonly used to treat severe inflammatory diseases, including sepsis [14]. GCs interact with a specific cytosolic glucocorticoid receptor to regulate downstream signaling pathway, which act as lifeguards to suppress IL-12 production and eliminate CD8+ dendritic cells during the hyperinflammatory phase of sepsis [15]. Moreover, exogenous GCs can inhibit myocar dial cell apoptosis by blocking the activation of Nuclear factor-κB (NF-κB), decreasing the generation of proinflammatory cytokines, and relieving inflammatory injury in heart tissues in a murine model of sepsis [16]. All these results suggest that GCs plays a key role in protection of organ injury from sepsis. Methylprednisolone (MP), a commonly used glucocorticoids, regulates the expression of Na-bile acid cotransport to regulate the bile acid metabolic homeostasis in normal and chronically inflamed rabbit ileal villus cells [17]. Given that bile acid metabolic homeostasis is critical to sepsis-induced ALI, we hypothesized that MP might protect mice against LPS-induced ALI, possibly related to bile acid metabolism in liver. In the present study, we investigated the hepatoprotective effect of MP on LPS-induced ALI in mice and explored the underlying molecular mechanisms in vivo.

Materials and methods

Chemicals and reagents

Lipopolysaccharide (LPS, E. coli 0111: B4) was purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Methylprednisolone (MP) was purchased from Pfizer. Serum total bilirubin (TBIL), total bile acid (TBA), alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were analyzed using commercial kits according to the manufacturer's protocols (Rsbio, Shanghai, China). Antibodies to BS-EP (Anti-ABCB11, ab112494) and NTCP (Anti-SLC10A1, ab97947) were purchased from Abcam. Immobilon Western chemiluminescent HRP substrate was purchased from Millipore (Massachusetts, USA).

Animals and drug treatments

Male C57BL/6J mice (8-week old) were purchased from the Shanghai Sippr-bk Laboratory Animals Co. Ltd. (Shanghai, China). Mice were maintained on standard laboratory mice chow in a 12-h light-dark cycle and access to dry pellets and sterile water ad libitum. The care and use of the mice were conducted according to a protocol that was approved by the ethics committee of the Children's Hospital Affiliated to Shanghai Jiao Tong University (Shanghai, China).

A total of 96 male C57BL/6J mice were randomly divided into four groups (n=24 per group) including NS group, LPS group, LPS+2 mg/kg MP group and LPS+20 mg/kg MP group depending on whether the mice received LPS or MP treatment. The mice were treated with vehicle (0.9% saline) (NS group, n=24) or LPS (10 mg/kg) (LPS group, n=72) by intraperitoneal injection. In order to test whether MP protected against LPS-induced liver injury in a mouse model, the LPS-induced mice were randomly assigned to receive an intraperitoneal injection of 0.9% saline (LPS group, n=24) or 2 mg/kg MP (LPS+2 mg/kg MP group, n=24) or 20 mg/kg MP (LPS+20 mg/kg MP group, n=24) three times at 1 h, 24 h, 48 h after LPS treatment. Serum and liver samples were collected at 24 h, 48 h and 72 h after LPS administration for further analysis.
Table 1. Primers for Ntcp, Bsep, Il-6, Tnf-α and Gapdh in RT-qPCR used in this work

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence (5’ to 3’)</th>
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<tbody>
<tr>
<td>Ntcp</td>
<td>F: TGGCTACCTCCTCCCTGAT</td>
</tr>
<tr>
<td></td>
<td>R: ATGCTGATGGTGGCTGCTG</td>
</tr>
<tr>
<td>Bsep</td>
<td>F: CCTCATACGGAACCCCAAGA</td>
</tr>
<tr>
<td></td>
<td>R: TGACTGGTAGACGGATGAG</td>
</tr>
<tr>
<td>Il-6</td>
<td>F: GATACCACTCCCAACGAC</td>
</tr>
<tr>
<td></td>
<td>R: CTTTCTTCATTCCACGAT</td>
</tr>
<tr>
<td>Tnf-α</td>
<td>F: CCCAGCTCTGTATCCTCTCTA</td>
</tr>
<tr>
<td></td>
<td>R: CCCAGCATCTTGTTTCTTCT</td>
</tr>
<tr>
<td>Gapdh</td>
<td>F: GGTTGCTCTGGCGACTTCA</td>
</tr>
<tr>
<td></td>
<td>R: TGGTCCAGGTTTCTACTCC</td>
</tr>
</tbody>
</table>


kg MP (LPS+20 mg/kg MP group, n=24) three times at 1 h, 24 h, 48 h after LPS treatment (Figure 1). To evaluate the effect of MP treatment on the expression of NTCP and BSEP under normal conditions, C57BL/6J mice were treated with MP at the dose of 20 mg/kg for 24 h, and the livers were collected for further study (n=5). The mice were anesthetized using pentobarbital (0.2 mg/kg) by intraperitoneal injection at 24 h, 48 h and 72 h after LPS administration (n=8 at each time point). Blood was collected by enucleation of eyeball and centrifuged at 3000 rpm for 10 minutes to separate serum for further analysis. Liver tissues were collected after in vivo heart perfusion with saline and were snap-frozen for further RT-qPCR or western blot analysis. The serum and liver samples were kept at -80°C for further analysis. In addition, liver tissues were also treated with 4% paraformaldehyde for histological analysis.

Histopathology

Liver samples were fixed in 4% paraformaldehyde overnight at 4°C, embedded in paraffin and sectioned at 4-μm thickness. Tissue sections were stained with hematoxylin and eosin (H&E) as previously described [19]. Then, the histopathological examination was performed under light microscopy.

Reverse transcription quantitative polymerase chain reaction (RT-qPCR)

Total RNA was isolated from livers using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer’s instructions. One microgram total RNA was reverse-transcribed to cDNA with random hexamer primer and M-MLV Reverse Transcriptase (Takara, Japan). Quantitative amplification by PCR was carried out using SYBR Green I Master Mix reagent by ABI 7500 system (Applied Biosystem, Foster, CA, USA). Primers of Ntcp, Bsep, Il-6, Tnf-α used for amplification are shown in Table 1. Amplification conditions were as follows: 95°C for 10 min, followed by 45 cycles of 10 sec at 95°C, 15 sec at 60°C, 15 sec at 72°C. Gapdh was used as an internal control for each gene of interests. Samples for each experimental condition were run in triplicate.

Western blotting analysis

Whole-cell lysates from frozen liver tissues were isolated using mammalian protein extraction reagent (Thermo SCIENTIFIC) and centrifuged at 12,000×g for 15 min at 4°C. The total protein concentration was measured using a Bradford Assay Kit (Bio-Rad, CA, USA). Then, the supernatant was mixed with isometric sodium dodecyl sulphate (SDS) buffer (125 mM Tris hydrochloride (pH 6.8), 10% mercaptoethanol (vol/vol), 4% SDS (wt/vol), 20% glycerol (vol/vol), and 0.002% bromophenol blue). The mixture was heated for 10 min at 100°C. Supernatants were subjected to 10% SDS-PAGE gels and blotted by wet blotting. Primary antibodies including anti-NTCP and anti-BSEP were incubated overnight at 4°C. After washing three times, horseradish peroxidase-conjugated secondary antibodies were incubated for 2 h at room temperature and visualized with Immobilon Western chemiluminescent HRP substrate. Band intensities were measured using the ImageJ® image processing program (National Institutes of Health, Bethesda, USA) and normalized to β-Tubulin. Images were taken using a C-Digit chemiluminescent Western blot scanner (LI-COR, Lincoln, USA).

Statistical analysis

All values in the figures and tables are expressed as means ± standard deviations (SD) values. Quantitative data were analyzed using the one way analysis of variance (ANOVA). The least significant difference (LSD) test was used for post hoc testing with SPSS 23.0 software (SPSS Inc, Chicago, IL, USA). Two-sided P values less than 0.05 were considered statistically significant.
Results

Methylprednisolone (MP) significantly improved LPS-induced cholestasis and liver injury in mice

Biochemical indicators were determined at 24 h, 48 h and 72 h after LPS administration with or without 2 or 20 mg/kg of MP therapy in mice. The serum TBIL and TBA levels, which are biochemical indicators of cholestasis, were significantly increased in LPS-induced mice, which were significantly reduced by MP treatment at either the 2 mg/kg or 20 mg/kg dose (Figure 2A, 2B). In addition, the serum ALT and AST levels, which are biochemical indicators of liver injury, were also significantly increased in LPS-induced mice, which were significantly reduced by MP treatment at either the 2 mg/kg or 20 mg/kg dose (Figure 2C, 2D).

Histological analysis provided direct evidence of the hepatoprotective effect of MP in LPS-induced acute liver injury

To evaluate the effect of MP on LPS-induced histological changes in liver by administration for 24 h, liver sections were stained with H&E and examined for bile duct proliferation and inflammatory cell infiltration. As shown in Figure 3A, the control group exhibited normal structures of liver tissue. By contrast, the liver in mice of LPS group was characteristic of liver injury, such as hepatic inflammation involves activation of sinusoidal endothelial cells, margination and migration of leukocytes. Moreover, focal necrosis, inflammatory cells infiltration and ductal proliferation and expansion were observed in mice treated with LPS for 24 h (Figure 3B). Interestingly, these changes were all reversed by MP treatment at either the 2 mg/kg or 20 mg/kg dose (Figure 3C, 3D).

MP stimulated the expression of NCTP and BSEP in liver of mice treated with LPS

Both NTCP and BSEP were suppressed at the mRNA and protein level following LPS administration (Figure 4A-C). As shown in Figure 4A, the Ntcp and Bsep mRNA levels were significantly decreased at 24 h after LPS administration, which were reversed by MP treatment at both 2 mg/kg and 20 mg/kg dose. It is noteworthy that 20 mg/kg MP treatment displayed significantly stronger effect on reversing the LPS-suppressed Ntcp and Bsep mRNA levels compared with 2 mg/kg MP administration. Furthermore, western blot assays indicated that LPS administration suppressed the protein levels of NTCP and BSEP in liver, which were significantly reversed by MP administration. In addition, NTCP, but not BSEP, displayed more
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Figure 3. Effects of methylprednisolone (MP) treatment on liver histopathology (200×) at 24 h after saline or LPS. Liver sections were stained with hematoxylin and eosin in control group (A), LPS-administration group (B), MP treatment at the dose of 2 mg/kg (C) and MP treatment at the dose of 20 mg/kg (D). (A) Normal control liver section showing normal arrangement of sinusoids and hepatocytes. (B) The LPS group liver section showing disorder of focal necrosis (yellow triangle), inflammatory cells infiltration (thin arrow), ductal proliferation and expansion (thick arrow). (C and D) 2 mg/kg (C) and 20 mg/kg (D) MP-treated liver section showing that the severe signs of liver damage induced by LPS are significantly attenuated.

Figure 4. Effects of methylprednisolone (MP) treatment on the expression of NTCP and BSEP in the livers of mice treated with LPS after 24 h. A: Relative mRNA levels of Ntcp and Bsep were detected by Reverse transcription quantitative polymerase chain reaction (RT-qPCR), normalized by Gadph. B: The protein levels of NTCP and BSEP were detected by western blot. C: The quantification analysis indicated that the expression of NTCP and BSEP were suppressed significantly by LPS, which were reversed by MP treatment. Data are presented as the mean ± SD (n=8). *indicates $P < 0.05$ as compared with the LPS administration (LPS) group. † indicates $P < 0.05$ between the LPS+2 mg/kg MP group and the LPS+20 mg/kg MP group.
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To assess the effects of MP on the expression of the inflammatory cytokines, the real-time PCR was performed to detect the IL-6 and TNF-α mRNA levels in livers. As shown in Figure 6, the mRNA levels of IL-6 and TNF-α were significantly up-regulated in the LPS group compared with control group. Moreover, LPS-induced IL-6 and TNF-α expression were significantly suppressed by MP treatment in the livers of mice (Figure 6A, 6B). Furthermore, the mRNA level of IL-6 (Figure 6A), but not TNF-α (Figure 6B), displayed more sensitive to 20 mg/kg MP treatment than that of 2 mg/kg MP treatment.

Discussion

Though GCs are administered usually in severe sepsis and septic shock, it is almost unknown its role and underlying molecular mechanisms in sepsis-induced ALI. In the present study, we indicated that MP treatment attenuated LPS-induced ALI and improved serum TBIL, TBA, ALT.
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and AST levels. Furthermore, the hepatoprotective effect of MP was associated with increased expression of the bile acid transporters NTCP and BSEP, as well as decreased IL-6 and TNF-α mRNA levels in the livers. These results suggested MP might improve LPS-induced ALI partially related to the regulation of bile acid transportation.

Corticosteroid therapy is frequently used in septic shock, but it need further study about the appropriate dose and the timing of therapy, as well as the underlying mechanisms involved in the process [18]. A short course of high-dose corticosteroid as an anti-inflammatory regimen was proved no evidence of a survival benefit [19, 20]. MP (30 mg/kg) by intravenous infusion every 6 hours for four doses could not improve occurrence rate of shock and recovery from shock, mortality rates and serum concentrations of creatinine and AST compared with placebo, but blood urea nitrogen was increased from baseline values significantly [21]. In contrast, more recent trials demonstrated that a longer course (≥ 5 days) of low-dose corticosteroid resulted in shock reversal [22] and improved mortality rate [23, 24]. Until now, the well-designed large randomized clinical trials are needed to give evidence to support the right dose used in sepsis [25].

Clinical evidence has shown that hepatic dysfunction is an early event in sepsis and is a specific risk factor for poor outcome [26]. There is no evidence to evaluate the effect of MP on LPS-induced liver injury at different doses in mice. In present study, we found that the serum levels of TBIL, TBA, ALT and AST had no difference in mice between 2 mg/kg/day MP group and 20 mg/kg/day MP group, which suggested that high-dose of MP did not bring more benefit for LPS-induced ALI. Consistently, low-dose MP (0.5 mg/kg/day) combined with antibiotic therapy is the optimal in the treatment of rats with fecal suspension-induced sepsis in rat [27]. In contrast, 10 mg of methylprednisolone sodium succinate (mPSL) administration (about 50 mg/kg/day) at 2 h after LPS and D-galactosamine (GalN) challenges in rat by tail vein or portal vein significantly increased the survival rate and reduced the serum values of ALT and apoptosis in liver [28]. In addition, slow-release MP mini-osmotic pumps with a calculated release of 20 mg/kg/day were implanted subcutaneously for 7 days to improve the bile acid homeostasis in bile duct ligation rat [29]. All these results indicated that MP therapy regulates bile acid homeostasis and plays a key role in hepatoprotective effect, but the accurate dose and the way of administration of MP therapy for sepsis-induced ALI need further study in clinical trials.

Sepsis induces cholestasis [4]. Recently, it is deemed that an adaptively altered bile acid production and transport back towards the systemic circulation would be beneficial for survival of patient with sepsis [30]. MP treatment regulates bile acid homeostasis by increasing the expression of NTCP and decreasing the expression of CYP7A1 [29]. In present study, MP administration decreased serum levels of TBIL and TBA at the dose of 2 mg/kg/day at first 24 h. Furthermore, the expression of both NTCP and BSEP were upregulated by MP treatment in the livers of LPS-induced sepsis mice. It is worth noting that the expression of BSEP was significantly increased in livers of LPS-induced mice treated with MP at the dose of 2 mg/kg, which was not further increased in livers of mice treated with MP at the dose of 20 mg/kg; but the expression of NTCP was significantly higher in livers of LPS-induced mice treated with MP at the dose of 20 mg/kg compared with 2 mg/kg. Furthermore, there was no change about the expression of NTCP and BSEP in response to MP treatment under normal conditions. Given that NTCP contributes to the uptake of bile acids into hepatocytes and BSEP promotes bile acids efflux from hepatocytes [31], we speculated that MP-stimulated the expression of NTCP and BSEP possibly through the suppression of IL-6 and TNF-α, which need further study. Furthermore, hepatic glucocorticoid receptor (GR) deficiency impaired liver bile acid uptake/transport via lower expression of NTCP

Endotoxin activates kupffer cells, which in turn secrete pro-inflammatory cytokines such as TNF-α, IL-1β, IL-6 and nitric oxide [33]. In present study, MP administration decreased the mRNA levels of IL-6 and TNF-α. Given that cecal ligation and puncture decreases expression of NTCP in an IL-6-dependent manner [34], we speculated that MP-stimulated the expression of NTCP and BSEP possibly through the suppression of IL-6 and TNF-α, which need further study. Furthermore, hepatic glucocorticoid receptor (GR) deficiency impaired liver bile acid uptake/transport via lower expression of NTCP
[34]. During sepsis, early cytokine activity activated or repressed the signal transducer and activator of transcription (STAT) 3, nuclear factor (NF)-κB, and hepatocyte nuclear factor (HNF) 1α [35-37]. Whether GR, STAT3, NF-κB and HNF1α are involved in the process of MP-mediated regulation of NTCP and BSEP expression need further study.

In conclusion, MP administration alleviated LPS-induced acute liver injury at the dose of 2 mg/kg/day associated with upregulating the expression of NTCP and BSEP and suppressing the expression of TNF-α and IL-6 in livers. Though it is still to further explore the molecular mechanisms underlying MP involved in the regulation of bile acid homeostasis under the condition of sepsis, these results suggested that MP therapy might play a critical role in stimulating liver bile acid excretion and transportation resulting in improvement of sepsis-induced liver injury.

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Disclosure of conflict of interest

None.

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