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Paeoniflorin protects against concanavalin A-induced hepatitis in mice ☆

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ABSTRACT

Paeoniflorin (PF) is one of the main effective components of the total glucosides of peony, which has been reported to have anti-inflammatory ability. However, the effects of paeoniflorin on concanavalin A (Con A)-induced hepatitis have not been carefully examined. The aim of this study was to investigate the protective effect of paeoniflorin and elucidate potential mechanisms of paeoniflorin on Con A-induced hepatitis. C57BL/6 mice were divided randomly into the following four experimental groups: PBS group, PF group, Con A group, and Con A + PF group. Mice received paeoniflorin (50 mg/kg) by tail vein before Con A intravenous administration. We found that paeoniflorin pretreatment can significantly reduce the elevated plasma aminotransferase levels and liver necrosis in Con A-induced hepatitis. Also, paeoniflorin pretreatment suppressed the secretion of proinflammatory cytokines (TNF-α, INF-γ, IL-6), compared with Con A group. Meanwhile, paeoniflorin pretreatment decreased $CD4^+$, $CD8^+$ and NKT cell infiltration in the liver. Besides, we observed that paeoniflorin pretreatment can decrease the expression level of Toll-like receptor (TLR) 4 mRNA or protein in liver tissues. Further results showed that paeoniflorin pretreatment was capable of suppressing the activation of the NF-κB pathway by inhibiting IκBα kinase and p65 phosphorylation in Con A-induced liver injury. These results suggest that paeoniflorin pretreatment protects mice against Con A-induced liver injury via inhibition of several inflammatory mediators and, at least in part, by suppressing $CD4^+$, $CD8^+$ and NKT cell infiltration in liver. The beneficial effect of paeoniflorin may be related to the downregulation of TLR4 expression and the inhibition of NF-κB activation.

1. Introduction

Liver diseases are still a major public health problem worldwide because of significant morbidity and mortality. The most common causes of liver damage in humans are infections with hepatitis B or C viruses, and autoimmune hepatitis [1–3]. It was estimated that one percent of people infected with hepatitis viruses develop fulminant hepatitis without intervention [4]. Concanavalin A-induced hepatitis in mice is a well established model that closely mimics the pathogenic mechanisms and pathological changes with viral and autoimmune hepatitis in human. In this model, inflammatory cells, such as T cells [5–7], and a variety of hepatotoxic cytokines, i.e., tumor necrosis factor (TNF)-α, interferon (IFN)-γ and interleukin (IL)-6, play a key role in hepatocyte damage [8–11].

Previous studies indicated that many therapeutic agents that abrogate liver injury might transect with the Toll-like receptor (TLR) 4 signaling pathway [12–14]. Moreover, some studies showed that TLR4 was critically involved in the pathogenesis of Con A-induced liver damage [15–17]. It has been reported that Con A also upregulated NF-κB expression in liver [18], and there was increasing evidence shown that Con A-induced liver injury was significantly attenuated via inhibiting NF-κB activation [19–21]. Therefore, inhibition of the expression of TLR4 and suppressing the activation of NF-κB pathway may well represent therapeutic targets for T cell-mediated hepatitis.

Paeoniflorin (PF) is a monoterpene glycoside isolated from the roots of *Paeonia lactiflora* Pall. which have been used for more than 1500 years in traditional Chinese medicine. Previous investigations of paeoniflorin exhibited many pharmacological effects such as anti-inflammation [22], anti-hyperglycemia [23], and neuroprotective effects [24]. Moreover, paeoniflorin has been proven to be effective in the treatment of many diseases in animal models, such as collagen-induced arthritis and acute lung injury [25,26]. Jiang et al. demonstrated that paeoniflorin inhibited systemic inflammation and improved survival in experimental sepsis via inhibiting the NF-κB activation [27]. But to our knowledge,

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it is unclear that whether paeoniflorin also provides a protective effect on Con A-induced hepatitis in mice.

Based on these studies, it is tempting to speculate that paeoniflorin might play an important role in alleviation of Con A-induced hepatitis. Therefore, the aim of this study was to investigate the effect of paeoniflorin and elucidate potential mechanisms of paeoniflorin on Con A-induced hepatitis.

2. Materials and methods

2.1. Reagents and mice

Paeoniflorin (CAS Number: 23180-57-6, purity >98%, molecular formula: $C_{23}H_{28}O_{11}$, molecular weight: 480.46) was purchased from Sigma-Aldrich Corporation. Con A was provided by Solarbio Corporation (Beijing, China). All of the other chemicals and reagents were standard commercially available biochemical quality. Deionized water was purified with a Milli-Q purification system and was used to prepare all solutions.

Six- to eight-week-old male C57BL/6 mice weighing between 20 and 25 g were obtained from the animal center of the Animal Experimentation Center of No. 113 Hospital of People's Liberation Army (Ningbo, China). The animals were kept in an environmentally controlled room (23 ± 2 °C, 55 ± 10% humidity) with a 12-h light and -dark cycle and allowed free access to food and water. All experiments were performed in accordance with the guidelines of Institutional Animal Ethics Committee of No. 113 Hospital of People's Liberation Army (Permit Number: 20120919-56).

2.2. Animal treatment

Paeoniflorin and Con A were dissolved in pathogen-free phosphate-buffered saline (PBS). Mice were administrated intravenously with paeoniflorin (50 mg/kg, 100 μl) or PBS (100 μl) as a control. The mice were injected intravenously with Con A (15 mg/kg, 100 μl). This dosage was proved effective by previous studies in mice [28].

Mice were randomly divided into four groups: PBS group, PF group, Con A group, and Con A + PF group. In PBS group mice, only the carrier solution (PBS) was injected. PF group mice were administrated paeoniflorin, without Con A treatment. Con A group mice were injected with Con A, without paeoniflorin treatment. Con A + PF group was a treatment group, in which mice were injected with paeoniflorin 3 h prior to Con A administration. The blood and liver tissue were harvested 12 h after Con A administration.

2.3. Measurement of serum aminotransferase and cytokine secretion

The blood was obtained 12 h after Con A administration. The blood was collected by heart puncture after sevoflurane anesthesia. Plasma was separated after centrifugation at 300 ×*g* for 5 min. Activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured spectrophotometrically using an automatic biochemical analyzer (Hitachi Auto Analyzer 7170, Japan). The concentrations of TNF-α, IFN-γ and IL-6 were detected by using enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's instructions (R&D system, USA). The thresholds of ELISA for TNF-α, IFN-γ and IL-6 were 0.36–7.21 pg/ml, 12.5–400 pg/ml, and 1.3–1.8 pg/ml, respectively.

2.4. Histopathological analysis

Liver samples were harvested 12 h after Con A administration. Tissue sections (thickness, 5 μm) were stained with hematoxylin and eosin (H&E) and were examined for liver damage by light microscopy. All sections were graded blindly by three pathologists under light microscopy according to the following criteria: 0, none; 1, individual cell necrosis; 2, ≤30% lobular necrosis; 3, ≤60% lobular necrosis; 4, >60% lobular necrosis [29].

2.5. Liver mononuclear cell preparation

Liver samples were harvested 12 h after Con A administration. In brief, liver tissues were infiltrated in collagenase type II (Life Technologies, USA) for 30 min. After flushing the liver with cold PBS, the livers were crushed through a stainless mesh (size 60, Sigma, St. Louis, MO) and suspended in RPMI 1640 medium (Gibco, BRL). Cell suspensions in PBS were centrifuged at 500 *g* for 5 min for removal of debris and placed through a nylon mesh presoaked in PBS. The supernatants containing hepatic mononuclear cells (MNCs) were collected and washed once with PBS, and then the cells were re-suspended in 40% Percoll (Sigma). The cell suspension was gently overlaid on top of 70% Percoll and centrifuged for 30 min at 800 *g*. MNCs were collected from the interphase, and washed twice in PBS. Approximately 1×10^7 cells/mouse liver was recovered.

2.6. Isolation of total RNA and detection of mRNA by reverse transcription

Liver samples were collected 12 h after Con A administration. Total RNA was isolated from the homogenate of the liver with Trizol reagent according to the protocol provided by the manufacturer. cDNA was synthesized from 2 μg of total RNA using PrimeScript™1st Strand cDNA Synthesis Kit (TaKaRa Biotechnology, China). The murine primer sequences are shown as follows:

TNF-α (forward, F) 5′-GGGCTACAGGCTTGTCACTCG-3′ and (reverse, R) 5′-ACTCCAGGCGGTGCCTATGTC-3′,

IFN-γ (F) 5′-CCTCAAACTTGGCAATACTCA-3′ and (R) 5′-CTCAAGTG GCATAGATGTGGA-3′,
IL-6 (F) 5′-AGTTGCCTTCTTGGGACTGA-3′ and (R) 5′-TCCACGATTTCC CAGAGAAC-3′,
TLR4 (F) 5′-GGTGTGAAATTGAGACAATTGAAAAC-3′ and (R) 5′-GTTT CCTGTCAGTACCAAGGTTGA-3′,
NF-κB (F) 5′-AGAAGGCTGGGGTCAATCTT-3′ and (R) 5′-CTCAGGCT TTGTAGCCAAGG-3′,
GAPDH (F) 5′-AGAGTGGGAGTTGCTGTTG-3′ and (R) 5′-GCCTTCCG TGTTCCTACC-3′.

Total RNA was treated with DNase I to eliminate genomic DNA contamination, followed by synthesis of the first-strand using reverse transcription system. Reverse transcription was carried out as follows: 42 °C for 60 min, 70 °C for 10 min and first-strand cDNA was stored at −20 °C. Real-time PCR was performed in a 20 μl of reaction solution containing SYBR Premix Ex Taq, primers, and cDNAs. The cycles for PCR were as follows: 95 °C for 2 min, 40 cycles of 95 °C for 15 s, 58 °C for 20 s, and 72 °C for 20 s. Melting curves were determined by heat-denaturing PCR products over a 35 °C temperature gradient at 0.5 °C/s from 65 to 99.5 °C. GAPDH was used as an internal control [30]. The relative amount of mRNA was determined using the ΔΔCT technique as described previously [31]. The levels of mRNA were expressed as fold changes after normalization to GAPDH.

2.7. Western-blotting analysis of NF-κB and TLR4

Livers were carefully excised and homogenized into lysis buffer (Thermo, USA) to yield a homogenate. After centrifugation, protein concentration was determined by BCA protein assay kit (Thermo, USA) with bovine serum albumin as a standard. Equal amounts of protein extracts separated discontinuously on 10% SDS-PAGE and transferred onto PVDF membranes. After blockade of nonspecific binding sites, membranes were incubated for 2 h at room temperature with various antibodies against IκBα, phospho-IκBα, NF-κB p65, phospho-NF-κB p65