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Effects of taurocholic acid on immunoregulation in mice

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ABSTRACT

Context: Currently, there is a dramatically growing interest in Chinese traditional medicines, especially in the therapy of inflammatory diseases. Taurocholic acid (TCA), as a kind of natural bioactive substance of animal bile acid, has medicinal applications to treat a wide range of inflammatory diseases.
Objective: The study was designed to evaluate the effects of TCA on cytokine secretion, such as TNF-α and IL-1β and on the ratio of $CD4^+/CD8^+$, which is beneficial for understanding the mechanism of TCA on immunoregulation preliminarily, and also will benefit our further research.
Materials and methods: The gene and protein expressions of TNF-α and IL-1β were measured by real time RT-PCR and ELISA in serum, spleen and lymphocytes respectively. The ratio of $CD4^+/CD8^+$ in peripheral blood and lymphocytes was measured by flow cytometry.
Results: Our present study has shown that lipopolysaccharide (LPS) and cyclosporin A (CsA) could increase or decrease the gene and protein expressions of TNF-α and IL-1β respectively. TCA (0.25 g/kg, 0.125 g/kg) could recover the suppressed expressions of TNF-α and IL-1β and increase the ratio of $CD4^+/CD8^+$. In vitro, TCA (15 μg/mL) could inhibit the increased production of TNF-α and IL-1β; TCA (0.15 μg/mL–15 μg/mL) could inhibit the increased gene expressions of IL-1β and TNF-α. TCA (0.15 μg/mL) could recover the suppressed expressions of TNF-α and IL-1β.
Conclusion: The function of immunoregulation of TCA may be accomplished through modulating the gene and protein expressions of TNF-α and IL-1β and elevating $CD4^+/CD8^+$ T-cell ratio.

1. Introduction

Bile acids, which are generated by the oxidation of cholesterol, are synthesized in the liver and stored in the gallbladder as the main constituent of bile. Bile is predominantly composed of cholesterol, phospholipids, bilirubin and bile acids. Chenodeoxycholic acid (CDCA) and cholic acid (CA) which are mainly conjugated to glycine (G) and taurine (T) and are called glycocholic acid (GCA) and taurocholic acid (TCA), are the two primary bile acids in humans [1]. TCA is one of the principal bioactive substances of animal bile.

It is clear that bile acids do not only facilitate digestion, absorption, and excretion of dietary lipids, but also are involved in numerous cellular signaling pathways [2–5]. It was proved that bile acids had functions in the regulation of cellular ions, cAMP levels, and cellular kinases [3,4], and especially, chemically synthesized TCA had marked bioactive effects such as an inhibitory potential against hepatic artery ligation induced biliary damage by upregulation of VEGF-A expression [6]. Animal bile has been widely used virtually for treatment of acute tracheitis, winter cough, pneumonia and whooping cough [7] because of its favorable anti-inflammatory and immunoregulatory actions, however, its mechanism of anti-inflammatory and immunoregulatory was not clear.

Based on the feature of TCA, we aimed to examine whether the regulatory effects on immune functions of TCA were relative with the secretion of cytokines such as tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β). The present study was undertaken to determine the immunoregulatory property of TCA in mice. We investigated the effects of TCA on the production of TNF-α, IL-1β in serum and lymphocytes, and gene expression in spleen and lymphocytes of mice. We also investigated the effect of TCA on the ratio of $CD4^+/CD8^+$ in peripheral blood and lymphocytes. These studies will help in understanding the mechanism of TCA on immunoregulation preliminarily, which will be the basis for our further research.

2. Materials and methods

2.1. Reagents

Levamisole was purchased from North China Pharmaceutical Group Corporation (Shijiazhuang, China). Cyclosporin A (CsA) was purchased

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from Bio Basic (Markham Ontario, Canada). Lipopolysaccharide (LPS) was purchased from Sigma (St. Louis, MO, USA). Lymphocyte separation medium for mice and fetal bovine serum (FBS) were purchased from TBD (Tianjin, China). ELISA kits for TNF-α and IL-1β were purchased from Neobioscience Technology Co., Ltd (Beijing, China). Tripre™ RNA regent, M-MulV reverse transcripts and SYBR® Premix Ex Taq™ were purchased from TaKaRa (Dalian, China).

2.2. TCA dissociated and depurated

Fresh bovine and/or sheep galls were collected from a slaughterhouse. The bile was deproteinated using alcohol after filtered by filter paper, and then it was condensed using rotary evaporator after depigmented by activated carbon. Crude bile acids were obtained after salting out, extracting and dewatering. TCA was dissociated and depurated from crude bile acid by chromatography techniques and the purity was detected by high performance liquid chromatography. Its purity was >98.7%.

2.3. Animals and drug treatment

The study was approved by the institute's Animal Ethical Committee and conformed to the national guidelines on the care of laboratory animals. Kunming mice (half male and half female), weight 20±2 g, were obtained from the experimental center, Inner Mongolia University. All animals were maintained at a controlled temperature (22±2 °C), and a regular light/dark cycle (7:00 am–7:00 pm, light) and all animals had free access to food and water. The animals were divided into 7 groups of 8 each (Table 1). All animals were treated orally by administration of intra-gastric gavage (i.g.) once daily and sacrificed after 7 days of treatment. Peripheral blood, serum and spleen were prepared for flow cytometry, ELISA and mRNA extraction respectively.

2.4. Isolation and culture of lymphocytes from the spleen of mice

Splenic lymphocytes from normal Kunming mice were harvested according to the procedures described previously [8] but with modification. Briefly, donor mice were killed by cervical dislocation. Each spleen was removed atraumatically, minced with fine scissors, and compressed through a 200-gauge stainless-steel wire mesh. The pooled cells were washed once in D-Hank's medium (Hyclon, USA) and filtered through a 200-gauge wire mesh. The cells were rewashed in RPMI-1640 (Hyclon, USA) medium and resuspended in the same medium, and then 1 mL suspension was added into a centrifugal tube within 2 mL lymphocyte separation medium. After centrifuging the tubes at 2000 rpm for 15 min, the middle layer of liquid was drawn with a pipette and washed once in RPMI-1640 medium. Cell counts and percent viability were determined in 0.05% trypan blue-PBS. Finally, suspension was seeded in cell culture flask (Corning, USA) at 37 °C in a 5% CO_2 humidified atmosphere.

2.5. Preparation of splenic lymphocytes supernatants and total RNA

Splenic lymphocytes were suspended in RPMI-1640 medium supplemented with 3 mM L-glutamine (Bio Basic, Canada), 10 mM hepes (Promega, USA) buffer, 100 U/mL penicillin and streptomycin (Gibco, USA) and 10% FBS at a concentration of 1×10^6 cells/mL which was added to six-well culture plate (2 mL/well) with LPS (final concentration 10 μg/mL) or CsA (final concentration 0.01 μg/mL). The cells were randomly divided into 6 groups: control group (normal mice lymphocytes), LPS/CsA group (cells with LPS/CsA only); the remaining 4 groups were treated with different concentrations of TCA (0.015 μg/mL, 0.15 μg/mL, 1.5 μg/mL, and 15 μg/mL). After incubation for 48 h, lymphocyte supernatants and total RNA were prepared in corresponding methods.

Table 1
Groups and drug treatment.

Groups	1	2	3	4	5	6	7
Levamisole (g/kg)	–	0.1	–	–	–	–	–
CsA (g/kg)	–	–	0.08	–	–	0.08	0.08
TCA (g/kg)	–	–	–	0.25	0.125	0.25	0.125

2.6. Measurement of related cytokine concentration in serum and supernatant of lymphocytes

The concentration of TNF-α, IL-1β in serum and supernatant of lymphocytes was measured by ELISA according to the manufacturer's instructions. The analytic sensitivities for these assays were both 7 pg/mL.

2.7. Real time RT-PCR assay for relative cytokine gene expression in the spleen and lymphocytes

Spleen and lymphocytes were collected and kept at −80 °C until RNA extraction after the mice were sacrificed. Total cellular RNA was extracted using Tripre™ RNA regent and both the ratios of $OD_{260/280}$ and agarose gel electrophoresis were generated to ensure the quality of RNA. Synthesis of cDNA was performed using an M-MulV reverse transcriptase with random 9-mers according to the manufacturer's protocol. cDNA was amplified using SYBR® Premix Ex Taq™ (perfect real time) kit with specific oligonucleotide primers for target sequences in a total of 25 μL reaction mixture (2 μL of cDNA, 12.5 μL of 2× SYBR® Premix Ex Taq™, 1 μL of each 10 μM forward and reverse primers and 8.5 μL of H_2O). The real-time quantitative PCR thermal cycling conditions were 95 °C for 30 s, followed by 95 °C for 5 s and Tm for 30 s for 35 cycles. The primer sequences and Tm are shown in Table 2. All primers were synthesized by TaKaRa Biotechnology (China). Data were analyzed according to the comparative Ct method [9] and were normalized by β-*actin* expression in each sample. Relative gene expression levels were calculated based on the Ct values, corrected for β-*actin* expression, according to the equation: $2^{-\Delta Ct}$ [ΔCt = Ct (*TNF*-α or *IL-1*β) − Ct (β-*actin*)]. Melting curves for each PCR reaction were generated to guarantee the purity of the amplification product.

2.8. Flow cytometry

Fluorescent-Activated Cell Sorting (FACS) analysis was performed according to the procedures described previously [10] and the following monoclonal antibodies (mAbs): FITC anti-mouse $CD3^+$, PE anti-mouse $CD4^+$, and PE anti-mouse $CD8^+$ (Miltenyi Biotec GmbH, Germany) were used. Antibodies were titrated to maximum staining efficiency

Table 2
Primer, temperature and amplified products size and reference in this study.

Primer	Temperature	Amplified products size	Sequence
β-Actin	65.3 °C	171 bp	Sense:5′-CAT CCG TAA AGA CCT CTA TGC CAA C-3′ Antisense:5′-ATG GAG CCA CCG ATC CAC A-3′
TNF-α	62.5 °C	199 bp	Sense: 5′-TAT GGC CCA GAC CCT CAC A-3′ Antisense: 5′-GGA GTA GAC AAG GTA CAA CCC ATC-3′
IL-1β	64.8 °C	105 bp	Sense: 5′-TCC AGG ATG AGG ACA TGA GCA C-3′ Antisense: 5′-GAA CGT CAC ACA CCA GCA GGT TA-3′