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Cryptotanshinone inhibits LPS-induced proinflammatory mediators via TLR4 and TAK1 signaling pathway

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ABSTRACT

Cryptotanshinone (CTN), one of the major constituents of tanshinones, was investigated for anti-inflammatory activity in the murine macrophage cell line RAW 264.7. CTN inhibited the production of nitric oxide (NO) production, as well as expression of inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2) in lipopolysaccharide (LPS)-stimulated macrophages. Since CTN was considered as inhibiting LPS-triggered phosphorylation of mitogen-activated protein kinase (MAPK) and nuclear factor (NF)-κB activation, we consequently evaluated the expression of toll-like receptor 4 (TLR4) and CD14, as well as phosphorylation of TGF-β-activated kinase 1 (TAK1). CTN reduced the expression of CD14 and TLR4, and suppressed LPS-induced phosphorylation of TAK1. Furthermore, CTN significantly increased the survival rate against LPS challenge in D-galactosamine-sensitized mice, which was in line with *in vitro* results. These results suggested that CD14/TLR4 and TAK1 might be the potential molecular targets for addressing the protective effects of CTN on LPS-induced inflammatory effects in macrophages.

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1. Introduction

LPS is a major component of the outer membrane of gram-negative enteric bacteria [1]. Humans are constantly exposed to low levels of LPS through infection. Many different cell types like neutrophils and macrophages can respond to LPS. LPS-stimulated macrophages initiate the immune defense reaction and the inflammatory reaction. In the host defense reaction macrophages are recruited to inflammatory sites, and are activated by various signals that stimulate many intracellular cascades of cytokines and chemokines [2]. During inflammatory processes, LPS induces the production of the proinflammatory cytokines and small mediators, such as nitric oxide (NO) and PGE_2 [3]. NO· is also involved in immune regulation and acts as a radical in the development of cell and tissue injury, and it has been suggested that an overproduction of NO· plays an important role in the pathogenesis of several inflammatory diseases. NO is synthesized from the amino acid L-arginine by a family of NO synthases. Especially, iNOS is known to be the main NOS involved in producing NO. COX-2, together with iNOS, is two major inflammatory mediators. iNOS-based inflammation pathway has been found to cross-link with COX-2 pathway that is the target of COX-2 inhibitor [4]. COX-2 is induced by pro-inflammatory stimuli, including LPS and cytokines in cells *in vitro* and in inflamed sites *in vivo*. Furthermore, COX-2 is believed to be responsible for the synthesis of prostaglandin in various models of inflammation [5].

Based on the recent evidences, we believe that there is a connection between host immunity and the TLR-mediated immunity apparently plays an important role. TLRs are essential elements in host defense against invasive infection by activating the innate immunity, a prerequisite to induction of adaptive immunity. TLRs require the presence of a co-receptor to initiate the signaling cascade, meanwhile TLR4 requires CD14 to participate in the process of LPS-induced signaling, including NF-κB activation [6]. When stimulated by bacterial LPS, many intracellular signaling pathways are activated, and ultimately lead to NF-κB activation, which in turn promotes pro-inflammatory cytokine production and release [7].

Salvia miltiorrhiza, which is called 'Danshen' in China, is a traditional herb to improve blood circulation and treat chronic hepatitis and hepatic fibrosis. Tanshinone I, tanshinone IIA and cryptotanshinone are the major constituents of abietane-type norditerpenoid quinone natural products (tanshinones) isolated from *S. miltiorrhiza* BUNGE [8]. Cryptotanshinone (CTN, Fig. 1A) was reported to have multiple pharmacological activities, such as anti-inflammatory [9], antitumor [10], antioxidative [11] and antiplatelet aggregation activities [12]. Previously, Tang et al. [13] reported that CTN markedly inhibited the phosphorylation of MAPK

Abbreviations: COX-2, cyclooxygenase-2; DMSO, dimethyl sulfoxide; D-GlaN, D-galactosamine; IL-1, interleukin-1; IκB-α, inhibitory kappa B-α; IKK, IκB kinase; iNOS, inducible nitric oxide synthase; IRAK, interleukin-1 receptor-associated kinase; LPS, lipopolysaccharide; LBP, lipopolysaccharide-binding protein; MAPK, mitogen-activated protein kinases, MAPKKK, MAPK kinase kinase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; MyD88, myeloid differentiation primary response gene 88; L-NIL, L-N^6-(1-Iminoethyl)lysine hydrochloride; NF-κB, nuclear factor-κB; NO, nitric oxide; PGE2, prostaglandin E2; TAK1, TGF-beta activated kinase 1; TAB, TAK1-binding protein; TIRAP, toll/IL-1 receptor domain containing adaptor protein; TLR4, toll-like receptor 4; TNF-α, tumor necrosis factor-α; TRAF6, TNF receptor associated factor 6; TRAM, TRIF-related adaptor molecule; TRIF, toll/IL-1 receptor domain containing adaptor inducing interferon-β.

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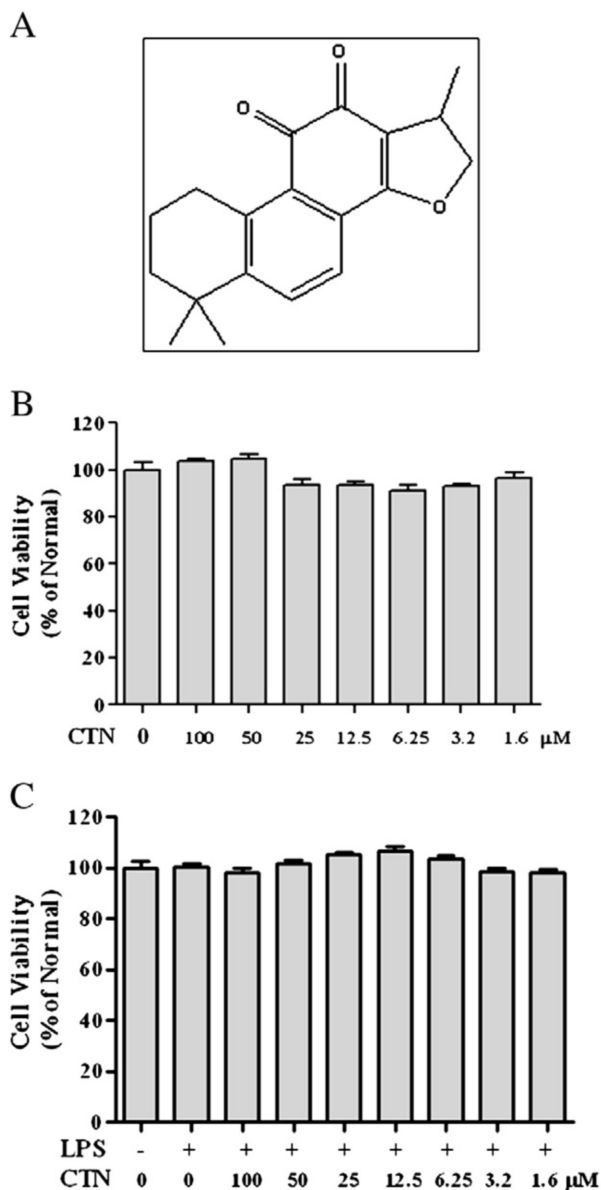

Fig. 1. Effect of CTN on the viability. A) Chemical structure of CTN. B) RAW 264.7 cells ($n=6$) were cultured with various concentrations of CTN for 24 h. C) RAW 264.7 cells ($n=6$) were pretreated with CTN or N-NIL for 1 h, followed by stimulating with LPS (1 μg/mL) for 24 h. Cell viability was determined by MTT assay. Data are expressed as means ± SD.

and abolished completely LPS-triggered NF-κB activation on LPS-stimulated macrophages. However, the mechanism of TLR4 and TAK1-mediated signaling has remained elusive, and thus, they were encouraged to evaluate CD14/TLR4 expression and TAK1 phosphorylation in RAW 264.7 macrophage cells, which can be stimulated by LPS to mimic conditions of infection and inflammation.

2. Materials and methods

2.1. Materials

Cryptotanshinone was obtained from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). The purity of cryptotanshinone reached 99%. All cell culture reagents were from Gibco, Stockholm, Sweden. LPS, DMSO and L-NIL were purchased from Sigma Chemicals Co. (St Loius, MO, USA). Silymarin, which was used as reference drug for *in vivo* experiments, was purchased from Aldrich Chemical Co. Inc. (MW, USA). All reagents were purchased from Beyotime Institute of Biotechnology (Jiangsu, China).

2.2. Cell culture

RAW 264.7 cells, a mouse macrophage cell line, were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin and incubated at 37 °C in 5% CO_2.

2.3. Measurement of cell viability by MTT assay

RAW 264.7 cells were seeded at 1×10^5 cells/ml in 96-well plates containing 100 μl of DMEM medium with 10% FBS and incubated overnight. CTN was dissolved in DMSO, and DMSO was added to all plates to compensate the same volume of DMSO. After 24 h, the cells were pretreated with different concentrations of CTN (0–100 μM) for 1 h, followed by stimulation with LPS for 24 h. Subsequently, cells were cultured with MTT solution (5 mg/ml) for 3 h. The viable cells converted MTT to formazan, which generated a blue-purple color after dissolving in 150 μl of DMSO. The absorbance at 570 nm was measured by an ELISA plate reader.

2.4. Quantification of NO production

The nitrite accumulated in culture medium was measured as an indicated of NO production based on the Griess reaction [14]. Briefly, 100 μl of cell culture medium was mixed with 100 μl of Griess reagent (1% sulfanilamide in 5% phosphoric acid and 0.1% naphthylethylenediamine dihydrochloride in water) in a separate 96-well plate. After an incubation of 15 min at room temperature, the optical density was determined at 540 nm with a microplate reader. Fresh culture medium was used as the blank in all experiments. The concentration of nitrite in the samples was measured with the serial dilution standard curve of $NaNO_2$.

2.5. Protein extraction and western blot analysis

For whole-cell extract preparation, cells were lysed in Cell lysis buffer for Western and IP (Beyotime Institute of Biotechnology, Jiangsu, China). Nuclear and cytosolic extracts were prepared using a Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime Institute of Biotechnology), according to the manufacturer's instructions. Forty micrograms of cellular protein from treated and untreated cell extracts was electroblotted onto a PVDF membrane following separation on a 10% SDS-polyacrylamide gel electrophoresis. The immunoblot was incubated with blocking solution (5% skim milk) for 1 h at room temperature, followed by incubation for overnight at 4 °C with specific primary antibodies that recognize iNOS, p-TAK1 (Cell Signaling Technology, Danvers, MA, USA), COX-2, CD14, TLR4 (Santa Cruz Biotechnology, CA, USA) and α-tubulin (Sigma, St.Louis, MO, USA). Blots were washed four times with PBS containing 0.05% Tween 20 (PBST), and incubated with a 1:2000 dilution of horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology) for 1 h at room temperature. Immunoreactive protein was visualized by the BeyoECL plus kit (Beyotime Institute of Biotechnology). The membranes were then stripped and reproved with α-tubulin antibody for the loading control. Band intensities were quantified by Quantity One software (Bio-Rad, USA).

2.6. Determination of lethality

Male C57BL/6 mice (20–22g) were purchased from the Animal Division of Jilin University (Jilin, China), fed with a standard chow diet and given tap water *ad libitum*. Animals were divided into five groups. CTN (20 or 40 mg/kg body weight) or Silymarin (100 mg/kg body weight) was administered orally to mice at 12 h and 1 h before LPS/D-GalN administration. One hour after the last doses of CTN or Silymarin

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