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Thymoquinone attenuates liver fibrosis via PI3K and TLR4 signaling pathways in activated hepatic stellate cells

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ABSTRACT

Thymoquinone (TQ) is the major active compound derived from the medicinal *Nigella sativa*. In the present study, we investigated the anti-fibrotic mechanism of TQ in lipopolysaccharide (LPS)-activated rat hepatic stellate cells line, T-HSC/Cl-6. T-HSC/Cl-6 cells were treated with TQ (3.125, 6.25 and 12.5 μM) prior to LPS (1 μg/ml). Our data demonstrated that TQ effectively decreased activated T-HSC/Cl-6 cell viability. TQ significantly attenuated the expression of CD14 and Toll-like receptor 4 (TLR4). TQ also significantly inhibited phosphatidylinositol 3-kinase (PI3K) and serine/threonine kinase-protein kinase B (Akt) phosphorylation. The expression of α-SMA and collagen-I were significantly decreased by TQ. Furthermore, TQ decreased X linked inhibitor of apoptosis (XIAP) and cellular FLIP (c-FLIP$_L$) expression, which are related with the regulation of apoptosis. Furthermore, TQ significantly increased the survival against LPS challenge in D-galactosamine (D-GlaN)-sensitized mice, and decreased the levels of serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST), which were in line with in vitro results. Our data demonstrated that TQ attenuates liver fibrosis partially via blocking TLR4 expression and PI3K phosphorylation on the activated HSCs. Therefore, TQ may be a potential candidate for the therapy of hepatic fibrosis.

1. Introduction

Liver fibrosis is a common consequence of chronic liver injury that is induced by a variety of etiological factors that lead to liver cirrhosis [1]. This progressive pathological process is characterized by the accumulation of extracellular matrix (ECM) proteins. Prolonged liver injury results in hepatocyte damage, which triggers activation of hepatic stellate cells (HSCs) [2,3]. HSCs are recognized as the primary cellular source of matrix components in chronic liver disease, and play a critical role in the development and maintenance of liver fibrosis [4]. Following a fibrogenic stimulus, HSCs lose their retinoid store, proliferate and express excessive smooth muscle α-actin (α-SMA), and produce large amounts of ECM proteins, including type I collagen. In the liver, phosphatidylinositol 3-kinase (PI3K) represents a key signaling molecule that controls many cellular functions including proliferation, survival, adhesion and migration [5,6]. PI3K is composed of an 85-kDa regulatory subunit and a 110-kDa catalytic subunit, which is activated by platelet derived growth factor (PDGF) receptor following HSC activation and growth factor stimulation [5]. Hepatocyte-associated PI3K regulates the activation of serine/threonine kinase-protein kinase B (Akt). The importance of PI3K/Akt signaling during the fibrogenic response in HSCs is becoming clearer. Therefore, the interruption of PI3K signaling could suppress the activation and proliferation of HSCs.

Lipopolysaccharide (LPS), the primary component of the outer membrane of Gram-negative bacteria, is responsible for the overwhelming innate immune response of the sepsis syndrome. CD14, a cell surface glycoprotein, is the main LPS receptor of leukocytes and contributes to host sensitivity [7]. In the liver, Toll-like receptors (TLRs) are expressed in many different cell types including Kupffer cells, hepatocytes and HSCs. Due to the powerful effects of TLRs expressed in the liver, there is a significant hepatic exposure to TLR ligands from the intestinal microbiota, even in early stages of liver disease. It suggests that TLRs act as an important link between hepatic inflammation, injury and fibrosis. Recent studies have identified TLR4 as a membrane cofactor in LPS-mediated transmembrane signaling in cytokine induction [8]. 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 [9]. TLR4 signaling activates NF-κB and JNK/AP-1 pathways through MyD88 and TRIF [10]. The activation of HSCs that express TLR4 is associated with the progression of liver fibrosis. These facts suggest a strong contribution of LPS-TLR4 interaction in the development of liver fibrosis. As mentioned above, HSCs are direct targets of LPS in vitro and in vivo [11,12].

Abbreviations: Akt, serine/threonine kinase-protein kinase B; ALT, alanine aminotransferase; AST, aspartate aminotransferase; α-SMA, α-smooth muscle actin; c-FLIP, cellular FLIP; D-GlaN, D-galactosamine; ECM, extracellular matrix; HSCs, hepatic stellate cells; LPS, lipopolysaccharide; MyD88, myeloid differentiation factor 88; NF-κB, nuclear factor-κB; TLR4, Toll-like receptor4; PI3K, phosphatidylinositol 3-kinase; XIAP, X linked inhibitor of apoptosis.

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Thymoquinone (TQ) is the main active ingredient from the seeds of *Nigella sativa* Linn (Fig. 1A), which has been traditionally used in the Middle East and Southeast Asian countries to treat ailments including asthma, bronchitis, rheumatism, cancer and related inflammatory diseases [13,14]. It was also reported that the oral administration of TQ in bile duct ligated rats maintained antioxidant defenses and reduces liver oxidative damage, and ductular proliferation as well [15]. However, the mechanism of anti-fibrotic effects of TQ has remained elusive, and thus, we were intrigued to evaluate TLR4 expression and PI3K/Akt phosphorylation involved in HSCs, which can be stimulated by LPS to mimic conditions of infection and inflammation.

2. Materials and methods

2.1. Materials

Thymoquinone, was purchased from Sigma Chemical Co. (St. Louis, MO, USA). The purity of thymoquinone reached 99%. LPS, D-GlaN, DMSO and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl thiazolium bromide (MTT) were purchased from Sigma Chemicals Co. (St. Loius, MO, USA). LY294002, a PI3K inhibitor, was purchased from Beyotime (Jiangsu, China). Anti-CD14, anti-TLR4, anti-PI3K, anti-Akt, anti-p-PI3K, anti-p-Akt antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz Biotechnology, CA, USA). Anti-collagen-I, anti-α-SMA, anti-β-actin antibodies were purchased from Abcam (Cambridge, MA, USA). Anti-c-FLIPL and anti-XIAP antibodies were from Cell Signaling Technology (Beverly, MA, USA). Anti-rabbit and anti-mouse IgG conjugated to horseradish-peroxidase (HRP) were purchased from Santa Cruz Biotechnology. The BCA protein assay kit was obtained from Beyotime (Jiangsu, China). All cell culture reagents were from Gibco/Invitrogen (Grand Island, NY, USA).

2.2. Cell culture

T-HSC/Cl-6 is an immortalized rat HSCs, which are transfected by the large T-antigen of SV40 vector containing a Rous sarcoma virus promoter. Normal human Chang liver cells derived from normal liver tissue are an ideal hepatic cytotoxicity experimental target and belong to hepatic serial subcultivation human cells with a high tolerance. All cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin G and 100 mg/ml streptomycin at 37 °C under 5% CO_2. The cultures were passaged by trypsinization every three days and cells were plated in 100 mm culture dishes at a density of 1×10^6 cells per dish in DMEM. Then cells were treated

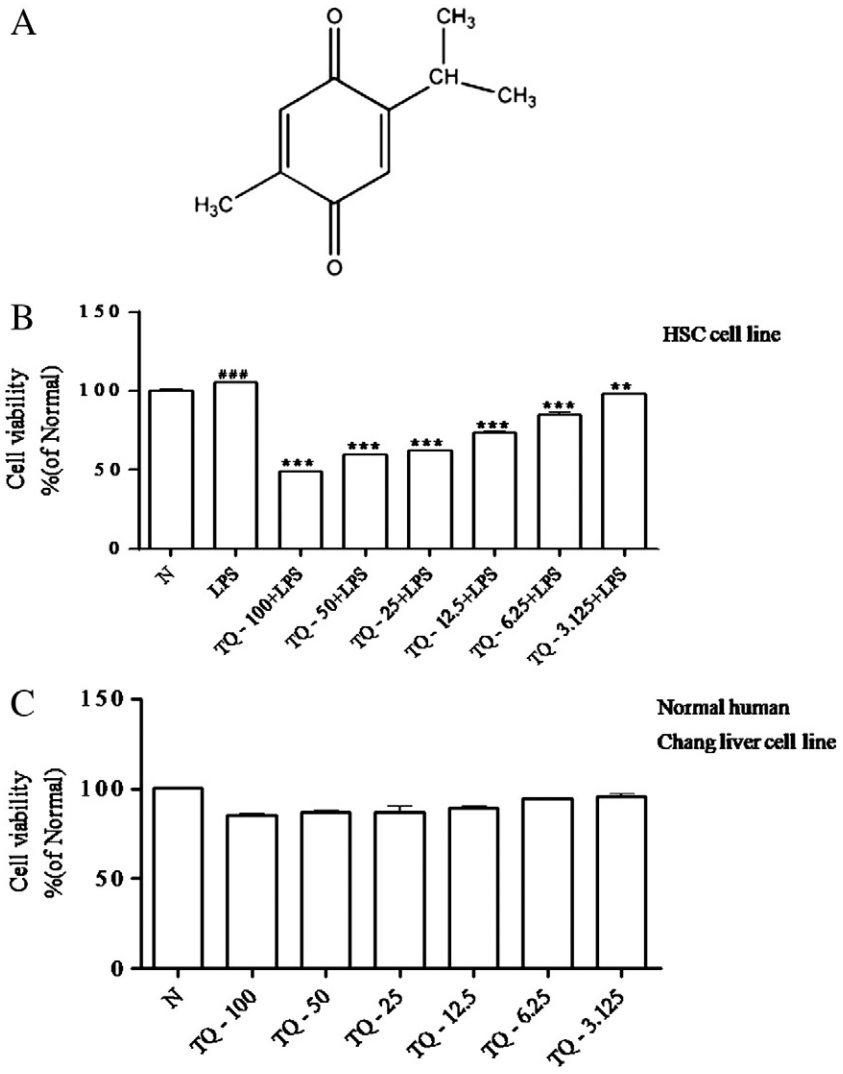

Fig. 1. The effect of TQ on the cell viability. (A) Chemical structure of TQ. (B)The viability of T-HSC/Cl-6 cells treated with TQ after induction of LPS in 24 h via MTT assay. (C) The cell viability of normal human Chang liver cells 24 h after TQ treatment via MTT assay. Normal T-HSC/Cl-6 cells were encoded with "N". Data are represented as the mean ± SD, (n = 6). $^{###}p<0.001$, compared with the normal group; $^{***}p<0.001$, $^{**}p<0.01$, significantly different compared with the LPS-activated T-HSC/Cl-6 cells.