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/CJ-6. T-HSC/CJ-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/CJ-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) 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.

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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].
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. Louis, 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-FIPL 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% CO2. 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...
with various concentrations of TQ 1 h prior to LPS (1 μg/ml) stimulation for the indicated time course.

2.3. Measurement of cell viability by MTT assay

Cell viability was assessed with MTT assay. T-HSC/Cl-6 cells or Chang liver cells were seeded in 96 well plates (1 × 10^4 cells per well). T-HSC/Cl-6 cells were treated with TQ plus LPS (1 μg/ml) and Chang liver cells were treated with TQ. MTT solution (5 mg/ml) was added after TQ or LPS treatment for 24 h, and the cells were then incubated for another 3 h. The extent of reduction of MTT to formazan was read at 540 nm by using a microplate reader.

2.4. 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). 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 membrane was incubated with a blocking solution (5% skim milk) for 1 h at room temperature, followed by a specific primary antibody incubation. Blots were washed with PBS containing 0.05% Tween 20 (PBST), and then incubated with HRP-conjugated secondary antibody for 1 h at room temperature. Immunoreactive protein was visualized by the BeyoECL plus kit (Beyotime Institute of Biotechnology). The membrane was then stripped and reprobed with β-actin antibody for the loading control. Band intensities were quantified by Quantity One software (Bio-Rad, USA).

2.5. Animals and treatment

Male Kunming mice (6 weeks old; body weight 18–23 g) were purchased from Yanbian University Laboratory Animal Centre. All mice were acclimatized to the laboratory environment, maintained at 22 ± 2 °C and 50–60% relative humidity, with 12-hour light-dark cycles throughout the experiment. Animal handling was performed in accordance with the National Institute of Health guidelines (National Research Council, 1996). All efforts were made to minimize the number and suffering of the animals used.

Animals were divided into five groups (n = 10). TQ (20 or 5 mg/kg body weight) or silymarin (100 mg/kg body weight) was administered orally to mice at 12 h and 1 h before LPS or GalN administration. One hour after the last doses of TQ or silymarin except the normal control group, mice were given an intraperitoneal injection of LPS (10 μg/kg body weight), immediately followed by intraperitoneal injection of GalN (700 mg/kg body weight). The survival rate of mice was monitored for 24 h after LPS or GalN injection. All mice were randomly divided into the following five groups (n = 10): normal group, LPS or GalN group, silymarin + LPS or GalN group, TQ (20) + LPS or GalN group, TQ (5) + LPS or GalN group. The normal group was given an equivalent volume of saline. TQ (20 or 5 mg/kg body weight) or silymarin (100 mg/kg) was administered orally to mice at 8 and 1 h before LPS or GalN administration. At 1 h after the last doses of TQ, except the normal group, mice that were induced by fulminant hepatic failure were given an intraperitoneal injection of LPS or GalN. After the administration of LPS or GalN, animals were sacrificed; and blood was collected from the carotid artery at 8 h. Blood samples were allowed to coagulate at 4 °C for 30 min and serum was then separated by centrifugation at 3000 rpm for 30 min at 4 °C. The liver of each mouse was removed immediately and then was kept at −80 °C for subsequent analysis.

2.6. Statistical analysis

All values are expressed as mean ± SD. A comparison of the results was performed with one-way ANOVA and Tukey’s multiple comparison tests. Calculations were performed by using the GraphPad Prism program (GraphPad Software, Inc, San Diego, USA). Statistically significant differences between groups were defined as p-values less than 0.05.

3. Results

3.1. The effect of TQ on the viability

Firstly, TQ (12.5–100 μM) significantly inhibited T-HSC/Cl-6 cell viability in a dose-dependent manner within 24 h after LPS activation, and the IC50 value was 28.91 μM (Fig. 1B). To determine whether TQ causes cytotoxicity in normal hepatocytes, we chose normal human Chang liver cells as normal hepatocyte to test the cell viability at various concentrations of TQ within 24 h. TQ (3.125–100 μM) exhibited scarcely little toxicity in human Chang liver cells (Fig. 1C).

3.2. The effects of TQ on protein expression of CD14 and TLR4

In response to LPS, HSCs express inflammatory and fibrogenic features, and these molecules were reported to activate HSCs directly [16]. Given that TLR4 is the major receptor for bacterial LPS on immune and some epithelial cells, as well as endothelial cells, we examined whether the TLR4 signaling pathway was involved in the T-HSC/Cl-6 cells with TQ. Therefore, we determined the expression of CD14 and TLR4. First of all, TQ alone could slightly increase the expression of CD14, and TQ alone treatment also could increase TLR4 expression at a high concentration (12.5 μM) (Fig. 2A). As expected, LPS induced high expressions of TLR4 and CD14 after 24 h. The increasing expressions of CD14 and TLR4 were decreased by TQ pretreatment. Furthermore, LY294002, a PI3K inhibitor, reduced the increase in CD14 and TLR4 expressions by LPS, indicating that blocking PI3K may suppress TLR4 signaling (Fig. 2B).

3.3. The effects of TQ on HSC apoptosis

HSC apoptosis is thought to be essential for the resolution phase of fibrosis. Induction of HSC apoptosis would be expected to be anti-fibrogenic by inhibiting the accumulation of the activated HSCs within the liver. Due to TQ showing cell toxicity on T-HSC/Cl-6 cells (Fig. 1B), we confirmed whether TQ-induced death was associated with apoptosis on activated HSCs. In LPS-activated T-HSC/Cl-6 cells, TQ significantly decreased the expression of c-FLIP, a dose dependent manner (Fig. 3). And the expression of XIAP markedly decreased with TQ treatment (Fig. 3). Therefore, TQ might trigger the apoptosis of activated T-HSC/Cl-6 cells. And these observations may suggest that TQ, at least in part, induced HSC apoptosis, and may be related with the decrease in TLR4 and CD14 expressions.

3.4. The effects of TQ on protein expression of collagen-I and α-SMA

The activation of HSCs is one of the central pathophysiological mechanisms of liver fibrosis, and α-SMA is an established marker of HSC activation [17]. Collagen I, the principal collagen responsible for cirrhosis, is generated within the liver by activated HSCs[18]. The levels of α-SMA and collagen-I were highly expressed in LPS-activated T-HSC/CI-6 cells indicating HSC activation upon LPS. In contrast, TQ decreased the levels of α-SMA and collagen-I to an almost quiescent HSC level. In addition, LY294002 reduced the increasing collagen I and α-SMA expression by LPS.
3.5. The effects of TQ on LPS-induced protein expression of PI3K/Akt phosphorylation

To confirm the anti-fibrotic mechanism of TQ with regard to the regulation of the PI3K/Akt signaling pathway, we firstly observed PI3K/Akt expression in activated T-HSC/Cl-6 cells. PI3K and Akt phosphorylations were highly expressed after LPS stimulation, but the levels were dose-dependently reduced by TQ treatment (Fig. 5).

3.6. Effects of TQ on lethality in mice with LPS/D-GalN-induced liver injury

To determine the protective effects of TQ on lethality, TQ or silymarin were orally and intraperitoneally administered to mice 12 and 1 h before LPS/D-GalN treatment. Mice began to die within 6 h of LPS/D-GalN injection. However, pretreatment with TQ reduced the lethality. Briefly, 24 h after LPS/D-GalN administration, the survival rates were 60% and 40% in the mice pretreated with 20 and 5 mg/kg TQ, respectively, as listed in Fig. 6A. Therefore, we selected 20 and 5 mg/kg of TQ for the following study.

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**Fig. 2.** Effects of TQ on TLR4 and CD14 expression. (A) T-HSC/Cl-6 cells were pretreated with indicated concentrations of TQ for 24 h. (B) T-HSC/Cl-6 cells were pretreated with indicated concentrations of TQ or LY294002 1 h prior to incubation of LPS (1 μg/ml) for 24 h. The expression of TLR4 and CD14 proteins were detected via Western blotting with specific antibodies. β-Actin was used as a loading control; data were normalized to the β-actin signal. ###p < 0.001, **p < 0.01, *p < 0.05, significantly different when compared with the LPS-stimulated cells.

**Fig. 3.** Effects of TQ on c-FLIP and XIAP expressions. T-HSC/Cl-6 cells were pretreated with indicated various concentrations of TQ for 1 h prior to incubation of LPS (1 μg/ml) for 24 h. C-FLIP, and XIAP proteins were detected via Western blotting with specific antibodies. β-Actin was used as a loading control; data were normalized to the β-actin signal. ***p < 0.001, **p < 0.01, *p < 0.05, significantly different when compared with the LPS-stimulated cells.

**Fig. 4.** Effects of TQ on collagen-I and α-SMA expression. T-HSC/Cl-6 cells were pretreated with indicated concentrations of TQ or LY294002 1 h prior to incubation of LPS (1 μg/ml) for 24 h. Collagen-I and α-SMA proteins were detected via Western blotting with specific antibodies. β-Actin was used as a loading control; data were normalized to the β-actin signal. ***p < 0.001, **p < 0.01, significantly different when compared with the LPS-stimulated cells.
3.7. Effects of TQ on serum ALT and AST levels in LPS/o-GalN-induced liver injury

Serum ALT/AST levels were determined as an index of hepatic function. To confirm the effect of TQ on serum enzymes, we investigated whether TQ regulates serum ALT and AST levels in LPS/o-GalN-induced liver injury. Both ALT and AST levels significantly increased in the LPS/o-GalN group after 8 h (Fig. 6B). In comparison, TQ or silymarin pretreatment significantly reduced the activity increases of serum ALT and AST occurring 8 h after LPS/o-GalN administration. As shown in Fig. 6B, the TQ pretreatment groups showed significantly inhibitory effects in a dose-dependent manner.

4. Discussion

The present study was undertaken to elucidate the biological effects of TQ, which is the major active compound derived from N. sativa, focusing on inhibiting HSC activation. It was previously reported that TQ suppressed LPS-induced cytokine production via the activation of NF-κB [19,20]. Nevertheless, the molecular mechanisms involving TLR4 and PI3K signaling have not been fully addressed. Therefore, we decided to characterize TLR4 and PI3K, the mechanism of two major signal transduction pathways and to find out the relation between HSC activation and the regulation of inflammatory response. As expected, TQ could suppress liver fibrosis, accompanied with induced HSC apoptosis and inhibiting LPS-TRL4 signaling and PI3K/Akt phosphorylation.

Many investigators have shown that the growth inhibitory effects of TQ are specific to cancer cells [21]. In the present study, TQ significantly inhibited the activated HSC viability within 24 h (Fig. 1B). So we concluded that TQ exhibited significantly anti-proliferative effect on HSCs.

Activated macrophages liberate cytokines at the site of inflammation and are involved in the progression of disease states resulting from chronic inflammation. LPS activates monocytes/macrophages by binding to its receptor, TLR4 [22,23]. Then, TLR4 activates the intracellular signaling cascade by recruiting MyD88, IL-1 receptor-associated kinase (IRAK)-1, and IRAK-4 to the membrane. IRKs associate with the receptor complex transiently. Once released, IRAKs can associate with and activate TRAF6, causing the activation of the IκB kinase (IKK) complex and MAPK [24–26], which are known to be involved in the regulation proinflammatory cytokine secretion [27,28]. The activated IKK complex induces phosphorylation of IκB, causing degradation of IκB and liberation of the transcription factor, NF-κB, which promotes the transcription of inflammatory cytokines. NF-κB activation plays an important role in pro-fibrogenic signaling pathways in response to LPS-mediated TLR4 activation such as HSC activation [29]. On LPS-activated HSCs, TQ inhibited the expressions of CD14 and TLR4 to a certain degree, which would partially contribute to anti-inflammation (Fig. 2). In addition, LY294002 reduced CD14 and TLR4 expressions, suggesting that PI3K signaling may be involved in TLR4 signaling. Furthermore, TQ decreased α-SMA and collagen-I, and apoptosis of HSCs was observed 24 h after TQ treatment (Figs. 3 and 4). Also HSCs treated with LY294002 expressed extremely less α-SMA and collagen-I after LPS stimulation, suggesting that PI3K signaling may contribute to liver fibrosis progress. Our in vitro data illustrated that TQ-induced apoptosis of HSCs is synergistic with suppressing TLR4 signaling to reduce α-SMA and collagen-I expression. But the underlying mechanism needs to be further studied.

PI3K represents a key signaling molecule that controls many cellular functions such as proliferation, survival, adhesion, and migration [6,30]. In the liver, macrophage-associated PI3K activation promotes cytokine production following partial
In the present study, inhibition of fibrogenesis was associated with reduced α-SMA expression, and collagen production as well (Fig. 4). Several mechanisms may exist by which PI3K might promote this activation. PI3K/Akt are also involved in the transduction of extracellular signals [32]. LY294002 was shown to act as a highly selective inhibitor of PI3K (Fig. 5). Application of LY294002 interpreted well that inhibiting of PI3K successfully alleviated liver fibrosis progress, represented by reduced α-SMA and collagen I. In a similar way, TQ blocked PI3K phosphorylation, then consequently suppressed TLR4 signaling and HSC activation. In view of the critical role of PI3K on HSC activation and collagen production, the ability of TQ to inhibit PI3K may be tightly related with its anti-fibrogenic effect.

Recently, cellular FLIP (c-FLIP), also known as FLICE-inhibitory protein, has been identified as a major resistance factor and critical anti-apoptotic regulator that inhibits death receptor-mediated apoptosis [33]. c-FLIP is expressed as the isoforms c-FLIP L and c-FLIP S ("long" and "short") and the recently discovered closed relative of c-FLIP S, c-FLIP Lα. c-FLIP L expression may serve as a finely tuned apoptosis regulator in normal tissues and can decide a cell towards apoptosis or survival [34]. The expression level of c-FLIP L is responsible for the dual functions, deciding whether it inhibits or enhances Fas-induced caspase-8 activation [33]. In the present study, c-FLIP L is shown as a function of inhibitor of apoptosis after TQ treatment in activated HSCs. Otherwise TQ suppressed the expression of XIAP (Fig. 3), indicating that TQ induced HSC apoptosis with the combined inhibition of FLIP and XIAP, which was in line with the previous report [35].

To confirm whether TQ has any therapeutic effect on LPS-induced liver injury in mice, mice was introduced to LPS combined with α-GalN. Experimental models of liver injury are largely based on animal exposure to endotoxin (LPS). Since rodents are constitutively resistant to LPS, mice treated with α-GalN were dramatically sensitized to LPS, allowing reduction of more than 2500-fold the lethal dose of endotoxin [36,37]. Based on this observation, we designed by using the LPS/α-GalN model. As expected, TQ showed a significant decrease in mortality, TQ pretreatment groups showed significant inhibitory effects in a dose-dependent manner, which can indirectly illustrate the anti-fibrosis effect of TQ (Fig. 6). However, further work should focus on LPS/α-GalN induced liver injury in more detail.

In conclusion, our present study suggests that the anti-fibrotic mechanism of TQ may be partially mediated by downregulating TLR4 signaling and blocking phosphorylation of PI3K/Akt. Furthermore, TQ exhibited apoptosis-inducing activity against activated HSCs (Fig. 7). Considering these results, TQ may represent a potential new source of medicine for the therapy of hepatic fibrosis, targeting to the activated HSCs. However, currently we do not have any evidences that NF-κB is involved in the anti-fibrotic effect of TQ towards liver fibrosis. Further investigation is needed.

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