International Immunopharmacology 19 (2014) 351–357

Contents lists available at ScienceDirect

International Immunopharmacology

journal homepage: www.elsevier.com/locate/intimp

Thymoquinone alleviates thioacetamide-induced hepatic fibrosis and inflammation by activating LKB1–AMPK signaling pathway in mice

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ARTICLE INFO

Article history:
Received 4 September 2013
Received in revised form 3 February 2014
Accepted 5 February 2014
Available online 22 February 2014

Keywords:
Thymoquinone
Hepatic fibrosis
LKB1
AMPK
PI3K

ABSTRACT

The current study was conducted to investigate the anti-fibrotic effect and its possible underlying mechanisms of thymoquinone (TQ) against hepatic fibrosis *in vivo*. TQ is the major active compound derived from the medicinal *Nigella sativa*. Liver fibrosis was induced in male Kunming mice by intraperitoneal injections of thioacetamide (TAA, 200 mg/kg). Mice were treated concurrently with TAA alone or TAA plus TQ (20 mg/kg or 40 mg/kg) given daily by oral gavage. Our data demonstrated that TQ treatment obviously reversed liver tissue damage compared with TAA alone group, characterized by less inflammatory infiltration and accumulation of extracellular matrix (ECM) proteins. TQ significantly attenuated TAA-induced liver fibrosis, accompanied by reduced protein and mRNA expression of α-smooth muscle actin (α-SMA), collagen- and tissue inhibitor of metalloproteinase-1 (TIMP-1). TQ downregulated the expression of toll-like receptor 4 (TLR4) and remarkably decreased proinflammatory cytokine levels as well. TQ also significantly inhibited phosphatidylinositol 3-kinase (PI3K) phosphorylation. Furthermore, TQ enhanced the phosphorylation adenosine monophosphate-activated protein kinase (AMPK) and liver kinase B (LKB)-1. In conclusion, TQ may reduce ECM accumulation, and it may be at least regulated by phosphorylation of AMPK signaling pathways, suggesting that TQ may be a potential candidate for the therapy of hepatic fibrosis.

1. Introduction

Hepatic fibrosis, defined by excessive accumulation of extracellular matrix (ECM) and resultant loss of pliability and liver function, is the result of wound-healing responses triggered by either acute or chronic liver injury [1,2]. Chronic liver disease and cirrhosis represent a major global health concern [3,4]. Currently, no antifibrotic agents for chronic liver disease have been approved by the Food and Drug Administration [5]. Therefore, a greater understanding of molecular mechanisms regulating the hepatic fibrosis in liver is needed for the identification of novel targets for successful antifibrotic therapies. Thioacetamide (TAA) is a toxic agent that is commonly used for fibrosis induction in rodents [6,7]. Based on biochemical and morphological observations, the TAA-induced mouse liver cirrhosis closely resembles the important features of human liver disease [5]. Also it can be used as a suitable animal model for studying the mechanisms of liver fibrosis reversibility and testing of anti-fibrotic and regulating agents [8].

Prolonged liver injury results in hepatocyte damage, which triggers activation of hepatic stellate cells (HSCs) [5,9]. 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. Hepatic fibrosis occurs during most chronic liver diseases and is driven by inflammatory responses to injured tissue. Chronic inflammation eventually causes scar tissue to form, a condition known as fibrosis. Controlling liver inflammation can control progression to fibrosis. Inflammatory response is triggered by similar factors such as allergens, leading to the increased production and release of inflammatory mediators, including interleukin (IL)-4, IL-5, prostaglandins and thymus and activation-regulated chemokine [10].

TLRs are a family of pattern-recognition receptors that recognize pathogen-derived molecules termed pathogen-associated molecular patterns (PAMPs), which are structural components unique to bacteria, fungi and viruses [11]. These ligands bind to TLRs, leading to signaling and activation of innate and adaptive inflammatory responses and are expressed in many different cell types, including Kupffer cells, hepatocytes and HSCs. The extraordinarily marked effects of TLRs on inflammation suggest that TLRs act as an important link between hepatic inflammation and fibrosis. Recent studies have shown that activation of adenosine monophosphate-activated protein kinase (AMPK) in HSCs leads to the reduction of induced proliferation and migration of

Abbreviations: α-SMA, α-smooth muscle actin; Akt, threonine kinase-protein kinase B; ALT, alanine aminotransferase; AST, aspartate aminotransferase; AMPK, adenosine monophosphate-activated protein kinase; ECM, extracellular matrix; HSCs, hepatic stellate cells; IL, interleukin; LKB-1, liver kinase B-1; NF-κB, nuclear factor-κB; PI3K, phosphatidylinositol 3-kinase; TAA, thioacetamide; TIMP-1, tissue inhibitor of metalloproteinase-1; TLRs, toll-like receptors.

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http://dx.doi.org/10.1016/j.intimp.2014.02.006
1567-5769/

HSCs [12]. Liver kinase B-1 (LKB1) is the upstream kinase capable of AMPK and a critical mediator of cellular response to low energy [13,14]. LKB1 dictates most of the AMPK activation in all tissues examined where LKB1 has been genetically knocked out [15]. Recent studies have shown that a biologically active lignin sauchinone activates AMPK in hepatocytes, the activation of AMPK depends on upstream LKB1 activation to protect the liver or hepatocyte toxicity [16]. As LKB1–AMPK axis may impact to hepatic fibrosis.

Thymoquinone (TQ) is the main active ingredient from the seeds of *Nigella sativa* Linn, which has been traditionally used in the Middle East and Southeast Asian countries as Habbatul Baraka or the 'seed of blessing', has been shown to produce multiple health beneficial activities, including antihistaminic, antibacterial, antihypertensive, hypoglycemic, antiinflammatory, immunopotentiating and antiarthritic activities [17–19]. In our previous study, TQ represents a potential new source of medicine for treating hepatic fibrosis, targeting at the activated HSCs *in vitro* [20]. Current study aims to investigate the anti-fibrotic effect of TQ on hepatic fibrosis and inflammation induced by TAA and to find a distinct mechanism involving TLR4 and AMPK signaling pathway.

2. Materials and methods

2.1. Materials

Thymoquinone was purchased from Sigma Chemical Co. (St. Louis, MO, USA). The purity of TQ reached 99%. Thioacetamide was purchased from Sigma Chemicals Co. (St. Louis, MO, USA). Anti-TLR4, anti-PI3K, and anti-p-PI3K antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz Biotechnology, CA, USA). Anti-collagen-I, anti-α-SMA, anti-TIMP-1, and anti-β-actin antibodies were purchased from Abcam (Cambridge, MA, USA). Anti-AMPK, anti-p-AMPK, anti-LKB1 and anti-p-LKB1 antibodies were from Cell Signaling Technology (Beverly, MA, USA). Horseradish peroxidase (HRP)-conjugated goat anti-mouse and goat anti-rabbit antibodies were purchased from Santa Cruz Biotechnology. The BCA Protein Assay Kit was obtained from Beyotime (Jiangsu, China).

2.2. Animals and treatments

Male Kunming mice (6 weeks old; body weight 18–23 g) were purchased from Yanbian University Laboratory Animal Centre [SPF, SCXK (J) 2011-0007]. 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. All efforts were made to minimize the number and suffering of the animals used. All mice were randomly divided into the following five groups: normal group, TAA group, TQ (20) + TAA group, TQ (40) + TAA group and TQ (40) group. Each group contained 8 mice. The normal group was given an equivalent volume of saline. The TAA group was intoxicated with TAA three times per week for 5 weeks. TQ (20 mg/kg and 40 mg/kg) was suspended with 2% (v/v) Tween 80 in saline. The TQ (20) + TAA and TQ (40) + TAA groups of mice were daily gavaged 20 or 40 mg/kg of TQ and also intraperitoneally injected 200 mg/kg TAA three times per week for 5 weeks. No mice died during the entire experimental period. All animals were sacrificed days after the last dose of TQ. Blood was obtained from all animals *via* cardiopuncture at the time of sacrifice. Blood samples were allowed to coagulate at room temperature for 30 min. Serum was then separated by centrifugation at 3000 rpm for 30 min at 4 °C, and serum ALT and AST levels were measured using commercial assay kits (Nanjing Jiancheng Bioengineering Institute, China) according to the manufacturer's instructions. The liver of each mouse was removed immediately and then kept at −80 °C for subsequent analysis. The experiment was performed in accordance with the guidelines of the Animal Care Committee of Yanbian University.

2.3. Histopathological and immunohistochemistry examination

Liver tissues were fixed in 15% neutral buffered formalin, processed routinely and embedded in paraffin. Serial paraffin sections of 4 μm were stained with hematoxylin–eosin (H&E) or Masson-trichrome staining (Fuzhou Maixin Biotechnology Development Co. Ltd., Fuzhou, China) and observed under an Olympus light microscope.

For immunohistochemistry, mouse liver was embedded in optimal cutting temperature compound and stored at −80 °C until use. Cryosections (5 mm) were fixed in acetone/methanol (1:1) and incubated with normal goat serum to reduce non-specific binding. Tissue sections were incubated with anti-collagen-I and anti-α-SMA monoclonal antibody overnight in a humidified chamber at 4 °C. Horseradish peroxidase (HRP) labeled secondary antibody included in the MaxVision™ HRP-Polymer anti-mouse/rabbit IHC kit (Fuzhou Maixin, China), was applied for 30 min at room temperature, followed by incubating at room temperature with diaminobenzidine (DAB) Chromogen for color development. Tissue sections were counterstained with Mayer's Hematoxylin (Sigma-Aldrich).

2.4. Reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was extracted from liver tissue samples by the Trizol (Beyotime Institute of Biotechnology, China) according to the manufacturer's protocol. cDNA was prepared using 1 μg of total RNA. RT-PCR was carried out on the ABI Veriti® Thermal Cyclers (Applied Biosystems, Foster City, CA). The primer sequences used in PCR are shown in Table 1. PCR products were run on a 2% agarose gel stained with ethidium bromide. The expression levels of all the transcripts were normalized to that of the GAPDH mRNA in the same tissue samples.

2.5. Western blot analysis

After treatments, the liver tissue was collected and lysed with lysis buffer. Fifty micrograms of extracted protein was electroblotted onto a PVDF membrane following separation on a 10% or 12% SDS-polyacrylamide gel electrophoresis. The membrane was incubated with blocking solution (5% skim milk) for 1 h at room temperature, followed by specific primary antibody incubation. The dilutions of primary antibodies are as followed: anti-collagen-I (1:500), anti-α-SMA (1:500), anti-TIMP-1 (1:500), anti-β-actin (1:5000) antibodies from Abcam; anti-TLR4 (1:500), anti-PI3K (1:500), anti-p-PI3K (1:500) antibodies from Santa Cruz Biotechnology; anti-AMPK (1:500), anti-p-AMPK (1:500), anti-LKB1 (1:500) and anti-p-LKB1 (1:500) antibodies from Cell Signaling Technology. 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

Table 1
Primer sequences for RT-PCR.

Target genes	Accession no.	Primer sequences (5′–3′)	Product length (bp)
Collagen-	NM_007742.3	TGAGTCAGCAGATTGAGAAC TACTCGAACGGGAATCCATC	301
α-SMA	NM_007392.2	CATCAGGGAGTAATGGTTGG CACAATACCAGTTGTACGTC	339
TIMP-1	NM_001044384.1	GGAAAGCCTCTGTGGATATG AACAGGGAAACACTGTGC	200
TLR4	NM_021297.2	TCCCTGCATAGAGGTAGTTC ACTCTGGATAGGGTTTCCTG	268
IL-1α	NM_010554.4	CTTGAGTCGGCAAAGAAATC GAGATGGTCAATGGCAGAAC	107
IL-1β	NM_008361.3	GTACATCAGCACCTCACAAG CACAGGCTCTCTTTGAACAG	268
IL-18	NM_008360.1	GATCAAAGTGCCAGTGAACC AACTCCATCTTGTTGTGTCC	233
GAPDH	NM_008084.2	CTTGTGCAGTGCCAGCC GCCCAATACGGCCAAATCC	233