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ROS-JNK1/2-dependent activation of autophagy is required for the induction of anti-inflammatory effect of dihydroartemisinin in liver fibrosis

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ABSTRACT

Accumulating evidence identifies autophagy as an inflammation-related defensive mechanism against diseases including liver fibrosis. Therefore, autophagy may represent a new pharmacologic target for drug development to treat liver fibrosis. In this study, we sought to investigate the effect of dihydroartemisinin (DHA) on autophagy, and to further examine the molecular mechanisms of DHA-induced anti-inflammatory effects. We found that DHA appeared to play an essential role in controlling excessive inflammation. DHA suppressed inflammation in rat liver fibrosis model and inhibited the expression of proinflammatory cytokines in activated hepatic stellate cells (HSCs). Interestingly, DHA increased the autophagosome generation and autophagic flux in activated HSCs, which is underlying mechanism for the anti-inflammatory activity of DHA. Autophagy depletion impaired the induction of anti-inflammatory effect of DHA, while autophagy induction showed a synergistic effect with DHA. Importantly, our study also identified a crucial role for reactive oxygen species (ROS) in the facilitation of DHA-induced autophagy. Antioxidants, such as glutathione and N-acetyl cysteine, significantly abrogated ROS production, and in turn, prevented DHA-induced autophagosome generation and autophagic flux. Besides, we found that c-Jun N-terminal kinase1/2 (JNK1/2) was a downstream signaling molecule of ROS that mediated the induction of autophagy by DHA. Down-regulation of JNK1/2 activity, using selective JNK1/2 inhibitor (SP600125) or siJNK1/2, led to an inhibition of DHA-induced autophagy. Overall, these results provide novel implications to reveal the molecular mechanism of DHA-induced anti-inflammatory effects, by which points to the possibility of using DHA based proautophagic drugs for the treatment of inflammatory diseases.

1. Introduction

Liver fibrosis occurs as compensatory responses to tissue repairing process in a wide range of chronic liver injures, and its end-stage cirrhosis is responsible for high morbidity and mortality worldwide [1–4]. In recent years, the use of natural products as a realistic option for the treatment of liver fibrosis has broadly been accepted. Novel anti-fibrosis compounds from herbal components represent an attractive alternative for drug development. Dihydroartemisinin (DHA), an antimalarial drug recommended by the World Health Organization (WHO) in response to problems of drug resistance, exhibits an ample array of pharmacological activities such as anti-tumor, anti-bacterial, and anti-fibrosis properties [5–7]. We previously reported that DHA alleviated bile duct ligation-induced liver fibrosis in vivo [8,9], and inhibited activation and contraction of hepatic stellate cells (HSCs) in vitro [10]. Interestingly, whether DHA improves the inflammatory microenvironment of liver fibrosis has never been investigated.

Inflammatory pathways play a crucial role in regulation of a wide range of cellular processes involved in induction, initiation, progression, and aggravation of liver fibrosis [11,12]. Growing evidence links improvement of inflammatory microenvironment to liver diseases reversal [13]. Previous studies [14,15], including ours [14], have

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase; Atg, autophagy-related gene; α-SMA, alpha smooth muscle actin; Cat, catalase; DHA, dihydroartemisinin; ERK1/2, extracellular regulated kinase1/2; GSH, glutathione; GSSG, glutathione disulfide; HSCs, hepatic stellate cells; IFN-γ, Interferon-γ; IL, Interleukin; JNK, c-Jun N-terminal kinase; LC3, microtubule-associated protein 1 light chain 3; MAPK, mitogen activated protein kinases; MitoPY1, Mito Peroxy Yellow 1; NAC, N-acetylcysteine; Na Py, Na-pyruvate; NLRP3, NACHT, LRR and PYD domains-containing protein 3; PDGF-βR, platelet derived growth factor-β receptor; ROS, reactive oxygen species; TGF-βR1, tumor growth factor-β receptor1; TNF-α, tumor necrosis factor α; TEM, transmission electron microscopy

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reported that inhibition of hepatic inflammation by blocking NLRP3/caspase-1 pathway improved the pathological changes of liver fibrosis. Recently, Liu et al. also showed that Dioscin alleviated alcoholic liver fibrosis by attenuating HSC activation via the TLR4/MyD88/NF-κB signaling pathway [16]. Besides, recent clinical and basic studies have confirmed that autophagy has been investigated for its involvement in inflammatory diseases [17]. Because of its unique feature to engulf part of cytoplasm in double-membrane cup-shaped structures, which further fuses with lysosomes, autophagy pathway and related proteins may play important roles in regulating immune response and controlling excessive inflammation [18]. Autophagy directly suppresses pro-inflammatory complexes, and also indirectly allows efficient clearance of damaged organelles or intracellular pathogenic microorganisms that both constitute potent inflammatory stimuli [19]. Naturally, regulatory networks that control autophagy activity are able to sense output signals from various inflammatory mediators-associated signaling, allowing a proper modulation of the process according to inflammation state.

Several lines of evidence indicate that reactive oxygen species (ROS) are early autophagy inducers upon various extreme conditions [20–22]. The proper ROS production mediates growth adaptation and survival, while the excessive accumulation of ROS could break cellular homeostasis, resulting in oxidative stress and mitochondrial dysfunction [20–22]. Meanwhile, oxidative stress could promote the formation of autophagy. Autophagy, in turn, may contribute to reduce oxidative damages by engulfing and degrading oxidized substance [23]. Interestingly, accumulating evidence suggests that mitogen activated protein kinases (MAPKs), including extracellular regulated kinase1/2 (ERK1/2), c-Jun N-terminal kinase1/2 (JNK1/2), and p38, are the critical kinases that regulate a variety of biological process, such as cell survival, apoptosis, and autophagy, which are regulated by intracellular ROS levels [24,25]. ROS can induce sustained activation of ERK1/2 by direct oxidation of its up-stream regulators [24]. ROS also can promote JNK1/2 and P38 activation by inhibition of their specific phosphatases [25]. Attractively, whether ROS-MAPKs-dependent autophagy contributes to the improvement of inflammatory microenvironment is worth to further study.

In the present study and for the first time, we elucidated the molecular mechanisms of DHA-induced anti-inflammatory effects in vivo and in vitro. We found that ROS-JNK1/2-dependent activation of autophagy was required for the induction of anti-inflammatory effect of DHA in liver fibrosis. Our study provides a rationale for the identification of DHA as a potent chemotherapeutic agent for the treatment of liver fibrosis.

2. Materials and methods

2.1. Chemicals and antibodies

Dihydroartemisinin (DHA), colchicine, glutathione (GSH), N-acetyl cysteine (NAC), hydrogen peroxide (H_2O_2), sodium pyruvate (Na Py), catalase (Cat), superoxide dismutase (SOD), platelet derived growth factor-BB (PDGF-BB), chloroquine(CQ), dimethyl sulfoxide (DMSO), Mito Peroxy Yellow 1 (MitoPY1), anti-rabbit IgG, and anti-mouse IgG were purchased from Sigma-Aldrich (St Louis, MO, USA). Oxidation sensitive 2′,7′-dichlorodihydrofluorescin diacetate (DCFH-DA) was purchased from Molecular Probes (Eugene, OR, USA). Dulbecco's modified essential medium (DMEM), Opti MEM medium, phosphate buffered saline (PBS), trypsin-EDTA, and fetal bovine serum (FBS) were bought from GIBCO BRL (Grand Island, NY, USA). Antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). GFP-RFP-LC3 (tf-LC3), Atg5 siRNA, JNK1/2 siRNA, negative control siRNA, Atg5 plasmid constructs and negative control vectors were purchased from Hanbio (Shanghai, China). MegaTran 1.0 transfection reagent was from OriGene (Rockville, MD, USA).

2.2. Animals

Male Sprague-Dawley rats weighing approximately 180–220 g were procured from Nanjing Medical University (Nanjing, China). All rats were bred and maintained in a specific pathogen-free condition. All experimental protocols were approved by the Institutional Animal Care and Use Committee of Nanjing University of Chinese Medicine (Nanjing, China). A mixture of CCl_4 (0.1 mL/100 g body weight) and olive oil [1:1 (w/v)] was used to induce liver fibrosis in rats. Forty-eight rats were randomly divided into six groups of eight animals each with comparable mean bodyweight. Rats of Group 1 were served as a vehicle control and intraperitoneally (i.p.) injected with olive oil. Rats of group 2 were i.p. injected with CCl_4. Rats of Groups 3, 4, and 5 were served as treatment groups and i.p. injected by CCl_4 and DHA with 3.5, 7 and 14 mg/kg, respectively. Rats of group 6 were served as a positive control and i.p. injected by CCl_4 and colchicine with 0.1 mg/kg. Rats of groups 2–6 were i.p. injected with CCl_4 every other day for 8 weeks. DHA was suspended in sterile phosphate buffered saline (PBS) and given once daily by intraperitoneal injection during weeks 5–8. Colchicine was suspended in sterile PBS and given once daily by gavage during weeks 5–8. At the end of the experiment, rats were sacrificed after anesthetization with an injection of 50 mg/kg pentobarbital. Blood was collected for ELISA assay. A small portion of the liver was removed for histopathological and immunohistochemical studies by fixation with 10% formalin and subsequent embedment with paraffin. The remaining liver was cut in pieces and rapidly frozen with liquid nitrogen for extraction of total RNA and hepatic proteins.

2.3. Cell isolation, cell culture conditions and drug treatment

Primary rat HSCs were isolated from male Sprague-Dawley rats weighing approximately 200–250 g (Nanjing Medical University, Nanjing, China) as described previously [26]. Isolated HSCs were cultured in DMEM with 10% fetal bovine serum, 1% antibiotics, and maintained at 37 ℃ in a humidified incubator of 5% CO_2 and 95% air. Cell morphology was assessed using an inverted micro-scope with a Leica Qwin System (Leica, Germany). HSCs at passages 2–4 were used in experiments. DHA was dissolved in DMSO at a concentration of 10 mM and was stored in a dark colored bottle at −20 ℃. The stock was diluted to required concentration with DMSO when needed. Prior to the DHA treatment, cells were grown to about 70% confluence, and then exposed to DHA at different concentrations (0–20 μM) for different period of time (0–24 h). Cells grown in a medium containing an equivalent amount of DMSO without DHA were served as control.

2.4. Plasmid transfection

Atg5 siRNA, JNK1/2 siRNA, negative control siRNA, Atg5 plasmid constructs, negative control vectors, and mRFP-GFP-LC3 plasmid were transfected into HSCs using MegaTran 1.0 transfection reagent according to manufacturer's instructions [27]. The transfection efficiency was confirmed by immunoblot analysis.

2.5. RNA isolation and real-time PCR

Total RNA was isolated by RNeasy Mini Kit followed by performing a qPCR using the QuantiTect SYBR Green PCR Kit (Qiagen, Valencia, CA. USA) in accordance to the manufacturer's instructions [14]. Actin levels were determined for normalization and fold change was calculated using 2^{-ddCt}. Primer Sequence available on request.

2.6. Immunoblotting analysis

Cells or tissue samples were lysed using a mammalian lysis buffer (Sigma St. Louis, MO, USA) and immunoblotting was performed according to the manufacturer's guidelines (Bio/Rad, Hercules, CA,