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Activation of autophagy is required for Oroxylin A to alleviate carbon tetrachloride-induced liver fibrosis and hepatic stellate cell activation

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ABSTRACT

Liver fibrosis is a reversible pathophysiological process correlated with intense repair and cicatrization mechanisms, and its end-stage cirrhosis is responsible for high morbidity and mortality worldwide. Interestingly, the use of natural products as a realistic option for the treatment of liver fibrosis has broadly been accepted. Oroxylin A, a safe and natural product, shows a wide range of pharmacological activities such as anti-inflammatory, anti-oxidant, and anti-tumor properties. However, the effects of Oroxylin A on liver fibrosis remain poorly understood. In the present study, we sought to determine the effect of Oroxylin A on carbon tetrachloride (CCl_4)-induced liver fibrosis, and to further examine the molecular mechanisms. We found that treatment with Oroxylin A markedly decreased the level of liver injury markers, alkaline phosphatase (ALP), aspartate aminotransferase (AST), and alanine aminotransferase (ALT), in a dose dependent manner. Moreover, Oroxylin A treatment remarkably inhibited extracellular matrix (ECM) deposition, and significantly down-regulated the mRNA and protein expression of liver fibrosis markers including α1(I)collagen, fibronectin, alpha-smooth muscle actin (α-SMA), PDGF-βR, and TGF-βR1 in CCl_4-induced murine model of liver fibrosis. Furthermore, experimental results in vitro showed that Oroxylin A treatment reduced the mRNA and protein expression of HSC activation markers, α-SMA, desmin, α1 (I) collagen, fibronectin, TGF-β, and TNF-α, in a dose dependent manner. Attractively, Oroxylin A treatment also markedly up-regulated the expression of autophagy makers, LC3-B, Atg3, Atg4, Atg5, Beclin1/Atg6, Atg7, Atg9, ATG12, and Atg14, and apparently reduced the expression of autophagy substrate p62 in both CCl_4-induced murine model of liver fibrosis and PDGF-BB-treated HSCs. Importantly, inhibition of autophagy by specific inhibitor 3-methyladenine (3-MA) completely abolished Oroxylin A-induced anti-fibrosis effect, indicating that activation of autophagy was required for Oroxylin A to alleviate liver fibrosis. Overall, these results provide novel implications to reveal the molecular mechanism of Oroxylin A-induced anti-fibrosis properties, by which points to the possibility of using Oroxylin A for the treatment of liver fibrosis.

1. Introduction

Liver fibrosis occurs as a compensatory response to the process of tissue repair in a wide range of chronic liver injures [1–3]. As the pathogenesis progresses without effective management, advanced liver fibrosis can seriously damage the normal function of the liver, trigger many serious complications, and finally lead to liver cirrhosis and hepatocellular carcinoma [1–3]. Importantly, given that liver fibrosis and early cirrhosis are dynamic and reversible, efforts to modulate the fibrogenesis process are important for preventive treatment of cirrhosis and hepatic failure [4]. Interestingly, natural products recently have attracted extensive attention in the prevention and treatment of liver fibrosis [5]. We previously have identified a number of natural products that can improve liver fibrosis, including curcumin [6], dihydroartemisinin [7], tetramethylpyrazine [8], and diallyl trisulfide [9]. In the current study, we aimed to evaluate the effect of Oroxylin A on liver fibrosis, and to further elucidate the underlying mechanisms.

Oroxylin A, a natural monoflavonoid isolated from *Scutellariae*

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radix, shows a wide range of pharmacological activities such as anti-inflammatory, anti-angiogenesis, anti-oxidant, and anti-tumor properties [10–14]. Ni T et al. recently reported that Oroxylin A inhibited the development and growth of colorectal cancer through reprogram of HIF-1α-modulated fatty acid metabolism [10]. Moreover, Shen L et al. showed that Oroxylin A treatment lowered the generation of Tregs in non-small cell lung cancer [11]. Furthermore, Han Q et al. found that treatment with Oroxylin A suppressed H_2O_2-induced oxidative stress in PC12 cells [12]. Besides, Zhou W et al. demonstrated that Oroxylin A treatment significantly reduced dextran sulfate sodium (DSS)-induced murine colitis through targeting NLRP3 inflammasome [13]. Additionally, Li J et al. revealed that Oroxylin A treatment inhibited cigarette smoke-induced oxidative stress and lung inflammation via activating Nrf2 signaling pathway [14]. Attractively, it is interesting to explore the effects of Oroxylin A on liver fibrosis.

Autophagy serves as an evolutionarily conserved and genetically programmed catabolic pathway, and is also a pivotal player in a variety of physiological and pathological processes in cells and tissues [15]. This degradative process is mediated by a group of proteins coded by autophagy-related genes (ATGs) that are widely conserved from yeasts to plants and mammals [15]. Large parts of cytoplasmic components including misfolded proteins, damaged organelles and excessive lipid droplets can be enwrapped by a double-membraned vesicle called an autophagosome. The formed autophagosome then fuses with a lysosome containing degrading enzymes and leads to the digestion of the autophagosome content [15]. It is now widely accepted that the appropriate autophagy level is considered as a self-protection mechanism, whereas excessive autophagy induction can lead to cell death. We previously reported that dihydroartemisinin can improve the inflammatory microenvironment of liver fibrosis by inducing autophagy pathway [7]. Furthermore, we also demonstrated that autophagy maintains the dynamic balance of lipid droplets in a ROS-Rab25-dependent mechanism in activated hepatic stellate cells (HSCs) [16]. Recently, we further revealed that excessive autophagy can lead to HSC senescence, and dihydroartemisinin can improve the pathological changes of liver fibrosis by inducing HSC senescence [17]. Additionally, Yue F et al. reported that orally administered spermidine can prolong lifespan and prevent liver fibrosis via inducing MAP1S-mediated autophagy pathway [18]. In addition, Qu Y et al. showed that exosomes derived from miR-181-5p-modified adipose-derived mesenchymal stem cells (ADSCs) down-regulated the expression of Stat3 and Bcl-2, and activated autophagy in the HST-T6 cells, which is the potential mechanism of its improvement of hepatic fibrosis [19]. Interestingly, whether Oroxylin A can improve liver fibrosis by regulating autophagy pathway is worth to further study.

In the current study, we elucidated the effect of Oroxylin A on liver fibrosis, and further determined the mechanism in this molecular context. We found that Oroxylin A alleviated CCl_4-induced liver fibrosis and HSC activation by modulating autophagy pathway. Our results indicate that Oroxylin A is expected to be a new choice for the treatment of liver fibrosis.

2. Materials and methods

2.1. Reagents and antibodies

Carbon tetrachloride (CCl_4) (#488488), dimethyl sulfoxide (DMSO) (#D2650), platelet derived growth factor-BB (PDGF-BB) (#P8147), chloroquine (CQ) (#C6628), and 3-methyl adenine (3-MA) (#M9281) were obtained from Sigma-Aldrich (St Louis, MO, USA). Fetal bovine serum (FBS) (#10099-141-FBS), trypsin-EDTA (#25200056), phosphate buffered saline (PBS) (#10010023), Opti MEM medium, and Dulbecco's modified essential medium (DMEM) (#12491) were purchased from GIBCO BRL (Grand Island, NY, USA). Oroxylin A was provided as a gift from Dr. Qinglong Guo (China Pharmaceutical University, Nanjing, China). Primary antibodies against α-SMA (#8456), LC3-B (#2775), LC3-A/B (#12741), p62 (#23214), PDGF-βR (#3169), and desmin (#4024) were purchased from Cell Signaling Technology (Danvers, MA, USA). Primary antibodies against TGF-βR1 (#ab31013), Atg5 (#ab108327), Atg6 (#ab62557), Atg12 (#ab155589), Atg14 (#ab139727), α1(I)collagen(#ab34710), and fibronectin (#ab2413) were purchased from Abcam Technology (Abcam, Cambridge, UK). Control siRNA and Atg5 siRNA were obtained from Hanbio (Shanghai, China).

2.2. Animals and experimental procedures

Male ICR mice aged approximately 6–8 weeks were from Yangzhou University (Yangzhou, China). All mice 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 olive oil (1:9 (v/v)) and CCl_4 (0.5 ml/100 g bodyweight) was used to induce liver fibrosis [17]. 40 mice were randomly divided into five groups of eight animals each. Mice of Group 1 were served as a vehicle control and intraperitoneally (i.p.) injected with olive oil. Mice of group 2 were served as a model group and i.p. injected with CCl_4. Mice of Groups 3, 4, and 5 were served as treatment groups, and i.p. injected with CCl_4 and Oroxylin A at 20, 30 and 40 mg/kg. Mice of groups 2–5 were i.p. injected with CCl_4 every other day for 8 weeks. Oroxylin A was given once daily by intraperitoneal injection during weeks 5–8. At the end of the experiment, blood samples and liver tissues were collected for subsequent experiments.

2.3. Histological analysis

Liver slices of 4-μm thick were prepared and stained with Sirius Red, Masson, and Hematoxylin and eosin (H&E) using standard methods [17]. H&E, Sirius Red, and Masson-stained areas from 10 fields were quantified with Image J software (NIH, Bethesda, MD, USA).

2.4. Hepatic enzyme measurement

Serum ALT, AST, and ALP levels were determined using enzyme-linked immunosorbent assay methods according to the kit protocols [8] (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

2.5. Cell isolation and cell culture conditions

Primary mouse HSCs were isolated from male ICR mice (Yangzhou University, Yangzhou, China) as described [17]. Isolated HSCs were cultured in DMEM with 10% FBS and 1% antibiotics, and maintained at 37 °C in a humidified incubator of 95% air and 5% CO_2.

2.6. RNA isolation and Real-time PCR

The total RNA was isolated from HSCs and fibrotic tissue using TRIzol (Life Technologies, Waltham, MA). Real-time PCR was performed using the QuantiTect SYBR Green PCR Kit (Qiagen, Valencia, CA. USA) in accordance to the manufacturer's instructions [17]. Actin levels were determined for normalization and fold change was calculated using 2^{-ddCt}. Primer sequences are available on request.

2.7. Western blot analysis

Tissue or cells samples were lysed using mammalian lysis buffer (Sigma St. Louis, MO, USA) and Western blot was performed as per the manufacturer's guidelines [17] (Bio/Rad, Hercules, CA, USA). Densitometry analysis was performed using Image J software (NIH, Bethesda, MD, USA).