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Activation of imidazoline I_1 receptor by moxonidine regulates the progression of liver fibrosis in the Nrf2-dependent pathway

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ABSTRACT

Imidazoline I_1 receptor (I_1R) has been recognized as a promising target in the treatment of many diseases, but little is known about its function in liver fibrogenesis. This study aimed to investigate the effect of I_1R activation on the development and progression of liver fibrosis. The results showed that I_1R expression was decreased in the livers of both patients and mice with liver fibrosis, and in TGF-β-treated hepatic stellate cells (HSCs). Activation of I_1R by moxonidine (MOX) significantly inhibited the progression of liver fibrosis in carbon tetrachloride-induced mice and attenuated the activation of HSCs and kupffer cells. MOX also suppressed the activation of TLR4/NF-κB and TGF-β/Smad signaling, however, knockdown of I_1R abrogated the inhibitory effects of MOX. Additionally, MOX activated Nrf2 signaling *in vivo* and *in vitro*, but knockout or knockdown of Nrf2 ameliorated the anti-inflammatory and anti-fibrotic effects of MOX. Taken together, activation of I_1R negatively regulates the progression of liver fibrosis in the Nrf2-dependent pathway, which suggests that specifically targeting I_1R may be a potential therapeutic strategy for the treatment of liver fibrosis.

1. Introduction

Liver fibrosis is a result of advanced liver diseases such as viral hepatitis, non-alcoholic steatohepatitis and alcoholic liver disease [1]. It is characterized by replacement of live tissue by fibrosis and regenerative nodules; these changes lead to loss of liver function [2]. Without appropriate treatment, liver fibrosis eventually leads to cirrhosis or liver cancer. Despite the high incidence of liver fibrosis worldwide, current anti-fibrotic therapies are still unsatisfactory.

Recent studies have demonstrated a direct relationship between the inflammation and the progress of live fibrosis [3]. In chronic liver injury, frequent and uncontrolled inflammatory processes result in the constant activation of Kupffer cells (KCs), which produce a variety of pro-inflammatory and pro-fibrotic cytokines, such as transforming growth factor-β (TGF-β) and platelet-derived growth factor (PDGF) [4]. In response to these stimuli, the quiescent hepatic stellate cells (HSCs) proliferate and transdifferentiate into fibrogenic myofibroblast-like cells, which produce ECM component proteins including collagen type-I, α-smooth muscle actin (α-SMA), TGF-β, and matrix metalloproteinase (MMP), which all contributes to liver fibrosis [5]. Among these proteins, TGF-β is considered to the key activator in mediating the deposition of ECM components in liver through the canonical TGF-β/Smad pathway [6]. Therefore, inhibition of KCs and HSCs activation was considered to be an important therapeutical strategy to attenuate hepatic fibrosis.

The nuclear factor kappa-B (NF-κB), a family of transcription factors, regulates the induction and resolution of inflammation [7]. NF-κB activation has been demonstrated with the survival of HSCs and the development of liver fibrosis [8]. TLR4, which can recognize multiple danger signals, is expressed on many cell types in the liver, including KCs, HSCs and hepatocytes [9]. TLR4 activation in KCs and HSCs can trigger the myeloid differentiation factor 88 (MyD88)-dependent pathway and lead to the rapid activation of NF-κB, which causes the release of pro-inflammatory cytokines including interleukin (IL)-1, IL-6 and TNF-α as well as profibrogenic cytokines including TGF-β [10]. Thus, inhibition of NF-κB may deserve investigations for the treatment of hepatic fibrosis.

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Oxidative stress has been reported to play a predominant pro-fibrogenic role in liver fibrosis [11]. Nuclear erythroid 2-related factor 2 (Nrf2) is an essential transcription factor that regulates an array of detoxifying and antioxidant defense genes expression in the liver [12]. Xu et al. reported that knockout of Nrf2 could aggravate CCl_4-induced liver inflammation and fibrosis [13]. Several pharmacological studies showed that increased Nrf2 expression and nuclear translocation induced by natural products could inhibit experimental hepatic fibrosis [14–16]. All these discoveries suggested that Nrf2 might be a novel therapeutic target for liver fibrosis.

The imidazoline receptors are available throughout the body and they have been reported to possess various functions [17]. Based on the differences in biological characters, the imidazoline receptors were divided into three subunits: imidazoline I_1 receptor (I_1R), imidazoline I_2 receptor (I_2R) and imidazoline I_3 receptor (I_3R). I_1R is involved in the regulation of behavioral disorders and the pathogenesis of many diseases, such as hypertension, obesity, and Huntington's disease [18–20]. Modulators of I_1R have been reported to exhibit multiple pharmaceutical potentials including suppressing inflammation, anti-diabetes and cytoprotection [21,22]. However, to date, the potential role of I_1R in treatment of liver fibrosis has not been reported.

Moxonidine (MOX) is a centrally-acting sympatholytic imidazoline compound that shows high affinity to I_1R [23]. MOX is widely used as an anti-hypertensive drug, especially in treating hypertension complicated with left ventricular hypertrophy (LVH) [24,25]. Treatment of hypertensive rats with MOX can reduce left ventricular IL-1β, TNF-α and IL-6 levels, as well as inhibit inducible nitric oxide synthase (iNOS) expression through Akt and p38 MAPK signaling pathways [26]. What's more, MOX can attenuate noradrenaline-induced cardiomyocyte apoptosis and fibroblast proliferation, and thus restrain myocardial fibrosis and ventricular remodeling [27]. These results suggested that MOX might be a potential anti-inflammatory and anti-fibrotic agent.

The current study discovered that I_1R expression is down-regulated in patients and mice with liver fibrosis. Activation of I_1R by MOX reduced CCl_4-induced liver fibrosis in mice through inhibiting TLR4/NF-κB and TGF-β/Smad signaling pathway. By utilizing I_1R and Nrf2 knockdown cells and Nrf2-knockout (KO) mice, we elucidated that I_1R activation by MOX negatively regulates liver fibrosis in the Nrf2-dependent pathway. In conclusion, our studies have revealed the anti-liver fibrosis property of I_1R and uncovered underlying mechanisms.

2. Materials and methods

2.1. Reagents

Moxonidine (MOX, Y0000226), Lipopolysaccharides (LPS, L2880), transforming growth factor-β (TGF-β, SRP0300) were purchased from Sigma-Aldrich (St.Louis, MO, USA). Carbon tetrachloride (CCl_4) was purchased from Xilong Chemistry Plant (Shantou, China). Anti-Nrf2 (ab62352), anti-HO-1(ab68477), anti-NQO-1 (ab34173), anti-α-SMA (ab5694) and anti-Col1 (ab84956) were purchased from Abcam (Cambridge, UK). Anti-p-IκB (9246), anti-IκB (4814), anti-p-p65 (3033) and anti-p65 (8242) were obtained from Cell Signaling Technology (CA, USA). Anti-Smad2 (sc-101153), anti-p-Smad2 (sc-101801), anti-Smad3 (sc-101154), anti-p-Smad3 (sc-130218), anti-keap1 (sc-365626), anti-iNOS (sc-8310), anti-COX2 (sc-23984), anti-GAPDH (sc-293335) and anti-PCNA (sc-9857) were obtained from Santa Cruz (CA, USA). IL-1β, IL-6, TNF-α and TGF-β enzyme-linked immunosorbent assay (ELISA) kits were obtained from Boster Biotechnology (Wuhan, China). Total Superoxide Dismutases Assay Kit and glutathione perosidase (GPx) Assay Kit were purchased from Beyotime Biotechnology (Shanghai, China).

2.2. Human liver samples

The study included 24 unrelated subjects who had undergone a liver biopsy at the Second Affiliated Hospital of Chongqing Medical University. 16 subjects have proved as liver fibrosis histopathologically and 8 were control subjects. Control liver samples were collected from living donors who received liver resection due to liver hemangioma or hepatic cyst. Each participant provided written informed consent after receiving an explanation of the nature and potential risks of the study. All procedures that involved human samples were approved by the Second Affiliated Hospital of Chongqing Medical University and conducted in accordance with the Declaration of Helsinki.

2.3. Animals

Male C57BL/6 mice (weight, 18–22 g; age, 6 weeks) were purchased from the Center of Experimental Animals of Chongqing Medical University (Chongqing, China). Nrf2-/- (Nrf2 KO; $Nfe^{2l2tm1Ywk}$/J) mice and Nrf2+/+ (Nrf2 WT; C57BL/6J) mice were purchased from Jackson Laboratory (No. 017009; Bar Harbor, Maine, USA). The mice were housed in an environmentally controlled room (temperature, 24 ± 1° C; humidity, 40–80%) under a 12 h dark/light cycle with free access to food and water. All animal experimentation described in the present study was performed according to the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (NIH Publication, 8th edition, 2011). The study was approved by the committee on the Ethics of Animal Experiments of Chongqing Medical University.

After 1 week of acclimatisation, mice were randomly divided into four groups (n = 8 per group) as follows: control group, MOX group, CCl_4 group, and CCl_4 + MOX group. Eight mice in CCl_4 group or CCl_4 + MOX group received intra-peritoneal injection of 10% CCl_4 dissolved in olive oil (0.02 ml/g) biweekly. Eight mice in MOX group or CCl_4 + MOX group were treated with MOX at a dose of 3 mg/kg body weight biweekly. Eight control mice received an isovolumetric dose of olive oil as that of the CCl_4. The body weight of mice were measured every week. At 8 week, the mice were sacrificed. The weight of livers were measured, and the liver tissues were harvested and stored at −80 °C for further experiments.

2.4. Isolation of hepatic satellite cells from mice liver tissue

Hepatic satellite cells were isolated and purified using a modified version of the collagenase perfusion method as previously described. Briefly, mice were injected with 10% chloral hydrate anesthesia (ip). The portal venous system was intubated and perfused first with a calcium-free HEPES buffer and then with HEPES buffer containing collagenase (100 units/ml; type IV; Sigma). Then the liver was excised, minced and filtered through 0.8 mm sterile cotton gauze, placed over a stainless steel mesh to release the hepatocytes. Then the sample was centrifuged through an 18% Nycodenz gradient (Sigma, St. Louis, MO, USA) to obtain the high-purity HSCs at the gradient interface. The concentration of HSCs was adjusted to 1×10^9 cells/L in DMEM containing 20% FBS and then inoculated in a plastic culture flask.

2.5. Cell culture and transfection

RAW264.7 cells and LX2 cells were purchased from MEIXUAN Biotechnology Company (Shanghai, China) and cultured at 37 °C in a normoxic atmosphere containing 5% CO_2 with high-glucose