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Isorhamnetin attenuates liver fibrosis by inhibiting TGF-β/Smad signaling and relieving oxidative stress

Ji Hye Yang [a,1], Sang Chan Kim [b,1], Kyu Min Kim [a], Chang Ho Jang [a], Sam Seok Cho [a], Seung Jung Kim [a], Sae Kwang Ku [b], Il Je Cho [b], Sung Hwan Ki [a,*]

[a] College of Pharmacy, Chosun University, Gwangju 61452, Republic of Korea
[b] MRC-GHF, College of Korean Medicine, Daegu Haany University, Gyeongsan, Gyeongsangbuk-do 38610, Republic of Korea

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ABSTRACT

Hepatic fibrosis is considered integral to the progression of chronic liver diseases, leading to the development of cirrhosis and hepatocellular carcinoma. Activation of hepatic stellate cells (HSCs) is the dominant event in hepatic fibrogenesis. We investigated the ability of isorhamnetin, the 3′-*O*-methylated metabolite of quercetin, to protect against hepatic fibrosis *in vitro* and *in vivo*. Isorhamnetin inhibited transforming growth factor (TGF)-β1-induced expression of α-smooth muscle actin (α-SMA), plasminogen activator inhibitor-1 (PAI-1), and collagen in primary murine HSCs and LX-2 cells. The TGF-β1- or Smad-induced luciferase reporter activity of Smad binding elements was significantly decreased by isorhamnetin with a concomitant decrease in Smad2/3 phosphorylation. Isorhamnetin increased the nuclear translocation of Nrf2 in HSCs and increased antioxidant response element reporter gene activity. Furthermore, isorhamnetin blocked TGF-β1-induced reactive oxygen species production. The specific role of Nrf2 in isorhamnetin-mediated suppression of PAI-1 and phosphorylated Smad3 was verified using a siRNA against Nrf2. To examine the anti-fibrotic effect of isorhamnetin *in vivo*, liver fibrosis was induced by CCl_4 in mice. Isorhamnetin significantly prevented CCl_4-induced increases in serum alanine transaminase and aspartate transaminase levels, and caused histopathological changes characterized by decreases in hepatic degeneration, inflammatory cell infiltration, and collagen accumulation. Moreover, isorhamnetin markedly decreased the expression of phosphorylated Smad3, TGF-β1, α-SMA, and PAI-1. Isorhamnetin attenuated the CCl_4-induced increase in the number of 4-hydroxynonenal and nitrotyrosine-positive cells, and prevented glutathione depletion. We propose that isorhamnetin inhibits the TGF-β/Smad signaling pathway and relieves oxidative stress, thus inhibiting HSC activation and preventing liver fibrosis.

1. Introduction

Liver fibrosis represents a significant and globally prevalent healthcare problem, ultimately leading to end stage of liver diseases such as cirrhosis and hepatocellular carcinoma (Bataller and Brenner, 2005). The progression of liver fibrosis is a dynamic process involving many cells in the hepatic sinusoids. It is characterized by disturbed hepatic architecture and excessive deposition of extracellular matrix (ECM), mainly collagen. Activation of hepatic stellate cells (HSCs) in response to hepatic injury is the central event in hepatic fibrogenesis, and entails the transformation of quiescent vitamin-A rich cells into proliferative, fibrogenic, and contractile myofibroblasts (Yin et al., 2013).

Transforming growth factor (TGF)-β is a key mediator of HSC activation and ECM accumulation that leads to fibrosis. The release and activation of TGF-β stimulates the synthesis of various ECM components such as collagen and fibronectin (Casini et al., 1993; Ramadori et al., 1992). In addition, TGF-β decreases ECM degradation by increasing the production of protease inhibitors such as plasminogen activator inhibitor-1 (PAI-1) (Sawdey and Loskutoff, 1991). TGF-β expression has been significantly correlated with progression and is used as a non-invasive biomarker of hepatic fibrosis in patients

Abbreviations: ALT, alanine transaminase; ARE, antioxidant response element; AST, aspartate transaminase; COL1A1, collagen 1A1; ECM, extracellular matrix; GCL, glutamate-cysteine ligase; GSH, glutathione; 4-HNE, 4-hydroxynonenal; HO-1, heme oxygenase-1; HSCs, hepatic stellate cells; Nrf2, NF-E2-related factor 2; PAI-1, plasminogen activator inhibitor-1; PPARγ, peroxisome proliferator-activated receptor gamma; SBEs, Smad binding elements; SMA, smooth muscle actin; TAK1, TGF-β-activated kinase 1; TGF, transforming growth factor

* Corresponding author.
E-mail address: shki@chosun.ac.kr (S.H. Ki).
[1] These authors contributed equally to the work.

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(Anscher et al., 1993; Nagy et al., 1991). Once activated, TGF-β binds to a heteromeric complex of type I and type II serine/threonine kinase receptors that phosphorylate and activate receptor-regulated Smads (R-Smads, e. g., Smad2 and Smad3) (Inagaki and Okazaki, 2007). Nuclear translocation of activated Smads is essential for TGF-β-dependent gene regulation. TGF-β receptors also activate Smad-independent pathways that not only regulate Smad signaling, but also allow Smad-independent TGF-β responses (Derynck and Zhang, 2003).

Recently, we successfully isolated isorhamnetin from water dropwort and reported its anti-inflammatory and antioxidant effects. Isorhamnetin decreased inflammatory gene induction and cytokine expression in macrophages (Seo et al., 2014; Yang et al., 2013a). Treatment with isorhamnetin specifically suppressed NF-κB activation, and significantly reduced acute carrageenan-induced inflammation in mice. Moreover, isorhamnetin can decrease the LPS-induced production of reactive oxygen species and apoptosis through the induction of basal expression of heme oxygenase-1 (HO-1) in macrophages (Seo et al., 2014). We have also reported that isorhamnetin can increase the nuclear translocation of Nrf2 and its target gene expression in hepatocytes (Yang et al., 2014a). Furthermore, isorhamnetin attenuated the reactive oxygen species production, mitochondrial dysfunction, and cell death induced by *tert*-butyl hydroperoxide or a combination of arachidonic acid and iron (Dong et al., 2014; Yang et al., 2014a). However, the role of isorhamnetin in liver fibrosis has not yet been elucidated.

Therefore, the aim of the current study was to evaluate the effects of isorhamnetin on liver fibrosis and HSC activation. Our results demonstrate that isorhamnetin inhibits TGF-β/Smad signaling and relieves oxidative stress, thus inhibiting the activation of HSCs and protecting against liver fibrosis.

2. Materials and methods

2.1. Materials

Antibodies against Nrf2 and Lamin A/C were provided by Santa Cruz Biotechnology (Santa Cruz, CA). PAI-1 was obtained from BD Biosciences (Becton, Dickinson and Company, NJ). Phospho-Smad2, Smad2, phospho-Smad3, and Smad3 antibodies were purchased from Cell Signaling (Danvers, MA). HO-1 antibody was provided by Enzo Life Sciences (Plymouth Meeting, PA), and GCL antibody was provided by Abcam (Cambridge, MA). Horseradish peroxidase-conjugated goat anti-rabbit and anti-mouse antibodies were purchased from Invitrogen (Carlsbad, CA). The α-smooth muscle actin (α-SMA) and β-actin antibodies were obtained from Sigma Chemicals (St. Louis, MO), and TGF-β1 was purchased from R&D Systems (Minneapolis, MN).

2.2. Preparation of isorhamnetin

Isorhamnetin was isolated and prepared as described previously (Yang et al., 2013a). Briefly, air-dried stems and leaves of *Oenanthe javanica* were extracted three times with MeOH and then concentrated. The methanolic extract was suspended in water and partitioned successively with $CHCl_3$ and *n*-BuOH. The *n*-BuOH fraction was adsorbed onto a silica gel column (15 × 80 cm, 70–230 mesh), and eluted with $CHCl_3$ followed by a gradient of $CHCl_3$-MeOH. The $CHCl_3$-MeOH (25:1) fraction was concentrated to give a dark brown residue. The obtained residue was further fractionated by silica-gel column chromatography using a gradient of *n*-hexane-EtOAc [20:1 (5 L), 10:1 (3 L), 4:1 (3 L), 1:1 (2 L), each fraction volume 250 ml]. Fractions 35–40 from this column were combined and evaporated to give an isorhamnetin mixture, and then successively washed with diethylether for further purification. An ultra performance liquid chromatography system equipped with a BEH C18 column (1.7 μm, 2.1 mm × 100 mm) and photodiode array detector (Waters ACQUITYTM, Milford, MA) was used to evaluate the purity of the isorhamnetin. The output signal of the detector was recorded using an Empower Data System. The structure of the purified isorhamnetin was confirmed by spectroscopic analyses including HPLC-ESI-MS (Agilent 6120 LC/MS system, Agilent Technologies, Palo Alto, CA) and NMR spectroscopy (Fig. 1A). ^1H- and ^{13}C NMR spectroscopy was carried out in a JEOL ECA-500 spectrometer (Tokyo, Japan) operating at 500 MHz and 125 MHz, respectively. The dimethylsulfoxide solvent signal was used as an internal standard.

2.3. Cell culture

LX-2 cells (immortalized human activated HSCs) were kindly provided by Dr. S. L. Friedmann (Mount Sinai School of Medicine, New York, NY). Cells were maintained in DMEM containing 10% FBS, 50 units/ml penicillin/streptomycin at 37 °C in a humidified 5% CO_2 atmosphere.

2.4. Animals

The protocols for the animal studies were approved by the Animal Care and Use Committee of Chosun University. Male ICR mice (6 weeks old) were obtained from Oriental Bio (Sung-nam, Korea) and acclimatized for 1 week. Mice ($n=6$ per group) were housed at 20 ± 2 °C with 12 h light/dark cycles and a relative humidity of 50 ± 5% under filtered, pathogen-free air, with food (Purina, Korea) and water available ad libitum.

2.5. CCl_4-induced hepatic fibrosis

To induce liver fibrosis, CCl_4 dissolved in olive oil (10%) was injected intraperitoneally (0.5 mg/kg) into the mice three times per week for 4 weeks as described previously (Ki et al., 2013). Isorhamnetin was administered orally 5 days per week.

2.6. Blood chemistry

Plasma alanine transaminase (ALT) and aspartate transaminase (AST) levels were analyzed using spectrophotometric diagnostic kits (Young-Dong Diagnostics, Yongin, Korea).

2.7. Histology and immunohistochemistry

Livers were excised, fixed in 10% neutral buffered formalin, then embedded in paraffin, sectioned (3–4 μm), and stained with hematoxylin and eosin for general observation or with Sirius red for visualizing collagen fibers. For more detailed changes, the percentage area of degenerative regions (% mm^{-2}) in the lateral lobes showing centrilobular necrosis, congestion, and inflammatory-cell infiltration into hepatic lobules was calculated using a computer-based image analyzer (*i*Solution FL ver 9.1, IMT *i*-solution Inc., Vancouver, Quebec, Canada). The percentage area with collagen fibers around central veins was expressed as % mm^{-2} of hepatic parenchyma with Sirius red staining. In addition, the number of hepatocytes showing any degenerative changes (mainly necrosis, acute cellular swelling (ballooning), and severe fatty changes) and inflammatory-cell infiltrates were also calculated using a digital image analyzer, and expressed as cells per 1000 hepatocytes and cells mm^{-2} of liver parenchyma, according to our previously established methods (Yang et al., 2015).

Immunohistochemical staining using TGF-β1 (Novus Biologicals, Littleton, CO), phospho-Smad3 (Abcam), 4-hydroxynonenal (4-HNE) (Abcam), or nitrotyrosine (Millipore, Temecula, CA) antibody was conducted as previously described (Park et al., 2012). Briefly, tissue sections were deparaffinized and then pretreated with 10 mM citrate buffer (pH 6.0). After inactivation of endogenous peroxidase and blocking with normal horse serum (Vector Labs Inc., CA, USA), tissue