Pharmacological Research xxx (2017) xxx–xxx

Contents lists available at ScienceDirect

Pharmacological Research

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

Perspective

Carnosol-mediated Sirtuin 1 activation inhibits Enhancer of Zeste Homolog 2 to attenuate liver fibrosis

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ARTICLE INFO

Article history:
Received 14 September 2017
Received in revised form 20 October 2017
Accepted 25 October 2017
Available online xxx

Chemical compounds studied in this article:
carnosol (PubChem CID:442009)
resveratrol (PubChem CID:445154)

Keywords:
liver fibrosis
EZH2
SIRT1
carnosol
aHSCs
EMT

ABSTRACT

Quiescent hepatic stellate cell (HSC) activation and subsequent conversion into myofibroblasts is the central event in hepatic fibrosis pathogenesis. Epithelial–mesenchymal transition (EMT), another vital participant in liver fibrosis, has the potential to initiate HSC activation, which promotes abundant myofibroblast production. Previous studies suggest that Enhancer of Zeste Homolog 2 (EZH2) plays a significant role in myofibroblast transdifferentiation; however, the underlying mechanisms remain largely unaddressed. Carnosol (CS), a compound extracted from rosemary, displays multiple pharmacological activities. This study aimed to investigate the signaling mechanisms underlying EZH2 inhibition and the anti-fibrotic effect of CS in liver fibrosis. We found that CS significantly inhibited CCl_4- and TGFβ1-induced liver fibrosis and reduced both HSC activation and EMT. EZH2 knockdown also prevented these processes induced by TGFβ1 in HSCs and AML-12 cells. Interestingly, the protective effect of CS was positively associated with Sirtuin 1 (SIRT1) activation and accompanied by EZH2 inhibition. SIRT1 knockdown attenuated the EZH2 inhibition induced by CS and increased EZH2 acetylation, which enhanced its stability. Conversely, upon TGFβ1 exposure, SIRT1 activation significantly reduced the level of EZH2 acetylation; however, EZH2 overexpression prevented the SIRT1 activation that primed myofibroblast inhibition, indicating that EZH2 is a target of SIRT1. Thus, SIRT1/EZH2 regulation could be used as a new therapeutic strategy for fibrogenesis. Together, this study provides evidence of activation of the SIRT1/EZH2 pathway by CS that inhibits myofibroblast generation, and thus, CS may represent an attractive candidate for anti-fibrotic clinical therapy.

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1. Introduction

Liver fibrosis is a reversible course in many chronic liver diseases but can lead to mortality via the development of cirrhosis. Central to the pathogenesis of the disease is hepatic stellate cell (HSC) activation and subsequent conversion into myofibroblasts, which increases the capacity of cells for excessive extracellular matrix (ECM) production. However, the relevant mechanisms in this pathological process remain underexplored, and thus far, there is not an effective treatment for liver fibrosis. It has been reported that the MeCP2/EZH2 pathway promotes myofibroblast activation via inhibition of PPARγ in hepatic fibrosis [1,2]. EZH2 is a core catalytic subunit of polycomb repressive complex 2 (PRC2) that primarily catalyzes trimethylation of lysine 27 on histone H3 (H3K27me3), which is important for direct repression of target genes [3,4]. It has been recently demonstrated that liver fibrosis can be inhibited by 3-deazaneplanocin A (DZNep), an efficient EZH2 inhibitor [5]. However, the molecular mechanism by which EZH2 regulates liver fibrosis remains largely unclear.

EZH2 is known to be frequently overexpressed in solid tumors, and enhanced EZH2 levels are correlated with epithelial-

Abbreviations: DZNep, 3-Deazaneplanoci A; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CS, carnosol; CCl_4, carbon tetrachloride; H3K27me3, catalyzes trimethylation of lysine 27 on histone H3; CTGF, connective tissue growth factor; EZH2, Enhancer of Zeste Homolog 2; EMT, epithelial-mesenchymal transition; ECM, extracellular matrix; H&E, hematoxylin and eosin staining; HSC, hepatic stellate cell; PPARγ, peroxisome proliferator-activatied receptor γ; PRC2, polycomb repressive complex 2; siRNA, small interfering RNA; SIRT1, Sirtuin 1; TGFβ1, transforming growth factor β1; TIMP1, tissue inhibitor of metalloproteinase 1; α-SMA, αsmooth muscle actin.

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https://doi.org/10.1016/j.phrs.2017.10.013
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mesenchymal transition (EMT) [6,7], which plays a vital role in the pathogenesis of liver fibrosis [8]. In this pathological progress, epithelial cells lose their phenotype and acquire mesenchymal characteristics and increased migration capacity [9]. Several studies have verified that hepatocytes, cholangiocytes and HSCs have the potential to undergo EMT, which is an important process that stimulates myofibroblast generation [10–12]. Thus, we reasoned that EZH2 may promote EMT in hepatic fibrosis, which could induce HSC activation and subsequent myofibroblast generation.

Post-translational modifications of EZH2 are critical in modulating its functions and silencing target genes. Acetylation is an important form of EZH2 that controls its stability and biological functions. EZH2 is primarily deacetylated by Sirtuin 1 (SIRT1), a NAD-dependent class III histone deacetylase, and deacetylation has been shown to inhibit lung cancer cell migration and invasion in lung adenocarcinoma [13]. SIRT1 was recently confirmed to play an important role in modulating liver fibrosis [14–16]. Therefore, we inferred that SIRT1 may inhibit EZH2 through deacetylation, which affects the stability of EZH2 and prevents myofibroblast generation in liver fibrosis.

SIRT1 can be activated by polyphenolic compounds, such as resveratrol and quercetin [17]. Carnosol (CS), a phenolic diterpene compound found in Rosmarinus officinalis, has been shown to have anti-inflammatory, anti-oxidant, anti-adipogenic, and anti-cancer properties by modulating relevant molecular pathways [18–22]. Because CS shares structural similarity with the above polyphenols, we postulated that CS may activate SIRT1, inhibit the activation of HSCs and reverse EMT in liver fibrosis.

The aims of this study were as follows: (1) to investigate whether SIRT1-mediated EZH2 deacetylation can modulate the stability of EZH2 during the progression of liver fibrosis and (2) to explore whether CS can protect against liver fibrosis via the SIRT1/EZH2 pathway.

2. Materials and methods

2.1. Reagents and experimental animals

CS (90% purity) was purchased from Hainan Super Biotech Co., Ltd. (Hainan, China) and dissolved in olive oil. Carbon tetrachloride (CCl_4) was obtained from the Department of Assets Management of Dalian Medical University (Dalian, China). Male Sprague-Dawley rats (180–220 g) were purchased from the Experimental Animal Center of Dalian Medical University (Dalian, China). The rats were kept in standard laboratory conditions, allowed to adapt to the new environment for one week, and then randomly divided into 5 groups: (1) control; (2) control + CS (30 mg kg^{-1} day^{-1}); (3) CCl_4; (4) CCl_4 + CS (15 mg kg^{-1} day^{-1}); and (5) CCl_4 + CS (30 mg kg^{-1} day^{-1}). We generated CCl_4-induced liver fibrosis via intraperitoneal injection of CCl_4 (0.5 mg kg^{-1}, diluted 1:10 in olive oil) twice weekly in the corresponding rats [39]. CS and olive oil were then administered via gavage every day. After 4 weeks, all animals were euthanatized, and liver and blood samples were harvested for further analysis. All procedures were performed in compliance with the Institute's guidelines and with the Guide for the Care and Use of Laboratory Animals. The study was approved by the institutional animal care committee of Dalian Medical University.

2.2. Cell isolation, culture and treatment

HSCs were isolated as previously described [5]. Briefly, HSCs were isolated from normal livers of 350 g adult male Sprague-Dawley rats by sequential perfusion with collagenase and pronase, followed by discontinuous density centrifugation in 11.5% Optiprep (Life Technologies). HSCs were cultured on the Collagen Type 1 cellcoat plates in DMEM supplemented with penicillin 100 U/mL, streptomycin 100 mg/mL and 10% (v/v) fetal bovine serum (FBS) with 5% CO2 at 37 °C. Activated HSCs were generated by continuous culture of freshly isolated cells on the Collagen Type 1 cellcoat plates for 7 days. AML-12 and LX-2 cell lines were purchased from American Type Culture Collection (Rockefeller, USA). AML-12 cells were grown in DMEM/F12 (1:1) containing 10% (v/v) FBS, 0.005 mg mL-1 insulin, 0.005 mg mL^{-1} transferrin, 0.005 mg mL-1 selenium and 40 ng mL-1 dexamethasone in a humidified incubator with 5% CO2 at 37 °C. LX-2 cells were grown in DMEM containing 10% (v/v) FBS in a humidified incubator with 5% CO2 at 37 °C. DMEM, DMEM/F12 (1:1), FBS, insulin, transferrin, selenium and dexamethasone are all Invitrogen products that were obtained from Life Biotechnologies (Carlsbad, CA, USA).

CS (98% purity) was purchased from Sigma-Aldrich (St. Louis, MO, USA), and TGFβ1 was obtained from PeproTech (Princeton, USA). Resveratrol (98% purity) was purchased from Shanghai Winberb Medical Science Co., Ltd. (Shanghai, China). To establish the *in vitro* model of fibrosis, the cells were pretreated with 10 μM CS or 10 μM resveratrol for 6 h before being exposed to TGFβ1 (4 ng mL^{-1}) for another 12 h.

2.3. Biochemical assays

Serum was separated from blood samples after centrifugation at 3000 rpm for 15 min. The serum ALT and AST levels were determined with commercial kits from Nanjing Jiancheng Bioengineering Institute (Nanjing, China) according to the manufacturers' instructions.

2.4. Liver histology

The left lateral segment of the liver lobes was isolated and fixed in 4% paraformaldehyde. Paraffin-embedded liver tissue samples were cut into 3- to 5-μm-thick sections for hematoxylin and eosin (H&E) staining and Masson trichrome staining as described previously [40]. Then, sections were observed with light microscopy.

2.5. Western blot analysis

Equal amounts of proteins were separated by 10% SDS-PAGE and transferred to PVDF membranes (Millipore, Bedford, MA, USA). After blocking in 5% skim milk for 2 h at 37 °C, the membranes were immunoblotted with primary antibodies that were specific for SIRT1, acetyl-lysine (SIRT1, ab110304, and acetyl-lysine, ab21623, both from Abcam Ltd., Cambridge, UK), EZH2, Collagen I, α-SMA, E-Cadherin, Vimentin and β-actin (EZH2, 21800-1-AP; Collagen I, 14695-1-AP; α-SMA, 23081-1-AP; E-Cadherin,20874-1-AP; Vimentin, 10366-1-AP and β-actin, 66009-1-lg all from Proteintech, Wuhan, China) overnight at 4 °C. Then, the blots were incubated at 37 °C with suitable secondary antibodies. The membranes were exposed to enhanced chemiluminescence-plus reagents (Advansta Corporation, CA, USA), and the emitted light was captured with a BioSpectrum 410 multispectral imaging system equipped with a Chemi 410 HR camera. The results were analyzed with Gel-Pro Analyzer Version 4.0 (Media Cybernetics, MD, USA).

2.6. Immunoprecipitation analysis of acetylated EZH2

A sufficient amount of anti-EZH2 antibody (Proteintech, Wuhan, China) was added to 50 μL of binding buffer with the already prepared Protein A/G Magnetic Beads (Bimake, Selleck Chemical, Houston, USA), and the mixture was gently rotated at 4 °C for 4 h. Next, the tubes were placed on a magnet for 5–10 s, and the supernatant was removed. The bead-Ab complexes were resuspended in

Please cite this article in press as: H. Zhao, et al., Carnosol-mediated Sirtuin 1 activation inhibits Enhancer of Zeste Homolog 2 to attenuate liver fibrosis, Pharmacol Res (2017), https://doi.org/10.1016/j.phrs.2017.10.013

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