



Contents lists available at ScienceDirect

Molecular and Cellular Endocrinology

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

Curcumin inhibits hepatic stellate cell activation via suppression of succinate-associated HIF-1 α induction

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ARTICLE INFO

Keywords:

Succinate

HIF-1 α

Hepatic fibrosis

Curcumin

ABSTRACT

Purpose: Aberrant succinate accumulation emerges as a unifying mechanism for inflammation and oxidative stress. This study aims to investigate whether curcumin ameliorates hepatic fibrosis via blocking succinate signaling.

Methods: We investigated the effects of curcumin on hepatic succinate accumulation and liver fibrosis in mice fed a high-fat diet (HFD). Meanwhile, we stimulated mouse primary hepatic stellate cells (HSCs) with succinate and observed the inhibitory effects of curcumin on succinate signaling.

Results: Oral administration of curcumin and metformin combated mitochondrial fatty acid oxidation and reduced hepatic succinate accumulation due to the inhibition of succinate dehydrogenase (SDH) activity and demonstrated inhibitory effect on hepatic fibrosis. In mouse primary HSCs, curcumin prevented succinate- and CoCl₂-induced hypoxia-inducible transcription factor-1 α (HIF-1 α) induction via suppression of ROS production and effectively reduced gene expressions of Col1 α , Col3 α , fibronectin and TGF- β 1 with inflammation inhibition. Knockdown of HIF-1 α with small interfering RNA blocked the action of succinate to induce HSCs activation, indicative of the essential role of HIF-1 α in succinate signaling.

Conclusions: Hepatic succinate accumulation served as a metabolic signal to promote liver fibrosis through HIF-1 α induction. Curcumin reduced succinate accumulation by combating fatty acid oxidation and prevented HSCs activation by blocking succinate/HIF-1 α signaling pathway.

1. Introduction

Chronic liver injury of any etiology is linked to liver fibrosis. Nonalcoholic steatohepatitis (NASH) is tightly associated with lipid disorders and characterized by fibrosis and inflammatory response (Anderson and Borlak, 2008). Myofibroblast activation is a hallmark of liver fibrosis, because embedding myofibroblasts not only produce excessive extracellular matrix (ECM) but also generate stress fibers, α -smooth muscle actin (α -SMA), fibronectin and collagens during liver injury. Although the composition of fibrogenic myofibroblasts varies dependent on the etiology of liver injury, liver resident hepatic stellate cells (HSCs) are the major source of myofibroblasts, responsible for the initiation of fibrosis (Xu et al., 2014; Iwaisako et al., 2014). Under physiological conditions, HSCs are in a quiescent state; however, in

response to cellular stress, quiescent stellate cells are activated and differentiated into myofibroblasts, characterized by α -SMA expression (Lua et al., 2014; Friedman, 2010). Therefore, the activation of HSCs in the liver is considered as a major cause for the development of fibrosis.

Inflammation is a driving force behind fibrosis and the recent study elucidates the contribution of succinate to inflammation (Tannahill et al., 2013). Succinate is an intermediate of the mitochondrial citric acid cycle (CAC). However, aberrant succinate accumulation works as a metabolic signaling to induce ROS production (Chouchani et al., 2014). In macrophages, succinate accumulation induces IL-1 β production in a manner dependent on hypoxia-inducible transcription factor 1 α (HIF-1 α) (Tannahill et al., 2013). As a transcriptional factor, HIF-1 α reprograms adaptive response for anoxic metabolism. HIF-1 α also exerts the ability to induce fibrosis in special tissues, such as the kidney, adipose

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<https://doi.org/10.1016/j.mce.2018.05.002>

Received 7 January 2018; Received in revised form 2 May 2018; Accepted 3 May 2018
0303-7207/ © 2018 Published by Elsevier B.V.

Abbreviations

α -SMA	α -smooth muscle actin
CAC	Citric acid cycle
ECM	Extracellular matrix
HFD	High-fat diet
HIF-1 α	Hypoxia-inducible factor-1 α

HSCs	Hepatic stellate cells
PA	Palmitate
PHDs	Prolyl hydroxylase domain dioxygenases
ROS	Reactive oxygen species
SDH	Succinate dehydrogenase
TMZ	Trimetazidine
2-MeOE2	2-Methoxyestradiol

tissue and synovium (Haase, 2012; Li et al., 2016a, 2016b). Although the contribution of succinate to hepatic fibrosis has been documented (Correa et al., 2007; Li et al., 2015a; Nguyen et al., 2018), more information is needed to elucidate the involvement of altered metabolism in succinate accumulation.

Curcumin is a polyphenolic compound found in *Curcuma longa*. Curcumin suppresses endoplasmic reticulum stress and oxidative stress with anti-inflammatory potency, demonstrating the ability to protect cellular homeostasis (Wang et al., 2016; Li et al., 2015b). Curcumin also ameliorates adipose dysfunction and reduces hyperglycemia via improving insulin sensitivity (Wang et al., 2016; Ding et al., 2016a, 2016b), exerting the ability to improve metabolism. Although curcumin is shown to inhibit fibrosis in special tissues, such as the liver and kidney (Zhao et al., 2014; Sun et al., 2017), the implication in metabolism is not known. To address this issue, in the present study, we studied the effect of curcumin on the activation of HSC with the emphasis on lipid metabolism. Our work showed that curcumin reduced hepatic succinate accumulation and prevented stellate cell activation via blocking succinate/HIF-1 α signaling.

2. Materials and methods

2.1. Reagents

Curcumin (purity $\geq 98\%$) was obtained from Nanjing Zelang medical technology Co., Ltd (Nanjing, China). Metformin was provided by Beijing Jingfeng Pharmaceutical Technology (Beijing, China). Palmitate (PA) was offered from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China) and dissolved in ethanol and diluted with medium containing 10% of bovine serum albumin (BSA) at the ratio of 1:19 to 10 mM before use. Trimetazidine dihydrochloride tablets were from Servier Pharmaceutical co., LTD (Tianjin, China). 2-Methoxyestradiol (2-MeOE2) was purchased from Selleck (USA). Oligomycin was from Dalian Meilun Biotech Co., Ltd. Dimethyl succinate (V900547), dimethyl malonate (136441) and Cobalt (II) chloride hexahydrate ($\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, C8661) were obtained from Sigma-Aldrich (St. Louis, MO, USA). The following items were purchased from the cited commercial sources: anti-mouse-IL-1 β antibody (503501) (BioLegend, USA); anti-HIF-1 α (ab179483), Goat Anti-Rabbit IgG H&L (Alexa Fluor 488) (ab181448), Abcam (Cambridge, MA, USA); anti-Hydroxy-HIF-1 α (Pro564) (Cell Signaling Technology, #3434); anti-GAPDH (AP0063), anti-TGF- β 1 (BS1361), anti- α -SMA (BS70000), Goat Anti-Rabbit IgG (H + L) HRP (BS13278), Bioworld Technology (St. Paul, MN, USA).

2.2. Animals

Eight-week-old male ICR mice were purchased from the Experimental Animal Center of Yangzhou University (Yangzhou, China) and housed in individually ventilated cages with 12 h dark-light cycles at 22 °C and free access to water and food. Mice were fed with HFD (71% fat, 11% carbohydrates, 18% protein, Cat. #MD12033) for 10 weeks with administration of curcumin (50 mg/kg) or metformin (200 mg/kg) by gavage every day, while mice in the control group were fed with standard rodent diet (10% fat, 70% carbohydrate and 20% protein, Cat. #MD12031). The oral doses of curcumin and metformin used were based on the previous studies (Li et al., 2016a; Wang et al.,

2016). All experiments involving experimental animals were approved by the Animal Ethics Committee of China Pharmaceutical University.

2.3. Cell isolation and culture

Primary HSCs were prepared as previously described (Mederacke et al., 2015). Briefly, mice were anaesthetized via intraperitoneal injection of sodium pentobarbital (50 mg/kg) and the liver was perfused with pronase E/collagenase IV (Sigma, USA) through the inferior vena cava. After digestion in vitro with prewarmed pronase E/collagenase solution, the HSCs were harvested by centrifugation in density gradient and then cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 20% fetal bovine serum (FBS) (Gibco, NY).

The human hepatic stellate cell line LX-2 was supplied by Xiangya Central Experiment Laboratory, Central South University, China. LX-2 cells were cultured in DMEM with 2% fetal bovine serum.

2.4. Pathological staining analysis and immunohistochemistry

The livers were removed from the control or HFD-fed mice and fixed in 4% paraformaldehyde, embedded in paraffin, and then cut into sections at 4 μm thickness. For the histological evaluation, the slides were stained with Hematoxylin and Eosin (H&E) staining and Masson's trichrome staining according to standard procedures, and then viewed by fluorescence microscope (Olympus, Japan). For immunohistochemistry analysis, after being subjected to 3% hydrogen peroxide, the slides were incubated overnight at 4 °C with specific antibodies. After washing, sections were incubated with HRP-secondary antibody for 1 h at 37 °C. Images were detected by fluorescence microscope (Olympus, Japan). Nonalcoholic fatty liver disease (NAFLD) activity score (steatosis, 0–4; inflammation, 0–4; ballooning degeneration, 0–4) was determined by a blinded pathologist. Masson's trichrome staining, α -SMA positive and TGF- β 1 positive were evaluated with the Image-pro Plus (Media Cybernetics Inc., Silver Spring, MD, USA) software.

2.5. Oil Red O staining

Liver slides were incubated with 60% filtered Oil Red O solution for 3 min at room temperature and then counterstained with hematoxylin staining for 5 min. They were viewed by fluorescence microscope (Olympus, Japan) and were calculated with the Image-pro Plus (Media Cybernetics Inc., Silver Spring, MD, USA) software.

2.6. Pimonidazole staining detection

The HFD-fed mice were injected with Hypoxyprobe™-1 solution at a dose of 60 mg/kg (Hypoxyprobe, Inc, Burlington, MA, USA) for 25 min prior to sacrifice. Fresh livers were formalin-fixed and embedded in paraffin. The sections were immunoblotted with 1:50 Hypoxyprobe-1 Mab1, followed by incubation with biotin-conjugated F(ab')₂ anti-mouse secondary antibody (1:100). The image was observed by confocal microscopy (LSM 700, zeiss, Germany).

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