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Succinate causes α-SMA production through GPR91 activation in hepatic stellate cells

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ABSTRACT

Succinate acts as an extracellular signaling molecule as well as an intermediate in the citric acid cycle. It binds to and activates its specific G protein-coupled receptor 91 (GPR91). GPR91 is present in hepatic stellate cells (HSCs), but its role in hepatic fibrogenesis remains unclear. Cultured HSCs treated with succinate showed increased protein expression of GPR91 and α-smooth muscle actin (α-SMA), markers of fibrogenic response. Succinate also increased mRNA expression of α-SMA, transforming growth factor β (TGF-β), and collagen type I. Transfection of siRNA against GPR91 abrogated succinate-induced increases in α-SMA expression. Malonate, an inhibitor of succinate dehydrogenase (SDH), increased succinate levels in cultured HSCs and increased GPR91 and α-SMA expression. Feeding mice a methionine- and choline-deficient (MCD) diet is a widely used technique to create an animal model of nonalcoholic steatohepatitis (NASH). HSCs cultured in MCD media showed significantly decreased SDH activity and increased succinate concentration and GPR91 and α-SMA expression. Similarly, palmitate treatment significantly decreased SDH activity and increased GPR91 and α-SMA expression. Finally, C57BL6/J mice fed the MCD diet had elevated succinate levels in their plasma. The MCD diet also decreased SDH activity, increased succinate concentration, and increased GPR91 and α-SMA expression in isolated HSCs. Collectively, our results show that succinate plays an important role in HSC activation through GPR91 induction, and suggest that succinate and GPR91 may represent new therapeutic targets for modulating hepatic fibrosis.

1. Introduction

Hepatic stellate cells (HSCs) constitute approximately 8–14% of cells in the normal liver, and HSC activation is crucial for the development of liver fibrosis. Following liver injury, HSCs become activated into contractile and highly proliferative myofibroblast-like cells to promote increased extracellular matrix (ECM) production and hepatic fibrosis [1,2]. This is accompanied by the upregulated expression of cytoskeletal protein such as α-smooth muscle actin (α-SMA) [1]. The molecular signals activated during HSC activation are not completely understood, but transforming growth factor β (TGF-β) and platelet-derived growth factor (PDGF) are known to play important roles [1,3–5].

Succinate is an intermediate in the citric acid cycle (or Krebs cycle). As part of this cycle in the mitochondrial matrix, succinate is produced by the oxidation of succinyl-CoA by the enzyme succinyl-CoA hydrolase and is further converted into fumarate by succinate dehydrogenase (SDH) [6]. In addition, it acts as an extracellular circulating signaling molecule that binds to and activates its specific G protein-coupled receptor (GPCR), G protein-coupled receptor-91 (GPR91) [7].

GPR91 activation triggered by local succinate accumulation increases the release of renin in the glomerular endothelium [8] and in the luminal membrane of the macula densa [9]. GPR91 mRNA is expressed in the polarized cells of the thick ascending limb of Henle's loop and the cortical and inner medullary collecting ducts, and its activation triggers the release of arachidonic acid and prostaglandins in the distal nephron [10].

In the retinal ganglion cells, GPR91 is involved in retinal angiogenesis [11] and modulates the release of vascular endothelial growth factor (VEGF) induced by high levels of glucose [12]. In dendritic cells, succinate triggers GPR91 activation, which is involved in helper T-cell activation and proinflammatory cytokine

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production [13]. GPR91 has been found in several highly vascularized tissues, including kidney, heart, liver, white adipose tissue, and retina [6,14,15].

In the liver, GPR91 protein is expressed in quiescent HSCs [16]; its mRNA is highly expressed in quiescent HSCs but less expressed in LPS-activated HSCs [17]. In a previous study [16], HSCs treated with succinate showed increased HSC activation, suggesting that succinate may be a novel HSC activator.

However, the roles of succinate and its receptor in the development of fibrosis have not been investigated extensively. In the present study, we determined whether succinate, malonate (an SDH inhibitor), MCD media, or palmitate regulate HSC activation and examined plasma levels of succinate and the expression of succinate and GPR91 by isolating HSCs induced in a nonalcoholic fatty liver disease (NAFLD) mouse model.

2. Materials and methods

2.1. Materials

Upregulation of α-SMA, a hallmark of myofibroblastic transdifferentiation, was used as a marker for HSC activation [3]. Completely deficient of methionine and choline (MCD medium) and methionine- and choline-supplement (MCS medium, control medium) were purchased from WELGENE (Kyeongsan, Korea). Succinate, malonate, and palmitate were purchased from Sigma (St. Louis, MO, USA).

2.2. Cell culture

LX2 cells are immortalized human stellate cells and they were kindly provided by Professor Ja June Jang, Seoul National University. The cells were cultured in DMEM (HyClone, South Logan, UT, USA) with 10% fetal bovine serum supplemented with 1% penicillin/streptomycin antibiotic solution.

2.3. Western blot analysis

Cells were lysed and the protein samples were analyzed by the bicinchoninic acid protein assay (Thermo Scientific Pierce, Rockford, IL, USA). The equal amounts of protein samples were resolved on a SDS/PAGE. Gels were transferred to a PVDF membrane and the membranes were probed with antibodies. Membranes were further incubated with secondary antibodies conjugated to HRP which were detected by the Westsave Star Detection Reagent system (AbFrontier, Seoul, Korea).

Sources of antibodies were as follows: anti-GPR91 (Santa Cruz Biotechnology, Dallas, USA); anti-α SMA and anti-GAPDH (GeneTex, Irvine, USA).

2.4. GPR91 siRNA transfection

Human siRNAs of scrambled GPR91 (SUCNR1) (siRNA; 5′-GGAACAGCAGUAAUAUUUA-3′) and control (siRNA; a universal negative control) were from Thermo Scientific (Dharmacon, Lafayette, CO, USA).

2.5. Animals and isolation of HSCs and hepatocytes

Six-to eight-week-old male C57BJ6 mice weighing 18–20 g were purchased from Central Animal Laboratory (Korea). All mice were housed at ambient temperature (22 °C ± 1 °C) with 12:12-h light:dark cycles and free access to water and diet. The mice were fed the methionine and choline deficient diet (MCDD group) as an animal model of NAFLD or control diet (Control group) for 4 weeks.

Primary mouse HSCs and hepatocytes were isolated from the livers of 10- to 12-week-old mice. *In situ* liver perfusion and digestion was performed with Pronase E (2.4 mg/mL, Roche Molecular Biochemicals, Indianapolis, USA) and Collagenase B (0.3–0.45 mg/mL, Roche Molecular Biochemicals, Indianapolis, USA). The resulting liver cell suspension was purified using a density gradient. Cells were grown on standard plastic tissue culture dishes in DMEM medium with 10% fetal calf serum and antibiotics. Primary cells were incubated at 37 °C and used 3 days after plating.

2.6. Succinate dehydrogenase assay and succinate assay

SDH activity in the supernatant was determined using ab109908-Complex II enzyme activity microplate assay kit and analysis of 2,6-dichlorophenol-indophenol (DCPIP) reduction at 600 nm for 60 min using a microplate reader. The data are expressed as mOD.min^{-1}.

The intracellular concentration of succinate was determined using the succinate colorimetric assay kit (BioVision Milpitas, CA, USA). The succinate was measured using spectroscopy at 450 nm and each measurement was performed in triplicate.

2.7. RT-PCR

Total RNA was isolated from LX2 cells using the Rneasy Mini Kit (Qiagen, Hilden, Germany). cDNA was isolated using the PrimeScript 1st strand cDNA Synthesis Kit (Takara Bio, Shiga, Japan). After the reverse transcription reaction, the cDNA template was amplified using quantitative RT-PCR with Maxima SYBR Green/ROX qPCR Master Mix (2X) (Thermo) using standard protocols. PCR was performed with 10 pmol specific primers for α-SMA (5-CCACCGCAAATGCTTCTAAGT-3) and (5- GGCAGGAATGATTT GGAAAGG-3), TGF-β1 (5-TCGACATGGAGCTGGTGAAA-3) and (5-GAGCCTTAGTTTGGACAGATCTG-3), collagen type 1 (5-GAACG CGTGTCATCCCTTGT-3 and 5-GAACGAGGTAGTCTTTCAGCAACA-3), and GAPDH (5-GGCATGGACTGTGGTCATGAG-3 and 5-TGCACCACCAACTGCTTAGC-3). Forty cycles were performed using an ABI Prism 7000 instrument (Applied Biosystems, Foster City, CA, USA). All amplified PCR products were confirmed via 2% agarose gel electrophoresis and photographed using ultraviolet illumination.

2.8. Hematoxylin-eosin stain

Samples of mouse liver were fixed in 10% (wt/vol) phosphate buffered formalin for 18–20 h. After standard histological processing and embedding in paraffin, transverse paraffin sections (5 μm thick) were deparaffinized in xylene twice for 30 min each, hydrated gradually through a graded series of alcohol (100% ethanol twice for 5 min each followed by 95%, 85%, and 75% ethanol for 5 min each) and rinsed in distilled water for 1 min and then counterstained with hematoxylin-eosin (H&E) (Merck, Mumbai, India).

2.9. Statistical analysis

All data are expressed as mean ± SEM. Data analyses for the two groups were performed using the *t*-test. P values <0.05 were considered significant.

3. Results

3.1. Succinate as a GPR91 agonist activates HSCs

The activation of HSCs by succinate was monitored using Western blotting and RT-PCR. To investigate the expression pattern of GPR91 in HSCs, Western blotting was performed using LX2 cells.